Cultured equine satellite cells as a model system to assess leucine stimulated protein synthesis in horse muscle

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ABSTRACT: Leucine has been shown to stimulate the mammalian/mechanistic target of rapamycin (mTOR) signaling pathway which plays numerous key regulatory roles in cell growth, survival, and metabolism including protein synthesis in a number of species. However, previous work with equine satellite cells has suggested distinct species differences in regards to physiological effects and the magnitude of responses to growth factors and regulators. Because there is limited research available regarding the role of leucine in regulating equine skeletal muscle protein synthesis, the objective of this study was to evaluate the effect of leucine on the mTOR signaling pathway in cultured equine satellite. Protein synthesis was evaluated by measuring the incorporation of [3H] Phenylalanine (³HPh) in equine satellite cell myotube cultures treated with a leucine titration ranging from 0 to 408 μ M. Our results show a 1.8-fold increase ($P < 0.02$) in protein synthesis at levels slightly greater than those found in the general circulation, 204 and 408 μ M when compared to a no leucine control (0 μ M). Puromycin incorporation, a nonradioactive surface sensing of translation (SUnSET) methodology, was also measured in cells treated with leucine (LEU; 408 μ M), a no-leucine control (CON), and a puromycin-negative

vehicle (PURO-). These results demonstrated a 180% increase ($P = 0.0056$) in puromycin incorporation in LEU compared to CON cultures. To evaluate the mTOR signaling pathway, equine satellite cell myotube cultures were treated with leucine (LEU; 408 μ M) or a no-leucine control (CON) in the presence or absence of rapamycin (LR and CR, respectively), an inhibitor of mTOR. The mTOR inhibitor, rapamycin, suppressed phosphorylation of mTOR ($P < 0.01$) and rS6 ($P < 0.01$) with an increase in phosphorylation of rS6 in leucine-treated cultures observed when compared to control cultures ($P < 0.05$). Similarly, there was a 27% increase ($P < 0.005$) in the hyperphosphorylated γ -form of 4E-BP1 compared to total 4E-BP1 in LEU compared to CON cultures with leucine-induced phosphorylation of 4E-BP1 completely blocked by rapamycin with a smaller decrease observed in CR compared to CON cultures. The major finding of this study was that leucine activated the mTOR translation initiation pathway and increased transcription of global proteins in cultured equine satellite cells. Use of the cell culture system with primary equine muscle cell lines provides the opportunity to distinguish the impact of leucine on muscle and protein synthesis, independent of systemic interactions.

Key words: horse, mTOR pathway, rS6, 4E-BP1, puromycin, protein synthesis

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INTRODUCTION

Skeletal muscle is critically important to a horse's (*Equus caballus*) athletic ability and

comprises approximately 50% of the live weight of the horse (Gunn, 1987). The amount of skeletal muscle mass is determined by the dynamic balance between skeletal muscle protein synthesis and protein degradation. Multiple factors influence that balance including factors such as amino acids, hormones, growth factors, and cellular energy. In addition to being a substrate for protein synthesis, the amino acid leucine has been shown to regulate the key mammalian/mechanistic target of rapamycin (mTOR) pathway that integrates and responds to environmental cues to promote cell growth.

While leucine's role in stimulating protein synthesis has been well established in the human (*Homo sapiens*; Drummond and Rasmussen, 2008), rat (*Rattus norvegicus*; ; Anthony et al., 2000; Crozier et al., 2005), mouse (Funabiki et al., 1992), and pig (*Sus domestica*; Han et al., 2008; Boutry et al., 2013), there has been little to no data related to the activating role of leucine in equine skeletal muscle. The lack of research may pose a problem as previous research using equine myogenic satellite cells has suggested distinct species differences in regards to physiological effects along with the magnitude of responses to growth factors and regulators (Greene et al., 1995).

Research focused on the requirements and roles for amino acids in horses has provided valuable information; however, few studies have been conducted. Research utilizing muscle satellite cell cultures in other species has allowed for controlled, focused alternatives to whole animal investigations. Therefore, the objective of this study was to use cultured equine satellite cells to evaluate the effect of leucine as a regulator of protein synthesis in equine skeletal muscle.

MATERIALS AND METHODS

Satellite Cell Isolation

Satellite cells were isolated from three horses using previously described procedures (Hathaway et al., 1991; Frey et al., 1995; Yi et al., 2001; Kamanga-Sollo et al., 2004). The horses were chemically euthanized for reasons not associated with a muscle disease/disorder, using procedures approved by the University of Minnesota's Institutional Animal Care and Use Committee at the University of Minnesota's College of Veterinary Medicine. Immediately postmortem semimembranosus muscle tissue was dissected using sterile techniques and transported to a cell culture laboratory where all subsequent procedures were conducted in a laminar

flow hood. After removal of obvious connective tissue, the muscle was passed through a sterile meat grinder. Within 30 min of euthanasia, the ground muscle was incubated with 0.1% pronase (Sigma-Aldrich, St. Louis, MO) in $\text{Ca}^{+2}\text{Mg}^{+2}$ free Earl's Balanced Salt Solution (117 mM NaCl, 10 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 5.4 mM KCl, 5.6 mM d-Glucose, pH 7.4) for 1 h at 37°C with frequent mixing. After incubation, the mixture was centrifuged at $1,500 \times g$ for 4 min at 25°C, the pellet was suspended in phosphate-buffered saline (PBS:140 mM NaCl, 1 mM KH_2PO_4 , 3 mM KCl, 8 mM Na_2HPO_4 , pH 7.4), and the suspension was centrifuged at $500 \times g$ for 10 min at 25°C. The resultant supernatant was centrifuged at $1,500 \times g$ for 10 min at 25°C to pellet the mononucleated cells. The PBS wash and differential centrifugation were repeated two more times. The resulting mononucleated cell preparations from each $1,500 \times g$ centrifugation were pooled together, suspended in cold (4°C) Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY) containing 20% horse serum (HS; GE Healthcare Life Sciences, Logan, UT) and 10% (vol/vol) dimethylsulfoxide. Equine satellite cell preparations were stored frozen in liquid nitrogen for later use.

Satellite Cell Characterization

The equine satellite cells were thawed and then plated on culture dishes precoated with 0.024 mg/cm² reduced growth factor basement membrane Matrigel (Becton Dickinson, Franklin Lakes, NJ). All culture media contained 1× antibiotic/antimycotic (Sigma-Aldrich). Cells were plated in DMEM containing 20% HS and incubated at 37°C, 5% CO₂ in a water-saturated environment. Cells were refed 20% HS at 72 h and again at 96 h postplating so that cultures achieved 85% to 100% confluency by 120 h, at which time they were incubated in differentiation medium consisting of DMEM containing 3% HS. To characterize the cultures, cell morphology, percent fusion, and the expression patterns of myogenic regulatory factors (Myf-5, MyoD, Myogenin, and Myostatin) were assessed during the differentiation process. Total RNA was isolated at various time points (72, 96, 120, 144, and 168 h in culture). The total RNA was isolated using an Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA). After a phenol chloroform extraction of the cell lysate, RNA was isolated following the protocol recommended by the manufacturer. Samples were treated with DNase while bound to the fiber matrix during the isolation. Quantitative reverse transcription polymerase chain reaction (RT-PCR)

was used to measure the quantity of each specific mRNA relative to the quantity of glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) mRNA in the total RNA isolated from the cells. The GAPDH mRNA was used to correct for sample concentration and reverse transcription efficiency because its abundance is relatively stable in these cells at all time points under our experimental conditions ($P > 0.05$). cDNA was made from equine skeletal muscle RNA for use as a positive control in all quantitative polymerase chain reaction assays. Total RNA (1 µg) was reverse transcribed to cDNA with Taq-Man reverse transcription Reagents (Applied Biosystems, Foster City, CA) using random hexamer primers. Measurements were carried out using 1 µL of the cDNA mixture, SYBR Green PCR Master Mix (Applied Biosystems), and 300 nM of the appropriate forward and reverse primers (Table 1).

Assays were performed using the 7300 Real Time PCR System (Applied Biosystems) using cycling parameters recommended by the manufacturer (40 cycles of 15 s at 95°C and 1 min at 60°C). Titration of primer pairs for each growth factor (300 nM forward and reverse primers) against increasing amount of cDNA gave a linear response. In each assay, measurements were made on triplicate cell cultures and values obtained were averaged. In all cases, data from at least three assays where each assay used equine satellite cells obtained from a different horse, were pooled.

An additional set of cultures from each satellite cell preparation was Giemsa stained at each time point and later photographed so that percent fusion could be determined. Fusion into myotubes was defined as three or more nuclei within a contiguous sarcolemma. The data collected from RT-PCR for

all genes was compared to the visual observations of the satellite cells.

Protein Synthesis Assay

Incorporation of radioactive phenylalanine into cellular proteins was used to assess the impact of increasing concentrations of leucine in culture medium. A custom formulated DMEM (Gibco), which did not contain any essential amino acids, served as the foundation for the washout medium and the test media so that the amount and ratio of supplemental essential amino acids (Sigma-Aldrich) could be controlled. With the exception of leucine, the amount of each supplemental essential amino acid in the medium was calculated based on [Bryden's \(1991\)](#) analysis of the amino acid composition of horse skeletal muscle relative to lysine (Table 2).

Equine satellite cells were grown and induced to fuse as described above. Cells isolated from each animal were plated in separate experiments for a total of three experiments with quadruplicate wells per treatment. At peak fusion, the differentiation medium was removed and replaced with washout medium consisting of custom formulated DMEM for 6 h. Subsequently, washout medium was removed and replaced with treatment media. All treatment media consisted of the custom formulated DMEM plus the essential amino acids (Table 2) and the growth factors bovine fibroblast growth factor (bFGF; 5 ng/mL) and human epidermal growth factor (hEGF; 5 ng/mL) plus a dose titration of leucine. After 3 h, 3 uCi ³H-Phenylalanine, L-[2,3,4,5,6-³H] (³H-Phe; Perkin Elmer, Waltham, MA) were added to each culture and incubation was continued for an additional 3 h. At the end of the 6 h treatment period, media was removed. The

Table 1. Accession numbers, primer pairs, efficiency of quantitative polymerase chain reaction

Accession	Accession and Primer pairs	PCR efficiency
Myf5	XM_001490003 F: CCACCTCCAAGTCTCAGATG R: GCTGTCAAACTGCTGCTTTTTC	105.43%
Myogenin	NM_001317250.1 F: TCACGGCTGACCTACAGATG R: GGTGATGCTGTCCACAATGG	92.17%
MyoD	XM-001504953 F: ACGGCTCTCTCTGCAACTTTG R: GAGTCGAAACACGGGTCATCA	90.02%
Myostatin	AB033541 F: TGGCTCTTTGGAAGATGATGATTA R: CACTTGCAATAGAAGATCAGACTCTGT	97.43%
GAPDH	Primer sequence in Kawal et al., 2013 F: CAAGGCTGTGGCAAGGT R: GGAAGGCCATGCCAGTGA	90.19%

Table 2. Essential amino acid concentrations derived from empirical calculations based on a ratio determined by [Bryden \(1991\)](#) from amino acid proportions in the muscle

Amino Acid	µM
Lysine	188
Methionine	54
Cysteine	19
Threonine	116
Tryptophan	35
Isoleucine	105
Histidine	122
Phenylalanine	113
Valine	118
Arginine	139

Figure 1. Relative Myogenin, MyoD, Myf-5, and Myostatin mRNA expression levels at 72, 96, 120, 144, and 168 h in culture of equine satellite cell differentiation in vitro. The mRNA levels relative to glyceraldehyde-3-phosphate dehydrogenases mRNA were measured using real time PCR. cDNA was made from equine skeletal muscle RNA for use as a positive control in all quantitative polymerase chain reaction assays. Data is representative of triplicated cell cultures for three independent assays where each assay used satellite cells isolated from a different horse.

cultures were rinsed three times with cold DMEM and incubated in cold (4°C) 5% trichloroacetic acid (TCA) for at least 12 h. Following TCA precipitation of proteins, cultures were washed two times with fresh, cold 5% TCA and then lysed in 0.5N NaOH at 37°C for 30 min. Cell lysate was collected, triplicate subsamples were used for protein determination (Pierce BCA Protein Assay; ThermoFisher Scientific) and the remainder of the lysate was counted in a Beckman LS-3801 liquid scintillation counter to quantify ^3H -Phe incorporation into proteins. Protein synthesis was calculated as ^3H -Phe incorporated into a μg of protein for each culture. There were quadruple determinations per treatment within each assay for the three assays; each

assay used satellite cells isolated from a different horse. Results from the three assays were averaged and SEM calculated (Figure 2).

SUnSET Assay

The SUnSET method was used to determine if leucine treatment impacted de novo protein synthesis. Equine satellite cells were grown and induced to fuse as described above with triplicate wells per treatment. The differentiation medium was replaced with washout media which consisted of the custom formulated DMEM for 6 h after which treatment media was applied. Treatment media consisted of the custom formulated DMEM plus the essential

Figure 2. Effect of L-leu titration (0–408 μM) on protein synthesis in equine satellite cell myotube cultures treated for 6 h measured by ^3H phenylalanine ($^3\text{HPhe}$) incorporation. Quadruple determinations were averaged for each assay for three assays, where each assay used satellite cells isolated from a different horse. Results are expressed as mean $\pm$ SEM of CPM of $^3\text{HPhe}$ per μg protein. Bars containing no letters in common are statistically different ($P < 0.02$).

amino acids (Table 2) and growth factors bFGF (5 ng/mL) and hEGF (5 ng/mL) with 204 μ M leucine (LEU) or without leucine (CON). After 2 h of treatment, with the exception of the puromycin negative controls (PURO–) which received only vehicle (custom-formulated DMEM), puromycin (540411; Calbiochem, Billerica, MA) was added to the media in each culture for a final concentration of 5×10^{-7} M. Cultures were incubated for an additional hour, after which they were collected in lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM ethylenediaminetetraacetic, 1% IGEPAL, 1 mM sodium fluoride, 1 mM polymethanesulfonyl fluoride, 1 mM sodium orthovanadate, 104 mM AEBSF, 80 μ M aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM leupeptin, and 1.5 mM Pepstatin). Cell lysate was centrifuged at $10,000 \times g$ for 10 min at 4°C; the supernatant was collected and frozen at –80°C for later analysis.

Protein concentrations were determined with a Micro-BCA protein assay kit (Thermo-Fisher Scientific) and equivalent amounts of protein (7 μ g) of each sample were dissolved in Laemmli buffer and subjected to electrophoretic separation on a tris/glycine 4% to 15% gradient gel (Mini-Protean TGX; Bio-Rad; Hercules, CA). Proteins were transferred to nitrocellulose membrane in high molecular weight protein transfer buffer (242 mM Tris, 58 mM glycine, and 20% v/v methanol). The

membrane was blocked in 5% powdered milk in Tris-buffered saline (TBS; 50 mM Tris-HCl [pH 7.5], 150 mM NaCl) with 0.1% Tween 20 (TBST) for 1 h followed by an overnight incubation at 4°C with 1:1,000 anti-puromycin (3RH11; Kerafast, Boston, MA). The membrane was then washed in TBST and incubated for 1 h at 20°C with 1:10,000 horseradish peroxidase-conjugated goat anti-mouse IgG (sc-2005; Santa Cruz Biotechnology, Dallas, TX). After TBST washes, membranes were developed by chemiluminescence and viewed by C-DiGit gel imaging system (LI-COR, Lincoln, NE). Band densities of the entire lane for each of the three lanes per treatment were quantified by a computer software program (Image Studio Ver. 3.1) and then averaged to compare LEU versus CON cultures. Afterward, a reversible protein stain (Pierce Reversible Protein Stain Kit—for nitrocellulose membranes; Thermo-Fisher Scientific) was used to stain the membrane to visually verify equal protein loading of all lanes (Figure 3).

Western Immunoblot of Translation Initiation Factors

Equine satellite cells were grown and induced to fuse as previously described. Cells isolated from each animal were plated in separate experiments for a total of three experiments with triplicate

Figure 3. Representative image of western blot analysis for puromycin incorporation into nascent proteins in equine myotube cell cultures treated with 204 μ M leucine (LEU) or without leucine (CON) for 2 h before they received puromycin for 1 h. Molecular weight markers can be found the left of the image. The specificity of the anti-puromycin blot was demonstrated by a sample that was not incubated with puromycin (PURO–). Integrated optical density units of the entire lane for each of the three lanes per treatment were quantified and averaged to compare LEU vs. CON cultures. Leucine treated cells increased puromycin incorporation by 1.8-fold ($P < 0.01$). The reversible protein stain was then used to verify equal loading.

wells per treatment. Treatments consisted of a no leucine control (CON), 408 μ M leucine (LEU), no leucine control plus 100 nM rapamycin (CR), and 408 μ M leucine plus 100 nM rapamycin (LR). At peak fusion, the differentiation media was removed and replaced with washout medium consisting of the custom formulated DMEM plus the essential amino acids (Table 1) with the exception of leucine. After 5.5 h in the washout media, rapamycin (EMD Millipore, Billerica, MA), a naturally occurring antifungal which potently inhibits mTOR signaling, was added to the washout media for a final concentration of 100 nM rapamycin in wells predetermined to be treated with rapamycin (CR and LR). After 6 h in the washout medium and 30 min of rapamycin pretreatment, the washout medium was removed and replaced with treatment media. Cultures were incubated in treatment media for 10 min after which cultures were immediately placed on ice, test media was removed, cultures were rinsed one time with ice cold PBS and then lysed with ice-cold lysis buffer and frozen as described above. Each treatment was assayed in triplicate in each of the three experiments with each experiment utilizing satellite cells isolated from a different horse.

Cell lysate protein concentrations were determined with a Micro-BCA protein assay kit (Thermo-Fisher Scientific). Based on protein concentration, equivalent amounts of protein (7 μ g) from each lysate sample were dissolved in Laemmli buffer and subjected to electrophoretic separation on a tris/glycine 4% to 15% gradient gel (Mini-Protean TGX; Bio-Rad Laboratories). Proteins were electrophoretically transferred to nitrocellulose membrane in high molecular weight protein transfer buffer (242 mM Tris, 58 mM glycine, and 20% v/v methanol). Subsequently, membranes were cut into four horizontal sections corresponding to the molecular weight regions for mTOR, tubulin, rS6, or 4E-BP1; each section was then assessed independently of the other sections. After blocking with 5% powdered milk in TBST for 1 h, membranes were incubated with the primary antibodies overnight at 4°C. The primary antibodies were mTOR (total and phosphorylated Ser²⁴⁴⁸; 1:1,000), rS6 (total and phosphorylated Ser^{235/236}; 1:1,000), 4E-BP1 (total and phosphorylated Thr^{37/46}; 1:1,000) and β -tubulin (2128; 1:2,000). All antibodies were purchased from Cell Signaling Technology (Danvers, MA). Membranes were washed for 30 min in TBST and then incubated at room temperature (20°C) for 1 h with goat anti-rabbit IgG conjugated to horseradish peroxidase (7074; Cell Signaling). After washing in TBST, membranes were developed by

chemiluminescence (LI-COR) and viewed by a gel imaging system (LI-COR). Quantification of band densities were calculated by a computer software program (Image Digits Studio Ver. 3.1) to determine the abundance of pmTOR, mTOR, prS6, rS6, p4E-BP1, 4E-BP1, and β -tubulin.

Triplicate cultures of each treatment for each animal were run on a single, 15-well gel for the examination of all proteins. The nitrocellulose membrane was first blotted for the phosphorylated protein abundance and then stripped with a commercial western blot stripping buffer (Restore-Plus, Thermo-Fisher Scientific) and probed again for total protein abundance. Membranes were tested following stripping to verify that all signals had been removed. Membranes were only reprobed once which minimized loss of protein and intra-assay variation while stripping and reprobing the same membrane acted to minimize inter-assay error resulting from the quantification of total and phosphorylated protein abundances on different membranes.

While gels were loaded based on protein concentration, β -tubulin abundance was assessed to further verify equal protein loading and efficiency of protein transfer; no difference in β -tubulin abundance was observed across any of the treatments ($P = 0.6923$; data not shown). Additionally, phosphorylated protein abundance was normalized to the nonphosphorylated counterparts for mTOR and rS6. The triplicate bands on the 4E-BP1 antibody corresponded to the level of phosphorylation in the proteins with the upper band (γ) indicating the hyperphosphorylated form and the lower band indicating the unphosphorylated or least phosphorylated form. As a result, the phosphorylated γ 4EBP-1 was normalized to total 4E-BP1.

Statistical Analysis

Data were analyzed using the PROC MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). For each study, an individual culture was considered the experimental unit, and statistical significance was set at $P \leq 0.05$. Replicate and animal were considered a random effect and treatments were designated as a fixed effect. Variables analyzed included CPM/ μ g protein in the protein synthesis assay, total protein abundance of LEU and CON treatments for the puromycin western immunoblot, and protein abundance for pmTOR, mTOR, prS6, rS6, γ 4E-BP1, total 4E-BP1, and β -tubulin for the translation initiation factor western immunoblots. The effects of treatment and rapamycin on western

blot initiation factors were evaluated according to a 2×2 factorial design. To meet analysis of variance assumptions, a square root transformation of western blot initiation factors was used. These transformations were back transformed for means separation using a Tukey's HSD test ($\sigma = 0.05$).

RESULTS AND DISCUSSION

Cultured equine satellite cells exhibited the expression patterns of myogenic regulatory factors, Myf-5, MyoD, and myogenin and muscle-specific myostatin during myogenesis expected of myogenic cell progression of differentiation. As anticipated, MyoD, Myf-5, and myostatin peaked during the mid-late proliferative period and then declined while Myogenin was low initially but continued to increase throughout differentiation (Figure 1). Peak fusion, as observed in stained cultures occurred at approximately 168 h in culture with 75% fusion on average. By 196 h in culture myotubes were detaching.

Leucine activated downstream effectors of the mTOR translation initiation pathway and increased transcription of proteins in cultured equine satellite cells. Those effects were further corroborated by the leucine-induced increase in equine satellite cell protein synthesis.

Protein Synthesis Assay

An increase in protein synthesis was observed at 204 μM as well as at 408 μM leucine but not at 102 μM leucine ($P < 0.02$; Figure 2). Average plasma leucine concentrations in the horse range between 80 and 160 μM (Westermann et al., 2011; Peters et al., 2013; Mastellar et al., 2016) just below the concentration at which statistical differences were observed in this study. No effect was observed between 25 and 102 μM leucine, suggesting that in these experiments those concentrations did not reach the threshold needed to detect an increase in ^3H -Phe incorporation into equine satellite cell proteins. There was an overall 1.8-fold increase in protein synthesis at the greatest concentrations of leucine compared to control cultures. No difference in ^3H -Phe incorporation was observed between 204 and 408 μM leucine-treated cultures. It was not possible from these results to determine whether the leucine's stimulatory effect on protein synthesis had plateaued, as was seen by Kimball et al. (1999) in C2C12 cells at much higher concentrations of leucine (1.5 to 16.5 mM). The concentration of leucine, which stimulated protein synthesis in the equine satellite cells, was only slightly greater than

the normal physiological range of leucine concentrations in horse plasma. Han et al. (2008) used primary porcine satellite cells to evaluate the effect of leucine at much higher concentrations than were used in this study (2 mM leucine) and reported a numerical but not significant increase in protein synthesis ($P = 0.058$). Much of the previous research, aimed at determining whether leucine enhanced muscle protein accretion or de novo protein synthesis in myogenic cell cultures, used muscle cell lines, either the mouse C2C12 or the rat L6. While some researchers using cell lines have reported that leucine stimulated protein synthesis (Kimball et al., 1999; Du et al., 2007), others have reported that it did not (Deldicque et al., 2008; Haegens et al., 2012; Areta et al., 2014). The variability in experimental results may be due to differences in protocols for the washout period, pretreatment conditions, and/or presence of sera or anabolic growth factors in the test media or concentration and length of leucine treatment or species differences. Interestingly, regardless of whether leucine enhanced muscle protein synthesis per se was observed, leucine activated mTOR and its downstream targets 4E-BP1, S6K1, and p70^{S6} (Kimball et al., 1999; Du et al., 2007; Deldicque et al., 2008; Han et al., 2008; Haegens et al., 2012; Areta et al., 2014). Because mTOR is impacted by numerous pathways that regulate cell growth, metabolism and survival, activation of translation initiation may be more sensitive than those used to measure total protein accretion or de novo protein synthesis, especially across a wide range of experimental protocols.

SUnSET Assay

The impact of leucine treatment on mRNA translation of de novo protein synthesis was quantitatively assessed via the nonradioactive technique, SUnSET, which relies on the use of the antibiotic puromycin and antipuromycin antibodies (Goodman and Hornberger, 2013; Goodman et al., 2016). Puromycin is a structural analog of aminoacyl-transfer RNA which is incorporated into elongating peptide chains, blocking subsequent peptide bonds from being formed. The result is the premature termination of peptide elongation and subsequent release of the shortened puromycin-bound peptides from the ribosomes. LEU had an average lane band integrated optical density of 2,106,667 in comparison to CON at 1,180,000 with a pooled error variance of $\pm 57,783$. The LEU cultures contained significantly ($P = 0.0056$; Figure 3) more puromycin in nascent proteins

compared to CON cultures; a 1.8-fold increase. Both LEU and CON puromycin treated cultures had significantly greater ($P = 0.0036$) incorporation of puromycin when compared to the puromycin negative control.

Translation Initiation Signaling

In numerous reports, the importance of leucine in skeletal muscle has been established as a result of its ability to stimulate skeletal muscle protein synthesis by initiating signal transduction pathways leading to translation initiation (Buse and Reid, 1975; and Li and Jefferson 1978; Buse et al., 1979; Anthony et al., 2000). Nutrient availability, specifically amino acid sufficiency or deficiency, impacts mTOR activity. In an amino acid rich environment, by a poorly characterized mechanism, mTOR stimulates protein synthesis (Jewell et al., 2013).

Phosphorylation of mTOR (pmTOR) at Ser²⁴⁴⁸ was suppressed in cultures treated with the mTOR inhibitor, rapamycin (LR; CR; $P < 0.01$; Figure 4A); however, there was no significant difference in phosphorylation of mTOR in control compared to leucine-treated cultures ($P = 0.76$; Figure 4A). While Du (2007) did not see an effect of 2 mM leucine on pmTOR abundance in C2C12 cells after only 15 min of treatment, he did observe a difference after 30 min. The current study differed from other reports of leucine stimulated pmTOR expression when C2C12 cultures were treated for 30 min with leucine at 1.5 mM (Areta et al., 2014), 2 mM (Du et al., 2007), or 5 mM (Deldicque et al., 2008; Haegens et al., 2012). While we observed a nonstatistically significant trend in pmTOR with LEU, it was not different from CON cultures. The current results may differ from previous studies that used super-physiological concentrations of leucine and/or a longer treatment period. It may also be that primary equine satellite cells respond differently compared to porcine satellite cells or rodent cell lines. The effect of rapamycin treatment to completely block the upregulation of pmTOR was consistent with other reports (Haegens et al., 2012; Dai et al., 2015) although it is also possible that since rapamycin inhibited all three markers independently of leucine treatment, leucine could be acting through another signaling pathway.

Phosphorylation of rS6, a component of the 40S ribosome subunit, downstream of mTOR, is used as an indicator of translation initiation (Nandagopal and Roux, 2015). In the current study, an increase in phosphorylation of rS6, at Ser^{235/236} in leucine-treated cultures was observed with a 36%

increase in relative intensity was compared to control cultures ($P < 0.054$; Figure 4B). Additionally, treatment with rapamycin suppressed phosphorylation of rS6 ($P < 0.01$) Treatment with rapamycin has been shown to dephosphorylate targets downstream of mTOR very quickly, although it is still unclear how mTOR complex 1 regulates the phosphorylation of those targets. Translation can be maintained in some cells following an extended treatment with rapamycin. As a consequence, solely relying on changes in prS6 may not be an appropriate as a downstream effector when assessing rapamycin inhibition of mTOR (Ma and Blenis, 2009). However, prS6 can be used to assess the impact on the status of translation initiation processes. Similar to results presented here, Areta et al. (2014) observed that 1.5 mM of leucine for 30 min stimulated phosphorylation of rS6 by approximately 2.3-fold in C2C12 cells and Deldicque (2008) observed an increase in prS6 after 30 min of treatment of 5 mM leucine.

The phosphorylation of 4E-BP1 is important due to its relationship with eIF4E (Thoreen, 2017). Recognition and unwinding of mRNA to allow the 40S ribosome to bind is an important regulatory site of translation initiation requiring the participation of eIF4E together with eIF4A and eIF4G into a complex termed eIF4F. The availability of eIF4E to bind eIF4G is dependent upon the phosphorylation of 4E-BP1 as 4E-BP1 and eIF4G compete for the same binding site. The phosphorylation of 4E-BP1 through the mTOR complex inhibits its ability to sequester eIF4E into an inactive complex (Hay and Sonenberg, 2004; Ma and Blenis, 2009; Polak and Hall, 2009). Consequently, 4E-BP1 governs the release of the translation initiation factor eIF4E.

Phosphorylation of 4E-BP1 at Thr^{37/46}, which inhibits its ability to suppress protein synthesis, was greater ($P < 0.01$) in LEU cultures compared to CON with a 359% relative intensity. When the mTOR inhibitor, rapamycin, was added into the culture media, leucine induced phosphorylation of 4E-BP1 was completely blocked while a smaller decrease on phosphorylation levels of 4E-BP1 in CR compared to CON cultures was observed ($P < 0.01$; Figure 4C)

There have been a limited number of in vivo studies focused on investigating the regulatory mechanisms evaluating the impact of amino acids and proteins on translation initiation in equine skeletal muscle. However, the results available are consistent with those observed in other species. Following an 18 h fast, Urschel et al. (2011) observed an increase in phosphorylation of 4E-BP1

Figure 4. Effects on the mTOR signaling pathway in equine satellite cell myotube cultures treated with a no leucine control (CON), 408 μ M leucine (LEU), no leucine control plus 100 *nM* rapamycin (CR), and 408 μ M leucine plus 100 *nM* rapamycin (LR). A pretreatment of rapamycin was added after a 5.5 h washout for 30 min before wells were rinsed and treatment media was applied for 10 min. Phosphorylated and nonphosphorylated initiation factors were assayed by western blot, in triplicate for each assay for three assays, where each assay used satellite cells isolated from a different horse. Phosphorylated protein abundance was normalized to the nonphosphorylated counterparts for mTOR and rS6 and the upper band (y) of 4E-BP1 was normalized to total 4E-BP1. Results are expressed as mean $\pm$ SEM. (A) Phosphorylation of mTOR (pmTOR) at Ser²⁴⁴⁸ was suppressed in cultures treated with the mTOR inhibitor, rapamycin (LR; CR; $P < 0.01$). ^{a,b}Within a graph, means without a common superscript differ by the main effect of rapamycin ($P < 0.05$). (B) An increase in phosphorylation of rS6 at Ser^{235/236} in leucine-treated cultures was observed compared to control cultures ($P < 0.05$). Additionally, treatment with rapamycin suppressed phosphorylation of rS6 ($P < 0.01$). ^{x,y}Within a graph, means without a common superscript differ by the main effect of treatment ($P < 0.05$). (C) Phosphorylation of 4E-BP1 at Thr37/46 was greater ($P < 0.01$) in LEU cultures compared to CON with a 359% relative intensity. When the mTOR inhibitor, rapamycin, was added into the culture media, leucine-induced phosphorylation of 4E-BP1 was completely blocked while a smaller decrease on phosphorylation levels of 4E-BP1 in CR compared to CON cultures was observed ($P < 0.01$). ^{a,b,c}Within a graph, means without a common superscript differ by the interaction of rapamycin $\times$ treatment ($P < 0.05$).

and rS6 in the middle gluteal muscle of horses after they consumed a high protein meal. When [Wagner et al. \(2013\)](#) compared the effects of horse age on activation of the mTOR signaling pathway in skeletal muscle they observed a lower activation of S6K1,

which acts through rpS6 to enhance protein synthesis, in aged (22 to 26 years) vs mature (7 to 14 years) horses. However, they did not observe a change in the phosphorylation of 4E-BP1. [Matsui and coworkers \(2006\)](#) assessed protein synthesis of skeletal

muscle after exercise by infusing horses with a 10% amino acid mixture (containing 107 mM leucine) immediately postexercise and assessing protein synthesis rates using an isotope dilution method (Matsui et al., 2006). The rate of protein synthesis was greater ($P < 0.05$) for horses infused with the amino acid mix compared to horses infused with saline. However, because the researchers used a mix of amino acids it was not possible to attribute the effect observed solely to leucine.

CONCLUSIONS

To the best of our knowledge, this is the first study to examine leucine-stimulated translation initiation in cultured equine satellite cells. The present study revealed the activation of downstream effectors of mTOR, 4E-BP1 and rS6, in response to near physiological levels of leucine treatment in equine skeletal muscle in vitro. Leucine treatment also increased protein synthesis in cultured equine satellite cells as measured by radioactive ^3H Phe and non-radioactive puromycin incorporation. These results show that leucine stimulates protein synthesis in equine skeletal muscle through activation of translation initiation factors S6 and 4E-BP1, downstream of mTOR. Activation of the same initiation factors were completely blocked by rapamycin, an mTOR inhibitor. Additionally, mTOR activation was inhibited by rapamycin in the presence of leucine. Use of the cell culture system with primary equine satellite muscle cells provided the opportunity to distinguish the impact of leucine on muscle and protein synthesis, independent of systemic interactions.

Conflict of interest statement. None declared.

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