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Effect of gluteus medius muscle sample collection depth on postprandial mammalian target of rapamycin signaling in mature Thoroughbred mares

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Abstract

Objective—To determine the effect of biopsy collection depth on the postprandial activation of mammalian target of rapamycin (mTOR) signaling factors, particularly protein kinase B, ribosomal protein S6 kinase, ribosomal protein S6, and eukaryotic initiation factor 4E binding protein 1 in middle-aged horses.

Animals—6 healthy Thoroughbred mares (mean $\pm$ SD age, 13.4 $\pm$ 3.4 years).

Procedures—Horses were fed a high-protein feed at 3 g/kg. Sixty minutes after horses were fed, the percutaneous needle biopsy technique was used to collect biopsy specimens from the gluteus medius muscle at 6, 8, and 10 cm below the surface of the skin. Muscle specimens were analyzed for the activation of upstream and downstream mTOR signaling factors, myosin heavy chain (MHC) isoform composition, and amino acid concentrations.

Results—A 21% increase in MHC IIA isoform expression and a 21% decrease in MHC IIX isoform expression were identified as biopsy depth increased from 8 to 10 cm below the surface of the skin; however, no significant change was evident in the degree of MHC I expression with muscle depth. Biopsy depth had no significant effect on the phosphorylation of any of the mTOR signaling factors evaluated.

Conclusions and Clinical Relevance—Postprandial mTOR signaling could be compared between middle-aged horses when biopsy specimens were collected between 6 and 10 cm below the surface of the skin. Optimization of muscle biopsy techniques for evaluating mTOR signaling in horses will facilitate the design of future investigations into the factors that regulate muscle mass in horses.

Collection of percutaneous muscle biopsy specimens from the gluteus medius muscle is commonly used to investigate skeletal muscle physiology and metabolism in horses.^{1–10} However, no standardized depth has been established for sample collection, and typical

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gluteus medius biopsy depths range from 1 to 9 cm below the muscle fascia.^{2,3} The gluteus medius muscle in horses is composed of mixed fiber types, where MHC isoforms vary with depth.^{2,3,11,12} Although the proportions of various fiber types in the gluteus medius muscle vary with breed of horse,¹³ in general, there is an increase in type I fibers and a decrease in type II fibers in the deep versus superficial aspect of the gluteus medius muscle in Thoroughbreds,^{2,3} stock-type horses,^{2,11} and Andalusians.¹²

The depth-related differences in muscle MHC content have been associated with differences in various metabolic properties of the muscle,¹⁴ including activities of enzymes involved in aerobic and anaerobic energy production pathways.^{2,3} The increase in type I fibers with increasing muscle depth has also been associated with an increase in capillary density,¹¹ which aids in the delivery of oxygen and substrates to these oxidative fibers. Therefore, a need exists to determine whether biopsy depth influences other metabolic properties, such as protein metabolism.

In an athletic species, such as horses, development and maintenance of muscle mass are important. Protein is the largest nonwater component of skeletal muscle in horses¹⁵; therefore, skeletal muscle mass is ultimately determined by the balance between the rates of muscle protein synthesis and breakdown.¹⁶ Muscle protein synthesis is regulated through the mTOR-mTORC1 signaling pathway. The upstream regulators of mTOR signaling include insulin (which acts through Akt), amino acids, and exercise, which activate mTOR through independent pathways.^{17,18} Once activated, mTOR is then able to phosphorylate its downstream targets, including 4EBP1 and S6K1. Ribosomal protein S6 kinase subsequently phosphorylates rpS6. Increases in mTOR signaling are associated with an increase in muscle protein synthesis rates,^{19,20} provided that sufficient amino acids are present.²¹

Although little research has been conducted regarding the regulation of mTOR signaling in equine skeletal muscle, research performed by our group has shown that mTOR signaling in that muscle type increases after feeding in growing⁴ and mature^{4,5} horses and that the magnitude of the signaling response to feeding is greater in yearlings than in 2-year-old horses.⁴ In 2 previous studies of mTOR signaling in horses, biopsy specimens of the gluteus medius muscle were obtained from a standardized depth of 6 cm⁵ or at 50% of the muscle's depth,⁴ but muscle fiber types at the site of biopsy were not characterized in either study. Research evidence suggests that variation in muscle fiber type can alter the degree of mTOR signaling activation in response to anabolic stimuli (exercise in rodents^{22,23} and humans^{24,25} and feeding in piglets²⁶), with type II fibers being more responsive to exercise and feeding stimuli. Because of the role of mTOR signaling in the development and maintenance of muscle mass, there is a need to understand the factors that regulate this signaling pathway in horses. Through determination of whether mTOR signaling in response to the anabolic stimuli of feeding varies with sampling depth in the equine gluteus medius, valuable information can be obtained for use in the design of future studies of mTOR signaling in horses.

The objective of the study reported here was to determine whether biopsy depth would affect the postprandial activation of mTOR signaling in the gluteus medius muscle of middle-aged horses. We hypothesized that differences would be evident in the degree of

mTOR signaling activation (ie, amount of phosphorylation of the mTOR signaling proteins) because of variations in fiber type composition as biopsy depth varied from 6 to 10 cm below the surface of the skin.

Materials and Methods

Animals

Six healthy Thoroughbred mares (mean $\pm$ SD age, 13.4 ± 3.4 years; mean body weight, 536 ± 45 kg) of moderate body condition (body condition score, 5/9 to 7/9²⁷) were used in the study. Mares were housed in partially covered dry lot pens measuring 3×15 m, with crushed limestone provided for footing. Free access to water and salt was provided. Diets were designed to meet all nutrient requirements of idle adult horses,²⁸ and all feeds were analyzed at a commercial laboratory.^a Horses were allowed to acclimate to the diet and housing for 1 week before muscles biopsies were obtained. All procedures used in the study were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Experimental procedures

Individual meals were provided twice daily (8 AM and 3 PM). Diets consisted of alfalfa hay cubes (mean $\pm$ SD digestible energy, 9.84 ± 0.12 MJ/kg; crude protein, $17.0 \pm 0.2\%$; acid detergent fiber, $35.2 \pm 0.8\%$; neutral detergent fiber, $44.9 \pm 1.3\%$; crude fat, $2.3 \pm 0.1\%$; and ash, $9.5 \pm 0.2\%$) and a ration balancer (digestible energy, 9.79 ± 0.04 MJ/kg; crude protein, $14.9 \pm 0.2\%$; acid detergent fiber, $22.5 \pm 0.8\%$; neutral detergent fiber, $43.2 \pm 0.4\%$; crude fat, $3.6 \pm 0.1\%$; and ash, $12.9 \pm 0.1\%$)^b provided at a rate of 1.75% and 0.2% of body weight/d, respectively.

On the day prior to obtaining muscle biopsy specimens, horses were fed their afternoon meals of hay cubes and ration balancer at 3 PM and all horses were finished eating by 5 PM. On the morning of obtaining muscle biopsy specimens (approx 15 hours after feeding), horses were weighed on an electronic scale^c and then fed a high-protein pellet at 3 g/kg of body weight (digestible energy, 13.02 ± 0.12 MJ/kg; crude protein, $36.3 \pm 0.7\%$; acid detergent fiber, $6.8 \pm 0.3\%$; neutral detergent fiber, $13.8 \pm 0.8\%$; crude fat, $3.9 \pm 0.1\%$; and ash, $17.2 \pm 0.3\%$).^d This time point was designated 0 minutes. Horses were not offered hay cubes during this time. The amino acid composition of the high-protein pellet has been reported elsewhere.⁵ A feeding protocol similar to the one used in the present study resulted in a 4-fold increase in plasma insulin concentration, an approximately 25% to 125% increase in plasma concentrations of indispensable (essential) amino acids, and an increase in the degree of upstream and downstream mTOR signaling in the gluteus medius muscle in the postprandial versus postabsorptive state.^{4,5}

^aDairy One Forage Laboratory, Ithaca, NY.

^bTriple Crown Lite, Southern States Cooperative Inc, Richmond, Va.

^cTI-500, Transcell Technology Inc, Buffalo Grove, Ill.

^dTriple Crown 30% Performance Supplement, Southern States Cooperative Inc, Richmond, Va.

At 60 minutes, each horse was lightly sedated with xylazine hydrochloride (0.3 mg/kg, IV) and positioned in a set of equine stocks.^e The biopsy site was standardized as the point of intersection of 2 imaginary lines: one between the tuber sacrale and the tuber ischii and the other between the tuber coxae and the tail head. The site was aseptically scrubbed and a local anesthetic (12 mL of 2% lidocaine solution) was injected into the area. Muscle biopsy specimens (approx 400 to 500 mg each) were collected via an incision in the gluteus medius muscle at depths of 6, 8, and 10 cm below the surface of the skin with a custom-made Bergstrom needle (length, 18 cm; depth, 6 mm), as described elsewhere.¹ The cutting window of the Bergstrom needle was 1.4 cm in length, so muscle specimens were actually collected from 4.6 to 6 cm, 6.6 to 8 cm, and 8.6 to 10 cm below the surface of the skin for the 6-, 8-, and 10-cm biopsy depths, respectively. The deepest 100 mg of each sample was immediately processed for Western blot analysis, and the remaining muscle tissue was frozen in liquid nitrogen and stored at -80°C until analysis for amino acid concentrations and MHC isoforms. Phenylbutazone (1 g, PO, q 12 h, for 48 hours) was administered to each horse after biopsy specimen collection to alleviate any potential discomfort associated with the procedure.

Amino acids analysis

Concentrations of free amino acids in muscle specimens were measured through reverse-phase high-performance liquid chromatography^f of phenyl isothiocyanate derivatives as described.⁵

Muscle homogenate preparation for Western blot analysis

A portion (approx 100 mg) of each biopsy specimen was homogenized at 7 $\mu\text{L}/\text{mg}$ wet tissue weight in buffer solution (20mM HEPES, 2mM ethylene glycol tetraacetic acid, 50mM NaF, 100mM KCl, 0.2mM EDTA, and 50mM β -glycerophosphate [pH, 7.4]), containing 20 μL of protease inhibitor^g/mL. After centrifugation of homogenates at $10,000 \times g$ for 10 minutes at 4°C , supernatant was harvested and frozen in aliquots at -80°C until subsequent analysis.

Prior to performance of Western blot analysis, the protein concentration of the homogenates was measured with a Bradford assay kit.^h Fifty microliters of supernatant was added to 25 μL of 3 $\times$ Laemmli buffer (125mM Tris hydrochloride [pH, 6.8], 4% [wt/vol] SDS, 20% [vol/vol] glycerol, 100mM dithiothreitol, and 0.01% [wt/vol] bromophenol blue), and then 1 $\times$ Laemmli buffer was added to each sample mixture to yield a protein concentration of 2 $\mu\text{g}/\mu\text{L}$. Each sample mixture was boiled for 5 minutes and placed on ice prior to gel electrophoresis.

Western blot analysis

The amount of each of 4EBP1 (total and phosphorylated at Thr^{37/46}), Akt (total and phosphorylated at Ser⁴⁷³), S6K1 (total and phosphorylated at Thr³⁸⁹), and rpS6 (total and

^eHS equine stocks, Priefert Rodeo & Ranch Equipment, Mount Pleasant, Tex.

^f3.9 $\times$ 300-mm PICO-TAG reverse-phase column, Waters Corp, Milford, Mass.

^gSigma P8340, Sigma Aldrich Corp, St Louis, Mo.

^hThermo Scientific, Rockford, Ill.

phosphorylated at Ser^{235/236} and Ser^{240/244}) in biopsy specimens of the gluteus medius muscle was determined with a reported Western blot protocol.⁴ Briefly, proteins were separated in electrophoretic gels containing 8% acrylamide-N,N'-methylene-bisacrylamide (29:1; Akt, S6K1, and rpS6) or 12% acrylamide-N,N'-methylene-bisacrylamide (19:1; 4EBP1) via electrophoresis. Samples were standardized by the amount of protein loaded per well for electrophoresis (20 µg of protein was loaded for Akt, rpS6, and 4EBP1, and 35 µg of protein was loaded for S6K1). A positive control cell extract (calyculin A-treated Jurkat cells)ⁱ was also loaded on each gel.

After electrophoresis, proteins were transferred to 0.45-µm polyvinylidene difluoride membranes. Individual rabbit polyclonal antibodiesⁱ were applied that recognized total Akt and Akt at Ser⁴⁷³, (1:2,000 dilutions); total S6K1 (1:1,000 dilution) and S6K1 at Thr³⁸⁹ (1:500 dilution); and rpS6 at Ser^{235/236} and Ser^{240/244} (1:2,000 dilutions). Rabbit monoclonal antibodiesⁱ specific to total 4EBP1 and 4EBP1 at Thr^{37/46} (1:1,000 dilutions) and total rpS6 (1:10,000 dilution) were also used. The secondary antibody used was a goat anti-rabbit IgG (heavy and light chains) with conjugated horseradish peroxidase (1:10,000 dilution).^j Membranes were developed with a chemiluminescence kit,^k and patterns were made visible on x-ray film through the use of a film processor.^l Band densities were quantified as the mean band intensity multiplied by the number of pixels with a computer software program.^m

Electrophoretic gels were assayed in duplicate, and the same membrane was used to probe for the total and phosphorylated forms of the proteins with different primary antibodies by stripping the membrane following phosphorylated protein measurement and then incubating and reprobing the membrane for the total protein.⁴

Myofibrillar protein preparation

The samples of muscle tissue that were flash-frozen at the time of obtaining muscle biopsy specimens were pulverized prior to myofibrillar protein analysis. After tissues were thawing on dry ice, the muscle that was flash-frozen was divided into (approx 50-mg) portions and homogenized with a manual Dounce homogenizer in 9 µL/mg tissue wet weight of buffer solution (250mM sucrose, 25mM NaCl, and 20mM Tris [pH, 7.4]). Following centrifugation (20,000 × g for 30 minutes at 4°C), the supernatant was removed and the pellet resuspended in homogenizing buffer (250 µL). After determination of protein concentrations,^h 40 µL of each sample was added to 20 µL of 3× Laemmli buffer (0.15M Tris [pH, 6.8], 6% [wt/vol] SDS, 75mM dithiothreitol, 0.06% [wt/vol] bromophenol blue, and 40% [wt/vol] glycerol). An amount of 1× Laemmli buffer was added to each sample mixture to yield a protein concentration of 0.2 µg/µL. The sample mixture was boiled for 2 minutes and immediately placed on ice prior to gel electrophoresis.

ⁱCell Signaling Technology Inc, Boston, Mass.

^jSilver Stain Plus, BioRad, Hercules, Calif.

^kAmersham ECL Plus western blotting detection reagents, GE Healthcare, Piscataway, NJ.

^lKodak X-OMAT film processor, Kodak Health Imaging Division, Rochester, NY.

^mAdobe Photoshop elements, version 8.0, Alpha Innotech, San Leandro, Calif.

Separation of MHC isoforms via electrophoresis

The percentage of MHC isoform in the gluteus medius muscle was determined with electrophoresis, followed by silver staining similar to described methods.²⁹ In short, myofibrillar proteins in the portions of muscle biopsy specimens that had been processed and stored in Laemmli buffer were separated via electrophoresis in gels containing 30% glycerol and 8% acrylamide-N,N'-methylene-bisacrylamide (50:1). Samples were standardized by the amount of protein loaded per well for electrophoresis (2 µg of myofibrillar proteins was loaded), and specimens of mouse gastrocnemius muscle served as a control sample on each gel. Gels were run at 4°C for 40 hours with a constant voltage of 70 V and then stained with a kit^l in accordance with the manufacturer's directions. Band densities were quantified as the mean band intensity multiplied by the number of pixels with a computer software program.^m

Statistical analysis

Amounts of mTOR signaling proteins are reported as a ratio of the phosphorylated to total forms for each protein (arbitrary units), with the ratios for each protein at the 6-cm depth set to 1. To correct for intergel differences in electrophoresis, blotting, and x-ray development conditions, prior to calculating the ratios, band densities were first standardized to the band density of the control sample that was run on each gel. Proportions of MHC isoforms are reported as percentages of total MHC.

Statistical softwareⁿ was used to perform repeated-measures ANOVA, with depth and block as the fixed effects and horse nested in block as the subject. The lowest value of the Schwarz Bayesian criterion was used to select the variance-covariance matrix for use in the model. When the fixed effect of depth was significant for a variable, all pairwise comparisons of the 6-, 8-, and 10-cm least squares means were made via a Tukey test with statistical software.^o Results are reported as least squares mean ± pooled SEM. Values of $P < 0.05$ were considered significant.

Results

All horses consumed the entire high-protein meal within 30 minutes after feeding. A significant ($P < 0.05$) effect of biopsy depth within the gluteus medius muscle was identified on muscle concentrations of free alanine, aspartate, glutamate, isoleucine, leucine, methionine, proline, and valine (Table 1). Biopsy depth had no effect on ratios for phosphorylation of Akt at Ser⁴⁷³ ($P = 0.06$), S6K1 at Thr³⁸⁹ ($P = 0.66$), rpS6 at Ser^{235/236} and Ser^{240/244} ($P = 0.21$), and 4EBP1 at Thr^{37/46} ($P = 0.62$) 60 minutes after the high-protein pellet was consumed (Figure 1). Biopsy depth also had no impact on total amounts of any of the proteins evaluated ($P > 0.05$).

Myosin heavy chain analysis revealed a significant effect of biopsy depth on the percentage of MHC IIA ($P = 0.03$) and IIX ($P = 0.02$) isoforms in muscle specimens (Figure 2). However, there was no effect ($P = 0.42$) of biopsy depth on the percentage of MHC I

ⁿProc MIXED, SAS, version 9.2, SAS Institute Inc, Cary, NC.

^oPDIF option in the LSMEANS statement, SAS, version 9.2, SAS Institute Inc, Cary, NC.

isoforms. The percentage of MHC IIA and IIX isoforms increased by 21% and decreased by 21%, respectively, as the biopsy depth increased from 8 to 10 cm. Individual horses differed in the actual percentage of each fiber type at each depth, as shown by SDs that were 10.6% at 6 cm, 8.6% at 8 cm, and 9.1% at 10 cm for MHC IIA; 9.5% at 6 cm, 9.1% at 8 cm, and 9.8% at 10 cm for MHC IIX; and 9.8% at 6 cm, 7.6% at 8 cm, and 6.0% at 10 cm for MHC I.

Discussion

The gluteus medius muscle was selected for use in the present study because it is the largest muscle of horses' hind limbs by mass,³⁰ it plays a key role in locomotion as a hip joint extensor and abductor, and biopsy samples are commonly obtained from this muscle in studies^{6–10} of muscle physiologic and metabolic properties in horses. Furthermore, changes in muscle fiber composition with biopsy depth have been extensively characterized in the gluteus medius muscle of various breeds of horses,^{2,3,11,12} and these depth-related changes have been associated with changes in the activities of enzymes involved in energy production in the skeletal muscle, with glycolytic (type IIX) fiber abundance being associated with lactate dehydrogenase activity and oxidative (type I and type IIA) fiber abundance being associated with citrate synthase activity.^{2,3}

The gluteus medius muscle is also the only muscle that has been evaluated with regard to mTOR signaling and its activation in response to feeding in horses.^{4,5} However, to the authors' knowledge, ours is the first study to examine the effects of gluteus medius muscle biopsy depth on the degree of mTOR signaling activation in fed horses.

No effect of biopsy depth was identified on the activation of downstream mTOR signaling 60 minutes after horses were fed, despite changes in proportions of MHC isoforms in the specimens. Therefore, provided that specimens of gluteus medius muscle are collected between 6 and 10 cm below the skin surface, the degree of mTOR signaling activation can be compared among middle-aged horses in a fed state.

Biopsy depth was determined from the skin surface rather than from the gluteal muscle fascia, as has been done in other studies^{2,11,12} of the effects of gluteus medius depth on fiber type composition and muscle metabolism. Although we did not measure rump fat depth, in other studies^{4,31} involving adult horses of a similar size to our horses, rump fat depth was approximately 2 cm. One can therefore infer that biopsy specimens were obtained 4, 6, and 8 cm below the muscle fascia, which is consistent with the muscle depths used in the previous studies.^{2,11,12} In another study,² the gluteus medius muscle was removed from stock horses (mean weight, 365 kg) after slaughter and its depth was measured to be approximately 8 cm. The Thoroughbred mares of the present study weighed almost 50% more than the stock horses did,² so the total depth of the gluteus medius muscle of the Thoroughbreds likely exceeded 8 cm. It follows that the 3 biopsy depths that we used represented locations from approximately one-third to one-half of the muscle depth (6-cm sample) to the near maximal depth (10-cm sample) of the gluteus medius muscle.

Extensive variation in proportions of fiber types in the gluteus medius muscle exists among breeds of horses.¹³ Previous measures of gluteus medius fiber type composition in Thoroughbreds revealed 0% to 35% type I fibers, 26% to 61% type IIA fibers, and 10% to 79% type IIX fibers in the gluteus medius muscle, depending on biopsy depth.^{3,13,32} In the Thoroughbred mares of the present study, the percentage of the various fiber types at each depth were near the midpoints the aforementioned ranges.^{3,13,32} Previous research has also shown that although the degree of intrasubject variation can be minimized through standardization of biopsy sites,^{32,33} a high degree of intersubject variation exists in fiber type composition at any given biopsy site or depth.^{2,3,33} We also found that individual horses of the same breed differed in the actual percentage of each fiber type at each biopsy depth.

Despite the high intersubject variation in the proportions of MHC isoforms at each depth, all horses had the same general response to increasing biopsy depth: as the depth of the biopsy increased from 6 to 10 cm below the surface of the skin, proportions of MHC IIA, MHC IIX, and MHC I isoforms in muscle tissues increased, decreased, and remained unchanged, respectively. The differences in proportions of MHC IIA and MHC IIX fiber types were consistent with previous reports,^{2,3,11,12} in which percentages of MHC IIA and MHC IIX isoforms (or percentage of type IIA and IIX fibers, respectively) increased and decreased, respectively, from the superficial to deep aspect of the gluteus medius muscle in horses. However, unlike in those reports,^{2,3,11,12} no significant increase in the percentage of MHC I isoform was identified with increasing biopsy depth in the present study. This lack of an increase could have been related to the biopsy protocol used. The greatest increase in the percentage of slow oxidative fibers (equivalent to MHC I) in another study² was identified in the deepest portion of the gluteus medius muscle. Because the deepest biopsy site in our study may not have been at the maximal muscle depth, any dramatic changes in the proportion of MHC I isoforms might have been overlooked.

Alternatively, the study¹² that showed the largest increase in the proportion of MHC I isoform with increasing muscle depth involved Andalusians, which appear to have had a much higher amount of MHC I in the deep region of the gluteus medius muscle (59% to 70% of total MHC quantity¹²) than was reported for Thoroughbreds at a comparable depth (0% to 35% of total muscle fibers as type I fibers³). The lack of a significant increase in MHC I proportion with increasing muscle depth in the present study might have been attributable to these breed differences. Another possible explanation relates to the actual site within the muscle where the biopsy was performed. Many external landmarks have been used to standardize the biopsy site of the gluteus medius in horses, including one-third to two-thirds of the distance between the tuber coxae and the tailhead,^{3,34} 10 to 25 cm caudodorsal from the tuber coxae,^{1,12,32} and in the region between the tuber sacrale and tuber coxae.^{34,35} A previous study³⁴ revealed variation in the MHC composition of the gluteus medius muscle, even at a constant depth, with biopsy specimens obtained from the muscle region.

Although changes in MHC isoform content were identified with increasing biopsy depth, no significant effect of biopsy depth was evident on the degree of activation of any of the mTOR signaling factors evaluated, even at the 8-cm depth where high muscle

concentrations of the branched chain amino acids, which stimulate mTOR signaling in other species,^{20,36–38} was identified. Generally, studies^{20,36–38} in which a beneficial effect on muscle mTOR signaling activation was identified for dietary supplementation with leucine or branched chain amino acids involved a rate of supplementation that resulted in at least a doubling of plasma leucine concentration. Although plasma amino acid concentrations were not measured in our study, previous studies^{4,5} involving similar horse feeding protocols showed that plasma branched chain amino acid concentrations were only 24% to 45% higher than preprandial concentrations 60 to 80 minutes after feeding. The muscle branched chain amino acid concentrations at the 8-cm biopsy depth were significantly greater than those at the other depths in the present study, but the 25% to 33% increase in their concentrations did not appear large enough to elicit an increase in the mTOR signaling response.

The present study is not the first to involve investigation of the association between muscle fiber type composition and mTOR signaling response to anabolic stimuli. In rodents, the phosphorylation of mTOR²² and S6K1²³ varies across different muscles in response to contractile activity, and different fiber types (type II vs type I) may be the cause for the greater mTOR signaling response in the predominantly type II muscle. Similarly, in humans, type II muscle fibers have a greater activation of S6K1 immediately following²⁵ and 1 hour²⁴ and 2 hours^{24,25} after resistance exercise than do type I fibers; however, the degree of Akt and mTOR phosphorylation in response to exercise appears unaffected by fiber type,²⁴ suggesting that the fiber-type specific increase in S6K1 activation is through an Akt- or mTOR-independent mechanism. In the present study, feeding rather than exercise was used to stimulate mTOR-related signaling. Activation of mTOR signaling and fractional muscle protein synthesis in piglets do not appear to be dependent on fiber type.²⁶ Rather, the muscle group with the highest proportion of fast twitch (type II) fibers has the greatest degree of mTOR signaling and protein synthesis after feeding. In human skeletal muscle; however, no correlation exists between muscle fiber type proportions and muscle fractional protein synthesis rates at rest before or following an infusion of an amino acid solution.³⁹ The relationship among muscle fiber types, mTOR signaling, and rates of muscle protein synthesis is clearly an area where additional research is warranted.

In the present study, although significant differences in proportions of MHC IIA and MHC IIX isoforms were evident with biopsy depth, the proportion of total MHC II isoform was similar across depths (87%, 89%, and 90% at 6, 8, and 10 cm, respectively), which may explain the lack of an effect of depth on downstream mTOR signaling in fed horses. The previous studies^{22–25} that found effects of fiber type composition on the degree of mTOR signaling did not differentiate between among type II fiber classes; therefore, our results are in general agreement with the previous findings.

Another factor that has been associated with variations in muscle fiber type and biopsy depth in horses¹¹ and that affects mTOR signaling in other species⁴⁰ is the amount of blood flow to the muscles, which would affect the delivery of amino acids and insulin to muscle tissue. Although muscle capillarity was not characterized in the present study, a previous study¹¹ that predominantly involved Thoroughbreds showed that the differences in muscle capillarity with biopsy depth were present at shallow gluteus medius depths (from 2 to 4 cm). However, in the range of muscle biopsy depths in our study (approx 4 to 8 cm below

the fascia), we also found no further changes in capillarity with increasing depth, providing additional support for the lack of differences in postprandial mTOR signaling among the depths of muscle from which biopsy specimens were obtained.

If the amount of anabolic stimulation (meal size or composition) or timing of the biopsy after feeding was inappropriate, then we may not have measured the maximal mTOR signaling response and may not have been able to detect depth-related differences in mTOR signaling activation. Although other studies^{4,5} involved obtaining muscle biopsy samples 90 minutes after feeding in horses, a study²⁶ involving piglets found that mTOR signaling and fractional synthesis rates in various types of skeletal muscle are activated from as early as 30 to 60 minutes after feeding. A feeding protocol similar to the one we used resulted in plasma glucose, insulin, and amino acid concentrations that were significantly higher than preprandial values from 40 to 60 minutes after feeding in another equine study.⁵ Therefore, it appears unlikely that the feeding protocol or biopsy timing contributed to the absence of depth-related differences in mTOR activation.

Another factor that may have affected the responsiveness of mTOR signaling in the gluteus medius muscle in the study horses was horse age, which was approximately 13 years. Although feeding activates mTOR signaling in the gluteus medius muscle of horses of various ages,^{4,5} the degree of activation is greater in yearlings than in 2 year olds,⁴ showing an age-related decline in the responsiveness of mTOR signaling to feeding. Because muscle mTOR signaling appears to be more sensitive to feeding in young horses, the amount of feeding stimulation in the present study may not have been sufficient to elicit detectable differences in the activation of mTOR signaling with muscle depth in middle-aged horses. Had younger horses been used, depth-related signaling differences may have been observed.

Although additional research is necessary to determine whether biopsy depth has a significant effect on the degree of mTOR signaling in response to anabolic stimuli other than feeding such as exercise or hormone administration and whether similar responses occur in other major muscle groups, the present study provided valuable information regarding the optimization of biopsy procedures for evaluating the regulation of mTOR signaling in the gluteus medius muscle of horses after feeding. Further knowledge of the factors that regulate muscle protein synthesis will allow for the design of management and feeding strategies to promote the development and maintenance of muscle mass in horses of all ages.

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Abbreviations

4EBP1 Eukaryotic initiation factor 4E binding protein 1

Akt	Protein kinase B
MHC	Myosin heavy chain
mTOR	Mammalian target of rapamycin
rpS6	Ribosomal protein S6
S6K1	Ribosomal protein S6 kinase

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Figure 1.

Least squares mean $\pm$ pooled SEM amount of muscle tissue phosphorylation of Akt at Ser⁴⁷³, S6K1 at Thr³⁸⁹, rpS6 at Ser^{235/236} and Ser^{240/244}, and 4EBP1 at Thr^{37/46} in biopsy specimens of the gluteus medius muscle obtained 6 (white bars), 8 (black bars), and 10 (gray bars) cm below the surface of the skin from six 9- to 19-year-old mares, 60 minutes after they were fed a high-protein pelleted feed (3 g/kg). Amounts of phosphorylated forms of the translation initiation factors were corrected through division by the total form amounts, with the value for the 6 cm depth set at 1.0 arbitrary units. One tissue sample from each horse was evaluated at each depth.

Figure 2.

Least squares mean $\pm$ pooled SEM percentages of MHC isoforms in the biopsy specimens of gluteus medius muscle described in Figure 1. ^{a,b}Different letters indicate a significant ($P < 0.05$) difference between depths for a given isoform. See Figure 1 for remainder of key.

Table 1

Least squares mean muscle amino acid concentrations (mmol/g of wet muscle tissue) in biopsy specimens of gluteus medius muscle obtained 6, 8, and 10 cm below the skin in 6 adult mares.

Amino acid	6 cm	8 cm	10 cm	Pooled SEM	P value
Alanine	697 ^a	944 ^b	744 ^{a,b}	102	0.05
Asparagine	60	60	59	6	0.97
Aspartate	70 ^a	94 ^b	68 ^a	9	0.02
Citrulline	41	50	46	6	0.48
Glutamate	305 ^a	442 ^b	390 ^{a,b}	49	0.03
Glutamine	246	303	257	35	0.46
Glycine	664	471	664	133	0.50
Histidine	58	68	53	8	0.24
Isoleucine	55 ^a	69 ^b	56 ^a	4	0.004
Leucine	105 ^a	135 ^b	111 ^a	10	0.007
Lysine	296	189	267	128	0.67
Methionine	36 ^a	50 ^b	39 ^a	4	0.004
Ornithine	15	26	17	9	0.59
Phenylalanine	48	64	104	29	0.39
Proline	94 ^a	122 ^b	100 ^a	9	0.004
Serine	144	150	137	12	0.45
Threonine	170	207	173	34	0.69
Tryptophan	18	20	18	2	0.56
Tyrosine	61	104	62	24	0.36
Valine	111 ^a	148 ^b	119 ^a	9	0.005

^{a,b} Values with different superscript letters are significantly ($P < 0.05$) different.