Gene expression of proteolytic systems and growth regulators of skeletal muscle in horses with myopathy associated with pituitary pars intermedia dysfunction

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Objective—To investigate gene expression of the major proteolytic systems and growth regulators in skeletal muscle of horses with myopathy associated with pituitary pars intermedia dysfunction (PPID).

Animals—14 horses with PPID-associated myopathy and 7 healthy control horses.

Procedures—Horses with PPID and controls were age matched (15 to 28 years old). Muscle biopsy specimens were collected from both groups and processed for RNA and cDNA extraction. Validation of the most stable housekeeping genes for skeletal muscle was performed and used to compare gene expression of the following proteolytic systems: cysteine aspartate protease–dependent systems (caspases), lysosomal-dependent systems (cathepsins), non–lysosomal calcium protease–dependent systems (calpains), and ubiquitin–proteasome–dependent systems (ubiquitins). Gene expression of negative regulators of muscle growth (myostatin and inflammatory cytokines interleukin-1β, interleukin-6, and tumor necrosis factor-α) was also determined.

Results—No significant difference between groups was detected in expression of the major proteolytic systems except for m-calpain, which was greater in horses with PPID. No differences in gene expression of myostatin and interleukin-1β, interleukin-6, and tumor necrosis factor-α were detected between groups.

Conclusions and Clinical Relevance—Greater expression of m-calpain may suggest that calpains play an important role in development of muscle atrophy in horses with PPID. However, because posttranslational events may alter protein activation, inactivation, and functions not studied here, other mechanisms of muscle atrophy cannot be excluded. (Am J Vet Res 2010;71:664–670)
atrophy. In addition, glucocorticoids depress glycolysis and induce an insulin-resistant state; this may make glycolytic-dependent myofibers more susceptible to the effects of glucocorticoids. Myonecrosis is not a feature of the myopathy associated with PPID in horses. Decreased muscle mass and strength can result in weakness and decreased physical activity and performance in horses with PPID.

Atrophy of skeletal muscle may be the result of local factors or systemic effects. Muscle atrophy is associated with withholding of food, disuse, ageing, and several pathological conditions. Examples of muscle-wasting conditions in humans include sepsis, endocrinopathy, cancer, cachexia, and critical illnesses. Muscle atrophy can also occur in inherited and acquired myopathies and neuromopathies. Triggering signals of muscle wasting include proinflammatory cytokines (TNF-α, IL-1β, and IL-6) that are potent activators of NFκB, catabolic hormones (myostatin and glucocorticoids), low concentrations of anabolic hormones (insulin and insulin-like growth factor-1), increased cytosolic calcium, and oxidative stress. Oxidative stress and NFκB are important regulators of proteolytic pathways leading to muscle atrophy. Oxidative stress plays an important role in the pathophysiologic mechanisms of PPID in horses.

There are 4 major proteolytic systems in skeletal muscle: cysteine aspartate protease–dependent systems (caspases), lysosomal-dependent systems (cathepsins), non–lysosomal calcium protease–dependent systems (calpains), and ubiquitin–proteasome–dependent systems (ubiquitins). These systems may be downregulated or upregulated by triggering signals. Ultimately, the activation of different signaling pathways will result in increased protein degradation, decreased gene expression, loss of myofibers by apoptosis or necrosis, or inability of satellite cells to counteract the decrease in fiber size and number.

Mechanisms of muscle atrophy have not been studied in horses and may differ depending on specific pathological disorders. The purpose of the study reported here was to investigate gene expression of the major proteolytic systems and triggering factors in skeletal muscle of horses with myopathy associated with PPID to gain understanding of the mechanisms of muscle wasting in diseased horses.

Materials and Methods

Control horses—Seven healthy horses from the University of California-Davis research herd that had been evaluated by use of an overnight dexamethasone suppression test or had plasma endogenous ACTH concentrations within reference ranges, tested during winter and spring months, were selected as controls. Horses were of Thoroughbred (n = 4) and Quarter Horse (3) breeds. Their age was matched to that of horses with PPID and ranged from 15 to 28 years with a median age of 20 years. Four horses were females and 3 were geldings.

PPID-affected horses—Fourteen horses referred to our institution with a clinical diagnosis of PPID and abnormal results of an overnight dexamethasone suppression test or abnormal plasma endogenous ACTH concentrations, tested during the winter and spring months, were selected for the study. These horses were used in a previous study and euthanized because of complications of PPID and severe degenerative joint disease, and their PPID status was further confirmed upon histopathologic evaluation of the pituitary gland. Horses were of Quarter Horse (n = 5), Thoroughbred (4), Standardbred (2), Arabian (2), and Appaloosa (1) breeds. The median age was 21 years with a range from 15 to 28 years of age. Eleven horses were females and 3 were geldings. The duration of the observed muscle atrophy in diseased horses ranged from a few months to 3 years. The study was performed according to guidelines of the University of California-Davis Institutional Animal Care and Use Committee.

Skeletal muscle biopsy—Muscle biopsy specimens were collected from the gluteus medius muscle of all control and PPID-affected horses. Horses were placed in stocks and sedated with 10% xylazine hydrochloride administered at 0.3 mg/kg, IV. Following subcutaneous local anesthesia with 2% lidocaine hydrochloride, a 1-cm incision through the skin and subcutaneous tissue was performed at a standardized site 20 cm along a line from the most dorsal aspect of the tuber coxae to the head of the tail. A 6-mm-diameter modified Bergström biopsy needle was inserted 6 cm deep below the gluteal fascia to collect a minimum of 300 mg of muscle. Two-thirds of the specimen was processed for histochemical and ultrastructural analysis to determine whether myopathic alterations were present for both groups of horses, as reported. The remaining third of the specimen was immediately frozen in liquid nitrogen and stored at −80°C in the Neuromuscular Disease Laboratory muscle bank until further processing. At the end of the biopsy collection, the surgical site was closed with absorbable suture by use of a single interrupted cruciate pattern. The horses were monitored after sample collection for the presence of inflammation or drainage from the surgical site. No antimicrobials were administered.

Muscle RNA and cDNA extraction—Frozen muscle samples in liquid nitrogen were pulverized by use of a mortar and pestle. The RNA was isolated by use of a commercial kit and quantified by use of a spectrophotometer. To avoid the presence of genomic DNA, the sample was passed through a genomic DNA elimination column after tissue disruption and homogenization. Furthermore, RNA was treated with DNase I following its extraction. To confirm the absence of genomic DNA, a non–reverse transcriptase PCR procedure that used primers encoding a single exon for a housekeeping gene was performed. The RNA was then transformed to cDNA by use of a reverse transcriptase assay according to manufacturer’s specifications.

Reference genes for skeletal muscle—To select the most stable reference genes for equine skeletal muscle, housekeeping genes belonging to different functional classes to avoid coregulation were studied. Primers for 5 equine housekeeping genes that have been published were included: GAPDH, S-9 ribosomal region, actin, HRPT1, and TUBA1. Equine-specific primers
for the housekeeping gene B2M were generated from equine cDNA sequence (GenBank accession No. NM_001082502). Consensus primers derived from various species (human [NM_004168], mouse [NM_023281], and rat [NM_174178]) were used to amplify 306 bp of the SDHA gene. Equine-specific primers were designed following sequencing of the PCR product.

The efficiencies of the 7 housekeeping genes were calculated by performing PCR assays by use of serial dilutions of cDNA from normal equine liver, which has high expression for the housekeeping genes under study. Resulting crossing point cycles (ie, Cp values) were plotted against the logarithm of the cDNA concentration, and a regression line was calculated. A slope for the reactions was obtained, and amplification efficiencies were calculated by use of the following equation: efficiency = \((10^{\text{slope}}) - 1\).

Real-time PCR assays were performed by use of a real-time detection system by detecting SYBR-green I dye intercalation. Reaction samples had a final volume of 20 µL, containing 10 µL SYBR-green I master mix, 0.5 µM of each primer, 0.5 U of uracyl glycosylase, 1 µL of cDNA, and 7.5 µL of water. The amplification conditions were 37°C for 10 minutes, 95°C for 5 minutes, and 45 cycles of 95°C for 5 seconds, 55°C for 10 seconds, and 72°C for 6 seconds. To ensure specificity of amplifications and detect primer-dimer formation, dissociation curves of melting temperatures were created and evaluated by heating the samples from 65° to 93°C in 0.5°C increments with a dwell time at each temperature of 10 seconds with continuous monitoring of fluorescence. All samples were run in triplicate, and nontemplate negative controls were included. To determine the stability of reference genes, PCRs were performed with the 7 housekeeping genes in cDNA from 2 control and 2 PPID-affected horses. The most stable genes were selected by use of commercial software as described. The software calculates the gene expression stability measure (M) for a reference gene as the mean pairwise variation (V) for that gene with all other tested reference genes. Stepwise exclusion of the gene with the highest M value allows ranking of the tested genes according to their expression stability. From the 2 most stable housekeeping genes, the one with the lowest Cp was selected as the reference gene for the study.

Real-time quantitative PCR assays—Muscle biopsy specimens from 21 horses (7 controls and 14 PPID-affected horses) were collected and processed as described. Potential genes involved in different pathways of muscle wasting were selected and included genes for caspase-3, cathepsin-D, m-calpain, UBC, myostatin, TNF-α, IL-6, and IL-1β. Horse-specific primers were designed from reported equine sequences (caspase-3, GenBank accession No. DQ174688; cathepsin-D, XM001493021; UBC, AF506969; and myostatin, AB033541). Consensus primers were designed to amplify 291 bp of equine m-calpain gene from conserved regions from human, rat, and sheep m-calpain gene (GenBank accession Nos. NM_001748.4, NM_009794, and NM_001112817, respectively). Specific primers were then designed to amplify a segment of the equine m-calpain gene. Published primers from proinflammatory cytokines TNF-α, IL-6, and IL-1β were also used. Efficiencies were calculated as described by use of tissue with high expression for each specific gene from our cDNA library as follows. Tissues included liver for caspase-3, cathepsin-D, and m-calpain; muscle for UBC and myostatin; and blood from a research horse that received an infusion of lipopolysaccharides (O55:B5 Escherichia coli [30 ng/kg]) over 30 minutes to induce expression of proinflammatory cytokines. After the infusion, blood was collected at various intervals. This horse was part of a concurrent research project that revealed increased expression of all inflammatory cytokines that peaked at 60 minutes. A blood sample was collected from a jugular vein catheter directly into a specialized RNA collection vacuum tube at 60 minutes following lipopolysaccharide infusion and then frozen at −80°C until further processing. The PCR assays were performed as described, and the specificity of the reactions was monitored by analysis of the melting curves, sepration of PCR products on a 2% agarose gel, and sequencing the PCR products. Samples were run in triplicate and in the presence of a nontemplate negative control and a calibrator. Relative quantification to the most stable housekeeping gene was performed with commercial software by use of the E-method as described. The E-method provides accurate relative quantification data by compensating for differences in target and reference gene amplification by use of specific gene efficiencies.

**Results**

Gene name and function, primer sequence, melting temperature, PCR product size, and calculated efficiency of the 7 housekeeping genes and target genes were determined (Table 1). Gene expression stability values for the housekeeping genes from the least to most stable genes in skeletal muscle were as follows: actin β (0.85), B2M (0.53), HRPT1 (0.45), SDHA (0.375), mitochondrial ribosomal protein S9 (0.3), and GAPDH and TUBA1 (0.225). Because skeletal muscle GAPDH had a lower Cp value than TUBA1, GAPDH was selected as the reference gene for the study. Analysis of the melting curves of the target genes’ PCR products revealed that only a single product was amplified without primer-dimer formation. In addition, specificity of the reaction was confirmed via direct sequencing. Low expression of genes for proinflammatory cytokines was found in a few horses with PPID (TNF-α [mean ± SD, 0.03 ± 0.08] in 2 horses, IL-1β [0.006 ± 0.01] in 3 horses, and IL-6 [0.001 ± 0.005] in 1 horse), and no expression was found in control horses. Genes for UBC, m-calpain, and cathepsin-D were expressed in all PIDD-affected and control horses. The gene for caspase-3 was expressed in 10 PIDD-affected and 5 control horses, and the gene for myostatin was expressed in 12 PIDD-affected and 5 control horses. There was a significant (P = 0.031) increase in gene expression for m-calpain in horses.
with PPID, compared with controls (Figure 1). Differences in the expression of other genes were not significant between groups.

Table 1—Forward and reverse primers for housekeeping and target genes in a study of gene expression of proteolytic systems and growth regulators of skeletal muscle in horses with myopathy associated with PPID.

<table>
<thead>
<tr>
<th>Gene type</th>
<th>Gene name</th>
<th>Forward primer (5′)</th>
<th>Reverse primer (5′)</th>
<th>Gene function</th>
<th>Tm (°C)</th>
<th>size (bp)</th>
<th>Efficiency</th>
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<tr>
<td>Housekeeping genes</td>
<td>Actin β</td>
<td>CCGAGACGATGAAGATCAAG</td>
<td>GGGGCTTCTGGAGATAGTG</td>
<td>Component of cytoskeleton</td>
<td>84.34</td>
<td>88</td>
<td>1.83</td>
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<td></td>
<td>β2M</td>
<td>GTCCATCCCGCTTGAATT</td>
<td>GGCGTCTTTCAGAGAGTG</td>
<td>Part of class I major histocompatibility complex</td>
<td>84.58</td>
<td>182</td>
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<td></td>
<td>GAPDH</td>
<td>AAGTGGATATTTGCTGCAAT</td>
<td>AACTTGCCCAGCTCTGAAT</td>
<td>Catalyses sixth step of glycolysis</td>
<td>79.71</td>
<td>86</td>
<td>1.83</td>
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<tr>
<td></td>
<td>HRPT1</td>
<td>GCAGAAGCCTATGACATT</td>
<td>CAAGGACATCCTTGAACAA</td>
<td>Purine biosynthesis in salvage pathway</td>
<td>81.43</td>
<td>183</td>
<td>2.31</td>
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<td>Mitochondrial ribosomal protein S9</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>SDHA</td>
<td>TGGAAGAGGAGGGCGCTG</td>
<td>ACACACTGCATCAATTCATG</td>
<td>Participates in citric acid cycle and respiratory chain</td>
<td>79.20</td>
<td>84</td>
<td>1.70</td>
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<td></td>
<td>TUBA1</td>
<td>GCCTCAACCTCCATTTGA</td>
<td>ATGGCTTCATTGTCACCA</td>
<td>Structural molecule</td>
<td>83.82</td>
<td>78</td>
<td>1.83</td>
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<td>Target genes</td>
<td>Caspase-3</td>
<td>AAATCGCATGAGAGCAGAC</td>
<td>CAGCATACAAAGACGACT</td>
<td>Role in execution phase of cell apoptosis</td>
<td>82.77</td>
<td>111</td>
<td>1.72</td>
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<tr>
<td></td>
<td>Cathepsin-D</td>
<td>CATCTTCTCTCTTACGGAGA</td>
<td>CTTTGGACTTTCGTAGGAGCT</td>
<td>Intracellular proteinase</td>
<td>86.87</td>
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<td>1.73</td>
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<td>IL-1β</td>
<td>GCCGATGTGACACAGTGA</td>
<td>TTTTGGCCATCTCTTCA</td>
<td>Proinflammatory cytokine</td>
<td>81.82</td>
<td>79</td>
<td>1.87</td>
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<td>IL-6</td>
<td>AGCATATTGAGATCGTGCC</td>
<td>CGGAGGACTGAGATCGCTC</td>
<td>Proinflammatory cytokine</td>
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<td>CAPN2</td>
<td>AGCTGATGAAGCTGAGAGA</td>
<td>GAAACCTGTAGTGACAAA</td>
<td>Calcium-activated cysteine protease</td>
<td>87.79</td>
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<td>Myostatin</td>
<td>GACATGACCCGAGCGCTT</td>
<td>GTTACACGAGATCATGACC</td>
<td>Negative regulator of muscle growth</td>
<td>81.22</td>
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<td>1.81</td>
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<td>TNF-α</td>
<td>GCCACAGCCTGATTATGCTTCCAT</td>
<td>CATTGACACGCGCCTCA</td>
<td>Proinflammatory cytokine</td>
<td>86.05</td>
<td>110</td>
<td>1.72</td>
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<tr>
<td></td>
<td>UBC</td>
<td>GCAAGACATCACCCTGGGA</td>
<td>CTAAAGCCACCTGAGAGCA</td>
<td>Ubiquitin pathway in protein turnover</td>
<td>86.74</td>
<td>206</td>
<td>1.71</td>
</tr>
</tbody>
</table>

Table 1: Forward and reverse primers for housekeeping and target genes in a study of gene expression of proteolytic systems and growth regulators of skeletal muscle in horses with myopathy associated with PPID.

Discussion

Skeletal muscle atrophy is a clinically important problem because decreased muscle mass is directly related to decreased muscle function, resulting in impaired physical activity or performance. Maintenance of muscle mass requires a balance between protein synthesis and degradation. Caspase-3, cathepsin-D, m-calpain, and UBC are proteins that represent the major proteolytic systems of skeletal muscle.10 Gene expression for these proteins, with the exception of m-calpain, was similar between controls and horses with PPID in this study. This suggests that calpains may be an important proteolytic system involved in the development of muscle atrophy in horses with PPID-associated myopathy. However, increases in RNA or protein expression may not necessarily correlate with increased activity of calpains.10 Therefore, the next step in the investigation of the role of calpains and other proteolytic and regulatory pathways in the development of muscle atrophy should include protein assays. This study did not investigate posttranscriptional and translational mechanisms that may affect protein production, activation or inactivation, and function. Lack of protein assays validated in horses specifically for the proteins under study here, cost of the development of such assays, and lack
of sensitivity or specificity of antibodies for assays such as ELISA, immunohistochemistry, or Western blot reactions, if using antibodies derived from other species, limited further investigation of such pathways. Also, expression of target genes and development of muscle atrophy at different stages of the disease (early vs late or chronic) were not evaluated. Therefore, other mechanisms of muscle atrophy cannot be entirely excluded.

The calpain family is a group of calcium-dependent proteolytic enzymes encoded by a minimum of 14 independent genes. Skeletal muscle expresses 3 distinct calpains: the ubiquitous calpains 1 and 2 (known as CAPN1 or mu-calpain and CAPN2 or m-calpain, respectively) and the skeletal muscle–specific calpain 3 (ie, CAPN3 or p94). Calpain substrates include myofibrillar proteins such as nebulin, titin, filamin, tropomycin, T, and desmin, which are essential for muscle integrity and function. Calpains require calcium for their activation in vitro; however, the calcium concentrations required for activation are much greater than physiologic concentrations. How activation occurs in vivo remains to be determined. Expression and activity of calpains are increased in glucocorticoid-induced muscle atrophy in laboratory animals and humans. Glucocorticoid receptors form a complex with the antiapoptotic protein Bcl-2 and translocate into the mitochondria in response to corticosteroid treatment in cortical neuronal cultures. However, chronic administration of corticosteroids results in the opposite. The protein Bcl-2 reduces the production of reactive oxygen species and increases calcium uptake by the mitochondria in response to corticosteroid treatment in cortical neuronal cultures. However, chronic administration of corticosteroids results in the opposite. The protein Bcl-2 reduces the production of reactive oxygen species and increases calcium uptake by the mitochondria in response to corticosteroid treatment in cortical neuronal cultures.

Muscle mass loss in horses with PPID is associated with atrophy of type 2A and 2B myofibers and loss of type 2B myofibers. The selective vulnerability of type 2 myofibers to atrophy in diseased horses may be explained by the deleterious effects of excess cortisol by depressing glycolysis in cells dependent on glycolysis for metabolism. However, type 2 myofiber atrophy is also observed with a variety of muscle-wasting diseases.

Oxidative stress plays an important role in the pathogenesis of PPID as indicated by the accumulation of 3-nitrotyrosine and lipofuscin in periventricular dopaminergic neurons, increased glutathione peroxidase activity in the pars intermedia, and reduction of antioxidant plasma thiols in diseased horses. It has been postulated that oxidative stress directs muscle cells into a catabolic state and that chronic exposure leads to muscle wasting by triggering proteolytic pathways and decreasing protein synthesis. States of hyperglycemia such as those observed in horses with PPID may be associated with an increased risk of laminitis caused by oxidative injury to the vascular endothelium as the result of auto-oxidation of glucose with free radical formation. Therefore, oxidative damage may also occur in the skeletal muscle vasculature, leading to further oxidative stress and muscle atrophy through the activation of proteolytic mechanisms. An important source of reactive oxygen species is the mitochondrion. An ultrastructural study in horses with PPID-associated myopathy revealed substantial subsarcolemmal accumulation of swollen mitochondria, compared with control horses. This alteration may be an indication of mitochondrial damage or oxidative injury. The role of calpains in oxidative stress was studied in vitro by use of skeletal muscle cell culture; calpains were required for hydrogen peroxide–induced myotube atrophy. The association of oxidative stress and increased gene expression of m-calpain in diseased horses remains to be elucidated.

An important regulator of muscle catabolism is NFκB, for which major activators are TNF-α and reactive oxygen species. Inflammatory cytokines such as IL-1β and IL-6 are also potent activators of NFκB.
This transcription factor plays a role in disuse atrophy and is particularly important in wasting diseases associated with inflammation, immune-mediated processes, and carcinogenesis. 40 In vitro study 41 in muscle cells has found that NFκB and glucocorticoids may have opposite effects in the induction of the proteasome pathway. The expression of proinflammatory cytokines IL-1β, IL-6, and TNF-α was low and not different between groups of horses in the present study.

Myostatin is a negative regulator of muscle growth, for which expression has been reported to increase with glucocorticoids. 42 However, in the present study, differences in expression of the myostatin gene were not detected between groups of horses.

Results of the present study suggest that overexpression of m-calpain may play an important role in the development of muscle atrophy in horses with PPID-associated myopathy. Gene expression of other proteolytic pathways of skeletal muscle such as caspasins, cathepsins, and ubiquitin-proteasome systems in horses with PPID was not different from that of age-matched control horses. However, these pathways cannot be excluded as important mechanisms of muscle atrophy because posttranscriptional and posttranslational mechanisms that may affect protein production, activation or inactivation, and function were not studied. Activation of the ubiquitin-proteasome pathway is a common end path for protein degradation in various disorders. However, significantly increased expression of the UBC gene was not identified in horses with PPID. Horses in the study had apparent muscle atrophy months to years prior to this investigation. It is possible that the ubiquitin-proteasome system was activated in the early stages of the development of muscle atrophy. Negative growth regulators of skeletal muscle such as myostatin and inflammatory cytokines were not overexpressed in this group of horses with PPID. Oxidative stress and increased endogenous glucocorticoids in diseased horses may disrupt the balance between protein synthesis and degradation in skeletal muscle through the expression and activation of catabolic pathways such as calpains that lead to muscle atrophy.

References


