Exertional rhabdomyolysis is a common cause of disability and poor performance in many horse breeds and has a variety of different underlying causes. One form of exertional rhabdomyolysis is PSSM, which is a glycogen storage disorder that affects American Quarter Horses and related breeds. Polysaccharide storage myopathy is characterized by severe muscle cramping, signs of pain, and cell damage following exercise. Samples of muscle obtained from PSSM-affected horses have a 1.5- to 4-fold increase in glycogen content, compared with findings in samples from clinically normal control horses, and contain amylase-resistant PAS-staining inclusions of abnormal polysaccharide in 1% to 30% of type II fibers. Many muscle fibers from horses with PSSM also contain subsarcolemmal vacuoles. No histologic abnormalities have been detected in the liver or adipose tissue of affected Quarter Horses, although some severely affected horses have abnormal polysaccharide in cardiac muscle. A familial basis for PSSM in Quarter Horses has previously been established from findings of a simulated pedigree analysis and a prospective breeding trial of PSSM-affected horses. Because deficiencies in enzymes of glycogenolysis and glycolysis in skeletal muscle of humans and other species result in histologic changes that are similar to those associated with PSSM, the activities of such enzymes have been investigated in muscles of PSSM-affected horses. However, compared with findings in clinically normal horses, no deficiencies or abnormalities in the activities of glycolytic or glycogenolytic enzymes have been identified in horses with PSSM.

**Biochemical and genetic evaluation of the role of AMP-activated protein kinase in polysaccharide storage myopathy in Quarter Horses**

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**Objective**—To evaluate whether biochemical or genetic alterations in AMP-activated protein kinase (AMPK) play a role in the development of polysaccharide storage myopathy (PSSM) in Quarter Horses.

**Animals**—30 PSSM-affected and 30 unaffected (control) Quarter Horses.

**Procedures**—By use of an established peptide phosphotransfer assay, basal and maximal AMPK activities were measured in muscle biopsy samples obtained from 6 PSSM-affected and 6 control horses. In 24 PSSM-affected and 24 control horses, microsatellite markers identified from the chromosomal locations of all 7 AMPK subunit genes were genotyped with a fluorescent DNA fragment analyzer. Alleles of 2 of the AMPK γ subunit genes were genotyped via DNA sequencing. Allele frequencies of DNA markers in or near the AMPK subunit genes were measured in isolated genomic DNA.

**Results**—No differences in basal or maximal muscle AMPK enzyme activities between PSSM-affected and control horses were detected. There were also no differences in allele frequencies for microsatellite markers near any of the 7 AMPK subunit genes between the 2 groups. Furthermore, previously known and newly identified alleles of 2 equine AMPK γ subunit genes were also not associated with PSSM.

**Conclusions and Clinical Relevance**—These results have provided no evidence to indicate that AMPK plays a causative role in PSSM in American Quarter Horses. (Am J Vet Res 2007;68:1079–1084)

**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>PSSM</td>
<td>Polysaccharide storage myopathy</td>
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<tr>
<td>PAS</td>
<td>Periodic acid–Schiff</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<td>cM</td>
<td>Centimorgan</td>
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horses, and contain amylase-resistant PAS-staining inclusions of abnormal polysaccharide in 1% to 30% of type II fibers. Many muscle fibers from horses with PSSM also contain subsarcolemmal vacuoles. No histologic abnormalities have been detected in the liver or adipose tissue of affected Quarter Horses, although some severely affected horses have abnormal polysaccharide in cardiac muscle. A familial basis for PSSM in Quarter Horses has previously been established from findings of a simulated pedigree analysis and a prospective breeding trial of PSSM-affected horses.

Because deficiencies in enzymes of glycogenolysis and glycolysis in skeletal muscle of humans and other species result in histologic changes that are similar to those associated with PSSM, the activities of such enzymes have been investigated in muscles of PSSM-affected horses. However, compared with findings in clinically normal horses, no deficiencies or abnormalities in the activities of glycolytic or glycogenolytic enzymes have been identified in horses with PSSM. During IV glucose tolerance testing and euglycemic hyperinsu-
linemic clamping, glucose is cleared from the blood in Quarter Horses with PSSM 1.5 to 2 times as fast as the rate of clearance in control horses; thus, PSSM-affected horses are more susceptible to the effects of insulin. Enhanced glucose uptake into PSSM muscle fibers is most likely responsible for the increased cellular glycogen concentration in affected horses (compared with unaffected horses), although the cause of the accumulation of abnormal polysaccharide and muscle cell damage is unknown. Draft horses and related breeds can also have a form of PSSM. Although that form of PSSM is similarly characterized by the presence of abnormal PAS-staining inclusions in skeletal muscle, exertional rhabdomyolysis is not as consistent a clinical feature in affected draft horses as it is in affected Quarter Horses. This suggests the possibility of different etiologies for the conditions in Quarter Horse- and draft horse-related breeds.

The heterotrimeric AMPK enzyme is a vital sensor of cellular energy status that is activated in response to low cellular ATP concentrations by at least 2 upstream kinases: an upstream regulatory serine-threonine kinase (the product of the STK11 gene known as LKB1) and a Ca2+-calmodulin-dependent kinase kinase. In turn, AMPK regulates several evolutionarily well-conserved pathways including the stimulation of glucose uptake, activation of insulin-stimulated PI-3-kinase, and stimulation of glycolysis concurrent with an inhibition of glycogen formation.

Two genes encoding α catalytic subunits (PRKAA1 and PRKAA2), 2 genes encoding beta subunits (PRKAB1 and PRKAB2), and 3 genes encoding gamma subunits (PRKAG1, PRKAG2, and PRKAG3) of AMPK are used to produce functional heterotrimers in different tissues. Evidence to date suggests that much of muscle AMPK is composed of α2, β2, and γ3 subunits. Mutations in the PRKAG2 gene encoding the AMPK γ2 subunit are associated with a human glycogen storage disease that accompanies a form of hypertrophic cardiomyopathy, and mutations in the PRKAG3 gene encoding the AMPK γ3 subunit result in the excessive skeletal muscle glycogen content and postmortem glycolysis associated with the Rendement Napole phenotype in swine. The role of AMPK as a metabolic master switch and its role in other glycogen storage disorders have made this protein a logical candidate for involvement in PSSM.

The purpose of the study reported here was to evaluate whether biochemical or genetic alterations in AMPK play a role in PSSM in Quarter Horses. To this end, basal and maximal AMPK activities in PSSM-affected muscle biopsy samples were measured, and microsatellite markers flanking each of the 7 genes encoding AMPK subunits were assessed in an allele-association analysis of PSSM-affected and healthy (control) Quarter Horses.

Materials and Methods

Horses—Six PSSM-affected and 6 unaffected (control) Quarter Horses were used for AMPK enzyme activity measurements. The genetic association study included 24 PSSM-affected and 24 control Quarter Horses. The PSSM-affected horses were all descended within 4 generations from a stallion that had clinical signs of exertional rhabdomyolysis consistent with PSSM. The degree of relatedness of horses used in the association study was controlled by selecting PSSM horses in this family that did not share common grandparents. Horses from which biopsies were taken were university owned, whereas most horses used in the association study were client owned and samples were collected from these horses with the owners’ informed consent. The study protocols conformed to University of Minnesota guidelines and were approved by the University Animal Care and Use Committee.

Diagnosis of PSSM—For AMPK assays, gluteal muscle biopsies were obtained by use of a 6-mm modified Bergstrom biopsy needle at a standardized site. Half of the muscle sample was rolled in talcum powder to prevent freeze artifacts and immersed in liquid nitrogen for later histochemical analysis. Half of the sample was frozen directly in liquid nitrogen and stored at −80°C for later biochemical analysis. For the allele association study, a diagnosis of PSSM was made on the basis of histologic evaluation of specimens obtained via open biopsy of the semimembranosus muscle or needle biopsy of the gluteal muscle.

After PAS staining with or without additional amylase-PAS staining, 10-µm transverse sections were evaluated for the presence of abnormal PAS-staining inclusions in at least 2 muscle fibers/biopsy specimen. Glycogen concentrations were determined fluorometrically in 10 to 20 mg of snap-frozen muscle (where available) that was boiled in 1M HCl for 2 hours to generate glucose residues.

Protein extraction from snap-frozen skeletal muscle lysates—Approximately 0.1 g of horse or rat skeletal muscle (control specimen) was crushed in a pestle and mortar under liquid nitrogen. Then, 3 volumes of ice-cold homogenization buffer (50mM Tris-HCl [pH, 7.4], 50mM sodium fluoride, 5mM sodium pyrophosphate, 0.25M sucrose, 1mM EDTA, 0.1mM phenylmethyl sulphonyl fluoride, and 1mM dithiothreitol) was added before homogenization with a tissue homogenizer (30-second bursts at full power until mixture was homogeneous). The suspension was centrifuged (15,000 X g) for 15 minutes at 4°C, and the supernatant was recovered. Polyethylene glycol 6,000 precipitates (2.5% to 6%) were prepared as previously described. The protein concentration was determined by use of a Bradford assay.

Immunoprecipitation—Anti-AMPK pan β antibody was adsorbed to protein A–sepharose (50% resin slurry) and used to immunoprecipitate AMPK complexes from the 2.5% to 6% polyethylene glycol extracts. Typically, 200 µg of total protein was incubated with 10 µL of 50% antibody-resin slurry for 2 hours at 4°C. The resin was then washed 3 times with 1 mL of buffer A containing 1% Triton X-100.

Western blot analysis—One hundred micrograms of rat or horse skeletal muscle (2.5% to 6% polyethylene glycol lysate) was immunoprecipitated with the anti-AMPK pan β antibody cross-linked to protein A–sepharose, and the immune complexes were resolved via SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The membranes were blocked by.
incubation in HST buffer (10mM Tris-HCl [pH 7.4], 0.5M NaCl, and 0.5% Tween 20) containing 5% low-fat milk powder for 30 minutes at room temperature (approx 22°C) and then incubated with polyclonal rabbit AMPK α2 (1:1,000 dilution) in the same buffer overnight (approx 16 hours) at 4°C. After extensive washing with HST buffer, the membrane was incubated for 1 hour at room temperature with protein A–IgG conjugated to horseradish peroxidase. After further extensive washing in HST buffer, the blots were developed by use of enhanced chemiluminescence.

AMPK activity measurement—Following immunoprecipitation, the pellet was divided into 2 aliquots for either direct assay of basal AMPK activity or assay of the maximal AMPK activity through addition of superphysiologic quantities of recombinant form of the upstream kinase (CAMKKβ). For the latter experiments, CAMKKβ was incubated with the immunoprecipitated AMPK in the presence of 0.2mM AMP, 0.2mM MgCl₂ with specific radioactivity of approximately 200 cpm/pmol, 5mM MgCl₂, 1mM dithiothreitol, and buffer (50mM HEPES [pH 7.5], 10% glycerol, and 1mM EDTA) at 37°C for 15 minutes. The resin was then washed 3 times with 1 mL of HEPES-glycerol-EDTA buffer to remove residual CAMKKβ and assayed by use of the SAMS peptide assay. Allele association analysis—Genomic DNA was isolated from whole blood of PSSM-affected and unaffected control horses. The chromosomal locations of 7 equine AMPK subunit genes were derived via radiation hybrid mapping in a previous study. Two to 5 of the closest flanking microsatellite markers to each of these genes were selected, and a tail sequence was attached to the forward primer of each microsatellite primer pair. The PCR reactions were performed in 15-µL reaction volumes including 12.5 ng DNA, 1.5mM MgCl₂, 1 pmol of the forward-tailed primer, 5 pmol of the reverse primer, 5 pmol of a color-specific dye primer that was complementary to the tail sequence, 30 µM dNTP, and 0.3 units of a modified Taq DNA polymerase. The PCR amplifications were comprised of 20 minutes at 95°C, 40 cycles of 30 seconds at 94°C, 30 seconds at 56°C, 30 seconds at 72°C, and a final extension of 15 minutes at 72°C. The PCR products were prepared for capillary gel electrophoresis and run on an automated fluorescent DNA fragment analyzer. Electropherograms were analyzed by use of the DNA fragment analyzer’s software, and alleles were manually confirmed. Allele frequencies were calculated for each marker in both the PSSM-affected and control groups, and minor alleles that were detected <10% of the time in the combined groups were eliminated from the analysis. The allele counts of each marker in the PSSM-affected and control groups were analyzed by use of a χ² test. A value of P < 0.05 was considered indicative of a significant association.

cDNA preparation and sequencing—Skeletal muscle mRNA was isolated, and cDNA was generated. Reverse transcription–PCRs were prepared in 50-µL reaction volumes consisting of 1 µL of concentrated cDNA, 5mM MgCl₂, 20 pmol of each the forward and reverse primers, 30µM dNTP, and 0.5 units of modified Taq DNA polymerase. The PCR primers for the amplification of segments of the equine PRKAG1 and PRKAG3 genes were derived from previously generated cDNA sequences (DQ318140 and DQ318144, respectively). The PCR products were run on a 1% agarose gel, and DNA bands were excised, purified, and submitted for sequencing. The cDNA from 2 PSSM-affected and 2 control horses were examined for sequence polymorphisms.

cDNA polymorphism genotypes—The PCR primer pair used to amplify a polymorphic site within exon 10 of the PRKAG1 gene was 5′-TTTGTATG TTCGCACCTAC–3′ and 5′-GCTTTGGGTCAAGACAC CATC–3′, and the primer pair used to amplify 2 polymorphic sites within exon 3 of the PRKAG3 gene was 5′-TAACAAAA CAGGCCCCATTT–3′ and 5′-GCAGTTGACCTTTCCCA CAT–3′. The PCR reactions were prepared in 30-µL reaction volumes consisting of 12.5 ng DNA, 3mM MgCl₂, 20 pmol of each the forward and reverse primers, 30µM dNTP, and 0.5 units of a modified Taq DNA polymerase. The PRKAG1 PCR products were digested with the restriction enzyme Nla III at 37°C for 2.5 hours. Digested products were run on a 2% agarose gel, and the single nucleotide polymorphism was genotyped on the basis of the banding pattern. The single nucleotide polymorphism genotypes for the PRKAG3 gene were obtained via sequencing with the forward PCR primer.

![Figure 1](image.png)

Figure 1—Detection of the catalytic AMPK α2 subunit in equine muscle and assessment of AMPK activity in muscle obtained from control (unaffected) and PSSM-affected horses. A—Results of western blot analysis of proteins of the AMPK β immunocomplex from rat and horse skeletal muscle (2.5% to 6%) lysates that were treated with an anti-AMPK α2 antibody after AMPK β immunoprecipitation. The equine α2 subunit migrated at the same molecular weight (63 kd) as the rat subunit and was detected at a similar concentration following immunoprecipitation. IP = Immunoprecipitation. WB = Western blot. B—Mean ± SEM activity of immunoprecipitated AMPK complex (detected by use of an anti-AMPK pan β antibody) in lysates of skeletal muscle obtained from 6 control (white bars) and 6 PSSM-affected (gray bars) horses. **P < 0.05. Basal AMPK activity (-CAMKKβ) and AMPK activity (+CAMKKβ) were measured by use of a SAMS peptide assay. There were no significant differences in either basal or maximal AMPK activity between muscle samples from PSSM-affected or control horses.
Results

Muscle glycogen concentrations and histopathologic findings—The mean ± SEM muscle glycogen concentration for the 6 PSSM-affected and 6 control horses was 170.5 ± 11.4 mmol glucose/kg and 100.7 ± 9.1 mmol glucose/kg of wet wt, respectively. These values were in the range previously reported for clinically normal and PSSM-affected muscle.

AMPK enzyme activity—The equine catalytic α2 AMPK subunit was detected at the expected molecular weight and at an expression level similar to that detected in rat muscle after immunoprecipitation of equivalent amounts of lysate by use of the anti-AMPK pan β antibody (Figure 1), confirming that both the anti-AMPK α2 antibody and anti-AMPK pan β antibody have cross-species epitope recognition. However, there was no difference in either basal or CAMKKβ (maximally activated) AMPK activity between control and PSSM-affected skeletal muscle lysates following immunoprecipitation.

Microsatellite allele association—Microsatellite DNA markers that were estimated to lie within 10 cM of each of the equine AMPK subunit genes on their respective chromosomes were analyzed in 24 PSSM-affected and 24 control horses (Table 1). At least 1 marker was located within 3 cM of each of the desired genes, and most of the flanking microsatellites were ≤ 5 cM from the desired genes. Most of the markers analyzed had 2 to 9 alleles and were informative in both PSSM-affected and control populations. No microsatellite marker was associated with significant differences in allele frequencies between PSSM-affected and control horses.

Identification and use of polymorphisms in the PRKAG1 and PRKAG3 cDNAs—Because several glycogen storage disorders in other species result from mutations in the AMPK γ subunits, we specifically investigated DNA sequence polymorphisms in the orthologous equine genes in cDNAs prepared from PSSM-affected and control Quarter Horse muscles. A G to A polymorphism in exon 10 of the equine PRKAG1 gene that resulted in an amino acid change from arginine (CGT) to histidine (CAT) at codon 236 (position 698 in sequence DQ318140) was identified. In addition, 2 of the 5 previously identified equine PRKAG3 polymorphisms were detected in Quarter Horse DNA samples. Both of these polymorphisms were within exon 3 (positions 301 and 371 in sequence DQ318144). The first was a G to A transition that resulted in the conversion of glycine (GGA) to arginine (AGA) at codon 51, and the second was an A to T transition that resulted in the conversion of proline (CCG) to leucine (CTG) at codon 74. Among the PRKAG1 and PRKAG3 polymorphisms, there was no significant difference in allele distribution between the PSSM-affected and control groups (Table 2).

Discussion

The accumulation of glycogen and abnormal polysaccharide in muscle fibers from PSSM-affected horses does not appear to be the result of an alteration in the glycolytic or glycogenolytic pathways, raising the possibility that PSSM is a novel glycogen storage disorder or that it is caused by alterations in other regulatory pathways controlling carbohydrate metabolism. These possibilities are supported by increased insulin sensitivity and enhanced glucose clearance (compared with findings in clinically normal muscle) that accompanies
the increased glycogen content of PSSM-affected skeletal muscle. The master regulatory role of AMPK in cellular energy metabolism and evidence that mutations in genes encoding subunits of AMPK cause glycogen storage diseases in other species strongly implicate AMPK's possible involvement in PSSM. We tested this hypothesis in the present study by measuring basal and maximal AMPK enzyme activities in PSSM-affected and control horse muscle samples and by examining microsatellite markers near the chromosomal loci of each AMPK subunit for possible allelic association with PSSM.

Traditionally, detection of AMPK activity from muscle lysates has been difficult because of its low activity state, unlike AMPK in liver samples, AMPK is not maximally activated during the muscle tissue harvesting process. To obtain meaningful AMPK activity measurements (including basal activities) and determine whether AMPK activity per se was altered in muscle from PSSM-affected horses, compared with muscle from control horses, we examined maximally activated enzyme activities by use of a recombinant upstream kinase preparation (CAMKKβ). There were no apparent differences, however, in either basal or maximal AMPK activity between muscle samples from control and PSSM-affected horses.

In vitro biochemical analysis of regulatory enzymes may not always accurately reflect in vivo activity, and not all proteins encoded by AMPK subunit genes that are actually expressed in equine muscle may have been measured in the enzyme assays reported here. The anti-AMPK pan β antibody used in the present study was expected to have immunoprecipitated all potential heterotrimeric combinations; however, the antibody's relative affinity for different equine heterotrimers is unknown. As such, we cannot exclude the possibility that the assay system was insufficiently sensitive to detect subtle alterations in activity of certain AMPK complex heterotrimers that were present at low concentration within the lysates.

A molecular genetic approach with DNA markers was therefore an attractive method with which to further investigate the candidacy of AMPK in PSSM. Microsatellite markers near each of the AMPK subunit genes were investigated for possible differences in allele frequency between PSSM-affected and control horses. As the PSSM-affected horses selected for this analysis were all descended from a common founder within 4 generations, a difference in allele frequency for a marker between the PSSM-affected and control groups would provide strong evidence that the chromosomal segment containing that marker is adjacent to the PSSM gene derived from the founder. However, the allele frequencies of the markers near any of the AMPK family genes were not different between the PSSM-affected and control groups. On the basis of the results of this analysis, we suggest that any contribution of these genes to the genetic susceptibility of Quarter Horses to PSSM can be excluded.

One of the more perplexing findings in the present study was that 3 DNA sequence polymorphisms identified in the Quarter Horse PRKAG1 and PRKAG3 cDNAs all resulted in amino acid changes within highly conserved regions of these γ subunits, yet no association between those sequence polymorphisms and PSSM was identified. All 3 of these codon changes were located within one of the cystathionine β-synthase domains, which are highly conserved structural regions of the protein within all 3 PRKAG isoforms. Therefore, although mutations in the cystathionine β-synthase domains of the PRKAG genes have previously been associated with a skeletal muscle disorder in swine and a form of cardiomyopathy in humans, a potential effect of these cystathionine β-synthase domain polymorphisms on PRKAG1 or PRKAG3 function in Quarter Horses could not be confirmed. Polymorphisms within other exons of the equine PRKAG3 gene have been identified in other breeds, also without a recognizable associated phenotype.

The data obtained via the biochemical approach or the candidate gene approach in the present study did not support a primary role for AMPK's association with the PSSM phenotype. However, the functional roles of different heterotrimERIC complexes of AMPK isoforms in muscle (and other tissues) are still poorly understood. The results of our study do not preclude the possibility of a subtle alteration in AMPK function that was not detected by the assay; such an alteration could perhaps be related to dysfunction of upstream regulators of specific isoforms, with important functional roles in muscle but with low expression. Further investigations of equine muscle energy regulatory pathways at the cellular level and a whole genome scan performed with either linkage analysis or linkage disequilibrium analysis would have the potential to identify the gene responsible for PSSM in American Quarter Horses.

References