Proglycogen, macroglycogen, glucose, and glucose-6-phosphate concentrations in skeletal muscles of horses with polysaccharide storage myopathy performing light exercise

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Objective—To determine concentrations of proglycogen (PG), macroglycogen (MG), glucose, and glucose-6-phosphate (G-6-P) in skeletal muscle of horses with polysaccharide storage myopathy (PSSM) before and after performing light submaximal exercise.

Animals—6 horses with PSSM and 4 control horses.

Procedures—Horses with PSSM completed repeated intervals of 2 minutes of walking followed by 2 minutes of trotting on a treadmill until muscle cramping developed. Four untrained control horses performed a similar exercise test for up to 20 minutes. Serum creatine kinase (CK) activity was measured before and 4 hours after exercise. Concentrations of total glycogen (G_t), PG, MG, G-6-P, free glucose, and lactate were measured in biopsy specimens of gluteal muscle obtained before and after exercise.

Results—Mean serum CK activity was 26 times higher in PSSM horses than in control horses after exercise. Before exercise, muscle glycogen concentrations were 1.5, 2.2, and 1.7 times higher for PG, MG, and G_t, respectively, in PSSM horses, compared with concentrations in control horses. No significant changes in G_t, PG, MG, G-6-P, and lactate concentrations were detected after exercise. However, free glucose concentrations in skeletal muscle increased significantly in PSSM horses after exercise.

Conclusions and Clinical Relevance—Analysis of the results suggests that glucose uptake in skeletal muscle is augmented in horses with PSSM after light exercise. There is excessive storage of PG and MG in horses with PSSM, and high concentrations of the 2 glycogen fractions may affect functional interactions between glycogenolytic and glycogen synthetic enzymes and glycosomes. (*Am J Vet Res* 2006;67:1589–1594)

Abbreviations

PSSM	Polysaccharide storage myopathy
GS	Glycogen synthase
GBE	Glycogen-branching enzyme
PG	Proglycogen
MG	Macroglycogen
G-6-P	Glucose-6-phosphate
CK	Creatine kinase
PAS	Periodic acid–Schiff
PCA	Perchloric acid
CV	Coefficient of variance
G_t	Total glycogen
kg DW	Kilograms of dry weight
GLUT4	Glucose transporter 4

Polysaccharide storage myopathy is a common, heritable condition affecting glycogen metabolism in Quarter Horse–related breeds.[1,2] Glycogen is stored in intracellular granules composed of noncovalently bound proteins and variable amounts of carbohydrate. This structure allows glycogen granules to be potentially regulated individually and regionally within a cell for metabolism.[3] Glycogen synthesis of new granules is catalyzed by a self-glucosylating protein primer called glycogenin. It generates an oligosaccharide primer of 7 to 11 glucosyl units, which serves as a substrate for GS.[4]

In combination with GBE, GS forms glycogen granules, which, in the initial stages, have a low molecular weight and a high protein-to-carbohydrate ratio and can precipitate in acid. These glycogen granules are termed PG and have a molecular weight of up to approximately 400 kd. When PG granules grow larger by the addition of glucose residues, they are termed MG. These MG granules are acid soluble and range in size from 400 to 10,000 kd.[5] Several studies[6-10] in humans and horses have revealed that these 2 fractions of glycogen granules respond differently during times of glycogen catabolism and anabolism. Several factors, such as exercise intensity, exercise duration, and initial glycogen concentration in the muscle, appear to influence the magnitude of degradation of the 2 pools, indicating that PG and MG differ metabolically in terms of regulation.

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Although horses with PSSM have glycogen concentrations that are up to 4-fold higher than glycogen concentrations in healthy horses, little is known about the distribution of PG and MG in the muscles of horses with PSSM and degradation during exercise. Separation of glycogen into PG and MG in muscle samples obtained from horses with PSSM may provide a deeper understanding of possible perturbations in glycogen synthesis or metabolism in affected horses. When glucose is taken up by muscle cells, it is rapidly phosphorylated by hexokinase to form G-6-P, which results in low intracellular concentration of free glucose. High concentrations of free glucose in muscle have been detected only after intense exercise.[9,11] A potential explanation for this is an intracellular increase in the concentration of G-6-P (a hexokinase inhibitor) as a consequence of increased glycogenolysis.

Horses with PSSM frequently develop exertional rhabdomyolysis following short-duration aerobic exercise.[12] Analysis of evidence suggests that rhabdomyolysis develops in specific muscle fibers as a result of inadequate energy metabolism, as indicated by an accumulation of inosine monophosphate within individual muscle fibers.[13] Although substantial glycogen use has been reported[14,15] after near-maximal exercise in horses with PSSM, little is known about glycogen use during light exercise. A study[16] of glycogen storage disorders in draft horses suggested that there was excessive glycogenolysis during rhabdomyolysis. Further investigation into the effects of light exercise on concentrations of glycogen, glucose, and G-6-P in muscle of horses with PSSM may clarify potential abnormalities in glucose uptake and glycogen use in muscles of affected horses during exercise. Therefore, the objective of the study reported here was to determine PG, MG, free glucose, and G-6-P concentrations in muscle specimens obtained from horses with PSSM before and after short-term submaximal exercise that induced rhabdomyolysis.

Materials and Methods

Animals—Ten horses (6 horses with PSSM and 4 control horses) were used in the study. All horses were owned by the University of Minnesota.

The 6 Quarter Horses with PSSM consisted of 5 mares and 1 gelding and ranged from 3 to 9 years of age (mean, 5 years). The criteria used to diagnose PSSM included episodes of exertional rhabdomyolysis; increases in CK activity in response to exercise; and detection of amylase-resistant, PAS-positive inclusion bodies in biopsy specimens obtained from the gluteal and semimembranosus muscles.

The control group consisted of 4 female Quarter Horses or Quarter Horse crossbreds that ranged from 10 to 16 years of age (mean, 9 years). None of these horses had a recorded history of any episodes of exertional rhabdomyolysis or increases in CK activity. In addition, there was no evidence of PAS-positive inclusion bodies resistant to amylase digestion in biopsy specimens obtained from the gluteal muscles.

All horses were housed in box stalls at the University of Minnesota and fed the same diet, which consisted of grass hay. At the start of the study, all horses were untrained but had previously been accustomed to exercising on a treadmill. The protocol for the conduct of this study was approved by the Institute for Animal Care and Use Committee at the University of Minnesota.

Exercise protocol—The exercise protocol was originally designed to cause subclinical rhabdomyolysis for use in a study[13] of adenine nucleotide degradation during exercise in horses with PSSM. All horses performed a light submaximal exercise test on a flat treadmill. The test consisted of repeated intervals of 2 minutes of walking (1.9 m/s) followed by 2 minutes of trotting (3 to 4 m/s), for a maximum of 20 minutes or until a horse had signs of a tucked up abdomen, stiffness or shifting lameness, muscle fasciculations, or a combination of these signs. On the basis of other studies[12,14,15] conducted by our laboratory group that involved use of these horses, it was anticipated that the exercise session would last for a maximum of 20 minutes. Serum CK activity was analyzed in blood samples obtained by venipuncture of a jugular vein before and 4 hours after exercise.

Collection of muscle specimens—Muscle biopsy specimens were collected percutaneously from the gluteus medius muscle at rest before exercise and immediately after the exercise test. Both specimens were collected by use of the same skin incision.[17] Muscle biopsy specimens were rapidly frozen in liquid nitrogen and stored at –80°C until analyzed. Before analysis, a portion of each specimen was cut; freeze-dried; and dissected free of visible blood, connective tissue, and fat. Two separate pieces of freeze-dried muscle, each of which weighed between 1.5 and 2 mg, were analyzed, and mean values for the 2 pieces were calculated.

Biochemical analysis of muscle specimens—The PG and MG fractions were separated on the basis of solubility in PCA in accordance with the method described elsewhere.[18] The glycogen fractions were boiled for 2 hours in 1M HCl, and the formed glucosyl units were assayed by use of flourometric methods.[19] Concentrations of free glucose and G-6-P were measured fluorometrically in aliquots of the PCA supernatants.[20] In the determination of MG, free glucose is included in the measurement. The obtained MG concentrations were therefore corrected by subtracting separately analyzed free glucose content.[9,18] Lactate content was analyzed in aliquots of the PCA supernatants by use of a commercially available kit.[a] All biochemical analyses were performed in duplicates, which enabled calculation of CV values.

Calculations and statistical analysis—The G_t concentration in each sample was calculated as the sum of the measured MG and PG concentrations. Results were analyzed by use of a computer software program.[b] Data for serum CK activity were logarithmically transformed to achieve an approximate Gaussian distribution. Comparisons between groups of horses were performed by use of Student *t* tests, whereas paired Student *t* tests were used to compare differences in mean values between duplicate analyses. Data were reported as mean ± SD. Differences were considered significant at values of $P < 0.05$.

Table 1—Reproducibility of biochemical analyses for duplicate analyses of each of 20 muscle biopsy specimens.

	Mean			
Metabolite	**Duplicate 1**	**Duplicate 2**	**SD**	**CV**
G_t (mmol/kg DW)	586	587	23	0.04
PG (mmol/kg DW)	388	397	17	0.04
MG (mmol/kg DW)	198	190	18	0.09
G-6-P (mmol/kg DW)	4.1	4.5	0.8	0.19
Free glucose (mmol/kg DW)	2.2	2.2	0.4	0.19
Lactate (mmol/kg DW)	30.1	32.2	8.1	0.26

Results

Exercise test—Mean ± SD duration of exercise for the horses with PSSM was 19 ± 9 minutes (range, 10 to 34 minutes). As mentioned previously, it was anticipated that the exercise test would last for a maximum of 20 minutes; however, it was not possible to induce signs of stiffness in 1 horse with PSSM, and the exercise test was therefore prolonged and terminated after 34 minutes. Mean serum CK activity for these horses was 605 ± 342 U/L (range, 246 to 958 U/L) before exercise and 5,607 ± 8,441 U/L (range, 255 to 22,265 U/L) 4 hours after exercise.

Mean ± SD duration of exercise for the control horses was 19 ± 1 minutes (range, 16 to 20 minutes). The exercise test was stopped after 16 minutes for 1 control horse because of an inability to maintain its pace while trotting on the treadmill. Mean serum CK value before and 4 hours after exercise for the control horses was 210 ± 66 U/L (range, 149 to 299 U/L) and 206 ± 64 U/L (range, 133 to 278 U/L), respectively. Serum CK activity was significantly higher in the horses with PSSM after exercise, compared with the value for the control horses.

Biochemical analysis of muscle specimens—Mean values, SD, and CV for muscle glycogen and metabolites did not differ significantly between duplicate analyses of the same muscle biopsy specimen (**Table 1**). Therefore, duplicate results were pooled and expressed as mean values of the duplicate analyses.

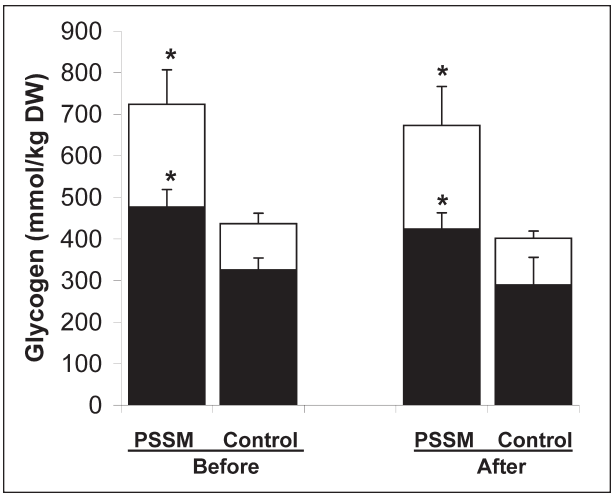

Figure 1—Mean ± SD concentrations of PG (black bars) and MG (white bars) in muscle specimens obtained at rest before and immediately after exercise for 6 horses with PSSM and 4 control horses. *Within a glycogen fraction within a time period, value differs significantly ($P < 0.05$) from the value for control horses.

Glycogen concentrations in skeletal muscle specimens obtained before exercise were significantly higher in horses with PSSM, compared with values for control horses. Values for horses with PSSM were 1.5-, 2.2- and 1.7-fold higher for PG, MG, and G_t, respectively, compared with results for control horses (**Figure 1**). Mean PG-to-MG ratio did not differ significantly between horses with PSSM (before exercise, 2.1 ± 0.7; after exercise, 2.0 ± 1.0) and control horses (before exercise, 3.1 ± 1.2; after exercise, 2.7 ± 1.0).

Concentrations of free glucose, G-6-P, and lactate before exercise did not differ significantly between horses with PSSM and control horses (**Table 2**). However, G-6-P concentrations before exercise in horses with PSSM were more variable (range, 0.7 to 9.5 mmol/kg DW), compared with concentrations in control horses (range, 2.2 to 4.8 mmol/kg DW).

After exercise, PG, MG, and G_t concentrations in muscle remained significantly higher in horses with PSSM, compared with concentrations in control horses. It was interesting that G_t, G-6-P, and lactate concentrations after exercise were all between 1.6 and 1.8 times higher in horses with PSSM, compared with values for control horses (Table 2). However, concentrations of G-6-P and lactate after exercise in horses with PSSM did not differ significantly from concentrations in control horses. The change in concentrations of PG, MG, G_t, G-6-P, and lactate before and after exercise for horses with PSSM and control horses was not significant for this small number of horses and with the light exercise conducted. However, concentrations of free glucose in muscle specimens obtained from the horses with PSSM increased significantly after light exercise and were higher than those found in control horses.

Discussion

In agreement with results of other studies,[14,21] G_t concentrations in muscle specimens obtained at rest before exercise were 1.7 times higher in horses with PSSM than in control horses. This was attributable to higher concentrations of both PG and MG in horses with PSSM. In fact, the mean PG concentration in horses with PSSM of approximately 500 mmol/kg DW was higher than the combined PG and MG content (ie, G_t concentration) of control horses. To our knowledge, the PG content of skeletal muscle from horses with PSSM reported here is the highest value ever described.

Glycogen stores in muscle can increase as a result of increases in the size of existing glycogen granules (MG) or increases in the number of glycogen granules through addition of glucose to additional glycogenin

Table 2—Mean ± SD concentrations of various metabolites in muscle specimens obtained before and after exercise in 6 Quarter Horses with PSSM and 4 control horses.

	Free glucose (mmol/kg DW)		G-6-P (mmol/kg DW)		Lactate (mmol/kg DW)	
Group	**Before**	**After**	**Before**	**After**	**Before**	**After**
PSSM	1.8 ± 0.5^{a}	3.4 ± 0.9^{b,A}	5.1 ± 2.8	5.25 ± 2.5	39.1 ± 23.9	51.4 ± 25.1
Control	2.0 ± 1.1	1.3 ± 0.7^{B}	3.1 ± 1.2	2.9 ± 1.6	23.6 ± 11.9	32.2 ± 7.9

^{a,b}Within a row, values with different superscript letters differ significantly ($P < 0.05$). ^{A,B}Within a column, values with different superscript letters differ significantly ($P < 0.05$).

protein (PG).[22] The first scenario would be accompanied by a decrease in the PG-to-MG ratio, whereas the latter would result in an increase in the PG-to-MG ratio as well as an increase in total muscle glycogenin. Free deglucosylated glycogenin does not exist in resting skeletal muscle.[23] In healthy horses and humans, the PG-to-MG ratio decreases as muscle glycogen concentrations increase, which indicates that the size of glycogen granules increases to a greater extent than does the number of glycogen granules.[18,24] The PG-to-MG ratio of 3.1 in control Quarter Horses (G_t concentration, 450 mmol/kg DW) contrasts with a PG-to-MG ratio of approximately 1 observed by one of the investigators in healthy Standardbred horses that had a G_t concentration of 600 mmol/kg DW. In humans with glycogen concentrations > 500 mmol/kg DW, glycogen is equally distributed between the PG and MG pools (ie, PG-to-MG ratio of 1). Interestingly, this distribution even persists in patients with McArdle disease who lack glycogen phosphorylase despite having G_t concentrations that exceed 800 mmol/kg DW.[3]

In the study reported here, however, horses with PSSM had a disproportionately high increase in PG concentration such that the PG-to-MG ratio was > 2.1 for a mean muscle G_t concentration of 724 mmol/kg DW. Thus, the high glycogen content in horses with PSSM was achieved by increases in the number of glycogen granules to a much greater extent than increases in the size of existing glycogen molecules. Smaller and more abundant glycogen molecules in the horses with PSSM would imply a higher glycogenin content in the skeletal muscle of those horses.

Currently, results[25-27,c] are conflicting with regard to the regulatory role for glycogenin in determining the extent of glycogen storage. However, there are indications that myoblasts that overexpress glycogenin increase total glycogen concentrations in cells stimulated by glucose and insulin.[c] It is therefore possible that the more numerous, smaller glycogen molecules (PG) in skeletal muscle of horses with PSSM are, in part, related to an overexpression of glycogenin.

The stimulus for both high PG concentrations and abnormal accumulation of polysaccharide in horses with PSSM may be an increase in the ratio of GS activity to GBE activity. Variably high G-6-P concentrations have been measured in skeletal muscle of horses with PSSM[14,21] as well as in patients with deficiency of phosphofructokinase enzyme.[28] In both disorders, it is believed that accumulation of an abnormal, less highly branched polysaccharide results from activation of GS without a corresponding increase in GBE.[21,29] This altered ratio of enzyme activity leads to longer outer glucose chains that have fewer α-1.6 branch points.[29]

Interestingly, overexpression of GS in mice leads to increased glycogenin concentrations and increased glycogen concentrations in skeletal muscle as a result of an increase in the number, rather than an increase in the size, of glycogen particles.[30] A higher GS-to-GBE ratio would create a less dense molecule because of longer chains and fewer branches.[31] This glycosome has a lower molecular weight per volume, and it is possible that this pattern favors the storage of glycogen in the PG form. The low molecular density of this glycosome would require considerably larger amounts of additional glucose to achieve the molecular weight of the MG fraction.

A glycogen molecule with less density is not a fully optimized molecule.[31] However, if a molecule becomes too densely branched, this could disturb interactions between enzymes in the glycosome.[3] Proglycogen is a smaller molecule and would therefore have less steric hindrance and possibly more favorable interaction with GS and glycogen phosphorylase, compared with results for the larger and more dense MG molecule. This hypothesis is supported by the fact that as exercise intensity increases, there is a preferential use of PG over MG.[7,8] In addition, after glycogen-depleting exercise in humans, the resynthesis rate of PG during early recovery is higher than that of MG.[6]

Increased sensitivity to insulin and increased uptake of glucose into resting skeletal muscle are believed to impact the high glycogen concentrations in muscles of horses with PSSM.[15,21,32] In addition, it was of interest to find that light exercise in horses with PSSM, but not in control horses, resulted in a significant accumulation of free glucose in skeletal muscle. The values for free glucose in the study reported here were determined by use of a glucose assay of muscle homogenates and were not extrapolated values for intracellular glucose. As such, some of the free glucose measured in this study was of extracellular origin in horses with PSSM and control horses.

Free glucose concentrations in the cytoplasm determine the intracellular glucose transport gradient, and this gradient is sustained by phosphorylation of glucose via hexokinase. The accumulation of glucose after light submaximal exercise in horses with PSSM suggests a limited or overwhelmed capacity to phosphorylate intracellular glucose. Possibly, hexokinase was inhibited by high intracellular concentrations of G-6-P; however, G-6-P usually accumulates only after rapid glycogenolysis with intense exercise, and horses with PSSM have limited glycogenolysis during a light exercise test.[33]

It is possible that increased basal concentrations of G-6-P could account for this inhibition because G-6-P concentrations were variably high before and after exercise but not significantly increased when compared with concentrations for the control horses. The lack of a significant difference could have been the result of inadequate statistical power because of the low sample size. However, a more likely explanation is that enhanced basal glucose transport in muscle of horses with PSSM is further augmented by additional translocation of GLUT4 into the plasma membrane during exercise. In another study,[21] investigators could not detect any differences in total GLUT4 content or in the sarcolemmal GLUT4-to-cytoplasmic GLUT4 ratio between horses with PSSM and control horses. It is possible that the sensitivity of the immunfluorescent technique used in that study was too low to detect differences in GLUT4 translocation. Furthermore, those measurements were performed only on muscle samples obtained during rest and did not evaluate differences in translocation of GLUT4 during exercise between horses with PSSM and control horses.

Muscle biopsy specimens from horses with PSSM generally have extremely low staining intensity for tetrazolium nicotinamide adenine dinucleotide reductase stain[21] and low citrate synthase activity,[34] which indicates an extremely low oxidative capacity. Citrate synthase activity of gluteal muscle in horses with PSSM in another study (8.1 ± 0.9 µmol/g/min)[34] was similar to that found in healthy untrained Quarter Horses (10.8 ± 1.5 µmol/g/min) but was only one fourth to one sixth the activity found in trained Standardbred or Thoroughbred horses[35,36] A low oxidative capacity could cause muscle fibers to rely to a large extent on anaerobic glycogenolysis and lactate production for maintaining energy production during exercise at lower speeds.

In addition, high glycogen content before exercise, such as in the group of horses with PSSM, may induce a higher rate of glycogenolysis during exercise.[37] However, a large difference in glycogen use was not detected between horses with PSSM and control horses after exercise. Analysis of the results reported here revealed that rhabdomyolysis in horses with PSSM was not associated with excessive glycogenolysis and lactate accumulation, as proposed by others in another report.[16] However, there was insufficient power to detect more moderate differences in glycogenolysis and glycolysis between horses with PSSM and control horses in the study reported here. It is likely that the duration of the exercise test was too short and the number of horses too few to be able to detect any use of glycogen as fuel for energy production in whole-muscle samples.

Substantial metabolic changes may develop in individual fibers, and metabolites were only measured in whole-muscle samples in the study reported here. In another study[13] in which we analyzed individual muscle fibers from horses used in the study reported here, noticeable variations in inosine monophosphate were observed within single fibers and within pools of single muscle fibers after exercise that were not apparent in whole-muscle samples. This subtle variability in metabolic response among single muscle fibers makes even rather large changes difficult to detect in muscle homogenates unless a critical mass of fibers is affected.

Our results of reproducibility (ie, SD and CV) for determination of G_t, PG, and MG content were in good agreement with those reported in other studies.[18,24] Results of reproducibility for G-6-P, free glucose, and lactate concentrations were slightly higher, compared with reproducibility for the glycogen values. Potential explanations for this could be higher variation of these metabolites among muscle fibers or a result of dilution effects from the extraction in PCA attributable to the analytic technique, which would increase the magnitude of the error.

Horses with PSSM appear to have enhanced glucose uptake during light submaximal exercise, which may contribute to particularly high concentrations of PG as well as high MG concentrations in skeletal muscle. There were no measurable changes in the concentration of PG, MG, or G-6-P in skeletal muscle during light aerobic exercise that induced rhabdomyolysis in horses with PSSM.

a. L(+) lactate kit, Sigma Aldrich Sweden AB, Stockholm, Sweden.
b. Minitab for Windows, Minitab Inc, State College, Pa.
c. Hansen BF. *Limits to glycogen storage*. PhD thesis, Laboratory of Human Physiology, August Krogh Institute, University of Copenhagen, Copenhagen, Denmark, 1998.

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Correction: Evaluation of the sensitivity and specificity of four laboratory tests for detection of occult blood in cockatiel (*Nymphicus hollandicus*) excrement

In "Evaluation of the sensitivity and specificity of four laboratory tests for detection of occult blood in cockatiel (*Nymphicus hollandicus*) excrement" (AJVR, Vol 67, pp 1326–1332), the legend for Figure 2 should read, "Mean and 95% CI for in vitro sensitivities of 3 chromogen tests (test A [horizontal-striped bars], test B [vertical-striped bars], and test C [gray bars], respectively) and cytologic examination (white bars) used for detection of whole blood mixed with cockatiel excrement to yield specimens containing various concentrations of Hb. *See* Figure 1 for remainder of key."

The legend for Figure 4 should read, "Mean and 95% CI for in vivo sensitivities of 3 chromogen tests and cytologic examination used for detection of occult blood in cockatiel excrement that was collected during the 24 hours after gavage administration of whole blood at various doses. *See* Figures 1 and 2 for remainder of key."