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The Glyco(geno)lytic Capacity of Skeletal Muscle in Horses with Exertional Rhabdomyolysis

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ABSTRACT. Glycogen storage diseases associated with glyco(geno)lytic enzyme defects are well defined causes of exertional rhabdomyolysis in man. The purpose of this study was to develop a screening test for glyco(geno)lytic enzyme defects in horses. Muscle biopsies from 16 horses with exertional rhabdomyolysis were obtained and glycogen concentrations were measured. Glyco(geno)lysis was evaluated by measuring lactate concentrations in muscle homogenates after incubation in a media containing either glycogen or hexose intermediates. In 9 horses, PAS positive inclusions resistant to amylase digestion were found in type II A and II B fibers. Mean glycogen concentrations were similar between controls and horses with exertional rhabdomyolysis but some horses with PAS positive inclusions had 30–50% higher glycogen levels than controls. While the amount of lactate production was less in homogenates with high glycogen concentrations and abnormal polysaccharide storage, under the conditions employed in this study our results did not suggest the presence of any specific enzyme deficiency associated with glyco(geno)lysis

Key words. Horses; muscle; glyco(geno)lysis; enzymes; rhabdomyolysis.

INTRODUCTION

Exertional myopathies involving rhabdomyolysis are common neuromuscular disorders in horses. Their clinical signs include variable expressions of muscle cramping, stiffness, swelling, weakness and myoglobinuria which develop during or after some form of physical exertion. The descriptive clinical term given to these disorders is "tying-up".

With the frequent use of the term tying-up to classify the common occurrence of those signs, it has become implied or assumed that a common factor or cause exists for their occurrence. As a consequence, subsets among such horses go largely unrecognized. For example, many performance horses experience an episode of exertional rhabdomyolysis at some point in their career. There is a smaller number of horses, however, which are unable to train intensively because they repeatedly develop exertional rhabdomyolysis. From a comparative perspective, nine glyco(geno)lytic disorders of skeletal muscle, causing rhabdomyolysis, have been identified for human beings and such disorders have also been recognized in dogs and cattle.[6,8] Specific diagnostic tests which define and characterize the myolytic processes in horses are needed for a better understanding of the pathogenesis of equine exertional rhabdomyolysis.

The purpose of this study was to evaluate the use of an assay for the measurement of *in vitro* lactate production as a method to screen for the presence of anaerobic glyco(geno)lytic enzyme deficiencies in horses with exertional rhabdomyolysis.

MATERIALS AND METHODS

Case material. Sixteen horses with a history of exertional rhabdomyolysis were selected from equine patients presented to the Veterinary Medical Teaching Hospital (VMTH), University of California, Davis, over the

Table 1. *Characteristics of horses with exertional rhabdomyolysis*

* = PAS positive inclusions, G = gelding, M = mare, S = stallion, SEMIT = semitendinosus, VASTUS = vastus lateralis, QH = quarterhorse, TB = thoroughbred, X = crossbred, APP = appaloosa

Horse	Age	Sex	Breed	Muscle	Glycogen (mmol kg^{-1})
1*	5	G	ARAB/QH	SEMIT	647
2*	10	M	QH	SEMIT	513
3*	9	M	TBXQH	GLUTEUS	757
4*	3	M	QH	SEMIT	716
5*	5	G	APP	GLUTEUS	569
6*	4	M	PAINT	GLUTEUS	541
7*	4	M	QH	GLUTEUS	505
8*	3	M	APP	GLUTEUS	650
9*	8	M	QH	GLUTEUS	548
10	5	G	TBX	SEMIT	459
11	10	G	MORGAN	VASTUS	297
12	3	M	TB	GLUTEUS	617
13	11	G	ARAB	GLUTEUS	580
14	6	M	QHX	GLUTEUS	475
15	11	S	QH	GLUTEUS	410
16	2	M	TB	GLUTEUS	463

past 12 years. A diagnosis of exertional rhabdomyolysis had been confirmed by a referring veterinarian. The sex, age and breed distribution for these horses are presented in Table 1. In addition, 9 healthy mares, including Arab and Thoroughbred breeds, were biopsied to provide a group of control horses.

Clinical examination procedures. The standard examination consisted of a physical examination, a complete blood count, a complete serum biochemical profile including assays of electrolyte concentrations and muscle-derived enzymes aspartate amino transferase (AST) and creatine kinase (CK), and a muscle biopsy examination.

Muscle biopsy procedures. Both open surgical biopsy techniques and needle muscle biopsy techniques[17] were used to obtain approximately 500 mg of muscle tissue. The gluteus medius muscle was biopsied in all control and 11 patient horses; the semitendinosus muscle was biopsied in 4 patients and the vastus lateralis in 1 patient.

Biochemical assay procedures. For all control horses and all but 2 patients, samples for biochemical analysis were frozen shortly after biopsy in liquid nitrogen and stored at −80°C for later analysis. Two muscle biopsies (Horses 2 and 4) were shipped (chilled) overnight to the VMTH and therefore were frozen within 24 hours of biopsy. Muscle glycogen was measured fluorometrically according to Lowry and Passonneau.[19] The modified Lowry procedure was used to analyze protein content in muscle homogenates (Sigma Kit No. 690-A).

The *in vitro* screening procedure for anaerobic glycolysis was modified after the technique developed by Layzer et al.[16] for screening human muscle samples. An aliquot of muscle was homogenized in 0.1 M potassium phosphate buffer. Following protein analysis of the homogenate, approximately 400 μg of muscle protein was incubated at 37°C for 30 min in 1 ml of incubation media which had a final composition of: 5 mM potassium phosphate pH 7.6, 53 mM potas-

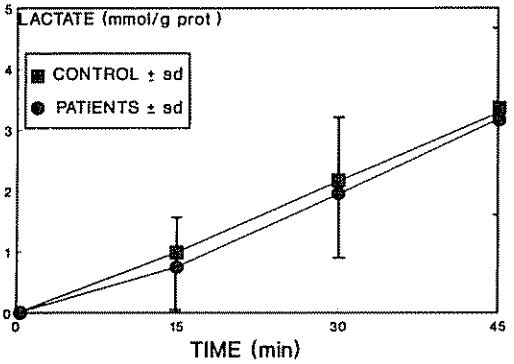

Fig. 1. The mean rate of lactate production in the presence of high glycogen concentrations. No significant difference in the rate of lactate formation was evident between horses with a history of exertional rhabdomyolysis and control horses.

sium bicarbonate, 10 mM ATP, 0.5 mM nicotinamide adenine dinucleotide, 40 mM nicotinamide, 4 mM magnesium sulfate, 1 mM cysteine and 2 mM potassium cyanide. To initiate the reaction one of the following substrates was added to the incubation media to give a final concentration of 0.3% glycogen, 0.3% glycogen and 1.5 mM AMP, 20 mM glucose-l-phosphate, 20 mM glucose-6-phosphate, 20 nM fructose-6-phosphate and 20 mM fructose-1,6-phosphate. The reaction was stopped through the addition of perchloric acid. After centrifugation, the supernatant fluid was removed, neutralized and assayed for lactate content fluorometrically using the technique of Lowry and Passonneau.[19] A 95% confidence interval was calculated in control horses for lactate accumulation per gram of muscle protein per min.

Histochemical procedures. The biopsy specimens to be used for histochemical examination were frozen in liquid freon cooled to −125°C with liquid nitrogen. Fresh frozen cryostat sections were then stained for myosin ATPase activity (without pre-incubation and after pre-incubation in acid media pH 4.30 and 4.47) and with periodic acid-Schiff (PAS) stain.[5]

In addition, muscle sections from control horses and horses with polysaccharide inclusions were cut on a cryostat microtome and incubated for 10 min in 0.2% EDTA in 50 mM potassium phosphate buffer. Subsequently, sections were incubated at 37°C for 5, 15 and 30 min in the incubation media described for the biochemical screening test. No substrate additions or AMP were included in the media. Sections incubated in phosphate buffer alone as well as sections digested by 3.5% amylase were used as positive and negative controls, respectively.

RESULTS

Blood chemistry

Serum enzymes for control horses ranged from 212 to 278 U l^{-1} for AST and 219 to 594 U l^{-1} for CK. The mean and range for serum CK in patient horses were 20 720 U l^{-1} (34–174 195) and the mean and range for AST were 5 706 U l^{-1} (468–37 110). Normal ranges for CK are 119–287 U l^{-1} and for AST 138–409 U l^{-1}. For each horse a CK and/or a AST value outside of normal limits was recorded in order to confirm that the horses had previously had rhabdomyolysis. Plasma electrolyte and glucose concentrations as well as liver enzymes (sorbitol dehydrogenase, alkaline phosphatase and gamma glutamyl transferase) were within normal ranges for all patient horses.

Muscle biochemistry

The mean (±SD) muscle glycogen concentrations for control horses was 520±96 mmol kg dry weight^{-1} (d.w.). All control samples were obtained from unexercised horses on a similar ration of oat and alfalfa hay. Individual values for glycogen concentrations in patients are shown in Table 1. The mean glycogen content of muscle from all patient horses was 551±94 mmol kg d.w.$^{-1}$ Muscle protein concentrations were similar in all horses. The mean concentration for controls was 454±67 g kg d.w.$^{-1}$ and for all 16 patients 478±40 g kg d.w.$^{-1}$

In the biochemical screening test, muscle samples were found to produce lactate at a constant rate for up to 45 min of incubation

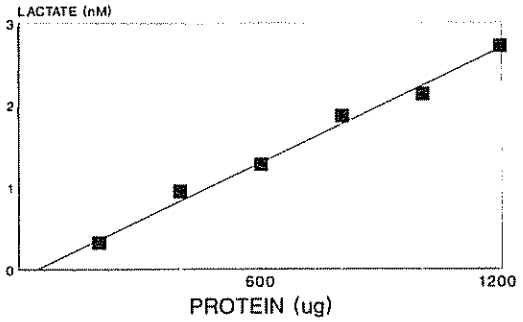

Fig. 2. The amount of lactate produced by different concentrations of the same muscle homogenate.

(Fig. 1). The linear correlation coefficient for lactate concentration on time was $r^2=0.95$ using glycogen as a substrate ($n=18$ horses). The screening test was also found to produce repeatable results for as little as 400 μg of protein or 0.5 mg of muscle d.w. (Fig. 2).

Fig. 3 shows the 95% confidence intervals for lactate production from various substrates by control horses and mean values for patient horses. The patient horses could utilize all of the substrates to produce lactate.

Muscle histochemistry

The muscle biopsies from control horses showed no histochemical abnormalities. During incubation in the screening media glycogen was first depleted in type II fibers and subsequently in type I fibers. By 30 min, glycogen depletion was complete in all control horses. Only a slight decrease in staining was seen in sections incubated in phosphate buffer. Nine of 16 patient horses had an accumulation of intensely PAS positive inclusions underneath the sarcolemma and between the myofibrils in type IIA and IIB fibers (Fig. 3). After 30 min of incubation, all except one patient had complete glycogen depletion. Sections from 1 horse required 45 min of incubation to deplete glycogen. The PAS positive inclusions were not affected by incubation in either the screening media or amylase.

DISCUSSION

In 1932, Carlstrom[7] investigated a commonly occurring exertional myopathy of draft horses and found that these horses had very high intramuscular glycogen concentrations prior to a rhabdomyolytic episode. Muscle damage was proposed to be due to an accumulation of muscle glycogen during a day of rest which, upon exercise, was rapidly metabolized anaerobically causing a lactic acidosis and muscle necrosis.

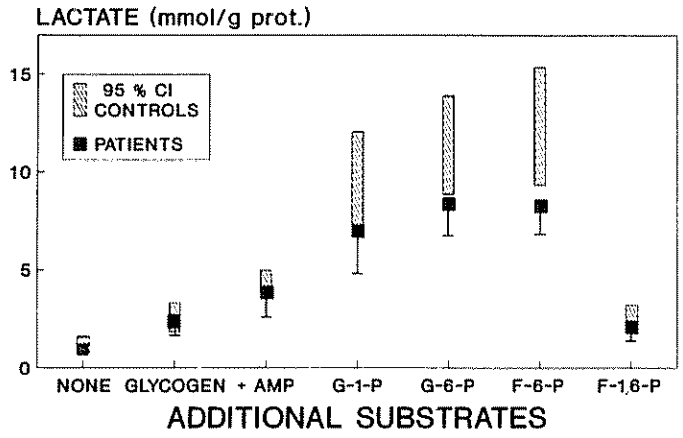

Fig. 3. The mean amount of lactate produced from various substrates by muscle samples from patient horses (±SD) and the 95% confidence intervals for control horses. The substrates include glycogen, and 1.5 mM AMP, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate.

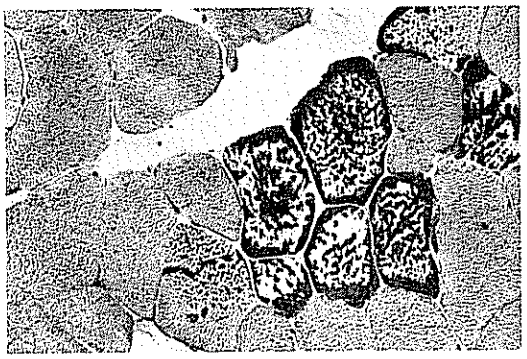

Fig. 4. A periodic acid Schiff-hematoxylin stain of a cross section of fibers from the gluteus muscle of Horse 3. Note the intensely stained inclusions and absence of normal glycogen staining pattern in some fibers.

Although not in complete agreement, many veterinarians feel that the exertional myopathy in pleasure horses is a similar but milder form of that described in work horses.[10,13,21] Several authors have found high muscle glycogen concentrations in horses with exertional rhabdomyolysis.[2,18,20,25] In the biochemical screening test used in this study the concentration of glycogen was far in excess of that normally found in muscle. Even in the presence of this relative glycogen overload, the amount of lactate produced from glycogen by horses susceptible to rhabdomyolysis was not different from that produced by healthy horses (Fig. 1). These findings are in agreement with other studies[15,25] and contradict the long held theory that rhabdomyolysis is due to an excessive anaerobic glycolysis with lactate accumulation induced by glycogen overload in skeletal muscle.

The glycogen concentrations measured in muscle from patient horses in this study should be interpreted with caution because no standardization was possible in terms of diet and exercise prior to biopsy. Some samples had a high proportion of necrotic fibers devoid of glycogen. In addition, some samples could not be frozen immediately so that a decrease in glycogen content could have occurred prior to freezing. In spite of these difficulties, some patients had a 30–50% higher muscle glycogen content than control horses. In one of the muscle samples that was chilled overnight, a glycogen concentration of 716 mmol kg d.w.$^{-1}$ was found. These findings were consistent with the intensity of PAS staining observed histochemically.

An accumulation of glycogen in skeletal muscle theoretically can only result from an inability to metabolize glycogen or from an increased synthesis of glycogen. In humans, defects in enzymes of anaerobic glycolysis including myophosphorylase, debrancher, phosphofructokinase and several distal enzymes, are all well defined causes of glycogen storage disorders and recurrent rhabdomyolysis.[8] The screening test used in this study did not detect any obvious defects in the enzymes of anaerobic glycolysis in the 16 horses examined. Possibly, factors which act to trigger rhabdomyolysis could cause a temporary block in anaerobic glycolysis but in the absence of these triggering factors the muscle can function normally. Several substances have been identified which can block anaerobic glycolysis and, through a depletion of high energy phosphates, produce a similar type of exertional rhabdomyolysis to that seen in horses.[4]

The presence of abnormal intramuscular polysaccharide accumulation in some of the horses in this study leads to further speculation about the integrity of the glyco(geno)lytic capacity of skeletal muscle in these horses. Several human patients with late onset polysaccharide storage myopathy have been reported in the literature.[1,3,9,11,12,14,22,23,24] Three patients with exertional rhabdomyolysis were found to have phosphofructokinase deficiency.[1,11] The biochemical defect responsible for the accumulation of this material in the 9 remaining patients with muscle weakness remains unknown.

In conclusion, using the present screening procedure a complete lack of any glyco(geno)lytic enzyme was not identified. Specific enzymatic analyses are needed in the horses with abnormal polysaccharide storage to

fully investigate the origin of this non-bioavailable storage product.

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