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MCT1, MCT4 and CD147 gene polymorphisms in healthy horses and horses with myopathy

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ABSTRACT

Polymorphisms in human lactate transporter proteins (monocarboxylate transporters; MCTs), especially the MCT1 isoform, can affect lactate transport activity and cause signs of exercise-induced myopathy. Muscles express MCT1, MCT4 and CD147, an ancillary protein, indispensable for the activity of MCT1 and MCT4. We sequenced the coding sequence (cDNA) of horse MCT4 for the first time and examined polymorphisms in the cDNA of MCT1, MCT4 and CD147 of 16 healthy horses. To study whether signs of myopathy are linked to the polymorphisms, biopsy samples were taken from 26 horses with exercise-induced recurrent myopathy. Two polymorphisms that cause a change in amino acid sequence were found in MCT1 (Val_{432}Ile and Lys_{457}Gln) and one in CD147 (Met_{125}Val). All polymorphisms in MCT4 were silent. Mutations in MCT1 or CD147 in equine muscle were not associated with myopathy. In the future, a functional study design is needed to evaluate the physiological role of the polymorphisms found.

1. Introduction

During intense exercise, lactate and protons accumulate in muscle cells and are subsequently transported to blood (Juel, 2008). Most of the lactate is transported together with protons by monocarboxylate transporters (MCTs; Juel, 2008). At least two isoforms of the transporter protein, MCT1 and MCT4 are expressed in muscle of various species, including horses (Wilson et al., 1998; Bonen et al., 2006; Koho et al., 2006). Both of these proteins need an ancillary protein CD147 (EMMPRIN, basigin, neurothelin) for their translocation to the cell membrane as well as for their activity (Kirk et al., 2000; Gallagher et al., 2007).

MCT1 and MCT4 are trans-membrane proteins. MCT1 has 12 membrane spanning domains and intracellular N- and C-termini (Poole et al., 1996). Topology predictions suggest similar structure of MCT4 (Juel and Halestrap, 1999). The ancillary protein, CD147, is a plasma membrane glycoprotein, which contains two extracellular immunoglobulin-like domains, a trans-membrane domain, and a small intracellular domain (Biswas et al., 1995). MCT1 and CD147 form a complex in the cell membrane, consisting of two MCT1 and two CD147 molecules (Wilson et al., 2002).

Several polymorphisms of MCT1 have been found in man, some of which are common in the healthy population (Merezhinskaya et al., 2000; Lean and Lee, 2009). A small study has also been performed examining polymorphisms in the coding sequence of MCT1 and partial coding sequence of CD147 of the horse (Reeben et al., 2006). One single nucleotide polymorphism (SNP) was found in the C-terminal area of MCT1 in a healthy horse. This mutation was in a different area than where mutations are found in human subjects (Merezhinskaya et al., 2000; Lean and Lee, 2009). Additionally, in several horses a polymorphism was found in the extracellular domain of CD147. Interestingly, in the horse, there are two expression levels of CD147, which have been shown to determinate the lactate transport activity in horse red blood cells (RBC; Koho et al., 2002).

The functional significance of the mutations in MCT1 has been demonstrated in studies that link several mutations of MCT1 with impaired lactate transport activity and signs of myopathy in humans (Fishbein, 1986; Merezhinskaya et al., 2000; Cupeiro et al., 2010). A large number of horse breeds suffer from myopathy and among athletic horses 3–15% of poor performance cases are due to recurrent exercise-induced myopathy (Morris and Seeherman, 1991; Martin et al., 2000; McCue et al., 2006). Some forms of recurrent myopathy are inherited, such as polysaccharide storage myopathy (PSSM) in Quarterhorse and draft related breeds and recurrent exertional rhabdomyolysis (RER) in Thoroughbreds (Dranchak et al., 2005; Finno et al., 2009). The small study of Reeben et al. (2006) included individuals which suffered from myopathy, but the SNPs found in MCT1 and CD147 could not be linked with the disease. The objective of the current study was to extend this earlier study and examine genetic variation in the complete coding sequence of MCT1 and CD147 in the horse and to study polymorphisms in the other MCT isoform, MCT4, that is found in

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the skeletal muscle. To do this we aimed to sequence horse MCT4 cDNA for the first time. Our final objective was to examine whether the polymorphisms could be linked to myopathy by using samples taken from horses that according to their owners had showed signs of myopathy, such as muscle stiffness or cramping after exercise on more than one occasion.

2. Materials and methods

Ethical approval was obtained by the institutional ethics committee.

2.1. Horses

Thirty Standardbreds and 12 Finnhorses aged from 2 to 20 years were included in this study.16 horses (10 Finnhorses and 6 Standardbreds) were reportedly clinically healthy and had no reported history of myopathy. The rest, 26 horses (2 Finnhorses and 24 Standardbreds), had according to owner reports suffered from repeated episodes of muscle stiffness post-exercise or other signs of recurrent myopathy. Prior to recruitment, all myopathy group horses had myopathy confirmed on at least one occasion by a veterinary surgeon and serum biochemistry that showed elevated serum aspartate aminotransferase (AST) and creatine kinase (CK). During the study all horses were in light to intense training.

Muscle biopsy samples were taken under local anaesthesia using a 5 mm modified Bergström biopsy needle from the middle gluteal muscle at depth of 6 cm (Lindholm and Piehl, 1974). Biopsy samples were immediately frozen in liquid nitrogen and stored at −80 °C until analyzed.

2.2. Blood samples

Serum and EDTA blood samples were taken from the jugular vein from all horses following a minimum of 12 h since intense exercise. Haematological values were measured within 3 h after collection from EDTA plasma. Another EDTA sample was centrifuged and red blood cells were separated and stored at −20 °C until analyzed for CD147. CK and AST were measured from serum with standardised methods (Konelab, Vantaa, Finland).

2.3. Sequencing of MCT1, MCT4 and CD147

Total RNA was extracted using QIAzol Lysis Reagent (QIAGEN, Valencia, CA, USA), after which mRNA was isolated using Poly(A)Purist MAG-kit (Ambion, Inc.; Austin, TX, USA) according to the manufacturer's instructions. RT-PCR was performed using PowerScript Reverse Transcriptase (Clontech Laboratories, Inc.; Mountain View, CA, USA) with an oligo dT primer. Ribolock was used as an RNAse inhibitor during first-strand cDNA synthesis reaction (Fermentas GmbH, St. Leon-Rot, Germany). The primers used for PCR are listed in Table 1. Sequence data of equine MCT1 and CD147 (GenBank Accession No. AY457175.1; EF564280.1; Reeben et al., 2006) was used to design primers for MCT1 and CD147. Because data on horse MCT4 was not available, primers were designed using homologous regions of the MCT4 of other species. PCR was performed using Advantage 2 PCR enzyme system (Clontech Laboratories, Inc.; Mountain View, CA, USA). The PCR-protocol for CD147 and MCT4 was set up according to the manufacturer's instructions: initial denaturation at 95 °C for 1 min, 35 cycles denaturation at 95 °C for 30 s and annealing at 68 °C for 1 min, and a final extension at 68 °C for 1 min. The PCR protocol for MCT1 was 5 cycles at 94 °C for 30 s and at 72 °C for 3 min, 5 cycles at 94 °C for 30 s and at 70 °C for 30 s and at 72 °C for 3 min, and 20 cycles at 94 °C for 30 s and at 68 °C for 30 s and at 72 °C for 3 min. One PCR product per sample was electrophoresed in 1.5% agarose gels and custom sequenced (16-capillary 3130*xl* Genetic Analyzer, Applied Biosystems, Foster city, CA, USA) in a sequencing facility (Biotechnical Institute, University of Helsinki, Finland) using both forward and reverse PCR amplification primers for the sequencing reactions. The data was analyzed using the software ClustalW2, available on the homepage of the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/clustalw2/).

2.4. Western blot

Plasma membranes of red blood cells (RBC) were isolated from frozen RBC as described by Koho et al. (2002). The amount of CD147 in plasma membranes of RBC was determined using western blot analysis (Koho et al., 2002). Horse-specific peptide was designed using the information available on the sequence of horse CD147 (GenBank Accession No. EF564280.1; Reeben et al., 2006) and the antibody against the peptide was raised in rabbits (Sigma Genosys, Cambridge, UK). Specificity of the antibody was tested by blocking the antibody reactivity with CD147 peptide. The optical intensity of the staining was quantified with AIDA image data analyzer (Raytest, Straubenhardt, Germany).

2.5. Histochemistry

Sections (20 μm) of frozen muscle were cut in a cryostat and stained with periodic acid–Schiff reaction for glycogen (Pearse, 1960). Parallel samples were first incubated for 30 min with amylase (1%) followed by periodic acid–Schiff stain, to study whether samples contained abnormal glycogen indicative of PSSM.

2.6. Statistics

Haematological values and age were distributed normally and are presented as mean ± SD. Muscle enzyme activities were not normally distributed and are presented as median (interquartile range). Differences between groups were calculated with a

Table 1
Forward and reverse primer sets used in sequencing of horse MCT1, MCT4 and CD147 cDNA.

Primer pairs	Sequence 5′ → 3′	Amplimer total bp	Base number
CD147 FOR	GGGGGACGGTGGCGACATG	867	1–19
CD147 REV	AGGCCTGTGCAGACTCCTCATCAGC		843–867
MCT1 FOR1	ACTGCATTTGGAATTCATCTACACTTAAAATGCC	1581	176–209
MCT1 REV1	CGATATCTTGGGTCACAAGTTGCTCCA		1730–1756
MCT1 FOR2	TCCCTGTTCACACACAGAGG	185	970–989
MCT1 REV2	CCCATAGAAGGTCTGGCTACC		1134–1154
MCT4 FOR1	CAGCCCTTAGGTGCCTCTCT	806	−46 to (−)27[a]
MCT4 REV1	GCCACAGCGTAGATCACAAA		741–760
MCT4 FOR2	GCTGCTCAACTGCTGTGTGT	1038	614–633
MCT4 REV2	GGCTTGGCTTCATCACAGAT		1632–1651[a]

[a] Numbers extend beyond the sequence of MCT4 in the GenBank Database Accession No. EF564279.