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Two levels of MCT1 and CD147 expression in the equine red blood cells and muscle

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ACADEMIC DISSERTATION

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To my Father and Sons

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ABSTRACT

Monocarboxylate transporters (MCTs), especially the isoforms MCT1 - MCT4, cotransport lactate and protons across the cell membranes. They are thus essential for pH regulation and homeostasis in glycolytic cells such as red blood cells (RBCs), and skeletal muscle cells during intense exercise. In 70% of the Standardbred horses the lactate transport activity (TA) in RBCs is high and transport is mediated mainly by MCTs. In the rest 30% of the Standardbreds MCT-mediated transport route is not active and the TA is low.

MCTs need an ancillary protein for their proper localization and functioning in the plasma membrane. The ancillary protein for MCT1 and MCT4 is a member of immunoglobulin superfamily, CD147. Here we determined the expression of MCT isoforms and CD147 in equine RBCs and gluteal muscle. We sequenced the cDNA of horse MCT1 and CD147 to achieve horse-specific antibodies and to reveal sequence variations that may affect the TA of RBCs. The amount of MCT1 and CD147 mRNA in muscle were also studied.

In all, 73 horses representing different breeds were used. Blood samples were drawn from the jugular vein and muscle samples were taken either from gluteal muscle using biopsy needle or during castration from expendable cremaster muscle. The TA of RBCs was studied using radiolabeled lactate and the amount of MCT isoforms and CD147 in the plasma membranes using Western blotting. The level of mRNA in muscle cells was determined using qPCR.

Isoforms MCT1 and MCT2 were found in the RBCs and isoforms MCT1 and MCT4 in the muscle cells of horses. The TA of RBCs was dependent on the expression of CD147 and MCT1 in the plasma membrane. Sequence variations were found in the cDNA of both MCT1 and CD147, but they did not explain the inactivity of MCT1- mediated transport route. The single nucleotide polymorphism (SNP) that causes $Met_{125}Val$ in CD147 and is found parallel with an SNP in 3´-untranslated region explained, however, decrease in CD147 expression in Standardbreds. A single mutation $Ile_{51}Val$ also decreased the expression of CD147 in one Warmblood.
The MCT1 and CD147 mRNA concentrations in the gluteal muscle were higher in horses with higher MCT1 and CD147 expression in RBCs and lower in horses with minor expression of CD147 and MCT1. This suggests that the bimodal distribution of TA is due to differences in transcriptional regulation that is functioning in parallel in *MCT1* and *CD147* gene.

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original articles, which are referred to in text by their Roman numerals:

I **Koho, N.M., Väihkönen, L.K. & Pösö, A.R.**, Lactate transport in red blood cells by monocarboxylate transporters. Equine Vet J, Suppl 34 (2002) 555-559

II **Koho, N.M., Hyyppä, S. & Pösö, A.R.**, Monocarboxylate transporters (MCT) as lactate carriers in equine muscle and red blood cells. Equine Vet J, Suppl 36 (2006) 354-358

III **Reeben, M., Koho, N.M., Raekallio, M., Hyyppä, S. & Pösö, A.R.**, MCT1 and CD147 gene polymorphisms in Standardbred horses. Equine Vet J, Suppl 36 (2006) 322-325

IV **Koho, N.M., Mykkänen, A.K., Reeben, M., Raekallio, M.R., Ilves, M., Pösö, A.R.** Sequence variations and two levels of MCT1 and CD147 in red blood cells and gluteus muscle of horses. *Accepted with revisions (Gene)*

Papers I – III are included in this dissertation as reprints with permission of their copyright holders. In addition, some unpublished material is presented (V).

ABBREVIATIONS

AE1	anion exchanger
AST	aspartate aminotransferase
ATP	adenosine triphosphate
AU	arbitrary unit
BCA	bicinchonic acid
Caco2-BBE	human intestinal epithelial cell line
CCD	charge coupled device
CHC	alpha-cyano-4-hydroxycinnamate
CK	creatine kinase
CoA	co-enzyme A
cRNA	RNA derived from cDNA through standard RNA synthesis
DIDS	4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid
cDNA	complementary DNA
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FADH	flavin adenine dinucleotide, reduced form is $FADH_2$
FRET	fluorescence energy transfer
FRTL-5	rat thyroid cell strain
HE	high expression
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	hypoxia induced transcription factor
HRP	horse radish peroxidase
HTA	high transport activity
Ig	immunoglobulin
K_m	Michaelis constant, an approximation of the affinity of the transporter for the substrate concentration at which the transport rate is half of V_{max}.
LDH	lactate dehydrogenase
LTA	low transport activity
MCT	monocarboxylate transporter
mMCT1	mitochondrial monocarboxylate transporter 1
NAD^+	nicotinamide adenine dinucleotide, reduced form is NADH
NE	negligible expression
pCMBS	p-chloromercuribenzene sulfonate
PCR	polymerase chain reaction
PCV	packed cell volume, hematocrit
qPCR	quantitative polymerase chain reaction
RBC	red blood cell
mRNA	messenger RNA
rRNA	ribosomal RNA
SCFA	short chain fatty acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
TCA	tricarboxylic acid
TM	transmembrane (segment)
UTR	untranslated region
V_{max}	maximal velocity, equal to the substrate concentration at which the transport rate of the substrate is maximal

1. INTRODUCTION

Lactate is the end product of anaerobic energy metabolism. It is produced in all cells that rely on glycolytic energy production, such as in red blood cells (RBCs) and smooth muscle cells. Lactate is also produced in other cell types during conditions in which their oxidative capacity is exceeded, e.g. in muscle cells during strenuous exercise (Nelson and Cox 2005).

The existence of a specific transport mechanism for lactate and pyruvate was first demonstrated in human RBCs (Halestrap and Denton, 1974, Halestrap, 1976). In 1980s three pathways for lactate transport in RBCs were described. One was passive diffusion, another the band 3 protein (later known as anion exchanger AE1), and the third was H^+-coupled monocarboxylate transporter (MCT) (Deuticke et al. 1982). MCT was purified and cloned for the first time in 1994 (Poole and Halestrap 1994, Garcia et al. 1994).

The path leading to this thesis began in 1995 when wide interindividual variation in plasma/whole blood lactate gradients was observed in horses after exercise (Pösö et al. 1995). The variation was similar, whether samples were taken from the same horses after maximal (competing in trotting races) or submaximal exercise (training). It was suggested that horses may have differing amounts of lactate transporters on their RBC membranes or the RBC membranes of different horses may vary in lactate permeability.

Some years later specific transport protein inhibitors were used to reveal the active lactate transport mechanism (Väihkönen and Pösö 1998). The influx of lactate into RBCs was studied in 89 Standardbred horses that were from 2 weeks to 9 years of age. The frequency distribution of this lactate transport activity in horses suggested that they could be divided into two groups; for 62 horses the mean rate of influx was 4.09 nmol mg^{-1} protein min^{-1} and for 27 horses 0.58 nmol mg^{-1} protein min^{-1} when RBCs were incubated at 10 mM lactate. In horses with high-lactate transport activity (HTA), MCT was the major transporter. In low-transport activity (LTA) horses no or very low MCT activity was detected. Interestingly, horses seemed to be the only species in which the distribution of lactate transport activity was bimodal. In humans, reindeer, and dogs the distribution was unimodal, although the transport activity in reindeer was as low as in LTA horses, in humans as high as in HTA horses, and in dogs almost twice as high as in HTA horses (Väihkönen et al. 2001).

The heritability of the transport activity was studied in a group of Standardbred horses that included 4 sires, 15 dams, and their 52 offspring. The heritability was evident and the result indicated that lactate transport activity was caused by a recessive allele in a single autosomal locus (Väihkönen et al. 2002). The transport activity was measured in these horses 2 – 5 times over several years and it was affected by age, training or both.

These changes were, however, relatively small and the horses remained in their original activity group.

In early 2000, we knew that there were two groups of horses, one with HTA and another with LTA in RBCs. We also knew that the activity was dependent on MCT proteins, was inheritable, was almost steady, and in most species studied normally distributed. Still, many questions were unsolved; e.g. which was the isoform of the transporter protein, how was it expressed, and what was the reason for its unusual distribution in horses?

2. REVIEW OF THE LITERATURE

2.1. Lactic acid

L(+)-lactic acid is the final product of anaerobic glycolysis. pKa of lactic acid is 3.86; thus it is dissociated into a lactate anion and a proton at physiological pH. Under aerobic conditions, glycolysis forms pyruvate that is transported into the mitochondrial matrix and converted to acetyl coenzyme A (CoA). Acetyl CoA functions as a fuel in the tricarboxylic acid (TCA) cycle, in which both reduced nicotinamide adenine dinucleotide and reduced flavin adenine dinucleotide (NADH and $FADH_2$) are produced. In oxidative phosphorylation, NADH and $FADH_2$ are oxidized to produce adenosine triphosphate (ATP) for the energy demands of the cell. If ATP utilization is high, the rate of glycolysis exceeds the mitochondrial capacity to oxidize pyruvate. Pyruvate is then reduced to lactate via lactate dehydrogenase (LDH). An elevated NADH /NAD^+ ratio, due to reduction of NAD^+ (nicotinamide adenine dinucleotide) to NADH by the glyceraldehyde dehydrogenase reaction, favors lactate formation. In lactate production, NADH is oxidized back to NAD^+, which is essential to maintain a high glycolytic rate. In RBCs, which lack mitochondria, anaerobic glycolysis is the only mechanism for energy production. However, all tissues are dependent on the glycolytic energy pathway during hypoxia or ischemia or when the oxidative capacity of the cell is exceeded (Nelson and Cox 2005).

At rest, lactate is removed from the blood at the same rate as it is produced, thus the blood lactate concentration in horses remains at approximately 1 – 1.5 mM (Higgins and Snyder 2006). In maximal exercise, lactate concentrations in the muscle of horses may increase to 23 – 46 mmol/kg (100 – 200 mmol/kg dry weight) and pH decrease to 6.5 (Lovell et al. 1987). Lactate concentrations in the blood may then increase by up to 30 mM (Lovell et al. 1987). Lactate that is transported out of the producing cells can be used by neighboring oxidative muscle fibers that have higher mitochondrial densities than glycolytic muscle fibers. Alternatively, lactate can be removed from blood by the liver and kidneys, which convert lactate to glucose in gluconeogenesis (Ahlborg et al.1974).

Lactate can also be delivered to the heart, brain, and other aerobic tissues in which it is used for energy production (Gertz et al. 1981, Dalsgaard et al. 2004).

The metabolism of lactate is one of the debated topics in research today. For decades `lactic acidosis´ has been a relevant explanation for the pH decline in muscles and generally the factor that induces pain and fatigue during strenuous exercise (Fitts 1994). This view still has support, but it has also been suggested that lactate production prevents acidosis, delays the onset of muscle fatigue, and may even protect the contraction force machinery (Lindinger et al. 1995, Nielsen et al. 2001, Posterino et al. 2001, Westerblad et al. 2002, Robergs et al. 2004, Juel 2008). However, to remain in homeostasis and prevent damage in muscle cells, the lactate and protons produced must be exported. MCTs cotransport both of these and are fundamental to pH regulation in working muscle cells.

2.2. Monocarboxylate transporters

2.2.1. History

During the 1970s, it was found that lactate and pyruvate transport across RBC plasma membranes was dependent on a chloride-independent carrier that was competitively inhibited with α-cyano-4-hydroxycinnamate (CHC) (Halestrap and Denton 1974, Halestrap 1976). Similar α-cyanocinnamate-sensitive MCTs were described functionally in multiple tissues, including hepatocytes, skeletal muscle fibers, thymocytes, tumor cells, and smooth muscle cells (see Edlund and Halestrap 1988). One decade later, the L-lactate carrier was reconstituted from rat and rabbit RBCs, and the protein was partially purified in 1992 (Poole and Halestrap 1988, 1992). The characteristics of the protein were well studied during the following years and similar proteins with different characteristics were found (Garcia et al. 1995, Price et al. 1998). The first cloned MCT (MCT1) was found in context with studies on met-18b-2 hamster ovarian cells that exhibited unusually high uptake of a dihydroxymonocarboxylate called mevalonate. The cloning and sequencing of the wild-type version of mevalonate transporter, coupled with the observation that wild-type transporter had more affinity for other monocarboxylates than mevalonate, identified this protein as being related to the lactate carrier found earlier in RBCs (Garcia et al. 1994).

2.2.2. MCT family

Later, 14 members of the MCT protein family (solute carrier family SLC16) were found (Meredith and Christian 2008). MCT proteins transport a wide variety of metabolically important monocarboxylates, such as lactate, pyruvate, acetate, butyrate, propionate, and ketone bodies, as well as many pharmaceutical agents (Halestrap and Meredith 2004, Enerson and Drewes 2003). The proton-coupled lactate transporters MCT1 – MCT4 are the main isoforms that are involved in lactate transport in mammalian cells (Halestrap

and Meredith 2004). MCT1, MCT2 and MCT4 are found in several tissues while MCT3 (SLC16A8, REMP) is found only in the retinal pigment epithelium and the choroid plexus epithelia to date (Yoon et al. 1997, Philp et al. 1998). In addition to MCT1 – MCT4, the other MCT isoforms characterized thus far are MCT8 (XPCT, SLC16A2) and MCT10 (TAT1, SCL16A10). They are closely related and transport their substrates in a Na^+- and H^+ -independent manner (Kim et al. 2001, Friesema et al. 2003). MCT8 is widely expressed and of major importance in the uptake of thyroid hormones into neurons, where they regulate normal development (Morreale de Escobar et al. 2000, Friesema et al. 2003). TAT1 is an aromatic amino acid transporter found in intestine, kidney, liver, skeletal muscle, heart, and placenta (Kim et al. 2001). It does not transport pyruvate or lactate. It should be noted that the transporters were named according to the order of finding and that MCT4 was originally called MCT3 (Wilson et al. 1998).

The expression patterns of the remaining isoforms, MCT5 – MCT7, MCT9 and MCT11 – MCT14, in humans are available (see Meredith and Christian 2008) and single studies and speculations on the physiological functions of MCT6, MCT7, and MCT13 have been presented (Yokel et al. 2002, Murakami et al. 2005, Hirai et al. 2007, Koho et al. 2007). The specific physiological roles of these isoforms await further elucidation.

All the isoforms studied so far have 10 – 12 conserved transmembrane (TM) segments with N- and C-termini located within the cytoplasm. A large intracellular loop (29 – 105 residues) is found between TM 6 and 7. Theoretical predictions indicate that MCT proteins are not glycosylated and experiments have proved this for isoforms MCT1 and MCT3 (Carpenter et al. 1996, Yoon et al. 1997, Halestrap and Price 1999). It has been proposed that the N-terminal domains (TMs 1 – 6) of MCTs are more important for H^+ or Na^+ coupling and membrane insertion, and/or correct structural maintenance, while the C-terminal domains (TMs 7 – 12) may be involved in substrate specificity determination (Saier 1994, Price et al. 1998)

2.2.3. MCT1, the housekeeping lactate transporter

MCT1 (SLC16A1) is the most widely distributed isoform of the MCT protein family. Thus far it has been found in the great majority of tissues of all species studied (Halestrap and Meredith 2004). MCT1 is quite well conserved, e.g. the protein sequences of rat and humans show 83% identity and 87% similarity (Jackson et al. 1997). It is also a nonspecific isoform: it transports short-chain unbranched aliphatic monocarboxylates such as acetate (K_m 3.5 mM) and propionate (K_m 1.5 mM), and short-chain monocarboxylates with several C2 and C3 substitutions, such as L-lactate (K_m 3 – 5 mM), pyruvate (K_m 0.7 mM), acetoacetate (K_m 4 – 6 mM) and D-β-3-hydroxybutyrate (K_m 10 – 12 mM). Bicarbonate, dicarboxylates, tricarboxylates, and sulfonates are not transported (Bröer et al. 1998, Manning Fox et al. 2000).

MCT1-mediated lactate transport is inhibited using bulky or aromatic monocarboxylates from which α-cyano-3-hydroxycinnamate and CHC are often used in research (Poole and Halestrap 1993). In addition, 4, 4´-diisothiocyanostilbene-2, 2´-disulfonate (DIDS) acts as a reversible inhibitor of MCT1, but it cannot be used as a specific inhibitor e.g. in RBCs, because it has a higher affinity for AE1 (Poole and Halestrap 1991). Especially sensitive MCT1 is to the organomercurial thiol reagent p-chloromercuribenzene sulfonate (pCMBS), which at concentrations of 0.3 – 1.0 mM blocks nearly completely the transport mediated by MCT1 (Poole and Halestrap 1994, Garcia et al 1995).

The essential amino acid residues that determine MCT protein trafficking to the plasma membrane, its substrate specificity, and transport activity are found mainly in the TM segments or nearby. Rahman et al. (1999) found that Phe360 in the complementary DNA (cDNA) of rat is important in substrate recognition and Arg306 in transport activity. A charge-pair Asp302 and Arg306 in TM 8 is essential for correct folding and substrate translocation. Site-directed mutations in these residues reduce activity of MCT1 in vitro (Manoharan et al. 2006). The activity is also inhibited or reduced when residues Arg143 or Phe360 are mutated, Phe360 being responsible for conformational changes that allow transport of bulkier monocarboxylates than wild-type MCT1 (Garcia et al.1994, Galic et al. 2003).

In mammalian cells, MCT1 plays an important role in transporting metabolites out of the producing cells and further into the cells in which continued metabolism occurs (described in detail in the chapter on lactate shuttles). In addition to transport of metabolites, the crucial function of MCT1 is pH regulation. MCT proteins transport the monocarboxylate anion across the cell membranes together with a proton (DeBrujine et al. 1983, 1985, Halestrap and Price 1999). It has been suggested that about two thirds of the proton release from muscles during exercise is coupled to lactate transport or diffusion, and only one third to Na^+/H^+ exchange or bicarbonate-dependent exchange systems (Juel 1996, 1998). The lactate/proton transport system contributes to the recovery from and prevents the development of fatigue in isolated mouse muscle cells (Westerblad and Allen 1992). In active skeletal muscle, MCT activity is of major importance for pH regulation (Juel 1996).

The direction of lactate transport via MCT is dependent on the proton gradient across the plasma membrane. Kinetic analysis suggested that the binding of H^+ followed by lactate preceded a conformational change that translocated the substrates across the membrane (Poole and Halestrap 1993). Recently, a detailed illustration of the proposed mechanism of lactate transport through MCT1 was presented (Wilson et al. 2009). In the mechanism proposed positively charged (protonated) Lys38 opens the extracellular side of the channel. Lactate from the extracellular side moves into the pore and forms an ion pair

with Lys38. The proton on Lys38 is transferred to Asp302 and this is followed by migration of lactate through the pore where it forms an ion pair with Arg306. The Phe360 together with the adjacent Asp302 and Arg306 controls channel selectivity (Manoharan et al. 2006). Once Lys38 is deprotonated, MCT1 relaxes back toward the closed state and releases lactic acid into the intracellular space

In human patients, signs of muscle injury during exercise and also subnormal erythrocyte lactate transport have been associated with the lack of proper functioning of MCT1. The reason for reduced MCT1 activity has been single nucleotide mutations either in the intracellular C-terminus or in the intracellular loop between TM6 and TM7 (Merezhinskaya et al. 2000, Cupeiro et al. 2010, Lean and Lee 2009).

2.2.4. MCT2, the high-affinity pyruvate transporter

The cDNA encoding MCT2 (SLC16A7) was first isolated from a hamster liver cDNA library (Garcia et al. 1995). The protein sequence of MCT2 appears to be less conserved than that of MCT1, showing about 65% identity and 79% similarity between rats and humans (Jackson et al. 1997). In some species the MCT2 gene produces a variety of mRNA transcripts with different molecular masses (Zhang et al. 2005). Expression of MCT2 is species- and tissue-specific. MCT2 is very abundant in hamster and pig muscle (Garcia et al. 1995, Sepponen et al. 2003), and liver, kidney, stomach, and skin preferentially express MCT2 rather than MCT1 (Garcia et al. 1995, Jackson et al. 1997).

Kinetic analysis shows that, depending on the species, MCT2 has higher or similar affinity for lactate (K_m 0.7 – 6 mM) in comparison to MCT1 and higher affinity for pyruvate (K_m 0.03 – 2.5 mM) (Lin et al. 1998, Bröer et al. 1999, Dimmer et al. 2000). The V_{max} of rat MCT2 for lactate is 20 times lower than that of rat MCT1 (Bröer et al. 1999). Such as MCT1, MCT2 is able to transport a wide variety of monocarboxylates and a 60-fold excess of lactate inhibits pyruvate transport completely and 1 mM DL-beta-hydroxybutyrate by 80% (Lin et al. 1998, Bröer et al. 1999). DIDS and CHC effectively inhibit the functioning of MCT2, but pCMBS has no effect on the activity of MCT2 (Garcia et al 1995, Lin et al. 1998).

The biological role of MCT2 is still unclear. On one hand, MCT2 is expressed in cells that use lactate or other monocarboxylates for their energy demands, such as hepatocytes and neurons (Garcia et al. 1995, Rafiki et al. 2003). On the other hand, MCT2 in pigs appears to be a housekeeping protein that exports lactate and regulates the acidification of glycolytic muscle cells at rest (Sepponen et al. 2003). A specific role in a facilitation of anaerobic glycolysis in cancer cells was also suggested (Lin et al. 1998)

2.2.5. MCT4, the low-affinity lactate exporter

MCT4 (SLC16A3) is a low-affinity transporter that is expressed abundantly in glycolytic cells, such as type IIB muscle fibers, astrocytes, and white blood cells (Wilson et al 1998, Meredith and Christian 2008). Significant amounts of messenger RNA (mRNA) were also detected in testis, small intestine, parotid gland, and lung of rat (Dimmer et al. 2000). No species differences in the expression of MCT4 were found (Dimmer et al. 2000). MCT4, as also MCT1 and MCT2, transports a wide variety of substrates, but has extremely low affinity for most of them. For example, the K_m for lactate is approximately 30 mM and values up to 153 mM have been calculated for pyruvate (Dimmer et al. 2000, Manning Fox et al. 2000). Under physiological conditions the preferred substrate for MCT4 is lactate (Dimmer et al. 2000). MCT4-mediated transport is inhibited by CHC, phloretin, and pCMBS (Dimmer et al. 2000, Manning Fox et al. 2000). DIDS reduces lactate transport, but cannot block it as effectively as it does for rat MCT1 (Dimmer et al. 2000).

It has been suggested that the role of MCT4 is to transport lactate out of cells that produce high amounts of lactate (Wilson et al. 1998). Contradiction between expression patterns of MCT4 in different muscles and species, however, occurs (Pilegaard et al. 1999, Hashimoto et al. 2005). MCT4 also transports other monocarboxylates, such as butyrate and propionate through epithelial cells of the intestine in species that have high microbial short-chain fatty acid (SCFA) production (Manning Fox et al. 2000, Kirat et al. 2005, Koho et al. 2005). To my knowledge, SCFA transport into the muscle fibers via MCTs is not thus far studied.

2.3. Ancillary proteins

Unlike the vast majority of TM proteins, MCTs are not glycosylated (Landolt-Marticorena et al. 1994, Carpenter et al. 1996). MCT need an ancillary protein for their proper localization and functioning in the plasma membrane (Kirk et al. 2000). For isoforms MCT1 and MCT4, the ancillary protein is CD147 (basigin) and for MCT2 probably gp70 (embigin)(Kirk et al. 2000, Wilson et al. 2005). In the present study, the focus is on the cooperation of CD147 with MCT isoforms 1 and 4.

2.3.1. CD147

CD147 is a widely expressed integral plasma membrane glycoprotein that belongs to the immunoglobulin (Ig) superfamily. It is expressed at particularly high levels in tumor cells, activated T cells, retinal pigment epithelium, and the neonatal blood-brain barrier (Kasinrerk et al. 1992, Seulberger et al. 1992, Biswas et al. 1995). CD147 has been identified during recent decades independently by a number of different laboratories and it has been named in the literature as basigin or gp42 in mouse (Altruda et al. 1989, Miyauchi et al. 1990), CE9 or OX-47 antigen in rat (Fossum et al. 1991, Petruszak et al.

1991), 5A11 antigen, neurothelin, or HT7 in chicken (Schlosshauer and Herzog 1990, Seulberger et al. 1990, Fadool and Linser 1993) and extracellular matrix metalloproteinase inducer (EMMPRIN) (Biswas 1995) and M6 antigen (Lagenaur et al. 1992) in humans. As the multiple names imply, CD147 has multiple tasks in tissue repairing, tumor progression, reproduction, inflammation, and in neural functioning (Muramatsu and Miyauchi 2003, Weidle et al. 2010). Targeted deletion of the *CD147* gene results in sterility, deficits in learning and memory, and blindness (Igakura et al. 1996, Naruhashi et al. 1997, Hori et al. 2000). In the present study, the name CD147 is used, regardless of the name used in the original studies.

The molecular mass of N-glycosylated CD147 is 54 kD, which is reduced to 28 kD with endoglycosidase F treatment (Kasinrerk et al. 1992). CD147 has an N-terminal extracellular domain, a single TM segment, and a short cytoplasmic tail (Fossum et al. 1991). Sequence comparison among species indicates that the TM segment is conserved and contains a leucine zipper motif and a charged glutamate residue, both of which are structures involved in protein association (Seulberger et al. 1990, Fossum et al. 1991, Kasinrerk et al. 1992). The N-terminus has two (or three) Ig-like domains that are linked with a five-residue-long flexible linker (Yu et al. 2008). In the CD147 the Ig-like domain in N-terminus is of the C2-type and proximal to the membrane is of the I-type (Yu et al. 2008). Cysteines, believed to stabilize the Ig-like domains by disulfide bonds, are also conserved (Kasinrerk et al. 1992).

mRNA splice variants have been found at least in mice and humans. Typically, these vary in the number of Ig-like domains and it was suggested that they may play different roles e.g. during development (Clamp et al. 2004).

Homo-oligomer formation plays a crucial role in the functioning of CD147, at least in mouse and chicken which CD147 can form homo-oligomers in vivo (Fadool and Linser 1996). An N-terminal Ig-like domain is necessary and sufficient for this function, and recently the mechanism in oligomerization was classified as β-strand swapping (Yoshida et al. 2000, Luo et al. 2009).

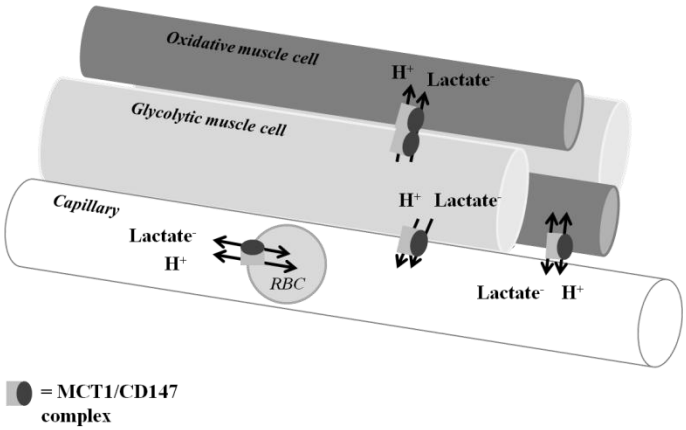

Figure 1. *The direction of the lactate transport by MCT1/CD147-complex is dependent on the concentration gradient of H^+. Glycolytic muscle cells mainly produce lactate. Oxidative muscle cells consume lactate as energy source, but during exercise they also may export lactate. Red blood cells (RBC) produce lactate, but they can also function as lactate sink during exercise.*

2.4. Coexpression of MCT1 and 4 together with CD147

The coexpression of MCTs and ancillary protein has been detected in several cell types of different species. Quite recent examples include MCT1 and CD147 in mouse kidney and in rat and pig liver and MCT1 and MCT4 together with CD147 in various tumor cells and in the gastrointestinal tract of ruminants (Koho et al. 2005, Kirat et al. 2007, Becker et al. 2010, König et al. 2010, Pinheiro et al. 2010). Studies of mammalian cell lines have shown that MCT1 and 4 transfected into cells alone do not effectively reach the plasma membrane, whereas when coexpressed with CD147, both MCT and CD147 are colocalized at the cell surface (Kirk et al. 2000). This has been proven with studies on MCT transfected *Xenopus* oocytes, in which endogenous CD147 expression was blocked with antisense cRNA (Manoharan et al. 2006). The study by Gallagher et al. (2007) shows clearly that knocking down CD147 expression in metastatic cancer cells results in MCT4 targeting to the endoplasmic reticulum (ER). Similar results were found in the retinal pigment epithelium of CD147-null mice, in which the MCT1 transcript is present, but the protein is not targeted to the plasma membrane (Philp et al. 2003). Interestingly, it was noted that vice versa maturation of CD147 is dependent on the coexpression of MCT1, while the silencing of MCT4 blocks the maturation of CD147 and prevents its cell-surface expression (Deora et al. 2005, Gallagher et al. 2007).

In the cell membrane, MCT and CD147 may function as an active tetrameric transporter complex that consists of two MCT1/CD147 heterodimers (Wilson et al. 2002). The

heterophilic interactions in CD147 may be located on N-linked glycosylation sites Asn44, Asn152 and Asn186, and the TM segment (Seulberger et al. 1990, Fossum et al. 1991, Yoshida et al. 2000, Yu et al. 2008). Based on fluorescence energy transfer (FRET) studies, the TM segments of MCT1 and CD147 are in continuing interaction and the C-termini of both proteins are in close proximity (Wilson et al. 2002). Recently, it was shown that regions near the N- and C-termini of the TM segment in CD147 interact with MCT1 (Finch et al. 2009). In contrast Glu218, which was earlier suggested to be a candidate for a member of a charge-pair forming between the TM segments of CD147 and MCT1, is not as important (Wilson et al. 2002, Manoharan et al. 2006, Finch et al. 2009).

In addition to MCT, a wide variety of proteins, such as integrins, caveolin-1, and cyclophilins interact with CD147 (Jiang and Tang 2007). CD147 thus plays important roles in proliferation, migration, adhesion, motion, and energy metabolism of the cell (Jiang and Tang 2007).

2.5. Expression of MCTs in muscle

At the whole-body level, muscle is the main lactate producer during exercise and the main lactate consumer at rest. The expression of MCT isoforms in muscle is dependent on the muscle fiber type. The oxidative muscle fiber composition of rat correlates with MCT1 expression, and lactate transport in rat and rabbit is 39 – 50% faster in oxidative muscle fibers than in glycolytic fibers (Juel et al. 1991, McCullagh et al. 1996). MCT1 transports lactate according to the H^+-gradient across the plasma membrane, thus at rest mainly out of the cells, but during exercise it may facilitate the influx of lactate into the oxidative muscle fibers for energy supply (Juel and Halestrap 1999). Glycolytic muscle fibers especially express isoform MCT4, which has low lactate affinity and high maximal transport velocity (V_{max}) (Wilson et al. 1998, Dimmer et al. 2000). MCT4 is needed during intense exercise, when high amounts of lactate are produced and the pH inside the muscle cells needs to be regulated (Wilson et al 1998). If lactate cannot be transported into the oxidative muscle fibers in the neighborhood, it may enter the circulation and be metabolized in other skeletal muscles, heart, and liver (see Gladden 2004).

Endurance training in humans increases the amount of MCT1 in muscle by 18 - 90%, but does not affect the amount of MCT4 (Pilegaard et al. 1999, Dubouchaud et al. 2000). The similar increase in the amount of MCT1, but not in that of MCT4 was also found in study, in which red and white muscles of rat were chronically stimulated (Bonen et al. 2000). High-intensity training increases the amount of both MCT1 and MCT4 by 11 – 76%, increasing MCT4, however, less than MCT1 (Pilegaard et al. 1999, Juel et al. 2004). The distinct role of MCT4 as a lactate exporter is supported by the up-regulation of the *MCT4* gene by hypoxia-inducible transcription factor (HIF)-1 (Ullah et al. 2006).

In the domestic pig, whose muscles are mainly glycolytic, MCT1 is expressed at very low amounts, while the expression of MCT2 and MCT4 is high (Sepponen et al. 2003). In pig muscles MCT2 may function as a housekeeping protein that prevents acidification at rest, while MCT4 may be needed to export the flood of lactate after exercise (Sepponen et al. 2003).

2.6. Lactate shuttles

MCT proteins are expressed in all tissues in the body. The effective transport route is a prerequisite for the lactate shuttle hypothesis that postulates lactate being utilized directly in the mitochondria of the producing cell (intracellular lactate shuttle) or in tissues that have more capacity to oxidize lactate than the producing tissue itself (cell-cell lactate shuttle) (Brooks 2000, 2009). The intracellular lactate shuttle is supported by the presence of a mitochondrially localized MCT1 (mMCT1) that is suggested to be a part of a mitochondrial lactate oxidation complex (Butz et al. 2004, Hashimoto et al. 2005). mMCT1 has been found also in horses (Mykkänen et al. 2010a). Cell-cell monocarboxylate shuttle is especially important in tissues that have high energy demands, such as muscle and brain (Bergersen 2007). Different cell types in a tissue may express characteristic isoforms and thus support each other's functioning. Cell-cell lactate shuttles were reported, e.g. between oxidative astrocytes and glycolytic neurons and also between oxidative and glycolytic muscle cells (Magistretti and Pellerin 1999, Hashimoto et al. 2005). Considerable interest has also been shown in the role of different MCT isoforms in the eye and gastrointestinal tract (Kirat et al. 2005, 2006a, b, Koho et al. 2005, Philp et al. 1998, Sepponen et al. 2007).

3. AIMS

In Standardbred horses, the lactate distribution between plasma and RBCs varies individually after exercise. This variation is due to the rate of lactate influx into RBCs and is dependent on the activity of MCT protein. According to the rate of lactate transport, horses can be divided into HTA or LTA groups. Although the transport activity can change slightly due to training or ageing, the inherent activity level remains in adulthood. In this thesis, the aim was to elucidate the mechanism that silences MCT-mediated transport in LTA horses, and also extend the studies to the muscles in which the effective lactate efflux is of major importance during exercise.

The specific aims of the studies were:

- to determine the expression of MCT isoforms in equine RBCs and to determine whether the LTA is due to lack of the MCT proteins or the ancillary protein CD147 (I).

- to examine the expression of CD147, MCT1, MCT2, and MCT4 in the middle gluteal muscle of horses and compare the results to those measured in RBCs (II).

- to obtain the coding sequences (cds) of horse MCT1 and CD147 cDNA to raise horse-specific antibodies and to determine whether a SNP results in differences in lactate transport activity in healthy and myopathic horses (III, IV).

- to obtain the sequences of the 3´- and 5´-untranslated regions (UTR) of MCT1 and CD147 cDNA, as well as the sequence of the CD147 promoter region, and to determine the mRNA concentrations of MCT1 and CD147 in horses with high and low levels of CD147 expression (IV).

4. MATERIAL AND METHODS

4.1. Ethical considerations

The protocols were approved by the Ethics Committee for Animal Experiments of the Faculty of Veterinary Medicine, University of Helsinki (I, III and IV) and by the Ethics Committee of Agrifood Research Finland (II).

4.2. Horses and samples

In all, 34 Standardbred horses were used, 19 of which were fillies or mares, 10 were colts or stallions, and 5 were geldings (I – III). The ages of the horses were 2 – 14 years (mean 6 years). The horses were privately owned and samples were taken during training at the track or at rest in the clinic or at their home stable. The owners' or trainers' permission was a prerequisite for sampling. A total of 24 horses, representing different breeds, were colts or stallions that were admitted to the Equine Hospital of the University of Helsinki for castration (IV). In addition, samples were taken from 15 Standardbreds in their home stables (IV). The horses were clinically healthy, except for 19 Standardbreds (III, IV) that were reported by their owners to have signs of myopathy after intense exercise.

Blood samples were drawn from the jugular vein into the tubes that contained ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The samples were cooled immediately in ice water or on ice. An aliquot of each sample was taken for lactate transport activity and lactate concentration analysis, and the rest of the sample was centrifuged at 2000 g to separate the plasma and RBCs. Long -term storage of plasma and RBCs was done at -80 °C. Short-term storage of RBCs for lactate transport activity analysis was done at 4 °C.

Muscle biopsy samples (approximately 200 – 400 mg) were taken under local anesthesia from the middle gluteal muscle at a depth of 6 cm, using modified 5-mm Bergström biopsy needle (Lindholm and Piehl 1974). The samples were blotted dry, frozen in liquid nitrogen, and stored at -80 °C until analyzed.

The cremaster muscle was partially removed during castration. Samples of the muscle tissue were cut from the bundles of muscle cells within 5 min of muscle removal. The samples were processed as biopsy samples.

I

In all, 10 Standardbred horses (5 fillies and 5 colts), 2 – 4 years of age (mean 3 years), were used. All horses were in regular training and just beginning their trotting careers. The horses were exercised on a straight 700-m track, two at a time. The horses trotted the

first bout at a speed of 10.8 m/s, the second at 11.4 m/s, and the last at 12.2 m/s. The time between bouts was about 5 min, during which the horses trotted slowly back to the beginning of the track. Blood samples were drawn at rest before the exercise, and after each of the three exercise bouts.

II

In all, 14 Standardbred horses (6 fillies or mares), 5 stallions or colts and 3 geldings, 2 – 14 years of age (mean 8 years) were used. The horses were either moderately trained (4 horses) or were regarded as race-fit (10 horses). The horses regarded as race-fit were participated in trotting races within 1 month from sampling. Blood samples were drawn at rest and muscle biopsy samples were taken later under local anesthesia.

III

Blood samples and muscle biopsy samples were collected from 10 Standardbred horses (8 fillies or mares and 2 geldings), at rest. The age of the horses was between 3 and 11 years (mean 5 years). Three of the horses were reported to be healthy and seven were reported by their owners to have muscle stiffness or other signs of muscle myopathy after intense exercise.

IV

Blood and muscle samples were collected from 24 colts or stallions representing the following breeds: Standardbred horse (n = 6), Finnhorse (7), Warmblood (10), and Icelandic horse (1). The age of the horses was 1.5 – 16 years (mean 4 years). Muscle samples were taken during castration from the expendable cremaster muscle. The castration was performed under general anesthesia according to the standard protocol of the Equine Hospital of the University of Helsinki.

In addition, blood and muscle (gluteus medius) samples were taken from 15 Standardbred horses (11 mares, 2 stallions, 2 geldings), that were 3 – 18 years of age (mean 7 years). The horses were selected beforehand to present both low (n = 7) and high (n = 8) levels of CD147 expression in the RBCs. 12 of the horses were reported by their owners to have signs of myopathy after intense exercise. The overview of the horses, samples and analysis is presented in Table 1.

Table 1. Overview of horses, samples and analyses in studies I – IV. Sex: stallions/colts + mares/fillies + geldings. Age is presented as the range of the years.

Study	Breed	Number	Sex	Age	Samples	Analyses
I	Standardbreds	10	5 + 5 + 0	2 – 4	RBC	Transport activity (lactate, pyruvate), Western Blot (MCT1, 2 and CD147)
II	Standardbreds	14	5 + 6 + 3	2 – 14	RBC, muscle (membranes)	Transport activity (lactate), Western Blot (MCT1, 2 and 4, CD147)
III	Standardbreds	10	0 + 8 + 2	3 - 11	RBC, muscle (DNA, mRNA)	Transport activity (lactate), histochemistry, sequencing
IV	Standardbreds Finnhorse Warmblood Icelandic horse	6 7 10 1	24 + 0 + 0	1 – 16	RBC, muscle (DNA, mRNA, membranes)	Western Blot (MCT1, CD147), sequencing
	Standardbreds	15	2 + 11 + 2	3 – 18	RBC, muscle (DNA, mRNA, membranes)	qPCR, Western Blot (CD147)

4.3. Methods

4.3.1. Blood/plasma lactate concentration analysis (I, IV)

For whole-blood lactate analysis, an aliquot of EDTA blood was transferred into tubes containing sodium fluoride and the rest of the blood was immediately cooled down in ice water. For the analysis of plasma lactate concentration, the plasma was separated rapidly by centrifugation. The lactate concentrations in blood and plasma were measured with a lactate analyzer (YSI 2300 STAT Plus, YSI Inc, Yellow Springs, OH, USA) within 24 h after sampling (I). The plasma lactate concentration was measured during the castration operation and recorded every half hour (ABL800 Flex, Radiometer, Copenhagen, Denmark) (IV).

4.3.2. Measurement of enzyme activities (IV)

The enzyme activities of creatine kinase (CK) and aspartate aminotransferase (AST) were measured in association with castration (KONE Pro Selective Chemistry Analyzer, Thermo Fisher Scientific, Vantaa, Finland). The activities were measured from venous blood samples before and 4 h after surgery, and 24 hours after surgery in 9 of the 24 horses.

4.3.3. Measurement of lactate and pyruvate transport activity (I, II)

The blood was centrifuged, and the RBCs washed, as described in detail by Väihkönen and Pösö (1998). The influx of radioactive lactate (sodium L-[U-^{14}C] lactate) into the RBC was measured in a N-[2-hydroxyethyl]piperazine-N'-2-ethanesulfonic acid] (HEPES) buffer suspension, in which the packed cell volume (PCV) of the washed RBC was adjusted to 30% (Väihkönen and Pösö 1998). The lactate concentration in the suspension was either 30 mM or 10 mM, representing the concentrations in equine plasma after maximal and submaximal exercise (Pösö et al. 1995). For the measurement of K_m values, lactate transport into the RBCs was measured at lactate concentrations of 0.1 – 5.0 mM. After 20-s incubation at 37 °C and washes in ice-cold buffer, the concentration of radioactive lactate in the RBCs was measured with a liquid scintillation counter (WinSpectral 1414, Wallac Oy, Turku, Finland). The procedure is described in detail by Skelton et al. (1995) and Väihkönen and Pösö (1998). Pyruvate transport activity was measured similarly as that of lactate at pyruvate concentrations of 0.1, 0.5, and 1 mM ([1-^{14}C] pyruvate).

The pyruvate and lactate transport activity measurements were also performed in the presence of inhibitors. CHC at a concentration of 5 mM was used in as a specific inhibitor of MCT1 and DIDS at a concentration of 0.2 mM as an inhibitor of AE1 (I). All transport activity measurements were performed in triplicate.

Table 2. Primary and secondary antibodies used in studies.

PRIMARY ANTIBODIES

Antibody	Made in / Against	Peptide	Manufacturer/Code	Used in studies
MCT1	Rabbit / Human	CKDTEGGPKEEESPV	Sigma Genosys	I, II
MCT1	Rabbit / Horse	CKGTEGDPKEESPL	Sigma Genosys	IV
MCT2	Chicken / Rat	NTHNPPSDRDKESSI	Chemicon/AB1287	I, II
MCT4	Rabbit / Horse =Human	CEPEKNGEVVHTPETSV	Sigma Genosys	I, II
CD147	Rabbit / Human	against Oka2 antigen	by Dr. Mawby, Bristol University, UK	I-IV
CD147	Mouse / Human	monoclonal	Sanbio/MON1071	II
CD147	Rabbit / Horse	CGHHVNDKDKNVRQRNAS	Sigma Genosys	IV

SECONDARY ANTIBODIES

Against	Host	Conjugate	Manufacturer/Code	Used in studies
Rabbit	Goat	HRP (horse radish peroxidase)	DAKO/P 0448	I-IV
Chicken	Rabbit	HRP	Chemicon/AP162P	I-II
Mouse	Goat	HRP	Zymed/81-6520	II

4.3.4. Isolation of RBC (I – IV) and muscle membranes (II, IV)

The RBC membranes for Western blotting were isolated either from fresh (I, II) or frozen RBCs (III, IV). The fresh RBCs were washed three times with tricine buffer (150 mM NaCl, 10 mM tricine, pH 8.0) and later hemolyzed with 5 mM sodiumphosphate buffer at pH 8.0. Hemolysed RBCs as well as thawed RBCs were washed with sodium phosphate buffer 3 – 4 times to remove hemoglobin from the samples. The plasma membranes of the muscle cells were isolated, using Percoll™ (P-1644, Sigma, StLouis, MO, USA) gradient centrifugation as adapted for biopsy scale samples from Jackson et al. (1997). All the plasma membranes were stored at -80 °C after determination of the protein concentration with a bicinchoninic acid (BCA) protein assay (Uptima BC Assay; Interchim, Montlucon, France).

4.3.5. Determination of MCT and CD147 expression (I – IV)

The amounts of MCT isoforms in the plasma membranes of RBCs and muscles were determined by Western blotting, as described in detail elsewhere (I, II). The primary antibodies used were commercially produced or ordered from Sigma Genosys (Cambridge, UK). The details of the antibodies are presented in Table 2. Unpurified primary antiserum against human CD147 was a generous gift from Dr. W. Mawby (University of Bristol, UK).

The custom-made antibodies were induced in rabbits against keyhole limpet hemocyanin-conjugated peptides. The peptides corresponded to the C-terminal region of the proteins. Two rabbits were immunized for each antibody and five antigen injections were included in the immunization protocol. Three test bleeds were taken during the 11-week protocol to confirm the production of the antibody that recognized the respective equine protein. The antibodies were affinity-purified from the terminal bleed, using affinity chromatography.

Secondary antibodies included horseradish peroxidase (HRP)-conjugated goat antirabbit antibody (DAKO, Glostrup, Denmark) for MCT1, MCT4, and CD147, HRP-conjugated goat antimouse antibody (Zymed, San Francisco, USA) for CD147 in muscles, and HRP-conjugated goat antichicken antibody (Chemicon International, Temecula, CA, USA; now Millipore, Billerica, MA, USA) for MCT2 (Table 2).

Immunodetection of Western-blotted proteins was performed by a chemiluminescence reaction (SuperSignal®, WestDura, Pierce, IL, USA) using X-ray films and Fluor-S densitometry (BioRad laboratories, Hercules, CA, USA) (I –II) or a charge coupled device (CCD) camera (Fuji LAS-3000; Raytest, Straubenhardt, Germany) (II –IV). The

signal intensities were quantified with Multianalyst Version 1.1. (BioRad) (I) or AIDA software Version 4.03 (Raytest) (II –IV).

4.3.6 Histochemistry (III, IV)

A piece of gluteus muscle was mounted on a specimen holder with optimal cutting temperature (OCT) embedding medium (Sakura Finetek, Zoeterwoude, the Netherlands). The 20-µm sections were cut in a cryostat and mounted on slides for histochemical staining. Muscle biopsy samples were stained with periodic acid-Schiff (PAS) reaction for glycogen (Pearse 1961). Parallel sections were incubated with amylase for 30 min before PAS staining.

Several 10-µm sections of cremaster muscle were stained with adenosine triphosphatase (ATPase) after acidic (pH 4.63) preincubation for fiber-type analysis (Brooke and Kaiser 1970).

4.3.7. Isolation of nucleic acids and sequencing of MCT1 and CD147 (III, IV)

Genomic DNA was extracted according to Miller et al. (1988). Total RNA was extracted, using a FastRNA Pro Green Kit (QBIOgene, Irvine, CA, USA) (III), QIAzol Lysis Reagent (Qiagen,Valencia, CA, USA) (gluteus samples in IV), or with a single-step guanidine method by Chomczynski and Sacchi (1987) (cremaster samples in IV). The mRNA fraction from the total RNA was isolated with a Poly(A)Purist^{TM} MAG kit according to the manufacturer´s instructions (Ambion, Cambridgeshire, UK).

Polymerase chain reactions (PCRs), described in detail elsewhere (III, IV), were used to amplify the regions for sequencing. The PCR kits used were from Clontech (Palo Alto, CA, USA) or Roche (Branchburg, NJ, USA) and the PCR reactions were performed in a reaction volume of 50 ul, using a PTC-200 thermocycler (MJ Research, Watertown, MA, USA). The PCR products were sequenced with an ABI 3130*xl* Genetic analyzer (Applied Biosystems, Foster City, CA, USA) at the DNA Sequencing and Genomics Laboratory, Institute of Biotechnology, University of Helsinki.

4.3.8. Quantitative PCR (IV)

The total RNA content of the samples was quantified with a NanoDrop fluorospectrometer (NanoDrop, Wilmington, DE, USA). The cDNA first strand was synthesized from 0.3 µg of total RNA, using a RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania). Quantitative PCR (qPCR) was performed, using a DyNAmo™ Flash Probe qPCR Kit (Finnzymes, Espoo, Finland) and an Mx3000P qPCR system with MxPro qPCR Software (Stratagene, La Jolla, CA, USA). Hydrolysis probes and primers were designed with the aid of the Primer Express program (Applied Biosystems) from the sequences of horse CD147 (Genbank AY457175.1) and

MCT1 (Genbank EF564280.1). The ribosomal protein gene 18S ribosomal RNA (rRNA) was used as an endogenous control. The sequences of the primers and the probe were designed from the partial 18S rRNA of the horse (AJ311673.1). The expression levels of the 18S rRNA were checked so as to be similar in all horses studied to ensure that the results were comparable and valid. The efficiencies of amplification varied between 94.2% and 108.8% and the R^2 of the calibration curve between 0.908 and 0.994. The differences in the concentration of samples were determined with the $\Delta\Delta C_q$ method.

4.3.9 Statistical analysis

The results are shown as mean ± standard deviation (II, III) and mean ± standard error (I, IV). Statistical differences between the groups were calculated with the Student´s t-test or Mann-Whitney U-test. The effect of inhibitors in lactate transport activity studies were calculated by paired t-test. Linear regression analysis and Spearman´s correlation analysis was used to analyze the correlations. For SNP analysis Haploview software (Barrett et al. 2005) was used. The results were regarded as significant at $P < 0.05$.

5. RESULTS

5.1. Red blood cells

5.1.1. Lactate transport activity

The lactate transport activity of the RBCs was measured as the influx of radioactive-labeled lactate across the RBC membrane (I - III). The influx varied between 176 and 1736 nmol ml $^{-1}$ cells min^{-1} at 10 mM lactate (n = 31) and between 299 and 6593 nmol ml $^{-1}$ cells min^{-1} at 30 mM lactate (n = 22) (Table 3). The K_m values for lactate transport in the RBCs were 0.63 ± 0.18 mM in LTA horses (n = 5) and 0.88 ± 0.38 mM in HTA horses (n = 3). The lactate transport activity was reported as nmol mg^{-1} protein min^{-1} in studies I and II.

As mentioned earlier, the horses could be divided into two groups according to their lactate transport activity (Figure 2). The boundary between the HTA horses and LTA horses was set at a lactate transport activity of 1.0 nmol mg $^{-1}$ protein min^{-1} at a10-mM lactate concentration that corresponds approximately to 370 nmol ml^{-1} min^{-1} (Väihkönen and Pösö 1998). The mean transport activity in HTA horses (n = 19) was 1188 ± 53 nmol ml $^{-1}$ min^{-1} and in LTA horses (n = 11) 214 ± 9 nmol ml^{-1} min^{-1} at a 10-mM lactate concentration ($P < 0.001$) (V). One horse showed an intermediate value of 478 nmol ml^{-1} min^{-1} and was not included in either group (II).

In HTA horses, 54 – 62% of the lactate transport could be inhibited with CHC, while in LTA horses the percentage was 12 – 26% and in one horse the effect of CHC was

positive. DIDS inhibited 7 – 23% of the lactate transport activity in LTA horses and in HTA horses both activation and inhibition was observed (I).

After moderate exercise 46 – 68% of the whole-blood lactate was in the RBCs in HTA horses, while in LTA horses the percentage was between 26% and 52% ($P < 0.001$)(I).

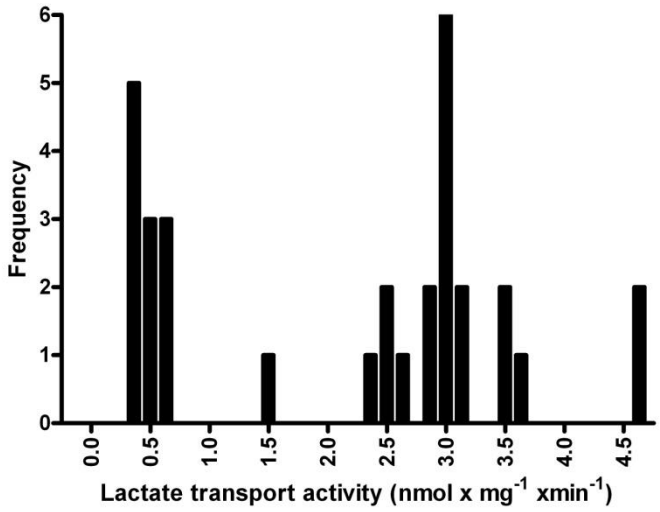

Figure 2. *Frequency distribution of the lactate transport activity in the RBCs of all horses (I – III). Lactate transport activity was measured at a lactate concentration of 10 mM.*

5.1.2. Pyruvate transport activity

The pyruvate transport activity of the RBCs was measured as the lactate transport activity described above at 0.1, 0.5, and 1.0 mM pyruvate concentration (I). The activity of pyruvate transport into the RBCs varied between 26 and 81 nmol ml^{-1} min^{-1} at a 1.0-mM pyruvate concentration (Table 3). The transport activity was higher in HTA horses ($n = 3$) than in LTA horses ($n = 5$) at a 1.0-mM concentration ($P < 0.01$), but no differences were found at 0.1 mM and 0.5 mM. Comparison of the pyruvate transport activity and lactate transport activity at 0.5-mM concentrations showed that the ratio of pyruvate transport/lactate transport was higher in LTA horses than in HTA horses ($P < 0.001$). The result was also similar in other concentrations, except at 0.1 mM. At a 1.0-mM pyruvate concentration, CHC inhibited 46 – 71% of the pyruvate transport in both groups of horses. DIDS inhibited pyruvate transport by 70 – 85% in LTA horses, but activated it by 54 – 79% in HTA horses.

Table 3. Lactate and pyruvate transport activities and inhibition of transport with 5 mM α-cyano-4-hydroxycinnamate (CHC) and 0.2 mM 4, 4'-diisocyanostilbene-2, 2'-disulfonic acid (DIDS) in the RBCs. In all, 5 high transport activity (HTA) and 3 low transport activity (LTA) Standardbred horses was included (I). * = P < 0.05; ‡ = P < 0.01; † = P < 0.001 for the corresponding activity without inhibitors. § = P < 0.05; ¤ = P < 0.01; # = P < 0.001 for the corresponding value in the HTA group. (V)

		HTA		LTA	
Lactate (mM)	**Inhibitor**	**Transport activity (nmol x ml^{-1} x min^{-1})**	**Inhibition%**	**Transport activity (nmol x ml^{-1} x min^{-1})**	**Inhibition%**
0.5	-	63 ± 5		13 ± 2	
10	-	999 ± 105		225 ± 24	
10	CHC	427 ± 26*	57 ± 4	197 ± 13	11 ± 13#
10	DIDS	864 ± 131	12 ± 24	189 ± 20*	15 ± 6
Pyruvate (mM)					
0.5	–	53 ± 19		35 ± 2	
1	–	59 ± 29		37 ± 3¤	
1	CHC	21 ± 8‡	57 ± 13	12 ± 2†	68 ± 3
1	DIDS	86 ± 29	-66 ± 13	9 ± 2†§	76 ± 6#

5.1.3. Blood chemistry

The plasma lactate concentrations during anesthesia were 1.2 ± 0.1 mM before the surgery, 1.5 ± 0.1 mM at 30 min and 1.6 ± 0.1 mM at 60 min. The mean activity of AST did not change in the 24 hours after the surgery and was 361 ± 9 U/l. The CK activity was 194 ± 12 U/l before the surgery, 286 ± 31 U/l four hours after the surgery and 280 ± 24 U/l 24 hours after the surgery (IV). At 24 hours the activities were measured from nine horses.

5.1.4. CD147 expression in RBCs

CD147 was found in the RBC membranes at a molecular mass of approximately 50 kD. The results that were obtained using horse-specific antibody and those that were obtained using unpurified serum against human CD147 correlated with each other ($R = 0.69$, $P < 0.01$). The amount of CD147 in RBC membranes was highly variable and was significantly higher in HTA horses than in LTA horses ($P < 0.001$). Horses could be divided into the same two groups according to their lactate transport activity and according to the amount of CD147 in their RBC membranes (II, IV). The horse groups that were distributed according to their CD147 expression were named high-expression (HE) and negligible-expression (NE) horses and the amounts of CD147 were 79 ± 16 arbitrary units (AU) and 0.1 ± 0.1 AU, respectively (IV).

5.1.5. MCT expression in RBCs

MCT isoforms MCT1 and MCT2 were found in the RBCs of horses. With antihuman antibody, both of these proteins gave the strongest signal at a molecular mass of 76 – 80 kD. When studied with horse-specific anti-MCT1 antibody the strongest signal was found at a molecular mass of 50 kD, but weaker bands at greater molecular masses were also found. Since the results obtained with antihuman MCT1 antibody (I, II) differed from the results obtained with horse-specific antibody (IV), the former results need to be re-evaluated. While tested with the horse-specific antibody the amount of MCT in RBCs was 397 ± 55 in HE horses and 6 ± 3 in NE horses (IV). A positive correlation between the amounts of MCT1 and CD147 in RBCs was found ($R = 0.82$, $P < 0.001$). This correlation was not observed using antihuman antibodies.

MCT2 was detected in these studies with antirat MCT2 antibody (I, II). MCT2 was found in the RBC membranes of all horses at a molecular mass of 80 kD and the amount showed no large interindividual variation. The expression of MCT2 in the RBCs of horses was later confirmed with a horse-specific anti-MCT2 antibody (Mykkänen et al. 2010b).

5.2. Muscles

5.2.1. Antibodies

Expression of MCT1 and CD147 in muscle samples was studied using antihuman (II) and horse-specific antibodies (IV). The anti-MCT2 antibody was raised in chicken against the C-terminal peptide of rat MCT2. The antibody against MCT4 was raised in rabbit against a

peptide synthesized according to the C-terminus of human MCT4. The human C-terminal sequence of MCT4 is identical to that of horse.

5.2.2. Gluteus muscle

In the gluteus muscle, MCT1 was found, using human-specific antibody, at a molecular mass of 55 kD and MCT4 at 45 kD (II). MCT2 was detected at a molecular mass of 41 kD, but at very low amounts. The ancillary protein CD147 was found in the gluteus muscle at a molecular mass of 52 kD. No correlation was found between MCT1 and CD147. The expression of CD147 and MCT1 was similar in HTA and LTA horses, although the tendency for higher expression was in HTA horses. The amounts of MCT4 and CD147 correlated positively with each other ($R = 0.80$, $P < 0.01$).

5.2.3. Cremaster muscle

The expression of MCT1 and CD147 in cremaster muscle was studied with horse-specific antibodies (IV). Both proteins were detected at a molecular mass of 48 kD. The amount of MCT1 in cremaster muscle was 113 ± 25 AU in HE horses ($n = 6$) and 19 ± 18 AU in NE horses ($n = 2$). The amount of CD147 was 43 ± 9 AU in HE horses and 13 ± 6 AU in NE horses. A positive correlation between CD147 and MCT1 expression was found ($R = 0.91$, $P < 0.01$). In the cremaster muscle the expression of MCT2 and MCT4 was not studied.

5.2.4. Effect of training and/or age

The expressions of MCT1, MCT4, and CD147 in gluteus muscle were compared between the race-fit horses ($n = 10$) and horses that were moderately trained ($n = 4$) (II). The race-fit horses showed higher MCT4 expression than moderately trained horses ($P < 0.05$). The amount of MCT1 in muscle or in RBC was not dependent on training status. Since the more intensively trained horses were also older (median 9 years, range 7 – 14 years) than the moderately trained horses (median 3.5 years, range 2 – 5 years), the training status and age cannot be distinguished in this study.

5.3. Correlations between RBCs and muscle

The amount of CD147 in the gluteus muscle correlated with the amount of CD147 in RBCs ($P < 0.05$, $R = 0.62$) (II). The amount of MCT1 in the cremaster muscle correlated with the amount of MCT1 in RBCs ($P < 0.05$, $R = 0.74$) (IV). No correlation between the amounts of CD147 in cremaster muscle and RBC was found (IV).

5.4. Sequence variations

The cDNA sequences of *CD147* and *MCT1* were determined in 34 horses (III, IV). The aim was to find possible SNPs in MCT1 or in CD147 that may affect the activity of the transport complex. cDNA-derived amino acid sequences were used to produce horse-specific antibodies.

5.4.1. MCT1

The entire coding sequence (cds) of MCT1, corresponding to nucleotides 202 – 1739 at GenBank database entry AY457175.1, was sequenced from the cDNA of 24 horses. The sequencing revealed two SNPs (Table 4). The first was 1498G>A which causes a $Val_{432}Ile$ change in TM12. This SNP was heterozygous in five horses. The second was 1573A>C, which causes the change $Lys_{457}Gln$ in the C-terminus of MCT1. This homozygous SNP was found in three Finnhorses. SNP 1498G>A was in Hardy-Weinberg equilibrium, but SNP 1573A>C was not.

5.4.2. CD147

The promoter region of CD147, including nucleotides - 425 – 57 from the transcription initiation site, was sequenced from the genomic DNA of 24 horses (IV); no mutations were found in this region. The entire cds, the 5´-flanking region, and the 3´-UTR from the cDNA of CD147 were also sequenced (III, IV). These sequences were submitted to GenBank and assigned accession number EF564280.1. The extracellular part of the equine CD147 molecule consisted of two Ig-like domains. SNPs in cds were found two (Table 4). One SNP was 389A>G, which causes the change $Met_{125}Val$ in the Ig-like domain proximal to plasma membrane. This SNP was homozygous in four horses and heterozygous in nine horses (III, IV). The other SNP was a silent 511G>A, which encodes serine at nucleotide 164. This SNP was homozygous in two of the horses and heterozygous in seven horses. At the 3´UTR, the SNP 888G>C was homozygous in four horses and heterozygous in nine and 990C>T homozygous in one horse and heterozygous in four horses. All the SNPs in CD147 were in Hardy-Weinberg equilibrium.

In one Warmblood a mutation 168A>G in CD147 was found. This mutation caused change $Ile_{51}Val$ in an N-terminal Ig-like domain. In comparison to other Warmbloods, this horse expressed less CD147 and MCT1 in the RBC membrane. The amounts of CD147 and MCT1 were 22 AU and 78 AU, respectively, in comparison to the amounts of 82 ± 17 AU and 414 ± 55 AU in other Warmbloods. This horse also showed low expression of CD147 (91 AU) in the cremaster muscle in comparison to other HTA Warmbloods (519 ± 101 AU). Interestingly, this horse showed elevated plasma lactate concentrations during anesthesia (2.3 mM at 30 min and 2.6 mM at 60 min) and also elevated plasma CK activities after surgery (838 U/l).

Table 4. Nucleotide changes in the cDNA of MCT1 and CD147, and corresponding changes in amino acid sequences in castrated horses (V).

	MCT1		CD147			
Nucleotide	**1498**	**1573**	**389**	**511**	**888**	**990**
Breed/No.						
Warmbloods						
1	G	A	A	G	G	C
2	R	A	A	G	G	C
6	R	A	A	R	S	C
12*	G	A	A	G	G	C
15	G	A	A	G	S	C
17	G	A	A	A	C	C
18	R	A	A	G	S	C
19	G	A	A	R	S	C
20	R	A	A	R	S	C
22	G	A	A	G	S	C
Finnhorses						
3	G	A	A	G	G	C
5	G	C	A	R	S	C
9	R	A	A	R	S	C
10*	G	A	A	A	C	C
21	G	C	A	G	G	G
23	G	A	A	G	G	C
24	G	C	A	G	G	C
Standardbreds						
4	G	A	R	R	S	Y
7*	G	A	R	R	C	Y
8	G	A	G	G	G	T
13	G	A	R	G	G	Y
14	G	A	R	G	G	C
16	G	A	A	G	G	C
Icelandic horse						
11*	G	A	A	G	C	C
	$Val_{432}Ile$	$Lys_{457}Gln$	$Met_{125}Val$	$Ser_{164}Ser$	-	-

Numbering of nucleotides is corresponding to the GenBank database entries AY457175.1 for MCT1 and EF564280.1 for CD147. Respective protein accession numbers are AAR21622.1 and ABQ53583.1 Horses with negligible CD147 and MCT1 expression are marked with asterisk.

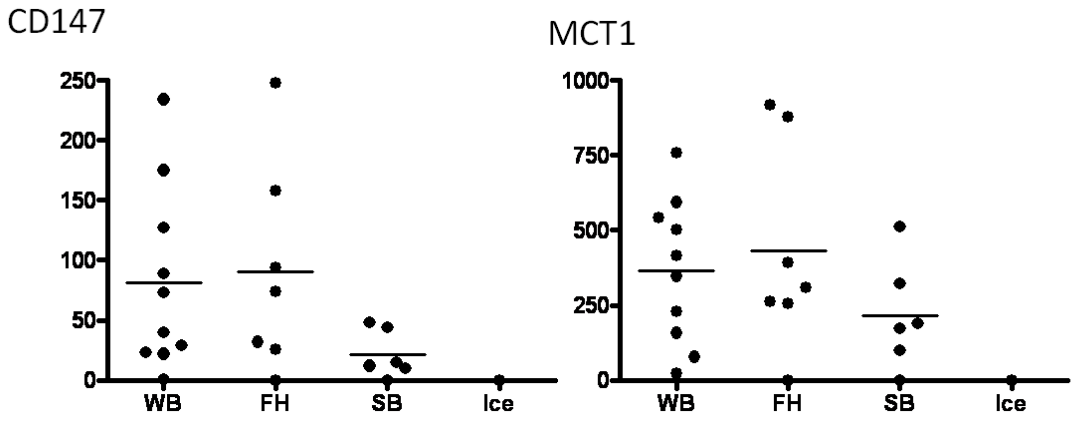

Figure 3. *CD147 and MCT1 expression in the RBCs of different breeds (V). Differences are non-significant.*

5.4.3. Differences between the breeds

Horses with high and negligible expression of CD147 and MCT1 were found in all the breeds (Figure 3). The heterozygous SNP 1498G>A in the cDNA of MCT1 was found in one Finnhorse and in four Warmblood horses. SNP 1573A>C was found only in three Finnhorses. The synonymous SNP 511G>A as well as SNP 888G>C were evenly distributed among horse breeds. The single Icelandic horse was not included in this examination. The SNP 389A>G was found only in Standardbreds, as well as the SNP 990C>T that was found in parallel with the SNP 389A>G.

The four Standardbreds that had the heterozygous SNPs 389A>G and 990C>T expressed the lowest amounts of CD147 in their RBCs although only one of them was included in the NE group (IV). The amounts of CD147 were 9 ± 3 AU in the above-mentioned four heterozygous Standardbreds in comparison to 46 ± 2 AU in two homozygous (A/A and G/G) Standardbreds. The 389A/A homozygotes in the other breeds showed the amount of 80 ± 17 AU. If the NE horses were excluded, the expression of CD147 in the heterozygote Standardbreds was 12 ± 1 AU in comparison to 46 ± 2 AU in homozygote Standardbreds and 95 ± 18 AU in homozygotes in other breeds. The differences between the groups were statistically significant ($P < 0.001$).

5.5. qPCR

qPCR was performed from the cDNA of the cremaster muscle of four HE and four NE horses. All the breeds in the study were representative. The mean efficiency of amplification in each individual probe/primer pair was 105% for MCT1 and 99% for CD147 and 18S RNA. In the cremaster muscle, the mRNA concentrations of CD147 and MCT1 did not differ significantly between HE and NE horses. However, there was a tendency toward higher concentrations of both mRNAs in HE horses. The qPCR from the cDNA of the gluteus

muscle was performed from eight HE Standardbreds and seven NE Standardbreds. The mRNA levels of CD147 and MCT1 were significantly higher in HE horses ($P < 0.05$). The statistics was similar if the C_q values were analyzed instead of the $\Delta\Delta C_q$ values.

5.6. Fiber-type analysis of the cremaster muscle

The fiber-type composition of the cremaster muscle was analyzed histochemically with ATPase staining. The percentage of muscle fiber type I, IIA, and IIB was 25 ± 7%, 70 ± 10%, and 6 ± 9%. In 12 of the 24 horses, no IIB fibers were found.

5.7. PAS staining

In all horses, the amylase treatment digested glycogen in the cremaster muscle samples.

5.8. Conclusion of the results

- The main lactate transporter in equine RBC is MCT1, which needs the ancillary protein CD147 for its function. In addition to MCT1, another isoform, MCT2, was found in the RBCs of horses. (I)

- In muscle, the isoforms MCT1 and MCT4 were found in abundance. Only traces of MCT2 were found. Training and/or ageing increased the amount of MCT4 in the gluteus muscle. The correlation of MCT1 and CD147 between RBCs and muscles was found, but additional studies are needed. (II)

- Sequence variations were found in the *MCT1* and *CD147* gene, but they were not the reason for negligible protein expression or the signs of myopathy. Sequence variations in the *CD147* gene may, however, decrease the expression of CD147 in RBC membranes and muscles and induce physiological problems, e.g. during anesthesia. (III, IV)

-The mRNA levels of both MCT1 and CD147 in the gluteus muscle were higher in HE horses and lower in NE horses. This suggests that the expression of MCT1 and CD147 is regulated in parallel manner. (IV)

6. DISCUSSION

6.1 Antibodies

One of the aims here was to determine, which MCT isoforms are expressed in the RBCs and muscles of horses. The isoforms were detected, using antibodies against MCT and CD147 proteins in Western blot analysis. The amino acid sequences of MCTs and CD147 of the horse were not yet known and antibodies against human (CD147, MCT1, MCT4) or rat (MCT2) proteins were used (I – III). The antibodies were ordered from SigmaGenosys or were chosen from several alternative commercially available antibodies. The antibodies chosen gave the strongest signal at the appropriate molecular mass and the minimum number of bands. They could also be blocked with the peptides against which they were raised, and the blockage made the appropriate bands in the Western blot disappear. All the antibodies that were used were tested with human RBCs or rat tissues and the molecular masses of the detected bands were compared with those in the horse. The antibodies used are shown in Table 2.

Evaluation of the specificity of the antibodies was complicated, because MCTs form dimers and complexes with their ancillary proteins. These complexes resulted in bands that in Western blots had double or quadruple molecular masses in comparison to the known molecular masses of MCTs and CD147. The intensity of the bands that were supposed to be homo- or heterodimers or heterotetramers of MCT and CD147 could be attenuated, e.g. by freezing and thawing the plasma membranes before the Western blotting. Correspondingly, the intensity of the band at the appropriate molecular mass was increased.

The cDNAs of horse MCT1, MCT2, MCT4, and CD147 were sequenced to obtain the specific antibodies against the respective proteins. The peptides against which the antibodies were raised are shown in Table 2. The horse-specific antibodies were used in studies IV and Mykkänen et al. 2010a,b,c. The results obtained were comparable to those obtained using unpurified serum against human CD147. This suggests that the antibody against CD147 was appropriate for all studies in the thesis.

The antibody against MCT1 that was used (I – III) was raised against the C-terminal peptide of human MCT1 (Swiss-Prot ID: P53985.2). In Western blotting of the RBC membrane, the antibody detected a band at a molecular mass of approximately 80 kD and also a weak band at a mass of 43 kD. In Western blotting, muscle tissue bands at an approximate molecular mass of 55 kD and a weaker band at 40 kD were detected. The differences between the tissues were probably due to the slight differences in the tertiary/quaternary structure, caused by different methods in membrane isolation. This could further affect the protein migration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The results obtained with antihuman MCT1-antibody showed that the expression of MCT1 in the RBCs was similar in HTA and LTA horses. Horse-specific antibody, which produced a band at a molecular mass of 50 kD, showed, however, a significant difference between the MCT1 expression in HTA and LTA horses. Since the results between the human- and horse-specific

antibodies differed, we preferred to rely on those obtained with the horse-specific antibody. The results obtained, using human-specific antibody (I-III) should be rejected or at least discussed with great care.

Both the antibody against the C-terminal peptide of horse MCT2 and the antibody against the C-terminal peptide of rat MCT2 detected a band at a molecular mass of approximately 80 kD. The predicted molecular mass of MCT2 is approximately 40 kD. The band at 80 kD may be an MCT2 homodimer or heterodimer that consists of MCT2 and its ancillary protein. The occurrence of a band at a molecular mass of approximately 80 kD as well as the difficulties in attenuating it, were also reported elsewhere (Garcia et al. 1995). In spite of the differences in the rat and horse peptides, against which the antibodies were raised, the two MCT2 antibodies gave similar results. Both antibodies showed that the expression of MCT2 in horse RBCs was normally distributed, and thus, we have no reason for not relying on them.

The C-terminal peptide of equine MCT4, against which the antibody was raised, has an identical sequence in humans. The results are thus also considered reliable in all studies of this thesis.

6.2. MCT-mediated substrate transport

MCT proteins are nonglycosylated and cannot reach the plasma membrane without an ancillary protein. The glycosylated ancillary protein for human MCT1 and MCT4 is CD147 (Kirk et al. 2000, Philp et al. 2003, Gallagher et al. 2007). The coexpression of MCTs and CD147 has been found in many cell types and there is also experimental evidence for the interactions between these proteins (Wilson et al. 2002, Pinheiro et al. 2009, Nakai et al. 2006).

The MCT isoforms MCT1 and MCT2 were found in the RBC membrane of horses. MCT1 is the major lactate transporter in the RBCs of several species, but the expression of MCT2 in RBCs has not been found in species other than horses (Poole and Halestrap 1993). Approximately 70% of the horses showed high MCT1 expression. The amount of MCT1 parallels that of CD147 (HE horses). In the rest (30%) of the horses, the expression of MCT1 and CD147 was significantly lower and these proteins were found only in traces or even not at all (NE horses). Thus, our studies suggest that in horses the ancillary protein for MCT1 is CD147. MCT2 was found equally in all horses. It is very probable that as well as in rats in horses, the ancillary protein of MCT2 is not CD147 but possibly its related protein gp70 (Wilson et al. 2005).

The role of MCT isoforms in lactate transport in RBCs was studied with specific inhibitors of lactate transporters: CHC that inhibits MCT-mediated transport and DIDS that inhibits especially the AE1-mediated route, but affects also the MCT proteins. At 10 mM and 30 mM lactate concentration, lactate transport was inhibited almost completely with CHC in 50 – 60% of the horses. These horses expressed MCT1 and CD147 in abundance and without inhibitors their lactate transport activity was high. In a previous study, approximately the

same percentage of Standardbred horses showed HTA (Väihkönen and Pösö 1998). Our results suggest that MCT1 is the main lactate transporter in HTA horses. In the rest of the horses, no CHC inhibition was shown and these horses expressed no or only traces of MCT1 and CD147. In these horses the normal lactate transport activity was low. Accordingly, in LTA horses lactate was transported by routes other than MCT1, such as AE1. Skelton et al. (1995) suggested that AE1 is the main lactate transport route in sedentary lifestyle animals. The inhibition of lactate and pyruvate transport with DIDS indicates AE1 activity in LTA horses. This together with a previous finding of LTA in racing reindeer (Väihkönen et al. 2001) suggests that the low expression of MCT1 rather than lifestyle directs lactate transport to AE1.

MCT1 has a K_m of 3 – 5 mM for lactate and 0.7 mM for pyruvate, whereas MCT2 has a K_m of 0.74 mM for lactate and of 0.08 mM for pyruvate (Bröer et al. 1999). Therefore, the role of MCT2 in RBCs was tested at low lactate and pyruvate concentrations. CHC inhibition of the pyruvate transport was detected in all horses, which indicates that MCT2 is active both in HTA and LTA horses. The transport of lactate in HTA and LTA horses was also similar until a concentration of 1 mM, at which the maximal capacity of MCT2 is exceeded and MCT1 takes over the role as the main transporter. The ratio of pyruvate transport activity/lactate transport activity was higher in LTA horses than in HTA horses. Human RBCs, which were used as controls in transport activity studies and are known to have only MCT1, showed the highest lactate and the lowest pyruvate transport activity (V).

Taken together, our results showed that in horses lactate transport in RBCs is mediated by two different MCT isoforms: MCT1 and MCT2 (in addition to AE1 and diffusion). HTA horses express both of these proteins. MCT2 is the most active isoform at concentrations below 1 mM and MCT1 at higher concentrations. In LTA horses, the only MCT isoform expressed in abundance in RBCs is MCT2. The activity of MCT2 is adequate to maintain homeostasis at low substrate concentrations, but at higher concentrations, LTA horses must rely on the nonspecific anion exchanger AE1. We speculate that the activity of MCT2 is sufficient to transport lactate out of the RBCs under normal physiological conditions, but while lacking MCT1, the RBCs of LTA horses are not as important plasma lactate equilibrators as those of HTA horses.

6.3. Muscles

The expressions of MCTs and CD147 were studied in two muscles, the gluteus medius and cremaster. In both muscles, the isoforms MCT1 and MCT4 as well as their ancillary protein CD147 were found in abundance. The expression of MCT2 was studied in the gluteus muscle and only traces were found. The findings are in accordance with previous studies showing that MCT1 and MCT4 are the major isoforms in the muscles of most species (Halestrap and Price 1999). MCT2 expression in muscles was reported only in pig and hamster (Garcia et al. 1995, Sepponen et al. 2003).

In the gluteus muscle, the amount of CD147 correlated with the amount of MCT4, but not with MCT1 (II). Some caution should, however, be taken with these results, because the expression of MCT1 was examined with human-specific antibody. Using horse-specific antibodies, a correlation between CD147 and MCT1 was found in cremaster muscle (IV). The different linkage between MCTs and CD147 in two studies may be due to several reasons. The linkage is a complicated one to examine in homogenized muscle samples, because the expression of MCT isoforms varies in different types of muscle fibers. MCT1 expression is correlated with the oxidative fiber composition of muscles and MCT4 is expressed most in glycolytic muscle fibers (McGullagh et al. 1996, Wilson et al. 1998). Coexpression should, thus, be studied in isolated muscle fibers or using immunohistochemistry. In addition, MCT4 expression varies species- and inter-individually (Wilson et al. 1998, Pilegaard et al. 1999). The correlations are further complicated by the fact that CD147 is also needed for the functioning of proteins other than MCTs (Jiang and Tang 2007). MCT1 is apparently also expressed in mitochondrial membranes and thus contamination of the sarcolemma preparations with mitochondrial membranes may affect the ratio of MCT1 and CD147 (Brooks et al. 1999, Butz et al. 2004, Mykkänen et al. 2010a).

The correlation of MCT expression in the gluteus muscle and the training status of the horses was studied in Standardbreds that were moderately trained or fit for racing (II). The study showed that race-fit horses expressed more MCT4 in their gluteus sarcolemma than moderately trained horses. The training status was not, however, the only variable in this study, because the moderately trained horses were younger than the race-fit horses. Thus, the effect of age on the MCT4 expression cannot be excluded. The training effect is, however, supported by the finding that the amount of MCT4 does not increase during growth from 2 to 24 months in Thoroughbreds (Kitaoka et al. 2010). In our study, the amount of MCT1 did not differ between the moderately trained and race-fit groups.

In general, the effect of training on anaerobic capacity is uncertain in horses (Hinchcliff et al. 2002, McGowan et al. 2002). The high intensity training, however, increase glycolytic enzyme activity in gluteus muscle, indicating the possibility to improve anaerobic capacity (Eto et al. 2004). The high intensity interval-type training with gradually increasing accumulation of lactic acid could be a trigger for increase in MCT4 expression in the muscle cells. The same effect may be achieved by hypoxia (Ullah et al. 2006).

Recently, a study on the effect of training and detraining on the expression of MCT1 and MCT4 in Throughbred horses was undertaken (Kitaoka et al. 2011). The expression of both MCT isoforms studied increased during 18 weeks of high-intensity training, while 6 weeks of detraining decrease the expression of MCT4. The results concerning MCT1 in that study as well as previously (II) should, however, be taken with caution, because a human-specific antibody was used.

6.4. Physiological and practical perspectives

Our results show that after moderate exercise, more lactate is accumulated in the RBCs of HTA than in those of LTA horses. The lactate concentration in RBCs varies individually after trotting races and is higher in horses that have HTA (Pösö et al. 1995, Väihkönen et al. 1999). Physiologically, this suggests that higher uptake of lactate into RBCs may lower lactate concentrations in plasma and thus increase the muscle-plasma lactate gradient and allow greater lactate efflux from the working muscles. We speculate that the more effectively the lactate (and H^+) is transported out of the glycolytic muscle, the more resistant the muscle is to fatigue. Thus, HTA should be beneficial for performance capacity, which was actually shown by Räsänen et al. (1995), but could not be confirmed by Väihkönen et al. (1999).

Secondly, lactate that is transported into the RBCs together with H^+ for contemporary storage decreases the pH of the cytoplasm in RBCs. Lower pH promotes the dissociation of oxygen from hemoglobin according to the Bohr effect. In addition, that effective lactate transport out of the muscles delays the fatigue, lactate transport into the RBCs may promote oxygen delivery from the RBCs into the exercising muscles.
Both plasma and whole-blood samples are used to study lactate accumulation after exercise. It is noteworthy that among horse populations, two distinct subgroups are found and these subgroups differ according to lactate transport into RBCs. In addition to Standardbreds the subgroups are also found in Finnhorses and Throughbreds (Mykkänen et al. 2010b). These results further support the view of Väihkönen et al. (1999) that whole-blood lactate concentrations give a more reliable picture of the accumulation of the lactate into the blood than that of plasma. For example, if two horses with different lactate transport activities in RBCs extrude equal amounts of lactate from muscles to the plasma, the one with higher lactate transport activity in RBCs, has lower plasma lactate concentration and higher lactate concentrations in RBCs. The other horse, which has lower lactate transport activity in RBCs, has almost all the lactate in the plasma; thus, plasma samples give a skewed picture of the blood lactate concentration.

6.5. Sequence variations

6.5.1. MCT1

Sequence variations in the cDNA of MCT1 and CD147 from the gluteus muscle were compared with the HE and NE of MCT1 and CD147 in the RBCs of horses (III, IV). The linkage with signs of myopathy was also examined. In all, 34 horses were used, of which eight were regarded as myopathic. In the cds of MCT1, two SNPs were found: 1498G>A and 1573A>C. The changes in the protein were, respectively, Val_{432}Ile in TM12 and Lys_{457}Gln in the C-terminus of MCT1. Both of them were also found in the study of Mykkänen et al. (2010c).

Comparison of the SNPs with the literature shows that our findings were not relevant for MCT activity. The amino acid sequences of various species show that both Val and Ile are common at the nucleotide corresponding to Val_{432}Ile in the horse. It was further suggested

that TM12 of MCT1 is not involved in the interaction with CD147 (Finch et al. 2009). In the MCT1 - CD147 transport complex, the intracellular C- and N-termini of MCT1 are close to the C-terminus of CD147 and thus the SNP 1573A>C could be important in complex formation (Wilson et al. 2002). However, in species other than horses both Gln and Lys are found at the site corresponding to $Lys_{457}Gln$ in the horse.
The SNPs in MCT1 were found equally in HE and NE horses and also in horses with and without myopathy. Neither of the SNPs found in our studies was reported in human subjects, in whom the SNPs in MCT1 decrease the activity of lactate transport (Merezhinskaya et al. 2000, Lean and Lee 2009). In these studies no SNPs were found in the TM segments that are important in hydrophobic interactions between MCT1 and CD147 (Finch et al. 2009).

6.5.2. CD147

In CD147, four sequence variations were found, two in the cds of and two in the 3´-UTR of cDNA. SNP 389A>G ($Met_{125}Val$) was found in the region that encodes the extracellular Ig-like domain that is proximal to the plasma membrane. When the complete sequence of equine CD147 was not yet known, the amino acid Met_{125} corresponded to the human Met_{239} (Swiss-Prot P35613) (III). Another SNP in the cds was 511G>A that was a synonymous $Ser_{164}Ser$. Both of these SNPs were also reported by Mykkänen et al. (2010c), the change $Met_{125}Val$ being very common in horses that showed signs of myopathy after strenuous exercise.
In my studies, the 3´-UTR was also sequenced and, interestingly, the horses that had the SNP 389A>G in the cds also had SNP 990C>T in the 3´-UTR. The heterozygosis also existed in parallel in these two loci. The SNP 888G>C showed no linkage with other SNPs. Thus far, functionally important SNPs in the cDNA of CD147 have not been found in other species.

6.5.3. Differences between the breeds

In this study, the SNP that causes $Val_{432}Ile$ in MCT1 was found in Warmbloods and in one Finnhorse, but not in Standardbreds and in a single Icelandic horse. However, in the study of Mykkänen et al. (2010c) this SNP was also found in Standardbreds. The SNP that causes $Lys_{457}Gln$ in MCT1 was found in three Finnhorses (IV). All the Finnhorses were homozygotes and thus this SNP was not in Hardy-Weinberg equilibrium. The change $Lys_{457}Gln$ was, however, also found in one Standardbred horse (III), thus this SNP was not breed-specific.
In the cDNA of the CD147 SNPs, 511G>A and 888G>C were found evenly in all horse breeds. Both homozygotes and heterozygotes existed. The SNPs 389A>G and 990C>T were found only in Standardbreds. The case/control test between Standardbreds and other breeds showed significant differences in the distribution of these SNPs (IV). The 389A>G changes an amino acid in CD147, which may affect the complex formation and furthermore the membrane expression of MCT1 and CD147. The SNP in the 3'-UTR may have a negative effect on the stability of mRNA of CD147.

An interesting linkage between the SNP and the expression of CD147 in the Standardbreds was found. Horses that were heterozygous for 389A>A/G and 990C>C/T had the lowest amounts of CD147 in their RBCs. The differences in CD147 expression were significant

between the heterozygote Standardbreds and the homozygous Standardbreds (A/A or G/G) or the A/A homozygotes in other breeds. Similar types of over/underdominance have been reported in several studies, but the exact mechanism for the effect is not clear (Morissette et al. 1998, Lindahl et al. 2009). For example, heterozygosity in a nonsynonymous missense SNP of the human *COL1A2* gene lowers bone mineral density, which may be due to disadvantageous protein interactions during the formation of collagen helices (Lindahl et al. 2009).

The highest MCT1 expression among Standardbreds was in the horse that had a homozygous SNP at nucleotide 389 in the cDNA of CD147. It is tempting to speculate that Standardbreds could counteract the effects of this common SNP by higher MCT1 expression. More samples are, however, needed to examine and prove this.

Standardbreds were bred in the late 16th and early 17th centuries in the USA from Thoroughbreds, Morgans, Hackneys, and other breeds to produce first of all fast horses for harness racing. Whether the SNPs 389A>G and 990C>T were already transmitted from the ancestors of Standardbreds is an interesting question. Even more interesting is why the SNPs remained in the Standardbred population and what are the benefits. The other breeds used in my studies were Warmbloods, which were bred from the old continental European horse populations and have not been mixed with Standardbreds for centuries, and Finnhorses, whose studbook was closed in the early 1900s. More material from the pure breed horses is also needed to trace back the origin of this SNP.

6.5.4. Single mutations

One Warmblood showed abnormally high plasma lactate concentrations during anesthesia (IV). Four hours after surgery, the CK activity also increased. In horses CK activity peaks at 6 – 12 h after acute, transient muscle injury (Higgins and Snyder 2006). Sequencing of cDNA showed that this horse had two mutations in CD147. The first one was 168A>G in the region encoding an N-terminal Ig-like domain of CD147, and another synonymous mutation in the region encoding proximal Ig-like domain. In comparison to other Warmbloods, the expressions of both CD147 and MCT1 in this horse were low. This $Ile_{51}Val$ change has not been reported before in CD147, but a similar Ile > Val change that was found in the TM region of the *HER2* proto-oncogen may affect the interaction between the proto-oncogene and a protein kinase (Kuraoka et al. 2003). In CD147 the N-terminal Ig-like domain is involved in the dimerization of CD147 proteins (Yoshida et al. 2000, Luo et al. 2009). Thus, the $Ile_{51}Val$ may affect the interactions between CD147 proteins and further affect the stability of the active transport complex.

6.6. qPCR

Studies of protein level showed that there was a linkage between the expressions of MCT1 and CD147. Horses with negligible expression of CD147 in RBCs also had very low expression of MCT1. Correlation of the expression of MCT1 and CD147 was also shown in muscles, but in contrast to the RBCs at least a small amount of both proteins was expressed in

the muscles of all horses. We showed that the sequence variations found are not the reason for the negligible expression of either protein, even if mutations may reduce their expression slightly (III, IV). Consequently, we examined whether the differences between the HE and NE horses were due to transcriptional regulation. We measured the concentrations of MCT1 and CD147 mRNA in the cremaster muscle cells of eight castrated horses that represented all the breeds studied and both HE and NE horses. Although no significant differences between the HE and NE groups were found, the mRNA concentrations of both proteins tended to be higher in HE horses. Since the linkage of the CD147 expression was shown between the RBCs and gluteus muscle (II), the mRNA concentrations were also determined in the gluteus muscle of 15 Standardbred horses. In these horses the significant difference was found: HE horses had higher concentrations of both MCT1 and CD147 mRNA than NE horses. The amounts of MCT1 and CD147 also correlated to each other. This suggests that HE and NE horses have differences in the transcriptional regulation of MCT1 and/or CD147 and, furthermore, that MCT1 and CD147 are regulated in a parallel manner. Similar suggestions were also presented in another recent study. MCT1 in rats and mice is up-regulated by peroxisome proliferator-activated receptor α (PPARα) (König et al. 2008). Concomitant with the up-regulation of MCT1, the mRNA level of CD147 was increased in the livers of pigs and rats (König et al. 2010). The mechanism of up-regulation remained unclear, however, because no induction of promoter activity with PPARα agonist was observed (König et al. 2010).

The amounts of mRNA in HE and NE horses differed statistically in the gluteus muscle but not in the cremaster muscle. This can be due to the heterogeneity of samples (different breeds) or the small number of NE horses in our study, but we speculate that also the distribution of the fiber type of the two muscles may play a role. We found that the percentage of IIB fibers in the cremaster muscle was only 6%, whereas in another study the percentage of IIB fibers in the gluteus muscle were reported to be 40% (Karlström and Essen-Gustavsson 2002). If the concentration of the regulatory factor(s) were different in IIB fibers, it would explain the difference between the two muscles studied.

7. SUMMARY OF FINDINGS

The principal aim of this thesis was to determine the reason for the bimodal lactate transport activity in the RBCs of horses. Horses which had high lactate transport activity (over 370 nmol ml^{-1} cells min^{-1}) also showed high levels of MCT1 and its ancillary protein CD147 in their RBCs. In horses with lower lactate transport activity, the expression of CD147 and MCT1 was negligible. The expression of CD147 and MCT1, however, varied widely, and variation was also found in the expression of CD147 in the gluteus muscle.

Four sequence variations in the cDNA that may affect the expression of CD147 were found. Two of them were SNPs commonly found in Standardbreds, and being heterozygous they appeared to attenuate the CD147 expression in RBCs. Another two SNPs were found only in one Warmblood. This horse had lower expression of CD147 and MCT1 in RBCs and muscle than the other Warmbloods, and in addition higher plasma lactate concentrations during

surgery and increased CK activity afterwards. qPCR showed that horses with minor expression of CD147 and MCT1 in RBCs, also had lower mRNA concentrations of CD147 and MCT1 in the gluteus muscle. Since both of these proteins were affected, they may be regulated by the same, thus far unknown, transcription factor.

Main conclusions:

- Lactate transport activity in the RBCs is dependent on the expression of MCT1 and CD147 in the plasma membrane. The bimodal distribution of the lactate transport activity in the RBCs of horses is due to the variable expression of MCT1 and CD147.

- SNPs in the *MCT1* and/or *CD147* gene are not the reason for negligible expression of MCT1 and CD147 in the RBC membrane, but...

- …the sequence variations in the *CD147* gene may decrease the CD147expression in the RBCs and muscle, and even induce physiological problems.

- The parallel decrease in MCT1 and CD147 mRNA levels in the gluteus muscle of horses, which have negligible MCT1 and CD147 expression in their RBCs, suggests that the reason for low lactate transport activity is to be found in the transcriptional regulation of MCT1 and CD147 expression.

8. PERSPECTIVES

During the last two decades, MCT proteins have proved to be essential in metabolism and pH regulation in various cell types. To date, the MCT proteins have been reported to be involved e.g. in homeostasis of cancer cells, pH regulation of malaria-infected RBCs, and in activation of lymphocytes (Elliott et al. 2001, Murray et al. 2005, Baba et al. 2008). It is currently believed that MCT proteins transport at least hormones, various metabolites, and several drugs, although the functions of most MCT isoforms remain unknown (Meredith and Christian 2008). The opportunities available in MCT research are thus limitless and basic knowledge of the regulation of expression and cooperation between MCTs and their ancillary proteins is needed.

Future perspectives in MCT research, using horses as subjects can be seen as bidirectional. Horses themselves, as super athletes, are an inexhaustible object of research. The linkage between MCT expression and exercise induced muscle problems is still open. The expression of MCT1 and CD147 is shown to be hormone dependent. In FRTL-5 cells thyroid stimulating hormone upregulates MCT1 and CD147 protein expression and in Caco2-BBE cells MCT-mediated uptake of butyrate is enhanced with leptin (Buyse et al. 2002, Fanelli et al. 2003). Therefore also sex hormones are worthy of study, because most horses suffering from muscle problems are mares. Neither have we yet executed a wider experimental layout in which muscle and blood samples could be collected from horses of the same age and different

training status, but representing only one breed. Here we could distinguish the effect of training on MCT expression from the age-dependent differences. The effect of MCT proteins on the anaerobic capacity of muscles of horses is unclear as is the linkage of performance capacity and lactate transport activity. In addition, the main questions remain unanswered: What is the final reason for the bimodal lactate transport activity in horses and why has it been maintained in horse populations?

The other direction in MCT studies is not currently focusing on the clinical problems of horses, but instead use horses as a natural model of high and low levels of expression of MCT1 and CD147. Using horses, it is possible to examine the necessity for MCT1 and study the differences in the monocarboxylate kinetics and metabolism, with and without MCT1-mediated monocarboxylate transport. Horses are also the only species that express MCT2 in their RBCs and are thus a good tool for studying the functioning of MCT2 and its ancillary protein. The recent accessibility to the entire genome of horses has also made it easier to examinate the noncoding regions of horse DNA, e.g. to study the differences in regulation sequences in HE and NE horses. Understanding the regulation of MCT1 and CD147 expression will be the next big challenge in our MCT research.

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