

Investigation of the expression and localization of glucose transporter 4 and fatty acid translocase/CD36 in equine skeletal muscle

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Objective—To investigate the expression and localization of glucose transporter 4 (GLUT4) and fatty acid translocase (FAT/CD36) in equine skeletal muscle.

Sample Population—Muscle biopsy specimens obtained from 5 healthy Dutch Warmblood horses.

Procedures—Percutaneous biopsy specimens were obtained from the vastus lateralis, pectoralis descendens, and triceps brachii muscles. Cryosections were stained with combinations of GLUT4 and myosin heavy chain (MHC) specific antibodies or FAT/CD36 and MHC antibodies to assess the fiber specific expression of GLUT4 and FAT/CD36 in equine skeletal muscle via indirect immunofluorescent microscopy.

Results—Immunofluorescent staining revealed that GLUT4 was predominantly expressed in the cytosol of fast type 2B fibers of equine skeletal muscle, although several type 1 fibers in the vastus lateralis muscle were positive for GLUT4. In all muscle fibers examined microscopically, FAT/CD36 was strongly expressed in the sarcolemma and capillaries. Type 1 muscle fibers also expressed small intracellular amounts of FAT/CD36, but no intracellular FAT/CD36 expression was detected in type 2 fibers.

Conclusions and Clinical Relevance—In equine skeletal muscle, GLUT4 and FAT/CD36 are expressed in a fiber type selective manner. (*Am J Vet Res* 2004;65:951–956)

Glucose and fatty acids are the main metabolic fuels for skeletal muscle; however, their use by skeletal muscle is contingent on their respective transport into the muscle cells. Glucose transport in mammalian skeletal muscle is almost exclusively mediated by the glucose transporter 4 (GLUT4) protein.^{1,2} In the soleus muscle of rats, GLUT4 proteins reside in intracellular subsarcolemmal groups of vesicles, Golgi-like structures at the nuclear poles, and between mitochondria and the myofibrillar region.^{3,4} Insulin and muscle contraction are 2 potent stimuli for GLUT4 translocation,

and both recruit GLUT4 from these intracellular storage sites. The exercise-sensitive GLUT4 pool is associated with an endosomal transferrin receptor, and the insulin-sensitive GLUT4 pool does not cotranslocate with this protein.^{4,5} There is still much debate about the exact nature of the GLUT4 pool subdivisions and translocation mechanism. McCutcheon et al⁶ determined the GLUT4 protein content in isolated muscle membrane fractions of homogenates of equine gluteal medius muscle. In rodents, GLUT4 protein content is higher in muscles that consist predominantly of myosin heavy chain (MHC) I (type 1, oxidative) fibers, compared with muscles that express predominantly MHC IIA and IIB (type 2, glycolytic) in their fibers.^{7,8} In biopsy specimens of human skeletal muscle, inconsistent results have been reported regarding the extent of GLUT4 protein expression per muscle fiber type,^{9–11} and it has been suggested that alternative factors such as motor unit firing rate, training status, and glycolytic activity may influence GLUT4 expression in skeletal muscle.^{9,12}

Trans-sarcolemmal membrane transport of fatty acids consists of passive diffusion (primarily for short- and medium-chain fatty acids) and a facilitated transport mechanism (for long-chain fatty acids [LCFA]). Findings of studies in rodents have provided firm evidence that membrane-associated proteins are involved in the cellular uptake of LCFA. Five different membrane-associated proteins have been identified as potential fatty acid receptors or transporters.^{13–19} Of these, an 88-kd integral membrane protein has been determined to be involved in fatty acid transport across the plasma membrane.¹⁸ This putative membrane fatty acid translocase (FAT) appears to be highly homologous (85%) to the human leukocyte differentiation antigen CD36 (glycoprotein IV)^{18,20} and shall be designated FAT/CD36 in this report.

The putative role of FAT/CD36 on LCFA uptake in rodent skeletal muscle has been studied extensively. The FAT/CD36 resides in intracellular membrane fractions and translocates to the plasma membrane of skeletal muscle cells after insulin or contraction stimuli.^{21,22} In rodent skeletal muscle, the expression of FAT/CD36 is higher in type 1 fibers, compared with its expression in type 2 fibers.²³

To our knowledge, no previous studies have been performed to investigate the fiber type selective expression of GLUT4 in the skeletal muscle of horses. The

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purpose of the study reported here was to investigate the expression and localization of GLUT4 and FAT/CD36 in equine skeletal muscle. It was our hypothesis that the fiber type specific expression of GLUT4 in different skeletal muscles (the vastus lateralis, triceps brachii, and pectoralis descendens muscles) of horses is comparable to that reported in human skeletal muscle (ie, higher expression of GLUT4 in glycolytic type 2 fibers, compared with that in oxidative type 1 fibers).⁹ Furthermore, we hypothesized that FAT/CD36 is expressed in equine skeletal muscle in a fiber type specific manner, supporting a functional role in fatty acid metabolism.²⁴

Materials and Methods

Animals—Five adult healthy Dutch Warmblood horses (4 mares and 1 gelding) were used in this study. Horses were 4.5 to 9.5 years old (mean \pm SD, 7.1 ± 1.9 years) and weighed 563 to 717 kg (mean weight, 632 ± 66 kg). The horses were owned by the Faculty of Veterinary Medicine of Utrecht University, The Netherlands. They did not have a history of myopathies, and none of the horses was involved in strenuous exercise in the 48 hours prior to the study. The horses were housed in tied standings and accustomed to frequent handling. The diet consisted of grass silage supplemented with concentrate feed and met nutrient requirements for maintenance and performance. The total diet contained 10% ash, 14.5% crude protein, 1.3% crude fat, 20% crude fiber, and 56.2% other carbohydrates. Water was provided ad libitum. All procedures were approved by the Institutional Animal Care and Medical Ethical Committee of Utrecht University and complied with the principles of laboratory animal care.

Muscle biopsies—Muscle biopsy specimens were obtained after application of local anesthesia by use of a modified Bergström needle^a (diameter, 7 mm). A 5-cm-deep biopsy specimen of the vastus lateralis muscle and 4-cm-deep biopsy specimen of the triceps brachii and the pectoralis descendens muscles were obtained from each of the 5 horses. Blood was carefully removed from the biopsy specimens before they were embedded in embedding medium for frozen tissue specimens^b and frozen in isopentane (cooled to the freezing point in liquid nitrogen) for immunohistochemical analysis. Muscle samples were stored at -80°C until analyzed.

Immunofluorescence staining for GLUT4, FAT/CD36, and muscle fiber type—Frozen muscle tissue was cryosectioned^c at -20°C . Transverse sections ($5\ \mu\text{m}$) were thaw-mounted on uncoated glass slides and air-dried. Sections were stored at -80°C and air-dried prior to staining procedures. For labeling, sections were fixed in methanol for 5 minutes, followed by acetone fixation for 1 minute, and air-dried. Sections were preincubated for 20 minutes with 10% normal horse serum in PBS solution. Some sections were subsequently incubated overnight (approx 16 hours) at 4°C with primary

mouse monoclonal antibodies against MHC I (A4.840^{25,d}; diluted 1:25) and MHC IIA (N2.261^{26,d}; diluted 1:25) and the rabbit polyclonal antibody GLUT4 (GLUT4-BW⁹; diluted 1:40), all diluted in PBS solution. Other sections were incubated overnight at 4°C with primary mouse monoclonal antibodies against MHC I (A4.840; diluted 1:25) and FAT/CD36 (131.4^{27,e}; diluted 1:25) in PBS solution. In serial sections, pri-

Table 1—Distribution of myosin heavy chain (MHC) isoforms (mean \pm SD% of fiber type/muscle) assessed via immunohistochemical staining in specimens of 3 skeletal muscles obtained from each of 5 horses.

Muscle	Muscle MHC distribution*		
	% MHC I-positive	% MHC IIA-positive	% MHC IIB-positive
Vastus lateralis	48 ± 3.5	38 ± 2.2	14 ± 4.0
Pectoralis descendens	34 ± 6.4	52 ± 3.7	14 ± 7.0
Triceps brachii	35 ± 4.1	37 ± 8.3	28 ± 12.1

*Approximately 200 fibers/biopsy specimen were counted and mean values calculated.

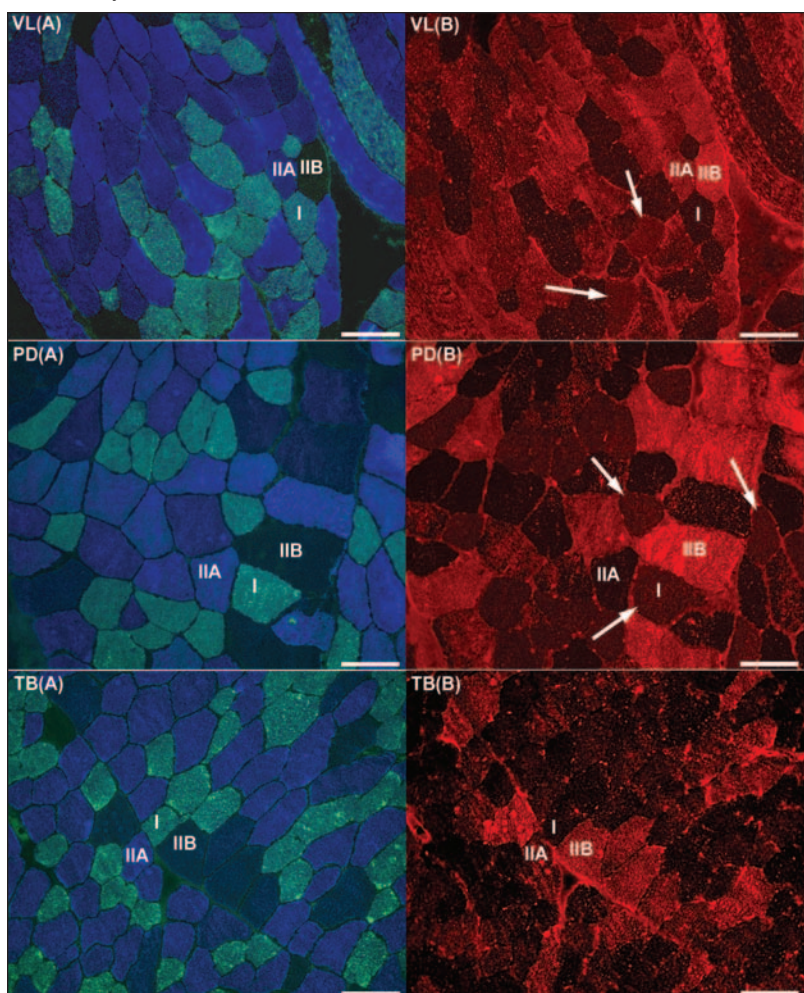


Figure 1—Results of immunofluorescence staining indicating glucose transporter 4 (GLUT4) distribution in sections of the vastus lateralis (VL), pectoralis descendens (PD), and triceps brachii (TB) muscles in horses. In the photomicrographs designated VL(A), PD(A), and TB(A), muscle fiber myosin heavy chain (MHC) isoforms are identified: MHC I-positive fibers appear green, MHC IIA-positive fibers appear blue, and MHC IIB-positive fibers have no staining (black). In the photomicrographs designated VL(B), PD(B), and TB(B), GLUT4 protein staining appears red; in all muscles examined, GLUT4 protein expression is highest in MHC IIB-positive fibers. Arrows indicate MHC I-positive fibers that also express GLUT4 protein. In all panels, bar = $100\ \mu\text{m}$.

many antibodies were omitted as a negative control. Sections were subsequently rinsed 3 times for a 5-minute period in PBS solution and incubated for 30 minutes at room temperature (approx 18°C) with secondary Alexa conjugated antibodies (goat anti-mouse IgM Alexa 488^f [diluted 1:200], goat anti-mouse IgG Alexa 350^f [diluted 1:130], and goat anti-rabbit IgG Alexa 555^f [diluted 1:400] for GLUT4 sections or goat anti-mouse IgM Alexa 488^f [diluted 1:200] and goat anti-mouse IgG Alexa 555^f [diluted 1:400] for FAT/CD36 sections) diluted in PBS solution. Finally, sections were rinsed 3 times for a 5-minute period in PBS solution. Sections prepared for investigation of GLUT4 expression were mounted in mowiol-Tris-HCl^g (pH, 8.5), whereas sections prepared for investigation of FAT/CD36 expression were mounted in mowiol with 4'-6'-diamino-2-phenylindole^h (DAPI; 0.5 µg/mL) to stain nuclei. Sections were examined by use of a fluorescence microscope^e coupled to a progressive scan color charge-coupled device cameraⁱ with a Bayer color filter at an output picture resolution of 1,300 × 1,030 pixels (horizontal × vertical) and a pixel size of 6.7 × 6.7 µm. Digitally captured images were processed and analyzed with computer software.^k

Results

Fiber type distribution—Skeletal muscle fibers are classified according to the expression of the dominant MHC isoform. In the study of this report, no functional analysis of myosin isoforms was performed and muscle sections were stained with MHC I (type 1) and IIA (type 2A) antibodies, such that the MHC IIB or IIX fibers remained unstained. For purposes of this report, the unstained fraction of muscle fibers will be referred to as type 2B muscle fibers.

Specimens of the vastus lateralis muscle contained a slightly higher portion of type 1 than type 2A muscle fibers and a minority of type 2B muscle fibers. The pectoralis descendens muscle also had few type 2B muscle fibers, whereas type 2A muscle fibers were most abundant. The triceps brachii muscle contained a relatively large portion of type 2B fibers, whereas type 1 and 2A fibers were equally distributed. Via fluorescence microscopy, a minimum of 200 fibers/biopsy specimen were manually and randomly selected and counted; mean percentages of each muscle fiber per muscle were calculated (Table 1).

Content of GLUT4 in equine muscle—Evaluation of immunofluorescence staining of sections of equine skeletal muscle revealed a distinct chessboard pattern of fibers stained either strongly or weakly for GLUT4 (Figure 1). Results of triple immunofluorescence staining of sections indicated that type 1 and 2A fibers had a low expression of GLUT4, compared with type 2B fibers in all 3 muscles (Figure 1). In control slides, no substantial background staining was observed. In fibers that were incidentally cut longitudinally to the

muscle fiber axis, a pattern of strings of GLUT4 aggregates distributed parallel to the fiber axis was detected (Figure 2), as has been observed previously in rat and human skeletal muscle.⁴ In sections of vastus lateralis muscle that had a high abundance of type 1 muscle fibers, a small portion of oxidative type 1 fibers expressed GLUT4 protein (Figure 1). In the pectoralis descendens muscle, GLUT4 was expressed predominantly in type 2B muscle fibers, but GLUT4 was expressed in all 3 muscle fiber types. Sections of triceps brachii muscle had low amounts of GLUT4 in type 1 and 2A muscle fibers and a high expression in type 2B fibers as well.

Content of FAT/CD36 in equine muscle—Immunofluorescent labeling of sections with antibodies against FAT/CD36 revealed strong expression of this protein at the sarcolemma of all equine skeletal muscle fibers and no substantial staining in control slides. A low intracellular expression of the FAT/CD36 protein was observed in type 1 fibers, whereas no intracellular FAT/CD36 expression could be detected in type 2 fibers (Figure 3). In both type 1 and type 2 fibers, FAT/CD36 aggregates were observed in close relation to the sarcolemma. These are most likely positioned near capillaries to aid in the uptake of LCFA

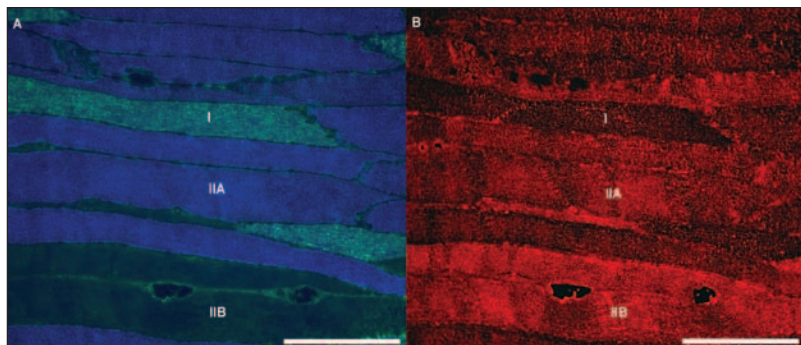


Figure 2—Photomicrographs of longitudinal sections of equine pectoralis descendens muscle after staining for MCH isoforms and GLUT4 protein. A—Results of staining for MCH isoforms. The MHC I-positive muscle fibers appear green, MHC IIA-positive fibers appear blue, and MHC IIB-positive fibers are not stained. B—Results of staining for GLUT4 protein. The GLUT4 protein (red) is distributed as dotted strings along the fiber axis. In both panels, bar = 100 µm.

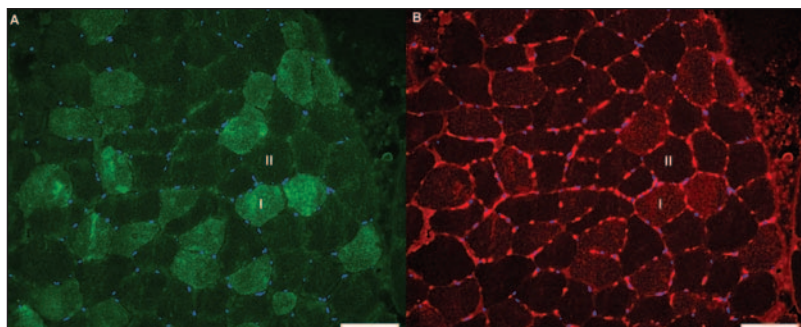


Figure 3—Photomicrographs of sections of equine pectoralis descendens muscle after staining for FAT/CD36 expression. A—The MHC I-positive muscle fibers (I) appear green, MHC IIA and IIB muscle fibers are unstained (II), and nuclei appear blue after staining with 4'-6'-diamino-2-phenylindole (DAPI). B—Distribution of FAT/CD36 (red) in muscle fibers; nuclei appear blue after staining with DAPI. Notice that MHC I-positive fibers have a low intracellular expression of FAT/CD36 (I), whereas MHC IIA and IIB fibers have no detectable intracellular expression of FAT/CD36 (II). FAT/CD36 is mainly clustered in sarcolemmal aggregates near capillaries in all fiber types. In both panels, bar = 100 µm.

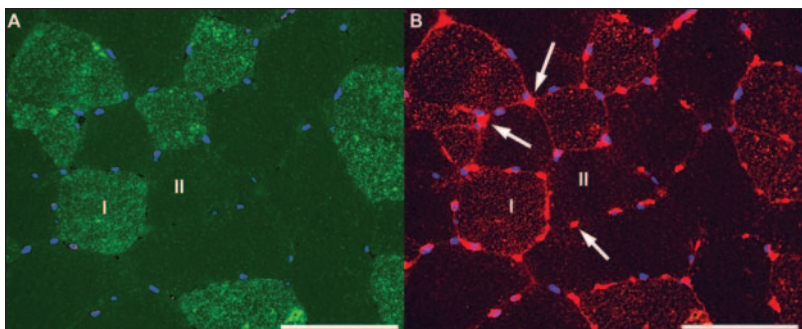


Figure 4—Photomicrograph of a section of vastus lateralis muscle from a horse illustrating FAT/CD36 expression in equine muscle fibers. The type 1 fibers (green; A) express low quantities of FAT/CD36 (red; B) intracellularly, but FAT/CD36 is mainly expressed in sarcolemmal aggregates in all fiber types, presumably near capillaries (example indicated by arrows). Also notice that sarcolemmal staining of FAT/CD36 is visible in type 1 muscle fibers. In both panels, bar = 100 μ m.

from the plasma. In the triceps brachii, pectoralis descendens, and vastus lateralis muscles, slight intracellular FAT/CD36 expression was limited to type 1 muscle fibers, although the sarcolemmal aggregates were observed in all muscle fiber types (Figures 3 and 4).

Discussion

Results of the indirect immunofluorescence assays used in the study reported here indicated that GLUT4 and FAT/CD36 protein expression in equine skeletal muscle is fiber type selective. Our findings indicated that equine skeletal muscle fiber type 2B has a higher expression of GLUT4 protein, compared with that of type 1 and 2A muscle fibers. In type 1 muscle fibers, FAT/CD36 is expressed intracellularly, but is mainly clustered in aggregates near muscle capillaries in all muscle fiber types.

In general, results of the GLUT4 immunofluorescence staining performed in our study are dissimilar to those of other studies involving rodent skeletal muscle extracts that indicated higher GLUT4 protein content in muscle which contained predominantly type 1 muscle fibers versus muscle which contained predominantly type 2 muscle fibers,^{7,8} but are consistent with data obtained from skeletal muscles of humans and rodents in a previous investigation in our laboratory.⁹ Species-specific differences in GLUT4 expression have been reported²⁸ in goat and calf muscles in which skeletal muscle GLUT4 content decreased with increasing oxidative capacity in different muscle groups. In a study by Katsumata et al,²⁹ an upregulation of GLUT4 was detected in the skeletal muscle of pigs after a period of mildly inadequate nutrition; concurrently, there was a decrease in insulin sensitivity in these pigs, compared with pigs receiving twice as much nutrition. These results were all obtained by analysis of mixed muscle homogenates, and although rodent skeletal muscle often contains a predominance of either MHC I or II fibers (thereby justifying its division into slow-twitch and fast-twitch muscle), other species exhibit a more mixed muscle fiber pattern. Similar to findings in human skeletal muscle, skeletal muscle of horses has a heterogeneous fiber type distribution and different fiber type distributions have been reported between biopsy

specimens obtained at sites only 2 cm apart.^{30,31} On the basis of these data, we selected a more direct method of assessment of the relation between GLUT4 and fiber type distributions; this method involved immunohistochemical triple staining of muscle sections for GLUT4 with MHC specific antibodies that, to our knowledge, had not been applied before in equine muscle studies. This technique has been used in investigations involving rodent and human skeletal muscle; results of a study involving this technique by Borghouts et al⁹ confirmed higher GLUT4 expression in type 1 muscle fibers (compared with that in other fiber types) only in rat gastrocnemius muscle; in the soleus,

extensor digitorum longus, cranial tibial, and vastus lateralis muscles, GLUT4 expression was higher in type 2 muscle fibers.⁹ In human skeletal muscle, some researchers have detected higher GLUT4 protein content in type 1 muscle fibers,^{10,12} whereas others identified higher GLUT4 expression in type 2 skeletal muscle fibers.⁹ From these findings and results of our study, in which GLUT4 was detected in some type 1 muscle fibers in vastus lateralis muscle of horses but most commonly in type 2B muscle fibers, it seems unlikely that a strict coupling between GLUT4 protein expression and muscle fiber type composition exists in mammalian skeletal muscle, and alternative factors contribute to GLUT4 expression in skeletal muscle.^{9,12}

Expression and localization of GLUT4 is not only dependent on muscle innervation and fiber type, but is also influenced by different stimuli. Insulin is a potent stimulus for GLUT4 translocation and transcription, and it increases glucose uptake and GLUT4 expression in rats.³² Muscular contraction is another stimulus for GLUT4 translocation, and exercise training also increases GLUT4 gene transcription and expression.^{33,34} In exercise-trained rats, primarily fast-twitch muscle fibers increase insulin-stimulated glucose uptake.³³ In rats, stimulation of AMP-activated protein kinase (an exercise linked activator of GLUT4 translocation) with 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) for 5 days increased GLUT4 protein in type 2 muscle fibers but not in type 1 muscle fibers.³⁵ Finally, diet can influence GLUT4 expression. A high-carbohydrate diet is associated with increases in GLUT4 expression in all muscle fiber types in rats,³⁶ whereas a high-fat diet is associated with a decrease in GLUT4 expression in rats, compared with control values.³⁷ These results also indicate that skeletal muscle can adapt fiber specific glucose uptake and GLUT4 expression to different stimuli.

By application of triple indirect immunofluorescence assays, our data have indicated that FAT/CD36 protein is expressed in equine skeletal muscle in a fiber type selective manner. The FAT/CD36 protein is expressed in sarcolemmal aggregates in all muscle fiber types and intracellularly in small amounts in type 1 skeletal muscle fiber types. This is in accordance with results of a study²³ involving rat skeletal muscle that

indicated higher expression of FAT/CD36 protein in red, oxidative skeletal muscle homogenates, compared with that in white, glycolytic skeletal muscle homogenates. Recently, this relationship was also confirmed in human skeletal muscle with experimental procedures similar to those used in the study of this report.³⁸ The FAT/CD36 is a 88-kD protein that is redistributed to the plasma membrane of muscle fibers after insulin stimulation²¹ or contraction²² in rats and correlates with an increased rate of palmitate uptake, compared with uptake in unstimulated cells.²¹⁻²³ In the same species, oxidative skeletal muscles have the highest potential for oxidation of fatty acids and the uptake (incorporation and oxidation) of LCFA is greatest in oxidative muscle strips.³⁹ In rats, palmitate uptake in giant vesicles obtained from red, oxidative (type 1) skeletal muscle was increased, compared with uptake in giant vesicles obtained from white, glycolytic (type 2) skeletal muscle.²³ Moreover, intramuscular lipid content (assessed by oil red O staining) is higher in human type 1 skeletal muscle fibers than in other muscle fiber types.⁴⁰ These observations suggest a higher capacity for uptake of LCFA in oxidative skeletal muscle fibers; therefore, the amount of FAT/CD36 is expected to be higher in these muscle fiber types. However, horses have a different dietary pattern than that of rodents and humans and derive more nutrients from fermentation of cellulose, which results in a larger metabolic dependence on plasma volatile fatty acids.⁴¹ These quantities of short- and medium-chain fatty acids make it questionable if horses need a facilitative LCFA transport system. Nevertheless, provision of supplemental fat in the diet increases the oxidative power of skeletal muscle in horses,⁴² and horses have a diurnal rhythm in plasma LCFA concentrations.⁴¹ In endurance-trained humans, a high-fat diet resulted in an increase in FAT/CD36 expression and a marked increase in fat oxidation, compared with a high-carbohydrate diet.⁴³ Geelen et al⁴⁴ reported an increase in carnitine palmitoyl transferase-1 activity in oxidative (masseter) skeletal muscle of horses fed a diet supplemented with soyabean oil; in humans, an increase in carnitine palmitoyl transferase-1 mRNA detected after training was correlated with an increase in FAT/CD36 mRNA in skeletal muscle.⁴⁵ Thus, the identification of FAT/CD36 in equine skeletal muscle in our study suggests a functional role of this transport protein in lipid metabolism in horses.

The findings of the study of this report indicated that GLUT4 protein is expressed in equine muscle in a fiber type specific manner that is analogous to GLUT4 protein expression in human muscle fibers, specifically with regard to the higher expression in type 2B muscle fibers, compared with that in type 1 muscle fibers. Intracellular FAT/CD36 is expressed primarily in type 1 muscle fibers in the skeletal muscle of horses, but FAT/CD36 is expressed in sarcolemmal aggregates in all muscle fiber types. Among the different muscle fiber types, these findings suggest a higher glucose uptake capacity in type 2B muscle fibers and a higher fatty acid uptake capacity in type 1 muscle fibers.

²Bergström needle, Maastricht Instruments, Maastricht, The Netherlands.
³Tissue-Tek, Sakura Finetek Europe bv, Zoeterwoude, The Netherlands.

⁴CM3050, Leica, Nussloch, Germany.

⁵Antibodies A4.840 and N2.261, obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa.

⁶Provided by Dr. N.N. Tandon, Otsuka Pharmaceutical Co, Rockville, Md.

⁷Alexa Fluor 488, 350, and 555, Molecular Probes Europe bv, Leiden, The Netherlands.

⁸Mowiol, Calbiochem, Omnilab International bv, Etten-Leur, The Netherlands.

⁹DAPI (0.5 µg/mL), Molecular Probes Europe bv, Leiden, The Netherlands.

¹⁰Nikon E800 fluorescence microscope, Uvikon, Bunnik, The Netherlands.

¹¹Basler A113 C progressive scan color CCD camera, Ahrensburg, Germany.

¹²Lucia 5.49 software, Nikon, Düsseldorf, Germany.

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