Effects of acute exercise and long-term exercise on total Na^+,K^+-ATPase content and Na^+,K^+-ATPase isoform expression profile in equine muscle

Maarten M. M. van den Burg, PhD; Karin Eizema, PhD; Ellen de Graaf-Roelfsema, DVM, PhD; Eric van Breda, PhD; Inge D. Wijnberg, DVM PhD; Johannes H. van der Kolk, DVM PhD; Maria E. Everts, PhD

Objective—To investigate the effects of acute exercise and long-term training on Na^+,K^+-ATPase content, mRNA isoforms, and protein concentration in equine muscle.

Animals—6 Standardbreds.

Procedures—Horses performed a bout of exercise on a treadmill before and after 18 weeks of combined interval and endurance training. Muscle biopsy specimens were obtained from vastus lateralis muscle (VLM) and pectoralis descendens muscle (PDM) before and after exercise. The Na^+,K^+-ATPase content, mRNA isoforms, and protein concentrations were determined by use of [^{3}H]ouabain binding, real-time PCR assay, and western blotting, respectively.

Results—6 Na^+,K^+-ATPase mRNA isoforms were present in equine muscle, but only α2 and β1 proteins were detected. Exercise before training resulted in increases of mRNA isoforms α1, α2, α3, and β2 in VLM and α1 and β3 in PDM. Training increased resting values for mRNA isoforms α3 and β1 in VLM and β3 in PDM. The Na^+,K^+-ATPase, [^{3}H]ouabain binding, and proteins of mRNA α2 and β1 increased in VLM, whereas in PDM, only α2 protein increased as a result of training. After training, effects of strenuous exercise on mRNA expression were no longer detectable.

Conclusions and Clinical Relevance—Equine muscle contained all Na^+,K^+-ATPase mRNA isoforms, but only α2 and β1 proteins could be detected. Expression of these isoforms changed as a result of strenuous exercise and long-term training, representing an adaptive response. Determination of Na^+,K^+-ATPase gene expression may be relevant for understanding alterations in excitability during neuromuscular diseases. (*Am J Vet Res* 2009;70:895–901)

Skeletal muscles have a high capacity to adapt to increases in loading, such as occurs during exercise and training. Studies[1,2] have detected increases in concentration of structural proteins, number of capillaries, and ion transport mechanisms in muscle after a single bout of exercise or prolonged training. The Na^+,K^+-pump is one of the variables that increase in concentration with increased activity.[2–4] The Na^+,K^+-pump is a ubiquitous enzyme located in the plasma membrane of the muscle fiber. After an action potential occurs, the pump becomes activated to restore the concentrations of the Na^+ and K^+ ions that determine the resting membrane potential, thereby maintaining excitability and force of the muscle.[2,5] The Na^+,K^+-pump comprises a catalytic α-subunit and a glycosylated β-subunit that operate as a heterodimer. Four α-isoforms of Na^+,K^+-ATPase can be distinguished (α1, α2, α3, and α4), and 3 β-isoforms can be distinguished (β1, β2, and β3). In mammalian skeletal muscle, isoform α1, α2, α3, β1, β2, and β3 genes are expressed and corresponding proteins are formed.[6,7] The α4-gene transcript has only been detected at low concentrations in human muscle so far.[8,9] Numerous combinations of different isoforms are possible, potentially meeting the specific demands of the working muscle cell.[10]

Several studies in human skeletal muscle reveal that these gene transcripts are responsive to exercise.

Abbreviations

BLAST	Basic Local Alignment Search Tool
HRmax	Maximal heart rate
PDM	Pectoralis descendens muscle
SET	Standardized exercise test
TBS-T	Tris-buffered saline Tween 0.05%
VLM	Vastus lateralis muscle

Received July 8, 2008.
Accepted September 9, 2008.
From the Departments of Pathobiology (van den Burg, Eizema, Everts) and Equine Sciences (de Graaf-Roelfsema, Wijnberg, van der Kolk), Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, NL-3584 CL Utrecht, The Netherlands; and the Department of Movement Sciences, Maastricht University, NL-6200 MD Maastricht, The Netherlands (van Breda).
The authors thank Dr. K. T. Murphy and P. W. A. Cornelissen for scientific and technical assistance.
Address correspondence to Dr. Everts.

Six minutes of knee extensor exercise increased the mRNA of α1, α2, α3, β1, β2, and β3.[7] Another study[9] revealed that 15 minutes of intermittent exercise increased α1, α2, β1, and β3 mRNA values. In rats, 1 hour of treadmill running increased α1 and β2 mRNA values, whereas isoforms α3 and β3 were not measured in that study.[11] This is in contrast to the protein production of Na^+,K^+-ATPase mRNA isoforms, which does not respond as fast as Na^+,K^+-ATPase mRNA isoforms to acute exercise. One bout of exercise was not sufficient to induce changes in Na^+,K^+-ATPase protein isoforms in humans.[6,7,12] Conversely, the study by Green et al[13] revealed increases of α2 protein after 2 bouts of 6 minutes of cycling within a time span of 1 hour and 6 minutes.

A considerable number of studies have detected increases of the enzyme concentration in skeletal muscle after training in a range of species via quantification of Na^+,K^+-ATPase content by measuring [^{3}H]ouabain binding, as reviewed by Clausen.[2] However, studies comparing both mRNA and protein isoform production are scarce. In 1 study,[14] endurance-trained athletes had lower resting mRNA copy-number expression of α1, α3, β2, and β3 isoforms than recreationally active persons, whereas the difference in Na^+,K^+-ATPase content was the opposite. Another study[15] found that 5.5 weeks of high-intensity training did not result in any changes in mRNA expression. Most training programs were of ≤ 8 weeks' duration, and changes in protein production by different isoforms attributable to training were often not consistent. Only Na^+,K^+-ATPase α2 isoform protein always increased in response to training.[15–19]

The Na^+,K^+-ATPase content in equine locomotory muscle has been found to increase in several training studies.[3,4,20–22] However, those data were not accompanied by analysis of the different isoforms of Na^+,K^+-ATPase via mRNA or protein analysis. Thus, the objective of the study reported here was to determine whether 2 equine muscles located in different regions of the body developed a different response in their Na^+,K^+-ATPase content during an exercise-training program; whether Na^+,K^+-ATPase isoforms present in other mammalian species are detectable in equine muscle; whether the effect of training on Na^+,K^+-ATPase content was associated with a proportional change in mRNA expression and protein production; whether a short exercise bout has an effect on Na^+,K^+-ATPase mRNA and protein values; and whether the effect of a short exercise bout on Na^+,K^+-ATPase isoforms is still present after training.

Materials and Methods

Animals—Six Standardbred geldings (mean ± SD, 20 ± 2 months of age; weight, 374 ± 23 kg) were trained for 18 weeks. At the end of the training period, mean weight was 388 ± 22 kg. Horses were owned by the Faculty of Veterinary Medicine of Utrecht University. The horses were individually housed; their diet consisted of grass silage supplemented with concentrate feed and vitamin supplements and met nutrient requirements for maintenance and performance. Salt blocks and water were available ad libitum. All procedures were approved by the Institutional Animal Care and Medical Ethical Committee of Utrecht University.

Training—All training sessions and exercise tests were performed on a high-speed treadmill.[a] Exercise intensity during the training was based on fixed percentages of the peak heart rate, as obtained in a previous study.[23] Training intensity was adjusted according to heart rate measurements obtained with a heart rate monitor[b] during the training sessions on a weekly basis. Subjects acted as their own controls. Each training session was preceded by 30 minutes of warm-up in a hotwalker followed by an 8-minute warm-up (4 minutes at 1.6 m/s and 4 minutes at 3.0 to 4.0 m/s, no incline) on the treadmill. Each training session ended with a cooling down period, consisting of a 5-minute walk on the treadmill followed by a 30-minute walk in a hotwalker. On rest days, horses walked in a hotwalker for 60 minutes.

A SET was performed in all horses at the beginning and end of the training period to monitor performance on a high-speed treadmill.[a] The SET started with a 4-minute warm-up period of walking at 1.5 m/s followed by 4 minutes of trotting at 4.5 m/s. Next, after 1 minute of additional walking at 1.5 m/s, horses trotted for 20 minutes. Finally, horses were allowed to cool down for 5 minutes at 1.5 m/s. Heart rate was measured by use of a heart rate meter[b] and continuous ECG monitoring.[c] During the 20-minute SET, speed and inclination of the treadmill were adapted to elicit a heart rate of 180 to 190 beats/min (approx 80% of HRmax). Horses were only allowed to trot. In the untrained horses, this corresponded with a speed of 6.5 to 7.0 m/s, whereas after training, horses trotted at a speed of 8.0 to 8.5 m/s with a treadmill inclination of 1% to 2.5%.

The 18-week training period consisted of 2 types of exercise, endurance running and interval running, on alternate days. The endurance running included alternating 20 to 24 minutes of continuous running at 60% HRmax (Tuesday) or 16 to 18 minutes at 75% HRmax (Friday). The interval training (Monday and Thursday) included three 3-minute bouts at 80% to 90% HRmax (beginning of the training period) or four 2-minute bouts at 80% to 90% HRmax (at the end of the training period) interspersed with 3-minute (or 2-minute) periods at 60% HRmax. The horses exercised 4 d/wk throughout the entire training period.

Muscle biopsy—Muscle samples were obtained before and directly after (ie, within 15 minutes) the SET at the beginning and end of the training period by use of local anesthesia (lidocaine hydrochlorine [2%] without epinephrine) administered with a modified Bergström needle (diameter, 7 mm).[d] A 5-cm-deep biopsy specimen of the VLM was taken at a point 15 cm ventral to the center of the tuber coxae and 7 cm caudal to the cranial border of the muscle. A 4-cm-deep biopsy specimen of the PDM was obtained at a point 20 cm caudal to a line extending through the shoulder joints in the middle of the muscle. Blood was carefully removed. Biopsy specimens were directly put into liquid nitrogen for analysis and stored at –80°C until analyzed.

mRNA—Total RNA was extracted from 20 to 50 mg of muscle and pulverized with a mortar and pestle by use of an RNA fibrous tissue kit[e] according to the manufacturer's recommendations. The RNA was eluted with

RNase-free water and stored at –80°C. Total RNA concentration was determined spectrophotometrically at 260 nm. For each sample, 1 µg of RNA was transcribed into cDNA by use of a cDNA synthesis kit[f] in a total volume of 20 µL according to manufacturer's protocol.

Real-time PCR (light cycler[f]) was run for 1 cycle (95°C for 3 minutes) and 40 cycles (95°C for 15 seconds, 57°C for 60 seconds). Fluorescence resulted from incorporation of SYBR green[f] to double-stranded DNA, and this fluorescence was measured after each repetitive cycle. Duplicate wells were run for each sample. Measurements included a no-template control. Primer sequence design was based on published equine Na^+,K^+-ATPase isoform sequences **(Appendix)**. Gene expression was quantified from fluorescence emission by use of a cycle threshold (ie, C_T) method. The relative expression of the genes was calculated by use of the expression $2^{-\Delta\Delta C_T}$, in which the expression of each gene was normalized for input cDNA by use of the housekeeping gene for glyceraldehyde-3-phosphate dehydrogenase.[24]

Quantifying mRNA copy numbers—All Na^+,K^+-ATPase isoform cDNA fragments were generated by use of real-time PCR. By use of PCR assay, partial sequences were isolated, cloned into a vector,[g] and transformed into DH5α competent cells. Cloned partial sequences of the specific isoforms were confirmed by use of sequencing. Plasmid DNA was obtained by use of a DNA isolation kit,[e] following manufacturer's instructions. To quantify the amount of copy numbers of the isoform, specific plasmids were measured via spectrophotometry. The corresponding copy numbers were calculated by use of the following equation:

$$\frac{N_A \text{ (copies/mol)} \times \text{concentration (g/mL)}}{\text{(number of base)} \times \text{(340 Da/base) (g/mol)}} = \text{amount (copies/mL)}$$

where Da represents daltons and N_A represents Avogadro's number. The copy numbers were calculated on the basis of the molecular mass of the plasmid. A dilution series of 1 to 10^8 copies/reaction was made. This was used in a real-time PCR assay where the expression of each isoform was plotted into a dilution curve.

Measurement of Na^+,K^+-ATPase in muscle—The Na^+,K^+-ATPase content was quantified by measuring [^{3}H]ouabain binding capacity in presence of vanadate (VO_4),[h] as described by Nørgaard et al.[25,26] This method allows the quantification of total content of Na^+,K^+-ATPase in small muscle samples, corresponding to the total population of functional Na^+,K^+ pumps.[2] The method has been validated for quantification of Na^+,K^+-ATPase content in muscles of young[22] and adult[4] horses. For the present study, incubations were performed at 37°C in buffer containing 0.6 µCi/mL of [^{3}H]ouabain[i] and unlabeled ouabain added to a final concentration of 10^{-6}M for 120 minutes under continuous gassing with air. Further processing was performed exactly as described by Suwannachot et al.[4,22] On the basis of the specific activity of [^{3}H]ouabain in the incubation medium, the amount of [^{3}H]ouabain taken up and retained in the muscle samples was calculated and after correction (for unspecific uptake, isotopic impurity, and the minute loss of specifically bound [^{3}H]ouabain during the washout) expressed as picomoles per gram of wet weight.[26]

Protein concentration—Muscle samples (50 mg) were homogenized by pottering in a 1.5-mL tube in a 1:5 dilution with extraction buffer (25mM Tris-HCl; pH, 6.8; 1% SDS; 5mM EGTA; 50mM NaF; 1mM sodium vanadate; 10% glycerol; and protease inhibitor). A portion of each sample was analyzed for total protein content,[j] with bovine serum albumin as the standard. The remaining samples were frozen at –80°C for immunoblotting.

The SDS-PAGE (10% separating gel, 5% stacking gel) was performed, and gels were loaded with 50 (β1) or 70 (α2) µg of protein. Following electrophoresis (20 minutes, 100 V; and 90 minutes, 150 V), the protein was transferred (45 minutes, 20 V) with a semidry blotting system[j] to a 0.45-µm nitrocellulose (β1) or 0.2-µm polyvinylidene fluoride (α2) membrane and blocked for 2 hours with blocking buffer (5% nonfat milk[j] in TBS-T). Membranes were incubated overnight at 4°C in primary antibodies diluted in blocking buffer. Membranes were washed in TBS-T and incubated for 1 hour in horseradish peroxidase–conjugated secondary antibodies (goat anti-mouse immunoglobulins or goat anti-rabbit immunoglobulins) diluted 1:10,000 in TBS-T buffer. Following 4 washes in TBS-T, membranes were dried and treated with chemiluminescent substrate.[k] The signal was captured and images obtained. Positive control samples included rat brain and kidney homogenates, and these were run on each gel to assess the reactivity and specificity of the antibody. The linearity of the blot signal versus protein loaded for the experimental conditions was established for each antibody.

Antibodies—Blots were probed with antibodies specific to each isoform for α1 (α6F[l]), α2 (AB9094[m]), α3 (MA3–915[n]), β1 (MA3–930[n]), β2 (catalog No. 610,914[o]), and β_3 (catalog No. 610,992[o]). Briefly, this involved the use of additional antibodies (ie, several other isoform-specific antibodies were tested), control samples (ie, equine brain, kidney, and additional muscle samples), and BLAST analysis of the antigen sequence of each antibody (when known) to evaluate cross-reactivity. The BLAST analysis revealed that the antigen sequence of β2 and β3 (published by the manufacturer) did not cross-react with non–Na^+,K^+-ATPase proteins, in agreement with Murphy et al.[7] Other antigen sequences could not be found. The BLAST analysis of the equine Na^+,K^+-ATPase protein sequences of α1-α3 and β1-β3 revealed a homology with human Na^+,K^+-ATPase proteins of at least 97%. Therefore, we decided to use the antibodies specific for human Na^+,K^+-ATPase protein isoforms to detect equine Na^+,K^+-ATPase isoforms. The selected antibodies recognized α1-α3 and β1-β2 isoforms in equine brain and the β3 isoform in equine kidney. Because all antibodies cross-reacted with rat tissues, as described by the manufacturer (for α1, α2, α3, β1, and β2) or Murphy et al[7] (for β3), rat brain and rat kidney homogenates were analyzed in parallel to the equine muscle samples on the same gel to check antibody specificity.

Statistical analysis—All data are given as mean ± SD values. Statistical analyses were carried out with

software[P] by use of a 1-way ANOVA with a Tukey post hoc test to detect differences in expression among Na^+,K^+-ATPase isoform mRNAs. General linear model repeated measures with factor time (within) was used to detect an effect of exercise, training, or both. As a second factor, muscle (within) was used to analyze differences between the PDM and VLM.

Results

Na^+,K^+-ATPase mRNA in resting equine skeletal muscle—In VLM and PDM, 3 α (1 to 3) and 3 β (1 to 3) isoforms of Na^+,K^+-ATPase mRNA were found (**Figure 1**). Quantification of the mRNA copy numbers revealed a comparable pattern of distribution of all isoforms in the 2 muscles. Furthermore, within a muscle, different levels of expression were found for α1, β1, and β3 ($P < 0.001$) and roughly equal expression for α2, α3, and β2 isoforms. The most abundant α subunits were α2 and α3. The β1 isoform had the highest mRNA expression in both muscles, compared with all other isoforms.

Exercise-induced changes in Na^+,K^+-ATPase mRNA—Acute exercise induced a significant increase at SET 1 (before training) in Na^+,K^+-ATPase mRNA expression of α1 (15.6 times, compared with baseline), α2 (2.7 times), α3 (2.1 times), and β2 (1.5 times) in VLM (**Figure 2**), whereas in PDM, an increase in α1 (3.6 times) and β3 (3.0 times) mRNA expression was found (**Figure 3**). After 18 weeks of training (SET 2), no significant effects of acute exercise could be detected in either of the 2 muscles. Training for 18 weeks significantly increased the basal values of Na^+,K^+-ATPase mRNA expression of α3 (1.7 times) and β1 (1.2 times) in VLM and β3 (2.1 times) in PDM.

Na^+,K^+-ATPase protein values—Only α2 and β1 were detectable as proteins by use of western blotting (**Figure 4**). The other isoforms were not detectable, although use of the antibodies did detect the isoforms in equine brain (α1, α3, and β2) or kidney (β3) tissues.

The basal protein values (before SET 1) of the α2 and β1 Na^+,K^+-ATPase isoforms were significantly lower in VLM than in PDM (Figure 4). Acute exercise did not induce changes in values of α2 and β1 Na^+,K^+-ATPase proteins in either of the muscles before or after training. Conversely, significant increases of α2 Na^+,K^+-ATPase protein in both VLM (2.2 times) and PDM (1.5 times) were found after 18 weeks of training. The protein value of the β1 isoform was only increased in VLM after 18 weeks of training (1.7 times; $P < 0.05$).

Na^+,K^+-ATPase content—The total Na^+,K^+-ATPase content quantified via [^{3}H]ouabain binding revealed that VLM contained significantly fewer Na^+,K^+-ATPase pumps than PDM. After 18 weeks of training, a significant increase in Na^+,K^+-ATPase content was measured in VLM (pretraining value, 170 ± 4 pmol/g of

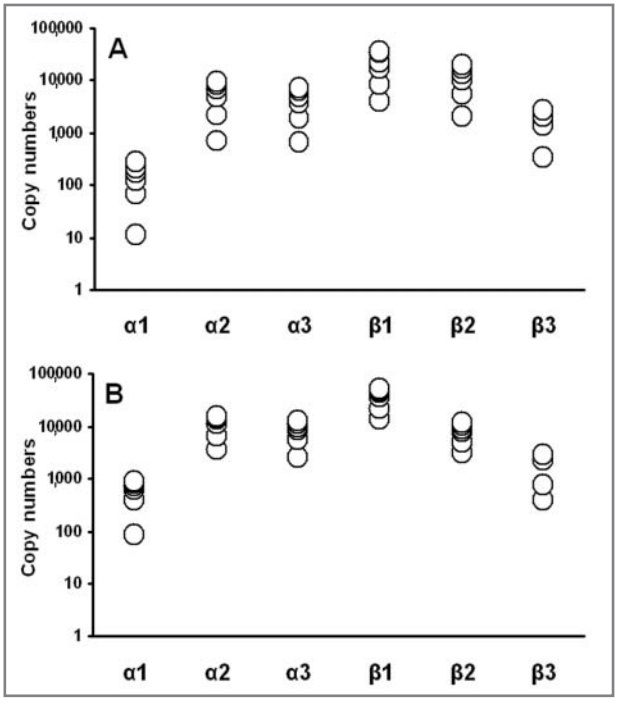

Figure 1—Expression (copy numbers) of Na^+,K^+-ATPase mRNA isoforms in resting untrained equine skeletal muscle specimens (n = 6 horses) from the VLM (A) and the PDM (B).

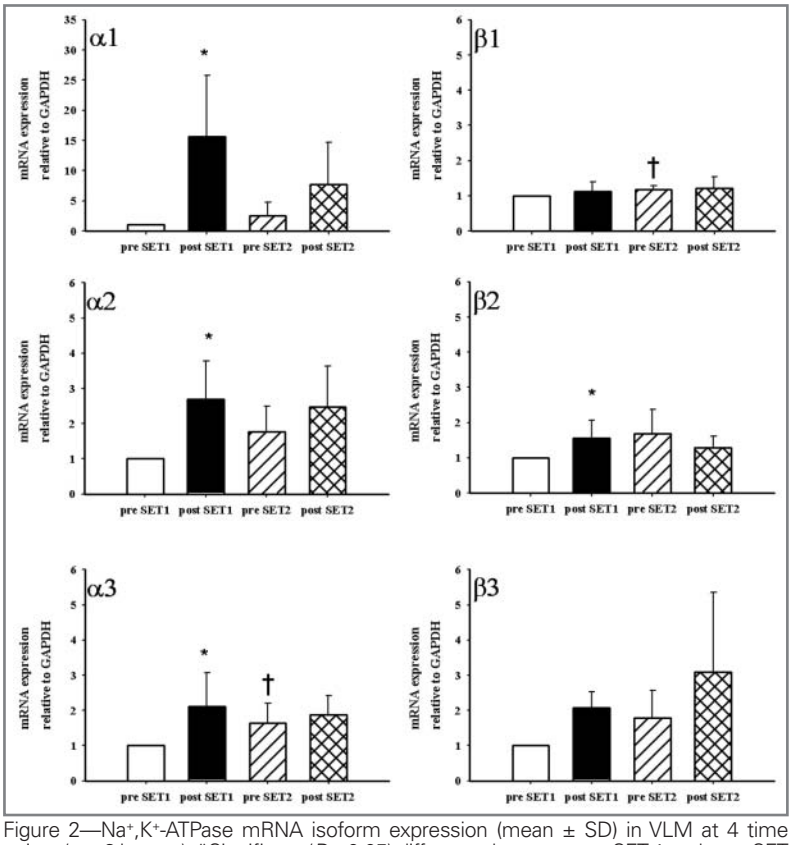

Figure 2—Na^+,K^+-ATPase mRNA isoform expression (mean ± SD) in VLM at 4 time points (n = 6 horses). *Significant ($P < 0.05$) difference between pre SET 1 and post SET 1 values. †Significant ($P < 0.05$) difference between pre SET 1 and pre SET 2 values. pre = Before. post = After. GAPDH = Glyceraldehyde-3-phosphate dehydrogenase.

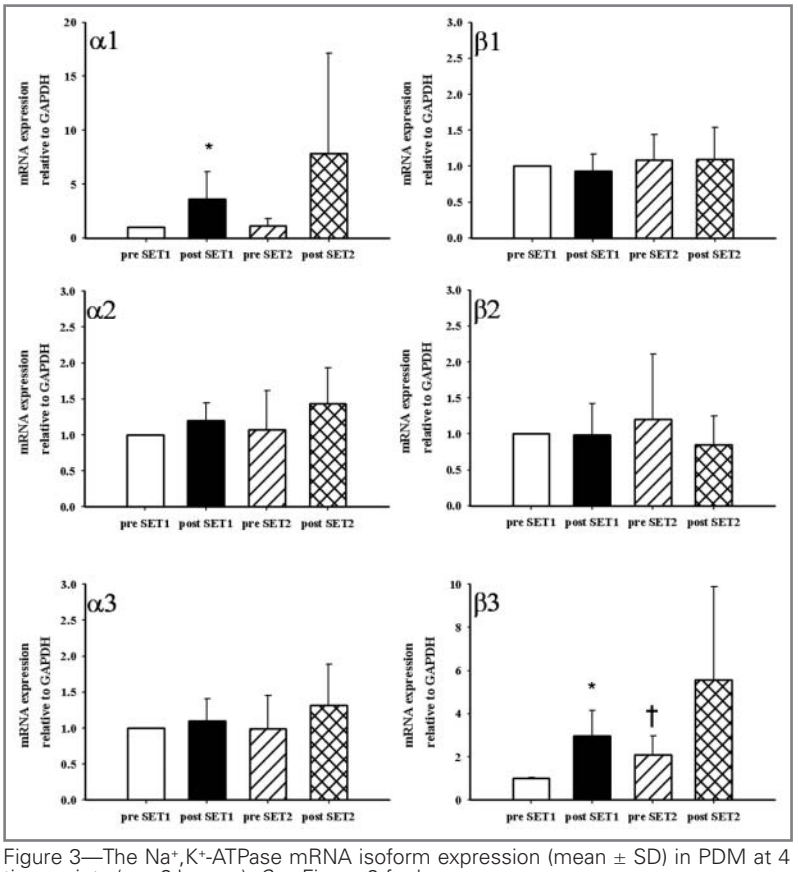

Figure 3—The Na^+,K^+-ATPase mRNA isoform expression (mean ± SD) in PDM at 4 time points (n = 6 horses). *See* Figure 2 for key.

wet weight vs post-training value, 212 ± 7 pmol/g of wet weight; $P < 0.05$). In the PDM, no significant changes were detected as a result of training (pretraining value, 223 +/- 15 pmol/g of wet weight vs posttraining value, 241 +/- 7 pmol of g wet weight).

Discussion

To the authors' knowledge, this is the first study of different isoforms of the Na^+,K^+-pump in equine skeletal muscle. The major findings of the present study were that 6 Na^+,K^+-ATPase mRNA isoforms were detectable in equine VLM and PDM, but only the α2 and β1 protein isoforms could be detected; a single bout of exercise increased mRNA expression of α1, α2, α3, and β2 and α1 and β3 Na^+,K^+-ATPase isoforms in VLM and PDM, respectively, without detectable changes in corresponding proteins; basal mRNA expression increased for α3 and β1 in VLM and β3 in PDM after 18 weeks of training; a bout of exercise after 18 weeks of training induced no further increases of mRNA isoform expression; and the responses of the 2 muscles to acute exercise and long-term training were different.

The mRNA isoforms α2, α3, and β1 were most abundant, whereas β2 was also expressed in high copy numbers. Isoforms α1 and β3 were detected but in low copy numbers. The present findings of α2 and β1 being most dominantly expressed were in agreement with studies in vivo (ie, human VLM)[9] and in vitro (ie, murine C2C12 cells).[27] In contrast, the finding of an equal amount of copy numbers for the α3 and the α2 isoforms in equine muscle was different from results found by Nordsborg et al[9] in human muscle, who reported much lower basal copy numbers of the α3 isoform mRNA. Unfortunately, we were not able to detect α3 protein in equine muscle; therefore, the importance of that finding is difficult to determine.

Although all 6 isoforms could be detected as protein in other equine tissues and as mRNA in equine muscle, only α2 and β1 proteins could be detected via Western blotting in muscle samples. These 2 isoforms were also those with the highest mRNA copy numbers. At this time, we can only conclude that the amount of α1, α3, β2, and β3 proteins in equine muscle is rather low in basal conditions. Despite comparable expression of the mRNA isoforms in VLM and PDM, concentrations of α2 and β1 proteins were higher in PDM. This is in agreement with the higher total Na^+,K^+-ATPase content in PDM as measured via [^{3}H]ouabain binding because this ligand primarily detects the α isoform.[25] The difference between VLM and PDM can be explained by the observation that PDM contains a higher number of fast-twitch muscle fibers,[28] which is associated with a higher number of Na^+,K^+-pumps.[2]

Results of the present study can only be compared with those of studies in humans[6,7,9,13-15,29] and rats.[11] The relative increases in expression of the different mRNA isoforms in VLM in response to SET 1 were in accordance with those detected in other studies,[7,9] apart from the 15-times increase in α1 mRNA expression. For the PDM, a response to acute exercise was detected only for α1 and β3 isoform mRNA, whereas for the VLM, a response to acute exercise was detected for α1, α2, α3, and β2 isoform mRNA and not for β3, indicating that the 2 muscles responded differently to exercise. This may be accounted for by differences in function or involvement in the exercise protocol. In a study[11] with rats, a different response to exercise was found for red and white hind limb muscles.

The increase of the α1 isoform after exercise has been observed in all muscles studied[7,15,30] and theoretically can easily be understood. Because of the low amount of copy numbers of α1 mRNA in resting muscle (Figure 1), a large increase in α1 mRNA may easily be achieved, compared with other mRNA isoforms that are present in larger copy numbers.

Unfortunately, we were not able to measure increases in mRNA isoform β1 in any of the muscles, although in several human studies,[6,7,15] isoform β1 also did not increase directly after exercise. Conversely, Nordsborg et al[9] detected an increase of mRNA isoform β1 in VLM, and Murphy et al[7] found positive effects on β1 when data from 3 biopsy specimens taken at different time points after exercise were pooled. The increase of mRNA isoform β2 in VLM is in accordance with findings in other studies.[6,7] However, in 1 study,[9] isoform β2

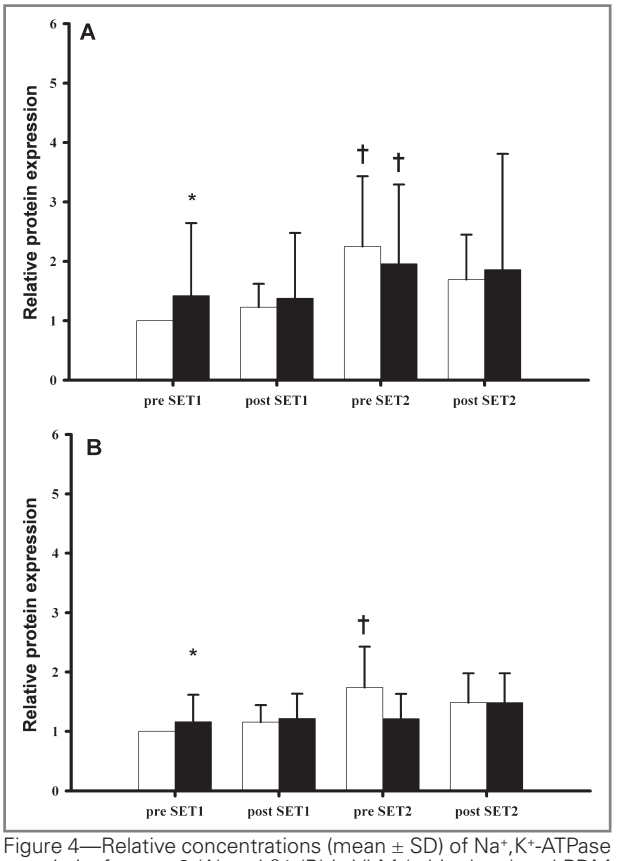

Figure 4—Relative concentrations (mean ± SD) of Na^+,K^+-ATPase protein isoforms α2 (A) and β1 (B) in VLM (white bars) and PDM (black bars) at 4 time points (n = 6 horses). *Significant ($P < 0.05$) difference between VLM and PDM. *See* Figure 2 for remainder of key.

did not increase with exercise. Presumably, differences in type, intensity, and duration of exercise as well as function explain differences in isoform adaptations to exercise. Surprisingly, mRNA isoform β3 was increased by a factor of 3 in PDM after a single bout of exercise, whereas this isoform did not change in VLM, which otherwise had the largest increases in mRNA isoforms. Also, results of studies[6,9] in humans are equivocal with respect to the changes of mRNA isoform β3 after exercise. Murphy et al[7] found only an increase after collecting results of biopsy specimens taken at different time points after exercise.

Protein production of the α2 and β1 isoforms did not change in response to a single bout of exercise in the horses studied here. None of the studies in humans detected increases in isoform-specific proteins directly after a single bout of exercise, apart from 1 study[6] in which increases in α2 and β1 proteins were detected 24 hours after cessation of the exercise, indicating that translation of the mRNA into protein requires several hours. However, another study[13] measured increases of α2 protein after 2 bouts of 6 minutes of cycling within a time span of 1 hour and 6 minutes. It could be either the number of bouts or the time lag between cessation of exercise and biopsy that determines whether changes in protein concentration can be detected. Unfortunately, we were not able to take biopsy specimens 24 hours after exercise because of the limitations of the project.

Although significant increases in several Na^+,K^+-ATPase mRNA isoforms were found after exercise in the untrained state, they were apparently transient because training only resulted in increased resting values for mRNA isoforms α3 and β1 in VLM and β3 in PDM. The effect of training on the expression of the isoforms was in the same order of magnitude as those induced by a single bout of exercise. Downregulation of Na^+,K^+-ATPase mRNA as detected by Murphy et al[14] was not evident, perhaps because training occurred for several years in that study. Conversely, well-trained athletes had no downregulation but did have upregulation of the mRNAs of some of the Na^+,K^+-ATPase isoforms after a 3-week period of training.[16] Values for β1 protein in VLM corresponded with the results for mRNA isoform β1 in this study.

Together with the increase of isoform α2 protein, the increased [^{3}H]ouabain binding detected after training corresponded well with results of other studies.[13,14] The increase of α2 isoform protein in PDM did not correspond with the data on Na^+,K^+-ATPase content by measurement of [^{3}H]ouabain binding, although a small but nonsignificant increase of Na^+,K^+-ATPase content was detected in PDM.

No effects of acute exercise on mRNA or protein values of the isoforms were detected after 18 weeks of training. The same workload in an exercise test after training for 18 weeks may not be sufficient to upregulate mRNA, compared with the untrained state. Nordsborg et al[15] reported similar data, albeit after 5.5 weeks of high-intensity training. Apart from the workload, it might be that further upregulation of mRNA expression in trained individuals occurs with a longer interval after the exercise test.

The mechanism of the increase in Na^+,K^+-ATPase mRNA isoforms that is evident after acute exercise has been discussed in several studies.[6,9,13,16,31] The common observation of these studies is that the reduction in Na^+,K^+-ATPase activity after a short bout of exercise is correlated with increased Na^+,K^+-ATPase gene expression, suggesting a signal transduction role for the decreased enzyme activity that occurs at fatigue. However, when Na^+,K^+-ATPase activity is increased after training, enzyme activity is still decreased after a short bout of exercise, although it is at a higher value. This would still trigger upregulation of mRNAs of the Na^+,K^+-ATPase, albeit to a smaller extent as compared with the upregulation observed after training. Our results that the upregulation of Na^+,K^+-ATPase gene expression was smaller after acute exercise in trained horses would be in agreement with this theory.

a. Mustang 2000, Kagra AG, Fahrwangen, Switzerland.
b. Polar electro Nederland BV, Antennestraat, The Netherlands.
c. Cardio perfect stress, version 4.0, Cardio Perfect Inc, Atlanta, Ga.
d. Maastricht Instruments, Maastricht, The Netherlands.
e. Qiagen GmbH, Hilden, Germany.
f. Bio-Rad Laboratories Inc, Hercules, Calif.
g. Promega Corp, Madison, Wis.
h. Sigma-Aldrich Corp, St Louis, Mo.
i. PerkinElmer, Boston, Mass.
j. BCA assay kit, Pierce Chemical Co, Rockford, Ill.
k. SuperSignal West Pico chemiluminescent substrate, Pierce Protein Research Products, Rockford, Ill.

l. Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City, Iowa.
m. Chemicon International, Bio-connect BV, Huissen, The Netherlands.
n. Affinity Bioreagents, Golden, Colo.
o. BD Biosciences Pharmingen, Franklin Lakes, NJ.
p. SPSS, version 12.0.1 for Windows, SPSS Inc, Chicago, Ill.

References

1. Rivero JL. A scientific background for skeletal muscle conditioning in equine practice. *J Vet Med A Physiol Pathol Clin Med* 2007;54:321–332.
2. Clausen T. Na+-K+ pump regulation and skeletal muscle contractility. *Physiol Rev* 2003;83:1269–1324.
3. Rietbroek NJ, Dingboom EG, Joosten BJ, et al. Effect of show jumping training on the development of locomotory muscle in young horses. *Am J Vet Res* 2007;68:1232–1238.
4. Suwannachot P, Joosten BJ, Klarenbeek A, et al. Effects of training on potassium homeostasis during exercise and skeletal muscle Na+,K(+)-ATPase concentration in young adult and middle-aged Dutch Warmblood horses. *Am J Vet Res* 2005;66:1252–1258.
5. Green HJ. Cation pumps in skeletal muscle: potential role in muscle fatigue. *Acta Physiol Scand* 1998;162:201–213.
6. Murphy KT, Petersen AC, Goodman C, et al. Prolonged submaximal exercise induces isoform-specific Na+-K+-ATPase mRNA and protein responses in human skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 2006;290:R414–R424.
7. Murphy KT, Snow RJ, Petersen AC, et al. Intense exercise up-regulates Na+,K+-ATPase isoform mRNA, but not protein expression in human skeletal muscle. *J Physiol* 2004;556:507–519.
8. Keryanov S, Gardner KL. Physical mapping and characterization of the human Na,K-ATPase isoform, ATP1A4. *Gene* 2002;292:151–166.
9. Nordsborg N, Thomassen M, Lundby C, et al. Contraction-induced increases in Na+-K+-ATPase mRNA levels in human skeletal muscle are not amplified by activation of additional muscle mass. *Am J Physiol Regul Integr Comp Physiol* 2005;289: R84–R91.
10. Blanco G, Mercer RW. Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. *Am J Physiol* 1998;275: F633–F650.
11. Tsakiridis T, Wong PP, Liu Z, et al. Exercise increases the plasma membrane content of the Na+ -K+ pump and its mRNA in rat skeletal muscles. *J Appl Physiol* 1996;80:699–705.
12. Juel C, Nielsen JJ, Bangsbo J. Exercise-induced translocation of Na(+)-K(+) pump subunits to the plasma membrane in human skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 2000;278:R1107–R1110.
13. Green HJ, Duhamel TA, Holloway GP, et al. Muscle Na+-K+-ATPase response during 16 hours of heavy intermittent cycle exercise. *Am J Physiol Endocrinol Metab* 2007;293:E523–E530.
14. Murphy KT, Aughey RJ, Petersen AC, et al. Effects of endurance training status and sex differences on Na+,K+-pump mRNA expression, content and maximal activity in human skeletal muscle. *Acta Physiol (Oxf)* 2007;189:259–269.
15. Nordsborg N, Bangsbo J, Pilegaard H. Effect of high-intensity training on exercise-induced gene expression specific to ion homeostasis and metabolism. *J Appl Physiol* 2003;95:1201–1206.
16. Aughey RJ, Murphy KT, Clark SA, et al. Muscle Na+,K+ATPase activity and isoform adaptations to intense interval exercise and training in well-trained athletes. *J Appl Physiol* 2007;103:39–47.
17. Dela F, Holten M, Juel C. Effect of resistance training on Na,K pump and Na+/H+ exchange protein densities in muscle from control and patients with type 2 diabetes. *Pflugers Arch* 2004;447:928–933.
18. Mohr M, Krustrup P, Nielsen JJ, et al. Effect of two different intense training regimens on skeletal muscle ion transport proteins and fatigue development. *Am J Physiol Regul Integr Comp Physiol* 2007;292:R1594–R1602.
19. Nielsen JJ, Mohr M, Klarskov C, et al. Effects of high-intensity intermittent training on potassium kinetics and performance in human skeletal muscle. *J Physiol* 2004;554:857–870.
20. McCutcheon LJ, Geor RJ, Shen H. Skeletal muscle Na(+)-K(+)-ATPase and K+ homeostasis during exercise: effects of short-term training. *Equine Vet J Suppl* 1999;30:303–310.
21. Suwannachot P, Verkleij CB, Kocsis S, et al. Specificity and reversibility of the training effects on the concentration of Na+,K+-ATPase in foal skeletal muscle. *Equine Vet J* 2001;33:250–255.
22. Suwannachot P, Verkleij CB, Weijs WA, et al. Effects of training on the concentration of Na+, K+-ATPase in foal muscle. *Equine Vet J Suppl* 1999;31:101–105.
23. Bruin G, Kuipers H, Keizer HA, et al. Adaptation and overtraining in horses subjected to increasing training loads. *J Appl Physiol* 1994;76:1908–1913.
24. Jemiolo B, Trappe S. Single muscle fiber gene expression in human skeletal muscle: validation of internal control with exercise. *Biochem Biophys Res Commun* 2004;320:1043–1050.
25. Nørgaard A, Kjeldsen K, Clausen T. A method for the determination of the total number of 3H-ouabain binding sites in biopsies of human skeletal muscle. *Scand J Clin Lab Invest* 1984;44:509–518.
26. Nørgaard A, Kjeldsen K, Hansen O, et al. A simple and rapid method for the determination of the number of 3H-ouabain binding sites in biopsies of skeletal muscle. *Biochem Biophys Res Commun* 1983;111:319–325.
27. Orlowski J, Lingrel JB. Differential expression of the Na,K-ATPase alpha 1 and alpha 2 subunit genes in a murine myogenic cell line. Induction of the alpha 2 isozyme during myocyte differentiation. *J Biol Chem* 1988;263:17817–17821.
28. van Dam KG, van Breda E, Schaart G, et al. Investigation of the expression and localization of glucose transporter 4 and fatty acid translocase/CD36 in equine skeletal muscle. *Am J Vet Res* 2004;65:951–956.
29. Green HJ, Chin ER, Ball-Burnett M, et al. Increases in human skeletal muscle Na(+)-K(+)-ATPase concentration with short-term training. *Am J Physiol* 1993;264:C1538–C1541.
30. Nordsborg N, Goodmann C, McKenna MJ, et al. Dexamethasone up-regulates skeletal muscle maximal Na+,K+ pump activity by muscle group specific mechanisms in humans. *J Physiol* 2005;567:583–589.
31. Petersen AC, Murphy KT, Snow RJ, et al. Depressed Na+-K+-ATPase activity in skeletal muscle at fatigue is correlated with increased Na+-K+-ATPase mRNA expression following intense exercise. *Am J Physiol Regul Integr Comp Physiol* 2005;289:R266–R274.

Appendix

The Na^+,K^+-ATPase genes $\alpha1$ to $\alpha3$ and $\beta1$ to $\beta3$ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) equine primer sequences used for mRNA analyses.

Gene	Genbank accesion No.	Sense primer (5′-3′)	Antisense primer (5′-3′)	Base pairs
$\alpha1$	EU_423853	TGCCGACAGAATTTGACC	CTTAGCCTTGATGAACTTCAG	149
$\alpha2$	EU_423854	GGGAAGGAGAGAAGATGC	GATGAGTTATCCACCTTACAG	133
$\alpha3$	EU_423856	CGAGATTGAGCACTTTATCC	ACAATGATGCCGATGAGG	129
$\beta1$	EU_423855	CGGCTACAAAGAGGGCAAACC	ACGGGCTGTACTTCATCACTGG	121
$\beta2$	EU_423857	CCAGAGCATGAATGTCACC	TTTGCCGTAGTAGGGGAAG	147
$\beta3$	EU_423858	GCACTCTTCTCGTTCACAATG	TGGATCAGACACACGGAATG	101
GAPDH	XR_036506.1	GCTGGTGCTGAATATGTTGTGG	AGCAGAAGGAGCAGAGATGATG	111