Exercise-induced increase in maximal in vitro Na-K-ATPase activity in human skeletal muscle

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Juel C, Nordsborg NB, Bangsbo J. Exercise-induced increase in maximal in vitro Na-K-ATPase activity in human skeletal muscle. Am J Physiol Regul Integr Comp Physiol 304: R1161–R1165, 2013. First published April 10, 2013; doi:10.1152/ajpregu.00591.2012.—The present study investigated whether maximal in vitro Na-K-ATPase activity in human skeletal muscle is changed with exercise and whether it was altered by acute hypoxia. Needle biopsies from 14 subjects were obtained from vastus lateralis before and after 4 min of intense muscle activity. In addition, six subjects exercised also in hypoxia (12.5% oxygen). The Na-K-ATPase assay revealed a 19% increase (P < 0.05) in maximal velocity (Vmax) for Na+-dependent Na-K-ATPase activity after exercise and a tendency (P < 0.1) toward a decrease in Km for Na+ (increased Na+ affinity) in both normoxia and hypoxia. In contrast, the in vitro Na-K-ATPase activity determined with the 3-O-MFPase technique was 11–32% lower after exercise in normoxia (P < 0.05) and hypoxia (P < 0.1). Based on the different results obtained with the Na-K-ATPase assay and the 3-O-MFPase technique, it was suggested that the 3-O-MFPase method is insensitive to changes in Na-K-ATPase activity. To test this possibility, changes in Na-K-ATPase activity was induced by protein kinase C activation. The changes quantified with the Na-K-ATPase assay could not be detected with the 3-O-MFPase method. In addition, purinergic stimulated Na-K-ATPase activity in rat muscle membranes; these changes could not be detected with the 3-O-MFPase method. Therefore, the 3-O-MFPase technique is not sensitive to changes in Na+ sensitivity, and the method is not suited to detecting changes in Na-K-ATPase activity with exercise. In conclusion, muscle activity in humans induces an increased in vitro Na+-dependent Na-K-ATPase activity, which contributes to the upregulation of the Na-K-ATPase in association with exercise both in normoxia and hypoxia.

Na-K pump; acute regulation; adaptation to exercise

ION GRADIENTS ACROSS THE MUSCLE membrane undergo pronounced perturbations during intense muscle contractions. These activity-induced changes in ion distribution affect muscle excitability and may lead to impairment of force development (muscle fatigue). The Na-K-ATPase (Na-K-pump) counteracts the rundown of transmembrane gradients for Na+ and K+. Regulation of Na-K-ATPase is therefore of importance for muscle function. It is generally accepted that the Na-K-ATPase is upregulated during muscle activity by a multifactorial process, and the Na-K-pump is sensitive to hormones and the intracellular Na+ concentration. Furthermore, purinergic stimulation may be involved (4, 24).

A number of studies in humans have reported that the maximal in vitro Na-K-ATPase activity, determined with the 3-O-MFPase technique, is reduced after exercise (14). Although there seems to be consensus about this reduction in maximal in vitro Na-K-ATPase activity after exercise, this is not supported by measurements using a more direct Na-K-ATPase assay. It has been demonstrated that treadmill running in rats significantly reduced the Km for Na+ (increased Na+ affinity) in membranes from glycolytic muscle, whereas the Km in membranes from oxidative muscle remained unchanged (9). The reduction in Km implies an increased in vitro Na-K-ATPase activity at physiological Na+ concentrations (10). For both fiber types, maximal velocity (Vmax) only tended to increase with exercise (9, 10). Of note, this mechanism is not due to the presence of hormones or different ion concentrations, since the membranes underwent a washing procedure before the measurements. Instead, changes in protein characteristics must be involved. It is not known whether similar changes in Na-K-ATPase activity take place in human skeletal muscle.

Hypoxia is another potent regulator of Na-K-ATPase activity. The Na-K ATPase activity can be inhibited by hypoxia (23), but this mechanism may not always be physiologically relevant (22). In humans, hypoxic exposure (inspired O2 fraction [FIO2] of 14%) has been reported to partly inactivate the Na-K-ATPase during 90 min of cycling exercise (18), whereas Na-K-ATPase activity was similar in hypoxia (FIO2, of 14%) and normoxia after progressive cycling exercise to fatigue (19). Thus it appears unclear whether Na-K-ATPase activity is affected by mild hypoxic exposure in human skeletal muscle.

Therefore, the aim of the present study was to examine the changes in in vitro Na-K-ATPase activity in human skeletal muscle with exercise. In addition, we investigated whether hypoxia during exercise influenced the in vitro ATPase activity.

For that purpose, human muscle biopsies were obtained before and immediately after 4 min of intense exercise. The in vitro Na-K-ATPase activity was quantified with both an ATPase assay and the 3-O-MFPase method.

MATERIALS AND METHODS

Subjects. Fourteen male volunteers participated in the study, which was approved by the regional ethics committee for the capital region of Denmark (H-A-2009-016).

All subjects gave written, informed consent to participate. Brief maximal intensity exercise was investigated to activate as many muscle fibers as possible. In a pretest, subjects performed two-legged knee extension exercise on a modified ergometer. Workload was determined in pretrials by having the subjects perform as much work as possible for 5 min. In the pretrial, braking force was constant (0.15 N/kg body wt), and the subjects were instructed to kick as fast as possible for the entire 5-min period. The resulting average power was used at the experimental workload. On the experimental day, the
workload was constant (98 ± 5 W), and the subject was instructed to keep the kicking rate at 60 RPM. The exercise period was 4 min.

A needle biopsy was obtained from m. vastus lateralis before and after exercise. Samples were stored at −80°C before use. Six subjects participated in two independent experiments; one experiment had subjects breathing normal air (normoxia), and one experiment had subjects breathing 12.5% oxygen (hypoxia).

Treatement of samples. Samples were homogenized for 30 s (Polytron PT 2100) in 250 mM mannitol, 30 mM t-histidine, 5 mM EGTA, and 0.1% deoxycholate, adjusted to pH 6.8 with Tris-base. Samples of the crude homogenate were stored for the 3-O-MFPase measurements. The remaining crude homogenates were centrifuged at 3,000 g for 30 min, and the resulting supernatant was centrifuged at 190,000 g for 90 min (at 4°C). The final pellets were used for both the ATPase assay and the 3-O-MFPase method.

Na+-ATPase assay. Na+-stimulated Na-K-ATPase activity was determined by measuring ATP hydrolysis. Released Pi was detected according to Fraser and McKenna (6). Briefly, samples (10 μl/H9262 protein) were incubated in assay medium (5 mM MgCl2, 1.25 mM KCl, 50 mM Tris-base, 5 mM EGTA, pH 7.4). Na+- was added to the samples to a final concentration of 0, 2, 4, 6, 10, 20, 40, and 80 mM (the ionic strength was kept constant by substituting NaCl with choline chloride). After 5 min of pre-incubation at 37°C, the reaction was started by adding Mg-ATP to a final concentration of 0.5 mM. After 30 min, the reaction was terminated by adding 1 ml of Biomol Green reagent. After 30 min of incubation, absorbance was read at 620 nm, and [Pi] was calculated from a standard curve. All samples were run in duplicate (0 mM Na+-Mg-ATPase value for each sample was obtained as mean of a triple measurement. P < 0.05 was considered to be statistically significant.

RESULTS

Protein yield. The ratio between the amount of total protein in the 190,000 g fraction and the amount of total protein in the crude homogenate was similar after exercise compared with before exercise (0.020 ± 0.005 vs. 0.019 ± 0.004). Western blotting experiments were performed with homogenate samples and 190,000 g samples on the same gel and with the same amount of protein to calculate the purification factors. The purification factor (protein in the 190,000 g fraction/protein in the homogenate) was 5.1 ± 1.0 (SD; n = 12, both pre- and postexercise samples included) for the Na-K-ATPase α1 subunit and 5.9 ± 2.2 for the α2 subunit. Thus the calculated recovery for the α1- and α2-isoform protein was 10.2% and 11.8%, respectively.

The Na-K-ATPase α-subunit protein expression in the 190,000 g fraction measured by Western blot was similar before and after exercise (103 ± 14 vs. 100 ± 10 arbitrary density units/mg of protein; n = 6).

Na+-dependent Na-K-ATPase activity before and after exercise. Figure 1 shows the Na+-dependent Na-K-ATPase activity at various Na+- concentrations in samples obtained before and after exercise. The mean values of Na+-dependent Na-K-ATPase activity differed after exercise compared with before exercise (n = 14; P < 0.001). Curve fits to the individual values showed Vmax being 19% higher (P < 0.05) after compared with before exercise (496 ± 19 vs. 415 ± 21 pmol·mg⁻¹·h⁻¹). Km for the Na+ activation of Na-K-ATPase was 6.4 ± 0.8 mM before exercise and tended (P = 0.1) to be lower after exercise (4.7 ± 0.6 mM).

The Na+-dependent Na-K-ATPase activity before and after exercise was quantified in six subjects both in normoxia and hypoxia. Data demonstrated that the Na-K-ATPase activity after exercise was higher (P < 0.01) than before exercise both in normoxia and hypoxia. The exercise-induced increase in Vmax was similar in normoxia and hypoxia. Changes in Km for Na+ could not be analyzed due to the low number of subjects in each group (Fig. 2).
3-O-MFP analysis. The 3-O-MFPase activity was quantified in the crude homogenates prepared from samples obtained before and after exercise both in normoxia and hypoxia. In normoxia, the 3-O-MFPase activity was lower ($P < 0.05$) in the homogenate samples obtained after exercise compared with before exercise, whereas only a tendency ($P < 0.1$) to a reduction was observed in hypoxia (Fig. 3).

The 3-O-MFPase activity was also measured in the 190,000 $g$ fraction used in the experiments depicted in Fig. 2. In normoxia, the 3-O-MFPase activity was lower ($P < 0.05$) in the samples obtained after compared with before exercise, and a tendency ($P < 0.1$) was seen in hypoxia (Fig. 3).

Validation of the 3-O-MFPase technique. 3-O-MFPase activity was measured in rat muscle material with and without 20-min pre-incubation with the stable ADP analog MeS-ADP, which has previously been reported to increase $V_{\text{max}}$ and decrease $K_m$ for Na$^+$ if quantified with the ATPase assay. All measurements were performed with three different potassium concentrations in an attempt to reveal changes in affinity. The 3-O-MFPase activity at 5, 10, and 15 mM potassium was not different in MeS-ADP incubated samples compared with control (Fig. 4). The 3-O-MFPase method therefore, seems not to be sensitive to the expected changes in Na-K-ATPase activity.

DISCUSSION

This is the first study to demonstrate that maximal in vitro Na-K-ATPase activity is elevated by exercise in human skeletal muscle when quantified with the Na-K-ATPase assay. In addition, acute hypoxia had no effect on exercise-induced changes in Na-K-ATPase activity.

Underlying mechanisms for changes in Na-K-ATPase activity. Treadmill running in rats has been shown to increase in vitro Na-K-ATPase activity. This effect is partly mediated by a decrease in $K_m$ for Na$^+$ (increased Na$^+$ affinity) (9). In addition, exercise has been shown to increase the association between the $\alpha$ and PLM subunits in rat (17), and exercise in humans has been demonstrated to increase the phosphorylation of the PLM unit (3, 21), which is known to modify the Na-K-ATPase ion affinity (13). Therefore, exercise-induced PLM phosphorylation and the resulting affinity changes are likely involved in the exercise-induced increase in ATPase activity revealed with the ATPase assay in the present study. Other mechanisms, such as phosphorylation of $\alpha$ subunits, could also be involved. Furthermore, the changes in affinity ($K_m$) and in $V_{\text{max}}$ may arise from different mechanisms, as previously suggested (24).

The changes reported here could not be related to differences in protein yield and Na-K-ATPase protein content between samples. The data in Figs. 1–3 is expressed per milligram of protein and not corrected for the $\sim 3\%$ lower Na-K-ATPase activity at 5, 10, and 15 mM potassium was not different in MeS-ADP incubated samples compared with control (Fig. 4). The 3-O-MFPase method therefore, seems not to be sensitive to the expected changes in Na-K-ATPase activity.

The magnitude and direction of changes in 3-O-MFPase activity and Na-K-ATPase activities in the 190,000 $g$ fraction of the human samples were compared in experiments known to influence Na-K-ATPase activity. Pre-incubation with 100 nM PMA (phorbol 12-myristate 13-acetate) increased the Na-K-ATPase activity at 5 mM Na$^+$ ($P < 0.05$) (Fig. 5A). The 3-O-MFPase activity was unaffected by PMA in identical samples (Fig. 5B).
protein expression (obtained by Western blot) after exercise. Furthermore, the recovery of α1 and α2 isoform protein in the membrane fraction used for the ATPase assay was similar, which suggests that the recovered protein does not represent a subtraction, such as T-tubular membranes.

In contrast to the finding in the present study that the Na-K-ATPase activity was increased with exercise, the 3-O-MFPase activity was reduced with exercise. A number of studies have shown that the maximal in vitro Na-K-ATPase activity quantified with the 3-O-MFPase method is reduced with exercise, a phenomenon usually referred to as inactivation of Na-K-pumps. The exercise protocols included submaximal cycling in humans (15), fatiguing knee extensor exercise of short duration (7, 16), high-intensity interval cycling (2), submaximal cycling (8, 12), incremental exercise in humans (1, 18), and prolonged treadmill running in rats (5). The reported depression of maximal Na-K-ATPase activity ranges from 5 to 15% in some studies (1, 5, 12, 16) to 24 to 35% in others (15, 19). It is noteworthy that one study using electrical stimulation in rats reported an increased Na-K-ATPase activity after contractions (20). A review paper listing these and other papers concluded that the maximal in vitro Na-K-pump activity in human and measured by the 3-O-MFPase method is reduced after exhaustive muscle activity (14). The present study is in line with these studies.

Methodological considerations. There may be a number of reasons for the different responses with the two ATPase quantification methods. The ATPase assay directly quantifies the release of P_i from the hydrolysis of ATP, whereas the 3-O-MFPase method uses an artificial substrate (3-O-methylflurorescein). In addition, the last method only quantifies the stimulatory effect of K^+ and does not allow the presence of Na^+.

Since modifications in Na-K-ATPase activity may involve changes in PLM phosphorylation (13) and resulting changes in Na^+ affinity (9), it may be that the 3-O-MFPase method is unable to detect changes in Na-K-ATPase activity caused by changes in Na^+ affinity. Another important issue is that the studies reporting reduced Na-K-ATPase activity (measured with the 3-O-MFP method) after exercise all used incubation with 10 mM K^+ (6). Since the K^+ dependency is bell shaped with the highest activity at 10 mM, an alternative hypothesis could be that if K^+ affinity changes take place, the activity curve is shifted along the concentration axis, which could result in an apparent lower activity value simply because 10 mM K^+ is not the optimal concentration for the measurements after exercise. To test this hypothesis, we performed measurements at three different potassium concentrations (5, 10, and 15 mM). The K^+ affinity was unchanged after exercise compared with before (Fig. 4).

Purinergic stimulation has been demonstrated to increase the Na-K-ATPase activity both in isolated rat muscle (4) and in rat muscle homogenates (24). The stable ADP analog 2-methylthio-ADP (2-MeS-ADP) has been reported to increase Na-K-ATPase V_max by 47% at 0.1 mM and by 78% at 0.5 mM in rat muscle homogenates (24). This effect was paralleled by a reduction in K_m for Na^+ (increased affinity) quantified with the same Na-K-ATPase assay as in the present study. We therefore used purinergic stimulation to test whether the 3-O-MFPase technique is able to detect such changes in Na-K-ATPase activity. Rat muscle material was used to ensure large uniform muscle samples. Incubation with MeS-ADP had no stimulatory effect on the activity level (Fig. 4). Furthermore, the direction and magnitude of changes in 3-O-MFPase activity and Na-K-ATPase activities in the 190,000 g fraction of the human samples were compared in experiments known to influence Na-K-ATPase activity. PMA [protein kinase C activation (24)] induced an increased Na-K-ATPase activity at low Na^+ (increased K_m). In contrast, the 3-O-MFPase activity was unaffected by PMA (Fig. 5). It is therefore concluded that the 3-O-MFPase method is not suitable to detect changes in Na^+ sensitivity and may therefore result in misleading conclusions about exercise-induced changes in in vitro maximal Na-K-ATPase activity.

Apparently, the Na-K-ATPase assay measuring P_i release is the most reliable method, and maximal in vitro Na-K-ATPase activity is increased with exercise.

In summary, the present study demonstrated that muscle activity increases maximal in vitro Na-K-ATPase activity and that this mechanism is not influenced by acute hypoxia. This is in contrast to the described reduction observed using the 3-O-MFPase assay (18) but is likely to be related to differences in the methods as outlined above. Moreover, the present findings demonstrate that the possibility of Na-K-ATPase inhibition by moderate hypoxia (Fi_o2 of 12.5%) appears to be
physiological irrelevant in human skeletal muscle during intense contractions.

**Perspectives and significance**

In vivo muscle activity induces an extra load on the Na-K-ATPase, which must be accelerated to counteract the ion-concentration changes due to membrane excitation. This requirement is met by several mechanisms, including hormonal stimulation, stimulation by increased intracellular Na\(^+-\), and purinergic activation. The present study shows that activity-induced changes in maximal Na-K-ATPase activity may contribute to the increased Na-K-ATPase activity after exercise.

**REFERENCES**