Prolonged submaximal exercise induces isoform-specific Na\(^+\)-K\(^+\)-ATPase mRNA and protein responses in human skeletal muscle

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This investigation evaluated the effects of prolonged submaximal exercise on Na\(^+\)-K\(^+\)-ATPase mRNA and protein expression, maximal activity, and content in human skeletal muscle. We also investigated the effects on mRNA expression of the transcription initiator gene, RNA polymerase II (RNAP II), and key genes involved in protein translation, eukaryotic initiation factor-4E (eIF-4E) and 4E-binding protein 1 (4E-BP1). Eleven subjects (6 men, 5 women) cycled at 75.5% (SD 4.8%) peak O\(_2\) uptake and continued until fatigue. A vastus lateralis muscle biopsy was taken at rest, fatigue, and 3 and 24 h postexercise. We analyzed muscle for Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\), \(\alpha_2\), \(\alpha_3\), \(\beta_1\), and \(\beta_3\), as well as RNAP II, eIF-4E, and 4E-BP1 mRNA expression by real-time RT-PCR and Na\(^+\)-K\(^+\)-ATPase isoenzyme activity using immunoblotting.

Muscle homogenate maximal Na\(^+\)-K\(^+\)-ATPase activity was determined by 3-O-methylfluorescein phosphatase activity and Na\(^+\)-K\(^+\)-ATPase content by \[^3\text{H}\]ouabain binding. Cycling to fatigue [54.5 (SD 20.6) min] immediately increased \(\alpha_3\) (\(P = 0.044\)) and \(\beta_2\) mRNA (\(P = 0.042\)) by 2.2- and 1.9-fold, respectively, whereas \(\alpha_1\) mRNA was elevated by 2.0-fold at 24 h postexercise (\(P = 0.036\)). A significant time main effect was found for \(\alpha_3\) protein abundance (\(P = 0.046\)). Exercise transiently depressed maximal Na\(^+\)-K\(^+\)-ATPase activity (\(P = 0.004\)), but Na\(^+\)-K\(^+\)-ATPase content was unaltered throughout recovery. Exercise immediately increased RNAP II mRNA by 2.6-fold (\(P = 0.011\)) but had no effect on eIF-4E and 4E-BP1 mRNA. Thus a single bout of prolonged submaximal exercise induced isoform-specific Na\(^+\)-K\(^+\)-ATPase responses, increasing \(\alpha_1\), \(\alpha_3\), and \(\beta_2\) mRNA but only \(\alpha_3\) protein expression. Exercise also increased mRNA expression of RNAP II, a gene initiating translation, but not of eIF-4E and 4E-BP1, key genes initiating protein translation.

gene expression; RNA polymerase II; 3-O-methylfluorescein phosphatase; \[^3\text{H}\]ouabain binding

IN SKELETAL MUSCLE, the Na\(^+\)-K\(^+\)-ATPase enzyme has major regulatory functions, including the maintenance of intra- to extracellular Na\(^+\) concentration ([Na\(^+\)]) and K\(^+\) concentration ([K\(^+\)]) gradients, membrane excitability, and contractility, as well as secondary transport processes (3, 6). The Na\(^+\)-K\(^+\)-ATPase comprises a catalytic \(\alpha\)-subunit (100–112 kDa) and a glycosylated \(\beta\)-subunit (40–60 kDa) and belongs to a multi-gene family, with different genes encoding four \(\alpha\)-isoforms (\(\alpha_1\), \(\alpha_2\), \(\alpha_3\), \(\alpha_4\)) and three \(\beta\)-isoforms (\(\beta_1\), \(\beta_2\), \(\beta_3\)) (3). Human skeletal muscle was recently shown to express each of the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\), \(\alpha_2\), \(\alpha_3\), \(\beta_1\), \(\beta_2\), and \(\beta_3\) gene transcripts and proteins (24). The \(\alpha_4\)-gene transcript has also been detected at low levels in human muscle in two studies (19, 29).

These Na\(^+\)-K\(^+\)-ATPase gene transcripts appear to be very responsive to intense exercise, with only ~6 min of knee extensor exercise elevating the mRNA expression of each of the \(\alpha_1\)-\(\alpha_3\) and \(\beta_1\)-\(\beta_3\) isoforms (24). Furthermore, 15–18 min of intense intermittent exercise elevated \(\alpha_1\) mRNA expression in one study (27) and elevated \(\alpha_1\), \(\alpha_2\), \(\beta_1\), and \(\beta_3\) mRNA expression in another (29). In comparison, isoform protein abundance appears much less responsive to acute exercise. There was no effect of brief intense exercise on the crude muscle homogenate protein abundance of any of the \(\alpha_1\)-\(\alpha_2\)-\(\alpha_3\)-isoforms and \(\beta_1\)-\(\beta_2\)-\(\beta_3\)-isoforms in one study (24) and no effect on the \(\alpha_1\), \(\alpha_2\), and \(\beta_1\) protein abundance in another (18). It is not known whether the lack of change in isoform protein abundance in the face of elevated mRNA expression reflects attenuated translation rates, increased protein degradation, or a combination of both. It is likely that exercise of more prolonged duration is required to increase isoform protein abundance (35). However, little is known about the adaptations in these Na\(^+\)-K\(^+\)-ATPase isoform gene transcripts and proteins in response to prolonged exercise. In rats, 1 h of treadmill running immediately increased \(\alpha_1\) and \(\beta_2\) mRNA expression in red, oxidative and in white, glycolytic muscles, respectively (45). In contrast, there was no effect of this exercise bout on \(\alpha_2\) and \(\beta_1\) mRNA expression, whereas \(\alpha_3\) and \(\beta_3\) mRNA expressions were not measured (45). The effect of a single bout of prolonged submaximal exercise on isoform protein abundance is unknown. Elevated levels of intracellular [Na\(^+\)] and [Ca\(^{2+}\)] have been implicated in regulation of Na\(^+\)-K\(^+\)-ATPase mRNA (40, 41, 44) and protein expression (20, 46) and increases in intracellular [Na\(^+\)] and in [Ca\(^{2+}\)] may also differ between submaximal and intense muscle contractions (15, 43). Hence, the mRNA and protein responses of the Na\(^+\)-K\(^+\)-ATPase isoforms might also differ between submaximal and intense exercise. We have previously determined increased mRNA expression but unchanged protein abundance of each Na\(^+\)-K\(^+\)-ATPase isoforms after intense exercise (24). The primary aim of this study was therefore to investigate the effects of prolonged submaximal exercise on Na\(^+\)-K\(^+\)-ATPase isoform

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It was hypothesized that prolonged submaximal exercise would also increase mRNA expression but not protein abundance of each of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase \( \alpha_1-\alpha_3 \) and \( \beta_1-\beta_3 \) isoforms.

Immediately after prolonged submaximal exercise, Na\textsuperscript{+}-K\textsuperscript{+}-ATPase maximal activity was depressed by \( \sim 13-35\% \) and content unchanged (2, 10, 21, 42). This suggests an exercise-induced structural alteration to the enzyme and/or to the membrane in which it is embedded, rather than a reduction in the number of functional Na\textsuperscript{+}-K\textsuperscript{+}-ATPase enzymes (2, 10, 21, 42). However, little is known about the effects of prolonged submaximal exercise on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase maximal activity and content in the 0- to 24-h period after exercise, when any exercise-induced increase in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase content might be expected to occur (35), and this was therefore a secondary focus of this study. A recent study found a negative correlation between the depression in maximal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity and the increase in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase mRNA expression with brief intense exercise (36). This was interpreted to suggest a possible modulatory role of depressed Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in muscle Na\textsuperscript{+}-K\textsuperscript{+}-ATPase mRNA expression. Whether a similar relationship is found between maximal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity and isoform mRNA expression after prolonged submaximal exercise is unknown and was investigated in this study. Therefore, the secondary hypothesis tested was that prolonged submaximal exercise would only transiently depress maximal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity and would not elevate Na\textsuperscript{+}-K\textsuperscript{+}-ATPase content in the 24-h period after exercise, with the depression in maximal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity being negatively correlated with the increase in isoform mRNA expression.

Gene transcription and protein translation are vital to the cellular adaptations to prolonged submaximal exercise. Under most circumstances, transcription and translation are controlled at the level of initiation (13, 32), which involves RNA polymerase II (RNAP II) and eukaryotic initiation factor 4E (eIF-4E) and 4E-binding protein 1 (4E-BP1), respectively. RNAP II directly initiates transcription by binding to the promoter region of a protein-coding gene and subsequently elongating the transcript, whereas initiation of translation involves binding of eIF-4E to the gene transcript and the ensuing recognition of a cap structure at the 5’-termini (33). However, recognition only occurs when eIF-4E interacts with other members of the eukaryotic initiation factor family, eIF-4A and eIF-4G, to form a heterotrimetric eIF-4F complex; in skeletal muscle, this event is compromised by the competing interaction of 4E-BP1 (14). Because prolonged submaximal exercise increases the mRNA expression of numerous metabolic genes (37), but inhibits protein synthesis (13), it is conceivable that prolonged submaximal exercise would increase mRNA expression of RNAP II and 4E-BP1 but not eIF-4E. However, this has yet to be investigated. Although there is no evidence that these key factors are directly involved in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase expression, these factors were also investigated here as global measurements of transcription and translation initiation. Therefore, the third hypothesis tested was that prolonged submaximal exercise would increase the mRNA expression of RNAP II and 4E-BP1 but reduce mRNA expression of eIF-4E.

### METHODS

#### Subjects

Eleven healthy subjects, 6 men and 5 women [age, 23.9 (SD 3.9) yr; height, 171.2 (SD 10.4) cm; body mass, 65.0 (SD 10.8) kg; mean (SD)], gave written informed consent and participated in this study. Most subjects were physical education students and participated in regular physical activity, such as jogging and cycling (30–60 min, 2–3 times/wk) but were not specifically trained in any sport. All protocols and procedures were approved by the Human Research Ethics Committee at Victoria University of Technology and conformed to the Declaration of Helsinki.

#### Experimental Design

Subjects performed three exercise tests cycling at 60 rpm on an electrically braked ergometer (Lode Excalibur, Groningen, the Netherlands), comprising an incremental exercise test, a familiarization step, and then an experimental, prolonged exercise trial. Each test was separated by \( \sim 10 \) days to minimize any training effect. Subjects refrained from vigorous exercise for 48 h and from caffeine, nicotine, and alcohol consumption for 24 h before each of the exercise tests. The experimental design was identical for men and women.

#### Incremental Exercise Test

Each subject completed an incremental exercise test to determine peak oxygen uptake (\( \dot{V}O_2 \text{peak} \)), comprising 4 min at each of 50, 80, 110, and 140 W, and then 25-W increments each minute until fatigue, defined as the inability to maintain pedal cadence above 55 rpm. The work rate corresponding to 75% \( \dot{V}O_2 \text{peak} \) was calculated from the linear regression of submaximal \( \dot{V}O_2 \) vs. work rate, for use in all subsequent exercise sessions. All equipment, procedures, and calibrations were as previously detailed (21).

#### Prolonged Exercise Test

The prolonged submaximal exercise test was conducted at a work rate corresponding to 75% \( \dot{V}O_2 \text{peak} \) and continued until fatigue. The intensity of 75% \( \dot{V}O_2 \text{peak} \) was selected because this intensity, lasting \( \sim 1 \) h, has previously been shown to both induce fatigue and to depress maximal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity (21). Muscle biopsy and venous blood sampling procedures were performed with the prolonged exercise test. Pulmonary \( \dot{V}O_2 \), \( \dot{CO}_2 \) output, and respiratory exchange ratio were measured during the periods 5–10, 25–30, and 40–45 min. Subjects consumed standardized meals composed of 62% carbohydrate, 22% fat, and 16% protein (total 2,452 kJ) on the day before, during, and the subsequent day after the trial. On the day of the invasive trial, subjects were fasted and meals were only consumed after the biopsy at 3 h postexercise was taken. On the day after the invasive trials, subjects were again fasted, with meals consumed after the biopsy at 24 h postexercise. Fluid intake was restricted to water and was given ad libitum throughout.

#### Blood Sampling

Before the prolonged exercise trial, a Teflon catheter (20 gauge; Jelco) was inserted into a superficial dorsal hand vein. The hand was placed in a waterproof plastic glove and was heated in a 45°C water bath throughout the test to allow sampling of arterialized venous blood (23). The arterIALIZATION technique is commonly employed to enable collection of blood of similar composition to arterial blood but without the need for difficult arterial cannulation and associated physical risks and ethical constraints and can be used under rest and exercise conditions (8). Good arterIALIZATION was evidenced by the high resting \( P_O_2 \) [78.2 (SD 15.6) Torr] (8). Samples were taken at rest, in the final 10 s of each 15 min during exercise, immediately after exercise, and at 1, 2, 5, and 10 min in recovery. The catheter was kept patent by regular infusions of sterile heparinized saline.
Blood Processing

The blood sample was transferred to a tube containing lithium heparin; 1 ml of blood was analyzed in duplicate for Hb concentration and Hct using an automated hematology analyzer (Sysmex K-800, Kobe, Japan), and another 1.5 ml were centrifuged at 4,500 rpm for 2 min with the plasma removed and frozen until later duplicate analysis of plasma [K$^+$] using an ion-selective electrode (Ciba Corning 865, Bayer, MA). Plasma [K$^+$] was corrected for changes in plasma volume as calculated by changes in Hb concentration and Hct (23). Analyzers were calibrated immediately before and during the analyses with precision standards.

Muscle Biopsy Sampling

A muscle biopsy was taken from the same leg at rest, immediately after prolonged exercise, and at 3 and 24 h postexercise. A local anesthetic (1% Xylocaine) was injected into the skin and subcutaneous tissue above the vastus lateralis, a muscle of mixed fiber type composition (31). A small incision was then made through the skin and fascia, and a muscle sample of ~120 mg was excised. Biopsies were taken from separate incisions in the same leg, with each sample taken from an incision ~1.5 cm distal to the previous incision. An experienced medical practitioner took all biopsies at approximately constant depth. The muscle sample was then blotted on filter paper to remove blood and immediately frozen in liquid N$_2$ and stored at −80°C until assayed later for Na$^+$-K$^+$-ATPase isoform mRNA and protein expression, maximal 3-O-methylflururine phosphatase (3-O-MFPase) activity, [3H]ouabain binding site content, and RNAP II, eIF-4E, and 4E-BP1 mRNA expressions.

Real-Time RT-PCR Measurement of mRNA

Total RNA was extracted from 5–10 mg of muscle with FastRNA reagents (BIO 101, Vista, CA) using methods previously employed in our laboratory (25). The resulting RNA pellet was dissolved in EDTA-treated water, and total RNA concentration was determined spectrophotometrically at 260 nm. RNA (1 µg) was transcribed into cDNA using the Promega AMV reverse transcription kit (Promega, Madison, WI), and the resulting cDNA was stored at −20°C for subsequent analysis.

Real-time PCR (GeneAmp 5700 sequence detection system; Applied Biosystems, Foster City, CA) was run for 1 cycle (50°C for 2 min, 95°C for 10 min) and 50 cycles (95°C for 15 s, 60°C for 60 s). Fluorescence resulted from incorporation of SYBR Green (SYBR green master mix; Applied Biosystems) to double-stranded DNA, and this fluorescence was measured after each repetitive cycle. All samples were run in triplicate, and measurements included a no-template control (no cDNA) as well as a human skeletal muscle sample endogenous control. Primer sequences were designed for cyclophilin (CYC) and the Na$^+$-K$^+$-ATPase $\alpha_1$, $\alpha_2$, $\alpha_3$, $\beta_1$, $\beta_2$, and $\beta_3$ genes, as previously detailed (24). Primer sequences were also designed for the Na$^+$-K$^+$-ATPase $\alpha_4$, and RNAP II, eIF-4E, and 4E-BP1 genes from published sequences (Table 1). Similar efficiencies of the Na$^+$-K$^+$-ATPase genes [−1.0 mean efficiency (10$^{-1}$/slope − 1) for each gene] and CYC [−1.0 mean efficiency] were obtained using calculations previously described (25). However, the Na$^+$-K$^+$-ATPase $\alpha_4$ gene was undetected by RT-PCR. This is most likely due to the low relative expression of this gene transcript in human muscle (29). Exercise had no significant effect ($P = 0.211$) on the mRNA expression of CYC, when expressed in the linear (2$^{{\text{CT}}}$) form (data not shown) and was therefore used as a control (housekeeping gene) to account for any variations in the amount of input RNA and the efficiency of reverse transcription. Gene expression was quantified using a cycle threshold (CT) method, whereby the relative expression of the genes compared with resting samples was made using the expression 2$^{-\Delta\Delta CT}$, in which the expression of each gene was normalized for input cDNA using the housekeeping gene CYC. The intra-assay coefficient of variation for each target gene was <15.0% for 2$^{-\Delta\Delta CT}$ (Table 2), which is within values previously reported (24, 25).

Western Blot Analysis

Immunoblotting methods were similar to those previously described (24). Muscle samples (20–30 mg) were diluted 1:40 with extraction buffer (25 mM Tris-HCl, pH 6.8, 1% SDS, 5 mM EGTA, 50 mM NaF, 1 mM sodium vanadate, 10% glycerol, 17.4 µg/ml PMSF, 10 µg/ml leupeptin, and 1 µg/ml aprotinin) and homogenized on ice for 15 s at a speed rating of 4 (Polytron PT1200, Kinematica, Lucerne, Switzerland). A portion of each homogenate was heated for 10 min at 90°C and analyzed for total protein content (BCA assay kit; Pierce, Rockford, IL), with BSA as the standard. Stabilizing buffer (0.75 M Tris-HCl, pH 6.8, 25% glycerol, 25 mM DTT, 5% SDS, and 5% 2-mercaptoethanol) was then added to the remaining homogenate in a 1:5 dilution, and each sample was subsequently frozen at −80°C for immunoblotting.

SDS-PAGE (10% separating gel, 5% stacking gel) was performed, and gels were loaded with 20 (α$_1$, β$_1$) or 70 (α$_1$, α$_3$, β$_2$, β$_3$) µg of protein. After electrophoresis (20 min, 100 V; 90 min, 150 V), the protein was transferred (90 min, 100 V) to 0.45-µm nitrocellulose membrane and blocked for 2 h with blocking buffer [5% non-fat milk in Tris-buffered saline-Tween 20 (TBST)]. Membranes were incubated overnight at 4°C in primary antibodies diluted in blocking buffer containing 0.1% NaN$_3$. Membranes were washed in 0.05% TBST and incubated for 1 h in horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse immunoglobulins or goat anti-

Table 2. Intra-assay variability of $2^{-\Delta CT}$ values

<table>
<thead>
<tr>
<th>Gene</th>
<th>$2^{-\Delta CT}$ CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>α$_1$</td>
<td>12.4</td>
</tr>
<tr>
<td>α$_2$</td>
<td>8.9</td>
</tr>
<tr>
<td>α$_3$</td>
<td>14.7</td>
</tr>
<tr>
<td>β$_1$</td>
<td>9.4</td>
</tr>
<tr>
<td>β$_2$</td>
<td>11.2</td>
</tr>
<tr>
<td>β$_3$</td>
<td>12.3</td>
</tr>
<tr>
<td>RNAP II</td>
<td>13.0</td>
</tr>
<tr>
<td>eIF-4E</td>
<td>9.9</td>
</tr>
<tr>
<td>4E-BP1</td>
<td>13.0</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>8.8</td>
</tr>
</tbody>
</table>

Each sample was run in triplicate wells in the same real-time PCR run; $n = 42$. CV, coefficient of variation; CT, cycle threshold.

Table 1. Na$^+$-K$^+$-ATPase $\alpha_4$ and RNAP II, eIF-4E, and 4E-BP1 gene primer sequences used for mRNA analyses

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession Number</th>
<th>Sense Primer (5’-3’)</th>
<th>Antisense Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_4$</td>
<td>NM_144699</td>
<td>CAACTGGTCGCGGAGGGTCTTC</td>
<td>GTCGACACAGAGCCCTTGTGTT</td>
</tr>
<tr>
<td>RNAP II</td>
<td>NM_000937</td>
<td>CGTAAAGCTCTCCAAAGAG</td>
<td>GAGGAGACCTCTGGCTGCTCT</td>
</tr>
<tr>
<td>eIF-4E</td>
<td>NM_001968</td>
<td>CTTGCCGCTTTTCGAGAGA</td>
<td>GACGCCAAAGACAAAGAGA</td>
</tr>
<tr>
<td>4E-BP1</td>
<td>NM_004095</td>
<td>GATACCCCTGTGGCTCTGCA</td>
<td>GAAGGTCGCGCTTCGCTGCA</td>
</tr>
</tbody>
</table>

Primer sequences were designed using Primer Express software (Applied Biosystems) from gene sequences obtained from GenBank. Primer specificity was determined using a BLAST search. RNAP II, RNA polymerase II; eIF-4E, eukaryotic initiation factor 4E; 4E-BP1, 4E-binding protein 1.
rabbit immunoglobulins) diluted 1:10,000 ($\alpha_1$, $\alpha_2$, $\alpha_3$) or 1:5,000 ($\beta_1$, $\beta_2$, $\beta_3$) in TBST buffer. After three washes in 0.05% TBST, membranes were dried and treated with chemiluminescent substrate (Pierce SuperSignal, West Pico, IL). The signal was captured and imaged (Kodak Digital Science Image Station 400Q, Eastman Kodak). Positive control samples included rat brain and kidney homogenates, and these were run on each gel to assess the reactivity of the probe. The linearity of the blot signal vs. protein loaded for our experimental conditions was established for each antibody.

**Antibodies**

Blots were probed with antibodies specific to each isoform: for $\alpha_1$, monoclonal $\alpha6F$ (developed by D. Fambrough and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA); for $\alpha_2$, monoclonal anti-HERED (kindly donated by T. Pressley, Texas Tech University); for $\alpha_3$, monoclonal MA3–915 (Affinity Bioreagents, Golden, CO); for $\beta_1$, monoclonal MA3–930 (Affinity Bioreagents); for $\beta_2$, monoclonal polyclonal 06-171 (Upstate Biotechnology, Lake Placid, NY); for $\beta_3$, polyclonal 06-817 (Upstate Biotechnology). We have previously established the specificity of these antibodies (24). Briefly, this involved the use of additional antibodies, human skeletal muscle cell cultures with minimal contamination of other tissues, control samples, and BLAST analysis of the antigen sequence of each antibody to evaluate cross-reactivity. In unpublished experiments, we also recently performed deglycosylation of the $\beta_2$-isofrom and observed a shift in molecular mass from ~50 to ~29 kDa, providing further support for the specificity of the anti-$\beta_2$ antibody.

**Maximal In Vitro 3-O-MFPase Activity**

Maximal in vitro 3-O-methylfluorescein phosphatase (3-O-MFPase) activity was measured using the K$^+$-stimulated 3-O-MFPase activity assay, as previously described (2, 11, 12). The specificity of this assay for the Na$^+$-$K^+$-ATPase has been previously shown by its almost complete (97%) inhibition with ouabain (2). Briefly, 5–15 mg of muscle were weighed and then homogenized (5% wt/vol) on ice for 2 minutes at 20,000 rpm (Omni 1000, Omni International, Marietta, GA) in homogenizing buffer (10 mM Tris, 2 mM EDTA, 250 mM sucrose; pH 7.4). Muscle homogenates were then exposed to one freeze-thaw cycle, after which homogenates were diluted one in five with ice-cold homogenizing buffer. We have previously established that four freeze-thaw cycles increased 3-O-MFPase activity by 24%, compared with non-freeze-thawed homogenates (12). Furthermore, we found that additional freeze-thaw cycles, of up to 12 cycles, did not further increase 3-O-MFPase activity (unpublished observations).

These results suggest that four freeze-thaw cycles are sufficient to permeabilize the membrane and unmask any hidden Na$^+$-$K^+$-ATPase enzymes. Diluted muscle homogenates were then freeze-thawed an additional three times. Thirty microliters of the diluted homogenate were incubated in 2.5 ml of assay medium (5 mM MgCl$_2$, 1.25 mM MgSO$_4$, 1 mM NaVO$_4$; pH 7.3). The final [H]ouabain binding site content was taken as the average of three or four pieces of muscle taken from the same biopsy. Muscle samples were then incubated for 2 h at 37°C in vanadate buffer with the addition of [H]ouabain (10$^{-6}$ M, 2.0 $\mu$Ci/ml, Amersham Pharmacia Biotech, Castle Hill, NSW, Australia). After incubation, the muscle samples were washed for 4 × 30 min in ice-cold vanadate buffer to remove unbound [H]ouabain, blotted on filter paper, and weighed before being soaked overnight in vials containing 500 $\mu$L of 5% TCA and 0.1 mM ouabain. The following morning, 2.5 ml of scintillation cocktail (Opti-Fluor, Packard) were added before liquid scintillation counting of the $^3$H activity. The content of [H]ouabain binding sites was calculated on the basis of the sample wet weight and the specific activity of the incubation medium and samples (expressed as pmol/g wet wt). The [H]ouabain binding site content was then calculated by subtracting the nonspecific [H]ouabain uptake (2.5%) (30) and multiplying by a correction factor of 1.13 to allow for impurity of the [H]ouabain (1.05; measured by supplier), loss of specifically bound [H]ouabain during washout (1.05) (30), and incomplete saturation (1.025) (T. Clausen, personal communication). The muscle [H]ouabain binding site content intra-assay coefficient of variation was 18.4% ($n = 41$).

**Calculations**

Based on the observation that the Na$^+$-$K^+$-ATPase also acts as a signal transducer in cardiac muscle, (for review, see Ref. 48), we recently found significant correlations between the percent change in maximal 3-O-MFPase activity from rest to fatigue and each of $\alpha_1$ and $\alpha_2$ mRNA expression at fatigue and average postexercise $\alpha_3$ mRNA expression after brief intense exercise in human skeletal muscle (36). In the present study, we therefore also determined the correlation between the percent change in maximal 3-O-MFPase activity from rest to fatigue and the percent changes in both mRNA at fatigue and the average postexercise mRNA.

**Statistical Analyses**

All data are presented as means and SD. Respiratory, blood, and muscle data were analyzed using a one-way repeated-measures ANOVA, with Newman-Kuels post hoc analyses. To account for individual variability in time responsiveness of mRNA to exercise, the average postexercise mRNA was also calculated as the mean of the fatigue and 3- and 24-h recovery samples for each individual (24). This average was contrasted against the resting value and analyzed using Student’s $t$-test for paired samples. Possible gender differences for time to fatigue, isoform mRNA and protein expression, [H]ouabain binding, and maximal Na$^+$-$K^+$-ATPase activity were analyzed using Student’s $t$-test for independent samples. Correlations were determined by least squares linear regression. Significance was accepted at $P < 0.05$.

**RESULTS**

**Exercise Results**

Incremental exercise $V_{02\text{peak}}$ was 4.05 (SD 1.21) l/min [61.8 (SD 12.6) ml kg$^{-1}$ min$^{-1}$], and $P_{02}$, $V_{02}$, $P_{CO2}$ output, and respiratory exchange ratio during prolonged submaximal exercise were 87.3 (SD 12.9) Torr, 3.05 (SD 0.93) l/min [75.5
Exercise Effects on Plasma Volume and [K\(^+\)]

Plasma volume decreased by 12.2 (SD 2.8)% after 15 min of exercise (\(P = 0.001\)), by 10.2 (SD 3.9)% at fatigue (\(P = 0.001\)), and remained 2.5 (SD 2.5)% below rest at 10 min postexercise (\(P = 0.012\)). ArterIALIZED venous plasma [K\(^+\)] fell rapidly, reaching values not significantly different from those at rest by 1 min postexercise and thereafter (Fig. 1).

Exercise Effects on Muscle Na\(^+\)-K\(^+\)-ATPase mRNA and Protein Expression

Effects of exercise differed for isoform gene transcripts and protein abundance. 

\(\alpha\)-Subunit isoforms. Prolonged submaximal exercise induced a delayed upregulation of \(\alpha_1\) mRNA, reaching values 2.0-fold greater than at rest at 24 h postexercise (\(P = 0.036\)) but had no effect on \(\alpha_1\) protein abundance (\(P = 0.628\), Fig. 2). There was no significant effect of prolonged submaximal exercise on either mRNA (\(P = 0.498\)) or protein expression of the \(\alpha_2\)-isoform (\(P = 0.549\), Fig. 3). In contrast, exercise elevated \(\alpha_3\) mRNA expression at fatigue by 2.2-fold (\(P = 0.044\)), which then declined (\(P = 0.016\)) to resting levels by 3 h postexercise (Fig. 4). A significant time effect was found for \(\alpha_3\) protein expression (\(P = 0.046\)), but post hoc analyses were unable to locate significant differences (Fig. 4).

\(\beta\)-Subunit isoforms. Prolonged submaximal exercise had no effect on \(\beta_1\) mRNA expression (\(P = 0.155\)) but tended to increase \(\beta_1\) protein abundance (\(P = 0.056\), Fig. 5). Exercise elevated \(\beta_2\) mRNA expression at fatigue by 1.9-fold (\(P = 0.042\)), which then declined (\(P = 0.049\)) to resting levels by 24 h postexercise (Fig. 6). In contrast, there was no effect of exercise on \(\beta_2\) protein abundance (\(P = 0.858\), Fig. 6). Exercise had no significant effect on the mRNA (\(P = 0.172\)) or protein expression of the \(\beta_3\)-isoform (\(P = 0.304\), Fig. 7).

Fig. 1. Arterialized venous plasma K\(^+\) concentration ([K\(^+\)]) at rest, during prolonged submaximal cycling continued to fatigue (Fat), and for 10 min recovery. Shaded bar represents exercise bout. Values were corrected for changes in plasma volume from rest. Data are means (SD); \(n = 11\), except during exercise at 30 min (\(n = 10\)) and at 45 min and in recovery at +1 min (\(n = 8\)). *\(P < 0.05\) greater than rest; #\(P < 0.06\) greater than rest.

Fig. 2. Muscle Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) mRNA and protein expression at rest and after prolonged submaximal exercise. Samples were collected at rest, fatigue (Fat), and at 3 h (+3 h) and 24 h (+24 h) postexercise. Samples were normalized against resting values. Data are means (SD); \(n = 11\). *\(P = 0.036\) greater than rest.

Fig. 3. Muscle Na\(^+\)-K\(^+\)-ATPase \(\alpha_2\) mRNA and protein expression at rest and after prolonged submaximal exercise. Samples were collected at rest, fatigue (Fat), and at 3 h (+3 h) and 24 h (+24 h) postexercise. All results were normalized against resting values. Data are means (SD); \(n = 11\).

Fig. 4. Muscle Na\(^+\)-K\(^+\)-ATPase \(\alpha_3\) mRNA and protein expression at rest and after prolonged submaximal exercise. Samples were collected at rest, fatigue (Fat), and at 3 h (+3 h) and 24 h postexercise (+24 h). Samples were normalized against resting values. Data are means (SD); \(n = 11\). *\(P = 0.044\) greater than rest; †\(P = 0.016\) greater than +3 h; **\(P = 0.046\) time main effect.
No Sex Differences for Exercise Time, Muscle Na\(^+\)-K\(^+\)-ATPase mRNA, or Protein Expression

There were no sex differences for time to fatigue (P = 0.630), mRNA (P = 0.232; \(\alpha_2\), P = 0.505; \(\alpha_3\), P = 0.624; \(\beta_1\), P = 0.381; \(\beta_2\), P = 0.079; \(\beta_3\), P = 0.163), and protein expression of any isoform (\(\alpha_1\), P = 0.089; \(\alpha_2\), P = 0.140; \(\alpha_3\), P = 0.093; \(\beta_1\), P = 0.170; \(\beta_2\), P = 0.237; \(\beta_3\), P = 0.250), [\(^3\)H]ouabain binding (P = 0.111), or maximal 3-O-MFPase activity (P = 0.087).

Individual Time Course Variability in mRNA Expression

Because intense exercise induced individual variability in isoform mRNA time responsiveness (24), we also investigated the average postexercise mRNA expression of each of the Na\(^+\)-K\(^+\)-ATPase isoforms. A tendency (P = 0.053) was observed for elevated average postexercise mRNA expression only for \(\alpha_1\) (rest, 1.00 (SD 0.00); average postexercise, 1.52 (SD 0.78) expression relative to rest), with no significant effect of exercise on mRNA expression of any of the \(\alpha_2\) (P = 0.14), \(\alpha_3\) (P = 0.64), \(\beta_1\) (P = 0.76), \(\beta_2\) (P = 0.09), or \(\beta_3\) isoforms (P = 0.15, data not shown).

Exercise Effects on Muscle Maximal In Vitro 3-O-MFPase Activity and [\(^3\)H]ouabain Binding Site Content

Maximal 3-O-MFPase activity was decreased by 18.9 (SD 15.5)% at fatigue (P = 0.004) but did not differ significantly from rest at 3 and 24 h postexercise (Fig. 8A).

There was no effect of exercise or 24-h recovery on muscle [\(^3\)H]ouabain binding site content (P = 0.804, Fig. 8B).

There was no significant correlation between the percent change in maximal 3-O-MFPase activity from rest to fatigue.
with the percent changes in both mRNA at fatigue, and the average postexercise mRNA, for any of the α₁-, α₂-, α₃-isofoms and β₁-, β₂-, β₃-isofoms (data not shown).

Exercise Effects on Muscle RNAP II, eIF-4E, and 4E-BP1 mRNA Expression

The mRNA expression of the transcription initiator gene RNAP II was elevated at fatigue by 2.6-fold (P = 0.011), with expression then declining (P = 0.045) to resting levels at 24 h postexercise (Fig. 9). In contrast, there was no effect of exercise on the mRNA expression of the key genes involved in protein translation [eIF-4E (P = 0.516) and 4E-BP1 (P = 0.278)] (Fig. 9).

Average postexercise mRNA expression of RNAP II was increased by 1.8-fold [1.79 (SD 0.89), P = 0.015] compared with rest (1.00), whereas there was no difference in average postexercise mRNA expression of eIF-4E [0.87 (SD 0.37), P = 0.284] and 4E-BP1 [1.15 (SD 0.58), P = 0.390].

DISCUSSION

This study investigated the effects of prolonged submaximal exercise on the mRNA and protein expression of the full complement of Na⁺-K⁺-ATPase isoforms expressed in human skeletal muscle. The first main finding was that prolonged submaximal exercise induced isoform-specific Na⁺-K⁺-ATPase mRNA and protein responses, with α₁, α₃, and β₂ mRNA expression increased, together with α₃ protein abundance. These findings provide evidence for exercise-specific effects, as they strongly contrast those of our earlier study, where brief intense exercise upregulated mRNA expression of each of the six Na⁺-K⁺-ATPase isoforms but did not change the protein abundance of any isoform (24). The second main finding was that prolonged submaximal exercise only transiently depressed maximal Na⁺-K⁺-ATPase activity and did not alter Na⁺-K⁺-ATPase content for up to 24 h after exercise, with the depression in maximal Na⁺-K⁺-ATPase activity not related to increased mRNA expression of the Na⁺-K⁺-ATPase isoforms. The third main finding was that prolonged submaximal exercise increased the mRNA expression of the transcription initiator gene (RNAP II) but not of key genes involved in translation (eIF-4E and 4E-BP1).

Prolonged Submaximal Exercise Induces Isoform-Specific Increases in Na⁺-K⁺-ATPase Isoform mRNA Expression

This is the first study to investigate prolonged submaximal exercise effects on mRNA expression of all six Na⁺-K⁺-ATPase isoforms expressed in human skeletal muscle. An isoform-specific effect was observed, with an immediate postexercise increase in α₃ and β₂ mRNA expression and a more delayed increase in α₁ mRNA expression detected at 24 h postexercise. Interestingly, the time course of increases for each of these α₁-, α₃-, and β₂-isoforms was similar to that seen after brief intense exercise (24). These findings of an isoform-specific increase in Na⁺-K⁺-ATPase mRNA expression with prolonged submaximal exercise is of considerable interest because they contrast with our previous study, where brief intense knee extensor exercise lasting ~6 min increased mRNA expression of all six isoforms (24). It is possible that the different exercise regimens (prolonged submaximal vs. brief intense exercise) may have induced time-dependent differences in isoforms during the recovery period that were undetected because of our limited number of postexercise biopsies. However, it is highly likely that different responses indeed occur because of differences in local intracellular responses, rather than simply after a different time course. Whether time-dependent differences did occur cannot be resolved here but would require more extensive postexercise biopsy sampling. This is however, difficult due to ethical constraints regarding the number of biopsies that can be performed on human volunteers.
Furthermore, prolonged submaximal exercise had no effect on the average postexercise mRNA expression of the Na\(^{+}-K^{+}\)-ATPase isoforms, which was elevated after brief intense exercise for each of the \(\alpha_1-\alpha_3\) and \(\beta_1-\beta_3\) isoforms (24). Although it is important to note that these values may not necessarily represent consistent mRNA expression between individuals over the 24-h period after expression, isoform mRNA adaptation to exercise appears to be exercise intensity and/or duration specific. This is consistent with the finding of elevated \(\alpha_1\), but not \(\alpha_2\) or \(\beta_1\), mRNA expression in the vastus lateralis muscle after 15 min of intense, intermittent knee extensor exercise (27). In that study, exercise effects on \(\alpha_3\), \(\beta_2\), and \(\beta_3\) were not measured. Only one other study has investigated prolonged exercise effects on skeletal muscle Na\(^{+}-K^{+}\)-ATPase mRNA expression, and this was performed in rats after 1 h of treadmill running (45). They reported increased \(\alpha_1\) mRNA expression in red, oxidative but not white, glycolytic muscles and \(\beta_2\) mRNA expression in white, glycolytic muscles in which it was exclusively detected (45). There was no effect of exercise on \(\alpha_2\) and \(\beta_1\) mRNA expression in red, oxidative muscles in which they were exclusively detected, whereas \(\alpha_3\) and \(\beta_3\) mRNA expression was not measured (45). However, interpretation of fiber-specific effects in the present study is complicated because our measures were in the vastus lateralis muscle, which, as with virtually all human muscles, are of mixed fiber-type composition (31). It is therefore not possible to determine whether the increases in \(\alpha_1\), \(\alpha_3\), and \(\beta_2\) mRNA expression were fiber-type specific. This can be done in animals using muscles of different fiber-type composition or by collecting single fibers for myosin heavy chain determination. These possibilities deserve to be investigated in future studies.

The consistent upregulation of \(\alpha_1\) and \(\beta_2\) mRNA expression after exercise in this and three other studies (24, 27, 45) raises speculation regarding the physiological significance of increased mRNA expression of each of these isoforms. The \(\alpha_1\)-isoform was recently shown to play a direct role in maintaining membrane potential and Na\(^{+}\) and K\(^{+}\) equilibrium gradients (39). A specific function of the \(\beta_2\)-isoform in skeletal muscle is not known, although the \(\beta\)-subunit facilitates correct processing, assembly, and membrane insertion of the Na\(^{+}-K^{+}\)-ATPase (17, 26). However, because there was no corresponding upregulation of \(\alpha_1\) and \(\beta_2\) protein, the significance of these isoform-specific increases in mRNA are uncertain. Presumably, repeated exercise bouts are required to induce protein upregulation.

The physiological mechanisms contributing to the elevated \(\alpha_1\), \(\alpha_3\), and \(\beta_2\) mRNA expression with prolonged submaximal exercise are unknown. However, these may involve raised muscle intracellular [Na\(^{+}\)] and/or [Ca\(^{2+}\)], both of which occur in mammalian muscle with exercise (34, 43) and which have each been associated with increased Na\(^{+}-K^{+}\)-ATPase mRNA expression, in cultured canine kidney (5), rat kidney (38, 40), and chick skeletal muscle cells (44).

Our laboratory has recently found that, with brief intense exercise, there were significant inverse correlations between the percent change from rest to fatigue in maximal 3-O-MFPase activity and the mRNA expression at fatigue for \(\alpha_1\) (\(r = -0.60, P = 0.05\)) and \(\alpha_3\) (\(r = -0.60, P = 0.05\)) (36). The lack of correlation here may indicate that the previous finding of a significant relationship between these variables (36) was coincidental. In contrast, the reduced mRNA responsiveness found with prolonged submaximal exercise may explain the lack of correlation between maximal 3-O-MFPase activity and mRNA expression in the present study or that different stimuli are involved with the two different exercise regimens, such as elevated intracellular [Na\(^{+}\)] and/or [Ca\(^{2+}\)] (36). The elevation in intracellular [Na\(^{+}\)] was less with submaximal exercise than with intense exercise (43), whereas the elevations in intracellular [Ca\(^{2+}\)] with submaximal exercise vs. intense exercise have not been directly compared.

Prolonged Submaximal Exercise Induces an \(\alpha_3\)-Specific Increase in Na\(^{+}-K^{+}\)-ATPase Isoform Protein Abundance

We previously showed that brief intense exercise had no effect on protein abundance of any of the Na\(^{+}-K^{+}\)-ATPase \(\alpha_1\), \(\alpha_2\), and \(\alpha_3\)-isoforms and \(\beta_1\), \(\beta_2\), and \(\beta_3\)-isoforms in crude muscle homogenates (24). In contrast, prolonged submaximal exercise had isoform-specific effects, increasing \(\alpha_3\) protein abundance, with a strong tendency toward elevated \(\beta_1\) protein abundance (\(P = 0.057\)). The lack of effect of prolonged exercise on \(\alpha_1\), \(\alpha_2\), \(\beta_3\), and \(\beta_1\) protein abundance is therefore consistent with our findings with brief intense exercise (24). The physiological significance of an elevated protein abundance of the \(\alpha_3\)-isoform is not clear because the \(\alpha_3\) protein has a low ion affinity, with the apparent affinity for Na\(^{+}\) of the \(\alpha_3\)-isoform being 2–3 fold lower than that of the \(\alpha_1\)- and \(\alpha_2\)-isoforms (7). Furthermore, the relative mRNA and protein expression of the \(\alpha_3\)-isoform in human muscle is likely to be low (29). The lack of statistical significance for the elevation in \(\beta_1\) protein abundance may reflect the interindividual variability in isoform protein abundance that is also seen in isoform mRNA expression (24, 29). Because the cause is unknown, further studies are required to investigate the source of this variability.

Because our subjects were not sedentary, we cannot completely rule out that the mRNA and protein responses observed in this group may be different from those observed in a sedentary population. However, to best mimic baseline conditions for these subjects, all refrained from exercise on the day before and day of the trial, with dietary control as well, to ensure that conditions were as close to “normal” as practically possible. Furthermore, we attempted to minimize any possible training adaptations to the incremental exercise test and to the familiarization trial, by ensuring a 10-day separation between each of these and the invasive trials. Finally, it is almost impossible to establish that mRNA and protein baseline conditions were “normal,” as there is a paucity of literature on these, with limited information on training and dietary effects.

The lack of change in \(\alpha_1\) and \(\beta_2\) protein abundance despite an elevated mRNA expression suggests that the time course of exercise stimulus (\(\sim 55\) min) was insufficient to induce detectable changes in the protein abundance of these isoforms. Indeed, \(^{3}H\)ouabain binding was not significantly changed with acute exercise up to \(\sim 72\) min in duration (21) and was only significantly increased when exercise duration was extended to \(\sim 10\) h (35). Thus increases in Na\(^{+}-K^{+}\)-ATPase isoform mRNA expression may occur very rapidly, whereas increases in isoform protein abundance require exercise of more prolonged duration. In contrast, the significant elevation in \(\alpha_3\) protein abundance after prolonged submaximal exercise suggests that this isoform may demonstrate different rates of
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translational and/or degradation than the other Na\(^{+}\)-K\(^{+}\)-ATPase isoforms. Although these rates for the \(\alpha_3\)-isoform are not known and the present study could not directly evaluate these mechanisms, since exercise also elevated \(\alpha_3\) mRNA expression, these results strongly suggest the involvement of an accelerated rate of translation. In contrast, the mechanism responsible for the lack of change in \(\alpha_1\) and \(\beta_2\) protein abundance in the face of elevated mRNA expression of these isoforms may reflect an attenuation in translation rate, an increase in protein degradation, or a combination of both.

Functional Effects of Prolonged Submaximal Exercise on Na\(^{+}\)-K\(^{+}\)-ATPase

The present study confirms that prolonged submaximal exercise immediately depresses maximal in vitro 3-O-MF\(\text{Pase}\) (Na\(^{+}\)-K\(^{+}\)-ATPase) activity but has no effect on \(^{3}\text{H}\)ouabain binding site (Na\(^{+}\)-K\(^{+}\)-ATPase) content (21). Additionally, it was demonstrated that Na\(^{+}\)-K\(^{+}\)-ATPase content remained unchanged from resting values at 3 and 24 h postexercise. This finding extends the lack of change in Na\(^{+}\)-K\(^{+}\)-ATPase content found previously immediately after (2, 21) and up to 24 h after acute exercise in humans (10) and with electrical stimulation in rat muscle (22). This is a 55-min period of exhaustive exercise with a 24-h recovery period was insufficient to increase Na\(^{+}\)-K\(^{+}\)-ATPase content, which may require ultraendurance exercise (35) or repeated bouts of exercise (16, 23). The lack of exercise effect on \(^{3}\text{H}\)ouabain binding suggests that the observed increase in \(\alpha_3\) protein abundance and tendency for increased \(\beta_1\) protein abundance may not necessarily reflect functional isoforms. Further studies are clearly required to explain the apparent discrepancy between isoform protein abundance and \(^{3}\text{H}\)ouabain binding site content.

Because of insufficient tissue, we were unable to determine whether responses of Na\(^{+}\)-K\(^{+}\)-ATPase maximal activity and content to prolonged submaximal exercise were similar, whether expressed per gram muscle wet weight or per gram of protein. However, in previous studies from our laboratory in which protein content was determined on a homogenate, a similar increase in protein degradation, or a combination of both. Further studies are clearly required to functional isoforms. Additionally, these studies strongly suggest the involvement of a transient depression of Na\(^{+}\)-K\(^{+}\)-ATPase activity. This was due to comparisons being made between nonexercised and exercised legs; thus any differences may not have necessarily been due solely to the exercise bout. Furthermore, comparisons of maximal in vitro Na\(^{+}\)-K\(^{+}\)-ATPase activity within the exercised leg were made only in the period after exercise and showed no difference between the end of exercise and at 1 and 4 h after exercise. This suggested that maximal in vitro Na\(^{+}\)-K\(^{+}\)-ATPase activity was either not depressed or did not recover in that study. The lack of change in Na\(^{+}\)-K\(^{+}\)-ATPase content and also the transient nature of the depression in maximal in vitro Na\(^{+}\)-K\(^{+}\)-ATPase activity suggest that this phenomenon does not reflect a decline in the number of functional Na\(^{+}\)-K\(^{+}\)-ATPase enzymes but rather a structural alteration to the enzyme and/or to the membrane in which it is embedded. Indeed, maximal in vitro Na\(^{+}\)-K\(^{+}\)-ATPase activity, as measured using the phosphate liberation assay, was recently found to be dependent on the membrane lipid composition in mesosomal membranes from the kidney of cattle and crocodiles (47). Previous studies have suggested that the depression in maximal in vitro Na\(^{+}\)-K\(^{+}\)-ATPase activity may involve elevated intracellular [Na\(^{+}\)], intracellular [Ca\(^{++}\)], and/or increased levels of intracellular reactive oxygen species (ROS) (2, 10, 11, 42). Indeed, when the nonspecific antioxidant N-acetylcysteine was infused intravenously, there was less of a reduction in the maximal in vitro Na\(^{+}\)-K\(^{+}\)-ATPase activity during prolonged submaximal exercise (McKenna M, Medved I, Goodman C, Brown M, Bjorksten A, Murphy K, Petersen A, Sostaric S, and Gong X, unpublished results), suggesting that increased ROS may inhibit Na\(^{+}\)-K\(^{+}\)-ATPase activity. It appears unlikely that the depression in maximal in vitro Na\(^{+}\)-K\(^{+}\)-ATPase activity was fiber-type specific, since in rats, maximal in vitro Na\(^{+}\)-K\(^{+}\)-ATPase activity was reduced by a similar magnitude in each of soleus, EDL and both white and red portions of the vastus lateralis following prolonged, submaximal exercise (9). Furthermore, since maximal in vitro Na\(^{+}\)-K\(^{+}\)-ATPase activity was measured in a crude muscle homogenate, it is possible that sarcosomal maximal in vitro Na\(^{+}\)-K\(^{+}\)-ATPase activity may have actually been increased due to any exercise-induced translocation of Na\(^{+}\)-K\(^{+}\)-ATPase isoforms (1, 18). However, there is no evidence for translocation in the present study since \(^{3}\text{H}\)ouabain binding, which appears to measure Na\(^{+}\)-K\(^{+}\)-ATPase enzymes of only sarcolemmal and T-tubule origin (6), was unchanged with exercise. Moreover, in rat skeletal muscle, we have previously found no evidence of translocation with electrical stimulation over a range of stimulation frequencies and durations (22). Thus it appears unlikely that the exercise protocol employed in the present study induced any increase in sarcosomal Na\(^{+}\)-K\(^{+}\)-ATPase activity.

A limitation in interpreting the importance of this finding is that depressed maximal in vitro activity does not directly reflect the functionally important in vivo activity, as discussed previously (2, 11, 21, 36). Nonetheless, the marked K\(^{+}\) fluxes in contracting muscle are consistent with a possible depressed maximal Na\(^{+}\)-K\(^{+}\)-ATPase activity also occurring in vivo. The depressed activity may augment the reduction in intracellular [K\(^{+}\)] (43) and the increase in interstitial [K\(^{+}\)] (28) that occur with exercise. Although there was only a modest rise in plasma [K\(^{+}\)] to ~5.6 mmol/l with prolonged submaximal exercise, a greater rise in muscle interstitial [K\(^{+}\)] is probable. Thus impaired maximal in vitro Na\(^{+}\)-K\(^{+}\)-ATPase activity may contribute to a transient loss of K\(^{+}\) homeostasis, reducing the transmembrane [K\(^{+}\)] gradient and membrane excitability (43) and subsequently impairing the development of force. Thus the relatively modest ~19% depression in maximal in vitro Na\(^{+}\)-K\(^{+}\)-ATPase activity immediately after exhaustive exercise may have important implications for the development of muscle fatigue.

Prolonged Submaximal Exercise Increases RNAP II mRNA Expression

Although there is no evidence that RNAP II, eIF-4E, and 4E-BP1 are directly involved in Na\(^{+}\)-K\(^{+}\)-ATPase expression,
these factors were investigated here as global measurements of transcription and translation initiation. We demonstrate for the first time that a single bout of prolonged submaximal exercise elevated the mRNA expression of the nonspecific transcription initiation gene, RNAP II, in human muscle. This is consistent with upregulated mRNA expression of numerous other genes in human skeletal muscle with acute prolonged exercise, including those involved in metabolism (37) and oxidative stress (37).

Prolonged Submaximal Exercise Has No Effect on Genes Involved in Protein Translation

We also found that a single bout of prolonged submaximal exercise had no effect on mRNA expression of the key genes involved in protein translation, eIF-4E and 4E-BP1. The lack of change in the average postexercise mRNA expression confirmed these findings. The effects of acute prolonged exercise on eIF-4E and 4E-BP1 have previously only been investigated at the protein level in rat skeletal muscle (13). In that study, measured 1 h after 2 h of treadmill running, there was a 250% increase in the amount of eIF-4E associated with 4E-BP1 and consequently a 26% reduction in net protein synthesis (13). Unchanged eIF-4E and 4E-BP1 mRNA expression in the present study suggests that the elevated association of eIF-4E and 4E-BP1 was not due to an increased abundance of either protein but rather may reflect a reduction in phosphorylation of 4E-BP1 (4). These mechanisms responsible for the subsequent inhibitory effect on net protein synthesis in mammalian muscle remain inconclusive and require further investigation.

Perspectives

The present study advances our understanding of the adaptiveness of the Na⁺-K⁺-ATPase isoforms, showing isoform-specific responses to prolonged submaximal exercise, which differ to brief intense exercise (24). Furthermore, different adaptabilities of the gene transcripts and proteins of each of the Na⁺-K⁺-ATPase isoforms suggest these are under different regulation and may not necessarily be regulated by the same stimuli. Such stimuli remain unknown in human muscle but may involve the intracellular concentrations of Na⁺ (20, 40, 46) and Ca²⁺ (41), the extracellular concentration of K⁺ (38), or the intracellular levels of ROS (49). The possibility of isoform-specific regulatory stimuli further supports the idea that evolutionary adaptations have occurred to render isoform-specific functions in muscle (3). Indeed, the upregulation of the α3 protein with prolonged exercise points to an as yet undefined but specific physiological function of this isoform.

In conclusion, a single bout of prolonged submaximal exercise induced isoform-specific Na⁺-K⁺-ATPase transcriptional and translational regulation in muscle, with increased mRNA expression of the α1-, α2-, and β2-isosforms, and increased protein abundance of the α3-isoform. Furthermore, prolonged exercise transiently depressed maximal in vitro Na⁺-K⁺-ATPase activity but was insufficient to upregulate Na⁺-K⁺-ATPase content in the 24-h period after exercise. Prolonged submaximal exercise also elevated mRNA expression of the transcription initiation gene RNAP II but had no effect on mRNA expression of key genes involved in protein translation, eIF-4E and 4E-BP1.

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