Metabolic, Enzymatic and Transporter Responses in Human Muscle During Three Consecutive Days of Exercise and Recovery

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Short title: Energy and Substrate Based Responses to Exercise

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ABSTRACT

This study investigated the responses in substrate and energy based properties to repetitive days of prolonged submaximal exercise and recovery. Twelve untrained volunteers ($\dot{V}O_{2_{\text{peak}}} = 44.8 \pm 2.0 \text{ ml.kg}^{-1}.\text{min}^{-1}$, mean$\pm$SEM) cycled (60 $\dot{V}O_{2_{\text{peak}}}$) on 3 consecutive days followed by 3 days of recovery. Tissue samples were extracted from the vastus lateralis both pre (Pre) and post (Post) exercise on day 1 (E1), day 3 (E3) and during recovery, (R1, R2, R3) and analyzed for changes in metabolism, substrate, enzymatic and transporter responses. For the metabolic properties (mmol.kg$^{-1}$ dry wt), exercise on E1 resulted in reductions (P<0.05) in PCr (80±1.9 vs 41.2±3.0), and increases (P<0.05), in IMP (0.13±0.01 vs 0.61±0.2) and lactate (3.1±0.4 vs 19.2±4.3). At E3, both IMP and lactate were lower (P<0.05) during exercise. For the transporters, the experimental protocol resulted in a decrease (P<0.05) in GLUT1 (29% by R1) and an increase in GLUT4 (29% by E3) and increases (P<0.05) for both monocarboxylate transporters (for MCT1, 23% by R2 and for MCT4, 18% by R1). Of the mitochondrial and cytosolic enzyme activities examined, cytochrome oxidase (COX), and hexokinase (HEX) were both reduced (P<0.05) by exercise at E1 and in the case of HEX and phosphorylase by exercise on E3. With the exception at COX, which was lower (P<0.05) at R1, no differences in enzyme activities existed at rest between E, E3 and recovery days. The results suggest that the glucose and lactate transporters are among the earliest adaptive responses of the substrate and metabolic properties studied to the sudden onset of regular low-intensity exercise.

Key words: Contractile activity, glucose, lactate, transporters, vastus lateralis, glycogen, energy metabolism, enzyme activity.
INTRODUCTION

In working skeletal muscle regular submaximal contractile activity promotes a better matching between ATP supply and ATP utilization, resulting in less of a disturbance in phosphorylation potential, increased delivery of fuels such as carbohydrate (CHO) to the metabolic pathways so as not to be limiting to pathway flux and attenuation of the by-products of metabolism, such as lactic acid, thereby minimizing their potentially disruptive effects (38, 39, 46, 56, 68).

It has also been reported that these altered responses depend on a series of adaptations that increase the abundance of a multitude of proteins and protein isoforms that are involved in oxidative phosphorylation, substrate availability and metabolic by-product management. The tighter metabolic coupling, as an example, has been attributed to the increase in the potential for oxidative phosphorylation (OXPHOS) which is believed to occur as a result of increases in mitochondrial density and the maximal activity \( V_{\text{max}} \) of enzymes and complexes of the citric acid cycle (CAC) and the electron transport system (ETS), (39, 68). According to current theory, the increase in OXPHOS capacity increases respiratory control sensitivity, allowing a given level of OXPHOS to be realized with less of a reduction in phosphorylation potential (39, 68). As a result of the improved phosphorylation potential and the attenuation of changes in the adenine nucleotide levels, the activation of phosphorylase and phosphofructokinase is reduced, leading to decreases in glycogenolytic and glycolytic flux rates (13, 56). These adaptations promote decreases in CHO utilization and lactate and hydrogen ion accumulation.

In recent years, the significance of increases in OXPHOS potential as the primary mechanism in mediating the metabolic response has been challenged using a variety of short-
term training studies. These studies indicate that training-like adaptations can be observed within the first few sessions of regular exercise in the absence of increases in Vmax of a range of mitochondrial enzymes (21, 28, 61). Current evidence indicates that the metabolic adaptations are mediated by events outside the muscle, namely increased $\dot{V}O_2$ kinetics during the non-steady rate (60) which occurs secondary to a more rapid adjustment in blood flow (71). Given the reports indicating increases in $V_{max}$ of citrate synthase during the exercise itself (50, 76), it remains possible that increases in OXPHOS can be mediated acutely via second messenger regulation (56).

Adaptations in the other proteins and protein isoforms also appear involved in regulating CHO utilization and accumulations of lactic acid. Training-induced increases in CHO delivery into the working muscle in the form of blood glucose also appears to be regulated by increases in the abundance of glucose transporters (GLUT) which facilitate the transport of glucose across the plasmalemma (38). Contractile activity, in itself, increases the translocation of an intracellular pool of one of the GLUT proteins, GLUT4, the principal isoform in skeletal muscle (38). The increase that has been reported to occur in GLUT4 with training (38) could potentially increase the amount of GLUT4 translocated, increasing glucose transport during exercise.

Improved management of the potentially inhibiting effects of the metabolic by-products with training also occurs in the working muscle cell (56). This is important since the accumulation of lactic acid, as an example, the terminal product of the overall process of glycolysis, could substantially increase the acidity of the cell (56) resulting in inhibition of one or more of the excitation-contraction processes (1, 18, 22, 55). This challenge is managed by a family of lactate transporters (MCT), which on binding to the lactate anion and the hydrogen proton, increase the diffusion across membranes (30). MCT4 is the principal isoform in skeletal
muscle while MCT1 is expressed in minor form (6, 46). Unlike GLUT4, the population of MCT4 resides in the plasmalemma and consequently does not appear to be involved in intracellular trafficking (30). As with GLUT, increases in MCTs are also commonly observed with training (47).

Unknown at present is the time-dependent interrelation between the development of the different mechanisms used to regulate substrate, metabolic and by-product behaviour following the onset of regular exercise. There is evidence that the transition can occur within the first few days, at least for the GLUT4 (62), MCT1 (7, 12, 26) and MCT4 (12, 26), as indicated by the increases in protein that have been observed. There is also evidence that the maximal activities of the mitochondrial enzymes respond with increases but later in training (73). The maximal activity of HEX, on the other hand, the enzyme involved in glucose phosphorylation, is known to increase very rapidly (57, 58). Changes in HEX, could be coupled with the up-regulation of GLUT4, which could promote the gradient necessary to exploit the increased potential for facilitated diffusion (38). Adaptations designed to minimize lactic acid accumulation also appear to be of high priority with suggestions of increases in MCT4 reported with just one or two exercise sessions (26). Adaptations in lactic acid transport could be important in shuttling the lactate to other tissues such as the liver where it can be used for gluconeogenesis, ensuring a greater protection to blood glucose homeostasis (14). Collectively, these studies suggest that the most rapid adaptations occur not to the metabolic pathways involved in ATP re-synthesis but to the proteins involved in muscle glucose transport, phosphorylation and lactic acid removal. In this regard, a common signal governing the expression of many of these adaptations may be the cellular energy status and the activation of AMP-activated protein kinase (31, 79).
The purpose of this study was to investigate the effects of 3 repetitive daily sessions of prolonged cycling on the metabolic response to prolonged exercise, the expression of the proteins involved in glucose and lactate transport in skeletal muscle and the maximal activities of a range of mitochondrial and cytosolic enzymes involved in ATP re-synthesis. We have hypothesized that 3 consecutive days of prolonged training would attenuate both the reduction in phosphorylation potential and the increase in lactate that occurs in working muscle. These adaptations would be accompanied by increases in GLUT4 and MCT4 protein levels and in HEX activity but not in the activity of enzymes directly involved in OXPHOS and glycolysis. In this study, we have also included 3 days of recovery in order to gain further insight into the development and/or a decrease of the various properties and their interrelationship. It should be emphasized that this study was part of a much larger study in which we have also investigated changes in excitation-contraction coupling properties (16) (23) and fatigue (75).

METHODS

Participants. Twelve volunteers met the inclusion criteria and were accepted into the study. The mean age and body mass were 19.2±0.27 yr and 71.1±3.4 kg, respectively. As a condition of acceptance into the study, all subjects were healthy and free of any medication (as determined by questionnaire), were not engaged in exercise on a regular basis and were prepared to meet the dietary and lifestyle restrictions during the experiment period. All participants were informed about the procedures, demands and risks associated with the study prior to obtaining written consent. As required, the study was first approved by the Office of Research Ethics at the University of Waterloo prior to subject recruitment.
Experimental Conditions. To investigate the effects of consecutive days of exercise and recovery on muscle properties, a 6 day protocol was utilized consisting of 3 days of cycle exercise followed by 3 days of recovery. Exercise on day 1 (E1), day 2 (E2) and day 3 (E3) was performed at the same relative intensity (approximately 60% $\dot{VO}_2^{peak}$) and the same duration (until fatigue or for a maximum of 120 mins). Each participant performed the same amount of work on each of the 3 days of cycling. For each individual, the relative intensity was determined from the relationship between $\dot{VO}_2$ and power output obtained during a progressive cycle test (41). The progressive cycle test was performed approximately 4 weeks prior to the short-term training sessions.

Peak aerobic power ($\dot{VO}_2^{peak}$) was assessed during the progressive exercise test to fatigue by a protocol previously used in our laboratory (41). Ventilation and gas exchange were measured continuously through the test by previously published methods (42). Exercise was performed on an electronically braked cycle ergometer (model 870 Quinton) calibrated during each testing day. For each individual, cycle seat height was established during the initial progressive test and remained standardized for all subsequent tests.

During the prolonged submaximal cycling sessions, ventilation and respiratory gas exchange measurements were made at rest and at regular intervals throughout the exercise. Arterialized venous blood samples were also extracted at regular intervals during the exercise from a catheter inserted into a pre-warmed dorsal region of the hand. The blood samples were processed and stored at -20°C pending the measurement of blood glucose, lactate and the catecholamines, norepinephrine (NEpi) and epinephrine (Epi). At E1 and E3, both pre (Pre) and post (Post), tissue samples were extracted from the vastus lateralis muscles in defined regions and according
to technique previously detailed (4). In the case of Post, the tissue was sampled immediately on cessation of the exercise. Tissue samples were also extracted during recovery day 1 (R1), day 2 (R2), and day 3 (R3). The seven biopsies were made from 7 separate sites which in the case of the exercise days, were prepared prior to the exercise. At each sampling time, 2 separate biopsies were performed from the same incision. The first sample was rapidly extracted and plunged into liquid nitrogen. This sample was stored at -80°C and was used for measurements of muscles metabolites. The second sample, extracted immediately after the first, was prepared for other measurements including enzyme activity, prior to freezing and storage at -80°C. On a given exercise day, tissue was obtained from a site on each leg, with the order of sites randomized between the legs. Given the large number of biopsies planned during the 6 day experimental protocol, it was decided to administer E1 some 3 to 4 weeks prior to the short-term training protocol to allow for complete recovery (unpublished). This exercise session represented the control or E1 condition. However we emphasize that E1 was repeated during our 3 day protocol. With the exception of the tissue samples, all other procedures and measurements were performed during the 3 day exercise protocol. We have previously reported that providing exercise patterns and dietary habits are not substantially altered, a wide range of muscle cellular properties remain stable over a similar time frame (15, 69).

All submaximal exercise sessions were performed at approximately the same time for each volunteer, approximately 24 h following the last session. Subjects were requested to maintain a normal diet throughout the experimental protocol and to refrain from the ingestion of caffeine-based beverages beginning at least 48 h prior to a last session. Approximately 2 h prior to exercise, participants ingested a can of Ensure (250 Kcal) meal replacement consisting of 9.4 g protein, 6-7 g fat and 38 g carbohydrate (Ross Products Division, Saint-Laurent, PQ, Canada).
We have used this as a standard procedure in similar exercise testing protocols in order to control nutrient intake prior to the experimental session. Water was permitted ad libitum during the exercise. Additional details regarding the management of the volunteers and nutrient profiles occur in earlier publications (16, 75).

Throughout the consecutive days of exercise, the cycle seat height was standardized for each individual. Pedaling rate was set at 60 rpm. All exercise sessions were performed under similar conditions with temperatures between 23-24°C and relative humidity between 50-60%.

**Analytical Procedures**

**Muscle glycogen and metabolites.** For measurement of glycogen, glycolytic intermediates, the high energy phosphates (ATP, PCr) and related metabolites (creatine, Cr, inorganic phosphate, Pi), the frozen tissue was initially freeze-dried and the constituents extracted according to procedures previously published (27, 32, 32). The adenine nucleotides (ATP, ADP, AMP) and IMP concentrations were measured using ion-pair reversed phase HPLC procedures originally developed by Ingrebretson et al (43) and subsequently modified by our group (27). The glycolytic intermediates and high-energy phosphates and related metabolites were measured fluorometrically (52). Total adenine nucleotides (TAN) represents the sum of ATP, ADP and AMP while phosphorylation potential (PP) is derived from PCr + TAN. Total creatine concentration (TCr), which represents the sum of PCr+Cr, was calculated and the average value for each individual (based on 7 individual samples) was used to correct all metabolite concentrations. This procedure, which is based on the assumption that TCr does not change with the experimental treatments, is used to minimize the contaminating effect of blood and connective tissue (34). Our results indicate no change in TCr either with exercise or between the
exercise and recovery days. Two of the metabolites assessed, namely glucose and lactate, also exist outside the cell in the extracellular space. Accordingly the values that we have determined, and used to represent the intracellular concentration, may be biased because of the extracellular concentration. Total glycogen and its 2 subtractions (proglycogen, PG, macroglycogen, MG) were measured using fluorometric techniques (53).

**Muscle enzymes.** The activities of a number of cytosolic and mitochondrial enzymes were determined. The mitochondrial enzymes were selected to represent the citric acid cycle [citrate synthase, (CS) and succinic dehydrogenase (SDH), the electron transport chain [ETC, cytochrome-c oxidase (COX)] and β-oxidation [3-hydroxy-CoA dehydrogenase (3-HAD)]. The cytosolic enzymes were selected to represent glycogenolysis [phosphorylase (PHOSPH)], glycolysis [phosphofructokinase (PFK)], glucose phosphorylation [hexokinase (HK)] and high-energy phosphate transfer [creatine phosphokinase (CPK)]. Enzymatic activities were performed on tissue samples hand homogenized in a phosphate buffer (pH 7.4) containing 5 mM mercaptoethanol, 0.5 mM EDTA, and 0.2% BSA. Homogenates were diluted in 20 mM immidazole buffer with 0.2% BSA. All assays, with the exception of SDH and PFK, were performed on frozen homogenates stored at -80°C. For PFK and SDH, the activities were measured on fresh homogenates prepared daily. The enzyme assays were performed at 24-25°C based on the procedures of Henriksson et al (35) as modified by our laboratory (28). Protein was determined by the Lowry technique as modified by Schacterle and Pollock (70). The maximal activity of the enzymes were measured as an end point assay at room temperature (11).

The assay for COX was performed at 37°C using a reaction medium that consisted of 10 mM potassium phosphate buffer (pH 7.0) and 1 mM solution of reduced cytochrome -c (Sigma C-2506). The sample homogenate was diluted 1:10 in the potassium phosphate buffer. The
assay was started by adding 1 ml at the diluted homogenate to the reaction medium and the
decrease in absorbance was measured spectrophotometrically (Shimadzu UV 160 U) at a wave
length at 550 nm for a period of 3 min. The units of activity per gram of tissue was calculated
using the measured slope and the milimolar extinction coefficient of reduced cytochrome-c. The
units of activity per gram of tissue was calculated and converted to units per gram protein.

For both the metabolites and enzymes, all tissue samples for a given subject regardless of
the property were measured during the same analytical session. For each property, the value
represents the average of duplicate measurements.

**Blood metabolites and hormones.** Whole blood for glucose and lactate was placed in a pre-
chilled tube containing 0.6 M perchloric acid and neutralized in a 1.25 mM KHCO₃, centrifuged
and the supernatant analyzed fluorometrically (24). Plasma Epi and NEpi were measured by
HPLC techniques using electrochemical detection as modified by our laboratory (25). For the
measurements of the catecholamines, whole blood was collected in a tube containing ethylene
glycol-bis (β-aminoethyl ether) N, N, N¹, N¹-tetra acetic acid and glutathione as antioxidant. The
blood was then centrifuged at 2000g at 2°C, the plasma removed and stored at -80°C prior to
analyses. All measurements for a given property and a given individual were performed in
duplicate during the same analytical session.

**Glucose and lactate transporters.** Electrophoresis and Western blotting for isolation of the
monocarboxylate transporters (MCT1 and MCT4) were performed in crude membrane
preparations essentially as described previously (7). Homogenates were prepared from samples
(30-40 mg) in 2 ml of buffer (in mM : 210 sucrose, 2 EGTA, 40 NaCl, 30 HEPES, 5 EDTA and
2 phenylmethylsulfonyl fluoride, pH 7.4) using a Polytron 2100-homogenizer (2x15 s at a setting
of 7). A pellet was isolated by centrifugation (230,000 g for 75 min at 4°C) and homogenized in
1-2 ml of buffer (10 mM Tris base, 1 mM EDTA, pH 7.4) using the Polytron protocol as described above. Protein was determined by the modified Lowry assay (70).

To resolve the MCT isoform protein content, Western immunoblotting was performed (50 µg of homogenate in 2% SDS buffer) using electrophoresis on 12% SDS-polyacrylamide gels (Mini-Protean 11, Biorad) essentially as described by Laemmli (48). For the analysis of each transporter, duplicate measurements using 2 different aliquots from each sample and two different gels were employed in conjunction with α-actin which was used as the standard to control for protein loading. An assumption associated with the use of α-actin as an internal sample is that no changes occur in content with the experimental protocol. It is known that some exercise models can case myofibrillar degradation (3). Once separated, the proteins were transferred to immobilon polyvinylidene difluoride membranes and incubated [20 mM Tris base, 137 mM NaCl, 0.1M HCl, pH 7.5, 0.1% (vol/vol) Tween 20 and 10% (wt/vol) nonfat dried milk] at room temperature on a shaker (~1h). Membranes were incubated (16h at 4°C) with diluted (1:400) polyclonal antisera (CHEMICON). For α-actin, the anti-α-sarcomeric actin antibody 5C5 was used. Thereafter, incubation took place in anti-rabbit immunoglobulin IgG horseradish peroxidase-conjugated secondary antibody (1:3000, Amersham, NA 934).

An enhanced chemiluminescence procedure was used to detect antibody content (Amersham, Buckinghanshue, UK). Blots were analyzed by use of a Chem Genius 2 model bioimaging system (SyngGene, Frederick, MD.) with Syne Gene software version 1.0.

The measurement of the protein content of GLUT 1 and GLUT 4 was essentially described for MCT1 and MCT4. GLUT4 and GLUT1 protein were detected by incubation (1:200) with anti-GLUT polyclonal antisera (Chemicon). These measurements have been described in detail previously (62, 64), including a recent study from our laboratory (20).
HSP70. The measurement of HSP70 was also made using Western blots and immunodetection, also using the general procedures described for the measurement of GLUT and MCT transporters. For this protein, 10 ug of homogenate in 2% SDS buffer was applied to a 10% polyacrylamide gel, separated using standard SDS-PAGE protocols and transferred to PVDF membranes. After blocking with a 10% skim milk suspension, the membranes were incubated for 16 h at 4°C with the anti-HSP70 monoclonal antibody SPA-810 (Stressgen Biotechnologies). After washing in Tris-buffered saline/0.1% Tween, the membranes were treated with horseradish peroxidase-conjugated anti-mouse secondary antibody for 1 h (Santa Cruz Biotechnology), the membranes washed in Tris-buffered saline/0.1% Tween, the signals detected and the densiometric analysis performed as described for the GLUT and MCT transporters. All samples were run in duplicate with the standard. HSP was included since it has been shown to be highly adaptable and rapidly upregulated with the stress of exercise (17, 51).

Individual values for both types of transporters were originally expressed as a percentage of the standard (α-actin) and then as a percentage of the initial pre-exercise (E1) value. The pre-exercise value was set at 100%. It should be noted that unlike the other cellular properties assessed, no measurements were made of the GLUT and MCT isoforms post-exercise at E1 and E3.

DATA ANALYSIS

A one way analysis of variance (ANOVA) for repeated measurements was used to examine differences at rest during E1 and E3 and the recovery days R1, R2, and R3 (glucose and lactate transporters, HSP 70) as well as between rest, exercise and recovery (metabolites, glycogen and
enzyme activities.) A two-way ANOVA for repeated measures was employed to determine the effect of time of exercise and days of exercise (blood glucose, lactate and the catecholamines). Where significance was found, the Neuman-Keuls technique was employed to locate which was significantly different. The probability for statistical significance was accepted at P<0.05. Where differences are indicated in the text, a probability of P<0.05 is implied. Where only main effects are found, the specific conditions involved are summarized in the legend. Data are represented as means ±SEM.
RESULTS

Respiratory Gas exchange. Peak aerobic power, as assessed during the progressive cycle test to fatigue, was 44.8±2.0 ml kg⁻¹•min⁻¹. During the submaximal exercise, $\dot{VO}_2$ increased at 15 min of exercise and then remained at steady-state at 30 and 60 min of exercise (Table 1). Carbon dioxide production showed the same pattern. Increases in RER were also observed by 15 min of exercise, and beyond, indicating a greater dependency on CHO as a fuel. No differences were observed between E1 and E3 for either $\dot{VO}_2$, $\dot{VCO}_2$, or RER (Table 1).

Metabolism. Prolonged exercise on either E1 or E3 failed to result in a reduction in ATP concentration (Table 2). In addition, no differences were observed in ATP at rest between E1 and E3 or between any of the recovery days. As expected, neither exercise nor recovery altered the concentration of the other adenine nucleotides, namely ADP and AMP (Table 2). Total adenine nucleotide concentration, which represents the sum of the concentrations ATP, ADP and AMP, was not different prior to or following exercise on E1 or E3, or between any of the exercise and recovery days. Significant increases were observed in IMP concentration but only during exercise on E1 (Table 2). At E3, no differences were found Pre versus Post exercise. Similarly, no differences in IMP concentration existed at rest between E1 and E3 and R1, R2, and R3.

Decreases in PCr were found at the end of exercise on both E1 and E3 with no differences observed between days in the magnitude of the response (Fig 1). The concentration of PCr was not different between any of the recovery days or between E1 and E3 prior to exercise. The Cr concentration increased with exercise on E1 and E3, with no differences in the response pattern observed between days (Fig 1). As predicted, based on the response of the PCr,
Cr was not different at rest across the exercise and recovery days. The response pattern for Pi was the same as for Cr, namely increases with exercise on E1 and E3 with no differences at rest over the 6 days of the experiment (Fig 1). Total creatine, represented by the sum of PCr and Cr, was not altered throughout the experimental period. Phosphorylation potential (PP) was decreased with exercise on E1 and E3, with no differences observed between days on the magnitude of the decrease (Table 2). No differences were observed in PP between days at rest.

Exercise on E1 elevated the concentrations of the 3 muscle metabolites of interest, namely glucose, G-6-P and lactate (Fig 2). By E3, no differences were observed pre and post exercise for glucose. However increases were observed for G-6-P and lactate on E3, which in the case of lactate was lower than observed on E1. A persistent elevation in glucose was observed at R1 compared with the Pre at E1 and E3. Both G-6-P and lactate recovered from the effects of exercise at R1 and then remained unchanged at R2 and R3. During the recovery days, the concentration of those metabolites were not different from E1 and E3 prior to the exercise.

Substrate. Total glycogen was reduced by 59.2% and 58.4% with exercise on E1 and E3, respectively (Fig 3). For MG (macro), the reductions were 55.7% and 53.2% while for PG (pro), the reductions were 70.3% and 78.7% at E1 and E3, respectively (Fig 3). No differences were observed between E1 and E3 for the exercise-induced depletion that occurred for either total, MG or PG. No differences existed prior to exercise on E1, E3 and R1 for any of the glycogen moieties examined indicating that replenishment of reserves were not compromised by the consecutive days of exercise. By R2, the second day of recovery, elevations in both PG and total glycogen were observed compared to R1. This effect persisted at R3. Although there was a trend for MG to follow the same pattern, the differences were not significant. At no time during recovery was glycogen, regardless of form, supercompensated compared to pre-exercise at E1.
**Transporters.** GLUT4, the main glucose transporter in human muscle, was rapidly elevated with the exercise protocol (Fig 4). At E3, as an example, GLUT4 was 29% higher than E1. The higher GLUT4 level persisted throughout the 3 recovery days. In contrast to GLUT4, GLUT1 was depressed by the repeated sessions of exercise (Fig 4). The decrease in GLUT1, first observed at R1, gradually recovered to E1 levels over the 2 additional days of inactivity.

Of the 2 lactate transporters measured, both MCT1 and MCT4 were elevated during our experimental protocol (Fig 5). For MCT 1, the increase was not observed until R2. The increase persisted at R3. For MCT 4 an increase of 18% elevation was observed at R1 compared to E3. Compared to E1, the apparent increase observed at R1 was not significant (P=0.08).

**Enzymatic activities.** Exercise, both acute and repetitive, altered selected enzyme activities. For all the mitochondrial enzymes, only COX, the enzyme used to characterize the potential of the ETS, was a reduction observed during exercise on E1 (Fig 6). With the exception of COX, no differences were found between the resting enzyme activities during the exercise and recovery days. For COX, activity was depressed at R1 compared to pre-exercise on both E1 and E3. By R2 and extending to R3, COX activity was higher than at R1. No differences were found pre-exercise between E1 and E3 and between pre-exercise and R2 and R3.

For the cytosolic enzymes, a significant reduction during exercise on E1 was observed only for HEX (Fig 7). For HEX, a reduction was also observed during exercise at E3. For PHOSPH but not PFK, lower enzyme activities were observed following exercise at E3. In the case of PHOSPH, the reduction persisted at R1. For all of the other cytosolic enzymes examined, no differences were observed between the values measured prior to exercise at E1 and E3 or between the recovery days.
Protein. The sample protein concentrations (mg/g) before (B) and after (A) exercise were at E1 (208±5.8 vs 212±10) and E3 (200±5.2 vs 202±5.1) and at R1 (197±4.7), R2 (204±6.4) and R3 (205±5.1).

Blood measurements. Differences in blood glucose were observed at the different exercise time points but not between conditions (Table 3). At 15 min of exercise, blood glucose decreased and then remained stable until fatigue. Blood lactate was observed to increase early in exercise at E1 and E3 and to remain elevated until fatigue. The consecutive days of exercise resulted in a general decrease in blood lactate, an effect that was not specific to any time point. Pronounced increases in the blood catecholamines, both NEpi and Epi, were found by 15 min of exercise, the first measurement point, at both E1 and E3 (Table 4). By the 60 min of exercise, further increases in both catecholamines were found. The repetitive days of exercise failed to elicit differences in the responses of both Epi and NEpi.

HSP70. Compared to E1 (100%), the relative changes in HSP70 were 104±3.9, 112±4.1, 105±6.1 and 105±5.9 at E3, R1, R2 and R3 respectively. No differences were found between any of the time points. Although differences between E1 and R1 are suggested, significance (P=0.11) was not observed.

DISCUSSION

As expected, given the exercise intensity employed (approximately 60% $V_{O2peak}$), the prolonged exercise on the first day, did not result in significant reductions in ATP. However, a small reduction may have occurred that was not detected, as indicated by the increase in IMP, recognized as a more sensitive measure of changes in ATP (37). The protection of ATP homeostasis was mediated by high energy phosphate transfer as illustrated by the approximate
50% reduction in PCr in association with increases in glycolysis and OXPHOS. At steady-state, given the intensity of exercise employed, OXPHOS has been estimated to account for in excess of 90% of the ATP synthesized (2). As expected, this form of exercise resulted in a 2-3 fold increases in muscle lactate, most of it probably accumulating during the non-steady state period when OXPHOS is progressively recruited. With the exercise protocol employed, it is known that lactate is also shuttled out of the cell, with diffusion facilitated across the plasma membrane by MCT4, the major transporter in skeletal muscle (30, 46). It should also be noted that the changes in the muscle cellular energy status during our exercise protocol is consistent with activation of AMPK, although not to the degree possible with more intense contractile activity (79).

By the third day of the prolonged exercise, although a small reduction in IMP occurred, there was no attenuation in the reduction in PCr and the accumulation of Cr and Pi (56). It is clear from these results that 2 days of exercise, prior to measurement on the third day, did not protect muscle phosphorylation potential as we have previously reported with short-term training. In our previous studies the shortest period of training employed using prolonged submaximal exercise was 3 days with the metabolic adaptations measured on the fourth day (21). It would appear that an additional day of prolonged exercise is needed to observe clear improvements in phosphorylation potential. In summary, contrary to our hypothesis, we found no effect of our short-term training protocol on the phosphorylation potential in working muscle.

The consecutive days of exercise resulted in a pronounced decrease in muscle lactate concentration during exercise. The decrease in lactate concentration could be due a decrease in glycolysis and/or increased trafficking of lactate to the mitochondria in the cell or to sites outside the cell (5, 8, 14). Since no changes were found at this time point in either MCT1 or MCT4 content, increased trafficking would have to occur in the absence of changes in the transporters.
Previous research has established a close correlation between increases in MCT4 and increases in the facilitated diffusion of lactate (46). Alternatively, decreases in lactate formation could have occurred secondary to decreased glycolysis or to increased pyruvate oxidation as a result of activation of pyruvate dehydrogenase (PDH), an inviting prospect given the rapid adaptation that occurs in this enzyme in response to contractile activity (74). The fact that we have observed reductions in lactate accumulation during the exercise in the general absence of changes in phosphorylation potential, suggests that management of this by-product is one of the earliest adaptations that need not be coupled to improved cellular energy homeostasis.

Based on the RER calculated at different exercise time points, it is clear that CHO represents the dominate substrate. As indicated by the large depletion of muscle glycogen this fuel represents a major source of the CHO metabolized. It has been estimated that in exercise of this intensity in the untrained, glucose oxidation can account for about 10% of the CHO utilized (67). Since we found only a small reduction in the blood concentration of glucose early in exercise, it appears that liver glycogenolysis and gluconeogenesis were generally able to keep pace with the utilization by muscle (78), thereby maintaining glucose homeostasis. It is well established that blood glucose entry into the muscle cell is facilitated by glucose transporters and, in particular, GLUT4 which is translocated to the plasma membrane following the onset of exercise (38). An unexpected and novel observation was the decrease in maximal HEX activity observed at the end of exercise. A decrease in maximal HEX activity would be expected to decrease maximal rate at which glucose can be phosphorylated (78). Given the relatively low intensity of the exercise protocol employed, it would not be expected that maximal rates of glucose utilization would be challenged (67).
It is clear that the amount of glycogen depleted between day 1 and day 3 was not altered, suggesting no change in net glycogenolysis. Since an increase in GLUT4 was found, increased glucose transport potential is suggested (38). Increased glucose transport during exercise could be used to increase the role of this substrate in glycolysis or as a fuel for glyconeogenesis (78). Expectedly, the increase in GLUT4 would be important to increase the availability of glucose for glyconeogenesis during the approximately 22 h period between exercise sessions (38). In this regard, it is significant that repletion of muscle glycogen was complete given the lack of a difference between resting concentrations on days 1 and 3. Regardless of the functional implications, it is clear that increases in GLUT4 represents one of the earliest adaptations, as hypothesized.

We have also examined the response of the mitochondrial and cytosolic enzymes to determine if the consecutive days of exercise resulted in alterations in $V_{\text{max}}$ at rest between E1 and E3 and the recovery days. We found no effect on either SDH, CS, or 3-HAD. However COX was depressed at R1 before recovering on R2 and R3. No change in $V_{\text{max}}$ was observed at rest for any of the cytosolic enzymes with the exception of PHOSPH which failed to recover at R1 following exercise at E3. Given the acute reductions observed for some enzymes such as COX and HEX during exercise, it is clear that the reductions are transitory, recovering between exercise days (HEX) or by the second day of inactivity. The failure to find increases in the maximal activities of the CAC and the rate limiting enzymes of glycogenolysis and glycolysis during the recovery days was not surprising given the results of several previous experiments which have reported no change in response to several sessions of exercise (21, 61). To our knowledge, decreases in COX have not been reported with either exercise or short term training. In fact, it appears to respond similar to the CAC enzymes, namely with increases if the training is
sustained (39, 59). The decrease in activity could occur as a consequence of increased degradation or possibly as a result of oxidation, mediated by reactive oxygen species (ROS). In the untrained, our exercise protocol is known to result in the accumulation of ROS (72) and COX is particularly sensitive to oxidation. There could be functional implications for decreases in COX during exercise since it is known to occupy a central position in terminal oxidation and respiratory control (68). In general, our results are consistent with what has been postulated, namely that changes in enzyme activities would be more delayed occurring after changes in the principal transporters.

An important issue is whether or not acute alterations occurred in $V_{\text{max}}$ of the mitochondrial and cytosolic enzymes to support the increased demand for OXPHOS and glycolysis. Our failure to observe an increase in CS, an enzyme of the CAC, is at odds with 2 earlier studies which have reported increases in activity following exercise (49, 76). In this regard, it is noteworthy that for all of the mitochondrial and cytosolic enzymes measured, with the exception of CPK, there are indications of a transient reductions. Only in the cases of COX and HEX were the reductions significant. Others have also reported acute reductions in enzyme activities with exercise in rats (44) as has our group in humans in response to intermittent exercise (29). Although many factors can alter enzyme activity (36), oxidative stress is believed to have special importance (45). The reasons underlying the discordant results between our study and the other studies are not clear. What is clear is that the difference cannot be due to differences in total protein since we found no effect of the prolonged exercise on protein concentration. However, it is possible that the protein content of individual enzymes, such as CS, was altered. In addition, it does not appear that the contradictory results can be attributed to the task demands since the prolonged exercise model has been employed previously (76).
The period of recovery resulted in several changes in the GLUT and MCT transporters examined. During this period, the increase observed in GLUT4 at E3 remained elevated, indicating a delayed reversal of the training induced up-regulation. In contrast, the consecutive days of exercise, resulted in a reduction in GLUT1, an effect that was fully manifested at R1. During the remaining days of recovery, GLUT1 content was gradually normalized. Although many studies have reported rapid increases in muscle GLUT4 soon after abrupt increases in contractile activity, few studies have examined the time course of change during inactivity. Of those that have focused on this issue, the persistence of the effects that we have observed were expected (40). For GLUT1, the general consensus is that the protein level is not responsive to training (38). However, isolated studies exist showing that increases can occur but with more extended periods of regular exercise (62, 63). There are reports that the increases observed at least in GLUT4 with training can be more readily reversed with CHO feeding (9, 10). Based on our results it would appear that the substantive depletion in muscle glycogen induced by exercise can be restored without reversing the increase in GLUT4 content. The replenishment of glycogen between exercise days would indicate that dietary CHO was adequate and that the mechanisms were in place to promote the facilitated diffusion of glucose across the plasma membrane. In this regard the increase in GLUT4 that occurred may have been of significance (80). The role of HEX in phosphorylating the intracellular glucose remains unclear. Previous studies have repeatedly demonstrated that HEX is a early responsive enzyme, reported to increase with as little as one session of exercise (57, 58). Our failure to find an increase was unexpected given that we have utilized a 3 day model of exercise. It is possible that the high CHO diet suppressed the transcription stimulus similar to what has been reported earlier (9).
Similar to the GLUT isoforms, the MCT isoforms examined also showed a different response during the recovery days. During this period, the increase in MCT4 observed at R1 appeared to be reversed (P=0.05) while delayed increases in MCT1 at R2 and R3 were found. It is well established that exercise is a potent stimulus for an up-regulation in both MCT1 and MCT4 (7, 12, 26). The relatively rapid reversal in MCT4 has been reported earlier (12), suggesting that the increase observed at R1 following the 3 days of training is transient. Unexpected was the delayed increase in MCT1, not observed until R2. Based on the $K_m$ measurements, it has been reported that MCT4 is primarily involved in facilitating the diffusion of lactate (and $H^+$) out of cells with a high concentration, such as working muscle while MCT1 is specialized for the diffusion into organelles and cells such as the mitochondria and liver that have low lactate levels (30, 46). If such is the case, a reversal of MCT4 would be expected during a period of inactivity while an increase in MCT1 during inactivity may increase the sensitivity to movement of lactate into other tissues when the concentration is low.

We have measured the blood concentrations of both Epi and NEpi since these hormones play key roles in a complex of functions and are recognized as important in meeting the many challenges faced during exercise (19). As expected, progressive increases occurred in both Epi and NEpi with the duration of exercise. Regular exercise results in a pronounced attenuation in the exercise-induced increase in the catecholamines, presumably because of adaptations that reduce the strain associated with maintaining homeostasis in a variety of cellular properties (19, 54). In this study, we observed no effect of the consecutive days of exercise on the catecholamine response, suggesting that no relevant adaptation had occurred. We have earlier demonstrated that depressions in the catecholamine response occur with short term training in association with improved cardiovascular and metabolic function (33).
A limitation of the current study was our failure to measure the GLUT and MCT protein isoforms following exercise on E1 and E3. It is possible that the acute effects of exercise itself could have altered one or more of these isoforms and be implicated in the time course response.

Changes in HSP70 with our exercise and recovery protocol were included because these proteins respond rapidly to the imposition of exercise (17) and are known to assist in the protection of a variety of cellular proteins and processes (51). We had reasoned that increases might serve to defend constituents involved in the metabolic and excitation-contraction coupling responses (51, 77). Although there was a clear trend towards an enhanced level of HSP70, the change was not significant. Since HSP70 has been shown to be up-regulated with just a single session of vigorous exercise (65), it would appear that the strain imposed by our relatively modest cycling protocol was not sufficient to stimulate changes in this protein.
PERSPECTIVES AND SIGNIFICANCE

In summary, we have shown that a 3 day model of prolonged submaximal exercise results in several adaptations. Among the most conspicuous are increases in the principal glucose and lactate transporters. These adaptations appear to occur in the absence of the classic adaptations which result in improved metabolic coupling and in the type of substrate used during exercise. The reduced accumulation of lactate in muscle suggests that decreased production and/or increased removal from the muscle is an important early response, dissociated from the improvement in phosphorylation potential and the increase in oxidative potential. Restoration of muscle glycogen levels following exercise also appears of high priority promoted at least, in part, by the rapid increase in GLUT4.

We have also found that early adaptations occur in the excitation-contraction coupling proteins and processes with our experimental protocol. Increases in the Na\(^+\)-K\(^+\)-ATPase pump abundance and in the $\alpha$, ($\alpha_1$, $\alpha_2$, $\alpha_3$) and $\beta$, ($\beta_1$, $\beta_2$, $\beta_3$) isoforms were found following the 3 days of repetitive exercise (23). The exercise protocol also resulted in increases in the sarcoplasmic reticulum Ca\(^2\)-ATPase SERCA1a but not the SERCA2a isoform (16). These changes were not accompanied by increases in $V_{\text{max}}$ of either cation pump (16, 23). In fact, acute reductions in the $V_{\text{max}}$ were observed following the exercise. An intriguing issue is the degree to which the remodeling that occurs to the ATPases is a consequence of demands imposed on cation transport by the exercise per se and/or the inactivation that occurs. It is tempting to speculate that changes in $V_{\text{max}}$ of the ATPases, which would have important implications to ATP utilization, may be coupled to changes in the potential of the metabolic pathways in the different cellular compartments. Surprisingly, few studies have addressed this possibility in training-based studies.
ACKNOWLEDGEMENTS

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Table 1. Respiratory gas exchange measured prior to and during exercise on day one and day three

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\dot{V}O_2$ (l.min$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>0.34±0.03</td>
<td>2.09±0.23</td>
<td>2.10±0.19</td>
<td>2.18±0.22</td>
</tr>
<tr>
<td>E3</td>
<td>0.40±0.02</td>
<td>2.04±0.20</td>
<td>2.05±0.21</td>
<td>2.08±0.19</td>
</tr>
<tr>
<td>$\dot{V}CO_2$ (l.min$^{-1}$)</td>
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<td></td>
</tr>
<tr>
<td>E1</td>
<td>0.28±0.03</td>
<td>2.00±0.22</td>
<td>1.99±0.19</td>
<td>2.02±0.22</td>
</tr>
<tr>
<td>E3</td>
<td>0.33±0.02</td>
<td>1.92±0.19</td>
<td>1.98±0.19</td>
<td>1.95±0.19</td>
</tr>
<tr>
<td>RER</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>0.82±0.02</td>
<td>0.96±0.01</td>
<td>0.94±0.01</td>
<td>0.92±0.01</td>
</tr>
<tr>
<td>E3</td>
<td>0.83±0.02</td>
<td>0.94±0.01</td>
<td>0.94±0.01</td>
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</table>

Values are $\bar{x} \pm SEM$ ($n=9$). E1 and E3, exercise on day one and day three, respectively. $\dot{V}O_2$, oxygen consumption, $\dot{V}CO_2$, carbon dioxide production, RER, respiratory exchange ratio. 0, 15, 30, 60, time of exercise. Main effects (P<0.05) of exercise were found for $\dot{V}O_2$, $\dot{V}CO_2$, and RER. For all properties, 0<15, 30, 60 min.
Table 2. Adenine nucleotide concentrations and IMP in working muscle during consecutive days of exercise and recovery

<table>
<thead>
<tr>
<th></th>
<th>E1</th>
<th>E3</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
</tr>
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<tbody>
<tr>
<td>ATP (mmol.kg⁻¹ dry wt)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>24.1±0.7</td>
<td>23.4±0.7</td>
<td>22.1±0.9</td>
<td>23.9±0.6</td>
<td>23.4±0.6</td>
</tr>
<tr>
<td>Post</td>
<td>23.4±0.8</td>
<td>23.4±0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP (mmol.kg⁻¹ dry wt)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>3.9±0.2</td>
<td>4.0±0.1</td>
<td>4.0±0.1</td>
<td>4.1±0.2</td>
<td>3.9±0.1</td>
</tr>
<tr>
<td>Post</td>
<td>4.0±0.2</td>
<td>3.9±0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP (mmol.kg⁻¹ dry wt)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>0.1±0.01</td>
<td>0.1±0.01</td>
<td>0.1±0.01</td>
<td>0.1±0.01</td>
<td>0.1±0.01</td>
</tr>
<tr>
<td>Post</td>
<td>0.2±0.03</td>
<td>0.1±0.01</td>
<td></td>
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<tr>
<td>TAN (mmol.kg⁻¹ dry wt)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>28.2±0.8</td>
<td>27.5±0.7</td>
<td>26.3±0.9</td>
<td>27.5±0.8</td>
<td>27.4±0.7</td>
</tr>
<tr>
<td>Post</td>
<td>27.6±0.9</td>
<td>27.5±0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP (mmol.kg⁻¹ dry wt)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>108±2.2</td>
<td>106±1.9†</td>
<td>103±3.5†‡</td>
<td>104±1.9†‡</td>
<td>102±1.2‡†</td>
</tr>
<tr>
<td>Post</td>
<td>68.7±3.6*</td>
<td>70.1±3.0*†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMP (mmol.kg⁻¹ dry wt)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>0.13±0.01</td>
<td>0.09±0.01†</td>
<td>0.11±0.02†</td>
<td>0.11±0.01†</td>
<td>0.09±0.01†</td>
</tr>
<tr>
<td>Post</td>
<td>0.61±0.2*</td>
<td>0.25±0.06†</td>
<td></td>
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</tr>
</tbody>
</table>

Values are $\bar{x} \pm SEM (n = 12)$. E1 and E3, exercise on day 1 and day 3, respectively. R1, R2 and R3, recovery days 1, 2 and 3 respectively. Pre, pre-exercise; Post, post exercise; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; TAN, total adenine nucleotide; PP, phosphorylation potential; IMP, inosine monophosphate.

*Significantly different (P<0.05) from Pre E1, †Significantly different (P<0.05) from Post E1, ‡Significantly different from Pre E3, * Significantly different (P<0.05) from Post E3.
Table 3. Blood lactate and blood glucose concentration on day one and day 3 of exercise

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>Fatigue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood lactate (mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>0.73±0.12</td>
<td>2.02±0.40</td>
<td>2.56±0.57</td>
<td>1.86±0.53</td>
<td>1.64±0.55</td>
</tr>
<tr>
<td>E3</td>
<td>0.64±0.07</td>
<td>1.42±0.24</td>
<td>1.44±0.26</td>
<td>1.28±0.33</td>
<td>1.29±0.33</td>
</tr>
<tr>
<td>Blood glucose (mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>4.64±0.25</td>
<td>4.15±0.14</td>
<td>4.34±0.22</td>
<td>4.19±0.12</td>
<td>4.02±0.13</td>
</tr>
<tr>
<td>E3</td>
<td>4.49±0.22</td>
<td>3.81±0.17</td>
<td>3.86±0.09</td>
<td>4.02±0.21</td>
<td>3.98±0.22</td>
</tr>
</tbody>
</table>

Values are \( \bar{x} \pm SEM \) \((n = 12)\). E1, exercise on day 1, E3, exercise on day 3, 0, 15, 30, 60, and fatigue, time of blood sampling during exercise. Fatigue, used to indicate the duration of exercise which was until fatigue (E1) or for a maximum of 2 h. Each subject performed the same amount of work each day. Main effects (P<0.05) of exercise were observed for both blood glucose and blood lactate concentrations. For blood glucose, 0> 15, 30, 60, and fatigue. For blood lactate, 0< 15, 30, 60, and fatigue. A main effect (P<0.05) of condition was found for blood lactate. For condition, Day 1 > Day 3.
Table 4. Blood catecholamines during prolonged exercise on day one and day 3 of consecutive days of exercise

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepi (pg.ml⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>379±60</td>
<td>1116±132</td>
<td>1235±136</td>
<td>1704±98</td>
</tr>
<tr>
<td>E3</td>
<td>410±34</td>
<td>1116±124</td>
<td>1274±96</td>
<td>1686±137</td>
</tr>
<tr>
<td>Epi (pg.ml⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>40.4±7.3</td>
<td>164±18</td>
<td>186±30</td>
<td>275±58</td>
</tr>
<tr>
<td>E3</td>
<td>46.9±5.8</td>
<td>154±23</td>
<td>160±29</td>
<td>211±28</td>
</tr>
</tbody>
</table>

Values are \( \bar{x} \pm SEM (n = 12) \). E1, exercise on day 1, E3, exercise on day 3, 0<15, 30, 60, and fatigue, time of blood sampling during exercise. Norepi, norepinephrine, Epi, epinephrine. A main effect (P<0.05) of exercise was found for both norepinephrine and epinephrine. For both hormones, 0<15, 30<60, min. Data for fatigue are not presented due to insufficient sample size.
LIST of FIGURES

Fig 1. High energy phosphates and related metabolites in vastus lateralis muscle during consecutive days of exercise and recovery. Values are $\bar{x} \pm SEM (n = 12)$. E1 and E3, exercise on day 1 and day 3, respectively. R1, R2, and R3, recovery days 1, 2, and 3, respectively. Pre, pre-exercise, Post, post-exercise, PCr, phosphocreatine, Cr, creatine, Pi, inorganic phosphate, TCr, total creatine. *Significantly different (P<0.05) from Pre E1. †Significantly different (P<0.05) from Pre E3, ‡Significantly different (P<0.05) from Post E1. *Significantly different (P<0.05) from Post E3.

Fig 2. Select metabolite concentrations in working vastus lateralis muscle during consecutive days of exercise and recovery. Values are $\bar{x} \pm SEM (n = 12)$. E1 and E3, exercise on days 1 and day 3, respectively. Pre, pre-exercise, Post, post-exercise. R1, R2 and R3, recovery days 1, 2 and 3, respectively. G-6-P, glucose-6-phosphate. *Significantly different (P<0.05) from Pre E1, †Significantly different (P<0.05) from Pre E3, ‡Significantly different (P<0.05) from Post E1. *Significantly different (P<0.05) from Post E3. *Significantly different from R1.

Fig 3. Total, macro and pro glycogen concentrations during consecutive days at exercise and recovery in vastus lateralis. Values are $\bar{x} \pm SEM (n = 12)$. E1 and E3, exercise on day 1 and day 3, respectively. Pre, pre-exercise, Post, post exercise. R1, R2 and R3, recovery days 1, 2 and 3, respectively. *Significantly different
(P<0.05) from Pre E1, †Significantly different (P<0.05) from Pre E3, ‡Significantly different (P<0.05) from Post E1. *Significantly different (P<0.05) from Post E3. *Significantly different from R1.

Fig 4. Representative immunoblots for GLUT1 and GLUT 4 and relative amounts of GLUT1 and GLUT4 protein contents in vastus lateralis muscle during consecutive days at exercise and recovery. A. Transporter isoform; B. α-actin internal control; C. % change in transporter isoform. Values are \( \bar{x} \pm SEM \) \((n = 12)\). E1 and E3, exercise on day 1 and day 3, respectively. R1, R2 and R3, recovery days 1, 2 and 3, respectively. %E1, relative changes based on standardizing the value pre-exercise at E1 to 100%. *Significantly different (P<0.05) from E1, ‡Significantly different (P<0.05) from E3, ‡Significantly different (P<0.05) from R3.

Fig 5. Representative immunoblots for MCT1 and MCT 4 and relative amounts of MCT 1 and MCT4 protein contents in vastus lateralis muscle during consecutive days of exercise and recovery. A. Transporter isoform; B. α-actin internal control; C. % change in transporter isoform. Values are \( \bar{x} \pm SEM \) \((n = 12)\). E1 and E3, exercise on day 1 and day 3, respectively. R1, R2 and R3, recovery days 1, 2 and 3, respectively. %E1, relative changes based on standardizing the value pre-exercise at E1 to 100%. *Significantly different (P<0.05) from E1. ‡Significantly different (P<0.05) from E3.
Fig 6. Mitochondrial enzyme activities assessed during consecutive days at exercise and recovery in vastus lateralis muscle. Values are $\bar{x} \pm SEM (n = 12)$. E1 and E3, exercise on day 1 and day 3 respectively. Pre, pre-exercise, Post, post exercise. R1, R2 and R3, recovery days 1, 2 and 3, respectively. SDH, succinic dehydrogenase, CS, citrate synthase, COX, cytochrome C oxidase, 3-HAD, 3-hydroxyl Co A dehydrogenase. *Significantly different (P<0.05) from Pre E1, †Significantly different (P<0.05) from Pre E3, ‡Significantly different (P<0.05) from Post E1. *Significantly different (P<0.05) from Post E3. •Significantly different (P<0.05) from R1.

Fig 7. Cytosolic enzyme activities during consecutive days of exercise and recovery in vastus lateralis muscle. Values are $\bar{x} \pm SEM (n = 12)$. E1 and E3, exercise on day 1 and day 3, respectively. R1, R2 and R3, recovery days 1, 2 and 3, respectively. Pre, pre-exercise, Post, post exercise. CPK, creatine phosphokinase, PHOSPH, total phosphorylase, PFK, phosphofructokinase, HEX, hexokinase. *Significantly different (P<0.05) from Pre E1, †Significantly different (P<0.05) from Pre E3, ‡Significantly different (P<0.05) from Post E1. *Significantly different (P<0.05) from Post E3.
Figure 1

**PCr**

![Bar chart showing PCr levels](image)

**Cr**

![Bar chart showing Cr levels](image)

**Pi**

![Bar chart showing Pi levels](image)

**TCr**

![Bar chart showing TCr levels](image)
Figure 2

**Glucose**

<table>
<thead>
<tr>
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<th>Post</th>
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<tbody>
<tr>
<td>E1</td>
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</tr>
<tr>
<td>E3</td>
<td>1.8</td>
<td>2.7</td>
</tr>
<tr>
<td>R1</td>
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</tr>
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<td>R2</td>
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<td></td>
</tr>
<tr>
<td>R3</td>
<td></td>
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</tbody>
</table>

**G-6-P**

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<tbody>
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<td>0.3</td>
</tr>
<tr>
<td>E3</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>R1</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>R2</td>
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<td></td>
</tr>
<tr>
<td>R3</td>
<td>0.3</td>
<td></td>
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</table>

**Lactate**

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<tbody>
<tr>
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<td>0.6</td>
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<tr>
<td>E3</td>
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</tr>
<tr>
<td>R1</td>
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<td>0.0</td>
</tr>
<tr>
<td>R2</td>
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<td>0.0</td>
</tr>
<tr>
<td>R3</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Asterisk indicates a significant difference compared to baseline.
† Diamond indicates a significant difference compared to previous time point.
‡ Double dagger indicates a significant change from the previous time point.
Figure 3

Macro

Pro

Total

(glucosyl units kg\(^{-1}\) dry wt)

(glucosyl units kg\(^{-1}\) dry wt)

(glucosyl units kg\(^{-1}\) dry wt)
Figure 4

A  Glut1
B  α-actin
C  % Change

% Change

GLUT1 (%E1)

E1  E3  R1  R2  R3

E1  E3  R1  R2  R3

% Change

GLUT4 (%E1)

E1  E3  R1  R2  R3

A  Glut4
B  α-actin
C  % Change
Figure 5

A  MCT1

B  α-actin

C  % Change

MCT1 (%E1)

MCT4 (%E1)

‡
Figure 6

**SDH**

- Pre
- Post

**CS**

- Pre
- Post

**COX**

- Pre
- Post

**3-HAD**

- Pre
- Post
Figure 7

**CPK**

![CPK Graph](image)

**PHOSPH**

![PHOSPH Graph](image)

**PFK**

![PFK Graph](image)

**HEX**

![HEX Graph](image)