REGULARLY PERFORMED EXERCISE induces a number of physiological adaptations in skeletal muscle. One of the most important adaptations to occur is the increase in the capacity of the oxidative pathways, reflected by an increase in mitochondrial density and increases in the maximal activities of a number of mitochondrial enzymes of the TCA cycle and β-oxidative pathways. Exercise at a given oxygen uptake after training results in less of a decrease in the high-energy phosphates, a smaller increase in P_i, creatine, and ADP (9–11), and this is believed to provide a reduced stimulus to glyco- genolysis and glycolysis and increase the reliance on fat catabolism during exercise (for review, see Refs. 8 and 13).

It is generally accepted that the increase in mitochondrial oxidative capacity that occurs with training leads to the altered metabolic response to exercise (for review, see Ref. 13). Spina et al. (21) have shown an increase in the activities of some mitochondrial enzymes during short-term training and have associated this with smaller increases in blood lactate and lower respiratory exchange ratio values during submaximal exercise after training. However, Green et al. (9, 11) and Phillips et al. (20) have shown the metabolic adaptations to occur before increases in enzyme activities during the same type of training. These findings are based on the use of mitochondrial enzyme activities as markers of mitochondrial potential. Increases in maximal activities of certain oxidative enzymes may not necessarily reflect changes in the flux through the metabolic pathways in which they participate.

The purpose of the present study was to use a technique for directly measuring mitochondrial ATP production rate (MAPR) in human skeletal muscle samples during a 10-day training program. The time course of changes in MAPR was then related to the time course of changes in the maximal activities of the mitochondrial enzymes citrate synthase (CS) and glutamate dehydrogenase (GDH).

METHODS

Subjects. Seven healthy volunteers [3 men, 4 women; 20.1 ± 2.0 (SD) yr, 66.0 ± 11.0 kg, 171 ± 13 cm] volunteered to participate in the study. Subjects were untrained and were not engaged in an endurance training program before the study. Subjects were informed of the procedures involved and any possible risks and discomfort associated with the experiment before giving written consent. The study was approved by the Human Research Ethics Committee of The University of Melbourne.

Experimental protocols. Subjects performed an incremental exercise test to fatigue on a cycle ergometer (Lode, Groningen, The Netherlands) to determine peak pulmonary oxygen uptake (V_O2peak) and the absolute workloads that corresponded to 75 and 95% V_O2peak for the subsequent training requirements. In all V_O2peak tests, subjects attained their age-predicted maximal heart rate, and the respiratory exchange ratio at fatigue exceeded 1.1. Subjects attended one training session per day over a period of 10 days, performing either 60 min of cycle exercise at 75% of pretraining V_O2peak (continuous) or six 5-min bouts of cycle exercise at 95% of pretraining V_O2peak separated by 2- to 3-min periods of exercise at 30–40% V_O2peak (interval). These sessions were alternated over the 10 training days. Muscle samples were obtained from vastus lateralis by percutaneous needle biopsy at rest on three occasions during the training program. The first was taken 1 wk after the initial V_O2peak assessment and a few days before training commenced. The second was taken after 5 days of training, and the third after 10 days, with no training performed on these days. Visible fat and connective tissue were dissected free from the muscle sample, and it was blotted to remove excess blood. The muscle samples were divided into two portions. The first portion (30–75 mg) was prepared for the immediate analysis of MAPR, and the remainder was frozen immediately in liquid nitrogen and stored at −80°C for the later determination of muscle GDH and CS activities. Both the first and second 5-day training periods began with a continuous session, so a total of six continuous and four interval sessions were completed over the 10 days. Relative intensities of the training sessions were...
verified by using heart rate monitors (Polar). In the 24-h period before each muscle sample was taken, subjects were instructed to abstain from alcohol, caffeine, and tobacco and reported to the laboratory after an overnight fast. During the entire experiment subjects were instructed not to perform any other exercise outside of the training sessions.

Analytic techniques. Oxygen and carbon dioxide contents of dried expiree were analyzed by Applied Electrochemistry S-3A/II and CD-3A analyzers (Ametek, Pittsburgh, PA), whereas volume was measured with a Parkinson-Cowan gas meter calibrated against a Tissot spirometer. MAPR was determined by using the bidimensional technique described for human muscle by Wibom and Hultman (23). Briefly, mitochondria were isolated from 30–75 mg of fresh muscle by a process involving gentle homogenization at low speed with a loose-fitting Teflon pestle and subsequent differential centrifugation. Isolated mitochondria in suspension were added to cuvettes containing ADP, Pi, metabolic substrates, and ATP-monitoring reagent (BioOrbit Oy, Turku, Finland). The ATP-monitoring reagent contained firefly luciferase, D-luciferin, L-luciferin, bovine serum albumin, magnesium acetate, and inorganic pyrophosphate. A stable light emission, proportional to the ATP concentration, is produced through the reaction of ATP and D-luciferase, which is catalyzed by firefly luciferase. MAPR was monitored in a luminometer (SLM Aminco, Urbana, IL) at 25°C by using the following two substrate combinations: pyruvate (1 mmol/l) + palmitoyl-L-carnitine (0.005 mmol/l) + α-ketoglutarate (10 mmol/l) + L-malate (1 mmol/l; PPKM) or palmitoyl-L-carnitine (0.005 mmol/l) + L-malate (1 mmol/l; PCM). The substrate combination PPKM has previously been shown to provide the highest MAPR values, whereas MAPR from PCM has been shown to increase the most in response to endurance training (23, 24).

A blank cuvette containing no metabolic substrate was also assayed to account for nonspecific ATP production (usually 10–20% of the activity measured with substrate). MAPR was first calculated in terms of mmol ATP·min"1"·kg wet wt"1" mitochondrial suspension"1". To express MAPR in terms of whole muscle (mmol·min"1"·kg wet wt"1"), MAPR was referenced to the ratio of GDH activity in intact mitochondria in the suspension to total muscle GDH activity. The coefficients of variation for the MAPR assay performed in duplicate samples from the same preparation (n = 12) were 2.1 (PPKM) and 3.0% (PCM). The coefficients of variation for MAPR determined from separate biopsy samples from the same muscle have been reported (23) as 10 (PPKM) and 11% (PCM). GDH activity was determined at 35°C by using the previously described spectrophotometric method (23) but was modified to allow for the assay to be done fluorometrically. A standard curve of various NADH concentrations was constructed to allow for the conversion of fluorescence signals to NADH concentrations. GDH activity of intact mitochondria in the suspension was determined by first assaying the extramitochondrial fraction in the suspension, then assaying the total GDH activity of the suspension after lysing mitochondria with 0.05% Triton X-100, the difference in these activities giving the intramitochondrial GDH activity. Total muscle GDH activity was assayed in a crude homogenate prepared from a different portion of the original muscle sample. CS activity was assayed according to the method of Srere (22). Total GLUT-4 was measured in crude membrane preparations in five subjects as has previously been described (17), except for the use of enhanced chemiluminescence detection. Absorbance was quantified by using densitometric scanning and normalized to the pretraining value for each subject. All samples from a single subject were run in the same gel.

**Table 1. Effects of 5 and 10 days of short-term training on maximal mitochondrial enzyme activities, total crude membrane GLUT-4, and VO2peak**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre</th>
<th>5 Days</th>
<th>10 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDH, mmol·min&quot;1&quot;·kg wet wt&quot;1&quot;</td>
<td>0.51 ± 0.06</td>
<td>0.78 ± 0.06*</td>
<td>0.80 ± 0.04*</td>
</tr>
<tr>
<td>CS, mmol·min&quot;1&quot;·kg wet wt&quot;1&quot;</td>
<td>18.1 ± 1.5</td>
<td>21.1 ± 1.3*</td>
<td>22.8 ± 1.4*</td>
</tr>
<tr>
<td>GLUT-4, au</td>
<td>1.0</td>
<td>1.6 ± 0.2</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>VO2peak, l/min</td>
<td>2.83 ± 0.30</td>
<td>3.09 ± 0.30*</td>
<td></td>
</tr>
</tbody>
</table>

*Values are means ± SE; n = 7 (for CS n = 6; for GLUT-4 n = 5).

**Fig. 1. Mitochondrial ATP production rate (MAPR) before and during 10 days of training.** Samples were collected before training (Pre) and after 5 and 10 days of training. MAPR was measured with substrate combinations pyruvate + palmitoyl-L-carnitine + α-ketoglutarate + malate (PPKM) and palmitoyl-L-carnitine + malate (PCM). Data are means ± SE in mmol ATP·min"1"·kg wet wt"1". Significantly different: *from Pre, P < 0.05; #from day 5, P < 0.05.
DISCUSSION

The results of the present study show that a 10-day training program resulted in 136 and 161% increases in MAPR measured with the substrate combinations PPKM and PCM, respectively. The activities of GDH and CS increased 53 and 16%, respectively, over the first 5 days but did not increase further over the final 5 days of training. V\(\text{O}_2\text{peak}\) was increased 9% from pretraining levels after 5 days of training. V\(\text{O}_2\text{peak}\) increased by 10.22 ± 0.33 on April 4, 2017 http://jap.physiology.org/ Downloaded from

Two factors that could contribute to the different enzyme responses observed in the aforementioned training studies are the initial fitness levels of the subjects and the training protocols employed. The pretraining \(\text{V}_2\text{peak}\) values for the group in the present study, and that reported by Spina et al. (21), were both below 3 l/min. These were lower than those of Phillips et al. (20) and Green et al. (9) (3.5–4 l/min), who reported no rapid increase in mitochondrial enzyme activities in response to training. Perhaps the initial level of fitness of the subjects is an important determinant of the mitochondrial enzyme response to training, the more well-trained subjects producing a slower enzyme response in these studies. The training protocol used in the present study differed from that employed by Green et al. (9, 11) and Phillips et al. (21), (2 h of cycling/day at 60–70% \(\text{V}_2\text{peak}\) for 5–7 days). Perhaps the high-intensity component in the present study, and subsequent differences in the pattern of muscle fiber recruitment, provided a greater stimulus for rapid enzyme upregulation. However, Chesley et al. (3) reported a rapid increase in CS activity, but the pretraining \(\text{V}_2\text{peak}\) for this group was 3.5 l/min, and the training protocol employed was similar to that used by Green et al. and Phillips et al.; so it seems that the discrepancies in these results remain unclear.

This discrepancy raises the question as to the validity of inferring increases in mitochondrial function from increases in the activities of certain mitochondrial enzymes with training. The measurement of MAPR provides an indication of the functional capacity of a number of processes leading to the production of ATP and is perhaps a more informative index of mitochondrial function than the measurement of enzyme activities in vitro. The results of the present study demonstrate no clear relationship between the increases in maximal activities of CS and GDH and the increase in MAPR with training. During the first 5 days of training, all activities increased; however, there was no

gen increase of the small sample size.

The activities of GDH and CS increased 53 and 16%, respectively, from pretraining levels after 5 days of training (Table 1). There was no further significant increase in the activities of GDH or CS over the final 5 days of training. There were no correlations between the increases in MAPR for either substrate and the increases in GDH and CS activity, except for MAPR measured with PCM and GDH activity over the first 5 days of training (\(r = 0.77, P = 0.04\)). Although total GLUT-4 increased 60% (Table 1), this failed to reach statistical significance (\(P = 0.06\)), most likely as a consequence of the small sample size.

DISCUSSION

The results of the present study show that a 10-day training program resulted in 136 and 161% increases in MAPR measured with the substrate combinations PPKM and PCM, respectively. The activities of GDH and CS increased 53 and 16%, respectively, over the first 5 days but did not increase further over the final 5 days of training. V\(\text{O}_2\text{peak}\) was increased 9% from pretraining levels after 5 days of training. V\(\text{O}_2\text{peak}\) increased by 10.22 ± 0.33 on April 4, 2017 http://jap.physiology.org/ Downloaded from
significant correlation between the enzyme response and MAPR increase, except for the increase in MAPR measured with PCM and the increase in GDH activity. During the final 5 days of training, when no further increases in enzyme activities were observed, MAPR still increased. This demonstrates a dissociation between the increase in the mitochondrial ability to generate ATP and the activities of these mitochondrial enzymes.

Furthermore, not all mitochondrial enzymes respond to training in the same way. The magnitudes of the increases in CS and GDH were different in the present study, and Holloszy et al. (14) have shown that many mitochondrial enzymes, including CS and GDH, do not increase in parallel in rat skeletal muscle with training. In addition, the maximal activities of CS and succinate dehydrogenase have been shown to have no correlation with the calculated maximal flux through the TCA cycle in isolated rat heart (5) and in human skeletal muscle (1). Thus it appears difficult to infer changes in mitochondrial function from changes in the maximal activities of certain mitochondrial enzymes. However, if we accept the directional changes in the activities of CS and GDH as markers of the size and number of mitochondria, the present findings suggest that increases in mitochondrial function can occur independently of increases in mitochondrial mass. This is indeed one possible interpretation of the findings of Green et al. (9, 11) and Phillips et al. (20). Perhaps there are mechanisms in addition to an increased mitochondrial mass contributing to an increase in mitochondrial function and, therefore, MAPR.

The larger increase in MAPR measured with PCM in the present study indicates an increased capacity for the mitochondria isolated from resting muscle to generate ATP from fat in vitro. The implications of this for the mitochondria isolated from exercising system in vivo are not clear. The catalytic ATP from fat in vitro. The implications of this for the mitochondria isolated from resting muscle to generate ATP and the activities of these mitochondrial enzymes.  

There is evidence to suggest the existence of subsarcolemmal and intermyofibrillar mitochondrial subpopulations in human skeletal muscle (7). With the use of morphometric analysis, these subpopulations have been shown to respond differently to training, subsarcolemmal mitochondria proliferating to a greater extent (15). However, it is unknown what bearing, if any, this may have on the results of the present study. It is unknown in what proportion each subpopulation was isolated in the present study, and it is possible this may be a source of variability in the comparison of whole muscle MAPR measurements throughout the training program.

In summary, the findings of the present study demonstrate a rapid increase in MAPR measured with the substrate combinations PPKM and PCM over a 10-day training period. The largest increase (161%) was measured with PCM. The activities of the mitochondrial enzymes CS and GDH and total crude membrane GLU4 increased significantly within the first 5 days of training but did not further increase thereafter. There was no clear relationship between the increase in the maximal capacity of mitochondria to generate ATP and the response of CS and GDH to 10 days of training. Furthermore, if CS and GDH are accepted as indicators of mitochondrial mass, our results suggest that mechanisms other than, or in addition to, an increase in mitochondrial size and number may be involved in the improvement of mitochondrial ATP-generating capacity after training.

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