Failure of hypoxia to exaggerate the metabolic stress in working muscle following short-term training

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Submitted 19 December 2008; accepted in final form 23 May 2009

Failure of hypoxia to exaggerate the metabolic stress in working muscle following short-term training. Am J Physiol Regul Integr Comp Physiol 297: R593-R604, 2009. First published May 27, 2009; doi:10.1152/ajpregu.91035.2008.—This study investigated the effects of hypoxia (experiment 1) and the effects of hypoxia following short-term training (experiment 2) on metabolism in working muscle. In experiment 1, eight males with a peak aerobic power (\(\dot{V}\)O\(_2\)\text{peak}\)) of 45 ± 1.7 ml·kg\(^{-1}\)·min\(^{-1}\) (\(\bar{x}\) ± SE) cycled for 15 min at 66.1 ± 2.1\% \(\dot{V}\)O\(_2\)\text{peak}\) while breathing room air [normoxia (N)] or 14\% O\(_2\) [hypoxia (H)]. In experiment 2, nine males with a \(\dot{V}\)O\(_2\)\text{peak}\) of 43.3 ± 1.6 ml·kg\(^{-1}\)·min\(^{-1}\) performed a similar protocol at 60.7 ± 1.4\% \(\dot{V}\)O\(_2\)\text{peak}\) during N and during H following 5 days of submaximal exercise training (H + T). Tissue samples extracted from the vastus lateralis before exercise and at 1, 3, and 15 min of exercise indicated that compared with N, H resulted in lower (\(P < 0.05\)) concentrations (mmol/kg dry wt) of creatine phosphate and higher (\(P < 0.05\)) concentrations of creatine, inorganic phosphate, and lactate, regardless of exercise time. When the exercise was performed at H + T and compared with N, no differences were observed in creatine phosphate, creatine, inorganic phosphate, and lactate, regardless of duration. Given the well-documented effects of the short-term training model on elevating \(\dot{V}\)O\(_2\)\text{kinetics}\) and attenuating the alterations in high-energy phosphate metabolism and lactate accumulation, it would appear that the mechanism underlying the reversal of these adaptations during H is linked to a more rapid increase in oxidative phosphorylation, mediated by increased oxygen delivery and/or mitochondrial activation.

The blunting of the decrease in the concentration of the high-energy phosphate compounds (phosphorylation potential) is specific to the exercise itself, since no effect of the short-term training has been observed in the resting concentration of any of the high-energy compounds (5, 18–20, 22, 49). Moreover, we have been able to determine that the metabolic adaptations are initially expressed during the transition from rest to steady-state exercise while oxygen consumption (\(\dot{V}\)O\(_2\)) is accelerating to a steady-state level (18, 20). We have shown that our short-term training model is also accompanied by an increase in \(\dot{V}\)O\(_2\)\text{kinetics}\) (45), which occurs in conjunction with an increase in femoral artery blood velocity kinetics (58) and ostensibly in oxygen delivery to the working muscle. At steady state, no differences are observed before and following the short-term training in the rate of oxidative phosphorylation (OXPHOS) as indicated by the lack of a difference in \(\dot{V}\)O\(_2\) (18–20). At steady state, an increase in regulatory sensitivity is indicated because OXPHOS is preserved in the face of a reduction in the putative modulators of OXPHOS such as one or more of the metabolites involved in high-energy phosphate transfer (40). The increase in OXPHOS during the transient phase following exercise onset would appear to be responsible for the improvement in metabolic stability.

Two hypotheses have been advanced to explain the early onset metabolic adaptations that occur. One hypothesis predicts that the mechanism is central in nature, mediated directly by increased delivery of oxygen to the mitochondria and consequently increased OXPHOS (62). The second hypothesis asserts that the mechanism has a peripheral locus due to adaptations in the muscle cell itself (13, 29).

The peripheral hypothesis asserts training results in a more rapid activation of OXPHOS as a result of mitochondrial adaptations that reduce metabolic inertia, allowing increases in \(\dot{V}\)O\(_2\)\text{kinetics}\). One of the most dominating and enduring hypothesis, initially popularized by Holloszy and Coyle (29) in the 1970s, was that the increase in metabolic stability could be explained by the increases in oxidative potential that occur with training. In response to an intensive 12-wk training program in rats, these investigators demonstrated that exercise was a potent stimulus to mitochondrial synthesis, resulting in an increased potential for OXPHOS, as indicated by the increases in the maximal activity of the enzymes involved in the citric acid cycle, the electron transport system, and \(\beta\)-oxidation (29). The precocious nature of the mitochondria to respond to regular activity has since been demonstrated on numerous occasions (30). Based on the mitochondrial adaptations (and the increase in the number of respiratory chains), Holloszy and Coyle (29) proposed that at a given level of OXPHOS less of an increase in one of more of the high-energy phosphates involved in the regulation of OXPHOS would be required, since the flux rate per respiratory chain would be reduced. The

IT IS NOW CLEAR based on the results of several studies published from our laboratory and from other laboratories that metabolic adaptations in working vastus lateralis muscle occur within days (5, 18, 19, 22, 49) and even hours (23) following the onset of repetitive exercise. The most conspicuous adaptations occur to the high energy phosphate transfer system where the reduction typically observed during submaximal exercise in phosphocreatine (PCr) concentration is blunted. This adaptation is also accompanied by reductions in the free AMP (AMPf) and ADP (ADPf) but not in total ATP (5, 18–20, 22, 49). The free ADP (ADPf) but not in total ATP (5, 18, 19, 23). The failure to observe a reduction in ATP, which would be expected given the near-equilibrium nature of the high-energy phosphate transfer reactions, has been attributed to lack of measurement sensitivity, given the small reduction expected relative to the concentration (27). It should also be noted that the short-term training models employed also result in a lower muscle lactate concentration during submaximal exercise (5, 18, 19, 22, 48).

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problem with this hypothesis is that we (18, 19, 22, 23, 49), and subsequently others (51), have shown that oxidative potential, as measured by the maximal activities of a range of mitochondrial enzymes, does not accompany the metabolic adaptations that occur in working muscle in response to short-term training.

The appeal of the central role of $O_2$ would be more inviting if it could be demonstrated that in humans engaged in voluntary exercise, acute manipulation of the oxygen availability to the working muscle induces alterations in both $V_\text{O}_2$ kinetics and the kinetics of the metabolic response. Several studies (50) have been published examining such responses to moderate exercise with both hypoxic and hyperoxic gas mixtures.

There is a general consensus that at moderate exercise intensity employing small muscle groups and $^{31}$P-MRS techniques, the kinetics of the high-energy phosphate response, typically measured by the changes in PCr, is a first order response that parallels $V_\text{O}_2$ kinetics (52, 53). Under these conditions, it has been demonstrated that the general magnitude of the PCr response is dependent on arterial $O_2$ content, with hypoxic gas mixtures clearly exaggerating the amount at which PCr is reduced (28). Moreover, hypoxia also results in a greater increase in $H^+$ concentration (28), suggesting a greater accumulation of lactic acid in hypoxia. The effects of hypoxia on metabolism have also been studied during moderate exercise such as cycling with large muscle groups, which greatly increases the demand on the central circulatory system. The metabolic changes have been characterized in tissue extracted from the vastus lateralis muscle, using the needle biopsy technique (1). During the steady-state, changes in phosphorylation potential and lactate accumulation are exaggerated in hypoxia (38, 42, 63). During the nonsteady state, when tissue was sampled at 1 min of exercise, a greater elevation in lactate concentration (25, 28), hyperoxia is without a significant effect on high-energy phosphate stability (38), although lactate concentration may be depressed (61).

Moderate exercise while breathing hypoxic gas mixtures depresses the fast $V_\text{O}_2$ response (9, 12, 31, 60, 62), while the effect on the slow component is controversial (12, 31). Interestingly, hypoxic gas mixtures appear to affect the $V_\text{O}_2$ response but only the slow phase (62). In general, other models used to increase $O_2$ delivery to muscle have not been effective in increasing $V_\text{O}_2$ kinetics (50). At present, the prevailing theory at least in moderate activity is that metabolic inertia and not $O_2$ availability regulates $V_\text{O}_2$ kinetics (15).

Collectively, the literature strongly indicates that during the nonsteady state of moderate intensity exercise, decreases in arterial $O_2$ content mediated by hypoxic gas mixtures exaggerate metabolic instability and that the exaggerated metabolic response is due to a depression in the onset kinetics of OXPHOS. Since our studies using the short-term training model support the notion that the attenuation of the metabolic stress occurs within the first 3 min of exercise and that increased $O_2$ utilization is involved, it is possible that training could reverse the effects of hypoxia on metabolism.

Our overall objective in this study was to assess whether the adaptation in metabolism that occurs soon after the onset of regular daily cycling occurred during the non-steady-state phase of exercise as a result of increases in the utilization of $O_2$ by the muscle cell. We have reasoned that exercise performed in hypoxia would exaggerate the metabolic response during the non-steady-state phase of exercise. We also postulate that if moderate exercise is performed in hypoxia following a brief training regime, then the metabolic response during the non-steady-state phase would not be different from that observed before training in normoxia, given the well-documented effects of training on the response. The failure to detect differences would provide compelling evidence that an increase in $O_2$ utilization in the working muscle cell represents the primary mechanism involved in the metabolic adaptation observed in training.

**METHODS**

**Experimental design.** Two separate experiments involving a different group and each involving a standard exercise protocol were performed as part of this study investigating muscle metabolic behavior. In **experiment 1**, the effects of acute hypoxia were investigated. Each participant volunteered for the two conditions employed in this experiment, namely normoxia (N) and hypoxia (H). The purpose of **experiment 2** was to determine the effects of short-term training while exercise was performed in H following training. For **experiment 2** to isolate the effects, a separate group of individuals performed exercise in normoxia before training (N) and again 24–36 h after the last training session while breathing an hypoxic gas mixture (H + T). We have also added a third condition in **experiment 2**, namely normoxia plus training (N + T). In this condition, which was performed 1 to 2 days following H + T, the intent was to document a training effect using heart rate and blood lactate criteria. Additional tissue sampling, which would have been desirable in N + T, was not possible given the number already performed. The training consisted of performing submaximal cycling for 2 h/day exercise at 66.1 ± 2.1% of peak aerobic power ($V_\text{O}_2\text{peak}$) for 5 consecutive days. For **experiment 1**, the H test was performed between 1 and 2 wk after the initial submaximal test in N.

The standard exercise protocol employed in **experiment 1** and **experiment 2** involved cycling for 15 min at between 66.1 ± 2.1% ($V_\text{O}_2\text{peak}$) and 60.7 ± 1.4% ($V_\text{O}_2\text{peak}$) of $V_\text{O}_2\text{peak}$. For each condition within an experiment, the absolute power output and exercise duration were identical. It should be emphasized that **experiment 1** was completed ~3 yr before **experiment 2**. In general, for each exercise test, regardless of condition, participants reported to the laboratory ~1 h early, during which time a catheter was inserted into a dorsal hand vein and used to sample blood just before exercise and at 2.5 and 14.5 min of the exercise. The blood was used for the measurement of lactate. During the preparatory period, the vastus lateralis muscles of each participant were also prepared for tissue sampling using the needle biopsy technique (1) under local anesthesia (2% xylcocaine with epinephrine) with suction to increase tissue yield. For each test, four sites were prepared, two on each leg. Tissue was extracted before exercise (0 min) and at three times during exercise (1, 3, and 15 min) from the four sites in random order. The same protocol was followed during the second test for each participant. Fields used for tissue sampling were free from nerves and blood vessels and widely separated. At each sampling, the volunteer remained seated on the cycle while quickly relaxing into the arms of an assistant while the targeted leg was simultaneously positioned to the horizontal by another assistant for the rapid sampling of tissue. These procedures were practiced before the study and could be completed in ~15 s. However, to remove any bias caused by the interruption of the exercise, the 15-min protocol was completed in two phases with a 30-min rest provided between phases. During the first phase, tissue was sampled at rest before exercise and following 3 min of exercise. In phase 2, tissue was sampled following 1 and 15 min of exercise. This latter protocol allowed for an additional 14 min of exercise to
minimize any disruptive effects of the short-term interruption at 1 min. A potential limitation with splitting the exercise into two phases is the possibility that the initial phase of exercise could act to alter $V_O_2$ kinetics during the second phase given the relatively brief recovery time provided between phases (4). However, given the moderate intensity and brief nature of the initial priming exercise, minimal effects would be expected (3).

Respiratory gas samples were obtained both before the start of exercise and for a 3- to 4-min period beginning at 10 min of exercise and used to calculate $V_O_2$ consumption. The actual power output used for the submaximal exercise tests for each participant was based on a percentage of the $V_O_2peak$ obtained during a progressive cycle test to fatigue, administered approximately 1 wk before the first submaximal test. The maximal exercise test was only administered during normoxia. Gas exchange was determined using an open-circuit system, commonly employed by our group (32). All exercise tests were conducted using an electronic cycle ergometer, calibrated on a daily basis (Quinton 870). Care was taken to ensure that the seat height was standardized for each subject on all testing occasions. The tests were conducted under standardized environmental conditions of 21–22°C and 54–55% relative humidity.

The submaximal N tests were performed while the subject was breathing a normoxic gas mixture (inspired O2 fraction = 0.21), while the H and H + T tests were performed while the subject was breathing an hypoxic gas mixture (inspired O2 fraction = 0.14). During both conditions, the volunteers inspired from a 350-l Tissot gasometer, beginning just before the start of exercise while positioned on the cycle and terminating at the end of exercise. The gas volume in the Tissot was maintained constant by manipulating the rates of flow into the Tissot from the appropriate gas mixture. All resting tissue samples regardless of condition were obtained only during normoxia.

Participant characteristics. For both experiments, all volunteers were healthy males and not involved in vigorous exercise on a regular basis (i.e., less than once per week). Further, the participants were requested not to participate in vigorous exercise, except as required, outside the experimental setting. For each group, the age, weight, and responses to maximal exercise are reported in Table 1. No differences between groups were found for $V_O_2peak$, either when expressed in absolute or relative terms. As required, both studies were approved by the Office of Research Ethics at the University of Waterloo. Before written consent was obtained by each participant, the purpose of study, the specifics of the experimental design, and the test protocols, including associated risks involved, were detailed as a required condition of ethics approval.

On a given experimental day during which the submaximal tests were scheduled, subjects were instructed to report to the laboratory after an overnight fast and without any nutrient intake, except water. The intent of this requirement was to standardize the nutrient habits during the day before the tests. In addition, no nutrient intake, including water, was permitted until after the exercise was completed.

Analytical procedures. Immediately following tissue extraction, the needle containing the tissue sample was immediately plunged in liquid N2 and stored at −80°C until analyses. Muscle tissue samples were freeze dried and subsequently measured for the adenine nucleotides (ATP, ADP, and AMP) and for inosine monophosphate (IMP) with HPLC using previously published procedures (33) as modified by our group (18). Measurements of high-energy phosphates (ATP and PCr) and related metabolites (Pi and Cr), as well as glycogen and selective glycolytic intermediates, were accomplished using fluorometric procedures (39) with modifications (18, 24). Glycogen was measured as glucosyl units in a separate piece of tissue after hydrolysis with hydrochloric acid (39). The calculation of the metabolite concentrations for each individual was adjusted to the total creatine content, a procedure that provides a more stable reference base, given the contamination of sample by blood, fat, and connective tissue that can occur. As a condition of this adjustment, it is assumed that no systematic change occurs in the value of total creatine as a result of either exercise or the experimental condition, an assumption that was tenable in this study.

Each metabolite was measured in duplicate with all samples for a given individual and for a given metabolite analyzed during the same experimental session.

The concentrations of ADP, and AMP, were calculated based on the measured values of ATP, PCr, and Cr the near-equilibrium constants for Cr kinase ($K_{ob} = 1.66 \times 10^{−10}$) and adenylation kinase ($K_{on} = 1.05$; Ref. 10). Muscle pH (and H+) was estimated from the regression equations previously established (56) using the concentrations of pyruvate and lactate. The concentration of free Mg2+ was assumed to be 1 mM (10). To assess the redox state in the cytosol, the lactate-to-pyruvate ratio was calculated (57). Provided that the nucleotide pool size remains constant as well as the related cations, given the near-equilibrium nature of the reactions involved in the phosphate energy system, the relative concentrations of ATP, ADP, AMP, and PCr are determined by one variable, which indicates the phosphorylation potential or the content of the high-energy phosphate bonds (8).

Blood lactate was analyzed fluorometrically (44) in supernatants that were obtained after centrifugation of blood that was placed in a prechilled tube containing 0.6 M perchloric acid and neutralized in 1.25 mM KHCO3.

Statistical procedures. A two-way ANOVA for repeated measures was used to assess the treatment and exercise effects for experiment 1 and experiment 2. Where significance was found, Newman-Keuls post hoc procedures were used to compare specific means. Significance was accepted at $P < 0.05$. In general, where differences are indicated in the text, significance is implied.

RESULTS

Respiratory gas exchange and blood lactate responses. Oxygen consumption and heart rate during submaximal exercise were measured both during experiment 1 and experiment 2. These measurements allowed response comparisons between conditions within each experiment and between experiments. During experiment 1, no differences were observed in the $V_O_2$ (l/min) measured during the later stage of exercise between N and H (2.20 ± 0.09 vs. 2.26 ± 0.10). At this time, the effects of H were clearly evident in the heart rate where H averaged 165 beats/min higher than during N (183 ± 5.8 vs. 183 ± 5.9). In experiment 2, measurements of $V_O_2$ (l/min) also assessed late in exercise were 2.04 ± 0.11 and 2.04 ± 0.10 in N and N +...
T, respectively. Unfortunately, technical problems precluded the measurement of VO2 in H + T. Measurements of heart rate late in the exercise demonstrated a clear effect of training, as indicated by the lower rate in N + T compared with N alone (148 ± 5.3 vs. 167 ± 4.8 beats/min). As expected, no difference was observed between N and H + T (167 ± 4.8 vs. 167 ± 4.0 beats/min).

Submaximal exercise in experiment 1, regardless of condition, resulted in a time-dependent increase in blood lactate concentration (Table 2). Compared with N, exercise in H resulted in a greater increase in lactate concentration. When exercise was performed in H following training (H + T), the increase in lactate observed was higher than during N alone (experiment 2). In contrast, exercise blood lactate concentration was lower during N + T compared with N, indicating that a training effect occurred.

Experiment 1. In this experiment, untrained volunteers performed moderate cycle exercise on two occasions, namely during N and during H. As expected, exercise during N did not result in changes in any of the adenine nucleotides, either ATP, ADP, AMP, or total adenine nucleotides (Table 3). These responses were not altered by H. Exercise in N was also without effect in altering IMP concentration. However, with H, IMP was elevated and different from N following 15 min of exercise.

Initial reductions in PCr were observed at 1 min of exercise in N remained stable at 3 min followed by a further decline at 15 min (Fig. 1). The same pattern was observed during exercise in H except that the reduction was more emphasized. At all exercise durations, PCr was lower in H than N. Changes in Cr with exercise were followed a similar pattern to PCr except that in contrast to decreases, increases were observed. As with PCr, the alterations in Cr with H were more pronounced, regardless of the duration of exercise. The near stoichiometric increases in Cr were also noted for Pi during the 15-min exercise protocol. However, unlike Cr the difference in Pi between the two conditions was a main effect and not specific to a sampling point.

The calculated changes in ADPf and AMPf displayed the same response to exercise in N, namely a time-dependent increase (Fig. 2). However, the point at which the increase was observed depended on the metabolite. In the case of ADPf, the initial increase was not observed until 3 min, which was followed by further elevations at 15 min. For AMPf, the increase was only observed at 15 min of exercise. H was observed to affect both metabolites but only at the end of exercise where the values in H were elevated to a greater extent than in N.

In general, H was without effect in modifying the exercise effect in N for most of the glycolytic intermediates examined, namely glucose, glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-phosphate (Table 4). The exceptions were fructose 6-phosphate and glucose-1-phosphate, which was consistently higher in H compared with N. It is important to emphasize that for the other metabolites a trend toward higher values with H was indicated but no significance was found. The metabolites, glucose, glucose-6-phosphate, and fructose-6-phosphate all displayed an increase in concentration with exercise.

H exaggerated the time-dependent increase in lactate concentration during exercise (Fig. 3). During this condition, a progressive increase in lactate was found with exercise duration. In contrast, with N, elevations in lactate were only found at the end of exercise. The lactate concentration was found to be lower at 3 and 15 min but not at 1 min of exercise in N compared with H. Neither exercise nor experimental condition altered pyruvate concentration. The calculation of the lactate-to-pyruvate ratio indicated the main effects of exercise but only at 15 min where the ratio was increased. In general, the lactate-to-pyruvate ratio was higher in H.

Exercise resulted in a reduction in glycogen, an effect that was not modified by H (Fig. 4). Glycogen concentrations were lower at 15 min of exercise than at rest and at 1 min of exercise.

Experiment 2. In this experiment, we have contrasted the metabolic response to moderate exercise during N before training and to the same exercise protocol during H following training. The exercise whether conducted during the untrained

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### Table 2. Blood lactate concentrations during submaximal exercise in normoxia and hypoxia for experiment 1 and experiment 2

<table>
<thead>
<tr>
<th>Exercise Time, min</th>
<th>0</th>
<th>3</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N + T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H + T</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE in mM. For experiment 1, n = 8. For experiment 2, n = 9. N, normoxia; H, hypoxia; N + T, normoxia plus training; H + T, hypoxia plus training. *Significantly different (P < 0.05) from N. †Significantly different (P < 0.05) from 0 min. ‡Significantly different (P < 0.05) from 3 min. §Significantly different (P < 0.05) from N + T.

### Table 3. Concentrations of adenine nucleotides and inosine monophosphate during submaximal exercise in normoxia and hypoxia

<table>
<thead>
<tr>
<th>Exercise Time, min</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>24.1 ± 0.91</td>
<td>4.94 ± 0.64</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>H</td>
<td>23.4 ± 0.39</td>
<td>5.23 ± 0.48</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>N</td>
<td>22.9 ± 0.52</td>
<td>5.44 ± 0.76</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>H</td>
<td>23.9 ± 0.80</td>
<td>5.45 ± 0.70</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>N</td>
<td>23.9 ± 0.80</td>
<td>5.45 ± 0.70</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>H</td>
<td>21.3 ± 1.6</td>
<td>5.21 ± 0.59</td>
<td>0.11 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 8) in mM/kg dry wt. TAN, total adenine nucleotides. IMP, inosine monophosphate. *Significantly different (P < 0.05) from N. †Significantly different (P < 0.05) from 0 min. ‡Significantly different (P < 0.05) from 1 min. §Significantly different (P < 0.05) from 3 min.
state in N or following training in hypoxia (H/T) failed to elicit changes in ATP, ADP, AMP, total adenine nucleotides, or IMP (Table 5). No changes between the two conditions were found regardless of the property examined or the time of measurement. Exercise under the two experimental conditions decreased PCr and increased Cr and Pi (Fig. 5). The time course of the exercise effect was the same for each property, namely a progressive alteration at each time point. Exercise whether performed in N in the untrained state or during H in the trained state did not affect the response regardless of the metabolite. Similarly, training failed to differentially affect the ADPf and AMPf responses (Fig. 6). For both ADPf and AMPf, the concentrations at 3 min of exercise were higher than before exercise and the concentration at 15 min of exercise was higher than before exercise and at 1 min of exercise.

Both pyruvate and lactate concentrations were increased by exercise with no differences in the time-dependent changes detected between experimental conditions (Fig. 7). For both conditions, pyruvate and lactate increased at 3 min and remained stable for the remainder of the protocol. In the case of lactate, the initial increase was observed after 1 min of exercise. Changes in the lactate-to-pyruvate ratio were also observed with exercise but not condition. The exercise-induced increase was found at 3 and 15 min.

In general, the concentration of endogenous substrate glycogen was lower at 3 and 15 min of cycling compared with rest, while the concentration at 15 min was lower than at 1 min (Fig. 8). In general, glycogen levels were persistently higher during hypoxic exercise after training than during N before training, an effect that could be attributed to the elevated resting level following training.

The relative intensities for the cycling tasks were 66.1 ± 2.1 and 60.7 ± 1.4% VO2peak for experiment 1 and experiment 2, respectively, a difference that was significant (P = 0.048). Blood lactate concentration at 15 min of cycling in N was low...
Table 4. Concentrations of glucose and selected glycolytic intermediates in vastus lateralis muscle during submaximal exercise performed in normoxia and hypoxia

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Exercise Time, min</th>
<th>Normoxia (N)</th>
<th>Hypoxia (H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0</td>
<td>3.71±1.4</td>
<td>2.62±0.48</td>
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<tr>
<td></td>
<td>1</td>
<td>3.29±0.80</td>
<td>3.53±0.25</td>
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<td></td>
<td>3</td>
<td>3.07±0.22</td>
<td>3.69±0.52</td>
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<tr>
<td></td>
<td>15</td>
<td>4.48±0.30</td>
<td>6.54±1.5</td>
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<tr>
<td>G-6-P</td>
<td>0</td>
<td>1.38±0.17</td>
<td>1.53±0.24</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.15±0.48</td>
<td>4.47±0.79</td>
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<td></td>
<td>3</td>
<td>3.37±0.47</td>
<td>5.21±0.81</td>
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<tr>
<td></td>
<td>15</td>
<td>3.36±0.37</td>
<td>3.54±0.50</td>
</tr>
<tr>
<td>G-1-P</td>
<td>0</td>
<td>0.09±0.01</td>
<td>0.13±0.04</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.14±0.02</td>
<td>0.18±0.04</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.11±0.03</td>
<td>0.24±0.03</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.15±0.02</td>
<td>0.15±0.02</td>
</tr>
<tr>
<td>F-6-P</td>
<td>0</td>
<td>0.21±0.03</td>
<td>0.22±0.04</td>
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<tr>
<td></td>
<td>1</td>
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<tr>
<td></td>
<td>15</td>
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<td>0.45±0.06</td>
</tr>
<tr>
<td>F-1,6-P</td>
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<td>0.45±0.08</td>
<td>0.35±0.04</td>
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<tr>
<td></td>
<td>1</td>
<td>0.56±0.08</td>
<td>0.56±0.08</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.49±0.07</td>
<td>0.60±0.10</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.44±0.07</td>
<td>0.63±0.17</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 8) in mmol/kg dry wt. G-6-P, glucose-6-phosphate; G-1-P, glucose-1-phosphate; F-6-P, fructose-6-phosphate; F-1,6-P, fructose-1, 6-phosphate. For glucose, G-6-P, and F-6-P, main effects (P < 0.05) were found for time. For glucose, 0, 1, 3, < 15 min. For G-6-P and F-6-P, 0 < 1, 3, 15 min. For G-1-P and F-6-P, a main effect (P < 0.05) of condition was found. For both metabolites, N < H.

and not different between conditions, suggesting that the metabolic stress induced by the task was comparable and of moderate intensity.

**DISCUSSION**

As postulated, our data clearly indicate that in the vastus lateralis muscle submaximal cycling performed in hypoxia after our training protocol did not result in differences in metabolic behavior compared with the performance of similar exercise before training in normoxia. Given the effect of hypoxia in decreasing O2 utilization during the nonsteady state, these findings suggest that some training-induced adaptation, external to or within the working muscle cell, occurs that serves to partially protect phosphorylation potential and related by-product accumulation. Since the adaptation in metabolic behavior occurs during the non-steady-state adjustment to the exercise challenge, the most probable mechanism to explain the changes observed is increased utilization of O2 by the contracting muscle cells. According to this theory, the increase in O2 utilization, occurring soon after the onset of exercise, results in an increase in OXPHOS, attenuating the dependence on high-energy phosphate transfer and glycolysis needed to satisfy the energy needs of the working muscle.

This theory suggests that during moderate-intensity cycling an early transient period exists where the mitochondrial respiratory flux rate is regulated either by limitations in O2 availability and/or by limitations in one or more of the other substrates and cofactors such as NADH, H+, ADP, and Pi.

The viability of the central hypothesis to explain the lack of an effect of training on muscle metabolism while exercising in hypoxia depends on three assumptions, namely that exercise in hypoxia in the untrained state would increase the disruptions in energy homeostasis during the early non-steady-state period, that the short-term training protocol that we have employed would result in less of a disturbance in energy homeostasis when the exercise is performed in normoxia, and that these responses would occur in the absence of adaptations in mitochondria and, in particular, increases in oxidative potential.

It is well known that exercise involving large muscle groups performed at moderate intensity in normoxia results in a reduction in PCr; an accumulation of Cr, Pi, lactate, and IMP; and a depletion of endogenous glycogen stores when measurements are performed in working muscle during the period following steady state (42). This is essentially what we have observed. However, in addition, we have been able gain insight into the time-dependent changes in metabolism that occurs during the nonsteady state by extracting tissue samples at 1 and 3 min after the onset of exercise in both normoxia and hypoxia.

**Fig. 3. Effects of submaximal exercise in normoxia and hypoxia on concentrations of lactate (A) and pyruvate (B) and the lactate-to-pyruvate ratio (C) in vastus lateralis muscle. Values are means ± SE (n = 8). *Significantly different (P < 0.05) from normoxia. †Significantly different (P < 0.05) from 0 min. ‡Significantly different (P < 0.05) from 1 min. ‡‡Significantly different (P < 0.05) from 3 min. For lactate-to-pyruvate ratio, main effects (P < 0.05) were found for both condition and exercise. For condition, hypoxia > normoxia. For exercise, 0 < 15 min.**
3 min of exercise. Our results reveal that initial reductions of
~20% occur in PCr within the first minute of exercise. Curiously,
no further reductions in PCr occurred during the ensuing
2 min of exercise. Over the next 12 min of exercise, additional
reductions in PCr were observed, ultimately reaching a con-
centration that was ~52% of preexercise. Near stoichiometric
but opposite changes were also observed in Cr and Pi, as would
be expected (41). In the case of Pi, the change that occurs is
tempered by the increase in the phosphorylated glycolytic
intermediates that results during exercise. In normoxia, our
protocol also elicited moderate increases in lactate concentra-
tion that were not observed until near termination of the
exercise. Increases in lactate are clearly suggested at 1 min of
effort; however, the individual variability in response complicat-
ed detection of significant effects. In a previous study (42),
also employing submaximal cycling exercise for 15 min in
normoxia, the modest reduction in PCr that occurred was fully
manifest by 1 min of exercise. The protocol also failed to
increase lactate concentration, a finding that might be ex-
plained by the use of a lower relative exercise intensity (55%
VO2peak) than the one used in the current study. The above
studies appear to be the only ones to have examined the
changes in metabolism during the early transient period after
exercise onset using whole body exercise and direct tissue
sampling.

Our hypothesis also depends on being able to demonstrate
that O2 availability in the mitochondria can compromise the
metabolic behavior during the transition period to steady-state
exercise. Our results with hypoxia are consistent with previous
studies (16, 50) employing exercise involving varying amounts
of muscle mass and both moderate and heavy exercise intensi-
ty. Moreover, we show, as have others (38), that the effect
can occur early, which in the case of our protocol is by 1 min
of exercise. We report that the effects of hypoxia on the PCr

Table 5. Concentrations of adenine nucleotides and inosine
monophosphate in vastus lateralis muscle during submaximal
exercise in normoxia and in hypoxia following training

<table>
<thead>
<tr>
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<th>Exercise Time, min</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>ATP</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>22.7±0.62</td>
</tr>
<tr>
<td>H + T</td>
<td>21.9±0.84</td>
</tr>
<tr>
<td>ADP</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>3.08±0.29</td>
</tr>
<tr>
<td>H + T</td>
<td>3.06±0.14</td>
</tr>
<tr>
<td>AMP</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>0.35±0.11</td>
</tr>
<tr>
<td>H + T</td>
<td>0.40±0.08</td>
</tr>
<tr>
<td>TAN</td>
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</tr>
<tr>
<td>N</td>
<td>26.3±0.85</td>
</tr>
<tr>
<td>H + T</td>
<td>26.2±0.78</td>
</tr>
<tr>
<td>IMP</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>0.18±0.06</td>
</tr>
<tr>
<td>H + T</td>
<td>0.12±0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 8) in mmol/kg dry wt.
hydrolysis were fully manifested during this time frame, with the differences sustained throughout the remainder of the exercise, a finding in contrast to an earlier study (42) that showed that an increased reduction occurs with hypoxia but only late in exercise and not at 1 min. In the above study, hypoxia produced a time-dependent acceleration in lactate concentration, similar to what we report.

Detailed responses to exercise in hypoxia have been reported in several recent studies using 31P-MRS procedures. In one of the first studies (26) examining metabolic responses using this technology and submaximal plantar flexion exercise, it was reported that the net hydrolysis of PCr was increased by hypoxia (10% O2) and reduced by hyperoxia (100% O2). The increased PCr hydrolysis in hypoxia occurred both in the fast and slow phases of exercise (26). In the case of the fast phase, the effect of hypoxia was not detected until after 30 s of contractile activity (26). As expected, increases in H+ also occurred during both the fast and slow phases when the exercise was performed in hypoxia (26). Collectively, these observations indicate that decreases in arterial O2 content are intimately linked to the increased disturbance in metabolism that occurs during the transition to steady-state exercise. Whether or not arterial O2 delivery to the working muscle cell is compromised during the early transient phase after exercise onset, particularly with large muscle groups, remains speculative. There is evidence to suggest, using small muscle groups and different contractile tasks, that increases in femoral arterial blood flow can compensate for reductions in arterial O2 content (9, 14, 34). The effects of hyperoxia are more difficult to demonstrate, since near complete saturation of hemoglobin with O2 occurs in normoxia.

A second assumption was that our training program would result in metabolic adaptations designed to protect phosphorylation potential and by-product accumulation and that at least part of this response would occur early in exercise. Although we have not investigated this issue in the current study, given the restrictions in the number of biopsies, we have examined this issue in many previous studies using a variety of exercise

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**Fig. 6.** Effects of submaximal exercise performed in normoxia and in hypoxia following training on the calculated concentrations of ADPf (A) and AMPf (B) in vastus lateralis muscle. Values are means ± SE (n = 9). Main effects (P < 0.05) of time were found for both ADPf and AMPf. For ADPf, 0 < 3, 15 min and 1 < 15 min. For AMPf, 0, 1 < 3, 15 min.

**Fig. 7.** Concentrations of lactate (A) and pyruvate (B) and the lactate-to-pyruvate ratio (C) in vastus lateralis muscle during submaximal exercise during normoxia and during hypoxia following training. Values are means ± SE (n = 9). Main effects (P < 0.05) of time were found for lactate, pyruvate, and the lactate-to-pyruvate ratio. For lactate, 0 < 1 < 3, 15 min. For pyruvate, 0 < 3, 15 min. For lactate-to-pyruvate ratio, 0 < 3, 15 min.
protocols and repetitive days of exercise. Collectively, these studies clearly indicate that providing the training intensity exceeds 60% $V_{\text{O}_2\text{peak}}$ and the daily exercise duration 2 h, less of a reduction in PCr and less accumulation of metabolites such as Cr, P$_i$, and lactate occur during submaximal exercise within the first few days (5, 18–20, 22, 49) and even following a prolonged session of heavy intermittent exercise (23). Moreover, these adaptations can be detected during the first 3 min of exercise (18, 20, 23), suggesting that the training effects occur during the transition period. When exercise is extended beyond this period, no further changes in phosphorylation potential occur (18, 22, 23). In the current study, the daily 2-h session of prolonged exercise was conducted for 5 days at 66% $V_{\text{O}_2\text{peak}}$. Although we did not perform a separate experiment to directly document the efficacy of our training program in improving metabolic stability during exercise in normoxia, we provide other evidence that the training program was successful in producing the desired effects. Short-term training is known to result in higher concentrations of muscle glycogen (5, 18, 47) and lower blood lactate (18, 46) in exercise, adaptations that we document in this study. In only one study (17) published to date by our group have we not found adaptations in high-energy phosphate metabolism within this time frame, a null effect we credit to the brief training period (2 days) in combination with the relatively low intensity (60% $V_{\text{O}_2\text{peak}}$).

Given that hypoxia creates a greater metabolic disturbance during the transition period from rest to steady-state exercise and given that our training protocol was effective in abolishing the metabolic effects of hypoxia during the transition period, it would appear that a common mechanism is involved. Several possibilities exist. The most inviting possibility is based on alterations in the regulation of OXPHOS. Flux rates in OXPHOS depends on the availability of $O_2$, NADH, H$^+$, ADP, and P$_i$ (41), all of which could be potentially limiting. Current regulatory theories stress the importance of phosphorylation potential and changes in one or more of the by-products of high-energy phosphate metabolism in the activation of the respiratory rate (40). Given the near equilibrium nature of the high energy phosphate transfer reactions and the coordinating nature of the changes in specific reactants, isolation of specific stimuli remains problematic (40).

It is known that changes in high-energy phosphate metabolism during moderate submaximal exercise occur during the non-steady-state transition period (16, 50), the period during which $V_{\text{O}_2}$ and OXPHOS reaches steady state (64). Recent studies (35, 52, 54) have been able to establish that the changes in both of these properties follow first order kinetics leading to the proposition that they are intimately related. Two theories predominate to explain the time delay needed to establish steady-state levels for both properties. In the case of $V_{\text{O}_2}$, the central hypothesis asserts that the time needed to deliver $O_2$ to the working muscle cells as a result of circulatory and diffusion factors is believed limiting (62). As a consequence, during the transition period, high-energy phosphate transfer not only provides the rapid increases in energy needed to meet the demand of the contracting muscle cells but, as well, provides the signals needed to increase glycolytic flux rate, both of which serve to optimize OXPHOS, given the $O_2$ that is available (40).

Others (16) have postulated that the limitation to OXPHOS during the transition period is not regulated by $O_2$ availability but by metabolic inertia and the delay in increasing metabolic flux either as a result of limitations in the availability of other substrates and cofactors or by the direct activation of the enzymes involved. There is extensive evidence, using a variety of models, that increasing $O_2$ availability to the contracting muscle cell has a minimal effect in increasing metabolic stability or increasing $V_{\text{O}_2}$ kinetics (16, 50). Accordingly, an increase in $O_2$ availability, which we have suggested as the mechanism underlying our metabolic adaptations to short-term training, would not appear tenable. However, our results must be put in the context of the exercise protocol employed, namely two-legged voluntary cycling at moderate intensity, which is known to depend on the activation of multiple muscles (43). Under such conditions, the temporal and spatial demands on blood flow in perfusing the working muscle cells and supplying adequate nutrients are fundamentally different from those generally employed in kinetic-based studies (50). It is possible that early adaptations simply involve a more appropriate regulatory distribution of nutrients (7), resulting in increased $O_2$ availability during the early transient phase of exercise. According to this possibility, measurements of $O_2$ delivery determined at the femoral artery would not be appropriate for detecting $O_2$ responses in the working muscle cell.

The results that we have obtained with the use of hypoxia provide evidence, albeit indirect, that limitations in $O_2$ availability to the electron transport system of the working muscle cell are responsible for the metabolic adaptations observed in short-term training. The hypoxia-based studies demonstrate that the greater depression in phosphorylation potential that occurs in hypoxia, which is mediated primary by a greater depression in PCr (42), similar to what we have observed in this study, is also accompanied by a slowing of $V_{\text{O}_2}$ kinetics (62). Moreover, we report that putative signals, namely ADP$_f$ and AMP$_f$, involved in the regulation of mitochondrial respiration (40) and glycogenolysis/glycolysis are also enhanced compared with normoxia. It would appear that the elevated lactate concentration observed with hypoxia is at least partly due to increased glycolytic flux, the final concentration primarily representing the balance between the amount of pyruvate entering the mitochondria via activation of pyruvate dehydrogenase (59) and the amount of lactate transported out of the cell via monocarboxylate transporters (36). We also report that
during the transition period the lactate-to-pyruvate ratio, a measure of cytosolic redox potential, is higher in hypoxia, as expected (37, 57). Increases in cytosolic redox potential could promote increases in mitochondrial redox potential (8, 55), an accommodation that could serve to protect OXPHOS in hypoxic environments (8, 65). Collectively, these studies provide evidence for an O$_2$-limited mitochondrial flux rate at least during part of the non-steady-state transition period during exercise in hypoxia. Unclear at present is whether the limitation is mediated by convective and diffusive transport to the mitochondria or entry into the mitochondria.

The mechanism whereby short-term training neutralizes the effects of hypoxia on energy metabolism may simply also involve an adaptation that improves mitochondrial oxygenation during the early transient period of exercise. It is known that training results in less of a perturbation in cellular phosphorylation potential, an adaptation that appears to be mediated during the non-steady-state phase of exercise and that persists during the steady state. Since no changes in V$_\text{O}_2$ occur during the steady-state following training (18, 20), for the central hypothesis to be valid, increases in V$_\text{O}_2$ kinetics are suggested. This is what we have found in an earlier study (45). The most probable implication of this finding is that increases in O$_2$ availability in the mitochondria enable a more rapid increase in the mitochondrial flux rate, resulting in less dependence on high-energy phosphate transfer and consequently a reduced intensification of activating signals, such as ADP$_\text{i}$ and AMP$_\text{i}$. This is what we have reported with our short-term training models. The lower muscle lactate concentration, typically observed with the short-term training regime, would appear to represent deceased glycolytic flux in combination with increased pyruvate removal by the mitochondria (59) and increased clearance from the cell (17, 48).

Since in normoxia near complete arterial hemoglobin saturation with O$_2$ occurs during submaximal exercise, the proposed increase in cellular oxygen utilization following training would have to be due to some other mechanism. One inviting possibility is an increase in blood flow. In support of this possibility, we (58) have reported faster femoral arterial blood flow kinetics during leg exercise following short-term training. Given this possibility, it might be expected that acute manipulations designed to increase O$_2$ delivery to working muscle during exercise would result in similar metabolic events to those observed with training. Although some effect with hypoxia has been observed, possibly as a result of improved diffusion, secondary to elevations in the partial pressure gradient for O$_3$, the effects are generally minimal (26, 61). Since arterial hemoglobin saturation is near complete in normoxia and techniques to increase blood flow are not well developed, the viability of the increased blood flow as the central mechanism mediating the metabolic adaptations remains tentative.

It is also possible that the primary adaptations are peripheral in nature and occur in the working muscle cells. A hypothesis of long-standing credibility is that increases in mitochondrial size and number, resulting in an upregulation of the citric acid cycle and electron transport chain, which can occur with training (29), represent the central event. According to this theory, the training-induced increase in the number of respiratory chains allows a given level of OXPHOS to be realized with less flux per chain and consequently less of an increase is needed in the signals activating mitochondrial respiration (29).

This hypothesis does not appear tenable with our short-term training model, since increases in the maximal activity of representative enzymes used to demonstrate increases in mitochondrial potential with training (29) do not occur with the short-term training model (6, 18, 19, 22, 23, 47, 51). Given the specifics of the training program used in this study, there is no reason to suspect that increases in oxidative potential would occur. However, it is possible that changes in other mitochondrial properties may have occurred, including changes in the regulatory behavior of enzymes (67).

**Perspectives and Significance**

The relative importance of central vs. peripheral factors in regulating mitochondrial respiration during the transition to steady-state voluntary exercise continues to remain in dispute, no doubt complicated by the complexity of factors involved (2). The results from our study and others using hypoxic gas mixtures support the possibility that decreases in mitochondrial O$_2$ availability compromise OXPHOS, even though the putative signals activating flux rates are amplified. Based on the experimental models employed to acutely increase cellular O$_2$ availability, it would appear that during normoxia O$_2$ availability is adequate to optimize OXPHOS. The application of hypoxia following a short-term training model suggests that OXPHOS can be enhanced during the early phase of exercise as a result of increases in O$_2$ availability. Time-course measures during the period need to be performed to determine where the metabolic adaptations occur and whether they are accompanied by increases in V$_\text{O}_2$ kinetics. Moreover, given the importance of muscle mass in the demands placed on the cardiovascular system, studies should also be performed examining tasks that depend on the activation of both small and large muscles and at different exercise intensities. Although we have demonstrated that the adaptations that occur need not be accompanied by increases in the mitochondrial potential as indicated by the lack of a change in the maximal activity of representative enzymes, further study is needed. These investigations need to address the sites and processes regulating mitochondrial flux rate in conjunction with intrinsic adaptations to mitochondria that may have occurred. Moreover, short-term training can also affect the catalytic activity of the cation pumps (11, 21), changes that have been proposed to be coupled to V$_\text{O}_2$ kinetics (66). Given the importance of adequate levels of OXPHOS in certain disease states, such as congestive heart failure and chronic obstructive pulmonary disease where cellular O$_2$ availability may be compromised during increased demands, identifying whether compensatory mechanisms exist in these populations would appear to be a high priority.

**GRANTS**

We acknowledge the financial support provided by the Natural Sciences and Engineering Research Council (Canada) for the research (to H. J. Green).

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