Effects of Endurance Training on the AMPK Response to Exercise.

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EFFECTS OF ENDURANCE TRAINING ON THE AMPK RESPONSE TO EXERCISE

by

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of a thesis submitted by

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This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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<thead>
<tr>
<th>Date</th>
<th>William W. Winder, Chair</th>
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<td>Allen C. Parcell</td>
</tr>
</tbody>
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ABSTRACT

EFFECTS OF ENDURANCE TRAINING ON THE AMPK RESPONSE TO EXERCISE

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Master of Science

Activation of AMP-activated protein kinase (AMPK) results in the upregulation of several intracellular systems which help to prepare a cell for a high energy challenge. The magnitude of the AMPK response to a 10 min bout of exercise has been found to decrease in red quadriceps (RQ) following training, while putative AMPK roles seem to be maintained; specifically, the biogenesis of mitochondria and higher levels of hexokinase II and glucose transporter 4 (GLUT4). If the AMPK response to exercise is responsible in part for these adaptations, how can they be maintained if the AMPK response is attenuated? The purpose of this study was to determine whether phosphorylation of AMPK in RQ increases during 2-hr training bouts after rats have trained for 8 wks. Male Sprague-Dawley rats ran up to 30 m/min up a 15% grade, 2 hr/day for 8 wks. On the final bout of exercise, trained rats ran for 0 (TRC), 30 (TR1), or 120 min (TR2) up a 15% grade at 30 m/min. Red quadriceps (RQ), soleus, and white quadriceps (WQ) were immediately collected and frozen for analysis. Citrate synthase activity increased in RQ (79 ± 3 vs. 37 ± 4 μmol/g/min) and
soleus (64 ± 4 vs. 35 ± 2 μmol/g/min) but not in WQ compared to non-trained controls. In trained rats, maximal increases in T-172 phosphorylation of AMPK occurred after 30 min of exercise (relative values = 1.29 ± 0.06 vs. 1.00 ± 0.06). AMPK phosphorylation did not change significantly in trained rats that ran for 2 hrs (1.31 ± 0.09) compared to rats that ran for 30 min. Similarly, maximal increases in AMPK activity in trained rats occurred after 30 min of exercise (pmoles/min/mg = 2.67 ± .05 vs. 1.09 ± .41) and AMPK activity did not change significantly in trained rats that ran for 2 hrs (2.79 ± .17) compared to rats that ran for 30 min. Previous studies demonstrated a 2–3 fold increase in AMPK activity in non-trained rats after 30 min of exercise at lower work rates. These results demonstrate that the AMPK response to exercise is attenuated even after two-hr bouts of exercise. This implies that the increase in mitochondrial oxidative enzymes, GLUT4, and hexokinase II may be maintained by signals other than the AMPK signaling system. The CREB signaling pathway is one such system. Western analysis of phospho-CREB (Ser133) showed a statistically significant increase in phospho-CREB content in trained rats relative to control. No change in phospho-CREB protein expression was observed between TRC, TR1, and TR2 rats. Significant increases of muscle phospho-CREB content in TRC relative to untrained rats suggest that CREB remains phosphorylated in trained rats even after 24 hrs of rest. Accordingly, chronically increased phospho-CREB in muscle of trained rats relative to controls may explain in part how increased levels of mitochondria are maintained in the face of reduced AMPK response. Alternatively, the attenuated AMPK response may still be above the threshold required for inducing adaptations to endurance training.
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# TABLE OF CONTENTS

## PROSPECTUS OF THESIS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>AMPK</td>
<td>2</td>
</tr>
<tr>
<td>AMPK Activation</td>
<td>3</td>
</tr>
<tr>
<td>Function of AMPK with Exercise/Training</td>
<td>4</td>
</tr>
<tr>
<td>AMPKK and Exercise/Training</td>
<td>5</td>
</tr>
<tr>
<td>Proposal</td>
<td>6</td>
</tr>
<tr>
<td>Training Protocol &amp; Anticipated Experiments</td>
<td>7</td>
</tr>
<tr>
<td>Methods</td>
<td>12</td>
</tr>
<tr>
<td>Estimated Costs</td>
<td>19</td>
</tr>
<tr>
<td>References</td>
<td>20</td>
</tr>
</tbody>
</table>

## THESIS: EFFECTS OF ENDURANCE TRAINING ON THE AMPK RESPONSE TO EXERCISE

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>28</td>
</tr>
<tr>
<td>Introduction</td>
<td>30</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>33</td>
</tr>
<tr>
<td>Results</td>
<td>38</td>
</tr>
<tr>
<td>Discussion</td>
<td>40</td>
</tr>
<tr>
<td>References</td>
<td>46</td>
</tr>
<tr>
<td>Figure Legends</td>
<td>55</td>
</tr>
<tr>
<td>Figures</td>
<td>58</td>
</tr>
<tr>
<td>Curriculum Vitae</td>
<td>67</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>58</td>
</tr>
<tr>
<td>Figure 2</td>
<td>59</td>
</tr>
<tr>
<td>Figure 3</td>
<td>60</td>
</tr>
<tr>
<td>Figure 4</td>
<td>61</td>
</tr>
<tr>
<td>Figure 5</td>
<td>62</td>
</tr>
<tr>
<td>Figure 6</td>
<td>63</td>
</tr>
<tr>
<td>Figure 7</td>
<td>64</td>
</tr>
<tr>
<td>Figure 8</td>
<td>65</td>
</tr>
<tr>
<td>Figure 9</td>
<td>66</td>
</tr>
</tbody>
</table>
INTRODUCTION

The 5′-AMP-activated protein kinase (AMPK) acts as a metabolic master switch regulating several intracellular systems including the cellular uptake of glucose, the β-oxidation of fatty acids and the biogenesis of glucose transporter 4 (GLUT4) and mitochondria (1, 2, 4, 5, 36). The energy-sensing capability of AMPK can be attributed to its ability to detect and react to fluctuations in the AMP:ATP ratio that take place during rest and exercise (muscle stimulation). During muscle contraction AMP increases while ATP decreases, which changes AMPK into a good substrate for activation via an upstream kinase, AMPKK. AMPKK is a complex of three proteins, STE-related adaptor (STRAD), mouse protein 25 (MO25), and LKB1 (a serine/threonine kinase). During a bout of exercise, muscle AMPK activity increases in response to metabolic stress brought about by an extreme cellular demand for ATP. This AMPK response to exercise has been found to notably decrease following training while putative AMPK roles seem to be maintained such as GLUT4 and mitochondrial biogenesis (3). One possible explanation for the attenuating effects of training on the AMPK response to exercise is that perhaps the immediate metabolic stress felt by a trained muscle cell during stimulation has been reduced due to the increase in GLUT4, blood supply, mitochondria and other adaptations that the endurance training itself induced upon the cell. This condition would bring about a delayed AMPK response to the exercise bout until the metabolic stress equates to that of an untrained cell. It has already been found that high-intensity work rates are needed for AMPK activation (6, 7, 8, 9). We hypothesize that these necessary high-
intensity conditions for AMPK activation will need to increase higher still with training. This study will investigate the effects that endurance training has on the AMPK response to exercise and its effects on other enzymes that take on key metabolic roles as well. This will include a comparative study that will examine the AMPK response to exercise at different stages of the final exercise bout following training. The foremost health related impact we hope to receive from this study includes the substantiation of an AMPK role in long-term exercise-induced adaptation mechanisms which may perhaps lead to improvements in certain metabolic aberrations in metabolic diseases.

**AMPK**

The heterotrimeric protein AMPK is composed of α, β, and γ subunits. Each of these three subunits takes on a specific role for both the stability and activity of AMPK (11). The γ subunit includes four particular cystathione beta synthase (CBS) domains giving AMPK its ability to sensitively detect shifts in the AMP:ATP ratio. The four CBS domains create two binding sites for AMP commonly referred to as Bateman domains. Binding of one AMP to a Bateman domain cooperatively increases the binding affinity of the second AMP to the other Bateman domain (49). As AMP binds both Bateman domains the γ subunit undergoes a conformational change which exposes the catalytic domain found on the α subunit. It is in the activation loop of the catalytic domain where AMPK becomes activated when phosphorylation takes place at threonine-172 by an upstream AMPK kinase (AMPKK) (19). The α, β, and γ subunits can also be found in different isoforms: α1 or α2, β1 or β2, and γ1, γ2, γ3.
Although the most common isoforms expressed in most cells are the $\alpha_1$, $\beta_1$, and $\gamma_1$ isoforms, it has been demonstrated that the $\alpha_2$, $\beta_2$, $\gamma_2$, and $\gamma_3$ isoforms are also expressed in cardiac and skeletal muscle (11, 12, 13).

**AMPK ACTIVATION**

Triggering the activation of AMPK can be carried out provided that two conditions are met. First, the $\gamma$ subunit of AMPK must undergo a conformational change so as to expose the active site (Thr-172) on the $\alpha$ subunit. The conformational change of the $\gamma$ subunit of AMPK can be accomplished by an increase in [AMP]. Increased concentrations of AMP will give rise to the conformational change on the $\gamma$ subunit of AMPK as two AMP bind the two Bateman domains located on that subunit. It is this conformational change brought about by increased concentrations of AMP that exposes the active site (Thr-172) on the $\alpha$ subunit. This critical role of AMP is further substantiated in experiments that demonstrate AMPK activation via an AMP analogue 5-amino-4-imidazolecarboxamide ribotide (ZMP) which is derived from 5-amino-4-imidazolecarboxamide riboside (AICAR) (14, 15, 16, 17). The second condition that must be met is the phosphorylation and consequent activation of AMPK on its activation loop at Thr-172 of the $\alpha$ subunit brought about by an upstream kinase (AMPKK) (19, 20). The complex formed between LKB1 (STK 11), mouse protein 25 (MO25), and the pseudokinase STE-related adaptor protein (STRAD) has of late been identified as the major upstream kinase responsible for phosphorylation of AMPK on its activation loop at Thr-172 (21, 22, 23). AMP also makes AMPK a poorer substrate for phosphatases (19, 20, 24).
Muscle contraction can provide the conditions mentioned above needed for AMPK activation (25). As muscles contract, ATP is hydrolyzed, forming ADP. ADP then helps to replenish cellular ATP by donating a phosphate group to another ADP, forming an ATP and an AMP. As more AMP is produced during muscle contraction, the AMP:ATP ratio dramatically increases, leading to the allosteric activation of AMPK (10, 26, 27). This fact is further authenticated with studies, such as those sited above, that used electrical stimuli as a means to contract muscle to facilitate AMPK activation (1, 6, 18, 28, 29).

FUNCTION OF AMPK WITH EXERCISE/TRAINING

Many biochemical adaptations of skeletal tissue take place in response to endurance training [increased mitochondrial biogenesis and capacity (31, 32), increased muscle glycogen (37), and an increase in factors which specialize in glucose uptake in cells such as GLUT4 and Hexokinase II (33, 35, 37) are thought to be mediated in part by AMPK when it is activated (2, 34). Additionally, recent discoveries can conceivably suggest a direct AMPK role in increasing blood supply to exercised/trained muscle cells by stimulating and stabilizing both vasculogenesis and angiogenesis (30). Taken together, these adaptations most likely transpire as a result of both temporary and maintained increases in AMPK activity brought about by increases in the AMP:ATP ratio during single bouts of exercise and long-term training.

During a single acute exercise bout, AMPK takes on immediate roles to allow the contracting muscle cells adapt to the energy challenges taking place by inducing GLUT4 translocation to the plasma membrane (28, 40, 41, 42) allowing for increased
glucose uptake. It has also been found that AMPK can help stimulate glycolysis in heart muscle (50). If exercise bouts continue through a long-term training regimen, AMPK and other signals will increase mitochondrial oxidative proteins, thus increasing capacity to produce ATP. AMPK accomplishes this transition to the oxidative mode of metabolism by upregulating and activating enzymes that are known to be involved (either directly or indirectly) in the oxidation of glucose and fatty-acids such as GLUT4, hexokinase II, PPARalpha, PGC-1, UCP-3, cytochrome C and TFAM, just to name a few (2, 35, 37, 38, 39, 43).

**AMPKK AND EXERCISE/TRAINING**

AMPK activity increases with exercise and the LKB1/MO25/STRAD complex is considered to be the major upstream AMPKK of the 5’-AMP-activated protein kinase phosphorylating the α subunit of AMPK at Thr-172 (19, 20, 21, 22). This fact is puzzling considering that although LKB1 protein abundance has been shown to increase in skeletal tissue with endurance training, AMPKK and AMPK activities have been shown to decrease with endurance training in both trained and untrained tissue (18, 29, 45, 46). Currently, the activity of AMPKK immediately following a 2-hr bout of exercise of an endurance trained rat is unclear. It is possible that there exists a direct link between the observed decrease in AMPKK activity in endurance trained skeletal muscle and the apparent decrease in the AMPK response to exercise with endurance training. AMPKK activity assays will be run to assess this possibility following training.
A seemingly paradoxical role of AMPK occurs when we take a closer look at the energy-sensing enzyme in relation to exercise and long-term training. Long-term endurance training triggers increases in muscle oxidative metabolic enzymes and increases in GLUT-4, mitochondrial size and quantity, and an increased capacity for oxidation of fatty acids; however, Winder et al. reported in 2002 that despite observing these increased oxidative biochemical adaptations to long-term endurance training (similar to those mentioned above), the AMPK response (activation of AMPK with the onset of exercise) to acute bouts of exercise decreased in red quadriceps (RQ) with training (3 – see Fig.1). Conversely, the study did not observe the same results in white quadriceps (WQ) and soleus (SOL) muscles that they did in RQ. The trained rats used for that endurance study ran on treadmills 5 days/wk in two 1-h sessions, morning and afternoon. The rats were also running up to 31m/min (grade 15%). Finally, following training, the rats were sacrificed either at rest or following 10 min. of exercise.

Because the AMPK response to exercise decreases with increased training duration, many questions arise that would challenge the AMPK role with respect to biochemical adaptations to exercise and endurance training. If the AMPK response to exercise is responsible in part for biochemical adaptations to training, how then can these adaptations to training be maintained if the AMPK response to exercise is being attenuated with training? It is hypothesized that in endurance trained rats, the increase in AMPK during each bout of exercise will be delayed as a result of the increase in mitochondrial oxidative enzymes. Therefore, we will run trained rats for a longer
duration (up to a 2-h bout of exercise) in order to determine if AMPK is activated late in the course of prolonged training bouts. Rats will be killed at rest and after running 30 min and 120 min. By doing this we hope to observe and capture the 5’-AMP-activated protein kinase (AMPK) in its now active state in RQ following a high intensity long-term training procedure.

TRAINING PROTOCOL & ANTICIPATED EXPERIMENTS

1) Rat Training Protocol & Tissue Homogenization- All rats will be trained appropriately according to the Institutional Animal Care and Use Committee of Brigham Young University. 30 male Sprague-Dawley rats (Sasco, Wilmington, MA) will be housed in a reasonably quiet, temperature controlled (21-22°C) room that cycles daily with a 12-h light and 12-h dark period. Rats will be kept in Institutional Animal Care and Use approved rodent shoebox compartments (two rats per compartment) with a metal grill top to allow for ventilation. Rodent bedding will be replaced in each shoebox up to two times a week and all rats will be weighed every week to keep a log of rat growth rate. Rats will be fed standard rat chow (Harlan Teklad rodent diet) and given water to drink ad libitum. Trained rats will be given 30 g of rat chow the night before they are killed to help measure blood glucose levels. 20 randomly assigned rats will run on a motor-driven rodent treadmill up a 15% grade 5 days/wk for 8-10 weeks in a temperature controlled room (16-17°C). Initially, the rats will run for 35-m the first day up a 5% grade at 16 m/min. Exercise bout
intensity will increase daily until the rats eventually progress to a single 120-m bout of exercise on a 15% grade at 32 m/min. Rat training at this point will be maintained for 4 more weeks. After 8-10 weeks, blood and tissue samples (left/right gastrocnemius, left/right red/white quadriceps, left/right Soleus, Liver and Heart) will be collected from trained rats anesthetized either at rest or immediately following a 30-m or 2-h bout of exercise by intravenous (jugular catheter) injection of pentobarbital sodium. The catheters will have been placed in the jugular veins (fastened tightly with sterilized string) of trained rats anesthetized with Isoflurane, USP (liquid for inhalation) 3 days prior to sacrifice. Once extracted, tissues will be compressed frozen with metal tongs at liquid nitrogen temperature (apprx. -196°C), wrapped immediately in labeled aluminum foil and then placed in a container filled with liquid nitrogen until they are stored later in an temperature ultra-low temperature (-90°C) freezer. Untrained rats will be anesthetized with pentobarbital sodium (50mg/kg body wt) and tissues (right red/white Quadriceps and right Soleus) will be extracted and clamp frozen at liquid nitrogen temperature. Untrained tissues will be used as a control to compare with trained tissues for proteins such as p-eEF2K, p-GS, p-IRS-1, p-TSC2, p-ACC hexokinase, cytochrome C, FAT/CD36, GLUT4, AMPK, ACC, PGC-1, MO25, STRAD, LKB1, AS160 and TFAM. Citrate synthase activity will be measured and used to confirm a training effect in the trained rats. All tissues will be ground with a mortar and pestle at liquid nitrogen temperature and then homogenized in a homogenization buffer
containing 250 mM mannitol, 50 mM Tris-HCl, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, and 1% triton all at a pH of 7.4. Dithiothreitol (DTT) (1 mM), 1 mM Benzamidine, 1µg/ml soybean trypsin inhibitor, and .5 mM AEBSF are added just prior to homogenization. The final concentration of the tissue in the homogenization buffer solution will be a 9 factor dilution (1 g muscle powder / 9 ml buffer). Approximately 300 µl of raw homogenate will be taken from the homogenate and the rest will then be centrifuged at 2100 rpm for 20-min at 4°C. Post-centrifugation, supernatant from the homogenate will be immediately taken from the tubes and aliquots will be dispensed into 1500 µl Eppendorf tubes.

2) **Western Blots** - Blots from trained rats will be performed on p-eEF2K, p-GS, p-IRS-1, p-TSC2, p-ACC GLUT4, AMPK, phospho-AMPK, ACC, PGC-1, cytochrome C, MO25, STRAD, LKB1, AS160, TFAM, FAT/CD36 and Hexokinase. From untrained rats we will run western blots for hexokinase, GLUT4 and citrate synthase. By looking at the abundances of these enzymes relative in the skeletal muscles at a range of different stages in the exercise training we will help elucidate the regulation of AMPK during a long-term exercise training protocol. Many of the other enzymes listed above, which also take on key roles for metabolic adaptations in muscle cells, have never been looked at immediately following a 2-h high-intensity exercise bout following an endurance training protocol.
3) **Citrate Synthase Assay**- Citrate synthase catalyzes the reaction between oxaloacetate, Acetyl-CoA and water to form citrate and can be used as a quantitative enzyme marker for the presence of mitochondria in trained muscle tissue. In this assay we will be able to confirm our rats were trained by measuring the citrate synthase activity. Citrate synthase activity in trained rats, when compared to untrained rats, will be significantly higher if in fact the rats are trained.

4) **Lactate Assay**- Formation of lactate in muscle cells is in part due to the enzyme lactate dehydrogenase (LDH) and rate of formation increases when the muscle cell cannot meet energy demands via aerobic respiration. Measuring the concentration of lactate in both muscle tissue and the blood will be able to tell us about the oxidative capacity of the tissue which we anticipate to be higher with the trained rats.

5) **Glycogen Assay**- Both liver and muscle cells contain stores of glycogen granules in their cytosol. Liver cells are unique because they carry the highest concentration of glycogen out of all of the tissues in the body and they have the ability to break down their glycogen stores into glucose molecules, which can be passed into the blood stream for use by other tissues that need them. Muscle cells do not have the ability to pass glucose directly into the blood stream, suggesting that their glycogen stores are intended as an immediate reserve source of glucose. Measuring levels of muscle glycogen concentration in our trained rats will tell us how much glycogen the trained rats are storing in muscle, and it will also help confirm
that muscle cells were utilizing the oxidative mode to metabolism later on in the exercise bouts.

6) **AMPKK Activity Assay** - One of the main objectives of this research is to capture AMPK in its activated state immediately following a 2-h high intensity exercise bout following training. Measuring AMPKK activity in this assay will be essential in confirming a possible link between the apparent reduction in the AMPK response to exercise following high-intensity endurance training. Alternatively, if in other experiments we observe an increased AMPK response to exercise immediately following an intense exercise bout, then we would also expect an increase in AMPKK activity. In addition, AMPKK activity will be measured from muscle homogenates taken at different stages of the final high-intensity exercise bout to observe the regulatory behavior of AMPKK activity during exercise.

7) **AMPK Activity Assay** - Measuring the activity of AMPK following training is the main experiment underlying the purposes of this study. Measuring p-AMPK via western blot will not be enough to know for certain just how active AMPK is following training. AMPK activity will be measured from trained muscle homogenates taken at different stages of the final high-intensity exercise bout to observe any fluctuations in AMPK activity in response to the exercise. If AMPK activation is delayed due to the physiological adaptations present in the contracting muscle, then we should observe an increase in AMPK activity in trained rats ran for the full
2 hrs on the final day of training (if at all) relative to the trained rats ran for 30 min or those trained rats that did not run at all on the final day.

METHODS

1) **Western Blot**- Samples (raw homogenate / supernatant) are mixed with 1 X loading buffer that has been heated to 50°C for appx. 5-m. The samples are then loaded into wells of an appropriate percent Tris-HCl, ready-made Criterion® gel. The proteins in the sample are then separated via SDS-PAGE for appx. 45-m at 200 V or until optimal separation is observed. Immediately after electrophoresis, gels are rinsed in Transfer Bx (4°C) and properly placed in a protein transfer sandwich (fiber paper, filter paper, gel, PVDF membrane, filter paper, fiber paper). Proteins will then be transferred to PVDF membrane in mixing temperature controlled (4°C) Transfer Bx for 1-h at 100 V. Blocking solution (1.25 non-fat dried milk / 25 ml PBST) is prepared (pH 7.6) while transferring is taking place. Immediately following transfer, membranes are stained with PONCEAU to confirm a successful transfer, washed and then allowed to incubate in blocking solution for 1 hr at room temperature. While blocking is taking place the correct primary antibody is prepared. After blocking, the membrane is washed for 30-m with PBST and PBS and then the membrane is incubated overnight with the primary antibody at 4°C. The next day the membrane is washed for 30-m with PBST and PBS and then allowed to incubate for one more hour with the correct secondary antibody with a
Horseradish Peroxidase (HRP) tag. Immediately following incubation, the membrane is washed one last time for 30-m with PBST and PBS. The membrane is then exposed for 1-m to a hydrolytic detecting solution (ECL) which will react with HRP and produce light on the membrane. Last of all, the membrane is quickly exposed to developing film in a dark room for the necessary time and the film is then developed, revealing the blot pattern. All blots will be summarized on blot reports and significance will be calculated.

2) **AMPKK Activity Assay**- The two-step process of the standard AMPKK activity assay includes the phosphorylation of AMPK and then the subsequent phosphorylation of AMARA peptide by phospho-AMPK (45, 48). In this assay we will be using the bacterially expressed heterotrimeric r-AMPK (recombinant AMPK) in the α2β2γ2 isoform as the AMPKK substrate. We will be able to measure activation of the r-AMPK substrate through the incorporation of a radioactive phosphate group (32P) from ATP into the AMARA peptide serving as the AMPK substrate (19). Tissue homogenates will be diluted 1:1 (vol/vol) in AMPK storage buffer and then 2 μl will be incubated with r-AMPK in 4 μl AMPKK assay buffer for 20-m. 15 μl of phosphorylation buffer will then be added, and the reaction will end by spotting 1-cm² pieces of pre-cut P81 filter paper (Whatman, Tewksbury, MA) with 15 ul of the final reaction mixture after 10-m. Finally, the filter papers will be washed 6 times in stirring 100 ml 1% phosphoric acid. The
phosphoric acid will be neutralized with Sodium Bicarbonate while the Whatman papers are rinsed with acetone and allowed to dry. After the papers are dry they will be placed in 3 ml of Ecolite (ICN, Irvine, CA) and then counted for 1-m on a scintillation counter.

3) **Muscle Glycogen Assay**- Frozen tissue samples will be ground to powder under liquid nitrogen with a mortar and pestle. 100 mg of ground tissue will be weighed out and incubated for 30-m in 0.5 ml cold 30% KOH with a marble cap. After 30-m the muscle mixture will be neutralized with 10.7 M acetic acid (~.55 ml). Once neutralized, 0.1 ml of mixture will be transferred to a 12 X 75 mm tube containing 0.9 ml amylloglucosidase buffer, capped and then incubated for 1-h at 55°C. Tubes are then centrifuged for 10-m prior to 0.1 ml being transferred to a cuvette containing 0.88 ml reaction buffer for an absorbance reading. Absorbance is read at an O.D. of 340 nm on a spectrophotometer. 15-m after the first readings, a final absorbance reading is measured with the addition of 0.02 ml G6PDH and HK enzyme mixture that has been mixed by inversion. From the O.D. results we will then be able to calculate μmoles glucose (from glycogen)/g tissue.

4) **Citrate Synthase Assay**- This is a comparative assay using two different amounts of the homogenate to assess linearity with the amount of enzyme. In general, a cuvette is filled with 0.60 ml 100 mM Tris (pH 8.0), 0.10 ml 3.0 mM Acetyl-CoA, 0.10 ml 1.0 mM DTNB, and 0.10 ml diluted red quadriceps homogenate. The cuvette is then covered with parafilm, mixed
by inversion several times and then placed in a thermostated (30° C) chamber of the spectrophotometer for 7-m. The change in O.D. is then recorded for 2-m at 30-s intervals at 412 mΦ. The reaction is started by adding 0.10 ml of 5 mM oxaloacetate and then mixed by inversion several times. This reaction mixture is then placed into the spectrophotometer to record the O.D. change over 3-m at 30-s intervals. Citrate synthase levels can then be calculated from the O.D. fluctuations observed on the spectrophotometer.

5) Muscle Lactate Assay- Like the muscle glycogen assay I will weigh out 100 mg muscle powder and add it to a cold homogenizing tube with 1 ml 6% PCA solution. The mixture is homogenized and centrifuged, and then 0.8 ml is added to a 12 x 75 mm tube. The resulting solution is neutralized to pH ~7 by adding approximately 0.35 ml 2 M KOH, 0.4 M KCl and 0.4 M imidazole. The solution is then centrifuged and frozen, and the supernatant is saved for analysis. The supernatant is measured for lactate by adding 0.10 ml neutralized muscle extract and 0.88 ml reaction mix. The mixture is inverted 20 times with a parafilm cap. After mixing, the solution’s initial O.D. is read. After the initial reading a final O.D. reading is taken after adding 0.01 ml LDH solution. With the initial and final O.D. readings, the µmoles lactate / g tissue can then be calculated.

6) IP AMPK Activity Assay- AMPK will be isolated by immunoprecipitation from spun cell homogenate of all rats participating in the study. G-sepharose bound to anti-AMPK-α2 will be evenly divided into
1.5 ml eppendorf tubes and allowed to incubate with spun homogenate taken from each rat overnight on a ferris wheel shaker at 4°C. This will allow for the immunoprecipitation of AMPK-α2 from the homogenate mixtures. The following day, the immunoprecipitates will be washed with ice-cold IP buffer + 1M NaCl to clear out non-specific proteins. Next, the immunoprecipitates will be evenly aliquotted in duplicate into 12 x 75 glass tubes. 25 μl working AMPK assay cocktail (SAMS peptide, AMP, ATP, DTT, and P<sup>32</sup>-ATP) is then added to the tubes and allowed to shake for 10 min at 37°C. After the reaction mixture has been allowed to shake, 15 μl from each tube will be transferred to labeled 1X1 cm Whatman P81 filter paper. The filter papers will then be rinsed 5 times in fresh 1% phosphoric acid, washed once with acetone, and allowed to dry. Dry papers are placed face up in scintillation vials containing 3 ml EcoLite scintillation cocktail (ICN Research Products Division, Costa Mesa, CA), placed in a Beckman scintillation counter, and counted for 5 min each. AMPK activity was measured based off of the counts measured per minute.

7) **Buffers**- Often DTT, Benzamidine, AEBSF and soybean trypsin inhibitor will be added to buffers just prior to use in order to inhibit protease activity.

**Muscle Homogenization Buffer:** 250 mM mannitol, 50 mM Tris-HCl, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 1% triton all at a pH of 7.4.

**4 x Loading Buffer (92% stock soln.):** 240 mM Tris-HCl, 8% SDS (sodium dodecyl sulfate), 40% Glycerol, .04% bromophenol blue all at a
pH of 6.8. Add 8% β-mercaptoethanol just prior to use to bring the buffer to 100% stock solution.

1 x Transfer Buffer (20% MeOH): 48 mM TRIS, 39 mM Glycine, .074% SDS, 20% Methanol, pH 9.0.

100 mM Tris Buffer, pH 8.0: 4.02 grams Trizma HCl + 2.97 grams Trizma Base diluted to 500 ml with distilled water. Adjust to final pH with HCl or KOH.

Amyloglucosidase buffer: (fresh daily - keep cold): Add 100 µg amyloglucosidase to 1 ml distilled water. Allow to dissolve. Add 0.1 ml from this solution to 10 ml, 50 mM acetate buffer pH, 4.7.

Phosphorylation Buffer: 40 mM Hepes, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM DTT, 5 mM MgCl₂, 0.2 mM AMP, 0.2 mM ATP, 0.33 mM AMARA peptide, 0.05 µCi/µl [γ³²]ATP, pH 7.0.

1 X Phosphate-buffered Saline (PBS): 138.6 mM NaCl, 2.71 mM KCl, 2.15 mM KH₂PO₄, 9.89 mM Na₂HPO₄, pH 7.3.

1 X PBST: Same as PBS but add 0.1% Tween20.

IP Buffer: 50 mM Tris-HCl, 150 mM NaCl, 50 mM NaF, 5mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 5 ug/ml soybean trypsin ingibitor, pH 7.4.

Hepes Buffer: 25 MM HEPES, 0.02% Brij, 1 mM DTT, pH 7.0.

Stock Solution: 100 mM HEPES, 200 mM NaCl, 20% glycerol, 2 mM EDTA, 12.5 mM MgCl₂, pH 7.0.
**Working Assay Cocktail:** 0.5 mM AMP, 2 mM DTT, 0.5 mM ATP in stock solution. 1 mM SAMS peptide and 10 mCi/ml P\textsuperscript{32}-ATP is added just before running the assay.
ESTIMATED COSTS

Western Blots-
- Developing reagents ~ $4,000.
- Antibodies net worth ~ $4,500.

AMPKK Activity Assay-
- Reagents ~ $1,000.

Muscle Glycogen Assay ~ $100.

Citrate Synthase Assay ~ $100.
- Acetyl-CoA ~ $400.

Muscle Lactate Assay ~ $100.

AMPK Activity Assay ~ $2000
- $^{32}$-ATP ~ $300
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39.) **Suwa M, Egashira T, Nakano H, Sasaki H, Kumagai S.** Metformin increases the PGC-1{alpha} protein and oxidative enzyme activities possibly


ABSTRACT

Activation of AMP-activated protein kinase (AMPK) results in the upregulation of several intracellular systems which help to prepare a cell for a high energy challenge. The magnitude of the AMPK response to a 10 min bout of exercise has been found to decrease in red quadriceps (RQ) following training, while putative AMPK roles seem to be maintained; specifically, the biogenesis of mitochondria and greater expression of hexokinase II and glucose transporter 4 (GLUT4) in the muscle fiber. If the AMPK response to exercise is responsible in part for these adaptations, how can they be maintained if the AMPK response is attenuated? The purpose of this study was to determine whether phosphorylation of AMPK in RQ increases during 2-hr training bouts after rats have trained for 8 wks. A maximized AMPK response after a 2-hr bout of exercise in endurance-trained rats could account for these adaptations. Male Sprague-Dawley rats ran up to 30 m/min up a 15% grade, 2 hr/day for 8 wks. On the final bout of exercise, trained rats ran for 0 (TRC), 30 (TR1), or 120 min (TR2) up a 15% grade at 30 m/min. Red quadriceps (RQ), soleus, and white quadriceps (WQ) were immediately collected and frozen for analysis. Citrate synthase activity increased in RQ (79 ± 3 vs. 37 ± 4 μmol/g/min) and soleus (64 ± 4 vs. 35 ± 2 μmol/g/min) but not in WQ compared to non-trained controls. In trained rats, maximal increases in T-172 phosphorylation of AMPK occurred after 30 min of exercise (relative values = 1.29 ± 0.06 vs. 1.00 ± 0.06). AMPK phosphorylation did not change significantly in trained rats that ran for 2 hrs (1.31 ± 0.09) compared to rats that ran for 30 min. Similarly, maximal increases in AMPK activity in trained rats occurred after 30 min of exercise (pmoles/min/mg = 2.67 ± .05 vs. 1.09 ± .41) and AMPK activity did not
change significantly in trained rats that ran for 2 hrs (2.79 ± .17) compared to rats that ran for 30 min. Previous studies demonstrated a 2–3 fold increase in AMPK activity in non-trained rats after 30 min of exercise at lower work rates. These results demonstrate that the AMPK response to exercise is attenuated even after two-hr bouts of exercise. This implies that the increase in mitochondrial oxidative enzymes, GLUT4, and hexokinase II may be maintained by signals other than the AMPK signaling system. The CREB signaling pathway is one such system. Western analysis of phospho-CREB (Ser133) showed a statistically significant increase in phospho-CREB content in trained rats relative to control. No change in phospho-CREB protein content was observed between TRC, TR1, and TR2 rats. Significant increases of muscle phospho-CREB content in TRC relative to untrained rats suggest that CREB remains phosphorylated in trained rats even after 24 hrs of rest. Accordingly, chronically increased phospho-CREB in muscle of trained rats relative to controls may explain in part how increased levels of mitochondria are maintained in the face of reduced AMPK response. Alternatively, the attenuated AMPK response may still be above the threshold required for inducing adaptations to endurance training.

Keywords: AMPK, GLUT4, hexokinase II, mitochondria, CREB
INTRODUCTION

The heterotrimeric, 5’-AMP-activated protein kinase (AMPK) is composed of \( \alpha, \beta, \) and \( \gamma \) subunits. Each of these three subunits takes on a specific role for both the stability and the activity of AMPK (40). The \( \gamma \) subunit plays a particularly important role during exercise because it includes four cystathione beta synthase (CBS) domains. The four CBS domains create two binding sites for AMP, commonly referred to as Bateman domains. Binding of one AMP to a Bateman domain cooperatively increases the binding affinity of the second AMP to the other Bateman domain (1). The \( \alpha, \beta, \) and \( \gamma \) subunits can be found in different isoforms: \( \alpha_1 \) or \( \alpha_2; \beta_1 \) or \( \beta_2; \) and \( \gamma_1, \gamma_2, \gamma_3. \) Although the \( \alpha_1, \beta_1, \) and \( \gamma_1 \) isoforms are the most common expressed in most cells, it has been demonstrated that the \( \alpha_2, \beta_2, \gamma_2, \) and \( \gamma_3 \) isoforms are also expressed in cardiac and skeletal muscle (7, 40, 49). The \( \alpha_2 \)-containing AMPK complex (AMPK\( \alpha_2 \)) is considered the foremost AMPK isoform responsible for the metabolic adaptations to training and exercise in contracting skeletal muscle (13, 41, 56).

The energy-sensing capability of AMPK can be attributed to its ability to detect and react to fluctuations in the AMP:ATP ratio that take place during rest and exercise (muscle contraction) (37). AMPK is sensitive to the fluctuating (increasing) AMP:ATP ratio during exercise because AMP acts as an allosteric activator of AMPK (52), while ATP inhibits AMPK activation by binding to the Bateman domains on the \( \gamma \) subunit (14). As AMP binds both Bateman domains, the \( \gamma \) subunit undergoes a conformational change that exposes the catalytic domain found on the \( \alpha \) subunit. Subsequently, this action allows AMPK to become activated in the activation loop of
the catalytic domain when phosphorylation takes place at threonine-172 by an upstream AMPK kinase (AMPKK) (16, 41, 54). Because increases in AMP concentrations in contracting skeletal muscle usually correlate with small decreases in ATP, AMPK will become activated either when energy demands needed for exercise are not met or when they are in short supply. Upon activation by AMPKK, AMPK serves as a metabolic master switch regulating several intracellular adaptations to muscle contraction.

Major responses to exercise and adaptations to endurance training effectuated by AMPK include the cellular uptake of glucose (17, 18, 25, 28), the β-oxidation of fatty acids, and the biogenesis of GLUT4 and mitochondria (4, 31, 48, 52, 53). Adaptations of skeletal tissue that take place in response to endurance training [increased muscle glycogen (21), increased PGC-1 (2, 45-47), hexokinase II (4, 21, 35), UCP-3 (42), cytochrome C (5, 6, 20), as well as increased activities of mitochondrial enzymes such as citrate synthase, succinate dehydrogenase, and malate dehydrogenase (55)] are also thought to be mediated in part by activated AMPK. Furthermore, recent discoveries suggest a direct AMPK role in increasing blood supply to exercised/trained muscle cells by stimulating and stabilizing both vasculogenesis and angiogenesis (32). Aside from upregulating these pathways that result in the generation of ATP during exercise, AMPK also acts to downregulate synthetic pathways that consume ATP (8, 9, 26, 29, 57). Together, these adaptations serve to decrease the high energy demands in muscle tissue felt during exercise and endurance training. Both the rapid/acute and chronic/long-term effects of AMPK activation most likely transpire in part as a result of both temporary and maintained
increases in AMPK activity brought about by increases in the AMP:ATP ratio during
single bouts of exercise and long-term training.

During a bout of exercise, muscle AMPK activity increases in response to
metabolic stress brought about by an extreme cellular demand for ATP. This AMPK
response to exercise has been found to notably decrease in red quadriceps (RQ)
following training, while putative AMPK roles such as increased GLUT4 and
hexokinase II expression and mitochondrial biogenesis are maintained (12). One
possible explanation for the attenuating effects of training on the AMPK response to
exercise is that perhaps the metabolic stress of trained muscle fibers during stimulation
has been reduced due to the cellular adaptations to exercise that took place during
training (increased GLUT4, blood supply, and mitochondria). Adaptations such as
these increase cellular capacity to produce ATP. Thus, the increased AMP:ATP ratio
required to maximize AMPK activation would be expected to be lower in trained rats
than in untrained rats that lack these adaptations.

It is also possible that the AMPK activity threshold needed to elicit such
changes is lower than expected or that it is decreased with training. Despite what is
actually taking place to account for the endurance-trained attenuation of the AMPK
response to exercise, it is also interesting to note that although AMPKK protein
abundance has been shown to increase in skeletal tissue with endurance training, its
level of activity has been shown to decrease with endurance training in both trained
and untrained tissue (22, 23, 44, 45). Currently, the activity of AMPKK immediately
following a 2-hr bout of exercise of an endurance-trained rat is unclear. It is possible
that there exists a direct link between the observed decrease in AMPKK activity in
endurance-trained skeletal muscle and the attenuated AMPK response to exercise with endurance training.

It has already been found that high-intensity work rates are needed for AMPK activation (13, 24, 33, 56). Metabolic adaptations in skeletal muscle brought about by endurance training may increase the work rate level of intensity needed to bring about maximized activation of AMPK. Accordingly, we hypothesized that the AMPK response to a given submaximal bout of exercise would be progressively increased during a 2-hr bout of exercise at a high work rate (12). This study investigated the effects of endurance training on the AMPK response to exercise; specifically, whether the phosphorylation and activity of AMPK in RQ increases in rats after a 2-hr training bout of exercise following an 8 wk training regimen. Results obtained from trained rats that ran for 2 hrs (TR2) were compared to results from trained rats that ran for 30 min (TR1) or not at all (TRC). Understanding patterns of AMPK activity during exercise and under conditions of training will help us to better understand its role in energy metabolism as well as the importance of exercise as a conditional treatment for metabolic diseases such as Type II diabetes.

MATERIALS AND METHODS

Animal care. All procedures were approved by the Institutional Animal Care and Use Committee of Brigham Young University. Thirty male Sprague-Dawley rats (Sasco, Wilmington, MA) were housed in a temperature-controlled (21–22°C) room that cycled daily with a 12-hr light and 12-hr dark period. Rats were kept in rodent shoebox compartments (two rats per compartment) with a metal grill top to allow for
ventilation. Rodent bedding was replaced in each shoebox two times a wk and all rats were weighed every wk to keep a log of rat growth rate. Rats were fed standard rat chow (Harlan Teklad rodent diet) and given water to drink ad libitum. Rats were given 30 g of rat chow the night before they were killed.

Muscle stimulation. 18 randomly assigned rats (n = 6/group) were run on a motor-driven rodent treadmill up a 15% incline 5 days/wk for 8-10 wks in a temperature controlled room (16–17°C). The rats were run for 35 min the first day up a 5% incline at 16 m/min. Exercise bout intensity was increased daily until the rats eventually progressed to a single 120 min bout of exercise on a 15% incline at 30 m/min. Rat training at this point was maintained for 4 more wks.

Muscle collection & homogenization. After 12 wks, blood and tissue samples (left/right red/white quadriceps, and left/right soleus) were collected from trained rats anesthetized either at rest or immediately following a 30-min or 2-hr bout of exercise by intravenous (jugular catheter) injection of pentobarbital sodium. The catheters were placed in the jugular veins of trained rats anesthetized with Isoflurane USP (liquid for inhalation) 3 days prior to being killed. Once extracted, tissues were compressed-frozen with metal tongs at liquid nitrogen temperature, wrapped immediately in labeled aluminum foil and then placed in a container filled with liquid nitrogen until they were moved to an ultra-low temperature (~90°C) freezer for storage. Untrained rats (n = 10) were anesthetized with pentobarbital sodium (50mg/kg body wt) and tissues (right red/white quadriceps and right soleus) were extracted and clamp-frozen at liquid nitrogen temperature. All tissues were ground with a ceramic mortar and pestle at liquid nitrogen temperature and then homogenized in a homogenization
buffer containing 250 mM mannitol, 50 mM Tris-HCl, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, and 1% triton, all at a pH of 7.4. 1 mM Dithiothreitol (DTT), 1 mM Benzamidine, 1µg/ml soybean trypsin inhibitor, and .5 mM AEBSF were added to the buffer just prior to homogenization. The final concentration of the tissue in the homogenization buffer solution was a 9 factor dilution (1 g muscle powder/9 ml buffer). Approximately 300 µl of raw homogenate was stored and the remainder was centrifuged at 2100 rpm for 20 min at 4°C. Post-centrifugation, supernatant from the homogenate was immediately taken from the tubes and aliquots were dispensed into 1500 µl Eppendorf tubes.

Western blots. Data obtained by comparison of protein content across all treatment groups were collected from RQ, SOL, and WQ. Western blots were performed as previously described (48). First antibody dilutions were cytochrome C, hexokinase II (1:1,000 and 1:2,000 resp.; Santa Cruz Biotechnology, Santa Cruz, CA); PGC-1 (1:1,000; Chemicon International, Temecula, CA); phospho-CREB (Ser133), phospho-ACC (Ser79), phospho-AMPK (Thr172) (1:5,000, 1:2,000, and 1:2,000 resp.; Cell Signaling Technology, Boston, MA); and UCP-3 (1:2,000; ABR Affinity BioReagents, Golden, CO). Second antibody dilutions were anti-mouse horseradish peroxidase (HRP)-linked antibody from donkey and anti-goat HRP-linked antibody from donkey (1:5,000 and 1:5,000 resp.; Santa Cruz Biotechnology, Santa Cruz, CA); and anti-rabbit HRP-linked antibody from donkey (1:5,000; Amersham Pharmacia, Piscataway, NJ). Blots were developed with ECL/ECL Plus Western blotting detection reagents (Amersham). Stripping and reprobing was carried out (when applicable) according to the manufacturer’s instructions (ECL, Amersham).
Citrate synthase assay. Aliquots from raw tissue homogenates of RQ, SOL, and WQ were slow-frozen at –20°C overnight and subjected to two additional freeze-thaw cycles. All tissue homogenates were prepared in homogenization buffer (250 mM mannitol, 50 mM Tris-HCl, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 1% triton all at a pH of 7.4). Proteolytic enzyme inhibitors were added immediately before preparation of the homogenates (1 mM Dithiothreitol (DTT), 1 mM Benzamidine, 1 μg/ml soybean trypsin inhibitor, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF)). Citrate synthase was assayed according to the method of Srere (39).

Blood glucose assay. Blood glucose (mM) was measured as previously described (3).

AMPK activity assay. All AMPK activity data were collected from RQ. AMPK was isolated by immunoprecipitation from spun (~950 x g) cell homogenate of all trained rats. G-sepharose bound to anti-AMPK-α2 incubated with muscle homogenates overnight on a vertically rotating mixer at 4°C. The following day, the immunoprecipitates were washed twice with ice-cold immunoprecipitation (IP) buffer (50 mM Tris-HCl, 150 mM NaCl, 50 mM NaF, 5mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 5 ug/ml soybean trypsin inhibitor, pH 7.4) + 1M NaCl to remove non-specific proteins and then once with IP buffer without the 1 M NaCl. Hepes buffer (25 mM HEPES, 0.02% Brij, 1 mM DTT, pH 7.0) was added to resuspend the IP pellet and prepare the immunoprecipitates for the AMPK assay. Next, the immunoprecipitates were evenly aliquotted (10 μl) in duplicate into 12 x 75 glass tubes. Working cocktail was then added to start the reaction. Final concentrations in the assay mix were 40 mM Hapes,
0.2 mM AMARA peptide (Zinsser Analytical, Maidenhead, Berkshire, UK), 0.2 mM AMP, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM DTT, 5 mM MgCl₂, 0.2 mM ATP, 2 μCi ³²P-ATP/tube, pH 7.0. After incubating for 10 min at 37°C, 15 μl from each tube was transferred to a labeled 1 x 1 cm Whatman P81 filter paper. The filter papers were then rinsed 5 times in fresh 1% phosphoric acid (5 min/wash), rinsed once with acetone, and allowed to dry. Dry papers were placed in scintillation vials containing 3 ml EcoLite scintillation cocktail (ICN Research Products Division, Costa Mesa, CA), placed in a Beckman scintillation counter, and counted for 5 min each. AMPK activity measured in pmol min⁻¹ mg⁻¹ was converted from the counts per min detected by the scintillation counter.

Statistics. Comparisons of relative protein abundances, blood glucose concentrations, or enzyme activities between treatment groups were made using one-way ANOVA. When main effects reached significance, Fisher's least significant difference multiple comparison test was used to determine the location. Student’s t-test was used to detect significant differences between trained and untrained muscle fiber protein abundances and citrate synthase activities in RQ, SOL, and WQ. For all tests, statistical significance was set at \( P < 0.05 \). Student’s t-tests were performed with Microsoft Office Excel 2003 technology (Microsoft, Redmond, Washington) while all other statistical procedures were performed with the Number Cruncher Statistical Systems program (NCSS, Kaysville, Utah). All data are reported as means ± SE.
RESULTS

Citrate synthase activity was measured to determine effectiveness of the training program. Endurance training resulted in a statistically significant increase in citrate synthase activity in both RQ and SOL but not WQ ($P < .05$) (Fig. 1A−C, $n = 10$ for untrained/control rats and $n = 18$ for trained rats).

Western blot analysis of phospho-AMPK (Thr172) in RQ revealed a statistically significant increase in trained rats that ran for 30 min (TR1) and 120 min (TR2) compared to trained/rested rats (TRC) ($P < .05$), whereas no significant change was observed between TR1 and TR2 (Fig. 2A, $n = 6$). In SOL, phospho-AMPK increased significantly ($P < .05$) between untrained/rested rats and trained rats, while no significant change was observed between TRC, TR1, and TR2 (Fig. 2B, $n = 10$ for UTR and $n = 6$ for TRC, TR1, and TR2). No significant change in phospho-AMPK protein content was found between any treatment groups in WQ (Fig. 2C, $n = 10$ for UTR and $n = 6$ for TRC, TR1, and TR2). Furthermore, an analysis of AMPK activity revealed a statistically significant increase in TR1 and TR2 compared to TRC ($P < .05$) showing that phospho-AMPK protein content correlated (to some extent) with AMPK activity (Fig. 3, $n = 6$).

Acetyl-CoA carboxylase (ACC) is a downstream target of AMPK. Since allosteric activation of AMPK cannot be detected by the AMPK activity assay, phospho-ACC was measured as an indicator of AMPK activation in the muscle. Phospho-ACC (Ser79) increased similarly to phospho-AMPK in RQ. In RQ, a statistically significant increase was found in TR1 and TR2 compared to TRC ($P < .05$), yet no significant change was observed between TR1 and TR2 (Fig. 4A, $n = 6$).
Phospho-ACC protein content was also similar to phospho-AMPK protein content in SOL. In SOL, we found a significant increase in phospho-ACC between UTR and TR ($P < .05$), while no significant change was observed between TRC, TR1, and TR2 (Fig. 4B, $n = 10$ for UTR and $n = 6$ for TRC, TR1, and TR2). No significant changes in WQ phospho-ACC protein content were observed between any of the treatment groups (Fig. 4C, $n = 10$ for UTR and $n = 6$ for TRC, TR1, and TR2).

CREB has been identified as a downstream target of many protein kinases, including AMPK. Phospho-CREB (Ser133) in RQ was significantly increased between UTR and TR ($P < .05$), but no significant change was observed between TRC, TR1, and TR2 (Fig. 5, $n = 10$ for UTR and $n = 6$ for TRC, TR1, and TR2).

Hexokinase II and cytochrome C genes have CRE in their promoter regions and serve as markers for CREB activity. In RQ and SOL, hexokinase II increased significantly ($P < .05$) in trained rats relative to untrained/rested rats (Fig. 6A, and 6, $n = 10$ for untrained/control and $n = 18$ for trained). Endurance training had no effect on hexokinase II levels in WQ relative to untrained/rested rats (Fig. 6C, $n = 10$ for untrained/control and $n = 18$ for trained). UCP-3 is a mitochondrial proton carrier and serves as a marker for mitochondrial content and oxidative capacity. UCP-3 also increased significantly ($P < .05$) in trained rats relative to untrained/rested rats in RQ (Fig. 7, $n = 10$ for untrained/control and $n = 18$ for trained).

Cytochrome C increased significantly ($P < .05$) in trained rats compared to untrained/rested rats in RQ and SOL (Fig. 8A–B, $n = 10$ for untrained/control and $n = 18$ for trained), while no change was observed in WQ (Fig. 8C, $n = 10$ for untrained/control and $n = 18$ for trained).
Measurement of blood glucose (mM) in trained rats revealed that blood glucose levels decreased significantly in TR1 and TR2 compared to TRC ($P < .05$). Beyond 30 min of treadmill exercise of trained rats, blood glucose levels appeared stable as no significant change in blood glucose was observed between TR1 and TR2 (Fig. 9, n = 6).

**DISCUSSION**

A previous study found that in RQ, the AMPK activity of endurance-trained rats decreased compared to sedentary controls in response to a 10 min bout of exercise (7). This observation suggested that, in some form or another, endurance training does have an attenuating effect on the AMPK response to exercise. We know that AMPK is activated in muscle fibers when the muscle fiber is experiencing a high energy challenge. We hypothesized that perhaps the attenuating effects that endurance training has on the AMPK response to exercise may be due to the cellular adaptations that take place during endurance training. The purpose of this study was to identify whether or not AMPK activity would increase significantly in trained rat muscle (RQ) after a 2-hr bout of exercise compared to trained rats that ran for 30 min. We found that although rats in our study ran for an additional 110 min compared to the rats ran in the previous study (12), no significant increases in AMPK activity were observed in RQ beyond the 30 min mark. Although RQ phospho-AMPK protein, and AMPK activity all increased significantly in trained/exercised rats relative to trained/rested rats, we did not observe a significant climb in AMPK activity or phospho-AMPK protein content in trained rats that ran for 2 hrs compared to those that ran for 30 mins.
This suggests that continued adaptations to training [increased hexokinase II, GLUT4, and mitochondria in rat RQ] are not due to a maximized AMPK response after 2 hrs of exercise.

Though no maximal response was observed, AMPK activity and phospho-AMPK content in our study did increase in response to the exercise. This response is notably lower than what can be observed in untrained muscle fiber in response to exercise (12). Despite these observations, we still observed marked increases in metabolic factors as well as mitochondrial activity (Fig. 1/Fig. 3a/Fig. 5a, 5c/Fig. 6a). It is likely that other signals, downstream of AMPK, are maintaining the AMPK signal to the extent that maximal AMPK activation is no longer required in endurance-trained muscle fiber. To investigate other downstream targets of AMPK that may be maintaining the AMPK signal in trained rats, we decided to investigate phospho-CREB content at the serine 133 position (phospho-CREB); the site phosphorylated by AMPK.

When activated, CREB acts as a transcription factor known to regulate the expression of several proteins involved in metabolic processes (2). It does this by binding to the cyclic AMP response element (CRE) of those genes involved in various cellular functions, including metabolism. Once bound to CRE sites, CREB can facilitate the transcription of these genes which will, in turn, yield proteins that can cause cells to differentiate, divide (30, 38, 43, 58), or adapt to cellular stresses, including those metabolic stresses that take place during exercise and endurance training.
A recent study has shown that endurance training results in increased phospho-CREB in skeletal muscle after 15 hrs of rest (36). Here, we also show that endurance training increases p-CREB and that this signal is maintained for at least 24 hrs after the most recent bout of exercise as we observed no significant change in phospho-CREB content between TRC, TR1, and TR2 rats. This did not correlate with phospho-AMPK expression as levels dropped after the 24 hr period of rest. This suggests that either AMPK is not responsible for the maintained phosphorylation of CREB at serine 133, or that the attenuated AMPK activity during rest is still above the threshold required for CREB phosphorylation. These data suggest that a long-lasting signal in the form of phospho-CREB may extend the signal for increasing training induced enzyme changes far beyond the end of each exercise bout.

It is already known that CREB expression correlates positively with PGC-1 expression, endurance training, mitochondrial respiratory capacity, exercise, and mitochondrial gene expression (2, 36, 50, 51). Our results show that CREB is phosphorylated in endurance-trained rats and that it remains phosphorylated during times of rest when AMPK is less active and during exercise when we show AMPK activity increased (see Fig. 4). Accordingly, it is possible that an extended CREB signal in endurance-trained muscle extends effects of contraction induced signals even during times of rest. Endurance-trained attenuation of the AMPK response to exercise would then be the result of less metabolic stress felt by the cell, and thus an attenuated demand for AMPK activity.

CREB also plays a part in coordinating the differentiation of several tissues, including adipose and endocrine (19, 34). Our study suggests a possible role for CREB
in which it is also differentiating muscle tissue into a phenotype consistent with cellular adaptations to endurance training when it is phosphorylated either by AMPK or by other upstream kinases such as PKA or CaMKII. Although other signaling pathways, such as CREB, may help to give an explanation for the endurance-trained attenuation of the AMPK response to exercise, we are still unclear whether these pathways are solely responsible. Other details fundamental to the activity and regulation of AMPK itself may help to give meaning to the marked increases in RQ adaptations to endurance training. Specifically, the reasons underlying AMPK attenuation in endurance-trained muscle fiber may help to account for these adaptations.

Although a 2-hr bout of treadmill running was not enough to maximize AMPK activation in endurance-trained RQ to the extent at which it is activated in untrained RQ (12), adaptations to the endurance training may have played a part in lessoning the demand for AMPK activation. Consequential to these adaptations to endurance training, muscle fiber capacity to produce ATP in this study will have increased in endurance-trained rats relative to control. It is likely that this will have resulted in an attenuation of the exercise-induced increases of the AMP:ATP ratio required for AMPK activation. Attenuation of the exercise-induced increase of the AMP:ATP ratio has already been shown in a previous study of the effects of short-term training on AMPK activation with prolonged exercise (27). That said, although AMPK activity and phospho-AMPK protein content did increase in trained RQ in response to exercise, maximal AMPK activity in RQ may only be required early in the training process prior to the onset of the increase in mitochondrial enzymes which act to increase ATP.
Accordingly, maximal AMPK activity may only be observed in muscle when adaptations to training are in maximal demand; untrained muscle exposed to high-intensity endurance training or trained muscle exposed to a training regimen with substantial increases in work rate intensity.

It is also possible that a 2-hr bout of treadmill exercise was not enough to sufficiently stress muscle fibers to the extent that maximal AMPK activation is required. Other tactics implemented on the final bout of exercise may be useful in activating AMPK in trained muscle to the same extent at which it is activated in untrained muscle (12). It has already been demonstrated that interval training regimens serve as an effective approach to AMPK activation (45). Perhaps subjecting trained rats to a high-intensity bout of interval exercise will prove enough to exhaust endurance trained RQ to the extent that maximal activation of AMPK is needed. Rats subjected to fasting may also elicit greater metabolic demands for maximal AMPK activation in trained RQ (10, 11, 15).

In summary, our results provide evidence that the continued adaptations to training in RQ, aside from the attenuated AMPK response to exercise, are not due to a delayed, yet maximal AMPK response at the end of a 2-hr bout of exercise; presumably where the metabolic demand for maximized AMPK activity would be the greatest. However, the premise that maximal AMPK activity is required in endurance-trained RQ in order to elicit such adaptations is a matter needing further investigation. To our knowledge, no investigation has been carried out to elucidate the necessary AMPK threshold required to yield endurance-trained adaptations in muscle, specifically in RQ. Nevertheless, studies have revealed that low-intensity exercise
(and presumably, relatively low AMPK activity) was sufficient to increase AMPK activity beyond the putative threshold needed to elicit adaptations to exercise such as increases in both PGC-1 mRNA and protein content in rat epitrochlearis, as well as increases in PGC-1 protein content in soleus (46, 47). Consequently, the putative threshold of AMPK activity required to elicit adaptations in skeletal muscle in response to training may be lower than expected. For that matter, an attenuated AMPK response to exercise in endurance-trained skeletal muscle may still be above the putative threshold needed for AMPK to elicit biochemical adaptations to endurance training. Alternatively, it is also possible that among the many adaptations to endurance training which take place in skeletal muscle, an increased cellular sensitivity to AMPK activation may also be prevalent. This would suggest that endurance-trained skeletal muscle would be poised to respond to AMPK activation during exercise to a greater extent than untrained skeletal muscle. Thus, an attenuated demand for maximal AMPK activation would be observed not only for the increased capacity of the muscle fiber to produce ATP, but also for an increased sensitivity to AMPK activation.
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FIGURE LEGENDS

Figure 1
Citrate synthase activity in rat RQ (A), SOL (B), and WQ (C) of control, and endurance-trained rats measured in µmol/g/min. Values are means ± SE (n = 10 for control and n = 18 for trained). *Citrate synthase values were significantly different from control value (P < 0.05).

Figure 2
Western blot analysis of the phosphorylation of AMPK as measured by relative protein quantities of phosphothreonine-172 AMPK (P-AMPK). P-AMPK content was measured either between the three trained groups (TRC, TR1, and TR2) or between all groups (UTR, TRC, TR1, and TR2). (A) Relative P-AMPK content in RQ. (B) Relative P-AMPK content in SOL. (C) Relative P-AMPK content in WQ. †Significantly different from TRC. *Significantly different from UTR (n = 6–10, P < 0.05).

Figure 3
Immunoprecipitation to determine AMP-activated protein kinase (AMPK) activity in endurance-trained rat RQ after running 0 min (TRC), 30 min (TR1), or 2 hrs (TR2) measured in pmol.min⁻¹.mg⁻¹. Values are means ± SE (n = 6). †AMPK activity was significantly greater compared to the TRC control (P < 0.05).
Figure 4
Western blot analysis of the phosphorylation of ACC by AMPK as measured by relative protein quantities of phosphoserine-79 ACC (P-ACC). P-ACC content was measured either between the three trained groups (TRC, TR1, and TR2) or between all groups (UTR, TRC, TR1, and TR2). (A) Relative P-ACC content in RQ. (B) Relative P-ACC content in SOL. (C) Relative P-ACC content in WQ. †Significantly different from TRC. *Significantly different from UTR (n = 6–10, P < 0.05).

Figure 5
Western blot analysis of the phosphorylation of CREB at the AMPK phosphorylation site (Ser133) as measured by relative protein quantities of phosphoserine-133 CREB (P-CREB). P-CREB content was measured in rat RQ all groups (UTR, TRC, TR1, and TR2). *Significantly different from UTR (n = 6–10, P < 0.05).

Figure 6
Hexokinase II protein relative abundance in rat RQ (A), SOL (B), and WQ (C) of control (UTR), and endurance-trained (TR) rats. Values are means ± SE (n = 10 for UTR and n = 18 for TR). *Hexokinase II values were significantly greater than UTR control value (P < 0.05).
Figure 7
UCP-3 protein relative abundance in rat RQ of control (UTR), and endurance-trained (TR) rats. Values are means ± SE (n = 10 for UTR/untrained and n = 18 for TR/trained). *UCP-3 value was significantly greater than UTR control value (P < 0.05).

Figure 8
Cytochrome C protein relative abundance in rat RQ (A), SOL (B), and WQ (C) of control (UTR), and endurance-trained (TR) rats. Values are means ± SE (n = 10 for UTR and n = 18 for TR). *Cytochrome C values were significantly greater than UTR control value (P < 0.05).

Figure 9
Blood glucose concentrations (mM) in TRC, TR1, and TR2 rats. †Significantly different from TRC (n = 6, P < 0.05).
Figure 1

A

[Graph showing C.S. Activity (µmol/g/min) for Control and Trained groups.]

B

[Graph showing C.S. Activity (µmol/g/min) for Control and Trained groups.]

C

[Graph showing C.S. Activity (µmol/g/min) for Control and Trained groups.]
Figure 2
Figure 3
Figure 4

A

B

C

p-ACC (Ser79)

(Relative Units)

TRC  TR1  TR2

UTR  TRC  TR1  TR2

UTR  TRC  TR1  TR2

p-ACC (Ser79)

(Relative Units)

TRC  TR1  TR2

UTR  TRC  TR1  TR2

p-ACC (Ser79)

(Relative Units)

TRC  TR1  TR2

UTR  TRC  TR1  TR2
Figure 5

![Graph showing p-CREB (Ser133) levels for different regions (UTR, TRC, TR1, TR2). The graph includes error bars and significance markers (*).](image-url)
Figure 6
Figure 7

![Graph showing UCP-3 levels in UTR and TR conditions.](image)

- UTR
- TR

**UCP-3 (Relative Units)**

- UTR
- TR

*Significance level marked with an asterisk.*
Figure 8
Figure 9

![Graph showing blood glucose levels for TRC, TR1, and TR2 conditions.](image)

- TRC
- TR1
- TR2

Blood Glucose (mM)
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ABSTRACTS & PUBLICATIONS


