Divergent cell signaling after short-term intensified endurance training in human skeletal muscle

Boubacar Benziane,1 Timothy J. Burton,2 Brendan Scanlan,3 Dana Galuska,4 Benedict J. Canny,5 Alexander V. Chibalin,1 Juleen R. Zierath,1 and Nigel K. Stepto2,3

1Section of Integrative Physiology, Department of Molecular Medicine and Surgery, Integrative Physiology, Karolinska Institute, Stockholm, Sweden; 2Centre for Aging, Rehabilitation, Exercise, and Sport, Victoria University, Footscray, Victoria; and 3Department of Physiology, Monash University, Clayton, Victoria, Australia

Submitted 9 May 2008; accepted in final form 29 September 2008


Endurance training represents one extreme in the continuum of skeletal muscle plasticity. The molecular signals elicited in response to acute and chronic exercise and the integration of multiple intracellular pathways are incompletely understood. We determined the effect of 10 days of intensified cycle training on signal transduction in nine inactive males in response to a 1-h acute bout of cycling at the same absolute workload (164 ± 9 W). Muscle biopsies were taken at rest and immediately and 3 h after the acute exercise. The metabolic signaling pathways, including AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR), demonstrated divergent regulation by exercise after training. AMPK phosphorylation increased in response to exercise (∼16-fold; P < 0.05), which was abrogated posttraining (P > 0.01). In contrast, mTOR phosphorylation increased in response to exercise (∼2-fold; P < 0.01), which was augmented posttraining (P < 0.01) in the presence of increased mTOR expression (P < 0.05). Exercise elicited divergent effects on mitogen-activated protein kinase (MAPK) pathways after training, with exercise-induced extracellular signal-regulated kinase (ERK) 1/2 phosphorylation being abolished (P < 0.01) and p38 MAPK maintained. Finally, calmodulin kinase II (CaMKII) exercise-induced phosphorylation and activity were maintained (P < 0.01), despite increased CaMKII expression (∼2-fold; P < 0.05). In conclusion, 10 days of intensified endurance training attenuated AMPK, ERK1/2, and mTOR, but not CaMKII and p38 MAPK signaling, highlighting molecular pathways important for rapid functional adaptations and maintenance in response to intensified endurance exercise and training.

adenosine 5′-monophosphate-activated protein kinase; protein kinase B; mitogen-activated protein kinases; calmodulin kinase II; mammalian target of rapamycin; glycogen

SKELETAL MUSCLE EXHIBITS remarkable malleability, as demonstrated by its ability to adapt to distinct patterns of contractile activity or exercise (7, 12, 70). Exercise represents a stimulus capable of inducing both acute and chronic adaptive responses, inducing specific changes that serve to minimize cellular stress in subsequent similar exercise sessions (14). Chronic adaptations to training are likely to represent the summation of each discrete bout of exercise, with these perturbations leading to cumulative alterations in gene and protein expression, and ultimately distinct phenotypic changes (13, 26, 70). Endurance training elicits metabolic and morphological responses promoting muscle fatigue resistance (13, 25) and includes mitochondrial biogenesis (1, 3, 29), a fast-to-slow twitch fiber phenotype transformation (70), a decreased reliance on carbohydrate-based fuels during submaximal exercise (28), and an improved whole body aerobic capacity (V̇O2max; see Ref. 52). Molecular events involved in this process include cell signaling pathway activation in response to exercise. These pathways integrate the multitude of inputs from neuromuscular activation, increased intramyocellular Ca2+, muscle force generation/tension, increased metabolic rates and endocrine/paracrine signals culminating in the appropriate changes in gene and protein expression in the hours after the exercise bout (22). The importance of understanding these molecular mechanisms driving adaptations to endurance exercise may aid in the development of strategies to prevent the development of insulin resistance, other muscle dysfunction (23), and enhance exercise performance.

A number of exercise-responsive signaling pathways have been identified in human skeletal muscle and are implicated in cellular metabolism and gene expression. Exercise/muscle contraction increases phosphorylation of stress and/or mitogen-activated protein kinases (MAPK), specifically the extracellular signal-regulated protein kinase (ERK) 1/2 (2) and p38 MAPK (61). Signal transduction via metabolic pathways, including 5′-AMP-activated protein kinase (AMPK) (20) and protein kinase B (PKB/Akt) (51), as well as Ca2++ or neuro-muscular activation-sensing pathway through Ca2++ calmodulin-dependent protein kinase II (CaMKII; see Ref. 47) is increased in response to acute exercise. Although the direct link between activation of these pathways and distinct metabolic and gene regulatory responses is only partly resolved, the adaptive response of these signaling pathways to chronic exercise training is unknown. Exercise-induced skeletal muscle signaling after training is attenuated and is a major contributing factor to muscular desensitization to endurance exercise (36, 69). Long-term endurance training (∼21 days) alters both metabolism (38) and cell signaling in human skeletal muscle (18, 46). Short-term training also leads to training-induced changes in human skeletal muscle metabolism (11, 36, 38). AMPK activity and phosphorylation is markedly attenuated after 10 days of intensified training (36); however, the effects of this training on the activation of stress and Ca2++ and
**Table 1. Subject characteristics**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pretraining</th>
<th>Posttraining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>23 ± 5</td>
<td>23 ± 5</td>
</tr>
<tr>
<td>Height, cm</td>
<td>178 ± 8</td>
<td>176 ± 8</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>79 ± 8</td>
<td>78 ± 9</td>
</tr>
<tr>
<td>(\text{VO}_{2\text{peak}}, \text{ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1})</td>
<td>44.1 ± 7.2</td>
<td>48.5 ± 5.3*</td>
</tr>
<tr>
<td>PPO, watts</td>
<td>248 ± 4</td>
<td>286 ± 31*</td>
</tr>
</tbody>
</table>

Data are means ± SE; \(n = 9\) experiments. \(\text{VO}_{2\text{peak}}\), maximal oxygen uptake; PPO, peak sustained power output. *Significantly different from pretesting \((P < 0.05)\).

**Table 2. Respiratory gas exchange and cardiovascular and venous plasma measures during the 60-min ride at \(\sim 70\%\) of \(\text{VO}_{2\text{peak}}\) before and after 10 days of endurance training**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Training Status</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{VO}_{2}, \text{l/min})</td>
<td>Pre</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>RER</td>
<td>Pre</td>
<td>1.01 ± 0.02</td>
<td>0.96 ± 0.01</td>
<td>0.94 ± 0.01</td>
<td>0.92 ± 0.01</td>
<td>0.91 ± 0.01</td>
<td>0.92 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>0.98 ± 0.01</td>
<td>0.95 ± 0.01</td>
<td>0.94 ± 0.01</td>
<td>0.92 ± 0.01</td>
<td>0.92 ± 0.02</td>
<td>0.92 ± 0.01</td>
</tr>
<tr>
<td>(\text{VE}, \text{l/min})</td>
<td>Pre</td>
<td>58 ± 3</td>
<td>59 ± 3</td>
<td>60 ± 4</td>
<td>60 ± 4</td>
<td>61 ± 4</td>
<td>62 ± 4</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>57 ± 3</td>
<td>58 ± 3</td>
<td>58 ± 4</td>
<td>56 ± 4*</td>
<td>57 ± 4*</td>
<td>57 ± 4*</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>Pre</td>
<td>156 ± 4*</td>
<td>163 ± 4*</td>
<td>166 ± 5*</td>
<td>167 ± 5*</td>
<td>168 ± 5</td>
<td>168 ± 5</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>147 ± 4*</td>
<td>150 ± 4*</td>
<td>152 ± 4*</td>
<td>150 ± 5*</td>
<td>152 ± 4*</td>
<td>154 ± 5*</td>
</tr>
<tr>
<td>Plasma lactate, mmol/l</td>
<td>Pre</td>
<td>6.7 ± 0.7</td>
<td>7.9 ± 1.1</td>
<td>7.8 ± 1.1</td>
<td>7.5 ± 1.3</td>
<td>7.3 ± 1.3</td>
<td>6.9 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>6.2 ± 0.6</td>
<td>6.7 ± 0.8*</td>
<td>6.3 ± 0.9*</td>
<td>6.2 ± 1.0*</td>
<td>6.0 ± 0.9*</td>
<td>6.1 ± 1.0*</td>
</tr>
<tr>
<td>FFA, mmol/l</td>
<td>Pre</td>
<td>0.20 ± 0.03</td>
<td>0.20 ± 0.03</td>
<td>0.23 ± 0.03</td>
<td>0.26 ± 0.04</td>
<td>0.32 ± 0.05</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>0.19 ± 0.03</td>
<td>0.19 ± 0.03</td>
<td>0.19 ± 0.03</td>
<td>0.21 ± 0.03*</td>
<td>0.27 ± 0.03*</td>
<td>0.35 ± 0.04</td>
</tr>
</tbody>
</table>

Data are means ± SE; \(n = 9\) experiments. \(\text{VO}_{2}\), volume of \(\text{O}_{2}\) consumed; RER, respiratory exchange ratio; \(\text{VE}\), ventilation; FFA, nonesterified free fatty acid. *Significantly different from the same time point pretraining, \(P < 0.05\).
weight was measured. An indwelling catheter was then inserted in the antecubital vein of one forearm for continuous blood sampling and kept patent with regular flushing of 0.9% sterile saline. After a 15-min rest period, a resting blood sample was drawn. The thigh was then prepared for the muscle biopsies, where an area on the thigh over the vastus lateralis was cleaned and sterilized. Three biopsy sites ~5 cm apart were then anaesthetized with lignocaine (5% xylocaine; AstraZeneca, North Ryde, Australia). Three incisions were made through the skin and muscle fascia. The first muscle biopsy (150–250 mg) was taken at rest from the most distal incision using the percutaneous needle biopsy technique with suction applied (17). Following the initial biopsy, subjects rode at 72 ± 1% \( V_{\text{O}_2\text{peak}} \) (164 ± 9 W) for 60 min. During the ride, expiratory gas samples were collected for the 5 min preceding each blood sample. Blood samples were taken every 10 min during exercise. A second muscle biopsy was taken immediately (<20 s) after the completion of the 1-h ride. The subjects were then rested in a supine position and instructed to keep as still as possible for 3 after which the final biopsy was taken. Drinking water (8 ml/kg) was provided to subjects to consume over the duration of the trial, and any additional water requested was recorded and repeated for the posttraining trial. Muscle biopsies were immediately frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until analysis. Blood samples were spun at 2,500 \( g \), and plasma was collected and stored at \(-30^\circ\text{C}\) until analysis.

**Training Protocol**

Training consisted of 10 days of endurance training on the Lode cycle ergometer, including 4 days of high-intensity interval training as described (36). Each subject performed the same relative level of exercise. Subjects rode at ~75% of their \( V_{\text{O}_2\text{peak}} \) for 45 min on days 1, 5, 6, and 10, for 60 min on day 3, and 90 min on day 8. High-intensity training took place on days 2, 4, 7, and 9, consisting of 6 × 5-min intervals at ~90–100% of subjects \( V_{\text{O}_2\text{peak}} \) with 2 min recovery at or below 40% \( V_{\text{O}_2\text{peak}} \) between exercise bouts.

---

Fig. 1. Changes in muscle glycogen content in response to exercise and training. Glycogen content at rest (0) immediately after 60 min of cycle exercise (164 ± 9 W; 60) and 3 h of recovery (240) pre (filled bars) and post (open bars) 10 days of training. The symbol \( \Delta \) represents the mean ± SE change in muscle glycogen content immediately postexercise. Data are means ± SE for \( n = 7 \) subjects. \( P < 0.05 \), significantly different from pre-0 (*), significantly different from post-0 (f), and significant training \( \times \) time interaction (†). dw, Dry weight.

---

Fig. 2. Changes in protein expression and signaling in the AMP-activated protein kinase (AMPK) pathway in response to exercise and training. Changes in AMPK (A) and acetyl-CoA carboxylase (ACC; C) phosphorylation at rest (0), immediately postexercise (60), and after 3 h of recovery (240) pre (filled bars) and post (open bars)-training in response to the same exercise bout (60; 164 ± 9 W). Representative immunoblots for phosphorylated AMPK (p-AMPK) and ACC (p-ACC) and loading control [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] are included. Protein expression of AMPK (B) and ACC (D) pre (filled bar)- and post (open bar)- is unaltered. Data are means ± SE for \( n = 8 \). *Significantly different from all other time points, \( P < 0.05 \). **Significantly different from all other time points, \( P < 0.01 \). †Significant training \( \times \) time interaction \( P < 0.01 \).
Analytical Techniques

Calculation of whole body substrate utilization. Whole body rates of carbohydrate and fat oxidation (g/min) were calculated from \( \dot{V}CO_2 \) and \( \dot{V}O_2 \) using the nonprotein RER (45). The grams per minute substrate oxidation rates were then converted to micromoles per kilogram per minute rates as previously described (57).

Blood analysis. Plasma was analyzed for glucose and lactate concentrations using the Synchron CX5 analyzer (Coulter, Fullerton, CA). Plasma free fatty acid (FFA) concentration was determined using a nonesterified FFA enzymatic colorimetric method, modified to a 96-well plate format (NEFAC code 279-75409; Wako, Tokyo, Japan).

Muscle tissue. Frozen muscle (50 mg) was freeze-dried and dissected free of blood and connective tissue. Muscle glycogen content was determined by standard fluorometric techniques (43). Skeletal muscle protein was extracted in ice-cold buffer (20 mM Tris, pH 7.8, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 0.5 mM Na₂VO₄, 1% Triton X-100, 10% glycerol, 10 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM dithiothreitol, 5 mM Na₃PO₄, 1 mM benzamidine, 1 µM/ml aprotinin, 1 µM/ml leupeptin, and 1 µM microcystin). All reagents were analytical grade (Sigma, St. Louis, MO), unless otherwise specified. Homogenates were rotated for 60 min at 4°C and centrifuged at 12,000 g for 10 min at 4°C, and protein concentration of the resulting supernatant was determined using a commercial kit (Bio-Rad, Richmond, CA).

Immunoblot analysis. Aliquots of muscle lysate were mixed with Laemmli sample buffer, and 40 µg of total protein/sample were separated by SDS-PAGE using gradient (5–20%) gels. The glucose transporters (GLUT) 1 and 4 were separated on 10% SDS-PAGE gels. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked in 10% nonfat milk for 2 h at room temperature, washed with TBST for 10 min, and then incubated with the appropriate primary antibody overnight at 4°C.

To determine the protein expression and phosphorylation, membranes were incubated with antibodies for 160-kDa Akt substrate (AS160; Abcam ab24469), phospho (Ser/Thr)-Akt substrate (PAS; Cell Signaling no. 9611), AMPKα (Cell Signaling no. 2532), phospho-AMPKα Thr172 (Cell Signaling no. 2531), acetyl-CoA carboxylase (ACC) and phospho-ACC Ser79 (Upstate no. 07-439 and Cell Signaling no. 3661), Akt (Cell Signaling no. 9272), phospho-Akt Ser473 (Cell Signaling no. 9271), phospho-Akt Thr389 (S6K1; Cell Signaling Technologies nos. 2317, 2211, and 4838), and phospho-S6 ribosomal protein kinase 1 Thr389 (S6K1; Cell Signaling Technologies no. 9101), mTOR and phospho-mTOR Ser2448 (Cell Signaling no. 2972 and 2971), p38 MAPK (Cell Signaling no. 9212), phospho-p38 MAPK Thr180/Tyr182 (Cell Signaling no. 9211), AMPKα/β Ser172 (Cell Signaling no. 9331), pan-ERK (BD Transduction no. 612641), phospho-p44/p42 MAPK Thr202/Tyr204 (ERK1/2; Cell Signaling no. 9351), mTOR and phospho-mTOR Ser2448 (Cell Signaling no. 2972 and 2971), p38 MAPK (Cell Signaling no. 9212), phospho-p38 MAPK Thr180/Tyr182 (Cell Signaling no. 9211), phospholamban (PLB) and phospho-PLB Thr17 (Cyclacel, UK; 010-14; Santa Cruz no. sc 17024-2), S6 ribosomal protein and phospho-S6 Ser235/236 and Ser42 (Cell Signaling Technologies nos. 2317, 2211, and 4835), and phospho-S5 ribosomal protein kinase 1 Thr389 (6SK1; Cell Signaling Technologies no. 9234). All membranes were normalized for loading with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz no. sc 25778). Following incubation with the primary antibodies, membranes were washed with TBST and incubated with the appropriate secondary antibody for 1 h at room temperature, followed by washing in TBST. Immunoreactive proteins were detected using enhanced chemiluminescence (ECL) reagents (Amersham, Arlington, IL) and quantified by densitometric scanning. Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse immunoglobulin G were from Bio-Rad Laboratories (Richmond, CA).

Statistical Analysis

Data are presented as means ± SE for nine subjects unless otherwise stated. Statistical analysis of plasma glucose, lactate, and FFA concentrations, as well as changes in protein phosphorylation, were calculated using a nonesterified FFA enzymatic colorimetric method, modified to a 96-well plate format (NEFAC code 279-75409; Wako, Tokyo, Japan).

AJP-Endocrinol Metab • VOL 295 • DECEMBER 2008 • www.ajpendo.org
were conducted using repeated-measures ANOVA (training × time). Correlations were conducted using the Pearson correlation. Analysis was conducted using the statistical package SPSS 15.0 for Windows (SPSS). The results from the ANOVA were considered significant when \( P < 0.05 \). A Fisher’s Least Significant Difference post hoc test was conducted to determine where specific differences occurred between results, with significance taken when \( P < 0.05 \). V˙O₂peak, PPO, and protein expression data were compared with a paired \( t \)-test, with significant differences taken as \( P < 0.05 \).

RESULTS

Physiological Responses to Exercise

The intensified training protocol resulted in a number of physiological or systemic adaptations, including changes in aerobic exercise capacity, where whole body V˙O₂peak increased by 10% \( (P < 0.05; \text{Table 1}) \), corresponding to a 15% increase in PPO \( (P < 0.05; \text{Table 1}) \). Furthermore, 10 days intensified training also altered the physiological responses to submaximal exercise at the same absolute work rate \( (164 \pm 9 \text{ W}) \). The participants exercised at \( 71 \pm 4 \) or \( 64 \pm 3 \% \text{ of pre- and posttraining V˙O₂peak}, \text{respectively} \). This was accompanied by reductions in mean heart rate and V˙E during the 60-min bout of cycling, decreasing from \( 164 \pm 4 \) to \( 151 \pm 4 \) beats/min and \( 60 \pm 4 \) to \( 57 \pm 4 \) l/min, respectively \( (P < 0.05; \text{Table 2}) \). RER \( (0.94 \pm 0.01) \) and V˙O₂ \( (2.4 \pm 0.1 \text{ l/min}) \) were unchanged during exercise before and after training. Mean whole body substrate utilization rates during exercise demonstrated a shift to fat oxidation, where the mean rate of fat oxidation increased from \( 9.8 \pm 2.2 \) to \( 38.1 \pm 1.9 \mu \text{mol·kg}^{-1}·\text{min}^{-1} \) \( (P < 0.01) \) after 10 days of training. A concomitant decrease in carbohydrate oxidation rate was not evident \( (188 \pm 8 \text{ vs. } 184 \pm 7 \mu \text{mol·kg}^{-1}·\text{min}^{-1}) \).
Blood glucose, lactate, and FFA were analyzed during the submaximal exercise trials. Blood glucose concentration was maintained at 5.3 ± 0.1 and 5.1 ± 0.1 mM ($P < 0.05$) during the pre- and posttraining exercise bouts, respectively. Blood lactate concentration increased rapidly at the onset of exercise and was maintained at 7.4 ± 0.2 mM in the pretraining bout and 6.3 ± 0.1 mM postraining ($P < 0.05$; Table 2). Plasma FFA concentration decreased markedly after the onset of exercise, reaching a nadir of 0.20 ± 0.01 mM at 10 min (Table 2). FFA plasma concentrations started to return to resting levels after 30 min of exercise and occurred more rapidly in the untrained state (Table 2; $P < 0.05$). Resting FFA in the postrained state was increased (0.33 ± 0.01 vs. 0.48 ± 0.01 mM; $P < 0.05$).

**Skeletal Muscle Metabolic and Signaling Responses to Submaximal Exercise**

Muscle glycogen content decreased in response to exercise and remained low during the 180 min of recovery ($P < 0.05$; Fig. 1). Resting muscle glycogen content increased after training from 600 ± 63 to 970 ± 54 mmol/kg dry wt ($P < 0.05$; Fig. 2). Glycogen utilization during pre- and postraining exercise bouts amount to 370 ± 64 and 320 ± 63 mmol/kg dry wt, respectively. Glycogen content after exercise postraining was the same as the pretraining resting muscle glycogen content (∼630 mmol/kg dry wt; Fig. 2).

During the pretraining exercise bout, AMPK α-subunit Thr172 phosphorylation was increased ∼15-fold ($P < 0.05$;
Fig. 2A) compared with rest and recovery. In the posttraining state, exercise increased AMPK α-subunit Thr172 phosphorylation approximately fourfold compared with rest and recovery (Fig. 3A). Similarly, exercise increased ACC phosphorylation ~14-fold in the pretrained state and 5-fold in the posttraining state (Pearson r = 0.73; P < 0.001; Fig. 2C; P < 0.01). AMPK and ACC expression was unaltered by training (Fig. 2, B and D).

AS160 phosphorylation was affected by both training and exercise (training status × time interaction; P < 0.05; Fig. 3A). Pretraining AS160 phosphorylation was not altered by the exercise bout but increased significantly after 3 h of recovery (Fig. 3B). After the 10-day training regimen, AS160 phosphorylation was unchanged in response to the exercise bout or 3 h of recovery (Fig. 3A). AS160 protein expression was increased 37 ± 7% after training (P = 0.06; Fig. 3B). GLUT4 protein was increased 64 ± 6% in response to the training regimen (P < 0.01; Fig. 3C). Conversely, GLUT1 was undetected in skeletal muscle, which is in agreement with previous investigators (21).

Akt/PKB phosphorylation was unaltered in response to exercise or training (Fig. 4, A and B). Akt phosphorylation on Thr308 (P = 0.12; Fig. 4A) and Ser473 (P = 0.16; Fig. 4B) tended to be reduced after training. Akt protein expression was unaltered between pre- and posttraining conditions (data not shown). GSK3α phosphorylation was unaffected by exercise either before or after the 10 days of training (Fig. 4C). GSK3β phosphorylation was unaffected by exercise before training (Fig. 4E). After 10 days of training, GSK3β phosphorylation was more responsive to exercise and recovery (training effect, P = 0.16; Fig. 4E), where exercise increased phospho-GSK3β 22%, which, following 3 h of recovery, drops to 30% of the resting levels (P < 0.05; Fig. 4E). The expression of GSK3α and β was unchanged (Fig. 4, D and F).

Phosphorylation of mTOR at Ser2448 was increased (121%) in response to the cycling exercise bout before the training regimen, but overall this phosphorylation for all time points was less than that during posttraining exercise (P < 0.05; Fig. 5A). Despite the total increase in mTOR phosphorylation (63%), posttraining exercise induced a further 58% increase in phosphorylation (P < 0.01; Fig. 5A). mTOR expression increased 166% in response to training (P < 0.01; Fig. 5B). Acute exercise increased S6K1 phosphorylated in response to exercise pretraining, and this response was attenuated in response to training such that after 3 h, or at recovery, S6K1 phosphorylation was reduced compared with all time points (P < 0.05; Fig. 5C). Cycle exercise pretraining increased phosphorylation of the ribosomal protein S6 on Ser240/244, which returns to baseline levels after recovery (P < 0.01; Fig. 5D). After 10 days of training, S6 Ser240/244 phosphorylation was substantially reduced (training, time, and training × time, P < 0.01; Fig. 5D). S6 Ser235/236 phosphorylation was reduced after 10 days of training (P < 0.01; Fig. 5E). S6 Ser235/236 phosphorylation correlated with ERK1/2 phosphorylation (Pearson’s r = 0.66; P < 0.001), but not S6K1 phosphorylation. Training was without effect on S6 protein expression (Fig. 5F).

Fig. 6. Changes in activation of the stress-activated pathways in response to exercise and training. Exercise induced changes to mitogenic activated protein kinase (MAPK) phosphorylation of p38 MAPK (A) and extracellular signal-regulated kinase (ERK) 1/2 (C) at rest (0), in response to the same 60-min cycling exercise bout (60; 164 ± 9 W), and after 3 h of recovery (240) pre (filled bars)- and post (open bars)-training. Representative immunoblots are included above each graph, also depicting the time course of these changes. Total protein expression changes of p38 MAPK and ERK1/2 are depicted in the representative immunoblots and in B and D. Data are means ± SE for n = 8. *Significantly different from all other time points, P < 0.05. †Significant time effect, P < 0.05. ‡Significant training × time interaction, P < 0.01.
Exercise increased phosphorylation of p38 MAPK both before and after training ($P < 0.05$ for the time effect; Fig. 6A). Phosphorylation of ERK1/2 MAPK was increased after exercise in the pretraining state, with its activation drastically reduced in response to the exercise posttrained state ($P < 0.01$; Fig. 6C). Protein expression of ERK1/2 MAPK and p38 MAPK were unchanged after 10 days of training (Fig. 6, B and D).

Exercise-induced phosphorylation of Thr$^{286}$ of total CaMKII was increased by 66% pretraining ($P < 0.05$; Fig. 7A). Similarly, CaMKII had a 44% increase in phosphorylation in response to posttraining exercise ($P < 0.05$; Fig. 7A). Protein expression of CaMKII was increased 48 ± 6% after 10 days of intensified training ($P < 0.05$; Fig. 7B). PLB, a substrate of CaMKII, demonstrated a similar phosphorylation pattern in response to exercise pre- and posttraining (Pearson $r = 0.83$; $P < 0.01$), which increased 135 ± 7 and 125 ± 8% immediately after exercise ($P < 0.05$; Fig. 7C), with no changes in PLB expression (Fig. 7D).

**DISCUSSION**

Here we provide evidence that endurance exercise leads to divergent signaling responses pre- and postintensified endurance training in the AMPK, ERK1/2 MAPK, and mTOR pathways. However, the exercise-induced signaling in CaMKII and p38 MAPK pathways is unaffected by short-term training. These signaling changes occurred in concert with attenuated whole body and muscle metabolite responses to exercise.

AMPK is a metabolic master switch that, when activated, switches off energy-consuming pathways and activates energy-producing pathways (65). AMPK activity and phosphorylation of Thr$^{172}$ on the $\alpha$-subunit isoform is increased in response to exercise intensity (20, 56) and attenuated by training (18, 36) in human skeletal muscle. We and others (36) report the AMPK $\alpha$-subunit is phosphorylated in response to exercise, and markedly attenuated posttraining. The reduced AMPK signaling may be due to the increased glycogen content posttraining (55, 66), but this should be viewed with caution, since identical signal attenuation has been reported in subjects with low starting glycogen levels posttraining (36). Collectively, AMPK signaling regulates gene expression (55) and RNA translation (32), a key response in early training adaptations to endurance training.

The Akt substrate AS160/TBC1D4 plays a role in GLUT4 translocation and glucose transport (10). Whereas the PAS antibody recognizes molecules phosphorylated by Akt and AMPK (14–16, 58), our methods to detect phospho-AS160 have been validated (14–16). Exercise and training interacted...
to alter AS160 phosphorylation only after 3 h of recovery pretraining. AS160 protein expression posttraining tended to increase (37%), which may account for the reduction in phosphorylation posttraining, as demonstrated earlier (19). The marked elevation in AS160 phosphorylation 3 h after the pretraining exercise bout potentially disassociates AS160 phosphorylation from Akt (30, 53) and AMPK (59). This may be physiologically relevant for GLUT4 translocation and glucose uptake, especially in the pretraining exercise bout when muscle glycogen content is low (~230 mmol/kg dry wt). Nevertheless, increased GLUT4 protein content may also account for the exercise training-induced improvement in glucose uptake (31, 41).

Akt is a Ser/Thr kinase that plays a pivotal role in glucose uptake (AS160), glycogen synthesis (GSK3α), and protein synthesis (mTOR, GSK3β; see Refs. 4, 9, and 39). Akt phosphorylation was unaltered in response to exercise either pre- or posttraining, despite activation of numerous of its substrates, as previously shown by some (35, 61, 67), but not all (14, 15, 51, 63, 64), workers. These discrepancies may arise from the different exercise protocols, dietary controls, and/or overall fitness levels of the subjects studied. Our data provide evidence that Akt may still be important in muscle adaptation to endurance exercise, since there were strong trends for lower overall phosphorylation at Ser473 and Thr308 posttraining. Further evidence for the importance of Akt signaling in endurance exercise adaptation is provided by the change in GSK3β sensitivity, which regulates RNA translation via eukaryotic initiation factor 2B (60). GSK3α is implicated in glycogen synthase regulation, but neither exercise nor training altered phosphorylation. Although this was unexpected, glycogen synthesis can be promoted without increases in GSK3α phosphorylation (8). Furthermore, over the duration of both pre- and postexercise trials, glycogen resynthesis was not altered.

Protein complexes involving mTOR sense diverse signals and elicit multiple responses, including mRNA translation, nutrient metabolism, cell growth, and ribosomal biogenesis (13, 54), as seen in muscle hypertrophy (4, 14). mTOR is regulated by many pathways, including Akt, AMPK, and p38 MAPK (5, 6, 16, 32, 33). We found that mTOR Ser2448 was phosphorylated immediately after exercise, consistent with some (35, 42, 63), but not all (14, 40), studies. The ERK1/2 pathway, which is sensitive to exercise intensity (62), has been proposed to play a role in the formation of slow muscle fibers (27) and in the general stress response (14). Our data show that, posttraining, the exercise-induced ERK1/2 activation is almost abolished, which occurred in conjunction with a drop in relative exercise intensity. With respect to the p38 MAPK pathway, our data showing no attenuation of phospho-p38 MAPK phosphorylation (68), which may partly explain the exercise-induced p38 MAPK phosphorylation. Our results suggest that the maintained activation of p38 MAPK in the posttraining exercise period plays a role in the maintenance of muscle adaptations.

CaMKII has been identified as the main multifunctional Ca²⁺-calmodulin dependent kinase expressed in human skeletal muscle (47, 48) and is implicated in contraction- and/or metabolic regulation (8). Activation of CaMKII is divergent from phospho-mTOR, indicating that downregulation of the mTOR-raptor signaling after training may have been masked by increased mTOR expression. S6, a downstream target of S6K1, demonstrated similar exercise-induced attenuation posttraining at Ser406/441 and Ser235/236. However, the regulation of S6 occurs via S6K1 at Ser240/244 and ERK1/2/p90RSK at Ser235/236 (44, 50). Our data highlight the mTOR pathway in the regulation of muscle function and adaptation in endurance exercise and training.

Different modes of exercise/contraction, mechanical stress, and training status increase MAPK signaling (14, 61, 68, 69). We found an exercise- and training-specific response in ERK1/2, but not p38, phosphorylation in accordance with some (68, 69), but not all (14, 40), studies. The ERK1/2 pathway, which is sensitive to exercise intensity (62), has been proposed to play a role in the formation of slow muscle fibers and in the general stress response (14). Our data show that, posttraining, the exercise-induced ERK1/2 activation is almost abolished, which occurred in conjunction with a drop in relative exercise intensity. With respect to the p38 MAPK pathway, our data showing no attenuation of phospho-p38 MAPK phosphorylation after training were unexpected. High mechanical stress induces p38 MAPK phosphorylation (68), which may partly explain the exercise-induced p38 MAPK phosphorylation. Our results suggest that the maintained activation of p38 MAPK in the posttraining exercise period plays a role in the maintenance of muscle adaptations.

CaMKII has been identified as the main multifunctional Ca²⁺-calmodulin dependent kinase expressed in human skeletal muscle (47, 48) and is implicated in contraction- and/or
exercise-mediated changes in carbohydrate metabolism, gene transcription, protein synthesis, and ion homeostasis (46–49). Our data demonstrate that, in the pre- and posttraining state, CaMKII is transiently phosphorylated on Thr287 in response to exercise and returns to resting levels after 3 h of recovery, which has not been demonstrated previously. Despite the identical phosphorylation patterns of CaMKII, training induced an ~50% increase in total CaMKII expression, consistent with an earlier report after 3 wk of single leg training (46). We assessed phosphorylation of CaMKII substrate PLB to determine the in vivo kinase activity (48) and show PLB phosphorylation directly correlates with phospho-CaMKII. CaMKs play an important role in ion homeostasis during excitation-contraction coupling (37, 51), but mechanistic data linking Ca2+ and CaMKII to gene expression are elusive, despite the coupling between CaMKII activity/phosphorylation with activation of serum response factor, class II histone deacetylases, and calcineurin-nuclear factor activated T cells (34, 48). Our data highlight that Ca2+ signaling during endurance exercise via CAMKII may play a role in the maintenance training adaptations, such as the transformation of fast to slow muscle fiber types in response to chronic endurance training (3, 70).

Here we report key signaling pathways are responsive to acute exercise and short-term intensified endurance training (Fig. 8). We highlight a potential role for the mTOR-protein kinase expressions as well as the convergence of metabolic and mi- togenic/gene regulatory signals along this pathway. The general and exercise-specific stress signals communicated via these complex and interconnected pathways ultimately influence gene and protein expression, which may account for short- and long-term phenotypic changes in human skeletal muscle in response to endurance exercise training. In conclusion, 10 days of intensified cycling training induced whole body physiological changes and skeletal muscle adaptations to exercise, which were accompanied by divergent desensitization of key kinases in metabolic, stress, and Ca2+-sensitive signaling pathways implicated in skeletal muscle adaptation to endurance exercise training. Phosphorylation and in vivo activity of AMPK, ERK1/2, and mTOR was reduced or even abolished in response to exercise posttraining, whereas exercise induced signaling via CaMKII and p38 MAPK posttraining. Taken together, these data highlight the signaling pathways that are sensitive to relative exercise intensity (general stress; AMPK, ERK1/2, and mTOR) and absolute exercise intensity (exercise-specific stress; CaMKII and p38 MAPK), providing molecular mechanisms for the rapid functional adaptation and maintenance of training-induced responses.

ACKNOWLEDGMENTS

We acknowledge Atul Deshmukh and Therese Griersmith for assistance in this study.

GRANTS

The Ian Potter Foundation, the European Foundation for the Study of Diabetes, Swedish Research Council, Swedish Diabetes Association, Strategic Research Foundation (INGVAR II), Knut and Alice Wallenberg Foundation, Novo Nordisk Research Foundation, and Commission of the European Communities (Contract No. LSHM-CT-2004-005272 EXGENESIS and Contract No. LSHM-CT-2004-512013 EUGENE2) supported this research.

REFERENCES


