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Selective activation of AMPK-PGC-1α or PKB-TSC2-mTOR signaling can explain specific adaptive responses to endurance or resistance training-like electrical muscle stimulation

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ABSTRACT

Endurance training induces a partial fast-to-slow muscle phenotype transformation and mitochondrial biogenesis but no growth. In contrast, resistance training mainly stimulates muscle protein synthesis resulting in hypertrophy. The aim of this study was to identify signaling events that may mediate the specific adaptations to these types of exercise. Isolated rat muscles were electrically stimulated with either high frequency (HFS; 6×10 repetitions of 3 s-bursts at 100 Hz to mimic resistance training) or low frequency (LFS; 3 h at 10 Hz to mimic endurance training). HFS significantly increased myofibrillar and sarcoplasmic protein synthesis 3 h after stimulation 5.3- and 2.7-fold, respectively. LFS had no significant effect on protein synthesis 3 h after stimulation but increased UCP3 mRNA 11.7-fold, whereas HFS had no significant effect on UCP3 mRNA. Only LFS increased AMPK phosphorylation significantly at Thr172 by ~2-fold and increased PGC-1α protein to 1.3 times of control. LFS had no effect on PKB phosphorylation but reduced TSC2 phosphorylation at Thr1462 and deactivated translational regulators. In contrast, HFS acutely increased phosphorylation of PKB at Ser473 5.3-fold and the phosphorylation of TSC2, mTOR, GSK-3β at PKB-sensitive sites. HFS also caused a prolonged activation of the translational regulators p70 S6k, 4E-BP1, eIF-2B, and eEF2. These data suggest that a specific signaling response to LFS is a specific activation of the AMPK-PGC-1α signaling pathway which may explain some endurance training adaptations. HFS selectively activates the PKB-TSC2-mTOR cascade causing a prolonged activation of translational regulators, which is consistent with increased protein synthesis and muscle growth. We term this behavior the "AMPK-PKB switch." We hypothesize that the AMPK-PKB switch is a mechanism that partially mediates specific adaptations to endurance and resistance training, respectively.

Key words: PKB (Akt) • signal transduction • skeletal muscle

"Why do endurance and resistance training cause different adaptations in skeletal muscle?" is a major question in exercise physiology. Endurance training and chronic electrical low-frequency stimulation promote mitochondrial biogenesis and a fast-to-slow muscle fiber phenotype transformation as can be seen in marathon runners. However, endurance training does not stimulate muscle hypertrophy and muscle weight decreases during chronic electrical low-frequency stimulation (1, 2). In contrast, resistance training and some forms of intermittent, high-intensity electrical stimulation have only small effects on fiber phenotype (3) but promote substantial hypertrophy due to the stimulation of protein synthesis (4–6). The muscles of body builders and weight lifters are examples for the specific adaptation to this type of exercise. The difference between resistance and endurance training adaptations suggests that different forms of contractile activity can induce different types of signaling responses. We are intrigued by this problem because the gross stimulus, skeletal muscle contraction, and the set of signals associated with it (i.e., increased tension, $[Ca^{2+}]$, energy turnover, nutrient usage) are similar whereas the adaptations are clearly not. An explanation could be that critical signals activate signal transduction pathways nonlinearly. To explain, high signal intensity or long signal duration might over proportionally activate a signal transduction pathway promoting growth or mitochondrial biogenesis, whereas low intensities or short durations have hardly any effect. Dolmetsch et al. were the first to demonstrate that signal transduction pathways could be selectively activated by different intensities of one signal. The authors found that a large, transient increase in $[Ca^{2+}]_i$ stimulated NF-κB and JNK whereas a low, sustained $[Ca^{2+}]_i$ rise activated NFAT in lymphocytes (7).

The major aim of the present study was to identify the specific signaling events that are induced by endurance training-like and resistance training-like electrical stimulation and that might explain the specific adaptations to both forms of exercise and stimulation patterns mimicking these. We applied 3 h of 10 Hz electrical stimulation to mimic endurance training and an intermittent 100 Hz protocol to mimic resistance training, because such stimulation patterns were shown to induce the known endurance and resistance training-like adaptations at least in fast skeletal muscles in vivo (4). We applied these stimulation protocols to "fast" (extensor digitorum longus, EDL) and "slow" (soleus) isolated rat muscles (8) in order to see whether the response depends on the muscle phenotype. We then carried out Western blot experiments measuring the phosphorylation state of major activity-related sites on AMP kinase (AMPK), protein kinase B (PKB; AKT), tuberin (TSC2), mammalian target of rapamycin (mTOR), p70 ribosomal S6 kinase (p70 S6k), 4E binding protein 1 (4E-BP1), glycogen synthase kinase 3β (GSK-3β), eukaryotic initiation factor 2B (eIF2B), eukaryotic elongation factor 2 (eEF2), extracellular signal regulated kinase 1/2 (ERK1/2), p38 MAPK (p38), and c-JUN-N-terminal kinase (JNK), and we also measured the concentration of the transcriptional cofactor peroxisome proliferator activated receptor gamma coactivator-1α (PGC-1α). The main findings of our study are that endurance training-like stimulation of muscle selectively activates AMPK-PGC-1α signaling and suppresses TSC2 and downstream regulators of translation initiation and elongation. In contrast, resistance training-like stimulation even of a fasted muscle induced a pronounced activation of PKB-TSC2-mTOR and of its downstream translational regulators.

MATERIALS AND METHODS

Animals

Male Wistar rats were maintained on a constant 12:12 h light-dark cycle. Animals were between 8 and 10 wk old and weighed 220 ± 12 g. Food and water were available ad libitum.

Muscle stimulation

The rats were killed by a blow to the head followed by cervical dislocation. EDL or soleus muscles were dissected and placed in Krebs Henseleit buffer (118.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$, 3.4 mM $CaCl_2$, 35 mM Mannitol, 5 mM glucose, 1 g/l^{-1} bovine serum albumin) perfused with 95% O_2 and 5% CO_2. Muscles were incubated at 25°C to aid tissue oxygenation (9). Pilot tests showed that EDL and soleus twitch tension rates did not change when left resting in the organ bath up to 12 h. Maximal contraction voltage was determined in preliminary experiments using a Grass isometric force transducer, Grass stimulator, and student Harvard chart recorder. Stimulation began 30 min after insertion of the muscle. The high-frequency stimulation (HFS) model was chosen based on its efficacy in inducing skeletal muscle hypertrophy while the low-frequency stimulation (LFS) model has been shown to be effective in inducing endurance-like adaptations when applied 5 days per week for 3 wk in rats (4). LFS was performed using isometric contractions at 50 V and 10 Hz with a 90 ms delay and 10 ms duration, continuously for 3 h. HFS was performed with isometric contractions involving 10 sets of six repetitions at 50 V and 100 Hz with a 7 ms delay on contractions. Each repetition was 3 s in length, and there was a 10 s recovery between repetitions and a 1 min rest period between sets. EDL and soleus muscles were either control (incubated in a separate organ bath for the same time period as their stimulated equivalent) or LFS and HFS at 0 h poststimulation or at 3 h poststimulation. At the end of the experiment, samples were quickly frozen in liquid nitrogen and stored at –80°C before processing.

Myofibrillar and sarcoplasmic fractional protein synthesis rate

To verify that HFS was capable of increasing protein synthesis relative to control and LFS, the fractional protein synthesis rate was measured in six control EDL, six EDL 3 h after LFS, and six EDL 3 h after HFS. The incubation media contained amino acids at fasting physiological levels (10), and protein synthesis was measured using a flooding dose of ^{13}C-labeled proline (20 atoms percent) over 15 min (11) . The labeling of proline in the incubation medium measured by gas chromatography-mass spectrometry and the incorporation of proline into myofibrillar and sarcoplasmic fractions was carried out as described in detail elsewhere using our standard techniques (12). Briefly, muscle (30–40 mg) was ground in liquid nitrogen to a fine powder and hand homogenized in a low-salt buffer; the myofibrils and collagen were pelleted by centrifugation. The sarcoplasmic fraction was aspirated off, and the myofibrils were separated from the collagen by dissolving in 0.7 M KCl. The incorporation of proline into the myofibrillar and sarcoplasmic protein fractions was then measured by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) after hydrolysis, purification, and derivatisation as their N-acetyl-*n*-propyl ester (NAP) derivative. The fractional synthetic rate (FSR) was calculated as the rate of increase of labeling of protein-derived ^{13}C proline compared with the average labeling of the proline in the incubation media.

After the completion of the main experiments, we carried out a control experiment to investigate whether the addition of amino acids at fasting physiological levels (10; the addition of amino acids was necessary for the measurement of protein synthesis) can increase mTOR Ser2448, 4E-BP1 Thr37/46, and p70 S6k Thr389 phosphorylation from their depression 3 h after LFS. Six EDL were collected either 3 h after LFS stimulation or 3 h after LFS stimulation with 15 min incubation with fasting physiological amino acid levels.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

We measured uncoupling protein-3 (UCP3) mRNA with RT-PCR in order to verify that LFS could induce a marker gene for the acute response to endurance training (13). RNA was extracted using Tri-reagent according to the manufacturer's protocol. Reverse transcription was performed with 1 μg of RNA as template using first strand cDNA synthesis kit (Roche, Indianapolis, IN; 1 483 188). PCR was carried out for 35 cycles using the lightCycler FastStart DNA master^{PLUS} SYBR Green 1 kit (Roche; 03 515 869). Primer sequences for rat UCP3 were designed using rat-specific sequence data (forward primer: 5′-GAACCATCGCCAGGGAAGAAGGAGTCAG-3′; reverse primer: 5′-GGGGGAGCGTTCATGTATCGGGTCTTTA-3′). Results were related to an internal GADPH control and then normalized to control EDL. Amplification products were separated on a 2.5% agarose gel and stained with ethidium bromide for visualization.

Protein extraction for Western blotting

Proteins were extracted from rat EDL and soleus muscles. Muscle (30 mg) was homogenized on ice in 0.6 ml of homogenization buffer (50 mM Tris-HCL; 0.1% Triton-X; 1 mM EDTA; 1 mM EGTA; 50 mM NaF; 10 mM β-glycerophosphate; 5 mM Na pyrophosphate; 0.1% 2-mercaptoethanol; 100 nM okadaic acid; 50 μM sodium orthovanadate). Samples were rotated for 60 min at 4°C before being centrifuged at 13,000 *g* for 10 min. Protein concentration was measured using the Bradford assay and adjusted to 2 mg ml^{-1} by diluting in SDS sample buffer (3.55 ml deionized water, 1.25 ml 0.5 M Tris-HCL, pH 6.8; 2.5 ml glycerol; 2 ml 10% [w/v] SDS; 0.2 ml 0.5% [w/v] bromophenol blue).

Western blotting

Samples were electrophoresed in running buffer (1% SDS; 192 mM glycine; 25 mM Tris base; pH 8.3) on a 10% SDS-PAGE gel at 100 V for 30 min through the stacking layer and then 200 V until the dye marker reached the bottom of the gel. Following conclusion of electrophoresis, the polyvinylidene difluoride (PVDF) membrane was permeabilized in 100% methanol for 1 min before both the gel and pre-wetted membrane were equilibrated in transfer buffer (192 mM glycine; 25 mM Tris base; 20% w/v methanol; pH 8.3) for 30 min. The transfer was run for 2 h at a constant 100 V. Upon completion of transfer, the uniformity of loading was checked with Ponceau S before the membrane was incubated in 30 ml of blocking buffer (TBS, pH 7.6; 0.1% Tween-20; 5% w/v nonfat milk powder) for 3 h. Following incubation with the blocking buffer, the membrane was washed three times with doubly distilled water.

Samples were exposed to the following antibodies overnight at 4°C: AMPK Thr172 (New England Biolabs, Beverly, MA, 2531; 1:2000); total AMPK (New England Biolabs, Beverly, MA, 2532; 1:1500); total PGC-1α (Chemicon, Temecula, CA, AB3242; 1:1000); PKB/Akt Ser473 (New England Biolabs, 9271; 1:2000); total PKB/Akt (New England Biolabs, 9272; 1:1500); p70S6k Thr389 (New England Biolabs, 9205; 1:2000); total p70 (New England Biolabs, 9202; 1:1500); 4E-BP1 Thr 37/46 (New England Biolabs, 9459; 1:2000); total 4E-BP1 (Courtesy C. G. Proud, University of Dundee; 1:1000); TSC2/tuberin Thr1462 (New England Biolabs, 3611; 1:2000); total TSC2/tuberin (New England Biolabs, 3612; 1:1500); mTOR Ser2448 (New England Biolabs, 2971; 1:2000); total mTOR (New England Biolabs, 2972; 1:1500); GSK3-α/β Thr21/Ser9 (New England Biolabs, 9331; 1:2000); total GSK-3α/β (New England Biolabs, 9332; 1:1500); eIF2B Ser535 (Courtesy C. G. Proud; 1:1000); total eIF2B (Courtesy C. G. Proud; 1:1000); eEF2 Thr56 (Courtesy C. G. Proud; 1:1000); total eEF2

(Courtesy C. G. Proud; 1:1000); ERK1/2 Thr180/Tyr182 (New England Biolabs, 9101; 1:2000); total ERK1/2 (New England Biolabs, 9102; 1:2000); p38 MAP kinase Thr180/Tyr182 (New England Biolabs, 9211; 1:2000); total p38 MAP kinase (Biocompare, South San Francisco, CA, S0096-01A; 1:2000); SAPK/JNK Thr183/Tyr185 (New England Biolabs, 9251; 1:2000); total SAPK/JNK (New England Biolabs, 9252; 1:2000).

The following morning the membrane was rinsed in wash buffer (TBS with 0.1% Tween-20) three times for 5 min. The membrane was then incubated for 1 h at ambient temperature within blocking buffer containing the appropriate secondary antibody, either horseradish (HRP)-linked anti-mouse IgG (New England Biolabs, 7072; 1:2000) or anti-rabbit IgG (New England Biolabs, 7074; 1:2000). The membrane was then cleared in wash buffer three times for 5 min. Membranes were exposed to ECL chemiluminescent detection HRP reagents (Amersham Biosciences, Piscataway, NJ, RPN2106) mixed 1:1 for 1 min. Membranes were partially dried, wrapped in Saran, and exposed to X-ray film. Examples of Western blot results are shown in Table 1.

Densitometry

Blots were scanned using a Bio-Rad (Temecula, CA) Imaging densitometer (model GS-670) to detect the relative band intensity. Each band was identified, and the optical density volume was adjusted by subtraction of the background. All values obtained from one blot were normalized to the average control band intensity, which was set to 1.

Statistics

All data are displayed as mean ± SE. Means were compared using an independent, three-factorial ANOVA (muscle: EDL, soleus; stimulation: HFS, LFS; time: control, directly after stimulation, 3 h control, 3 h after stimulation). A one-factorial ANOVA (control; 3 h after LFS; 3 h after HFS) was used to analyze the protein synthesis and RT-PCR data. Tukey's test was used as a post hoc test. $P < 0.05$ was used as a threshold for statistical significance.

RESULTS

Tension generated during stimulation

Different tensions were generated in response to LFS and HFS. EDL and soleus generated a tension of 5.3 ± 0.4 mN and 4.6 ± 0.4 mN during the first 3-s burst of the HFS protocol, which significantly decreased to 3.7 ± 0.3 mN and 4.0 ± 0.3 mN during the last (i.e., 60th) burst, respectively. During LFS, force stabilized in the EDL and soleus at 2.3 ± 0.2 mN and 2.2 ± 0.2 mN after 30 s of stimulation, and the force decreased significantly only in the EDL to 1.6 ± 0.2 mN but not in the soleus where it was 2.0 ± 0.2 after 3 h of stimulation. The tension generated during the LFS protocol was always significantly lower than in the HFS protocol.

Validation of LFS and HFS models by measuring protein synthesis and UCP3 expression

In a separate experiment, we measured myofibrillar and sarcoplasmic protein synthesis and UCP3 mRNA in control and 3 h after LFS and HFS, respectively, to see whether known adaptive responses to endurance and resistance training could be induced by LFS and HFS (n=6 EDL per group; amino acid supplemented; see Materials and Methods and Fig. 1*A*). Under these conditions, myofibrillar and sarcoplasmic protein synthesis were 5.32 and 2.65 times higher 3 h

after HFS compared with control, respectively ($P<0.05$). In LFS, protein synthesis was stimulated only to a small degree; this, however, did not reach significance. UCP3 mRNA increased significantly (Fig. 1*B*) to 11.70 ± 0.96 of control 3 h after LFS but remained at 1.25 ± 0.12 of control 3 h after HFS, showing that the increase in UCP3 mRNA is a specific, adaptive response to LFS.

AMPK-PGC-1α signaling

AMPK phosphorylation at Thr172 increased significantly in response to LFS but not to HFS (Fig. 2*A*; Table 1). LFS significantly increased AMPK phosphorylation 2.02 ± 0.11-fold directly after stimulation and 1.90 ± 0.06-fold 3 h later. In contrast to LFS, AMPK phosphorylation was significantly decreased to 0.69 ± 0.08 of control 3 h after HFS. The AMPK-activation pattern was matched directly after stimulation by a concentration change of PGC-1α: PGC-1α increased significantly 1.30 ± 0.04-fold directly after LFS and fell to 0.82 ± 0.03 of control directly after HFS (Fig. 2*B*; Table 1).

PKB-TSC2-mTOR signal transduction pathway

The signaling response of the PKB-TSC-2-mTOR-related signaling cascade (Fig. 3; Table 1) was a mirror image of the AMPK response. It increased after HFS but not LFS.

HFS effect on PKB, TSC2, mTOR, and GSK-3β phosphorylation

HFS increased PKB Ser473 (Fig. 3*A*; Table 1) and TSC2 Thr1462 (Fig. 3*B*; Table 1) phosphorylation significantly more in the EDL than in the soleus. HFS increased PKB phosphorylation 8.36 ± 1.52-fold and 2.09 ± 1.04-fold and TSC2 phosphorylation 3.66 ± 0.20-fold and to 2.6 ± 1.3-fold in the EDL and soleus directly after HFS, respectively. Neither PKB nor TSC2 phosphorylation were significantly changed 3 h after HFS. We also measured the concentration of total TSC2 because of the possibility of ubiquitin-dependent TSC2 degradation (see Discussion). Total TSC2 significantly decreased to 0.82 ± 0.02 of control after HFS and was not different from baseline 3 h later (Fig. 3*C*; Table 1). Similar to PKB, the phosphorylation of GSK-3β at Ser9 increased 3.08 ± 0.12-fold directly after HFS and returned to 1.13 ± 0.04 of control 3 h poststimulation (Fig. 3*G*; Table 1). Finally, the phosphorylation of mTOR at Ser2448 was significantly increased 1.75 ± 0.08-fold directly after HFS and was 1.23 ± 0.04 of control 3 h later (Fig. 3*D*; Table 1). To summarize, PKB Ser473, TSC2 Thr1462, and mTOR Ser2448 are acutely phosphorylated in response to HFS and the phosphorylation is not significantly different from control 3 h later.

HFS effect on translational regulators

All translational regulators were significantly activated directly and 3 h after HFS with the exception of 4E-BP1, which was only activated directly after stimulation. The phosphorylation of p70 S6k at Thr389 significantly increased 6.85 ± 0.94-fold and 9.76 ± 0.60-fold directly and 3 h after HFS, respectively (Fig. 3*E*; Table 1). The phosphorylation of 4E-BP1 at Thr37/46 significantly increased 3.20 ± 0.13-fold directly after HFS but was not significantly different from control 3 h later (Fig. 3*F*; Table 1). The phosphorylation of eIF2B at Ser535 (Fig. 3*H*; Table 1) and of the elongation factor eEF2 at Thr56 (Fig. 4*A*; Table 1) was 0.51 ± 0.05 and 0.76 ± 0.02 and 0.78 ± 0.06 and 0.38 ± 0.03 of control directly and 3 h after HFS, respectively (both factors are activated by dephosphorylation).

LFS effect on TSC2 and downstream translational regulators

From TSC2 downstream the signaling proteins and translational regulators related to the PKB-TSC2-mTOR-signaling cascade were deactivated in response to LFS (Fig. 3*E*, 3*F*, 3*H*, 4*A*; Table 1). The only exception was mTOR phosphorylation at Ser2448, which was not affected by LFS (Fig. 3*D*; Table 1). TSC2 Thr1462 phosphorylation significantly decreased to 0.24 ± 0.02 directly and 0.55 ± 0.06 of control 3 h after LFS (phosphorylation was significantly lower in soleus than EDL 3 h after LFS) (Fig. 3*B*; Table 1). LFS also significantly decreased the phosphorylation of p70 S6k to 0.22 ± 0.03 and 0.17 ± 0.03 of control and of 4E-BP1 to 0.36 ± 0.02 and 0.10 ± 0.03 of control directly and 3 h after LFS, respectively (Fig. 3*E*, 3*F*; Table 1). The phosphorylation of eIF2B was 1.19 ± 0.05-fold of control 3 h after stimulation (Fig. 3*H*; Table 1). LFS also increased the phosphorylation of eEF2 to 1.62 ± 0.06 and 1.65 ± 0.07 directly and 3 h after stimulation, respectively (Fig. 4*A*; Table 1).

In a control experiment, we found that addition of fasting levels of amino acids (10) used for the protein synthesis measurements increases mTOR Ser2448 phosphorylation 3 h after LFS by 45% (P=0.06), 4E-BP1 Thr37/46 phosphorylation by 7% (P=0.09), and p70 S6k Thr389 phosphorylation by 40% (P=0.02).

ERK1/2, p38, and JNK

In contrast to all other proteins, phosphorylation of the three MAPK studied here was not significantly different between the stimulation protocols at any point (Fig. 4*B–D*; Table 1). The only exception was ERK2 Thr180/Tyr182 phosphorylation, which increased in response to both protocols directly and 3 h after stimulation but significantly more so in response to HFS than LFS (Fig. 4*B*; Table 1). ERK1 Thr180/Tyr182 phosphorylation increased significantly after both protocols by ~10-fold. In contrast, neither stimulation protocol had a significant effect on p38 Thr180/Tyr182 phosphorylation (Fig. 4*C*; Table 1). JNK phosphorylation at Thr183/Tyr185 increased significantly by 1.16 ± 0.03-fold after HFS and then fell both 3 h after LFS and HFS to ~0.77 ± 0.05 (Fig. 4*D*; Table 1).

DISCUSSION

The major finding of this study is that AMPK-PGC-1α signaling can be specifically induced by LFS and that the PKB-TSC2-mTOR cascade and protein synthesis are specifically activated by HFS.

Selective AMPK-PGC-1α activation by LFS and inhibition by HFS

The LFS protocol but not the HFS protocol increased AMPK Thr172 phosphorylation via a yet unknown mechanism. The identity of the upstream kinase that has phosphorylated AMPK in response to exercise is unclear because it was recently shown that the known upstream kinase LKB1 does not alter its activity in response to in situ contraction (14). A possible explanation for the lack of AMPK activation by HFS is that [AMP] does not sufficiently change during the HFS protocol. Contractions at an intensity above the anaerobic threshold cause a steady decrease of [phosphocreatine] ([PCr]) (15) because glycolytic and aerobic ATP resynthesis cannot maintain a steady state. [PCr] is linked to [ADP] via the creatine kinase reaction and [ADP] to [AMP] via the myokinase reaction. Therefore, the 3 s stimulation bursts were probably too short to allow [PCr] to decrease enough to result in a large increase of [AMP]. [AMP] was also likely to

recover during the 10 s rests (or 1 min between sets) in-between. Longer stimulations at 100 Hz will eventually deplete [PCr] and markedly increase [AMP], which explains the increase of AMPK activity by 100 Hz stimulation in the minute range (14). The continuous LFS was likely to have led to a steady state with a constant, moderate decrease of [PCr] and increase of [AMP], which was sufficient for AMPK activation. AMPK has a glycogen-binding domain (16), and AMPK is more activated when the concentration of muscle glycogen is low (17). Therefore, the glycogen depletion associated with LFS was likely to have activated AMPK further. The increase in AMPK phosphorylation by LFS is consistent with a transient increase in the transcriptional cofactor PGC-1α, which is known to be induced by AMPK via an unkown mechanism (18, 19). An increased expression of PGC-1α occurs after endurance training (20) and can explain increased mitochondrial biogenesis and an up-regulation of slow motor proteins (21, 22), which are known adaptations to endurance training. The inhibition of PGC-1α directly after HFS can possibly be explained by the finding that PKB suppresses PGC-1α expression (23).

Selective activation of the PKB-TSC2-mTOR signaling cascade activation by HFS

HFS specifically increased the phosphorylation of the anabolic PKB-TSC2-mTOR signaling cascade, GSK-3β, and significantly activated the translation initiation regulators p70 S6k, 4E-BP1, and eIF2B and the translation elongation factor eEF2. LFS stimulated the opposite response by inhibiting this signaling network from TSC2 downstream.

The activation of the PKB-TSC2-mTOR signaling cascade by HFS appears to be related to the high intensity of the HFS protocol. We have recently hypothesized that high intensity contractile activates currently unknown "upstream" signal transduction events that regulate the expression of specific muscle growth factors such as IGF-1, its muscle-specific "mechanosensitive" splice variant mechano-growth factor (MGF), and myostatin (6, 24). IGF-1 splice variants then activate phosphoinositide 3-kinase (PI3K), which synthesizes phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3), which binds to the pleckstrin homology domain of PKB. The conformational change induced by this event turns PKB into a better substrate for its constitutively active upstream kinase 3-phosphoinositide-dependent protein kinase (PDK1,2) (25, 26). PDKs then phosphorylate PKB at Thr308 (not measured) and Ser473 (27). PDK1 and PKB in turn phosphorylate downstream targets, leading to an activation of regulators of translation initiation and elongation, which can explain the increase in protein synthesis measured after the HFS protocol (27–29). An increase in muscle protein synthesis lasting up to 24–48 h is a known adaptation to a bout of resistance training (6).

The HFS-related signal, its sensor, and the upstream signaling events that activate PKB, mTOR, translational regulators, and protein synthesis are unknown. Passive stretch, which is known to activate protein synthesis (30), PKB (31), and p70 S6k (32), cannot have been the signal because all contractions were isometric. The elusive signal must thus be related to the higher stimulation frequency, higher $[Ca^{2+}]_i$, greater than twofold higher tension generated or another factor related to the high intensity of the HFS protocol. The hypothesis that high intensity is necessary for PKB activation is supported by previous studies by Sakamoto et al., who reported PKB activation in response to 100 Hz stimulation in situ (33).

HFS but not LFS increased TSC2 Thr1462 and mTOR Ser 2448 phosphorylation. PKB is known to phosphorylate TSC2 at Thr1462, resulting in TSC2 inactivation (34). The authors of this study also found that PKB-phosphorylated TSC2 was more ubiquitinated, suggesting that PKB-

phosphorylation of TSC2 at Thr1462 leads to its degradation by ubiquitin-dependent proteolysis (34). We observed a significant 3.13 ± 0.23-fold increase in TSC2 Thr1462 phosphorylation (normalized to total TSC2) and a significant decrease to 0.82 ± 0.02 of control total TSC2 directly after HFS. These data are further support for the hypothesis that TSC2 is degraded after phosphorylation by PKB. In contrast, LFS significantly decreased phosphorylation of TSC2 at Thr1462 to 0.24 ± 0.02 and 0.55 ± 0.06 directly and 3 h after LFS, respectively. LFS also significantly increased the TSC2 concentration to 1.27 ± 0.02 of control directly after LFS, suggesting that TSC2 Thr1462 phosphorylation and TSC2 concentration are linked. Taken together, these findings suggest that TSC2 mediates the anabolic response to HFS and inhibits protein synthesis during LFS-induced catabolism. TSC2 partially controls activity of mTOR via the GTPase Rheb (35), which is likely to regulate mTOR phosphorylation via a yet unknown mechanism.

PKB can also directly phosphorylate mTOR at Ser2448, which is the site that we have investigated (36). We found a 1.75 ± 0.08-fold increase in mTOR Ser2448 phosphorylation directly after HFS but no effect of LFS, suggesting that this site is not dependent on TSC2-Rheb signaling, which was affected by LFS. AMPK-TSC2-Rheb effects could potentially affect mTOR activity via Thr2446 phosphorylation, which was shown to be affected by the TSC2 activator AMPK (37).

Effect on translational regulators

HFS significantly activated and LFS significantly inhibited the translational regulators p70 S6k, 4E-BP1, eIF2B, and eEF2. In contrast to their upstream regulators, translational regulators were activated also 3 h after HFS with the exception of 4E-BP1. These data suggest that HFS can activate a prolonged translation initiation and elongation response even in fasted muscles. The origin of this long-term effect is unknown.

The significant 6.85 ± 0.94-fold and 9.76 ± 0.60-fold increases of p70 S6k phosphorylation directly and 3 h after HFS can possibly explain the increased protein synthesis 3 h after HFS. p70 S6k can be directly phosphorylated by PDK1 at the Thr229 site (not measured) (38). In contrast, the phosphorylation of the Thr371 (not measured) and Thr389 sites of p70 S6k are mTOR-dependent (38). The inhibitory effect of LFS and the anabolic effect of HFS are reflected by the phosphorylation state of the Thr389 site, respectively. The long-term increase in p70 S6k phosphorylation is consistent with the observation that the phosphorylation of p70 S6k and protein synthesis in skeletal are activated long-term after resistance exercise (39, 40). It is unclear why p70 S6k Thr389 phosphorylation is further increased 3 h after HFS whereas phosphorylation of PKB at Ser473, TSC2 at Thr1462 and mTOR at Ser2448 are back to normal.

In the unstimulated state, 4E-BP1 binds and inhibits the eukaryotic translation initiation factor eIF4E. Phosphorylation of 4E-BP1 at Thr37/46 leads to a release of 4E-BP1 from eIF4E promoting cap-dependent translation initiation (41). An increase in 4E-BP1 phosphorylation was observed in response to resistance exercise in rats (42), which is in line with our observations of a significant 3.20 ± 0.13-fold increase in 4E-BP1 phosphorylation directly after HFS. However, 3 h after, HFS 4E-BP1 phosphorylation at Thr37/46 was not significantly different from control.

Another regulatory pathway affecting translation initiation is the PKB-GSK-3β-eIF2B cascade. Activated PKB phosphorylates GSK-3β at Ser9, which inhibits the activity of GSK-3β (43). An inhibition of phosphorylated GSK-3β will lead to a reduced phosphorylation of eIF2B at Ser535,

which in turn promotes translation initiation (44). Inhibition of GSK-3β by phosphorylation promoted hypertrophy in cultured muscle cells (45). The 3.08 ± 0.12-fold increase in GSK-3β Ser9 phosphorylation directly after HFS and decrease of eIF2B Ser535 phosphorylation to 0.51 ± 0.05 and 0.78 ± 0.06 of control directly and 3 h after HFS support the hypothesis that this pathway is involved in stimulating an increase in protein synthesis in response to HFS. It is unclear why GSK-3β Ser9 phosphorylation only changed acutely whereas eIF2B Ser535 was decreased directly and 3 h after HFS.

eEF2 catalyzes the translocation of peptidyl-tRNA during translation elongation and is regulated by mTOR via p70 S6k and p90 RSK1 (46). Phosphorylation of eEF2 at Thr56 by its upstream kinase inactivates eEF2 (47). Our data show a significant, prolonged decrease of eEF2 Thr56 to 0.76 ± 0.02 and 0.38 ± 0.03 directly and 3 h after HFS, respectively, suggesting an activation.

LFS affected phosphorylation of p70 S6k, 4E-BP1, eIF2B, and eEF2 at various sites, suggesting an inhibition of translation initiation and elongation that was still present 3 h after LFS. Recent studies have shown that AMPK can directly phosphorylate TSC2 at Thr1227 and Ser1345, leading to an inhibition of mTOR and regulators of translation initiation in various tissues, including muscle (48–51). In addition, the translation elongation regulator eEF2 is also inhibited by direct AMPK phosphorylation of the eEF2 upstream eEF2 kinase (52, 53). These results suggest that LFS inhibits energy-consuming protein synthesis as long as the muscle is kept in a fasted state. An inhibition of translational regulators by stimuli similar to LFS was noted by Gautsch et al. who reported a decrease of 4E-BP1 phosphorylation after endurance exercise, which was reversed by subsequent feeding (41). Activation of mTOR via a nutrient-sensitive pathway by the amino acid mix necessary for the measurement of protein synthesis can explain why myofibrillar and sarcoplasmic protein synthesis 3 h after LFS were not significantly lower compared with control (Fig. 1*A*). We carried out a control experiment in order to attempt to explain the discrepancy between the decreased phosphorylation of translational regulators (in medium without fasting levels of amino acids) and the unchanged protein synthesis 3 h after LFS [fasting levels of amino acids (10) had to be added for measuring protein synthesis]. We found that the addition of fasting concentrations of amino acids significantly increased p70 S6k Thr389 phosphorylation 3 h after LFS. Stimulation of p70 S6k and possibly other translational regulators by fasting levels of amino acids is thus a possible explanation for the apparent discrepancy between translational regulator phosphorylation and protein synthesis.

MAPKs

Confirming Nader and Esser's results (4) and in contrast to Wretman et al.'s and Martineau and Gardiner's conlusions (54, 55), we found no stimulation pattern-specific effects (i.e., no difference between LFS and HFS) on ERK1, p38, and JNK phosphorylation at the activity-related Thr/Tyr sites. A stimulation-specific effect was found for ERK2, which increased more, up to 17.75 ± 5.77-fold of control 3 h after HFS whereas the maximal response after LFS was a 4.81 ± 1.69-fold increase directly after LFS. However, phosphorylation did not change in opposite directions. Moreover, we did not detect any increase in p38 α/β phosphorylation in response to either LFS or HFS. These data confirm that p38 is not always phosphorylated in response to contractile activity. Wretman et al. equally failed to measure an increase in p38 phosphorylation in response to concentric contraction of isolated EDL (54). We have not investigated p38δ/γ phosphorylation, and thus it is unclear whether p38 isoforms are activated in our model.

The ERK1/2 pathway has been shown to promote the formation of slow fibers and to induce slow and to inhibit the expression of fast motor proteins (56, 57), suggesting ERK1/2 is involved in the fast-to-slow exchange of myosin heavy chain isoforms in response to exercise and electrical stimulation. This does not contradict the finding that ERK1/2 phosphorylation also increases in response to HFS. Limited fast-to-slow changes in the expression of myosin heavy chain isoforms from IIx to IIa are commonly reported in response to resistance training (58). The JNK response to HFS was small and not significantly different from the LFS response at any point contrary to the finding that JNK Thr183/Tyr185 phosphorylation is quantitatively related to the tension (55).

Summary

The major result of our study is that electrical muscle stimulation mimicking endurance or resistance training can switch signaling to either a AMPK-PGC-1α- or PKB-TSC2-mTOR-dominated state. We term this behavior the AMPK-PKB switch (Fig. 5). The increased expression of PGC-1α after LFS can potentially explain an increase in mitochondrial biogenesis and the progression toward a slower muscle phenotype (22). In a fasted muscle, AMPK activation will suppress translation initiation and elongation, which can explain why chronic, electrical 10-Hz stimulation does not stimulate muscle growth. Moreover, we hypothesize that HFS induces an anabolic state via a prolonged activation of regulators of translation initiation and elongation even in a fasted muscle without the need for systemic effectors. The observed effects of HFS on PKB alone can potentially explain the observed increase in protein synthesis 3 h after HFS: Expression of a constitutively active PKB construct was shown to markedly increase fiber size in regenerating rat fibers that expressed this construct compared with fibers that did not (59). Our hypothesis does not exclude that AMPK and PKB can both be activated at the same time by specific stimulation protocols. Finally, other pathways are likely to contribute to the specific adaptive events induced by LFS and HFS. These pathways include the calcineurin (60), CamK (61), and myostatin-Smad2/3 signal transduction pathways (62).

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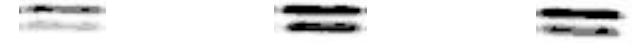
Table 1

Examples for Western blot bands[a]

Target (phosphorylation site)	Stimulation	Control	Poststimulation	3 h Poststimulation
AMPK (Thr172)	LFS (10 Hz)			
	HFS (100 Hz)			
Total PGC-1α	LFS (10 Hz)			
	HFS (100 Hz)			
PKB (Ser473)	LFS (10 Hz)			
	HFS (100 Hz)			
TSC2 (Thr1462)	LFS (10 Hz)			
	HFS (100 Hz)			
Total TSC2	LFS (10 Hz)			
	HFS (100 Hz)			
mTOR (Ser2448)	LFS (10 Hz)			
	HFS (100 Hz)			
p70 S6k (Thr389)	LFS (10 Hz)			
	HFS (100 Hz)			
4E-BP1 (Thr37/46)[b]	LFS (10 Hz)			
	HFS (100 Hz)			
GSK-3β (Ser9)	LFS (10 Hz)			
	HFS (100 Hz)			

Table 1 (cont.)

eIF2B (Ser535)	LFS (10 Hz)	
	HFS (100 Hz)	
eEF2 (Thr56)	LFS (10 Hz)	
	HFS (100 Hz)	
ERK1/2 (Thr180/Tyr182)	LFS (10 Hz)	
	HFS (100 Hz)	
p38 (Thr180/Tyr182)	LFS (10 Hz)	
	HFS (100 Hz)	
JNK (Thr180/Tyr182)[c]	LFS (10 Hz)	
	HFS (100 Hz)	

[a]All bands in one row have been obtained from one Western blot. [b]The density of all bands corresponding to the various 4E-BP1 isoforms was quantified. [c]Only JNK2 (p54) was quantified.

.

Fig. 1

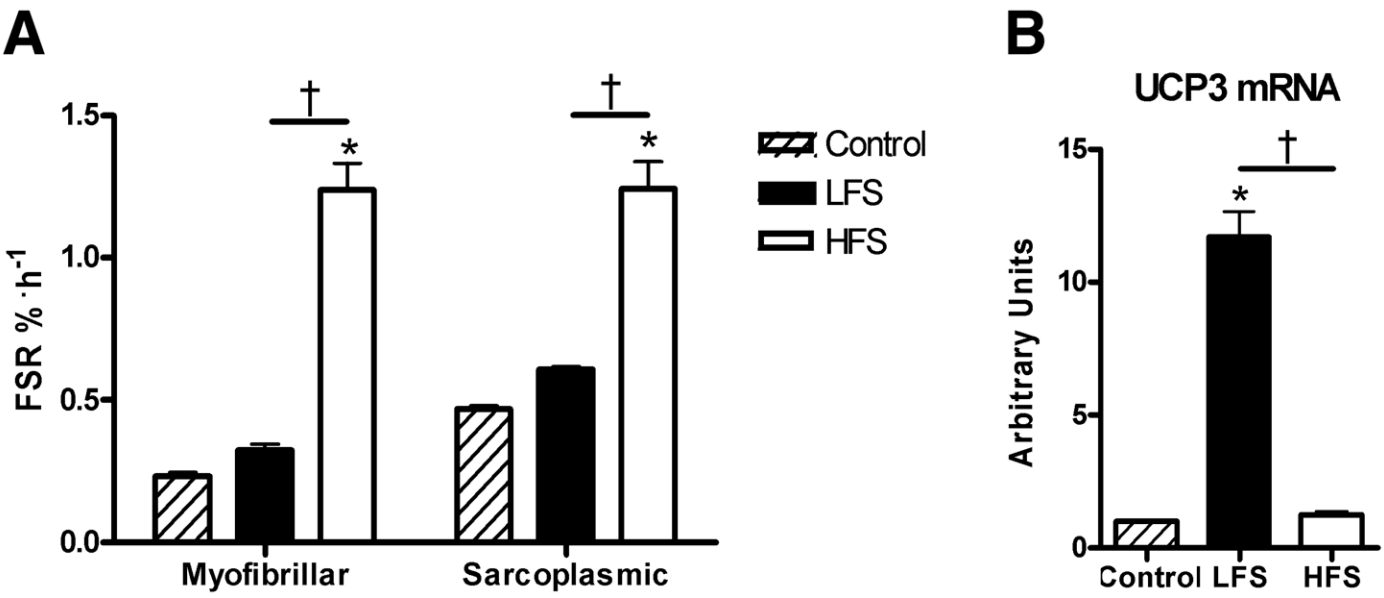


Figure 1. ***A)*** Myofibrillar and sarcoplasmic protein synthesis in rat EDL muscles incubated without stimulation and 3 h after LFS or HFS, respectively (n=6 EDL per bar; mean±SE). ***B)*** UCP3 mRNA relative to control (n=6 EDL per bar; mean± SE) *Significantly different from control; †significant difference between LFS and HFS stimulation protocols (ANOVA, Tukey's post hoc, P<0.05).

Fig. 2

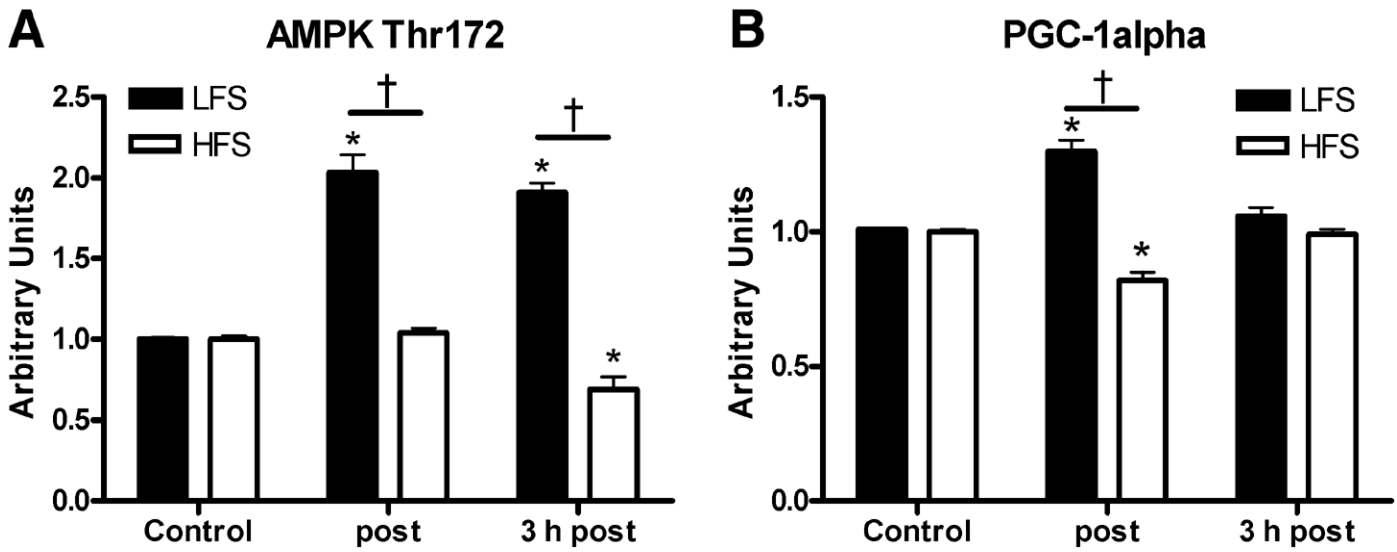


Figure 2. ***A)*** AMPK Thr172 phosphorylation relative to total AMPK. ***B)*** Total PGC-1α (*n*=8; 4 EDL and 4 soleus per bar; mean± SE) of resting muscle (control), directly after (post) and 3 h after (3 h post) LFS and HFS. All values were normalized to the relative intensity of the control bands. *Significantly different from control; †significant difference between LFS and HFS stimulation protocols (ANOVA, Tukey's post hoc, $P<0.05$).

Fig. 3

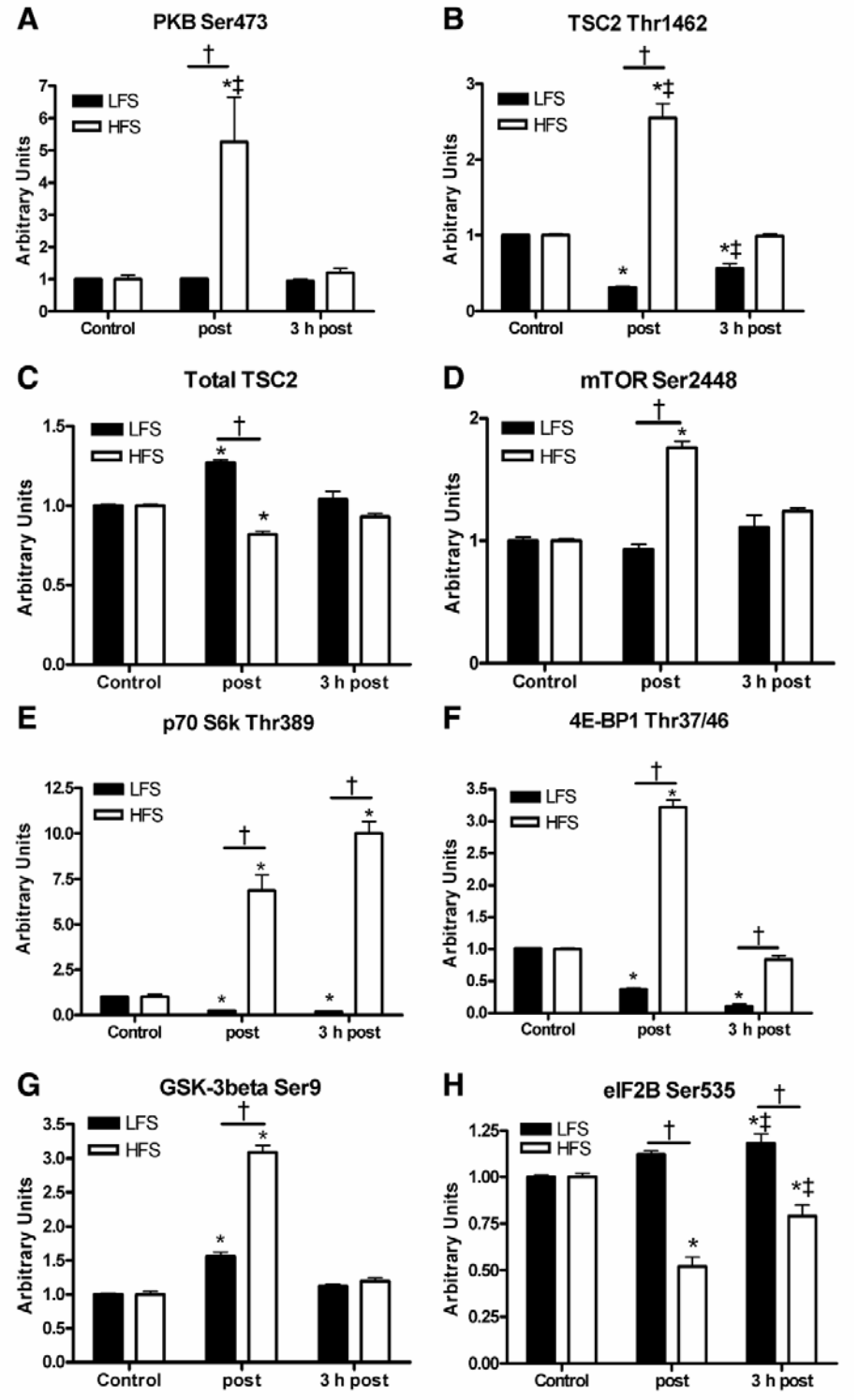


Figure 3. ***A*)** PKB Ser473 phosphorylation relative to total PKB. ***B*)** TSC2 Thr1462 phosphorylation relative to total TSC2. ***C*)** Total TSC2. ***D*)** mTOR Ser2448 phosphorylation relative to total mTOR. ***E*)** p70 S6k Thr389 phosphorylation relative to total p70 S6k. ***F*)** 4E-BP1 Thr37/46 phosphorylation relative to total 4E-BP1. ***G*)** GSK-3β Ser9 phosphorylation relative to total GSK-3β. ***H*)** eIF2B Ser535 phosphorylation relative to total eIF2B (all n=8; 4 EDL and 4 soleus per bar; mean± SE) of resting muscle (control), directly after (post) and 3 h after (3 h post) LFS and HFS. All values were normalized to the relative intensity of the control bands. *Significantly different from control; †significant difference between LFS and HFS stimulation protocols; ‡significant difference between EDL and soleus muscles (ANOVA, Tukey's post hoc, P<0.05).

Fig. 4

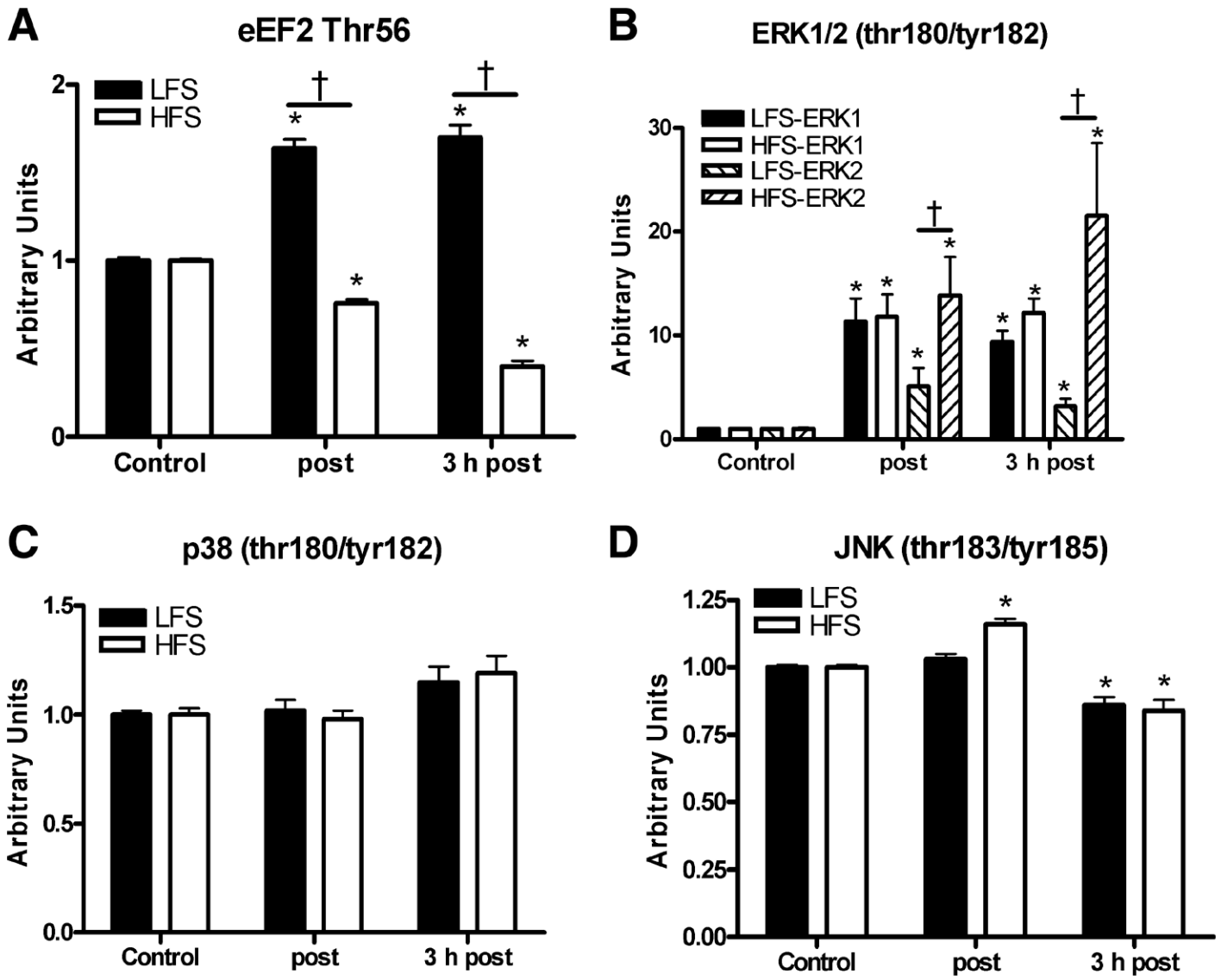


Figure 4. ***A*)** eEF2 Thr56 phosphorylation relative to total eEF2. ***B)*** ERK1/2 Thr180/Tyr182 phosphorylation relative to total ERK1/2. ***C)*** p38 Thr180/Tyr182 phosphorylation relative to total p38. ***D)*** JNK1/2 Thr183/Tyr185 phosphorylation relative to total JNK (all n=8; 4 EDL and 4 soleus per bar; mean± SE) of resting muscle (control), directly after (post) and 3 h after (3 h post) LFS and HFS. All values were normalized to the relative intensity of the control bands. *Significantly different from control; †significant difference between LFS and HFS stimulation protocols (ANOVA, Tukey's post hoc, P<0.05).

Fig. 5

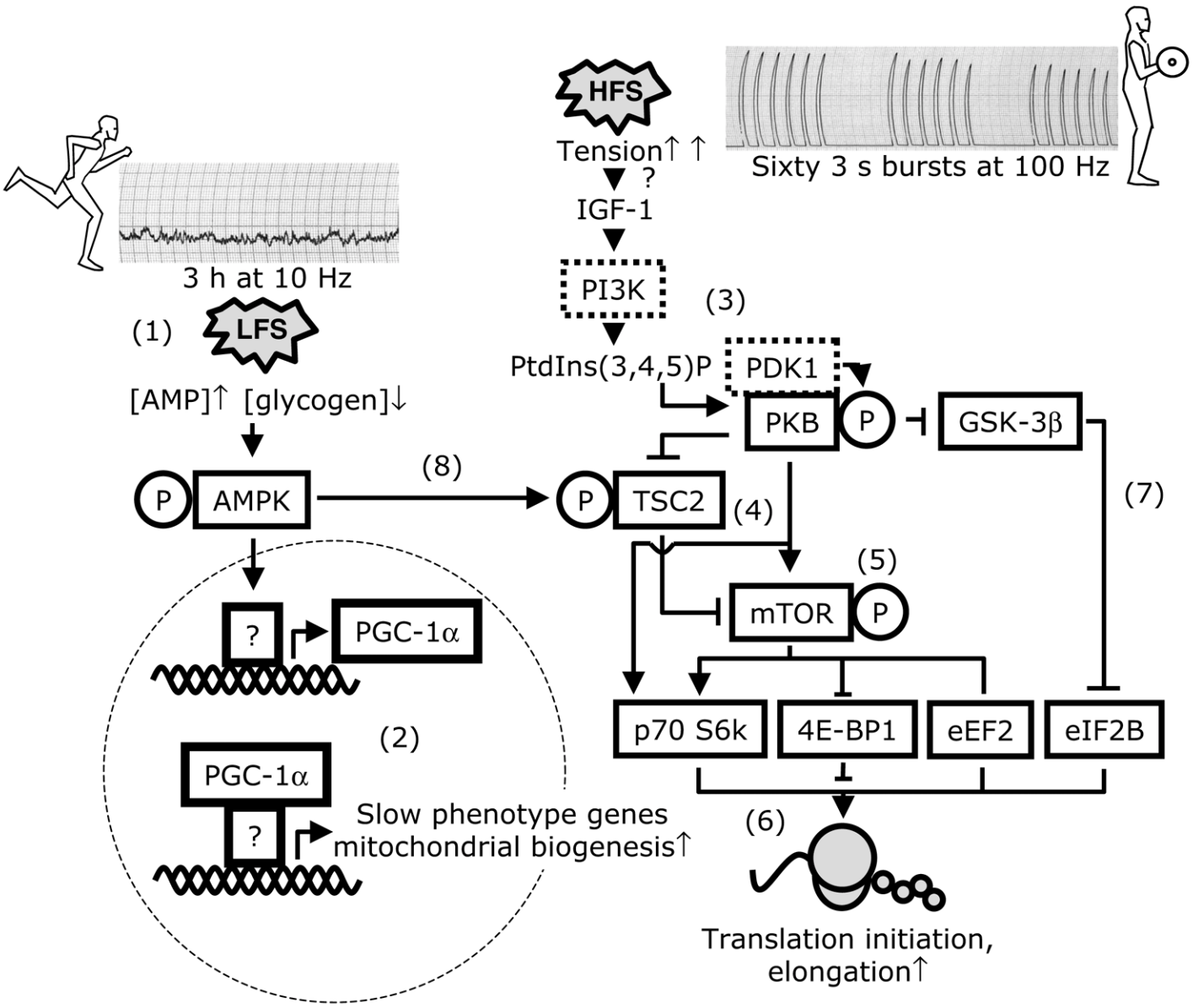


Figure 5. Schematic diagram summarizing the "AMPK-PKB switch" hypothesis, which can explain specific adaptations to LFS and HFS. ***1*****)** LFS specifically increases [AMP] and will reduce [glycogen], causing AMPK activation and ***2*****)** an increased expression of PGC-1α, which can partially explain a progression toward a slow phenotype and increased mitochondrial biogenesis. ***3*****)** In contrast, possibly the high tension generated as a result of HFS will induce IGF-1 and will via a PI3K-PDK1,2-dependent mechanism (not measured) activate PKB. ***4*****)** PKB will then directly or via TSC2 regulate the activity of mTOR, which also depends on ***5*****)** nutrients via a PI3K-independent pathway. ***6*****)** Regulators of translation initiation and elongation will be activated by PKB and mTOR and stimulate a prolonged increase in protein synthesis. ***7*****)** PKB also increases protein synthesis by inhibiting GSK-3β, which eliminates the inhibition of eIF2B, activating translation initiation further. ***8*****)** AMPK can also directly activate TSC2, which then inhibits mTOR and downstream regulators of translation initiation and elongation.