

Intracellular signaling specificity in skeletal muscle in response to different modes of exercise

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Received 16 October 2000; accepted in final form 13 December 2000

Nader, Gustavo A., and Karyn A. Esser. Intracellular signaling specificity in skeletal muscle in response to different modes of exercise. *J Appl Physiol* 90: 1936–1942, 2001.—The aim of this study was to understand better the specific signaling events resulting from different modes of exercise. Three different exercise protocols were employed based on their well-characterized, long-term training effects on either muscle hypertrophy or endurance phenotypes. Rats were subjected to a single bout of either a high-frequency electrical stimulation, a low-frequency electrical stimulation, or a running exercise protocol. Postexercise intracellular signaling was analyzed in the tibialis anterior and soleus muscles at 0, 3, and 6 h. A prolonged increase in p70^{S6k} and a transient increase in protein kinase B phosphorylation were only observed in response to a growth-inducing stimulus (e.g., tibialis anterior in high-frequency electrical stimulation). In contrast, extracellular regulated kinase and 38-kDa stress-activated protein kinase were activated in response to all forms of exercise at 0 h, but only extracellular regulated kinase phosphorylation was found significantly elevated at 6 h after running exercise. These results demonstrate that different exercise protocols resulted in the selective activation of specific intracellular signaling pathways, which may determine the specific adaptations induced by different forms of exercise.

hypertrophy; endurance; signal transduction; adaptation

ONE REMARKABLE FEATURE ABOUT skeletal muscle is its ability to adapt to different functional demands (4). These adaptations are specific to the exercise stimulus. Resistance training results in increased muscle mass, fiber hypertrophy, and strength (28, 42). In contrast, endurance training results in increased mitochondrial density, capillary supply, changes in key metabolic enzymes, and increased maximal oxygen uptake (17). However, whereas the physiological and biochemical adaptations induced by these modes of exercise have been well characterized, the molecular events underlying these specific adaptations remain poorly defined.

A way in which changes in external and internal environments are converted into the appropriate responses in the cell is through the activation of various signaling molecules (18). These in turn regulate specific targets involved in the molecular responses to

these perturbations. To date, several signaling pathways have been characterized, some of which are known to regulate gene expression at the level of transcription (18, 22) and mRNA translation (21).

The 70-kDa ribosomal S6 kinase (p70^{S6k}/S6K1) has emerged as an important factor in size regulation. Direct evidence for a role of p70^{S6k} in cellular growth control has been provided by experiments in which pharmacological and genetic interventions that blocked p70^{S6k} phosphorylation suppressed or reduced cellular growth (3, 30). Among the responses that p70^{S6k} is known to mediate are those induced by insulin (35), growth factors and hypertrophic agents (3), and anchorage-dependent signaling (26).

Another important kinase is the protein kinase B (PKB/Akt). This kinase is known to regulate p70^{S6k} indirectly through the activation of the mammalian target of rapamycin (mTOR) (33, 40). In addition, PKB is also known to mediate the mitogenic effects of insulin and insulin-like growth factor-I (1) by regulating gene expression at the translational and transcriptional levels (15, 35).

The mammalian mitogen-activated protein kinase (MAPK) family is composed of several signaling pathways [extracellular regulated kinases (ERKs) and stress-activated protein kinases (SAPKs)]. The ERK and the 38-kDa SAPK (p38) represent the best characterized members of this group of kinases. They function in two distinct pathways, and they are involved in the regulation of gene expression directly through the phosphorylation of nuclear transcription factors, (e.g., Myc, Fos) or indirectly through the activation of downstream kinases [e.g., p90^{RSK}, cAMP response element binding protein (CREB)] (5, 8). ERK and p38 also play a role in increasing capped mRNA translation through the phosphorylation of a mitogen and stress-activated kinase termed Mnk1 (36, 45).

These kinases have been studied in detail in a wide variety of model systems, but their specific responses to different forms of exercise are not clearly understood. For instance, several reports have shown that MAPK activity increased immediately after an acute bout of treadmill running (13), cycling exercise (47), and elec-

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trical stimulation (41). However, it is not clear whether or not these pathways are activated in response to high-resistance exercise. Conversely, our laboratory and others, using a model that induces skeletal muscle hypertrophy, showed that a single bout of a growth-inducing stimulus was sufficient to induce a prolonged increase in p70^{S6k} phosphorylation (2, 16). Nevertheless, the time course for p70^{S6k} activation in response to endurance exercise remains to be elucidated.

Therefore, the purpose of this study was to characterize the responses of several intracellular signaling pathways after an acute bout of contractile activity. We hypothesized that 1) a single bout of exercise would induce the activation of multiple signaling pathways, and 2) different modes of exercise would result in the activation of specific intracellular signaling pathways. To test these hypotheses, the phosphorylation states of p70^{S6k}, PKB, ERK, and p38 were analyzed in the tibialis anterior (TA) and soleus (Sol) muscles of the rat during recovery from an acute bout of high-frequency electrical stimulation (HFES), low-frequency electrical stimulation (LFES), or running (RN) exercise.

METHODS AND EXPERIMENTAL DESIGN

All procedures were approved by the Animal Care Committee of the University of Illinois at Chicago and were in accordance with the Guidelines for Care and Use of Laboratory Animals. Female Wistar rats (Charles River Laboratories, Wilmington, MA) were maintained on a constant 12:12-h light-dark cycle. On arrival, animals were allowed to acclimatize for 6 days before any intervention took place. Animals were age (6–7 wk) and weight (208 ± 16 g) matched in all experiments. Food and water were available ad libitum. The surgical and electrical stimulation interventions, as well as the tissue collections, were performed under anesthesia (pentobarbital sodium 50 mg/kg ip). After electrode implantation, animals were housed individually and were allowed to recover for 5 days before the experimental exercise bout.

Exercise protocols. The HFES model was chosen based on its efficacy in inducing skeletal muscle hypertrophy (2). Multistrand electrodes (Medwire, Mount Vernon, NY) were implanted on both sides of the right sciatic nerve above the anatomic branching point. Tetanic contractions were delivered with the use of a Grass S5 stimulator (Grass Instru-

ments, Quincy, MA) at a frequency of 100 Hz, 6–12 V, 1-ms duration, 9-ms delay, for 10 sets of 6 repetitions, with each repetition lasting 3 s. A 10-s recovery was given between repetitions and 1 min between sets, with the stimulation protocol lasting a total time of 20 min. This model takes advantage of the anatomic distribution of the hindlimb muscles of the rat. During each stimulation, all hindlimb muscles are recruited, and the dorsiflexor muscles are stimulated to contract against forces that are three times larger than the ones generated by the antagonistic plantar flexors (49). This type of stimulus, when adequately repeated, is sufficient to induce a hypertrophic response in the overloaded dorsiflexors (2, 49). The muscles from the contralateral limb served as control.

The LFES model has been shown to be effective in inducing endurance-like adaptations when applied 5 days/wk for 3 wk (34). Electrodes were implanted on both sides of the peroneal nerve, and stimulation was delivered at a frequency of 10 Hz, 5 V, 10-ms duration, 90-ms delay, for a total time of 30 min. With this stimulation paradigm, the TA muscle was recruited, resulting in twitching contractions with no direct recruitment of the Sol muscle. The muscles from the contralateral limb served as control.

Treadmill running was utilized as a model of endurance exercise. After a brief 5-day acclimation that consisted of treadmill walking at 10–15 m/min for 5 min, rats were subjected to a 30-min experimental run at 30 m/min with a 1.5% inclination. This intensity corresponds to ~83% of maximal oxygen uptake, which has been shown to induce characteristic endurance adaptations when performed 5 days/wk for 8 wk (9). At this intensity, both Sol and TA muscles have been shown to experience increased electrical activity (37). Muscles from an age- and weight-matched group served as controls. The control animals were placed in an adjacent treadmill while the other group performed the experimental running bout. The different characteristics and phenotypic adaptations induced by these protocols are depicted in Table 1.

Muscle glycogen analysis. To verify the efficacy of the different exercise protocols, muscle glycogen levels were determined as previously described by Lo et al. (25). Briefly, tissues were collected immediately postexercise, cut, weighed, and boiled for ~30 min in 1 ml of 30% KOH saturated with NaSO₄. Once tissues were completely digested, glycogen was precipitated with 2 ml of 95% ice-cold ethanol and incubated on ice for 20 min. Tubes were spun for

Table 1. Individual characteristics and phenotypic adaptations induced by the different exercise protocols

Muscle	Parameter	HFES	LFES	RN
TA	Stimulation frequency, Hz	100	10	≤4
	Duration of stimulus, min	20	30	30
	Type of contraction	Eccentric/tetanic	Concentric/twitch	Concentric/eccentric
	Tension	>P ₀	<P ₀	<P ₀
Phenotypic response	Hypertrophy	Increased oxidative capacity and capillary supply	N/A	
Sol	Stimulation frequency, Hz	100	N/A	~4
	Duration of stimulus, min	20	30	30
	Type of contraction	Concentric/tetanic	Passive stretch	Concentric/eccentric
	Tension	<P ₀	<P ₀	<P ₀
Phenotypic response	No change in size	N/A	Increased oxidative capacity and mitochondrial density	
References		2, 49	34	9

TA, tibialis anterior; Sol, soleus; HFES, high-frequency electrical stimulation; LFES, low-frequency electrical stimulation; RN, running exercise; P₀, maximal tetanic force; N/A, not applicable.

30 min at 500 *g*. The pellets were resuspended in 1 ml H₂O, and 1 ml of 5% phenol was added. A colorimetric reaction was obtained by adding 5 ml of sulfuric acid. After incubation on ice for 30 min, absorbance was determined at a wavelength of 490 nm.

Western blot analysis. The TA and Sol muscles from the exercised and control limbs were collected at 0, 3, and 6 h postexercise. Muscles from both limbs were carefully dissected, freeze clamped, and frozen in liquid nitrogen. Tissue harvesting was completed within ~5 min. Samples were stored at -80°C until processing. Muscles were homogenized in a buffer containing (in mM) 10 MgCl₂, 10 KH₂PO₄, 1 EDTA, 5 EGTA, 50 β-glycerolphosphate, and 10 okadaic acid; (in μg/ml) 10 phenylmethylsulfonyl fluoride, 10 leupeptin, 10 aprotinin, and 10 Na₃VO₄; and 1% Nonidet NP-40. Protein concentration was quantified with a detergent-compatible protein assay kit (DC Protein Assay, Bio-Rad, Hercules, CA). For immunoblots, aliquoted supernatants (10 μg for p70^{S6k}, 15 μg for p38, and 35 μg for ERK and PKB) were boiled for 5 min at 100°C in an equal amount of Laemmli sample buffer and resolved by SDS-PAGE (23). Running gel composition was 7.5% for p70^{S6k} and 12% for PKB, ERK, and p38. Proteins were transferred to nitrocellulose (PKB, ERK, and p38) and polyvinylidene difluoride (p70^{S6k}) membranes (43), blocked in 5% milk in Tris-buffered saline (pH 7.5) and 0.1% Tween 20 and probed with the corresponding antibodies as described below. Phosphorylated proteins were identified with antibodies that recognize specific amino acid residues in the activated form of these proteins. Antibodies for phospho-ERK (1:2,000), phospho-PKB (Thr³⁰⁸) (1:2,000), and phospho-p38 (1:2,000) were from New England Biolabs (Beverly, MA). Analysis of p70^{S6k} (1:5,000) (Santa Cruz Biotechnology, Santa Cruz, CA) phosphorylation was determined by using an antibody that detects both phosphorylated and unphosphorylated forms of the protein. Changes in p70^{S6k} phosphorylation were analyzed based on the characteristic mobility shift caused by the different phosphorylation states of the protein. After phospho-specific analysis, blots were stripped with 1 × Re-Probe (Geno Tech, St. Louis, MO) and incubated with the following antibodies: ERK (1:1,000) and p38 (1:1,000) from New England Biolabs, and PKB (1:5,000), which was a generous gift of Dr. Morris Birnbaum (Howard Hughes Medical Center, Univ. of Pennsylvania). Anti-rabbit secondary antibodies were from Vector Laboratories (1:5,000) and New England Biolabs (1:3,000). Membranes were subsequently stained to verify loading conditions and protein integrity. Muscles from insulin-treated animals were used as internal controls for each Western blot (data not shown).

Enhanced chemiluminescence and densitometric analysis. Protein immunoblots were visualized by enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech, Uppsala, Sweden), and quantification was performed by scanning densitometry (Alpha Scan, San Leandro, CA). The sizes of the immunodetected proteins were verified by using standard molecular-weight markers (Bio-Rad, Hercules, CA).

Statistical analysis. Means ± SD were calculated from five observations per group. The effect of treatment on protein phosphorylation was calculated from the mean changes in each group vs. the corresponding control values. A one-way ANOVA was used to determine statistical differences. Group differences were determined with Tukey's honestly significant difference post hoc analysis test. Statistical significance was established at *P* < 0.05. All data were analyzed using the Sigma Stat software (version 2.03, SPSS).

RESULTS

To verify the effectiveness of the different exercise protocols, we analyzed skeletal muscle glycogen levels immediately after an acute bout of exercise (Fig. 1). The HFES caused a significant reduction in muscle glycogen both in the TA (77.9 ± 8.8%) and in the Sol (51 ± 2.9%) (*P* < 0.05). A significant decrease was also observed in the TA after one bout of LFES (51 ± 4.8%). As expected, no changes in muscle glycogen concentration were found in the nonstimulated Sol. The RN protocol was also effective in reducing muscle glycogen levels in both TA (34.7 ± 5.2%) and Sol (57.2 ± 15.1%) (*P* < 0.05).

Intracellular signaling in response to HFES. The effects of the HFES protocol on intracellular signaling in the TA and Sol muscles are shown in Figs. 2 and 3, respectively. PKB phosphorylation was significantly elevated (266 ± 38%) from control levels in the TA immediately postexercise (*P* < 0.05), with phosphorylation values returning to control by 3 h. No effect on PKB phosphorylation was observed in the Sol muscle at any of the time points studied. As previously reported, the effects of the HFES protocol on p70^{S6k} phosphorylation were significant in the TA muscle at 3 h (450 ± 40%) and 6 h (380 ± 72%) postexercise (*P* < 0.05). No changes in p70^{S6k} phosphorylation were seen in the Sol muscle with this protocol. ERK and p38 showed similar changes in phosphorylation in both muscles and as a function of time. Both ERK (200 ± 50 and 180 ± 61%) and p38 (360 ± 90 and 200 ± 58%) were significantly elevated at 0 h in the TA and Sol muscles, respectively (*P* < 0.05), with the phosphorylation states of both kinases returning to control levels by 3 h postexercise.

Intracellular signaling in response to LFES. The LFES protocol was also sufficient to induce changes in intracellular signaling (Fig. 4). Like HFES, a single bout of LFES caused a significant increase in PKB phosphorylation (157 ± 40%) in the TA muscle only at 0 h. Similar to the HFES protocol, p70^{S6k} phosphorylation (415 ± 75%) in the TA muscle was significant

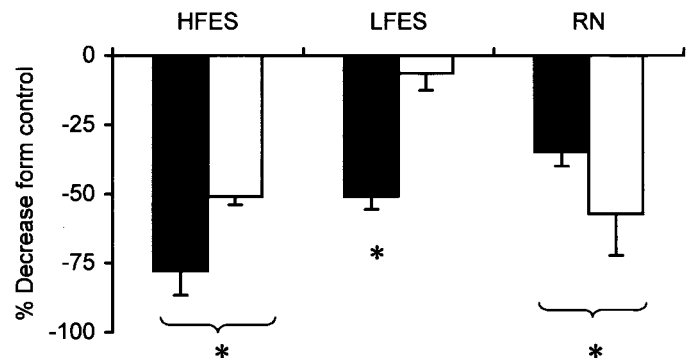


Fig. 1. Muscle glycogen levels in the tibialis anterior (TA; solid bars) and soleus (Sol; open bars) muscles after a single bout of contractile activity. Muscle glycogen was determined as described in METHODS AND EXPERIMENTAL DESIGN. HFES, high-frequency electrical stimulation; LFES, low-frequency electrical stimulation; RN, running exercise. *Significant from control (*P* < 0.05).

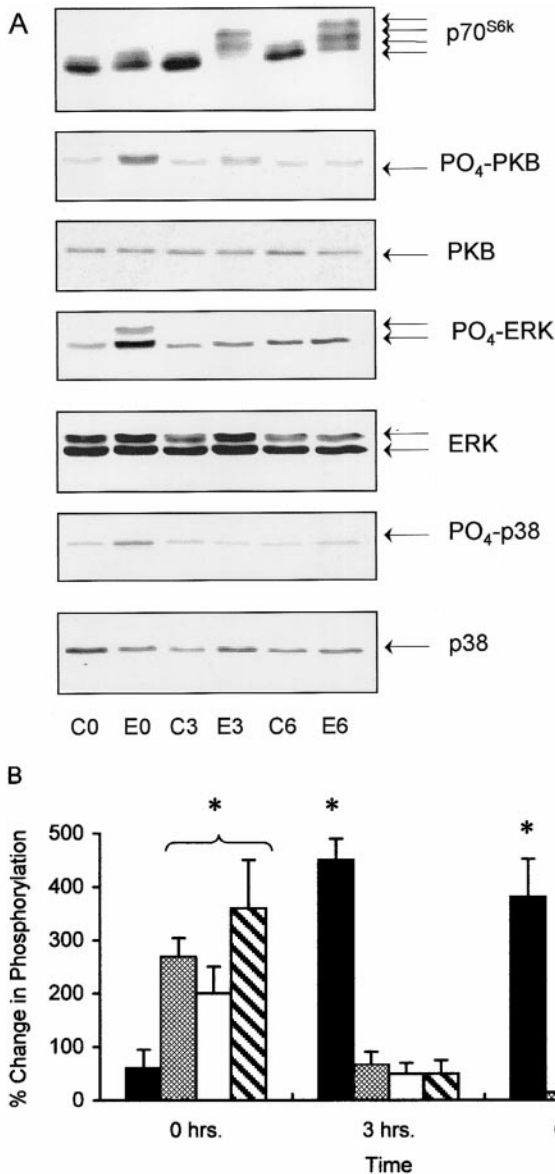


Fig. 2. A: representative Western blots from the TA muscle in response to an acute bout of HFES. C, control; E, exercise; 0, 3, and 6: hours after exercise; p70^{S6k}, 70-kDa ribosomal S6 kinase; PKB, protein kinase B; ERK, extracellular regulated kinase; p38, 38-kDa stress-activated protein kinase. B: protein phosphorylation changes in the TA muscle in response to an acute bout of HFES. Solid bars, p70^{S6k}; cross-hatched bars, PKB; open bars, ERK; and hatched bars, p38. *Significant from control ($P < 0.05$).

elevated at 3 h. However, this increase was transient, returning to basal levels by 6 h postexercise ($P < 0.05$). ERK ($200 \pm 54\%$) and p38 ($280 \pm 62\%$) phosphorylation were also elevated from control immediately post-exercise, with values not different from control thereafter ($P < 0.05$). As expected, no changes in protein kinase phosphorylation were detected in the Sol muscle with this model because this muscle did not receive any direct stimulation (data not shown).

Intracellular signaling in response to RN. No changes in PKB or p70^{S6k} phosphorylation were found after an acute bout of running in either TA (Fig. 5) or

Sol (Fig. 6) muscles. Nevertheless, this protocol resulted in significant increases in both ERK and p38 phosphorylation in the TA (125 ± 46 and $127 \pm 35\%$) and Sol (182 ± 25 and $208 \pm 31\%$) muscles at 0 h ($P < 0.05$). At 3 h postexercise, the phosphorylation levels of both kinases were not different from control. Surprisingly, at 6 h postexercise, ERK phosphorylation was significantly elevated from control levels in both TA ($102 \pm 23\%$) and Sol ($141 \pm 37\%$) muscles ($P < 0.05$). There was also a trend for p38 to be increased at 6 h postexercise in both muscles ($P < 0.1$).

DISCUSSION

Several reports have shown that exercise is capable of inducing the activation of different intracellular

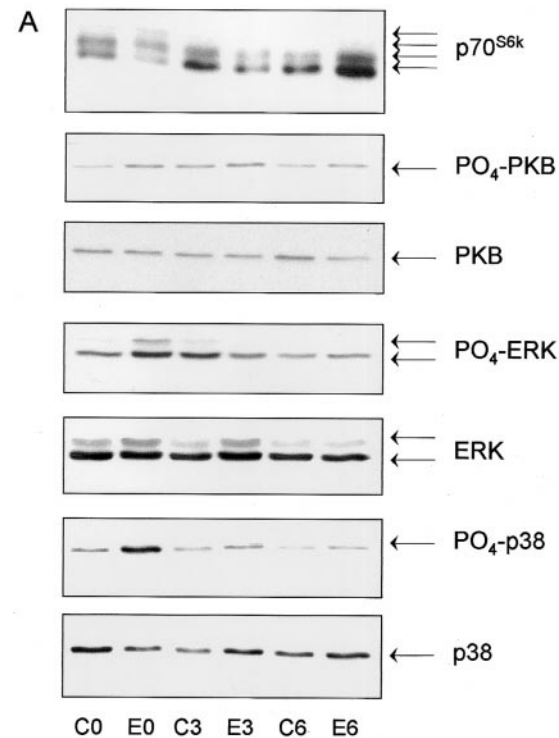


Fig. 3. A: representative Western blots from the Sol muscle in response to an acute bout of HFES. B: protein phosphorylation changes in the Sol muscle in response to an acute bout of HFES. Bars are as described in Fig. 2 legend. *Significant from control ($P < 0.05$).

Fig. 3. A: representative Western blots from the Sol muscle in response to an acute bout of HFES. B: protein phosphorylation changes in the Sol muscle in response to an acute bout of HFES. Bars are as described in Fig. 2 legend. *Significant from control ($P < 0.05$).

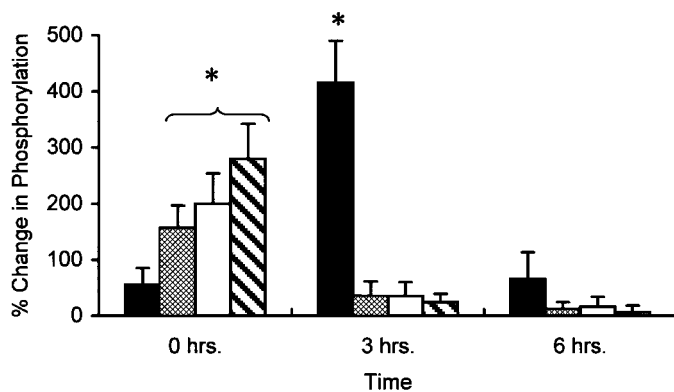


Fig. 4. Protein phosphorylation changes in the TA muscle in response to an acute bout of LFES. Bars are as described in Fig. 2 legend. *Significant from control ($P < 0.05$).

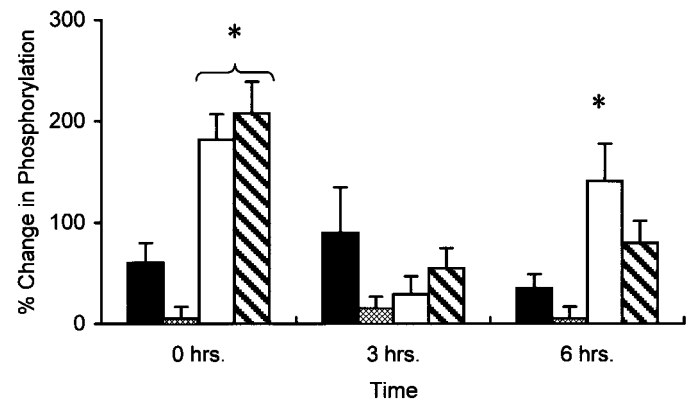


Fig. 6. Protein phosphorylation changes in the Sol muscle in response to an acute bout of RN. Bars are as described in Fig. 2 legend. *Significant from control ($P < 0.05$).

signaling pathways, but, despite these results, the specific intracellular signaling activation in response to different modes of exercise remains to be elucidated. A unique aspect of this study is the utilization of well-characterized exercise protocols known to induce distinct phenotypic adaptations (described in Table 1). These allowed us to determine 1) whether a single bout of exercise is capable of inducing the activation of multiple signaling pathways, and 2) whether different exercise protocols will result in the activation of specific intracellular signaling events.

By analyzing the responses of $p70^{S6k}$ to an acute bout of HFES, LFES, and RN in both TA and Sol, and based on the known phenotypic adaptations resulting from these exercise paradigms, we propose that prolonged $p70^{S6k}$ phosphorylation may represent one of the mechanisms mediating exercise-induced skeletal muscle hypertrophy. After an acute bout of HFES, an increased and prolonged $p70^{S6k}$ phosphorylation was observed in the TA but not in the Sol muscle. As previously shown by Baar and Esser (2), when a training program based on this type of stimulus is repeated for 6 wk, muscle hypertrophy results in the TA but not in the Sol muscle. Surprisingly, an acute bout of LFES caused a significant increase in $p70^{S6k}$ phosphorylation

in the TA muscle only at 3 h postexercise, whereas an acute bout of RN had no effect on $p70^{S6k}$ phosphorylation in either TA or Sol. A likely explanation for the differential responses of $p70^{S6k}$ in the TA muscle after an acute bout of LFES and RN might reflect the intrinsic characteristics (e.g., intensity) of these two models of endurance exercise (39).

The involvement of $p70^{S6k}$ in cellular hypertrophy was previously described in other cell types. A prolonged activation of this kinase has been observed in response to growth-inducing agents, such as overload (24), growth factors (3), and high-resistance exercise (2, 16). Along these lines, the pharmacological or genetic disruption of this kinase inhibits cellular growth. Administration of rapamycin-ameliorated cardiac myocyte growth in response to phenylephrine (3) and mutations in the drosophila $p70^{S6k}$ ($dS6k$) gene resulted in a reduced cell size with no effect on cell number (30). These results, together with our data, provide compelling evidence for a role of $p70^{S6k}$ in cellular growth regulation and exercise-induced skeletal muscle hypertrophy.

Because PKB is associated with the regulation of $p70^{S6k}$, it is not surprising that PKB phosphorylation was consistently increased in the TA muscle after an acute bout of HFES and LFES exercise. Similar results were reported by Turinsky and Damrau-Abney (44) in which PKB activity increased immediately after a single bout of exercise in the gastrocnemius muscle. Unlike the HFES and LFES protocols, RN exercise did not result in either $p70^{S6k}$ or PKB phosphorylation at any of the studied time points. This is, however, consistent with previous results in which RN exercise did not affect PKB or $p70^{S6k}$ activity (12, 27).

Among the factors that activate PKB are insulin and insulin-like growth factor-I, indicating its role in mediating the responses to mitogenic stimulation (1, 35). For instance, PKB plays a role in protein synthesis because it is the inactivating kinase that regulates glycogen synthase kinase-3 β (6), an inhibitor of the eukaryotic initiation factor-2B (19). PKB also regulates mTOR (33, 40), which in turn is known to be directly involved in the phosphorylation of $p70^{S6k}$ and the

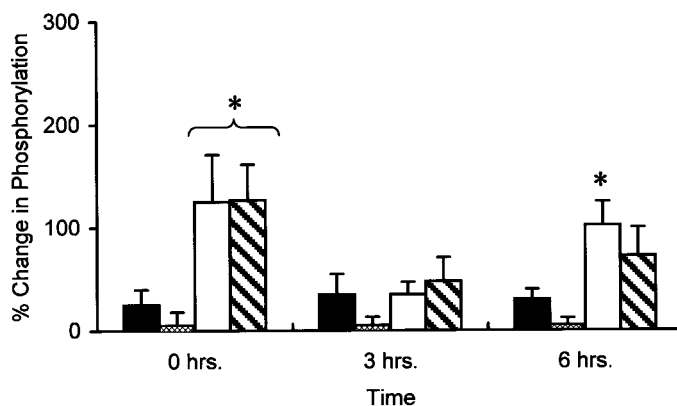


Fig. 5. Protein phosphorylation changes in the TA muscle in response to an acute bout of RN. Bars are as described in Fig. 2 legend. *Significant from control ($P < 0.05$).

eukaryotic initiation factor-4E binding protein 1 (4E-BP1/PHAS-1) (20, 31). Despite these observations, the responses of PKB to exercise remain controversial. This may result from the fact that different loads, stimulation patterns, tissue sampling procedures, and time points were utilized and analyzed in the different studies. Nevertheless, it is clear that the changes in PKB phosphorylation, as reported in this study, are also specific to the type of exercise stimulus.

The ERK and p38 pathways are implicated in the regulation of protein synthesis and gene transcription by a wide variety of stimuli (22, 45). In this study, all forms of exercise increased ERK and p38 phosphorylation immediately postexercise, suggesting that the activation of these pathways may be a component of a general response to exercise. Recent reports have shown that ERK and p38 are activated during exercise, indicating their involvement in the stress response caused by increased contractile activity (13, 47). The role of ERK and p38 in exercise-induced adaptations has not been directly determined yet, but some evidence suggests that these kinases may have multiple functions in regulating the response(s) to contractile activity. ERK and p38, via their downstream targets MAPK-activated protein-1 (p90^{RSK}) and MAPK-activated protein-2, activate the recently discovered mitogen and stress-activated protein kinase 1 (Msk 1) (8, 38), which, in turn, phosphorylates CREB and other transcription factors known to increase the activity of immediately early genes (22, 48). Moreover, immediately early gene expression has been shown to increase in response to several distinct forms of contractile activity (7, 29, 46), supporting the hypothesis that ERK and p38 are general mediators of the stress response to exercise.

A surprising and novel finding of this study is that treadmill running resulted in a significant increase in ERK phosphorylation at 6 h postexercise. This suggests that the activity of this kinase might be involved in specific adaptations induced by running exercise. Murakami et al. (32) reported that an acute bout of endurance running caused an increase in nuclear respiratory factor-1 mRNA levels in the Sol muscle 6 h postexercise (31). This observation may provide a link among ERK activation, nuclear respiratory factor-1 gene expression, and the adaptive response to endurance exercise. Another suitable mechanism that may link the ERK pathway to endurance adaptations is through the regulation transcription factors, such as activator protein-1 (AP1), CREB, and/or activating factor-2 (ATF) (48). For instance, the cytochrome *c* promoter contains functional binding sites for these transacting elements, suggesting potential ERK targets in the adaptations induced by endurance exercise (10, 14). Importantly, the expression of this gene is involved in mitochondrial biogenesis, a selective adaptation characteristic of endurance training (17). Because ERK and p38 were activated in a similar way immediately postexercise, we conclude that the activation of these pathways represents a general response to an exercise stimulus. However, the biphasic induction of ERK, as

reflected by its activation 6 h after endurance running, suggests that this kinase may play a role in the specific adaptations induced by endurance exercise. It is not possible to rule out whether this response was induced by a circulating or a local factor(s). The upstream effectors of ERK and p38 after a bout of endurance exercise remain to be identified. As mentioned earlier, the differences between RN and LFES may have occurred as a result of the different characteristics of these models (39).

The results reported in this study provide, for the first time, experimental evidence demonstrating that different forms of exercise result in the activation of specific signaling pathways. Because the adaptations to exercise training are specific to the exercise stimulus, we conclude that intracellular signaling selectivity is one of the mechanisms regulating specific exercise-induced adaptations in skeletal muscle.

The technical assistance of Dr. Keith Baar is greatly appreciated. The authors also thank Drs. Warren K. Palmer and Shann Kim for valuable suggestions on the manuscript.

This work was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-45617.

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