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Exercise Stimulates c-Jun NH_2 Kinase Activity and c-Jun Transcriptional Activity in Human Skeletal Muscle

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Exercise causes selective changes in gene expression leading to alterations in the structure and function of human skeletal muscle. However, little is known about the specific signaling pathways that enable exercise to modulate gene regulatory events. We determined the effects of exercise on c-Jun NH_2-terminal kinase (JNK) activity, a signaling molecule involved in the regulation of transcription. Biopsies of vastus lateralis muscle were taken from eight subjects at rest and after 60 min of cycle ergometer exercise. Exercise increased JNK activity in all subjects (5.9 ± 1.8 fold above basal). JNK activation was associated with an increased expression of its downstream nuclear target c-*Jun* mRNA. When two additional subjects were studied using a one-legged exercise protocol, JNK activity increased only in the exercising leg, indicating that exercise-induced JNK signaling represents an intrinsic response of the contracting muscle, rather than a systemic response to exercise. These studies demonstrate that the JNK pathway may serve as a link between contractile activity and transcriptional responses in human skeletal muscle. © 1998 Academic Press

Endurance exercise causes a selective increase in gene expression, leading to the synthesis of specific muscle proteins (1, 2). This reprograming of gene expression results in the induction of proteins involved in oxidative metabolism (3, 4), and an increase in the expression of the slow/cardiac isoforms of proteins of the contractile apparatus (5). The physiological consequences of these adaptations render the muscle resistant to fatigue during sustained contractile work.

Little is known about the molecular signaling mechanisms that convert skeletal muscle contractile activity into biochemical and gene regulatory responses. These putative mechanisms should be able to relay the "exercise" signals to the transcriptional machinery in the nucleus, thus modulating gene expression (6). Furthermore, although changes in the contractile properties of skeletal muscle result from the cumulative effects of repeated bouts of exercise, the initial signaling events that result in long-term adaptations should occur with each individual exercise bout.

The c-Jun-NH_2-terminal kinase (JNK) pathway typically mediates cellular responses to environmental stressors (7–10). JNKs are activated through phosphorylation at conserved Thr-183 and Tyr-185 residues (11) by the dual-specificity enzymes mitogen-activated protein kinase (MAPK) kinase 4 (MKK4) (9, 12), and MKK7 (13). MKK4 is in turn activated by MAPK kinase kinase 1 (MEKK1)(8, 14). JNKs can be stimulated by a variety of cellular stresses such as UV radiation (10), osmotic (15) and heat shock (10, 16). JNKs are also strongly activated by pro-inflammatory cytokines including TNF-α (17) and interleukin-1 (10). Once activated, JNKs can translocate to the nucleus (18), and their immediate downstream targets include a variety of transcription factors, notably c-Jun (10, 12) and ATF-2 (19).

Recent studies have noted the induction of early response (IE) genes, including c-*jun* and c-*fos* in response to contractile activity in rat (20), rabbit (21), and human (22) skeletal muscle. These gene products have been postulated to control the expression of downstream genes that define muscle phenotype ("late responsive genes", e.g. myosin isoforms)(1). We hypothesized that the JNK pathway may provide a plausible mechanism for the conversion of mechanical stimuli into transcriptional responses through the activation of IE genes (20). In this study we show that exercise stimulates JNK activity in human skeletal muscle, and that the activation of JNK signaling is temporally related to the upregulation of c-*jun* mRNA expression.

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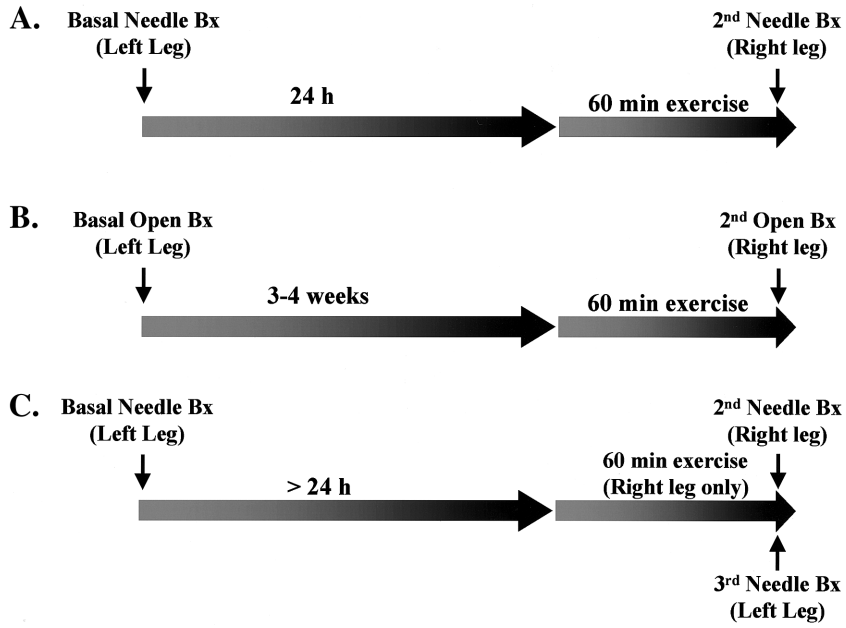

FIG. 1. Study protocols. Bx - biopsy.

METHODS

Experimental Protocols

The study population included 10 healthy volunteers (8 males and 2 females) age 19-51 y (30 ± 12; mean ± SD). The study was approved by the institutional Human Research Committees. The procedures involved in the study were fully described, and informed consent was obtained from each subject. To determine the appropriate intensity for the acute bout of exercise, subjects underwent maximal oxygen consumption ($VO_{2\ MAX}$) testing prior to participation in the study protocol. The study consisted of two experimental protocols (Figure 1).

Protocol 1. Four subjects were studied at rest and following 60 min of cycle ergometer exercise at a load corresponding to 70% of their VO_2 max. Each subject was instructed not to perform any exercise for 72 h before the study. Basal needle muscle biopsies were obtained from the left vastus lateralis muscle (see below). Twenty four hours later a second biopsy was taken from the right vastus lateralis muscle approximately 3–4 min after completion of 60 min of exercise (Figure 1A).

Protocol 2. Four additional subjects were studied using a similar protocol, except that open muscle biopsies (see below) were obtained and the interval between the basal and post-exercise biopsies was 3–4 weeks. The open biopsies provided a larger amount of tissue required for Northern blot analysis (Figure 1B).

Protocol 3. To determine whether the observed effects of exercise on the JNK signaling pathway occur as a result of the systemic release of hormones or cytokines during exercise, or are restricted to the exercising muscles, two subjects were studied using a one-legged exercise protocol (Figure 1). This protocol was similar to protocol 1 except that subjects exercised with one leg (right) at a relative work intensity of 70% of the one-legged VO_2 max. At the end of the exercise bout, biopsies of vastus lateralis muscle were taken from both the exercising and non-exercising leg (Figure 1C).

Muscle Biopsy Procedures

Needle biopsies. Samples of vastus lateralis muscle were obtained using a percutaneous needle-biopsy (23). The needle was inserted through an incision made in the skin using a local anesthetic (1% Lidocaine hydrochloride) and sterile conditions. Once the muscle tissue was removed, it was quickly dissected free from the surrounding fat and connective tissue, and immediately placed in liquid nitrogen and stored at −80°C.

Open biopsies. A local anesthetic was infiltrated subcutaneously down to, but not into the muscle. An incision was made and the subcutaneous tissue was dissected down to the muscle fascia. The fascia was opened and the vastus lateralis fibers carefully separated. A bundle of muscle fibers was dissected and clamped with a forked hemostat, cut, and immediately placed in liquid nitrogen.

Muscle Processing

For JNK activity assays, the muscles were powdered and then Polytron homogenized in ice-cold lysis buffer containing 20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 10 μM leupeptin, 3 mM benzamidine, 5 μM pepstatin A, 10 μg/ml aprotinin, and 1 mM phenylmethylsulphonyl fluoride (PMSF). Homogenates were rotated for 1 h at 4°C, and centrifuged at 14,000 × g for 60 min at 4°C to remove insoluble matter. Protein concentrations in the soluble extracts were estimated by the Bradford method (24). For Northern blotting, total RNA was extracted from muscle using TRI REAGENT according to the manufacturer's protocol.

Immunoblotting

Muscle proteins (300 μg) were solubilized in Laemmli's buffer and boiled for 5 min. Samples were then resolved on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose paper, and immunoblotted with anti-JNK. The filters were then probed with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2500) and antibody binding was detected via the method of enhanced chemiluminescence (ECL).

JNK Activity Assay

Aliquots (250 μg) of precleared muscle extracts were immunoprecipitated with 1.0 μg of polyclonal anti-JNK1. The immunoprecipi-

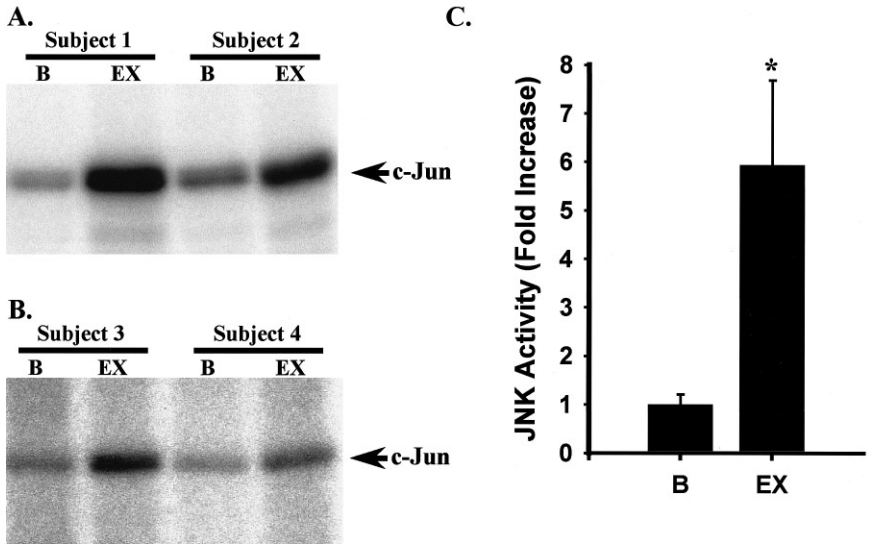

FIG. 2. Effects of an acute bout of exercise on JNK activity. Muscle extracts were immunoprecipitated with anti-JNK, and the immunoprecipitates were subjected to an *in vitro* kinase assay using GST-c-Jun-(1-135) as substrate. Phosphorylated proteins were separated by SDS-PAGE followed by autoradiography. (A) Representative immunoblot of 2 subjects that underwent needle biopsy (B) Representative immunoblot of 2 subjects that underwent open biopsy. (B) B, baseline; EX, post-exercise. (C) Quantitation of JNK activity showing combined results from protocol 1 and 2. Data are mean ± SE, n=8.

tates were washed twice with lysis buffer, twice with LiCl buffer, and twice with JNK buffer (20 mM Mops pH 7.2, 2 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100). The immunoprecipitates were resuspended in 50 μL JNK buffer containing 1 μg GST-Jun-(1-135), 50 μM ATP, and 10 μCi of [γ-^{32}P]ATP. The kinase reaction was performed at 30°C for 30 min and terminated with Laemmli sample buffer. The reaction products were resolved using 10% SDS-PAGE, gels were dried and the phosphorylated GST-c-Jun (42–44 kD) was quantitated by PhosphorImager and ImageQuant software (Molecular Dynamics, Inc.).

Northern Blot Analysis

Aliquots (10 μg) of total RNA were denatured and size-separated by electrophoresis in a 1% agarose-6.6% formaldehyde gel and transferred to a nylon membrane in 10X standard sodium citrate (SSC; 1X = 0.15 M sodium chloride and 0.015 M sodium citrate). Equal loading of RNA was confirmed by ethidium bromide staining of the gels. RNA was UV cross-linked to the membrane, and the blots were hybridized with 1.8-kb EcoRI fragment of the rat c-jun cDNA probe, labeled with [α-^{32}P]-dCTP to ~10^9 dpm/mg using a multiprime DNA labeling kit. The membranes were washed in 2× SSC/0.1% SDS, at room temperature. The blots were then quantitated by PhosphorImager.

Statistical Analysis

Differences between basal and post-exercise points were determined using a paired Student's t-test. $P < 0.05$ was considered statistically significant.

Reagents

γ-[^{32}P]-ATP (3000 Ci/mmol) was purchased from Du Pont-New England Nuclear (Boston, MA). A PGEX vector designed to express a glutathione S-transferase (GST)-c-Jun fusion protein (amino terminal residues 1-135) was provided by Dr. John Kyriakis, Massachusetts General Hospital. Anti-JNK1, was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and protein A-agarose was from Pierce (Rockford, IL). Zeta-Probe blotting membranes were from Bio-Rad laboratories (Richmond, CA). A multiprime DNA labeling kit was from Amersham (Arlington, IL), and TRI REAGENT was from Molecular Research Center, Inc. Protein concentrations were determined using a dye reagent from Bio-Rad. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Exercise stimulates JNK activity. JNKs are the only known protein kinases that efficiently phosphorylate Ser-63 and Ser-73 on the NH_2-terminal transactivation domain of c-Jun (10, 11, 25). To determine whether contractile activity increases JNK activity toward its physiologic substrate c-Jun, immune complex kinase assays were performed using c-Jun-(1-135) as substrate. In subjects participating in protocol 1, exercise increased JNK activity in all subjects, with the magnitude of stimulation ranging from 3.3- to 16.6-fold above basal (Figure 2A). No JNK activity was detected if anti-JNK were omitted from the assay or when the immunoprecipitation step was done using an irrelevant antibody (preimmune IgG). Similarly, in subjects who underwent open muscle biopsies, exercise increased JNK activity in all exercising subjects, with the degree of stimulation ranging from 2.0- to 10.0-fold above basal (Figure 2B). Overall, exercise resulted in a 5.9 ± 1.8 (mean ± SEM) fold increase in JNK activity ($p < 0.05$) (Figure 2C).

Because the basal and post-exercise biopsies were not taken on the same day, changes in JNK activity could result from altered expression of the JNK protein. To test this possibility we determined JNK expression in all muscle samples by immunoblotting.

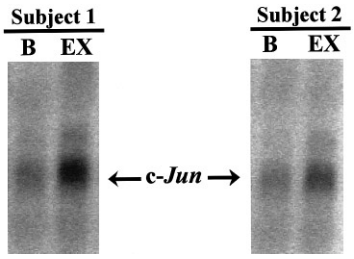

FIG. 3. Expression of c-*jun* mRNA in response to contractile activity. Total RNA was extracted from muscle and 10 μg of total RNA was separated by 1% agarose gels. Northern blot analysis was performed by using the ^{32}P cDNA probe for c-jun. B, baseline; EX, post-exercise.

These experiments demonstrated no difference in the amount of the JNK protein between the first and second biopsy (data not shown).

Effect of exercise activity on c-jun mRNA expression. JNKs phosphorylate two serine residues in the transactivation domain of c-Jun leading to an increase in its transcriptional activity (10, 11, 25). Thus, we determined if exercise-induced JNK activation is associated with an increase in c-*jun* mRNA levels. Northern blot analysis of muscle samples taken from the 4 subjects who underwent open biopsies revealed that exercise resulted in a 1.8 $\pm$ 0.4 fold increase in c-*jun* mRNA (Figure 3). These experiments demonstrate that exercise-induced JNK activation is accompanied by an increase in c-*jun* expression in human skeletal muscle.

Activation of the JNK signaling is restricted to exercising muscle. Physical activity is known to elicit an endocrine stress response that involves a surge like secretion of stress hormones and cytokines (26). Pertinent to the mechanism of exercise-induced JNK activation is the release of cytokines during exercise which are potent JNK activators, such as interleukin-1 (27). Thus, the observed exercise-induced JNK activation could be mediated through the systemic release of cytokines. We addressed this question by using a one-legged exercise protocol which allows matched comparisons within the same subject and controls for the systemic effects of physical activity.

In subjects performing protocol 3 the increase in JNK activity was limited to the exercising leg, whereas no significant effect on JNK activity was observed in the contralateral leg (Figure 4). These results suggest that activation of the JNK signaling cascade occurs primarily as a local event in the exercised muscle rather than as a systemic, cytokine-mediated response.

DISCUSSION

The functional adaptations that occur in skeletal muscle in response to the cumulative effects of repeated bouts of exercise entails a selective reprogramming of gene expression leading to the induction of critical muscle proteins (1, 2). Thus, an exercise-responsive signaling mechanism that enables skeletal muscle cells to regulate gene expression is necessary. Furthermore, the *initial* signaling events that result in long-term adaptations should occur with each individual exercise bout. Previous signaling studies in skeletal muscle have been done in the context of muscle hypertrophy (28, 29), and have not elucidated a signaling mechanism primarily involved in the regulation of gene expression.

Williams and Neufer (1) have proposed two major criteria for candidate signals that may control exercise-induced gene regulatory events. First, the putative effector and/or putative pathway must be altered by contractile activity in skeletal muscle, and second, the pathway must be known to regulate gene expression, if not in contracting muscle, at least in other well defined biological systems (1). We believe that a third criteria must be met: the effect of exercise on the putative signaling pathway should be confined to the exercising muscle and thus independent of the systemic effects of physical activity. This is based on studies showing that the signal or inducer for the adaptive processes in skeletal muscle appears to be endogenous rather than a systemic factor. For example, in human subjects, if only a single leg undergoes aerobic training on a cycle ergometer, mitochondrial density increases only in the trained leg (2). Likewise, increased specific gene expression (21) and protein synthesis (30) are restricted to the contracting or overloaded muscle.

The data presented in the current study demonstrate that exercise-stimulated JNK signaling meets all of these criteria. First, JNK activity consistently increased with exercise in all ten subjects. Second, the increase in JNK activity was associated with the induction of c-*jun* mRNA levels. It is well established that the general function of the JNK cascade is to relay environmental signals to the transcriptional

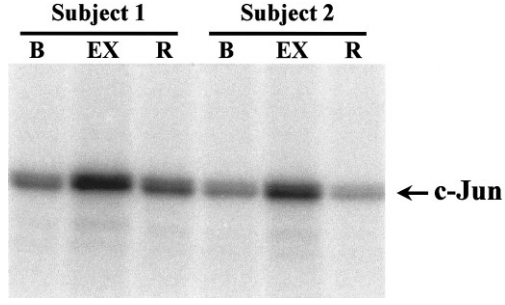

FIG. 4. Activation of JNK signaling is restricted to exercising muscle. JNK activity in the two subjects who underwent the one-legged exercise protocol. Exercise stimulates JNK activity only in the exercising leg. B, baseline; EX, post-exercise; R, rest.

machinery in the nucleus and involves the activation of c-*Jun* (31, 32). Finally, using the one-legged exercise protocol, we have shown that the stimulation of JNK activity is restricted to the exercising leg. These results suggests that exercise-induced JNK signaling represents an intrinsic response of the contracting muscle, rather than a systemic response to exercise. This concept of local activation of JNK in contracting muscles is also consistent with our previous findings showing increased JNK activity in hindlimb rat skeletal muscles contracted by electrical stimulation of the sciatic nerve (20).

We recently reported that exercise activates the mitogen activated protein kinase pathway in human skeletal muscle (33), which is also involved in the regulation of transcription factors (31, 32). The induction of genes encoding for transcription factors is the earliest response of skeletal muscle to contractile activity at the gene level (1, 2, 21), and presumably represents transduction of early nuclear signals to longer-term changes in gene expression (1). For example, c-*jun* and c-*fos* are involved in the induction of structural muscle proteins such as skeletal alpha-actin (34).

In summary, we have shown that an acute bout of exercise results in a contraction-dependent stimulation of JNK activity and the induction of its downstream nuclear target c-*Jun* in human skeletal muscle. These results suggest that JNK may serve as cytoplasmic element for contraction-related information transfer to the nucleus in human skeletal muscle. Our findings also emphasize the potential role of JNK signaling in physiological processes outside the context of extreme cellular stresses.

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REFERENCES

1. Williams, R. S., and Neufer, P. D. (1996) *in* Handbook of Physiology, Section 12: Exercise: Regulation and integration of multiple systems (Rowell, L. B., and Shephard, J. T., Eds), pp. 1124–1150, Oxford University Press, New York.
2. Booth, F. W., and Thomason, R. B. (1991) *Physiol. Rev.* **75,** 541–585.
3. Williams, R. S., Garcia-Moll, M., Mellor, J., Salmons, S., and Harlan, W. (1987) *J. Biol. Chem.* **262,** 2764–2767.
4. Williams, R. S., Salmons, S., Newsholme, E. A., Kaufman, R. E., and Mellor, J. (1986) *J. Biol. Chem.* **261,** 376–380.
5. Brown, W. E., Salmons, S., and Whalen, R. G. (1983) *J. Biol. Chem.* **258**(23)**,** 14686–14692.
6. Vandenburgh, H. H. (1992) *Am. J. Physiol.* **262,** R350-R355.
7. Kyriakis, J. M., and Avruch, J. (1996) *J. Biol. Chem.* **271,** 24313–24316.
8. Yan, M., Dai, T., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R., and Templeton, D. J. (1994) *Nature* **372,** 798–800.
9. Sánchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M., and Zon, L. I. (1994) *Nature* **372,** 794–798.
10. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. W. (1994) *Nature* **369,** 156–160.
11. Dérijard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M., and Davies, R. J. (1994) *Cell* **76,** 1025–1037.
12. Dérijard, B., Raingeaud, J., Barrett, T., Wu, T., Han, I.-H., Ulevitch, J., and Davis, R. J. (1995) *Science* **267,** 682–685.
13. Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1997) *Proc. Natl. Acad. Sci. USA* **94**(14)**,** 7337–7342.
14. Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Dérijard, B., Davies, R. J., Johnson, G. L., and Karin, M. (1994) *Science* **266,** 1719–1722.
15. Galcheva-Gargova, Z., Dérijard, B., Wu, I.-H., and Davies, R. J. (1994) *Science* **265,** 806–808.
16. Adler, V., Schaffer, A., Kim, J., Dolan, L., and Ronai, Z. (1995) *J. Biol. Chem.* **270,** 26071–26077.
17. Sluss, H. K., Barrett, T., Dérijard, B., and Davis, R. J. (1994) *Moll. Cell. Biol.* **14,** 8376–8384.
18. Cavigelli, M., Dolfi, F., Claret, F. X., and Karin, M. (1995) *EMBO. J.* **14,** 5957–5964.
19. Gupta, S., Campbell, D., Dérijard, B., and Davies, R. J. (1995) *Science* **267,** 389–393.
20. Aronson, D., Dufresne, S. D., and Goodyear, L. J. (1997) *J. Biol. Chem.* **272**(41), 25636–25640.
21. Michel, J. B., Ordway, G. A., Richardson, J. A., and Williams, R. S. (1994) *J. Clin. Invest.* **94,** 277–285.
22. Puntschart, A., Wey, E., Jostarndt, K., Vogt, M., Wittwer, M., Widmer, H. R., Hoppeler, H., and Billeter, R. (1998) *Am. J. Physiol.* **274**(1 Pt 1)**,** C129–137.
23. Bergström, J. (1962) *Scand. J. Clin. Lab. Invest. Suppl.* **68,** 1–110.
24. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
25. Minden, A., Lin, A., Smeal, T., Derijard, B., Cobb, M., Davis, R. J., and Karin, M. (1994) *Mol. Cell. Biol.* **14,** 6683–6688.
26. Kindermann, W., Schnabel, A., Schmitt, W. M., Biro, G., Cassens, J., and Weber, F. (1982) *Eur. J. Appl. Physiol.* **49,** 389–399.
27. Cannon, J. G., and Kluger, M. J. (1983) *Science* **220**(4597), 617–619.
28. Vandenburgh, H. H., Shansky, J., Karlisch, P., and Solerssi, R.-L. (1993) *J. Cell. Physiol.* **155,** 63–71.
29. Vandenburgh, H. H. (1992) *Am. J. Physiol.* **262,** R350-R355.
30. Chesley, A., MacDougall, J. D., Tarnopolsky, M. A., Atkinson, S. A., and Smith, K. (1992) *J. Appl. Physiol.* **73,** 1383–1388.
31. Treisman, R. (1996) *Curr. Opin. Cell. Biol.* **8,** 205–215.
32. Whitmarsh, A. J., and Davis, R. J. (1996) *J. Mol. Med.* **74,** 589–607.
33. Aronson, D., Violan, M., Dufresne, S. D., Zangen, D., Fielding, R. A., and Goodyear, L. J. (1997) *J. Clin. Invest.* **99,** 1251–1257.
34. Bishopric, N. H., Jayasena, V., and Webster, K. A. (1992) *J. Biol. Chem.* **267,** 25535–25540.