Differential regulation of MAP kinase, p70S6K, and Akt by contraction and insulin in rat skeletal muscle

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Differential regulation of MAP kinase, p70S6K, and Akt by contraction and insulin in rat skeletal muscle. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E870–E878, 1999.—To study the effects of contractile activity on mitogen-activated protein kinase (MAP kinase), p70 S6 kinase (p70S6K), and Akt kinase signaling in rat skeletal muscle, hindlimb muscles were contracted by electrical stimulation of the sciatic nerve for periods of 15 s to 60 min. Contraction resulted in a rapid and transient activation of Raf-1 and MAP kinase kinase kinase 1, a rapid and more sustained activation of MAP kinase and the 90-kDa ribosomal S6 kinase 2, and a dramatic increase in c-fos mRNA expression. Contraction also resulted in an apparent increase in the association of Raf-1 with p21Ras, although stimulation of MAP kinase signaling occurred independent of Shc, IRS1, and IRS2 tyrosine phosphorylation or the formation of Shc/Grb2 or IRS1/Grb2 complexes. Insulin was considerably less effective than contraction in stimulating the MAP kinase pathway. However, insulin, but not contraction, increased p70S6K and Akt activities in the muscle. These results demonstrate that contraction-induced activation of the MAP kinase pathway is independent of proximal signals in insulin and/or growth factor-mediated signaling, and that contraction and insulin have discordant effects with respect to the activation of the MAP kinase pathway vs. p70S6K and Akt. Of the numerous stimulators of MAP kinase in skeletal muscle, contractile activity emerges as a potent and physiologically relevant activator of MAP kinase signaling, and thus activation of this pathway is likely to be an important molecular mechanism by which contractile muscle cells transduce mechanical and/or biochemical signals into downstream biological responses.

signal transduction; muscle contraction; exercise

Contractile activity plays a critical role in determining the biochemical and morphological characteristics of skeletal muscle. Chronic increases in contractile activity can lead to muscle hypertrophy and increased expression of numerous muscle proteins (13). These activity-induced changes in muscle phenotype may be regulated in part by the induction of "immediate-early" genes such as c-fos, c-jun, and egr-1 (22). Contractile activity can also alter the metabolic and morphological profile of the muscle by directly increasing rates of protein synthesis (4). Despite these profound effects of contractile activity in regulating muscle phenotype, little is known about the intracellular signaling mechanisms that link the biochemical and mechanical events of muscle contraction with the activation of translation and transcription.

Signaling through the classical mitogen-activated protein (MAP) kinase cascade involves the sequential phosphorylation and increased catalytic activity of the Raf-1 kinase, the MAP kinase kinases (MEK1 and MEK2), the MAP kinase isoforms p44MAPK (ERK1) and p42MAPK (ERK2), and the cytosolic p90 ribosomal S6 kinase (RSK) (26). In rat hindlimb skeletal muscle, insulin has been shown to modestly increase MAP kinase activity (12, 41), whereas in rat diaphragm muscle epidermal growth factor (EGF) is more effective than insulin in stimulating MAP kinase activity (3). Furthermore, we have demonstrated that physical exercise can increase MAP kinase signaling in both rat (8) and human (2) skeletal muscle. The physiological consequences of insulin, EGF, or exercise-stimulated increases in MAP kinase activity in skeletal muscle are not known. However, because a role for growth factor-induced MAP kinase stimulation in controlling carbohydrate metabolism and protein synthesis (3) has been ruled out, a primary function of MAP kinase signaling in skeletal muscle may be to regulate transcription, which in turn may regulate the expression of critical muscle proteins. In cultured cardiac myocytes, mechanical stretch can stimulate MAP kinase signaling (30, 38, 39), with some studies providing evidence that this increase in activity plays a role in stretch-induced hypertrophy (7, 39).

Activation of the MAP kinase pathway can occur through ras-dependent and -independent mechanisms, including G protein-coupled receptors (28) and numerous growth factors (26). Growth factors stimulate this signaling cascade through activation of tyrosine kinase receptors, Shc phosphorylation, association of phosphorylated Shc with the adapter protein Grb2, leading to Sos-mediated activation of Ras (32). Insulin-induced activation of the MAP kinase pathway can also occur through the association of tyrosine-phosphorylated insulin receptor substrate 1 (IRS1) with Grb2 (36). It is not known whether these molecules are involved in exercise-stimulated MAP kinase signaling.

The p70 S6 kinase (p70S6K) and Akt kinase (also referred to as RAC/PI3K) are alternative signaling proteins that could potentially link the biochemical and mechanical events of muscle contraction to downstream biological responses. These two serine/threo-
nine kinases can be activated by growth factors via complex mechanisms involving phosphoinositide 3-kinase (PI-kinase) and 3-phosphoinositide-dependent kinase (PKD1) (6). Interestingly, certain stimuli such as hyperosmolarity (31), growth hormone (31), heat shock (21), and reagents that increase cAMP (29) can increase Akt activity through a PI 3-kinase-independent mechanism. p70SGK is thought to play an important role in regulating protein synthesis by controlling the translation of numerous mRNA transcripts that encode components of the translational apparatus (18, 24). Akt mediates diverse cellular functions, including the inhibition of apoptosis (6) and possibly the stimulation of muscle contraction also increases glucose transport through the translocation of GLUT-4, although the signaling mechanism that mediates this response is not known (11).

In the current investigation, we studied the effects of contractile activity on MAP kinase, p70SGK, and Akt signaling in rat skeletal muscle. Our results demonstrate that neither p70SGK nor Akt is altered by muscle contractions. In contrast, contractile activity rapidly and potently stimulates the MAP kinase signaling cascade in rat skeletal muscle, and this stimulation occurs immediately before an increase in expression of c-fos mRNA. Because the MAP kinase pathway has been implicated in the regulation of protein synthesis and gene transcription in numerous cell types, activation of this cascade in response to contraction is likely to be a key mechanism in the regulation of these processes in skeletal muscle.

MATERIALS AND METHODS

Animals. For insulin treatment, male Sprague-Dawley rats (Taconic) were fasted for 16 h and then injected intraperitoneally with 20 U of porcine insulin (maximal dose). Control (basal) animals were injected with saline. Rats were decapitated, and hindlimb muscles, including biceps femoris, gastrocnemius, and soleus, were rapidly dissected and frozen in liquid nitrogen. For contraction studies, both control (basal) and treated (contraction) rats were anesthetized with sodium amobarbital (50 mg/kg body wt ip). The sciatic nerves to both hindlimbs were exposed, and electrodes were attached. For contraction treatment, hindlimb muscles were stimulated for 15 s or for 2, 5, 10, 15, 30, or 60 min with one 500-ms train/s. Each train consisted of one to three repeated V pulses of 0.1 ms duration delivered at 100 Hz. When the stimulation period exceeded 5 min, each additional 5-min period was separated by 2 min of rest. Hindlimb skeletal muscles were removed and frozen in liquid nitrogen. For p70SGK mobility shift studies, hindlimb muscles were powdered and then homogenized in 50 mM MOPS (pH 7.4), 5 mM EGTA, 2 mM EDTA, 75 mM β-glycerophosphate, 2 mM diithothreitol (DTT), 1 mM NaF, 2% Triton X-100, 10% glycerol, and 5 µM leupeptin, 3 mM benzamidine, 5 mM pepstatin A, 10 µg/ml apropin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 200 µg/ml soybean trypsin inhibitor. For all other assays, powdered muscle was homogenized in lysis buffer containing 20 mM HEPES (pH 7.4), 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM NaF, 1% Triton X-100, 10% glycerol, 10 µg/ml leupeptin, 3 mM benzamidine, 5 µM pepstatin A, 10 µg/ml apropin, 1 mM PMSF, and 200 µg/ml soybean trypsin inhibitor. For all studies, homogenates were mixed for 1 h at 4°C and centrifuged at 15,000 g for 1 h at 4°C. Protein concentration was determined on the supernatants by use of the Bradford dye-binding procedure, with BSA as standard (Bio-Rad).

Kinase activity assays. For RSK2 activity assays, 1 mg of rat muscle protein was preincubated with protein A-Sepharose beads for 1 h at 4°C. Supernatants were incubated with 3.5 µg/ml of α-RSK2 (Upstate Biotechnology (UBI) 06–300) for 1 h at 4°C, followed by adhesion of fresh protein A beads and overnight incubation at 4°C. RSK2 activity was measured in the immune complexes using the 3R S6 peptide substrate (RRRLSSLRRA) as previously described (8).

To measure MEK1 activity, muscle proteins (250 µg) were incubated with 1.25 µg α-MEK1 (Transduction Laboratories M17020) at 4°C for 2 h, followed by incubation with 40 µl of protein A-agarose. The immunoprecipitates were washed three times in buffer containing 150 mM NaCl, 20 mM Tris (pH 8.0), 10 mM NaF, 100 µM Na3VO4, 10% glycerol, 1% Nonidet P-40, and 2 µg/ml leupeptin, and 3 times in MEK1 buffer (25 mM HEPES (pH 7.5), 10 mM MgCl2, and 2 mM DTT). The immune complexes were resuspended in 80 µl MEK1 buffer containing 50 µM ATP, 10 µl of [γ-32P]ATP (3,000 counts·min·µmol−1), and a recombinant kinase-inactive human GST-MAPK (1 µg/tube) as substrate. For the Raf-1 activity assay, muscle proteins (250 µg) were incubated with 1.25 µg α-Raf-1 (Santa Cruz SC227) at 4°C for 2 h, followed by incubation with 4 µg/ml of protein A-Sepharose beads. Immunoprecipitates were washed three times in buffer containing 150 mM NaCl, 20 mM Tris (pH 8.0), 2 mM EDTA, 1 mM PMSF, 100 µM Na3VO4, 10 µg/ml apropin, 10% glycerol, 1% Nonidet P-40, and 2 µg/ml leupeptin, and three times with Raf-1 buffer (30 mM HEPES (pH 7.4), 10 mM MnCl2, 5 mM MgCl2, 100 µM Na3VO4, 25 mM β-glycerophosphate, 1 mM DTT, and Brij 35 0.003%). The Raf-1 immune complexes were resuspended in 50 µl of Raf-1 buffer containing 20 µM ATP, 10 µCi of [γ-32P]ATP, and 1 µg of recombinant kinase-inactive GST-MEK1 as substrate. For both the MEK1 and Raf-1 assays, reactions were incubated for 30 min at 30°C and were terminated by adding Laemmli sample buffer. Products were boiled for 5 min and resolved on 10% SDS-PAGE. Gels were dried, and the phosphorylated GST-MAPK (70 kDa) or GST-MEK1 (71 kDa) was quantitated by PhosphoiImager and ImageQuant software ( Molecular Dynamics). For the p70SGK activity assays, aliquots of muscle protein (1 mg) were incubated with 5 µl of α-p70SGK (UBI 06–322) for 1 h at 4°C. The immunocomplexes were washed twice in lysis buffer; 2 times in high salt buffer containing 1 M NaCl, 10 mM Tris (pH 7.2), 0.1% NP-40, 2 mM DTT, and 40 µg/ml PMSF; and once in 150 mM NaCl and 50 mM Tris (pH 7.2). The pellets were resuspended in kinase buffer containing 200 mM HEPES (pH 7.2), 100 mM MgCl2, and 1 mg/ml BSA. The reaction was carried out at 30°C in final concentrations of 0.3 µg GST-S6 protein/µl reaction volume, 0.1 µCi [γ-32P]ATP/µl reaction volume, and 50 µM ATP. After 10 min, the reaction was stopped by addition of Laemmli sample buffer containing 200 mM DTT. The labeled GST-S6 protein was separated by 12% SDS-PAGE. Gels were stained in Coomasie Blue, destained in 30% CH3OH-20% CH2COOH, dried, and exposed to film. Bands corresponding to phosphorylated GST-S6 were quantitated by phosphorimagier.

For the Akt activity assays, muscle proteins (0.5 mg) were incubated for 16 h at 4°C in 3 µg α-Akt (UBI 06–558) coupled to protein A beads in a buffer containing 20 mM HEPES (pH 7.4), 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM NaF, 1% Triton X-100, 10% glycerol, 10 µg/ml leupeptin, 3 mM benzamidine, 5 µM pepstatin A, 10 µg/ml apropin, 1 mM PMSF, and 200 µg/ml soybean trypsin inhibitor. For all studies, homogenates were mixed for 1 h at 4°C and centrifuged at 15,000 g for 1 h at 4°C. Protein concentration was...
washed 3 times in 20 mM Tris (pH 7.4), 5 mM EDTA, 10 mM NaPO₄, 100 mM NaF, 2 mM Na₂VO₄, and 1% NP-40, and 2 times in 20 mM Tris (pH 7.4), 10 mM MgCl₂, and 1 mM DTT. The pellets were resuspended in reaction buffer containing 50 mM Tris (pH 7.4), 10 mM MgCl₂, 1 mM DTT, 1 µM protein kinase inhibitor (Sigma), 30 µM cycloheximide (UBI), 3 µCi [γ-32P]ATP, and 5 mM cold ATP. The reaction was carried out for 30 min at 30°C. The reactions were stopped by spotting aliquots onto P81 phosphocellulose papers (in duplicate) followed by extensive washing with 75 mM H₃PO₄. Papers were dried, and radioactivity was counted using a scintillation counter.

Immunoblotting. Phosphorylation of MAP kinase was assessed by immunoblotting muscle lysates with an antibody (α-MAPK-PP) that recognizes only the phosphorylated forms of p44MAPK and p42MAPK (Quality Controlled Biochemicals). Immunoblotting was also used to detect changes in the electrophoretic mobility of the RSK2, Raf-1, and p70S6K proteins. Muscle proteins (300 µg for RSK2 and p70S6K, 100 µg for Raf-1 and α-MAPK-PP) were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and blocked in Tris-buffered saline plus NaN₃ (TNA) containing 10% BSA (RSK2, Raf-1, p70S6K) or 5% milk (α-MAPK-PP) for 1 h at room temperature. The membranes were incubated overnight at 4°C with α-RSK2 (0.4 µg/ml), α-Raf-1 (2 µg/ml), α-p70S6K (1 µg/ml), or α-MAPK-PP (1 µg/ml). Bound antibodies were detected with rabbit Ig-horseradish peroxidase, and the membranes were washed in Tris-buffered saline plus 0.05% Tween 20 and then incubated for 1 min with enhanced chemiluminescence (ECL) reagents.

Immunoprecipitation/Immunoblotting. To measure tyrosine phosphorylation of IRS1, IRS2, and Shc, aliquots of muscle proteins were incubated with α-IRS1 [5 µg (10)], α-IRS2 [10 µl (23)], or α-Shc [5 µl (Transduction Laboratories S14630)] for 16 h at 4°C. Immunoprecipitated proteins were separated by SDS-PAGE (10% gel), and tyrosine-phosphorylated proteins were detected by immunoblotting with an anti-phosphotyrosine antibody preparation (α-PY) [2 µg/ml (9)]. To assess Grb2-associated proteins, muscle lysates (1 mg) were incubated with 20 µg of GST-Grb2 fusion protein coupled to agarose beads for 4 h at 4°C, or with 1 µg/ml of α-Grb2 (Transduction Laboratories G16720) for 16 h at 4°C followed by incubation with protein A-Sepharose beads for 2 h at 4°C. The beads were washed twice in lysis buffer. Proteins were separated by SDS-PAGE (10% gel) and immunoblotted with α-Shc or α-IRS1. To study Raf-1/Ras association, aliquots of muscle protein (2 mg) were incubated with 1 µg/ml of α-Raf-1 for 16 h at 4°C. The samples were then incubated with protein A-Sepharose beads for 2 h at 4°C, and the immune complexes were washed 2 times in 50 mM HEPES, 100 mM NaF, 1% Triton X-100, 2 mM Na₂VO₄, and 0.1% SDS. Immunoprecipitated proteins were separated by SDS-PAGE (10% gel), transferred to nitrocellulose, immunoblotted with α-Ras (1 µg/ml; Santa Cruz SC-35, Oncogene Science OP23), and antibody binding was detected using ECL.

Northern blotting. Total RNA was extracted by TRI REAGENT according to the manufacturer’s protocol (Molecular Research Center). Twenty micrograms of total RNA per sample were electrophoresed in a 1% agarose formaldehyde gel and transferred to a nylon membrane. RNA was immobilized by ultraviolet cross-linking, and the blots were hybridized with a 32P-labeled rat c-fos probe labeled with [α-32P]dCTP to ~10⁹ dpm/mg with a random hexamer priming kit (Amer sham).

RESULTS

The stimulation of MAP kinase can occur in response to the sequential phosphorylation and activation of Raf-1 and MEK (26, 40), although under some conditions MAP kinase and RSK2 activation may not correlate with Raf-1 and MEK activation (40). Figure 1 shows the time course of activation of Raf-1, MEK1, and MAP kinase in response to contraction. To study the effects of contraction on Raf-1, we assessed Raf-1 activation by electrophoretic mobility shift of the protein by immunoblotting with an anti-Raf-1 antibody. The representative immunoblot in Fig. 1A shows that with 15 s of contraction there was reduced electroph-
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Retic mobility of the Raf-1 protein, and with prolonged periods of contraction the change in mobility was no longer present. Similarly, when Raf-1 activity was measured using an immune complex assay, contraction resulted in a rapid increase in enzyme activity that was no longer present with longer periods of contraction (data not shown). Similar to the rapid activation of Raf-1, MEK1 activity was stimulated with only 15 s of muscle contraction and rapidly returned to baseline (Fig. 1 B). The effects of contractile activity on MAP kinase phosphorylation was measured by immunoblotting muscle lysates with a MAPK-PP, an antibody that recognizes only the phosphorylated MAP kinase proteins (Fig. 1 C). Similar to Raf-1 and MEK1 activity, MAP kinase phosphorylation was significantly increased with 15 s of contraction. However, in contrast to Raf-1 and MEK1 activity, there was a more sustained activation of MAP kinase in the muscle. Contraction significantly increased phosphorylation of p42MAPK and p44MAPK, indicating that both isoforms are activated in response to contraction in skeletal muscle.

Of the three known isoforms of the mammalian p90 RSK, RSK2 is abundantly expressed in muscle and has been implicated as a direct substrate of the p44 and p42 MAP kinases (34). RSK2 activity toward a S6 protein peptide substrate (3R S6 peptide) was measured in RSK2 immune complexes from the muscle lysates. Figure 2 shows the time course of RSK2 activity in response to muscle contractions. By 2 min of contraction, RSK2 activity was increased by more than twofold above basal, with peak kinase activity occurring with 10 min of contraction. Thereafter, RSK2 activity decreased, although there was still significant stimulation with 30 min of contraction. Insulin also increased RSK2 activity as early as 5 min after injection (1.5-fold above basal), with peak activity occurring 20 min after insulin injection. Figure 2 shows that the maximal insulin response was considerably less than the maximal contraction effect. The inset of Fig. 2 shows the effects of contraction on RSK phosphorylation as assessed by gel mobility shift. Skeletal muscle contraction caused a clear retardation in the electrophoretic mobility of the RSK protein, suggesting increased phosphorylation and confirming the increase in RSK activity by the contraction stimulus.

Because MAP kinase signaling has been implicated in the activation of immediate-early genes, we determined whether the stimulation of the MAP kinase cascade induced by this contraction protocol was also effective in increasing c-fos mRNA. Figure 3 shows that contraction dramatically increased c-fos mRNA by 25-fold above basal. Increased c-fos expression was present after only 15 min of contraction and was still elevated with 30 and 60 min of contraction. In contrast, insulin only increased c-fos mRNA by 4.5-fold above basal, and this effect was maximal 30 min after insulin injection.

One mechanism for the activation of the MAP kinase signaling cascade is via growth factor receptor stimulation and subsequent formation of Shc/Grb2/Sos and IRS1/Grb2/Sos complexes, resulting in increased Ras-GTP exchange and activation of Raf-1. We have assessed whether these proteins are mediators of the contraction-induced increase in MAP kinase signaling. The standard method to measure Ras activation involves the loading of cells with radiolabeled GTP. Because it is not possible to use this technique in intact animals, we used immunoprecipitation and immuno-
blotting techniques to assess the association of Raf-1 with Ras as a surrogate indication of Ras activation. Muscle lysates were immunoprecipitated with α-Raf-1, and the immune complexes were immunoblotted with α-Ras. For these experiments, two different antibodies made to unique epitopes of Ras were used. There was an increase in the abundance of p21 Ras in the Raf-1 immunocomplexes from the insulin and contracted samples (Fig. 4A). Interestingly, immunoblotting of the Raf-1 immunoprecipitates with this Ras antibody also recognized a 30-kDa protein that was significantly stimulated by insulin, contraction, and acute treatment of anesthetized rats with EGF (Fig. 4B). Immunoblotting of muscle lysates with the Ras antibody (with no prior immunoprecipitation) also revealed two bands with molecular masses of ~21 and 30 kDa (Fig. 4C). The identity of this 30-kDa protein is not known, but on the basis of its size and cross-reactivity with two different Ras antibodies, it may be a Ras-related GTPase. The ability of contractile activity to stimulate the association of Raf-1 with Ras and a putative Ras-related molecule suggests that Ras is involved in the activation of MAP kinase signaling by contraction in skeletal muscle.

Activation of MAP kinase signaling with contractile activity could result from tyrosine phosphorylation of one or more muscle tyrosine kinase receptors. A precedent for this hypothesis comes from studies of PC12 cells, where activation of MAP kinase signaling in response to calcium influx was shown to be mediated by tyrosine phosphorylation of EGF receptors (27). In skeletal muscle, there are transient increases in intracellular calcium concentrations with the initiation of each contraction. Therefore, we tested the possibility that tyrosine phosphorylation of the EGF receptor was involved in the contraction-induced stimulation of MAP kinase signaling in skeletal muscle. Lysates from rat muscles contracted for various periods of time were first immunoprecipitated with an EGF receptor antibody, and the immune complexes were immunoblotted with α-PY, an anti-phosphotyrosine antibody. Control experiments were done by injecting rats with a maximal dose of EGF (200 µg/kg body wt) and dissecting hindlimb skeletal muscles 15 min later. Figure 5A is a representative phosphorimage showing minimal, if any, effects of muscle contraction on EGF receptor phosphorylation. In contrast, EGF administration, which stimulated RSK2 activity and MAP kinase phosphorylation by considerably less than muscle contraction (~2-fold above basal; data not shown), caused a dramatic increase in EGF receptor phosphorylation (Fig. 5A). To determine whether another tyrosine kinase receptor may be involved in the stimulation of MAP kinase signaling by contraction, similar experiments were performed using insulin receptor antibodies. As with the EGF experiments, there was no effect of contraction on insulin receptor tyrosine phosphorylation (data not shown). The lack of an effect of contraction on EGF receptor and insulin receptor tyrosine phosphorylation is consistent with our previous study showing no effect of contraction on tyrosine phosphorylation of proteins in the molecular mass range of 80–200 kDa, when measured in α-PY immunoprecipitates (9). Taken together, these studies strongly suggest that secondary activation of receptor tyrosine kinases is unlikely to represent a mechanism for the stimulation of MAP kinase signaling by contraction in skeletal muscle.

Activation of the MAP kinase pathway by ras-dependent mechanisms can involve the association of Grb2 with tyrosine-phosphorylated IRS or Shc molecules. In addition to these complexes being formed in response to stimulation of receptor tyrosine kinases, some classes of G protein-coupled receptors can also activate the association of Shc with Grb2 (28). To determine whether these molecules are involved in the increase in MAP kinase signaling by contraction, we studied the effects of contractile activity on IRS1, IRS2, and Shc tyrosine phosphorylation, Shc/Grb2 association, and IRS1/Grb2 association. Immunoprecipitation of muscle lysates with α-IRS1 or α-IRS2 and immunoblotting with α-PY also showed no increase in IRS tyrosine phosphorylation in response to contraction (Fig. 5, B and C). In fact, there was a decrease in tyrosine phosphorylation of IRS1 with longer periods of contraction (Fig. 5B). Similar to the lack of effect of
contraction on IRS tyrosine phosphorylation, none of the Shc isoforms were tyrosine phosphorylated in response to contraction (Fig. 5D). To determine whether muscle contraction increased the association of Shc with Grb2, muscle lysates were incubated with an antibody to Grb2 or with a GST-Grb2 fusion protein coupled to agarose beads and immunoblotted with α-Shc. Both of these experiments demonstrated that there was no increase in immunodetectable Shc in the Grb2 immunoprecipitates from the contrated muscle, suggesting that muscle contractile activity did not increase Shc/Grb2 (data not shown). Furthermore, contraction did not increase immunodetectable Grb2 in IRS1 immunoprecipitates (data not shown). These results demonstrate that contraction activates MAP kinase signaling by a mechanism that is independent of IRS and Shc tyrosine phosphorylation and of formation of Shc/Grb2 or IRS1/Grb2 complexes.

Because p70S6K has been implicated in the regulation of protein synthesis (13), we hypothesized that this enzyme may also be activated by contractile activity in skeletal muscle. The effects of muscle contraction on p70S6K activity were assessed by molecular weight shift of the protein and by measuring p70S6K activity toward a GST-S6 protein substrate in p70S6K immune complexes. Figure 6A shows that muscle contractile activity for periods ranging from 15 s to 30 min did not alter the mobility of the p70S6K protein. In contrast, insulin induced a marked shift in the electrophoretic mobility of p70S6K. Muscle contraction had no effect on phosphorylation of the G7-S6 protein, whereas 20 min of insulin treatment significantly increased p70S6K-mediated GST-S6 protein phosphorylation by ~75% (Fig. 6, B and C).

Recent studies have suggested that Akt is part of the mechanism by which insulin increases glucose transport. Physical exercise can increase glucose transport in the contracting skeletal muscles, and therefore we determined whether muscle contraction can increase Akt activity. Figure 7 demonstrates that 5 min after insulin injection there was an approximately fivefold increase in Akt activity in the skeletal muscle. In contrast, contraction for as little as 15 s, or as long as 60 min, did not increase Akt activity. Similar to these results, we have also shown that exercising rats on a rodent treadmill for periods up to 60 min has no effect...
Contractile activity is a critical regulator of protein synthesis (4) and gene transcription (22) in skeletal muscle. Chronic changes in the level of contractile activity lead to structural remodeling of muscle, resulting in profound effects on the functional capacity of the tissue (13). To determine the underlying molecular mechanisms through which contraction causes these important changes in skeletal muscle, the signaling molecules that convert the mechanical/biochemical contraction stimulus into intracellular responses must be defined. The MAP kinase pathway is a candidate for such a signaling system in contracting skeletal muscle. This pathway is extensively used for transcytoplasmic signaling to the nucleus and is involved in a wide variety of cellular responses to external stimuli (26). Furthermore, several studies have shown that the MAP kinase pathway is activated in cultured cardiac myocytes in response to stretch (30, 38, 39). It is noteworthy, however, that treatment of cultured cardiac myocytes in vitro does not mimic the mechanical loading present in skeletal muscles contracting in the intact animal, nor does it replicate the hormonal and neural environment of the contracting skeletal muscle in situ.

The current findings demonstrate that sciatic nerve stimulation to produce muscle contractions in intact rat skeletal muscle causes a rapid and potent stimulation of the MAP kinase signaling cascade. The temporal activation of these molecules suggests that Raf-1, MEK1, MAP kinase, and RSK2 are sequentially phosphorylated and activated in response to contractile activity in the muscle. Our MAP kinase and RSK2 data also suggest that contractile activity is a considerably more potent activator of MAP kinase signaling in skeletal muscle than growth factor stimulation by EGF or insulin. Furthermore, contraction of isolated muscles in vitro is a much more potent stimulator of MAP kinase phosphorylation than incubation of isolated skeletal muscles with a maximal dose of insulin (T. Hayashi and L. J. Goodyear, unpublished data). Thus the major function of this cascade of signaling proteins in adult, fully differentiated skeletal muscle may be to mediate the biological responses to increased muscle activity rather than responses to growth factors.

Components of the MAP kinase signaling pathway are expressed in smooth (1, 37), cardiac (30, 39), and skeletal muscles (8). In cardiac myocytes, MAP kinase signaling has been shown to be involved in adaptations to stretch (30, 39), and emerging data indicate a similar response of MAP kinase in arterial smooth muscle cells subjected to mechanical load or hypertension (1, 37). Our studies of rat (8) and human (2) skeletal muscle have shown that physical exercise can also stimulate MAP kinase phosphorylation. Thus, although there are considerable differences in the nature of the mechanical stimuli to which different muscle cell types are normally exposed, the MAP kinase pathway emerges as a signaling system for the conversion of mechanical stimuli into intracellular signals, a system that is shared by all (skeletal, smooth, and cardiac) muscle cell types.

The physiological consequences of increased MAP kinase signaling in skeletal muscle in response to insulin (12, 41), EGF (3), and contraction (current study) are not known. In cardiac myocytes, activation of the MAP kinase signaling pathway by mechanical stretch may regulate the stretch-induced increases in protein synthesis and the transcriptional activation of several immediate-early genes (16). In skeletal muscle, the induction of the immediate-early genes c-fos, c-jun, and egr-1 has been shown to occur 4 h after the onset of sustained muscle contractile activity caused by low-voltage electrical stimulation (22). In the current study, we show that a period of only 15 min of tetanic contractions is necessary for a dramatic increase in c-fos mRNA in skeletal muscle. The temporal relationship of the peak activation of the MAP kinase signaling intermediaries and c-fos expression (Raf-1 = 15 s; MEK1 = 15 s; MAP kinase = 5–10 min; RSK2 = 10 min; c-fos = 15 min) suggests that MAP kinase signaling could be involved in the activation of this immediate-early gene. It is also interesting that, compared with the modest effects of insulin to increase both RSK2 activity and c-fos mRNA, contraction results in a much greater activation of both RSK2 and c-fos mRNA. Because the MAP kinase pathway has been implicated in the regulation of gene transcription in numerous cell types (26), it is likely that transcriptional regulation is a consequence of increased MAP kinase signaling with muscle contraction. Thus the control of gene transcription through the MAP kinase signaling pathway could be a major factor in the ability of repeated bouts of muscle contraction to alter muscle phenotype.

Activation of the MAP kinase signaling cascade by contraction could be mediated by systemic effects that occur in response to increased muscle contractile activ-
ity, local tissue-specific effects that are associated with the mechanical or biochemical events of muscle contraction, or a combination of both. We believe that there are limited, if any, systemic contributions to the activation of this pathway, because we have shown that one-legged exercise in human subjects stimulates MAP kinase signaling only in the exercising leg (2). Thus it is probably a local effect of contractile activity that results in increased MAP kinase signaling. Intrinsic changes in the skeletal muscle that could lead to increased MAP kinase activation include alterations in the energy status of the muscle fibers, osmotic changes, and mechanical or shear stresses. It is also possible that local activation of MAP kinase signaling occurs via an autocrine or paracrine mechanism. The current studies have made the important observation that contractile activity does not act via tyrosine kinase receptors Shc, IRS1, IRS2, and Grb2. These findings also suggest that G protein-coupled receptors that utilize Shc for the activation of MAP kinase signaling are not a mechanism for the effects of muscle contraction. One potential mechanism for activation of the MAP kinase pathway involves protein kinase C. Protein kinase C can activate the MAP kinase cascade in several cells types (15, 17), and muscle contraction has been shown to increase protein kinase C activity in rat skeletal muscle (25). Future investigations using isoform-specific protein kinase C inhibitors will be required to determine whether this enzyme mediates the effects of contraction to stimulate MAP kinase in skeletal muscle.

Contractile activity increases skeletal muscle glucose transport through a PI 3-kinase-independent mechanism (9, 11). Certain stress-stimulated stimuli have been shown to activate Akt in a PI 3-kinase-independent manner (21, 31), and several recent studies have implicated Akt in the regulation of glucose transport (33). However, the current data clearly demonstrate no effect of contractile activity on Akt activity in skeletal muscle, suggesting that this molecule does not play a role in mediating contraction-stimulated glucose transport. While our manuscript was in preparation, two other groups reported that contraction of isolated skeletal muscles contracted in vitro had no effect on Akt activity (5, 20). Similar to the lack of effect of muscle contraction on Akt activity, contraction had no effect on p70Sk activity or IRS2 tyrosine phosphorylation. The lack of stimulation of IRS2 tyrosine phosphorylation by contraction in skeletal muscle is in contrast to a recent report suggesting that contraction of ventricular cardiomyocytes in vitro increases IRS2 tyrosine phosphorylation (35). Thus, although some contraction-activated intracellular signaling responses (e.g., MAP kinase) are similar among different muscle types, the regulation of other signaling molecules is tissue specific.

In conclusion, contractile activity rapidly and potentially stimulates the MAP kinase signaling cascade in skeletal muscle. Our data suggest that the activation of MAP kinase signaling in skeletal muscle involves Ras, whether by contraction, insulin, or EGF. On the other hand, in contrast to the mechanisms by which insulin and EGF stimulate the MAP kinase pathway in skeletal muscle, contraction-induced stimulation of this cascade occurs by an IRS-, Shc-, and Grb2-independent mechanism. The temporal relationship for the activation of the MAP kinase cascade and c-fos expression suggests that signaling through this pathway may regulate the expression of immediate-early genes. These findings provide direction for future studies intended to determine the molecular basis for the acute regulation of gene transcription and subsequent changes in phenotype that occur in skeletal muscle in response to contractile activity.

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