MEF2 activation in differentiated primary human skeletal muscle cultures requires coordinated involvement of parallel pathways

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Al-Khalili, Lubna, Alexander V. Chibalin, Mei Yu, Bertil Sjödin, Carolina Nylén, Juleen R Zierath, and Anna Krook. MEF2 activation in differentiated primary human skeletal muscle cultures requires coordinated involvement of parallel pathways. Am J Physiol Cell Physiol 286: C1410–C1416, 2004. First published February 11, 2004.—The myocyte enhancer factor (MEF2) transcription factor is important for development of differentiated skeletal muscle, and regulating many myogenic genes. Given the critical role of MEF2 in the regulation of skeletal muscle gene expression, we investigated the regulation of MEF2 DNA binding in human skeletal muscle cultures and in isolated rat epitrochlearis muscle in response to insulin, AICAR, or cellular stress (hyperosmolarity or oxidative stress). The effect of muscle contraction or AICAR on MEF2 DNA binding was almost undetectable. Exposure of cells to 20 min to 120 mM insulin, 0.1 and 1.0 mM hydrogen peroxide, osmotic stress (400 mM mannitol), or 1.0 mM 5-aminimidazole-4-carboxamide-1-β-d-ribofuransoide (AICAR) led to a profound increase in MEF2 DNA binding. To study signaling pathways mediating MEF2 activity, we preincubated human skeletal muscle cell cultures or isolated rat epitrochlearis muscles with inhibitors of p38 mitogen-activated protein kinase (MAPK) (10 μM SB-203580), MEK1 (50 μM PD-98059), PKC (1 and 10 μM GF109203X), phosphatidylinositolositol (PI) 3-kinase (10 μM LY-294002), or AMP-activated protein kinase (AMPK; 20 μm compound C). All stimuli resulted primarily in activation of MEF2 DNA binding. Exposure of cells to osmotic or oxidative stress increased MEF2 DNA binding via pathways that were completely blocked by MAPK inhibitors and partially blocked by inhibitors of PKC, PI 3-kinase, and AMPK. In epithrochlearis muscle, MAPK inhibitors blocked contraction but not AICAR-mediated MEF2 DNA binding. Thus activation of MEF2 in skeletal muscle is regulated via parallel intracellular signaling pathways in response to insulin, cellular stress, or activation of AMPK.

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supplemented with 1% PeSt (100 U/ml penicillin-100 µg/ml streptomycin). Satellite cells were isolated and cultured as described previously (2, 3).

Animal characteristics. Male Wistar rats (90–110 g) were purchased from B & K Universal (Sollentuna, Sweden), housed in a 12:12-h light-dark cycle, and allowed free access to water and chow for 7 days before the day of the experiment. Rats were fasted overnight before the day of the experiment. The North Stockholm Animal Ethics Committee approved the animal experiments.

Antibodies, media, and reagents. Phosphospecific antibodies against Akt/PKB, ERK1/2, p38 MAPK, and nonphosphorylated Akt/ PKB, ERK1/2, and p38 MAPK protein were from New England Biolabs (Beverly, MA). The BMK1/ERK5 antibody was from Upstate Biotechnology (Waltham, MA). The phosphospecific antibodies against AMPK (Thr172) were from Cell Signaling Technologies (Beverly, MA). Antibodies for p90 rsk were prepared as previously described (1). Antibodies used in MEF2 gel shift analysis were purchased from B&K Universal (Sollentuna, Sweden), housed in a refrigerator, MA). Antibodies for p90 rsk were prepared as previously described (1). Antibodies used in MEF2 gel shift analysis were purchased from B&K Universal (Sollentuna, Sweden), housed in a refrigerator.

Human skeletal muscle culture protocol. Myotubes were grown on 10-cm petri dishes. At day 4 or 5 after differentiation, cells were serum-starved 16 h before use. Cells were preincubated for 30 min with either vehicle (0.37% DMSO) or 50 µM PD-98059 (MEK1 inhibitor), 10 µM SB-203580 (p38 MAPK inhibitor), 1 or 10 µM GF109203X (PKC inhibitor), 10 µM LY-294002 (PI 3-kinase inhibitor), or 20 µM compound C (AMPK inhibitor). After preexposure to DMSO or inhibitors, cells were stimulated with 120 nM insulin, 0.1 or 1 mM hydrogen peroxide, 1 mM AICAR, or 400 mM mannitol for 20 min. Myotubes were either washed and frozen immediately at −70°C for Western blot analysis or harvested immediately for nuclear extraction.

Epitrochlearis muscle dissection and ex vivo contraction protocol. Dissection of epitrochlearis muscles and subsequent ex vivo contraction was performed as described previously (32). All incubation media were prepared from a pregassed (95%O2-5% CO2) stock of Krebs-Henseleit buffer supplemented with 5 mM HEPES and 0.1% bovine serum albumin (RIA grade). When pharmacological inhibitors were used, an additional 30-min incubation was employed to preexpose the muscle to the inhibitor. Once added, inhibitors remained present for the duration of the experiment. The addition of labeled probes (0.5 ng) was incubated with the inhibitor for 30 min at room temperature. Thereafter, 20 µl aliquots were loaded on a nondenaturing 5% polyacrylamide (30% acrylamide/bisacrylamide) gel buffer followed by Tris-borate-EDTA (10 mM Tris, 90 mM boric acid, 1 mM EDTA) and subjected to electrophoresis at 40 mA for 1.5 h at room temperature. The dried gels were analyzed using a phosphoimager (Fuji model BAS-1800II). For supershift analysis of MEF2, nuclear extracts were incubated for 1 h with isofrom-specific MEF2 antibodies (MEF2A, MEF2C, and MEF2D). To exclude nonspecific binding, extracts were incubated with unlabeled mutant MEF2 oligo (5’-GAT CGC TGT AAA CAT AAC CCT GTC G-3’). The mixture was then incubated with radioactively labeled oligo for 30 min at room temperature. Samples were loaded as described above.

Western blot analysis. Myotubes were treated as described in Human skeletal muscle protocol and then washed twice with ice-cold PBS, frozen, and harvested by scraping into ice-cold buffer D (135 mM NaCl, 1 mM MgCl2, 2.7 mM KCl, 20 mM Tris, pH 8.0, 0.5 mM Na3VO4, 10 mM NaF, 1% Triton X-100, 100% (vol/vol) glycerol, 0.2 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotonin). Homogenates were rotated for 60 min at 4°C and subjected to centrifugation (20,000 g for 10 min at 4°C). Western blotting was carried out as described (22).

Kinase activity of p90 rsk. Myotubes were treated as described in Human skeletal muscle protocol and harvested into buffer E (50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 1 mM EDTA, 50 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 0.1% 2-β-mercaptoethanol, 0.2 mM PMSF, and 0.1 μM microcystine). Aliquots (100 µg) of protein were immunoprecipitated for 60 min at 4°C with antibody previously incubated with protein G-Sepharose in buffer E. p90 rsk activity was determined as described previously (32).

Kinase autophosphorylation of BMK1/ERK5. Autophosphorylation of BMK1/ERK5 was determined as described previously (35). Brieﬂy, myotubes were preincubated for 30 min with 50 µM PD-98059 or with an equal amount of 0.37% DMSO as a control. After 20-min stimulation with 1 mM hydrogen peroxide, myotubes were harvested into buffer E. Aliquots (200 µg) of protein were immunoprecipitated for 120 min at 4°C with antibody previously incubated with protein A-agarose and protein G-Sepharose (50 µl, 1:1 slurry) in buffer F (300 mM NaCl, 10 mM Tris-HCl, pH 7.6, 0.1% Triton X-100, and 1 mM DTT), once with buffer E, once with buffer F supplemented with 0.5 M NaCl, once with buffer F (500 mM LiCl, 100 mM Tris-HCl, pH 7.6, 0.1% Triton X-100, and 1 mM DTT), once with buffer G (20 mM HEPES, pH 7.2, 2 mM EGTA, 10 mM Na3VO4, 10 mM NaF, 1% Triton X-100, 100% (vol/vol) glycerol, 0.2 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotonin). Homogenates were rotated for 60 min at 4°C and subjected to centrifugation (20,000 g for 10 min at 4°C). Western blotting was carried out as described (22).

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mM MgCl₂, 0.1% Triton X-100, and 1 mM DTT), and once with buffer H (20 mM Tris/HCl, pH 7.5, 2 mM EGTA, 0.5 mM PMSF, and 2 mM DTT). Samples were resuspended in 30 μl of kinase buffer (20 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, and 2 Ci of [α-32 P] ATP). The reaction was incubated for 30 min at 30 °C with agitation (1,000 rpm) and terminated on ice. Beads were washed once with ice-cold buffer E and twice with buffer F. Laemmli sample buffer (30 μl, 1.5%) was added to each sample, and beads were boiled for 5 min. Samples were resolved on a 15% SDS-PAGE gel and transferred to polyvinylidene difluoride membrane, and autophosphorylated BMK1/ERK5 was visualized using a phosphoimager.

**Results**

**MEF2 DNA binding.** Differentiated myotubes from primary human skeletal muscle cultures were stimulated with insulin (120 nM), hydrogen peroxide (0.1 or 1.0 mM), AICAR (1.0 mM), or mannitol (400 mM) for 20 min. In unstimulated cultures, basal DNA binding activity of MEF2 was nearly undetectable. Each of the different stimuli robustly increased MEF2 DNA binding activity approximately four- to fivefold (Fig. 1, P < 0.01 vs. basal). Protein expression of MEF2A, MEF2C, or MEF2D was unaltered in cells after incubation with any of the stimuli (data not shown).

**Effects of p38 MAPK and MEK1 inhibitors on MEF2 DNA binding.** Preexposure of primary human skeletal muscle cells to the p38 MAPK inhibitor (SB-203580) completely inhibited insulin-, hydrogen peroxide-, and osmotic stress-induced MEF2 DNA binding activity (Fig. 2A). In contrast, preincubation of cells with SB-203580 was without effect on AICAR-stimulated MEF2 DNA binding activity (Fig. 2A). Similar results were obtained using the MEK1 inhibitor; preexposure of myotubes to PD-98059 completely inhibited MEF2 DNA binding activity induced by insulin, hydrogen peroxide, and osmotic stress. The MEK1 inhibitor was without effect on AICAR-mediated MEF2 DNA binding (Fig. 2A). We also investigated whether a combined exposure of SB-203580 and PD-98059 would inhibit AICAR-stimulated MEF2 DNA binding. However, no further inhibition above that observed for incubation with PD-98059 alone was noted (Fig. 2E).

**Effects of p38 MAPK and MEK1 inhibitors on MAPK signaling.** Phosphorylation of p38 MAPK in primary human skeletal muscle cells was increased in response to treatment...
with hydrogen peroxide (1.0 mM) or mannitol (400 mM) and decreased (40%) after preexposure to SB-203580. In contrast, no detectable increase in p38 MAPK phosphorylation was observed after treatment with 0.1 mM hydrogen peroxide. The MEK1 inhibitor PD-98059 did not alter p38 MAPK phosphorylation (data not shown).

As expected, basal phosphorylation of ERK1/2 was reduced by the MEK1 inhibitor PD-98059 (Fig. 3A). Treatment of cells with insulin, hydrogen peroxide, or osmotic stress increased ERK1/2 phosphorylation. The MEK1 inhibitor decreased ERK1/2 phosphorylation when cells were stimulated with either insulin or 400 mM mannitol (P < 0.05). In contrast, when cells were stimulated with 1.0 mM hydrogen peroxide, PD-98059 did not significantly reduce ERK1/2 phosphorylation (Fig. 3A). To determine whether the phosphorylated form of ERK1/2 mirrors the active form of this kinase, we analyzed the activity of the ERK1/2 downstream kinase p90rsk. Exposure of differentiated human muscle cells to 1 mM AICAR did not lead to an increased activation of p90rsk whereas exposure of cells to insulin, osmotic stress, or hydrogen peroxide increased p90rsk activity. Preexposure of myotubes to the MEK1 inhibitor significantly inhibited p90rsk activity in response to insulin, osmotic stress, or 0.1 mM hydrogen peroxide. In contrast, the fivefold increase in p90rsk activity after exposure to 1 mM hydrogen peroxide was not significantly inhibited by PD-98059 (Fig. 3B). Thus the ability of PD-98059 to inhibit hydrogen peroxide-mediated MEF2 DNA binding does not involve ERK1/2-p90rsk signaling.

Activation of BMK1/ERK5 signaling. Previous reports provide evidence that PD-98059 may also inhibit ERK5 (16, 35). We therefore assessed whether ERK5 is the PD-98059-sensitive kinase mediating MEF2 DNA binding in human skeletal muscle. Myotubes were preincubated with 50 μM PD-98059 or 0.37% DMSO for 30 min and treated for 20 min with or without 1 mM hydrogen peroxide. Autophosphorylation of ERK5 increased with hydrogen peroxide exposure; however, the PD-98059 inhibitor did not decrease ERK5 autophosphorylation (Fig. 4).

Effects of PKC and PI 3-kinase inhibitors on MEF2 DNA binding. The PKC inhibitor GF109203X has been shown to inhibit all PKC isoforms at high concentrations (10 μM) and novel and conventional (but not atypical) isoforms at lower (1 μM) concentrations (38). GF109203X did not inhibit MEF2 DNA binding activity in response to any of the stimuli studied when a low (1 μM) concentration of the inhibitor was used, whereas a high (10 μM) concentration completely inhibited insulin-mediated MEF2 activity and partially reduced osmotic stress- and hydrogen peroxide-stimulated MEF2 DNA binding. Neither concentration of the PKC inhibitor had any effect on AICAR-stimulated MEF2 activation (Fig. 5). A similar pattern of inhibition was noted when cells were exposed to the PI 3-kinase inhibitor LY-294002 (Fig. 5).

Effect of AMPK-inhibitors on MEF2 DNA binding. In contrast to the MAPK inhibitors, the AMPK inhibitor compound C completely inhibited AICAR-mediated activation of MEF2 DNA binding (Fig. 6A). Preexposure of cells to compound C also resulted in a partial inhibition of osmotic stress- and hydrogen peroxide-stimulated MEF2 DNA binding but was without effect on insulin-stimulated activation of MEF2 (Fig. 6A). We further measured the phosphorylation of AMPK by Western blot analysis. Similar to results for MEF2 DNA binding activity, compound C inhibited the AICAR-stimulated increase in AMPK phosphorylation and showed a partial effect.
on hydrogen peroxide- and osmotic stress-mediated AMPK phosphorylation (Fig. 6B). Insulin stimulation did not lead to increased AMPK phosphorylation (Fig. 6B).

**Insulin, AICAR, and cellular stress lead to activation of MEF2D DNA.** Nuclear extracts of primary human muscle cells exposed to either insulin or AICAR were incubated with antibodies specific for MEF2A, MEF2C, or MEF2D isoforms. Insulin, AICAR, and cellular stress all specifically increased MEF2D DNA binding (Fig. 7).

**Effect of AICAR and contraction on MEF2 DNA binding in isolated rat epitrochlearis muscle.** Electrically stimulated contraction of isolated rat epitrochlearis muscle led to an increase in MEF2 DNA binding. When muscles were preexposed to either SB-203580 or PD-98059, contraction-mediated MEF2 DNA activation was inhibited (Fig. 8A). We also determined MEF2 DNA binding in isolated rat epitrochlearis muscle after 20-min exposure to 1 mM AICAR. In contrast to the results for contracting muscle, and similar to results obtained in primary human muscle cultures, AICAR-mediated activation of MEF2 DNA binding in isolated rat epitrochlearis muscle was not affected by either of these two inhibitors (Fig. 8B), suggesting that contraction and AICAR mediate MEF2 DNA binding by different pathways. To determine the role of AMPK in contraction-mediated activation of MEF2 DNA binding, isolated epitrochlearis muscle was forced to contract in the presence or absence of compound C. Although muscle incubated with compound C had reduced activation of MEF2 DNA binding, the ability of the muscle to contract was also markedly impaired; thus it was not possible to determine whether the reduced MEF2 DNA binding was due to reduced contraction or direct inhibition of AMPK (data not shown).

**DISCUSSION**

The regulation of MEF2 DNA binding has been determined in primary human skeletal muscle and isolated rat epitrochlearis skeletal muscle. Muscle cultures were exposed to insulin, cellular stress (osmotic stress and hydrogen peroxide), or AICAR. These different stimuli broadly activate diverse signaling cascades that regulate MEF2 DNA binding. Activation of AMPK leads to increased MEF2 DNA binding via pathways that are independent of p38 MAPK-, p90 ribosomal S6 kinase, PKC-, or PI 3-kinase-mediated signaling. In contrast, insulin increased MEF2 DNA binding, via an AMPK-independent mechanism, that requires activation of p38 MAPK, PI 3-kinase, atypical PKCs, and a PD-98059-sensitive kinase. Activation of MEF2 DNA binding after cellular stress, induced by either osmotic stress or hydrogen peroxide, appears to be dependent on activation of p38 MAPK and a PD-98059-sensitive kinase. Activation of MEF2 DNA binding after cellular stress, induced by either osmotic stress or hydrogen peroxide, appears to be dependent on activation of p38 MAPK and a PD-98059-sensitive kinase and also partially sensitive to PI 3-kinase-, atypical PKC-, and AMPK-dependent signaling. Thus activation of MEF2 in skeletal muscle is regulated via parallel intracellular signaling pathways in response to insulin, cellular stress, or activation of AMPK.

Insulin is a potent activator of MEF2 DNA binding activity in primary human skeletal muscle cultures. Insulin-stimulated MEF2 DNA binding was completely blocked by the PI 3-kinase inhibitor LY-294002, as well as by 10 μM, but not 1 μM,
of the PKC inhibitor GF109203X. Thus insulin signals to MEF2 via activation of PI 3-kinase and atypical PKC. This is consistent with rodent muscle cell lines, whereby PI 3-kinase activates MEF2 during skeletal muscle differentiation (36, 41). Furthermore, regulation of MEF2 is correlated with insulin action in diabetes (37). In heart and skeletal muscle of diabetic mice, MEF2 DNA binding activity is reduced and positively correlated with the transcription rate of the GLUT4 gene. Importantly, in these animals, MEF2 binding activity is completely recovered to control levels after insulin treatment (37).

In this study we have demonstrated that insulin-mediated MEF2 activation is dependent on PI 3-kinase, p38 MAPK, and PD-98059-sensitive kinase. All three pathways appear to be necessary for MEF2 DNA binding activity. Inactivation of any pathway results in a complete inhibition of insulin-mediated MEF2 DNA binding activity. PD-98059 and SB-203580 have been widely used to delineate components of MAPK signaling cascades in cultured cells (15). Furthermore, LY-294002 and GF109203X are well-characterized inhibitors for PI 3-kinase (39) and PKC (38), respectively. SB-203580 is a highly specific for inhibition of p38 MAPK (15); however, a small but significant inhibition of Akt/PKB by SB-203580 has been reported (15) and was also noted in the current study (data not shown). Thus we cannot exclude the possibility that Akt/PKB signaling cascades are involved in the activation of MEF2 DNA binding.

We also assessed the effects of these different stimuli on signal transduction via the MAPK cascades in primary human skeletal muscle. The lack of an inhibition of ERK1/2 phosphorylation after exposure to 1 mM hydrogen peroxide and PD-98059 was an unexpected finding. Furthermore, PD-98059 did not inhibit activation of p90S6K in response to 1 mM hydrogen peroxide. Despite this, exposure of cells to PD-98059 completely inhibited hydrogen peroxide-mediated activation of MEF2 DNA binding. Two chemically distinct MEK1 inhibitors (PD-98059 and U0126) inhibit ERK5 activation (18). We therefore explored the possibility that ERK5 was the PD-98059-sensitive kinase required for hydrogen peroxide-mediated, and possibly insulin- and hyperosmosis-mediated, activation of MEF2. Activation of ERK5 has been shown to increase MEF2 activity in rat smooth muscle cells (19). Exposure of primary human skeletal muscle cells to PD-98059, however, did not prevent hydrogen peroxide-mediated increase in ERK5 autophosphorylation [an indication of ERK5 kinase activity (35)]. Thus the PD-98059-sensitive kinase involved in mediating MEF2 DNA binding in human skeletal muscle cultures is unlikely to be ERK5. The nature of the PD-98059-sensitive kinase remains unknown. The nucleoside analog AICAR is a compound widely used to pharmacologically activate AMPK. Treatment of primary human skeletal muscle cells with AICAR was associated with a profound stimulation of MEF2 DNA binding activity. PD-98059 and SB-203580 have been used to delineate components of MAPK signaling cascades in cultured cells (15). Furthermore, LY-294002 and GF109203X are well-characterized inhibitors for PI 3-kinase, PKC, p38 MAPK, or PD-98059-sensitive kinase activation. There are conflicting reports as to whether AICAR treatment of cells leads to activation of MAPK signaling (10, 27, 40); however, we did not observe any change in p38 MAPK or ERK1/2 phosphorylation after cell treatment with AICAR. AICAR-mediated activation of MEF2 DNA binding was completely inhibited by compound C, a recently described AMPK inhibitor (45). Treatment of cells with compound C also led to a partial reduction in MEF2 DNA binding.

In conclusion, MEF2 DNA binding activity is regulated in differentiated human skeletal muscle cultures via at least two distinct pathways involving AMPK and MAPK, respectively. Understanding the signal transduction pathways that regulate MEF2 DNA binding in skeletal muscle may be of clinical relevance for regulation of genes important for glucose metabolism in diabetes as well as the control of muscle development and aging-related disorders such as sarcopenia. This is particularly relevant because MEF2 consensus binding sequences have been identified in the promoter region of several genes that undergo hormonal and metabolic regulation. Activation of these genes through targeted approaches to MEF2 may specifically regulate protein expression in skeletal muscle.

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