

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

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Submitted 14 October 2003; accepted in final form 10 February 2004

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; 10.1152/ajpcell.00444.2003.—The myocyte enhancer factor (MEF)2 transcription factor is important for development of differentiated skeletal muscle. We investigated the regulation of MEF2 DNA binding in differentiated primary human skeletal muscle cells and isolated rat skeletal muscle after exposure to various stimuli. MEF2 DNA binding activity in nonstimulated (basal) muscle cultures was almost undetectable. Exposure of cells for 20 min to 120 nM insulin, 0.1 and 1.0 mM hydrogen peroxide, osmotic stress (400 mM mannitol), or 1.0 mM 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (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 μ M SB-203580), MEK1 (50 μ M PD-98059), PKC (1 and 10 μ M GF109203X), phosphatidylinositol (PI) 3-kinase (10 μ M LY-294002), or AMP-activated protein kinase (AMPK; 20 μ M 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 epitrochlearis 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.

myocyte enhancer factor 2; insulin; phosphatidylinositol 3-kinase; adenosine 5'-monophosphate kinase; cell stress

MYOCYTE ENHANCER FACTOR 2 (MEF2) is a transcription factor that plays a key role in skeletal muscle differentiation (5, 21, 28). In differentiated myotubes, MEF2 is localized to the nucleus, confirming the importance of this transcription factor in specific skeletal muscle gene expression (25). Four MEF2 isoforms (A–D) have been identified, and all except MEF2B are expressed in skeletal muscle (6, 8). MEF2 isoforms bind DNA as homo- and heterodimers but can also form protein interactions as members of the basic helix-loop-helix family of transcription factors, such as the myogenic basic helix-loop-helix transcription factor (MyoD) and myogenin. Each protein-protein association is suggested to act distinctively in the regulation of skeletal myogenesis (5, 9, 28, 30).

The signal transduction pathways leading to the regulation of MEF2 DNA binding have not been fully elucidated. The

serine/threonine mitogen-activated protein kinases (MAPK) have been implicated in regulation of MEF2 (for review, see Ref. 26), because they transduce environmental signals from the cell membrane to the nucleus in response to a variety of external signals, including growth factors, tumor promoters, and osmotic or cellular stress (12, 13, 31). There are four distinct MAPK pathways: ERK1/2, p38, JNK, and BMK1/ERK5. ERK5 and p38 MAPK stimulate MEF2 activity by direct association with MEF2 (20, 24). The p38 MAPK pathway promotes skeletal muscle differentiation, at least in part, via activation of MEF2C (43). However, the precise involvement of the other MAPK family members in the regulation of MEF2 is less clear.

Several MAPK-independent pathways have also been implicated in the regulation of MEF2. Protein kinase C (PKC) isoforms, phosphatidylinositol (PI) 3-kinase, AMP-activated protein kinase (AMPK), and calmodulin-dependent protein kinase transcriptionally activate MEF2 isoforms (36, 41). Although evidence supports the involvement of PKC in the activation of the ERK/MAPK pathway, PKC-mediated MEF2 DNA binding also may be independent of MAPK signaling (14, 33). Furthermore, PKC may also mediate MEF2 via PI 3-kinase-dependent pathways by forming complexes with the 3-phosphoinositide-dependent kinase PDK-1 (23).

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 also was determined in isolated rat epitrochlearis muscle. Our aim was to delineate the pathway(s) regulating MEF2 DNA binding in skeletal muscle. A detailed understanding of this pathway could be of clinical importance in targeting and regulating many myogenic genes.

MATERIALS AND METHODS

Subject characteristics. Muscle biopsies were obtained with the informed consent of the donors during scheduled abdominal surgery. Subjects (3 male and 4 female) had no known metabolic disorders. Mean age was 55 ± 7 yr (body mass index 26.2 ± 1.5 kg/m² and fasting blood glucose 5.2 ± 0.3 mM). The ethical committee at the Karolinska Institutet approved the study protocols.

Cell culture and differentiation. Muscle biopsies (rectus abdominus, ~1–3 g) were collected in cold phosphate-buffered saline (PBS)

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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 Santa Cruz Biotechnology (Santa Cruz, CA; MEF2A), Cell Signaling Technology (Beverly, MA; MEF2C), and BD Biosciences (Palo Alto, CA; MEF2D). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse immunoglobulin G were from Bio-Rad (Richmond, CA). Reagents for enhanced chemiluminescence were from Amersham (Arlington Heights, IL). PD-98059, SB-203580, GF109203X, and LY-294002 were from CalBiochem (La Jolla, CA). The AMPK inhibitor compound C (45) was obtained from Merck Research Laboratories (Rahway, NJ). All other reagents were analytic grade (Sigma, St. Louis, MO). Dulbecco's modified Eagle's medium, Ham's F-10 medium, fetal bovine serum, penicillin, streptomycin, and fungizone were obtained from GIBCO-BRL (Life Technologies, Stockholm, Sweden).

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 1.0 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 final concentration of DMSO was adjusted to 0.35% for each group (without or with inhibitor) in experiments in which PD-98059, SB-203580, or compound C was used. After incubation, epitrochlearis muscles were frozen immediately between aluminum tongs cooled to the temperature of liquid nitrogen. Skeletal muscle was stored at -70°C until processed for the DNA binding assay.

Nuclear extraction. The nuclear extraction procedure was essentially as described previously (4), with some modifications. Myotubes were grown in 10-cm petri dishes, stimulated as described in *Human skeletal muscle protocol*, and then washed immediately with ice-cold PBS. Cells were scraped in 600 µl of PBS and transferred to microcentrifuge tubes. After centrifugation at 350 g for 30 s, cells were resuspended in 300 µl of *buffer A* [10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)]. Cells were left to swell for 15 min. After centrifugation, the pellet, which contains the

nuclear proteins, was resuspended with 20 µl of *buffer B* (20 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 420 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF). The nuclear proteins were left to diffuse into the buffer for 30 min. The supernatant, with the nuclear proteins, was collected in microcentrifuge tubes supplemented with 50 µl of low-saline *buffer C* (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 0.5 mM DTT, and 0.2 mM PMSF). Homogenization, preparation of nuclear extracts, and processing for MEF2 DNA binding analysis for intact rat epitrochlearis muscles were performed as described previously (7). Protein concentration of the resulting supernatant was determined using a commercial kit (Bio-Rad).

Electrophoretic mobility shift assay. MEF2 DNA binding activity was determined by an electrophoretic gel mobility shift assay (EMSA) as described previously (34). A synthetic consensus oligonucleotide representing a high-affinity MEF2 binding motif (5'-GAT CGC TCT AAA AAT AAC CCT GTC G-3') (Santa Cruz) was end-labeled with T4 polynucleotide kinase and 2 µCi of [γ -³²P]ATP. Nuclear extracts (1.5–2.5 µg) were incubated for 20 min in reaction mixture containing 2 µg poly(dI-dC), 0.5 mM DTT, 25 mM Tris-HEPES, pH 7.5, 60 mM KCl, 1 mM EDTA, and 12% glycerol (15 min on ice and 5 min at room temperature). As a negative control, unlabeled competitor oligonucleotide was incubated with stimulated cell nuclear extract before the addition of labeled probe. ³²P-labeled probes (0.5 ng) were incubated with the protein-mixture complex for 30 min at room temperature. Thereafter, 20 µl aliquots were loaded on a nondenaturing 5% polyacrylamide (30% acrylamide/bisacrylamide) gel buffered with 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 phosphorimager (Fuji model BAS-1800II). For supershift analysis of MEF2, nuclear extracts were incubated for 1 h with isoform-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, 10% (vol/vol) glycerol, 0.2 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin]. 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, 1 mM EGTA, 50 mM NaF, 10 mM β-glycerolphosphate, 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 E*. Immunoprecipitates were washed once with *buffer E*, once with *buffer E* 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

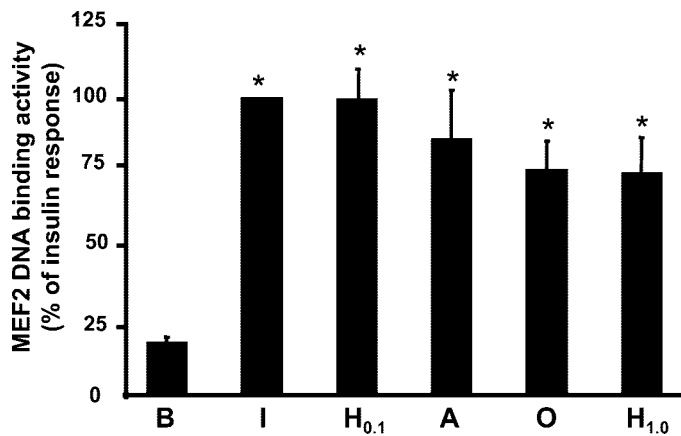


Fig. 1. Myocyte enhancer factor 2 (MEF2) DNA binding activity in differentiated human skeletal muscle cells. Gel mobility shift analysis (EMSA) was used. Cells were preincubated with 0.37% DMSO for 30 min. Cells were stimulated either without or with insulin (120 nM), hydrogen peroxide (0.1 and 1.0 mM), osmotic stress (400 nM mannitol), and 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR; 1.0 mM) for 20 min and harvested directly for nuclear extraction as described in MATERIALS AND METHODS. Each set of samples ($n = 7$) was analyzed at the same time. B, basal; I, insulin; H_{0.1}, hydrogen peroxide (0.1 mM); H_{1.0}, hydrogen peroxide (1.0 mM); A, AICAR; O, osmotic stress. * $P < 0.01$ vs. basal.

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 μ l of kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 2 μ Ci of [γ -³²P] 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 μ l, 1.5 \times) 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.

Statistics. Data are presented as means \pm SE. Differences were determined using ANOVA for multiple comparisons for different stimuli parameters. Student's *t*-test was used to determine differences between experimental conditions for each subject.

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. 2B). We also investigated whether a combined exposure of SB-203580 and PD-98059 would inhibit AICAR-mediated 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

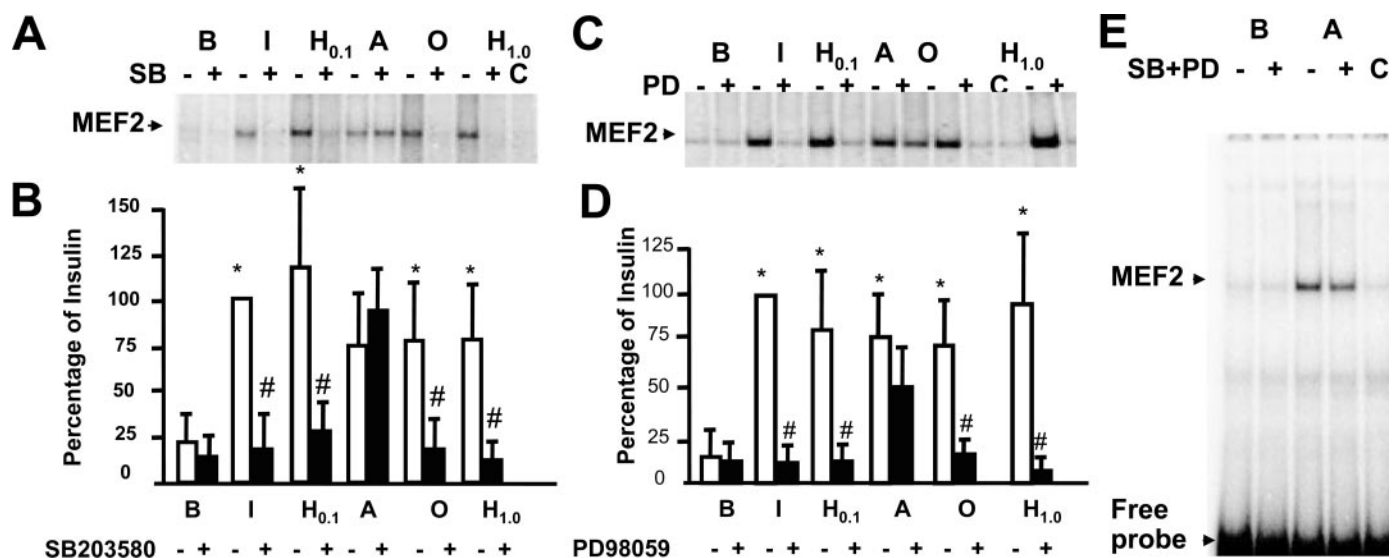


Fig. 2. MEF2 DNA binding in primary human muscle cells ($n = 7$) after incubation without (–) or with (+) inhibitors. **A:** EMSA showing effects of 30-min preexposure to 10 μ M SB-203580 (p38 MAPK inhibitor) on different stimuli-mediated MEF2 activity. **B:** means \pm SE for data presented in A. **C:** EMSA showing effects of 30-min preexposure to 50 μ M PD-98059 (MEK1 MAPK inhibitor) on different stimuli-mediated MEF2 activity. **D:** means \pm SE for data presented in C. **E:** EMSA showing effects of 50 μ M PD-98059 and 10 μ M SB-203580 on AICAR-stimulated MEF2 DNA binding. C, excess cold oligo. * $P < 0.05$ vs. basal. # $P < 0.05$ vs. stimulation.

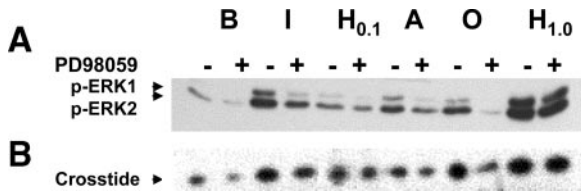


Fig. 3. ERK1/2 phosphorylation and p90^{rsk} activity in primary human skeletal muscle ($n = 7$). Myotubes were preincubated for 30 min with 50 μ M PD-98059 (+) or with 0.37% DMSO (-). *A*: phosphorylation of ERK1/2. *B*: effect of PD-98059 on stimulation of p90^{rsk} activity (crossside).

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 p90^{rsk}. Exposure of differentiated human muscle cells to 1 mM AICAR did not lead to an increased activation of p90^{rsk}, whereas exposure of cells to insulin, osmotic stress, or hydrogen peroxide increased p90^{rsk} activity. Preexposure of myotubes to the MEK1 inhibitor significantly inhibited p90^{rsk} activity in response to insulin, osmosis, or 0.1 mM hydrogen peroxide. In contrast, the five-fold increase in p90^{rsk} 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-p90^{rsk} 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 μ M PD-98059

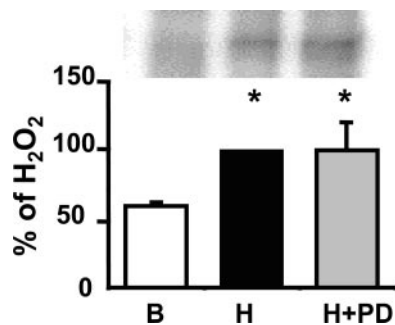


Fig. 4. Autophosphorylation of BMK1/ERK5 in primary human muscle cells ($n = 7$). Myotubes were preincubated for 30 min with 50 μ M PD-98059 (+) or with 0.37% DMSO (-). Values are reported in arbitrary units. H, Hydrogen peroxide (1.0 mM); PD, PD-98059. * $P < 0.01$ vs. basal.

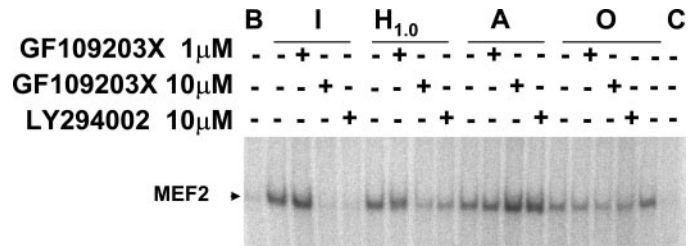


Fig. 5. MEF2 DNA binding in primary human muscle cells ($n = 4$) after 30-min preexposure without (-) or with (+) PKC inhibitor (1 or 10 μ M GF109203X) or phosphatidylinositol 3-kinase inhibitor (10 μ M LY-294002).

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 μ M) and novel and conventional (but not atypical) isoforms at lower (1 μ M) concentrations (38). GF109203X did not inhibit MEF2 DNA binding activity in response to any of the stimuli studied when a low (1 μ M) concentration of the inhibitor was used, whereas a high (10 μ M) 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

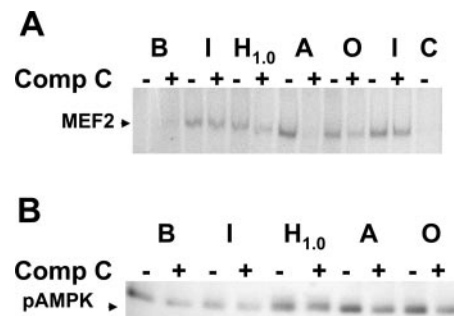
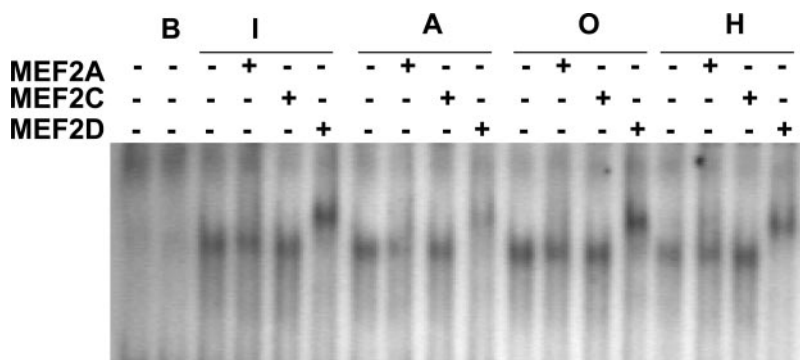


Fig. 6. MEF2 DNA binding in primary human muscle cells after incubation without (-) or with (+) AMPK-activated protein kinase (AMPK) inhibitor (20 μ M compound C) on MEF2 activity (*A*; $n = 4$) and AMPK phosphorylation (*B*). Cells were stimulated for 20 min without or with insulin (120 nM), hydrogen peroxide (1.0 mM), osmotic stress (400 nM mannitol), or AICAR (1.0 mM) and were harvested for Western blot analysis as described in MATERIALS AND METHODS. Comp C, compound C.

Fig. 7. Activation of MEF2 DNA binding in nuclear extracts from primary human muscle cells after incubation with isoform-specific antibodies MEF2A, MEF2C, and MEF2D ($n = 7$). Cells were stimulated for 20 min as described in Fig. 1 and harvested directly for nuclear extraction. Supershift assay was performed using EMSA.



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^{rsk}-, 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 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 μ M, but not 1 μ M,

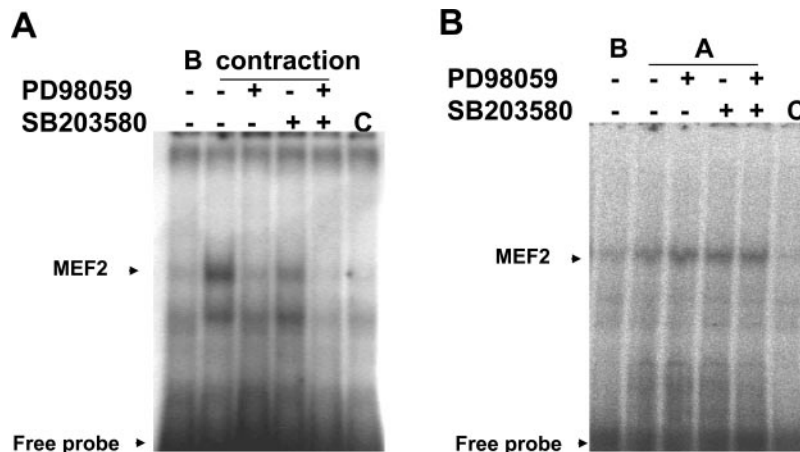


Fig. 8. Activation of MEF2 DNA binding in isolated rat epitrochlearis muscle ($n = 8$) in response to electrical stimulation (contraction) (A) or AICAR (B). Muscle was analyzed after incubation without (-) or with (+) inhibitors as indicated.

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 p90^{rsk} 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. In contrast to insulin-stimulated MEF2 DNA binding, AICAR-mediated MEF2 DNA binding was independent of 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

activation mediated by osmotic stress and hydrogen peroxide. This observation is consistent with the notion that these three stimuli increase AMPK activity in skeletal muscle (11, 17). In contrast to AICAR, hyperosmosis- and hydrogen peroxide-mediated MEF2 activation was inhibited by PD-98059 and SB-203580, indicating that these cellular stressors also activate different (AMPK independent) signaling pathways. Importantly, the fact that AMPK activation was noted in response to hyperosmosis and hydrogen peroxide further indicates that AMPK-mediated signals may not be sufficient for MEF2 DNA binding activation in skeletal muscle.

Contraction of skeletal muscle through exercise leads to a potent activation of AMPK. Although both AICAR and contraction increase AMPK activity, evidence is emerging that AICAR may not mimic all exercise- or contraction-induced responses, such as glucose transport (29). We therefore compared effects of AICAR and electrically stimulated contraction on MEF2 DNA binding in isolated rat epitrochlearis skeletal muscle. Incubation of isolated rat epitrochlearis muscle with AICAR increased MEF2 DNA binding activity, consistent with our present results observed in human skeletal muscle cultures and previous studies of in vivo AICAR treatment in rodents (34, 44). Furthermore, electrically stimulated muscle contraction also increased MEF2 DNA binding activity, consistent with our previous observations for exercise in humans (42). However, contraction-mediated, but not AICAR-mediated, MEF2 activation was reduced by MAPK inhibition. Although the role of AMPK in contraction-mediated signaling is still not completely elucidated, our results are consistent with the observation that contraction-mediated activation of MEF2 utilizes MAPK pathways in the regulation of MEF2 DNA binding, similar to our results observed for insulin or cellular stress (hyperosmolarity and hydrogen peroxide). Furthermore, our findings reveal that contraction is a direct physiological regulator of MEF2 DNA binding via MAPK pathways.

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.

ACKNOWLEDGMENTS

This study was supported by grants from the Swedish Research Council (12669, 12679, and 9517), Karolinska Institutet Foundation, Thuring's Foundation, Lars Hiertas Stiftelse, Tore Nilsons Stiftelse, Novo-Nordisk Foundation, Harald and Greta Jeansson's Stiftelse, Swedish Diabetes Association, Markus and Amalia Wallenberg Foundation, Wibergs Foundation, and Swedish Society for Medicine.

REFERENCES

1. Alessi DR, Cuenda A, Cohen P, Dudley DT, and Saltiel AR. PD 98059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. *J Biol Chem* 270: 27489–27494, 1995.
2. Al-Khalili L, Chibalin AV, Kannisto K, Zhang BB, Permert J, Holman GD, Ehrenborg E, Ding VDH, Zierath JR, and Krook A. Insulin

- action in cultured human skeletal muscle cells during differentiation: assessment of cell surface GLUT4 and GLUT1 content. *Cell Mol Life Sci* 60: 991–998, 2003.
3. Al-Khalili L, Krämer D, Wretenberg P, and Krook A. Enhanced insulin-mediated ERK1/2 MAPK and Akt/PKB phosphorylation in cultured human myocytes post differentiation. *Acta Physiol Scand* 180: 395–403, 2004.
 4. Andrews NC and Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res* 19: 2499, 1991.
 5. Black BL, Molkentin JD, and Olson EN. Multiple roles for the MyoD basic region in transmission of transcriptional activation signals and interaction with MEF2. *Mol Cell Biol* 18: 69–77, 1998.
 6. Black BL and Olson EN. Transcriptional control of muscle development by myocyte enhancer factor 2 (MEF2) proteins. *Annu Rev Cell Dev Biol* 14: 167–196, 1998.
 7. Blough E, Dineen B, and Esser K. Extraction of nuclear proteins from striated muscle tissue. *Biotechniques* 26: 202–204, 1999.
 8. Brand NJ. Myocyte enhancer factor 2 (MEF2). *Int J Biochem Cell Biol* 29: 1467–1470, 1997.
 9. Brand-Saberi B and Christ B. Genetic and epigenetic control of muscle development in vertebrates. *Cell Tissue Res* 296: 199–212, 1999.
 10. Chen HC, Bandyopadhyay G, Sajan MP, Kanoh Y, Standaert M, Farese RV Jr, and Farese RV. Activation of the ERK pathway and atypical protein kinase C isoforms in exercise- and aminoimidazole-4-carboxamide-1- β -D-ribose (AICAR)-stimulated glucose transport. *J Biol Chem* 277: 23554–23562, 2002.
 11. Choi SL, Kim SJ, Lee KT, Kim J, Mu J, Birnbaum MJ, Soo Kim S, and Ha J. The regulation of AMP-activated protein kinase by H₂O₂. *Biochem Biophys Res Commun* 287: 92–97, 2001.
 12. Cobb MH, Boulton TG, and Robbins DJ. Extracellular signal-regulated kinases: ERKs in progress. *Cell Regul* 2: 965–978, 1991.
 13. Cobb MH and Goldsmith EJ. How MAP kinases are regulated. *J Biol Chem* 270: 14843–14846, 1995.
 14. Corbit KC, Foster DA, and Rosner MR. Protein kinase C δ mediates neurogenic but not mitogenic activation of mitogen-activated protein kinase in neuronal cells. *Mol Cell Biol* 19: 4209–4218, 1999.
 15. Davies SP, Reddy H, Caivano M, and Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 351: 95–105, 2000.
 16. Dwivedi PP, Hii CS, Ferrante A, Tan J, Der CJ, Omdahl JL, Morris HA, and May BK. Role of MAP kinases in the 1,25-dihydroxyvitamin D₃-induced transactivation of the rat cytochrome P450C24 (CYP24) promoter. Specific functions for ERK1/ERK2 and ERK5. *J Biol Chem* 277: 29643–29653, 2002.
 17. Hayashi T, Hirshman MF, Fujii N, Habinowski SA, Witters LA, and Goodyear LJ. Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. *Diabetes* 49: 527–531, 2000.
 18. Kamakura S, Moriguchi T, and Nishida E. Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus. *J Biol Chem* 274: 26563–26571, 1999.
 19. Kato Y, Kravchenko VV, Tapping RI, Han J, Ulevitch RJ, and Lee JD. BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. *EMBO J* 16: 7054–7066, 1997.
 20. Kato Y, Zhao M, Morikawa A, Sugiyama T, Chakravorty D, Koide N, Yoshida T, Tapping RI, Yang Y, Yokochi T, and Lee JD. Big mitogen-activated kinase regulates multiple members of the MEF2 protein family. *J Biol Chem* 275: 18534–18540, 2000.
 21. Kaushal S, Schneider JW, Nadal-Ginard B, and Mahdavi V. Activation of the myogenic lineage by MEF2A, a factor that induces and cooperates with MyoD. *Science* 266: 1236–1240, 1994.
 22. Krook A, Björnholm M, Jiang XJ, Galuska D, Fahlman R, Myers M, Wallberg-Henriksson H, and Zierath J. Characterization of signal transduction and glucose transport in skeletal muscle from type 2 (non-insulin-dependent) diabetic patients. *Diabetes* 49: 284–292, 2000.
 23. Le Good JA, Ziegler WH, Parekh DB, Alessi DR, Cohen P, and Parker PJ. Protein kinase C isoforms controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* 281: 2042–2045, 1998.
 24. Lu J, McKinsey TA, Zhang CL, and Olson EN. Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. *Mol Cell* 6: 233–244, 2000.
 25. McKinsey TA, Zhang CL, Lu J, and Olson EN. Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. *Nature* 408: 106–111, 2000.
 26. McKinsey TA, Zhang CL, and Olson EN. MEF2: a calcium-dependent regulator of cell division, differentiation and death. *Trends Biochem Sci* 27: 40–47, 2002.
 27. Meisse D, Van de Castele M, Beauloye C, Hainault I, Kefas BA, Rider MH, Foufelle F, and Hue L. Sustained activation of AMP-activated protein kinase induces c-Jun N-terminal kinase activation and apoptosis in liver cells. *FEBS Lett* 526: 38–42, 2002.
 28. Molkentin JD, Black BL, Martin JF, and Olson EN. Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *Cell* 83: 1125–1136, 1995.
 29. Mu J, Brozinick JT Jr, Valladares O, Bucan M and Birnbaum MJ. A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Mol Cell* 7: 1085–1094, 2001.
 30. Naidu PS, Ludolph DC, To RQ, Hinterberger TJ, and Konieczny SF. Myogenin and MEF2 function synergistically to activate the MRF4 promoter during myogenesis. *Mol Cell Biol* 15: 2707–2718, 1995.
 31. Ryder JW, Chibalin AV, and Zierath JR. Intracellular mechanisms underlying increases in glucose uptake in response to insulin or exercise in skeletal muscle. *Acta Physiol Scand* 171: 249–257, 2001.
 32. Ryder JW, Fahlman R, Wallberg-Henriksson H, Alessi DR, Krook A, and Zierath JR. Effect of contraction on mitogen-activated protein kinase signal transduction in skeletal muscle. Involvement of the mitogen- and stress-activated protein kinase 1. *J Biol Chem* 275: 1457–1462, 2000.
 33. Schonwasser DC, Marais RM, Marshall CJ, and Parker PJ. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isoforms. *Mol Cell Biol* 18: 790–798, 1998.
 34. Song XM, Fiedler M, Galuska D, Ryder JW, Fernstrom M, Chibalin AV, Wallberg-Henriksson H, and Zierath JR. 5-Aminoimidazole-4-carboxamide ribonucleoside treatment improves glucose homeostasis in insulin-resistant diabetic (ob/ob) mice. *Diabetologia* 45: 56–65, 2002.
 35. Suzuki Y, Yoshizumi M, Kagami S, Koyama AH, Taketani Y, Houchi H, Tsuchiya K, Takeda E, and Tamaki T. Hydrogen peroxide stimulates c-Src-mediated big mitogen-activated protein kinase 1 (BMK1) and the MEF2C signaling pathway in PC12 cells: potential role in cell survival following oxidative insults. *J Biol Chem* 277: 9614–9621, 2002.
 36. Tamir Y and Bengal E. Phosphoinositide 3-kinase induces the transcriptional activity of MEF2 proteins during muscle differentiation. *J Biol Chem* 275: 34424–34432, 2000.
 37. Thai MV, Guruswamy S, Cao KT, Pessin JE, and Olson AL. Myocyte enhancer factor 2 (MEF2)-binding site is required for GLUT4 gene expression in transgenic mice. Regulation of MEF2 DNA binding activity in insulin-deficient diabetes. *J Biol Chem* 273: 14285–14292, 1998.
 38. Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, and Loriolle F. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J Biol Chem* 266: 15771–15781, 1991.
 39. Vlahos CJ, Matter WF, Hui KY, and Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* 269: 5241–5248, 1994.
 40. Xi X, Han J, and Zhang JZ. Stimulation of glucose transport by AMP-activated protein kinase via activation of p38 mitogen-activated protein kinase. *J Biol Chem* 276: 41029–41034, 2001.
 41. Xu Q and Wu Z. The insulin-like growth factor-phosphatidylinositol 3-kinase-Akt signaling pathway regulates myogenin expression in normal myogenic cells but not in rhabdomyosarcoma-derived RD cells. *J Biol Chem* 275: 36750–36757, 2000.
 42. Yu M, Blomstrand E, Chibalin AV, Krook A, and Zierath JR. Marathon running increases ERK1/2 and p38 MAP kinase signalling to downstream targets in human skeletal muscle. *J Physiol* 536: 273–282, 2001.
 43. Zetser A, Gredinger E, and Bengal E. p38 mitogen-activated protein kinase pathway promotes skeletal muscle differentiation. Participation of the Mef2c transcription factor. *J Biol Chem* 274: 5193–5200, 1999.
 44. Zheng D, MacLean PS, Pohner SC, Knight JB, Olson AL, Winder WW, and Dohm GL. Regulation of muscle GLUT-4 transcription by AMP-activated protein kinase. *J Appl Physiol* 91: 1073–1083, 2001.
 45. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, and Moller DE. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 108: 1167–1174, 2001.