Hypoxia stimulates via separate pathways ERK phosphorylation and NF-κB activation in skeletal muscle cells in primary culture

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Hypoxia stimulates via separate pathways ERK phosphorylation and NF-κB activation in skeletal muscle cells in primary culture. J Appl Physiol 106: 1301–1310, 2009. First published January 29, 2009; doi:10.1152/japplphysiol.91224.2008.—Mammalian cells sense oxygen levels and respond to hypoxic conditions through the regulation of multiple signaling pathways and transcription factors. Here, we investigated the effects of hypoxia on the activity of two transcriptional regulators, ERK1/2 and NF-κB, in skeletal muscle cells in primary culture. We found that hypoxia significantly enhanced ERK1/2 phosphorylation and that it stimulated NF-κB-dependent gene transcription as well as nuclear translocation of a green fluorescent protein-labeled p65 NF-κB isoform. Phosphorylation of ERK1/2- and NF-κB-dependent transcription by hypoxia required calcium entry through L-type calcium channels. Calcium release from ryanodine-sensitive stores was also necessary for ERK1/2 activation but not for NF-κB-dependent transcription. N-acetylcysteine, a general scavenger of reactive oxygen species, blocked hypoxia-induced ROS generation but did not affect the stimulation of ERK1/2 phosphorylation induced by hypoxia. In contrast, NF-κB activation was significantly inhibited by N-acetylcysteine and did not depend on ERK1/2 stimulation, as shown by the lack of effect of the upstream ERK inhibitor U-0126. These separate pathways of activation of ERK1/2 and NF-κB by hypoxia may contribute to muscle adaptation in response to hypoxic conditions.

Adaptive responses to hypoxia occur in the skeletal muscle of humans exposed temporarily or permanently to high altitude. A more widespread activity such as dynamic exercise also produces temporal and spatial hypoxia (8, 18, 25). The hypoxia-inducible factor HIF-1 is accumulated in human skeletal muscle after exercise (18, 25), while endurance-training results in the modification of several hypoxia-sensitive genes (25). As a result, skeletal muscle responds to hypoxia with changes in the expression of genes for structural proteins and enzymes of the energetic metabolism (18, 25). Moreover, local hypoxia may cause some of the responses displayed by muscle exposed to exercise (2, 12, 17, 26, 35). Yet, with the exception of the HIF-1 increase reported to occur in skeletal muscle under endurance exercise, other exercise-induced responses have not been linked to hypoxia (18, 25).

The generation of reactive oxygen species (ROS) increases in many cell types, including skeletal muscle, in response to hypoxia (8, 40, 42, 45). A rise in intracellular calcium concentration also occurs in many different cell types following a ROS increase; this calcium increase may be caused by ROS-mediated activation of calcium entry pathways or of calcium release from intracellular stores, mediated either by inositol 1,4,5-trisphosphate receptors (IP3Rs) or by ryanodine receptors (RyRs) (16). In skeletal muscle cells in primary culture, an exogenous ROS such as hydrogen peroxide (H2O2) induces a rise in intracellular calcium concentration that in turn promotes the activation of several calcium-dependent transcriptional regulators, including the mitogen-activated protein kinase ERK1/2 (10). The ERK1/2 proteins and the nuclear factor-κB (NF-κB) are considered major regulators of the response of skeletal muscle to stressful situations (21, 26). Moreover, exercise and sustained depolarization of skeletal muscle cells activate ERK1/2 and NF-κB via ROS generation and intracellular calcium increase (5, 6, 14, 17, 20, 26, 36, 43). NF-κB activation in skeletal muscle in response to exercise has been suggested to play a role in the increased expression of cell defense enzymes like superoxide dismutase, and of enzymes related to adaptation to exercise like inducible nitric oxide synthase and endothelial nitric oxide synthase (14). Yet, the effects of hypoxic conditions on these parameters have not been reported. Accordingly, we studied in this work the response to hypoxia of primary skeletal myotubes focusing on these two transcriptional regulators, ERK1/2 and NF-κB.

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MAMMALIAN OXYGEN HOMEOSTASIS must be tightly regulated to maintain the oxygen levels needed for critical oxygen-dependent processes. Hypoxia occurs when oxygen availability/delivery decreases below the levels required for sustaining the physiological oxygen tension of a particular tissue, i.e., when the tissue demand for oxygen exceeds its supply. Mammalian cells possess different mechanisms to sense oxygen levels and to respond and adapt to hypoxic conditions. The cellular responses to hypoxia are complex and involve the regulation of multiple signaling pathways and transcription factors that promote the coordinated expression of numerous genes (40, 42). In particular, the hypoxia-inducible factor-1 (HIF-1) is a critical transcription factor that under hypoxic conditions regulates the transcription of many genes in cell specific ways (39).
found that hypoxia enhanced ERK1/2 phosphorylation and NF-κB-dependent transcription and translocation by calcium-dependent mechanisms that engaged different cellular calcium sources. Thus ERK1/2 activation required both calcium entry and RyR-mediated calcium release from sarcoplasmic reticulum, whereas NF-κB activation only required extracellular calcium entry. In addition, our results strongly suggest that ROS play an important role in NF-κB activation but do not affect hypoxia-induced ERK1/2 phosphorylation.

MATERIALS AND METHODS

Materials. DMEM/F-12, ryanodine, nifedipine, and horseradish peroxidase (HRP)-conjugated anti-mouse antibody were from Sigma (St. Louis, MO). Fetal calf serum, calf serum, antibiotics, and antimycotics were from Invitrogen (Grand Island, NY). N-acetylcysteine (NAC) and U-0126 were from BIOMOL Research Laboratories (Plymouth Meeting, PA). Antibodies against dual phosphorylated forms of ERK-1 and ERK-2 and anti-ERK2 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against β-actin and HIF-1α were from Cell Signalling Technology (Beverly, MA) and Novus Biologicals (Littleton, CO), respectively. Secondary HRP-conjugated anti-rabbit antibody was from Pierce (Rockford, IL). Enhanced chemiluminescence reagents were from Amersham Pharmacia Biotech (Amersham, UK). Polyvinylidene difluoride (PVDF) membranes were from Millipore (Bedford, MA). All other reagents were obtained from Sigma, Merck (Darmstadt, Germany), or Invitrogen.

Cell cultures. The experimental protocol was approved by the Bioethics Committee for Investigation in Animals of the Facultad de Medicina, Universidad de Chile. Primary cultures of skeletal muscle cells were prepared from Sprague-Dawley neonate rats as previously reported (6). Briefly, muscle tissue dissected from the hindlimbs was minced, incubated with collagenase for 15 min at 37°C and grown in 60-mm plates in medium composed of F12-DMEM (1:1), 10% bovine serum, 2.5% FBS, 100 U/ml penicillin, and 10 mg/ml streptomycin. Two days after plating, fibroblast growth was inhibited with cytosine arabinoside (5 mM) that was maintained for 24 h. For differentiation, the growth medium was changed to serum-free medium at day 4 of culture. Six- to seven-day-old cultures were employed for the experiments.

Cell exposure to hypoxia. Cells were washed with Ca²⁺- and Mg²⁺-free PBS and maintained under resting conditions for 30 min in Krebs-Ringer (in mM: 20 HEPES-Tris, pH 7.4, 118 NaCl, 4.7 KCl, 3 CaCl₂, 1.2 MgCl₂, and 10 glucose). To attain hypoxic conditions, skeletal muscle cells were placed in a hypoxia chamber and infused with nitrogen. The oxygen pressure (PO₂) was measured with a gas analyzer (model ML 205, AD Instruments) connected to a computer, and quantified as oxygen contents (normoxia 21% oxygen; hypoxia 5%, and quantified as oxygen contents (normoxia 21% oxygen; hypoxia 1% oxygen).

For immunoblot experiments, cells were exposed to hypoxia for variable times (10–30 min) and then lysed immediately, and for immunocytochemistry, cells were exposed to hypoxia for 30 min, maintained in normoxic conditions for 60 min, and then fixed. For reporter vector experiments, cells were exposed to hypoxia during 30 min, the medium was changed after 15 min and subsequently grown for 12 h under normoxia, and then the cells were lysed. Cells were incubated with pharmacological inhibitors for 15 to 30 min prior to hypoxia, as specified in the text. All experiments were matched with vehicle-treated controls. Concentrations of DMSO or ethanol were kept <0.1%; these concentrations had no effect on the subsequent responses of skeletal muscle cells to different pharmacological inhibitors. Both control and experimental cells underwent the same bath changes to discard possible differences induced by handling.

Determination of ROS production. To determine ROS in cell cultures under hypoxic conditions, the hypoxia protocol was modified as follows: 30 min before the experiments and during the experiments, myotubes were perfused with Krebs solution constantly bubbled with 100% nitrogen. In control experiments, myotubes were handled identically except that exposure to hypoxic Krebs solution was omitted. All experiments were done at room temperature. Myotubes were loaded with 10.8 μM 5- and 6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-HeDCAFD) (Molecular Probes, Eugene, OR) for 30 min in Krebs solution. To allow deesterification, excess dye was washed and cells were incubated in Krebs solution for 15 min. Myotubes were transferred to the recording chamber and mounted on an inverted fluorescence microscope. CM-HeDCAFD fluorescence was detected using excitation at 488 nm and emission at 510–540 nm. Images were collected every 30.4 s and regions of interest were analyzed with the Image J program [National Institutes of Health (NIH)]. The ROS signals are presented as (F − F₀)/F₀ values, where F is fluorescence and F₀ corresponds to the basal fluorescence obtained at time 0.

Protein immunodetection. Western blotting was performed as previously reported (6, 43). Briefly, cells were solubilized at 4°C in 0.05 ml of lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 5 mM NaF, 0.1 mM Na3VO4, 10 μM sodium pyrophosphate and a protease inhibitor cocktail from Calbiochem-Novabiochem (San Diego, CA). Aliquots of lysates were suspended in Laemmli buffer, and proteins were resolved in 10% SDS-polyacrylamide gels and transferred to PVDF membranes. Primary antibody incubations using dilutions of 1:2,500 (phospho-ERK1/2 or total ERK), 1:2,000 (β-actin) and 1:500 (anti-HIF-1-α) were carried out at 4°C overnight. After incubation with HRP-conjugated secondary antibodies for 1.5 h, membranes were developed by enhanced chemiluminescence according to the manufacturer’s instructions. To correct for loading, the membranes were stripped and blotted for anti-ERK1/2 or anti-β-actin. After scanning the films, densitometry analysis of the bands was performed with the Image J program.

Transient transfection and reporter assay for NF-κB-dependent transcription. Cells in primary culture were transiently transfected with FuGene 6 (Roche Applied Science) according to the manufacturer’s specifications. Briefly, 2-day-old myoblasts plated in 60-mm culture dishes were transfected with 0.9 μg of the reporter vector DNA (6× NF-κB-pGL3) and 0.1 μg of the Renilla pRL-TK vector (Promega, Madison, WI) plus 3 μl FuGene 6 in 1.5 ml DMEM (43). The NF-κB reporter plasmid was produced cloning six tandem repeats of NF-κB binding sites inserted immediately upstream of the pGL3 promoter using XhoI restriction sites (Promega). The orientation of the insert was verified by PCR amplification of the isolated DNA of the different clones. After 12 h, the medium containing the DNA complexes was replaced by serum-free medium. Luciferase activity was determined using a dual-luciferase reporter assay system (Promega), and light detection was carried out in a Berthold F12 luminometer. Results were normalized for transfection efficiency and expressed as the ratio of firefly to Renilla luciferase. Transfection efficiency was 5%, and reporter expression was assessed by the analysis of luciferase activity at 12 or 16 h after hypoxia.

Localization of p65-eGFP in muscle cells by fluorescence microscopy. Primary skeletal muscle cells were grown on coverslips and transiently transfected with Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to the manufacturer’s specifications. Briefly, 4-day-old myoblasts plated in 60-mm culture dishes were transfected with 1 μg enhanced green fluorescent protein (eGFP)- or the p65-eGFP vector plus 3 μl of Lipofectamine in 1.5 ml of DMEM. Full-length p65 was subcloned from p65-GFP (32) into the pEGFP-N1 vector (Clontech) using HindIII and BamHI restriction sites. After 3 h, the medium-DNA complexes were replaced by serum-free medium. Seven-day-old myotubes transfected with the p65-eGFP plasmid were incubated for 30 min in Krebs-Ringer under resting conditions before stimulation and exposure to hypoxia. The effects of different compounds on the response of cells to hypoxia were tested; these included the general antioxidant agent NAC, the L-type calcium channel blocker nifedipine, the calcium chelating agent BAPTA, and the protein kinase C inhibitor GF109203X.
H2DCFDA fluorescence represented a cellular ROS increase; fluorescence was caused by hypoxia; this increase in CM-rescence. In these conditions, a significant increase in probe
above, cellular calcium can increase through enhanced calcium
hypoxia-induced ERK1 or ERK2 phosphorylation (Fig. 3).

Findings strongly suggest that the enhanced ERK1/2 phosphorylation produced by hypoxia requires both calcium entry and RyR-mediated CICR from intracellular stores. Accordingly, we tested whether calcium entry and/or calcium release was required for hypoxia-induced ERK1/2 phosphorylation. Incubation of myotubes in calcium-free medium and subsequent exposure to hypoxia for 10–30 min decreased significantly the stimulatory effects of hypoxia on both phosphorylated ERK1 and phosphorylated ERK2 (Fig. 4). These results suggest that extracellular calcium, presumably to ensure calcium entry, is an essential requisite for the hypoxia-induced ERK1/2 phosphorylation increase. To test whether calcium entry through L-type plasma membrane calcium channels is involved in this response, myotubes were preincubated with 10 μM nifedipine, a selective blocker of L-type voltage-dependent calcium channels, and subsequently exposed to hypoxia. Nifedipine effectively prevented the increase in the phosphorylation of both ERK1 and ERK2 after 20 or 30 min under hypoxic conditions (Fig. 5), suggesting that calcium entry through L-type calcium in response to hypoxia is a requisite step of hypoxia-induced ERK1/2 phosphorylation.

A joint increase in calcium and ROS stimulate RyR-mediated calcium-induced calcium release (CICR) from sarcoplasmic reticulum vesicles isolated from mammalian skeletal muscle (16). To test whether hypoxia-induced RyR-mediated calcium release also contributed to stimulated ERK1/2 phosphorylation, myotubes were preincubated for 60 min with 50 μM ryanodine and were then subjected to hypoxia in the continuous presence of 50 μM ryanodine. The presence of ryanodine prevented (P < 0.05) the enhanced phosphorylation of ERK1 (20 and 30 min) and ERK2 (20 min) induced by hypoxia (Fig. 6). These findings strongly suggest that the enhanced ERK1/2 phosphorylation produced by hypoxia requires both calcium entry and RyR-mediated CICR from intracellular stores.

**Effect of hypoxia on NF-κB activity.** The NF-κB proteins include the p65 and p50 NF-κB isoforms. In non-stimulated cells, these NF-κB proteins display a predominant cytoplasmic location and are associated with the inhibitory IκB proteins. A variety of NFκB activators stimulate the kinases that phosphorylate the IκB proteins and that are known as IKK. The phosphorylated IκBs are subsequently ubiquinated and degraded by the proteasome pathway (15). IκB dissociation allows the nuclear translocation of the NF-κB proteins; within the nucleus, other regulatory steps such as posttranslational modifications of NF-κB proteins and chromatin remodeling may occur, resulting in enhanced transcription of several genes (7).

Exercise and depolarization activate the nuclear transcription factor NF-κB in skeletal muscle cells (5, 14, 17, 20, 26, 43). In particular, the p65 NF-κB isoform translocates to the

**RESULTS**

To control for the hypoxic experimental conditions, we determined first the HIF-1 protein content of skeletal muscle cells exposed to hypoxia. Western blot analysis showed an increase in HIF-1 protein content after 10 min of hypoxia, the shortest time analyzed, with a further increase after 20 min to levels that remained constant for up to 90 min under hypoxic conditions (data not shown).

**Effect of hypoxia on ROS levels.** Previous reports in skeletal muscle and other cell types (8, 45) indicate that there is enhanced ROS generation during hypoxia. To determine whether hypoxia induces ROS generation in skeletal muscle cells in primary culture, it was necessary to induce hypoxia by directly bubbling nitrogen on the plates containing myotubes preloaded with CM-H2DCFDA while measuring probe fluorescence. In these conditions, a significant increase in probe fluorescence was caused by hypoxia; this increase in CM-H2DCFDA fluorescence represented a cellular ROS increase because it was completely prevented by 10 mM NAC (Fig. 1).

**Effects of hypoxia on ERK1/2 phosphorylation.** In the present study, ERK 1/2 phosphorylation was determined by Western blotting of total and phosphorylated ERK1/2 proteins. We found that myotubes exposed to hypoxia exhibited a significant increase in both ERK1 and ERK2 phosphorylation at 10, 20, or 30 min after establishing hypoxic conditions; ERK1/2 phosphorylation decreased after 60 min under hypoxia (Fig. 2) to reach at 90 min levels that were not significantly different from controls (not shown). To test the possible contribution of ROS to hypoxia-stimulated ERK1/2 phosphorylation, we preincubated cells with 10 mM NAC and maintained NAC during hypoxia. In these conditions, NAC did not modify significantly hypoxia-induced ERK1 or ERK2 phosphorylation (Fig. 3).

Hypoxia promotes an increase in the intracellular calcium concentration in several cell systems (40, 42). As discussed above, cellular calcium can increase through enhanced calcium entry or via stimulation of calcium release from intracellular stores. Accordingly, we tested whether calcium entry and/or calcium release was required for hypoxia-induced ERK1/2 phosphorylation. Incubation of myotubes in calcium-free medium and subsequent exposure to hypoxia for 10–30 min decreased significantly the stimulatory effects of hypoxia on both phosphorylated ERK1 and phosphorylated ERK2 (Fig. 4). These results suggest that extracellular calcium, presumably to ensure calcium entry, is an essential requisite for the hypoxia-induced ERK1/2 phosphorylation increase. To test whether calcium entry through L-type plasma membrane calcium channels is involved in this response, myotubes were preincubated with 10 μM nifedipine, a selective blocker of L-type voltage-dependent calcium channels, and subsequently exposed to hypoxia. Nifedipine effectively prevented the increase in the phosphorylation of both ERK1 and ERK2 after 20 or 30 min under hypoxic conditions (Fig. 5), suggesting that calcium entry through L-type calcium in response to hypoxia is a requisite step of hypoxia-induced ERK1/2 phosphorylation.

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Exercise and depolarization activate the nuclear transcription factor NF-κB in skeletal muscle cells (5, 14, 17, 20, 26, 43). In particular, the p65 NF-κB isoform translocates to the external nuclear compartment, and its steady-state level is positively regulated by phosphorylation. The NF-κB transcription factor is translocated via the IκBα ubiquitin-proteasome degradation mechanism (15). Upon stimulation, the NF-κB inhibitor, IκBα, is phosphorylated on Ser-32 and Ser-36 by an IKK complex consisting of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NEMO (15). The phosphorylation of IκBα allows for ubiquitination and proteasome-mediated degradation (15). The degradation of IκBα exposes the nuclear localization signal in NF-κB dimers (15). The NF-κB dimers translocate to the nucleus and bind to specific DNA sequences, known as κB sites, to activate transcription of target genes.

**Fig. 1.** Hypoxia increases reactive oxygen species in primary myotubes. Myotubes were perfused with hypoxic solution and 5- (and 6)-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate acetyl ester CM-H2DCFDA fluorescence was recorded during 25 min. ○, Hypoxic myotubes; ●, control myotubes. At 20 min, hypoxic myotubes were perfused with 10 mM NAC. Values are means ± SE; n = 3. NAC, N-acetylcysteine; F, fluorescence; F₀, basal fluorescence obtained at time 0. In control and under hypoxic conditions the calculated slopes from data were 0.017 ± 0.002 and 0.028 ± 0.002, respectively (P < 0.01).
nucleus in skeletal muscle cells in primary culture exposed to electrical stimulation (43). In this work, NF-κB activation was assessed by a Luciferase reporter gene assay that measures NF-κB-dependent transcription, and by studying the translocation of a GFP-labeled p65 (p65-eGFP) protein to the nucleus. In some cases, we also measured IκB degradation as an additional indicator of NF-κB activation following hypoxia.

The present results indicate that NF-κB-dependent transcription, assessed by the Luciferase reporter gene, increased greater than threefold in myotubes exposed to hypoxia (Fig. 7A). In primary skeletal muscle cells depolarized by exposure to high K⁺, there is a minor but significant contribution of ERK1/2 phosphorylation to NF-κB activation (43). To test whether ERK1/2 phosphorylation was also involved in hypoxia-induced NFκB activation, we inhibited MEK, the upstream ERK kinase. We found that the specific MEK inhibitor U-0126 did not affect hypoxia-enhanced NF-κB-dependent transcription, as determined by the reporter gene assay (Fig. 7A). Similarly, U-0126 did not affect the increase in IκBα degradation produced by 30- or 60-min incubation under hypoxic conditions (Fig. 7, B and C). These combined results strongly suggest that hypoxia enhances NF-κB-dependent transcription and that ERK pathway stimulation does not contribute to this response.

Preincubation of myotubes for 30 min with 10 μM nifedipine or removal of extracellular calcium produced a significant inhibition (P < 0.05) of hypoxia-induced NF-κB-dependent transcription (Fig. 8, A and B); in contrast, 30-min preincubation with 50 μM ryanodine did not affect NF-κB activation (Fig. 8C). Combined, these results suggest that NF-κB activation requires calcium entry through L-type calcium channels but is independent of RyR-mediated calcium release.

Fig. 2. Hypoxia enhances ERK1/2 phosphorylation in primary myotubes. Myotubes were maintained in hypoxic conditions for the times indicated and lysed, and proteins (50 μg) were analyzed by Western blot of phosphorylated (p) ERK1/2 and total ERKs. A: representative Western blots. B and C: bar graphs represent P-ERK1/ERK and P-ERK2/ERK ratios (means ± SE; n = 7), respectively. Statistical analysis was performed by one-way ANOVA test followed by Bonferroni’s post test. *P < 0.05, **P < 0.01, and ***P < 0.001 with respect to the control group (normoxia).

Fig. 3. NAC does not modify hypoxia-stimulated ERK1/2 phosphorylation. Myotubes were maintained under hypoxic conditions in the presence or absence of 10 mM NAC. The antioxidant was present for 15 min of preincubation and during the hypoxic treatment. Six to seven independent experiments were performed; a representative Western blot is shown in A. The bar graphs in B and C represent P-ERK1/ERKs and P-ERK2/ERKs ratios, respectively, expressed as means ± SE. Two-way ANOVA followed by Bonferroni’s post test was performed. *P < 0.05 and **P < 0.01 with respect to nonstimulated cells (normoxia).
NF-κB is considered a prototypical ROS-sensitive transcription factor, albeit this characteristic is cell and context dependent (13). In the particular case of myotubes, depolarization-induced NF-κB activation presumably involves ROS participation, as suggested by the inhibitory effects of NAC on this response (43). In myotubes exposed to hypoxic conditions, preincubation with 10 mM NAC for 15 min decreased to near control levels (*P < 0.05) the enhanced activity of the NF-κB reporter gene induced by hypoxia (Fig. 8D). In addition, reporter gene assay experiments performed in the absence of extracellular calcium and in the presence of NAC as above resulted in complete inhibition of hypoxia-induced NF-κB activation (Fig. 8E).

Complementing the above results, immunocytochemical analysis of skeletal muscle cells transfected with p65-eGFP indicated that 90 min under hypoxia effectively promoted the translocation of p65-eGFP to the nucleus (Fig. 9, panels 1 and 3). Cells exposed to hypoxia for 30 min and fixed immediately did not exhibit p65-eGFP translocation (not shown), suggesting that more time is needed for translocation to occur. Yet, p65-eGFP translocation to the nucleus in cells exposed to hypoxia for 30 min, incubated under normoxic conditions for 60 min, and then fixed was comparable to that seen in cells exposed to hypoxia for 90 min and fixed immediately (Fig. 9, panels 2 and 3). The absence of extracellular calcium, the addition of the general antioxidant NAC, or these two combined conditions inhibited p65-eGFP translocation to the nucleus in cells exposed to hypoxia for 30 min and fixed after incubation under normoxic conditions for 60 min (Fig. 9: panels 4, 5, and 6, respectively).

Fig. 4. Hypoxia-stimulated ERK1/2 phosphorylation requires extracellular calcium. Myotubes were maintained in hypoxic conditions either in the presence of 3 mM calcium or in the absence of calcium plus the addition of 0.5 mM EGTA. A: representative Western blots. B and C: bar graphs represent P-ERK1/2 and P-ERK2/ERK ratios (means ± SEM; n = 7–8). Statistical analysis was performed by two-way ANOVA test followed by Bonferroni’s post test. *P < 0.05 and **P < 0.01 in the presence of extracellular calcium with respect to the not stimulated cells; &P < 0.05 and &&P < 0.01 in the absence of extracellular calcium with respect to each control condition in the presence of calcium.

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Fig. 5. Blockade of L-type calcium channels by nifedipine inhibits hypoxia-enhanced ERK1/2 phosphorylation. Cells were preincubated with or without 10 μM nifedipine in the presence of extracellular calcium, and maintained in hypoxic conditions in the same incubation conditions. A: Western blots of P-ERKs and total ERKs. B and C: P-ERK1/2 and P-ERK2/ERK ratios (means ± SE; n = 6–7). Statistical analysis was performed by two-way ANOVA test followed by Bonferroni’s post test. *P < 0.05 for cells incubated without nifedipine, with respect to the not stimulated cells; &P < 0.05 for cells incubated with nifedipine with respect to each control in the absence of nifedipine.

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These combined results show that NF-κB activation, as measured by the reporter gene assay or by p65-eGFP translocation to the nucleus, requires calcium entry through L-type calcium channels and cellular ROS generation.

**DISCUSSION**

The main novel findings reported in this work are that hypoxia significantly enhanced ERK1/2 phosphorylation and activated NF-κB-dependent transcription and p65 translocation to the nucleus in primary muscles cells that follow separate pathways. Activation of both ERK1/2 and NF-κB by hypoxia required calcium entry through L-type calcium channels. Yet, ERK1/2 activation also required calcium release from ryanodine-sensitive stores and was unaffected by NAC added as a ROS scavenger, whereas NF-κB-dependent transcription was significantly inhibited by NAC but was not affected by ryanodine and did not depend on ERK1/2 stimulation.

**Calcium requirements for hypoxia-induced ERK1/2 and NFκB stimulation.** We found in this work that calcium entry through L-type calcium channels was required for the activation of ERK1/2 and NFκB induced by acute hypoxia, as indicated by the inhibitory effects of nifedipine. These results represent the first description of ERK1/2 and NF-κB activation...
by hypoxia-induced L-type calcium channel stimulation in skeletal muscle cells in culture. In other excitable cells, chronic hypoxia can induce changes in L-type calcium channel expression and activity (see Ref. 34), whereas by inducing membrane depolarization, acute hypoxia activates voltage-gated calcium channels and causes a significant increase in intracellular calcium concentration in PC12 cells (40). In addition, the signaling pathways that lead to stimulation of HIF-1 translation in PC12 cells require calcium influx from the extracellular environment through L-type channels (19).

**ERK1/2 activation.** There is ample evidence in the literature indicating that the ERK1/2 proteins are activated downstream of Ras through calcium activation of Ras-specific exchange factors (41). Our laboratory has described calcium-dependent transient increase of ERK1/2 phosphorylation in skeletal muscle cells in primary culture following depolarization (6, 36) or exogenous H$_2$O$_2$ addition (10); in both cases, ERK1/2 stimulation involved calcium release from intracellular stores. Our laboratory has also reported a calcium-mediated increase in ERK1/2 phosphorylation in N2A and hippocampal neurons exposed to exogenous H$_2$O$_2$, due to RyR-mediated calcium release (23).

The present results represent the first description that myotubes exposed to acute hypoxia displayed a transient ERK1/2 phosphorylation increase that required both calcium entry through L-type calcium channels and RyR-mediated calcium release from intracellular stores. Whereas calcium entry was required for both ERK1/2 and NF-κB activation, RyR-mediated calcium release was needed for ERK1/2 but not for NF-κB activation. These results suggest that hypoxia, by simultaneously enhancing calcium entry and ROS production, stimulates RyR-mediated CICR producing intracellular calcium signals that possess the right magnitude and/or location to stimulate ERK1/2 phosphorylation. Previous studies suggest participation of calcium release from intracellular stores in hypoxia-induced responses in smooth muscle cells and astrocytes (33). Additionally, calcium release from ryanodine-sensitive, but not from inositol 1,4,5-trisphosphate (IP$_3$)-sensitive stores, participates in the acute pulmonary vasoconstriction induced by hypoxia in perfused rat lungs (30). Similarly, in pulmonary artery smooth muscle cells the RyR3 isoform mediates calcium release and the ensuing hypoxia-induced contraction (44), while redox-activated RyR participate in the sustained phase of pulmonary vasoconstriction induced by hypoxia (9).

**NF-κB activation.** Calcium appears to stimulate NF-κB activity in neurons and muscle cells. In neurons, activation of NF-κB...
is triggered by a rise of intracellular calcium induced by either glutamate or depolarization (27, 29). In cerebellar granule neurons, NF-κB activation by depolarization requires calcium for both nuclear translocation and stimulation of the NF-κB p65 protein transactivating activity by phosphorylation (27). Furthermore, neuronal NF-κB activity is proportional to the concentration of extracellular potassium, and it involves the activity of L-type calcium channels and IP3R (27). Moreover, depolarization of skeletal muscle cells activates NF-κB, through IkBα degradation and p65 translocation to the nucleus, by a mechanism involving calcium release mediated either by RyR or IP3 receptors and that does not require extracellular calcium (43).

We report in this work that hypoxia produced a significant increase of NF-κB activity, as determined by a gene reporter assay, nuclear translocation of the p65 NF-κB isoform detected by immunocytochemistry, and detection of IkBα degradation by Western blots. The present results suggest that NF-κB activation requires calcium entry through L-type calcium channels but is independent of RyR-mediated calcium release. Yet, the potential contribution of IP3-dependent calcium release to hypoxia-induced NF-κB activation was not investigated in this work.

*Hypoxia, ERK1/2 phosphorylation, ROS, and NF-κB stimulation.* Acute as well as chronic hypoxic conditions promote the activation of the Ras/ERK1/2 cascade in several cell types.
Previous reports indicate that hypoxia also promotes NF-κB activation. In pancreatic cancer cells (Mia PaCa-2), long-term hypoxia (18 h) increases p65-NFκB DNA binding and NF-κB transactivation (37); NAC blocks these effects by decreasing p65-NF-κB binding to DNA without modifying NF-κB protein levels and NF-κB nuclear translocation during hypoxia. According to these authors, redox modulation of the NF-κB signaling pathway is a major protective mechanism against hypoxic apoptosis. In pulmonary artery smooth muscle cells exposed to acute hypoxia (30 min), the increase in NF-κB activity plays a critical role in regulating HIF-1α mRNA levels, which are also increased in this model (4). In these cells, the p65 and p50 NF-κB isoforms directly bind to the HIF-1α promoter under hypoxia, as shown by chromatin immunoprecipitation and electrophoretic mobility shift assay (4). A role for NF-κB activity at the level of HIF-1α transcription has been corroborated in hypoxia-mice lacking the β isoform of IKK (38).

Hypoxia-induced ROS generation may represent a normal physiological response in skeletal muscle, whereas chronic and extreme hypoxic exposure may prevent normal homeostasis, resulting in cell injury or dysfunction (8). Hypoxia strongly upregulates uncoupling protein 3 (UCP3), which is enriched in skeletal muscle and confers resistance against oxidative stress, whereas UCP3 absence in primary skeletal myocytes intensifies hypoxia-induced ROS generation (28). In isolated rat diaphragm strips, loaded with a fluorescent redox probe, the transition to low intracellular PO2 induces an early intracellular ROS burst, which is reversed on reoxygenation (45). Moreover, dynamic exercise produces skeletal muscle hypoxia (8, 18, 25), and exercise-induced free radical generation has been documented in humans (3).

Consequently, we investigated the eventual ROS contribution to hypoxia-induced ERK1/2 phosphorylation and NF-κB stimulation. The results indicate that the ROS scavenger NAC did not affect the hypoxia-induced increase in ERK1/2 phosphorylation. These findings contrast with the stimulation of ERK1/2 and cAMP response element binding protein phosphorylation and increased mRNA levels of the early genes c-fos and c-jun following H2O2 addition to skeletal muscle cells, which is mediated by H2O2-induced ryanodine-sensitive calcium signals (10). We propose that the calcium-dependent stimulation of ERK1/2 phosphorylation produced by hypoxia does not seem to involve ROS-dependent activation of RyR or L-type calcium channels.

In contrast to its lack of effect over ERK1/2 phosphorylation, NAC exerted a partial inhibitory effect on hypoxia-induced NF-κB activation determined by the reporter gene assay, suggesting ROS participation. The complete inhibition produced by incubation of myotubes with NAC in the absence of extracellular calcium suggests that NF-κB activation by hypoxia requires a joint increase in calcium and ROS, as occurs during depolarization of skeletal muscle cells (10, 43). The present results agree with previous reports on the effects of ROS on exercise-induced NF-κB activation (14, 43). Thus the xanthine oxidase inhibitor allopurinol blocks ROS generation and NF-κB activation in skeletal muscle of rats exercised until exhaustion (14). Furthermore, incubation of primary muscle cells with NAC (30 mM) reduced to about 50% of control values the NF-κB reporter gene activation induced by depolarization with high external K+ or by electrical stimulation (43). These two conditions produce a significant ROS increase in skeletal myotubes (10), which may be responsible for the stimulation of NF-κB produced by electrical stimulation (43).

In fact, activation of NF-κB by high micromolar H2O2 has been reported in two skeletal muscle cell lines (1, 24). In C2C12 cells, H2O2 causes increased NF-κB-dependent promoter activity and IkBα phosphorylation and degradation (24), while H2O2 increases p65 translocation in L6 skeletal muscle cells (1).

We found no significant effect of upstream ERK inhibition (with U-0126) on NF-κB activation by hypoxia, as determined by the reporter gene assay and by Western blot analysis of IκBα levels. In a study performed with C2C12 myoblasts, exposure to 0.1–3 mM H2O2 activated both ERK and NF-κB; p65 phosphorylation, but not NF-κB translocation or IκBα levels, was affected by pharmacological ERK inhibition (22). Exercise increases ERK1/2 phosphorylation and NF-κB activity in rat skeletal muscle; ERK inhibitors decrease somewhat the activation of NF-κB but the effects are not statistically significant; only the combination of U-0126 plus p38 MAPK inhibition decreases significantly the activation of NF-κB induced by exercise (17). Our laboratory has reported recently in skeletal muscle cells that ROS, calcineurin and protein kinase C participate in depolarization-induced NF-κB activation, whereas the contribution of ERK appears to be minor (43). Thus, in skeletal muscle cells, NF-κB activation induced by hypoxia, exercise, or depolarization seems to take place independently of the ERK pathway.

Concluding remarks. The present results show for the first time that short exposure of myotubes to hypoxic conditions leads to activation of ERK1/2 and NF-κB via distinct calcium-dependent pathways. Physical exercise elicits a number of changes in skeletal muscle gene expression, yet the signaling cascades and transcription factors involved are beginning to be unraveled (26). Exercise activates MAP kinases, including ERK1/2, and stimulates NF-κB (14, 17, 20, 26). It is conceivable that via activation of ERK1/2 and NF-κB, as reported here, hypoxic conditions produced by dynamic exercise (8, 18, 25) induce changes in gene expression that result in adaptive responses (e.g., increases in oxidative capacity and hypertrophy).

GRANTS

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