Nitric oxide facilitates NFAT-dependent transcription in mouse myotubes

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Drenning JA, Lira VA, Simmons CG, Soltow QA, Sellman JE, Criswell DS. Nitric oxide facilitates NFAT-dependent transcription in mouse myotubes. Am J Physiol Cell Physiol 294: C1088–C1095, 2008.—Intracellular calcium transients in skeletal muscle cells initiate phenotypic adaptations via activation of calcineurin and its effector nuclear factor of activated t-cells (NFAT). Furthermore, endogenous production of nitric oxide (NO) via calcium-calmodulin-dependent NO synthase (NOS) is involved in skeletal muscle phenotypic plasticity. Here, we provide evidence that NO enhances calcium-dependent nuclear accumulation and transcriptional activity of NFAT and induces phosphorylation of glycogen synthase kinase-3β (GSK-3β) in C2C12 myotubes. The calcium ionophore A23187 (1 μM for 9 h) or thapsigargin (2 μM for 4 h) increased NFAT transcriptional activity by seven- and fourfold, respectively, in myotubes transiently transfected with an NFAT-dependent reporter plasmid (pNFAT-luc, Stratagene). Cotreatment with the NOS-inhibitor Nω-nitro-l-arginine methyl ester (l-NAME; 5 mM) or the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM) prevented the calcium effects on NFAT activity. The NO donor diethylenetriamine-NONO (DETA-NO; 10 μM) augmented the effects of A23187 on NFAT-dependent transcription. Similarly, A23187 (0.4 μM for 4 h) caused nuclear accumulation of NFAT and increased phosphorylation (i.e., inactivation) of GSK-3β, whereas cotreatment with l-NAME or ODQ inhibited these responses. Finally, the NO donor 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPA-NONO; 10 mM) increased the phosphorylation of GSK-3β in a manner dependent on guanylate cyclase activity. We conclude that NOS activity mediates calcium-induced phosphorylation of GSK-3β and activation of NFAT-dependent transcription in myotubes. Furthermore, these effects of NO are guanylate cyclase-dependent.

Nitric oxide synthase; nuclear factor of activated t-cells; glycogen synthase kinase-3β; soluble guanylate cyclase; myosin heavy chain I/β;

ADULT VERTEBRATE SKELETAL MUSCLE consists of different fiber types, one slow (type I/β) and three fast (IIa, IIx, and IIb), which differ in their contraction speed, strength, fatigability, and insulin sensitivity. Skeletal muscle exhibits a high degree of plasticity with transformations in fiber type occurring in response to altered physiological demand and contractile load (3, 22). Tonic, low-frequency neural activity or electrical stimulation causes a shift from fast, glycolytic fibers toward the slow, oxidative phenotype (14, 19). The pathway by which low frequency muscle activation induces transcription of slow-twitch genes involves sustained calcium [Ca2+] levels sufficient to stimulate calcineurin phosphatase activity (6, 9, 27). Dephosphorylation of the nuclear factor of activated t-cells (NFAT) transcription factors by calcineurin promotes its translocation from the cytoplasm to the nucleus, where it will bind to a nucleotide recognition sequence and stimulate the transcription of target, slow-twitch genes (3, 11). Although this pathway explains activity-induced activation of NFAT, overall transcriptional activity, and therefore fiber type change, is determined by the balance between activation and deactivation of this transcription factor (1, 5). Recent studies suggest that glycogen synthase kinase-3β (GSK-3β) synergistically regulates nuclear export of NFAT in skeletal muscle fibers by phosphorylation of its serine residues (9, 24).

Nitric oxide (NO) is a ubiquitous signaling molecule produced enzymatically by NO synthases (NOS). Recently, it has been reported that NO is required for NFATc3 nuclear accumulation in mouse cerebral arteries in response to increased intravascular pressure and that this effect was dependent on inhibition of NFAT nuclear export (8). Since both constitutive isosforms of NOS (i.e., endothelial and neuronal isosfoms, eNOS and nNOS, respectively) are expressed by skeletal muscle cells (25) and are regulated by calcium-calmodulin binding (25), we postulated that a similar mechanism could be important for NFAT-dependent transcriptional adaptations in this tissue.

Our lab has recently reported that inhibition of NOS prevents induction of slow myosin heavy chain (MHC) gene expression during functional overload of the rat plantaris muscle (23). Since GSK-3β has been identified as an important regulator of NFATc1 nuclear export (24) and type I slow MHC expression (9) in muscle, we postulated that NO supports NFAT-dependent transcription, translocation, and nuclear accumulation in muscle cells by inhibiting GSK-3β activity. Therefore, we tested the following hypotheses: 1) endogenous NOS activity is necessary for calcium ionophore-induced slow MHC mRNA expression, 2) endogenous NOS activity is necessary for calcium ionophore-induced nuclear accumulation of NFAT and NFAT-dependent transcriptional activity, 3) NO activity is necessary for calcium-ionophore-induced nuclear accumulation of NFAT and NFAT-dependent transcriptional activity, 4) an NO donor is sufficient to affect GSK-3β phosphorylation, and 5) NO-dependent GSK-3β phosphorylation and calcium-ionophore-induced NFAT-dependent transcriptional activity are mediated by soluble guanylate cyclase (sGC) activity.

METHODS

Chemicals and Reagents

Calcium ionophore (A23187) and thapsigargin were purchased from Fisher BioReagents (Fair Lawn, NJ), and cyclosporin A (CsA) was from Sigma Chemical (St. Louis, MO). Nω-nitro-l-arginine methyl ester (l-NAME), 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1),

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diethylenetriamine-NONO (DETA-NO), methylene hexamethylene methylene-NONO (MAHMA-NO), and 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine-NONO (PAPA-NO) were obtained from Cayman Chemical (Ann Arbor, MI). Lithium chloride (LiCl) was purchased from Acros Organics (Morris Plains, NJ).

Cell Culture

Mouse C2C12 myoblasts were obtained from American Type Culture Collection (Manassas, VA) and cultured at 37°C in 5% CO2-95% atmospheric air.

Myoblasts were plated on 24-well collagen-coated plates and proliferated in Dulbecco's modified Eagle's medium (DMEM) growth media containing 10% fetal bovine serum and 1% penicillin-streptomycin. For the 4-aminooxy-5-methylamino-2,7'-difluorofluorescein (DAF-FM) experiment, C2C12 cells were plated on 24-well plates, grown to 70–80% confluency, and differentiation induced by switching to medium containing 2% horse serum for 5 days before treatment. For all luciferase experiments at 70–80% confluency, the growth medium was removed, and cultures were washed with serum-free media and transiently transfected with plasmid vectors as described below. For all Western blots and mRNA isolation, C2C12 myotubes were plated on six-well plates, grown to 70–80% confluency, and differentiation induced by switching to medium containing 2% horse serum for 7 days. Myotubes were treated with one or more of the following chemicals in media containing 2% serum: A23187, L-NAME, PAPA-NO, ODQ, YC-1, LiCl. Whenever treatments were used in combination, inhibitors of NOS (L-NAME) and guanylate cyclase (ODQ) were added 30 min before other treatments. Control groups were exposed to treatment vehicles in concentrations equal to experimental groups. When total protein extracts were harvested, cells were washed twice with ice-cold PBS and harvested in nondenaturing lysis buffer (NDL) containing 1% vol/vol protease inhibitors and 1% vol/vol phosphatase inhibitors. Concentration and purity of the extracted RNA were measured spectrophotometrically at A260 and A280 in 1× Tris-EDTA (TE) buffer (Promega). Reverse transcription (RT) was performed using the SuperScript III First-Strand Synthesis System for RT-PCR according to the manufacturer’s instructions (Life Technologies). Reactions were carried out using 5 μg of total RNA and 2.5 μM oligo(dT)20 primers. First-strand cDNA was treated with two units of RNase H and stored at −80°C. Primers and probes for slow MHC (GenBank NM_012751, 1m Assay no. Rn00562597_m1) were obtained from the ABI Assays-on-Demand service and consisted of Taqman 5' labeled FAM reporters and 3' nonfluorescent quenchers. Primer and probe sequences from this service are proprietary and, therefore, are not reported. Primer and probe sequences also consisting of Taqman 5' labeled FAM reporters and 3' nonfluorescent quenchers for hypoxanthine guanine phosphoribosyl transferase (HPRT) obtained from Applied Biosystems (Assays-by-Design) are the following: forward, 5'-GGTGGAATACAGGGCCAGTCTTGT-3'; reverse, 5'-AGTCAAGGGCATATCACAACAA -3'; Probe, 5'-ACTTGTCCTGGAAATTTCA-3'.

Quantitative real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (ABI, Foster City, CA). Each 25-μl PCR reaction contained 1 μl of cDNA reaction mixture. In this technique, amplification of the fluorescently labeled probe sequence located between the PCR primers was monitored in real time during the PCR program. The number of PCR cycles required to reach a predetermined threshold of fluorescence (CT) was determined for each sample. Samples were quantified relative to the CT (using the 2−ΔΔCT method, where CT is threshold cycle) (20) for a normalizing gene (HPRT) determined separately in the same sample.

Measurement of NO Production

Intracellular NO was monitored with DAF-FM (Invitrogen, Carlsbad, CA), a pH-insensitive fluorescent dye that emits increased fluorescence after reaction with an active intermediate of NO formed during the spontaneous oxidation of NO to NO2. C2C12 myotubes were incubated at 37°C for 30 min in phenol red-free, serum-free DMEM containing 10 mM of DAF-FM diacetate. After loading was completed, cells were rinsed three times with phenol red-free, serum-free DMEM and then placed in a SpectraMax M5 multi-detection reader (Molecular Devices, Sunnyvale, CA) for fluorometric analysis of live cells. NO fluorescence was measured using excitation and emission wavelengths of 488 and 520 nm, respectively. After measurement, cells were harvested for protein analysis and fluorometric intensity normalized to protein content for each well. Treatment groups before fluorometric analysis are described below in experimental treatments section.

Transient Transfections

Myoblasts were transfected with either a reporter plasmid containing the firefly luciferase gene driven by a promoter sequence containing four repeats of a consensus NFAT binding site or a negative control plasmid (pNFAT-luc or pCIS-CCK, Stratagene; 0.4 μg/well). Cells were cotransfected with a second plasmid (pRL-CMV, 0.02 μg/well; Promega, Madison, WI) to control for transfection efficiency. Plasmids were complexed with Lipofectin reagent (Invitrogen) and exposed to myoblasts in serum-free DMEM for 5 h. Parallel myotube cultures were transfected using identical procedures with a plasmid expressing green fluorescent protein (pEGFP-C1, BD Biosciences) to quantify transfection efficiency, which was consistently >60%. After transfection, cells were again placed in 10% FBS media for 16 h before being switched to differentiation media (DMEM supplemented with 2% horse serum and 1% penicillin-streptomycin). Differentiation medium was refreshed every 48 h until confluent myotubes were formed (4–7 days).

Dual Luciferase Assay

Immediately after treatment, myotube cultures were washed with ice-cold PBS and lysed by addition of 120 μl passive lysis buffer (Stratagene). Plates were rocked at room temperature for 15 min. The lysate was then transferred to microcentrifuge tubes and centrifuged for 5 min (4°C, 300 g) to sediment cellular debris. Supernatant was transferred to new tubes and kept on ice during the assay. Firefly luciferase (originating from transcriptional activity of the pNFAT-luc or pCIS-CCK vectors) and renilla luciferase activities (originating from the constitutively active uptake-control plasmid: pRL-CMV) were measured sequentially in the same 10-μl volume of cell lysate using the dual luciferase assay kit (Promega) according to the manufacturer’s instructions and a luminometer (model FB12, Berthold) set to measure average light intensity in relative light units (RLU) over a 10-s measurement period. NFAT-dependent transcriptional activity for each sample was taken as the raw firefly luciferase activity (RLU) divided by the renilla luciferase activity (RLU). For each experiment, all values were expressed relative to the average of the control group.

RNA Expression by RT-PCR

Concentration and purity of the extracted RNA were measured spectrophotometrically at A260 and A280 in 1× Tris-EDTA (TE) buffer (Promega). Reverse transcription (RT) was performed using the SuperScript III First-Strand Synthesis System for RT-PCR according to the manufacturer’s instructions (Life Technologies). Reactions were carried out using 5 μg of total RNA and 2.5 μM oligo(dT)20 primers. First-strand cDNA was treated with two units of RNase H and stored at −80°C. Primers and probes for slow MHC (GenBank NM_012751, 1m Assay no. Rn00562597_m1) were obtained from the ABI Assays-on-Demand service and consisted of Taqman 5' labeled FAM reporters and 3' nonfluorescent quenchers. Primer and probe sequences from this service are proprietary and, therefore, are not reported. Primer and probe sequences also consisting of Taqman 5' labeled FAM reporters and 3' nonfluorescent quenchers for hypoxanthine guanine phosphoribosyl transferase (HPRT) obtained from Applied Biosystems (Assays-by-Design) are the following: forward, 5'-GGTGGAATACAGGGCCAGTCTTGT-3'; reverse, 5'-AGTCAAGGGCATATCACAACAA -3'; Probe, 5'-ACTTGTCCTGGAAATTTCA-3'.

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Western Blotting

Protein concentrations were measured using the DC Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). Aliquots of cell lysates (8–15 μg) were run in 4–20% and 12% SDS-PAGE gels for phospho-GSK-3β, total GSK, NFA Tc1, β-actin, and histone. Nuclear extracts (11 μg) were run in 12% SDS-PAGE gels for NFAT blots. Protein was transferred to nitrocellulose membrane and blocked with Odyssey blocking buffer for 1 h. The primary antibodies used were the following: phospho-GSK-3β (Ser-9): sc-11757 (goat), 1:1,000 dilution, GSK-3β (H-76): sc-9166 (rabbit), 1:1,000 dilution, NFAT c1 (F-1) sc-8405 (mouse), 1:500 dilution, (Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (mouse), 1:4,000 dilution, (Abcam, Cambridge, MA) and anti-histone H2B, (rabbit), 1:5,000 dilution (Upstate, Lake Placid, NY). The membranes were incubated at 4°C overnight in primary antibody diluted with Odyssey blocking buffer (LI-COR, 1:5,000), IRDye 680 mouse anti-rabbit (LI-COR, 1:2,500), and IRDye 800CW rabbit anti-goat (LI-COR, 1:1,000 dilution). Membranes were washed four times with TBS-T and once with TBS before being scanned and detected using the Odyssey infrared imaging system (LI-COR).

Experimental Treatments

NO production. EXPERIMENT 1. One hour after DAF-FM loading, myotube cultures were treated with one of the following nine treatments: 1) DMSO vehicle control, 2) 1 μM MAHMA-NO, 3) 0.1 μM A23187, 4) 0.4 μM A23187, 5) 1 μM A23187, 6) 5 mM L-NAME, 7) 0.1 μM A23187 + l-NAME, 8) 0.4 μM A23187 + l-NAME, or 9) 1 μM A23187 + l-NAME. Groups 6–9 were pretreated with l-NAME for 30 min before addition of other compounds. Fluorometric analysis was then performed as described under “Measurement of NO Production”.

MHC-I/β expression. EXPERIMENT 2. Myotubes were treated for 24 h with one of the following four chemicals: 1) DMSO vehicle control, 2) 0.4 μM A23187, 3) A23187 + l-NAME, and 4) 5 mM L-NAME. RT-PCR was then performed as described in detail above.

NFAT transactivating potential. Dose and time course experiments were conducted to obtain the optimal calcium ionophore treatment to evoke NFAT transcriptional activity.

EXPERIMENT 3. Transfected myotube cultures were exposed to one of the following eight treatments for 9 h: 1) DMSO vehicle control, 2) 5 mM l-NAME, 3) 1 μM CsA to inhibit calcineurin activity, 4) 1 μM A23187, 5) l-NAME + A23187, 6) CsA + A23187, 7) 5 mM of GSK-3β inhibitor LiCl, or 8) LiCl + A23187 + l-NAME. The optimal dose of A23187 was used in this experiment (1 μM). However, all subsequent A23187 NFAT transactivating experiments were conducted at an effective but submaximal dose of 0.4 μM to increase sensitivity of the signaling pathway to potential modifiers.

EXPERIMENT 4. Transfected myotube cultures were exposed to one of the following four treatments for 9 h: 1) DMSO vehicle control, 2) 5 mM l-NAME, 3) 2 μM thapsigargin, or 4) l-NAME + thapsigargin.

EXPERIMENT 5. Transfected myotube cultures were exposed to a range of concentrations of the NO donor DETA-NO (0, 1, 10 and 50 μM) for 9 h, with or without the calcium ionophore A23187 (0.4 μM).

EXPERIMENT 6. Transfected myotube cultures were exposed to one of the following four treatments for 9 h: 1) DMSO vehicle control, 2) 0.4 μM A23187, 3) 10 μM of sGC inhibitor ODQ, or 4) A23187 + ODQ.

NFAT translocation. EXPERIMENT 7. Myotubes were treated with one of the following six treatments for 4 h: 1) DMSO vehicle control, 2) 0.4 μM A23187, 3) A23187 + l-NAME + LiCl, 4) A23187 + l-NAME, 5) 5 mM l-NAME, or 6) 5 mM LiCl. As described above, nuclear and cytosolic proteins were then separated for Western blot analysis (details above).

GSK-3β phosphorylation. EXPERIMENT 8. To determine the role of NOS in affecting GSK-3β phosphorylation, myotubes were treated for 4 h as follows: 1) DMSO vehicle control, 2) 0.4 μM A23187, 3) A23187 + l-NAME, or 4) 5 mM l-NAME. Total protein extracts were then measured by Western Blot analysis.

EXPERIMENT 9. The role cGMP plays downstream of NO in affecting GSK-3β was investigated by treating myotubes for 1 h with the following: 1) DMSO vehicle control, 2) 1 μM PAPA-NO, 3) PAPA-NO + ODQ, 4) PAPA-NO + YC-1, 5) 10 μM ODQ, or 6) 200 μM of sGC enhancer YC-1. PAPA-NO was used in this 1-h experiment due to its faster half-life for NO release (~15 min at 37°C, Cayman Chemical).

Statistical Analyses

Normalized values were analyzed for each experiment using either one-way or two-way ANOVAs. Tukey’s test was applied post hoc to determine individual group differences where main effects were found. Significance was established at P < 0.05.

RESULTS

Calcium Ionophore A23187 Effectively Induces NO Production

We measured NO production in myotubes treated with the calcium-ionophore A23187 (0.1, 0.4, and 1 μM), NO inhibitor L-NAME (5 mM), or NO donor, methylamine hexamethylene methyleneimine (MAHMA-NO, 1 μM). Fluorescent emission of DAF-FM indicated that A23187 increased NO production in a dose-dependent manner. Figure 1 shows that L-NAME abrogates the effect of A23187 at all concentrations, thus demonstrating the NOS specificity of the fluorescent response.

MHC-I/β mRNA Expression is NOS Dependent

We tested the responsiveness of MHC-I/β to calcium-ionophore with and without the NO inhibitor l-NAME, as well as l-NAME alone. Figure 2 shows results relative to the house-
keeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT) following 24 h treatment with the calcium ionophore A23187. A23187 induced a twofold increase in MHC-I/β expression. Treatment with L-NAME completely abrogated this induction.

L-NAME Inhibits Calcium-Induced NFAT-Dependent Transcriptional Activity

Treatment with the calcium ionophore A23187 (1 μM) caused a sevenfold increase in NFAT transcriptional activity consistent with previous findings (4, 21). Cyclosporin A (CsA), a calcineurin inhibitor, blocked the effect of the calcium ionophore, also in accordance with established data (1, 5). Our novel findings showed the nonisoform-specific NOS inhibitor L-NAME blocked the effect of A23187 on NFAT transcriptional activity. In the same experiment, the GSK-3 inhibitor LiCl also increased NFAT transactivating potential. Furthermore, the LiCl + A23187 effect was not inhibited by L-NAME (Fig. 3). Myotubes transfected with the negative control vector pCIS-CK, which contains the luciferase gene but lacks the NFAT-responsive promoter sequence, did not respond to any of the treatment groups in any of the transfection experiments (see Figs. 3–6). Treatment of myotubes with thapsigargin (2 μM) for 4 h to inhibit uptake of calcium by the sarcoplasmic reticulum also caused an increase in NFAT transcriptional activity, as previously reported (2). Similar to the calcium ionophore experiment, cotreatment with L-NAME inhibited this effect (Fig. 4).

DETA-NO Augments A23187-Induced NFAT-Dependent Transcriptional Activity

Treatment of myotubes with DETA-NO (1–50 μM) alone for 9 h did not affect NFAT transcriptional activity. However, cotreatment with 0.4 μM A23187 and DETA-NO produced a synergistic effect, increasing transcriptional activity of NFAT ninefold above baseline. Treatment with 10 μM DETA-NO doubled the effect of calcium ionophore on NFAT transactivating potential (Fig. 5).

Guanylate Cyclase Inhibition Prevents Induction of NFAT-Dependent Transcriptional Activity

Treatment of myotubes with the guanylate cyclase inhibitor ODQ (at both 5 and 10 μM) for 9 h prevented an increase in NFAT-dependent transcriptional activity with exposure to A23187 (Fig. 6). Of note, the induction of NFAT-dependent
transcriptional activity by 0.4 μM A23187 (Figs. 5 and 6) was approximately one-third to one-half the effect observed with 1 μM A23187 (Fig. 3).

NFAT Nuclear Translocation is NOS Dependent

Western blot analysis showed an increase in NFAT nuclear accumulation in A23187-treated cells (4 h) compared with control. However, L-NAME blocked the calcium ionophore effect. Consistent with our data from the pNFAT transfection experiments, treatment with A23187, L-NAME, and LiCl resulted in significantly higher NFAT nuclear accumulation than A23187 + L-NAME. Separation of nuclear and cytosolic protein fractions was confirmed by immunoblotting for histone and β-actin (Fig. 7).

NOS Activity is Necessary For A23187-Induced Phosphorylation of GSK-3β

Total GSK-3β and phospho-GSK-3β Western blot analysis showed a significant A23187-induced inactivation (i.e., increased phospho-to-total ratio) of GSK-3β at 4 h, as has been previously described (9, 24). The effects of A23187 on
GSK-3β phosphorylation were completely blocked with the NOS inhibitor L-NAME (Fig. 8).

GSK-3β Phosphorylation Occurs Via a NO-cGMP Pathway

Treatment of myotubes for 1 h with the NO-donor PAPA-NO increased the ratio of phospho-to-total-GSK-3β by twofold (Fig. 9). Cotreatment with the guanylate cyclase inhibitor ODQ completely prevented this effect. YC-1, which activates sGC independent of NO (10), also increased the ratio of phospho-to-total-GSK-3β by twofold demonstrating that activation of sGC is sufficient for this effect. Furthermore, cotreatment with PAPA-NO and YC-1 produced GSK-3β phosphorylation similar to either drug alone (Fig. 9).

DISCUSSION

NO and NFAT Activity

NO has been found through multiple studies to be an important signaling molecule in muscle (25, 26). It is produced enzymatically from NOS and increased during muscle contraction to support multiple acute and chronic adaptive responses, such as glucose transport and mitochondrial biogenesis (18). We recently reported that NOS activity is necessary for overload-induced expression of MHC-I/β mRNA in the rat plantaris (23). Additionally, Gonzalez-Bosc et al. (8) demonstrated that NO is required for NFATc3 nuclear accumulation in vascular tissue. Since NFAT nuclear translocation induces the slow-twitch phenotype in skeletal muscle (3, 11, 16), NO may be an important regulator of slow-twitch gene expression downstream of Ca2+. Although we did not quantify Ca2+ transients in response to the calcium ionophore A23187, this drug has been shown to increase intracellular Ca2+ influx in myotubes (11). Furthermore, our DAF-FM data confirms that A23187 is sufficient to increase NO production (Fig. 1).

Previous studies have described a calcium-calcineurin-NFAT-dependent pathway regulating fiber type-specific gene expression (11, 17). However, our study reports the novel finding that MHC-I/β expression is not only calcium dependent, but NOS dependent as well. In fact, our results show that the calcium ionophore A23187 requires NOS activity to induce MHC-I/β mRNA (Fig. 2). This is also the first evidence of a relationship between NO and NFAT-dependent transcriptional activity in skeletal muscle cells. We report that NO activity is necessary for calcium ionophore-induced nuclear accumulation of NFATc1 (Fig. 7) and induction of NFAT-dependent transcriptional activity (Fig. 3). Also, we found that the NOS inhibitor L-NAME showed a similar attenuating effect on NFAT transcriptional activity in myotubes treated with the calcium-releasing chemical thapsigargin (Fig. 4). Additionally, NO donor amplified the effect of a calcium ionophore to enhance NFAT-dependent transcriptional activity in cultured myotubes (Fig. 5). The current data extends our previous in vivo data (23) to a controlled cell culture environment and identifies regulation of NFAT-dependent transcription as a mechanism of the effect of NO on MHC-I/β expression.

Chin et al. (3) defined the pathway by which calcium signaling in muscle fibers will activate calcineurin to dephos-
phosphorylate NFAT and instigate its nuclear import and subsequent promoter activity of slow-specific genes. NO could influence MHC-I/β expression via involvement in one or more of the following steps: 1) dephosphorylation of NFAT and nuclear translocation/accumulation, 2) DNA binding and promoter activation, 3) rephosphorylation and nuclear export of NFAT, and/or 4) regulation of MHC-I/β mRNA stability or translational efficiency. Our data suggest that the involvement of NO in MHC-I/β gene regulation occurs at the transcriptional level via NFAT accumulation in the nucleus.

Meissner et al. (16) has recently described the assembly of a transcriptional complex, including NFATc1, MyoD, MEF2D, and p300, on the MHC-I/β promoter in response to calcium ionophore treatment. Although NO could affect assembly of this transcriptional complex, the current experiments show that NO affects activity of an engineered promoter driven only by four repeats of a consensus NFAT-binding element. Therefore, it is unlikely that our results, and the previously reported effects of NO on MHC I/β mRNA, are due to an NO effect on transcriptional complex formation. More likely, NO is involved in nuclear import or export of NFAT. Theoretically, NO could directly influence calcineurin phosphatase activity. An NO donor augmented the effect of a calcium ionophore on NFAT-dependent transcriptional activity (Fig. 5). However, in the absence of the calcium ionophore, NO donor treatment did not increase NFAT activity (Fig. 5). This suggests that NO (at the doses tested) works synergistically with calcium signaling but is not sufficient to independently augment the phosphatase activity of calcineurin.

**NO and GSK-3β**

Nuclear NFAT concentrations are dependent on a balance between import and export (activation or deactivation). Kinases are known to phosphorylate NFAT (24), cause its nuclear export (8, 24), inhibit DNA binding (9), and blunt its transactivating potential (24). Protein kinase G and AMP-associated protein kinase (13) are activated by NO and inhibit NFAT signaling in cardiac muscle (7, 12). Nevertheless, our data indicate that NO augments NFAT-dependent transcription, suggesting that NO effects on NFAT activity in skeletal muscle are not mediated by these kinases. Recent reports indicate that GSK-3β is an important inhibitor of NFAT in skeletal muscle (9, 24). Overexpression of GSK-3β in avian skeletal muscle promotes nuclear export of NFAT, whereas inhibition of GSK-3β augments NFAT transactivating potential and enhances MHC-I/β expression (9). Therefore, we investigated the role of NO in deactivation of GSK-3β. We report that an NO donor induces inhibitory phosphorylation of GSK-3β. Furthermore, inhibition of GSK-3β by LiCl causes nuclear accumulation of NFATc1 and stimulates NFAT-dependent transcription. It is unclear why LiCl treatment alone was sufficient to induce NFAT-dependent transcription, whereas DETA-NO treatment only augmented the calcium ionophore effect. We postulate that either LiCl has effects beyond inhibition of GSK-3β that stimulate NFAT or DETA-NO at the doses tested inhibited GSK-3β to a lesser extent than LiCl. Nevertheless, the effects of LiCl on NFAT, unlike A23187 effects, are not inhibited by l-NAME, suggesting that GSK-3β inhibition occurs downstream of NOS activity. Our current data are consistent with the hypothesis that calcium/calcineurin-dependent NFAT activation is accompanied by NO production, which lessens NFAT nuclear export (Fig. 5) via inhibition of GSK-3β (Fig. 6), enhancing its transactivation potential (Fig. 8).

**Influence of NO-cGMP Pathway**

Stimulation of sGC and the resultant accumulation of cGMP mediates many of the signaling functions of NO and regulates complex signaling cascades through immediate downstream effectors, including cGMP-dependent protein kinases, cGMP-regulated phosphodiesterases, and cyclic nucleotide-gated ion channels (15). Guanylate cyclases and cGMP-mediated signaling cascades play a central role in the regulation of diverse physiological processes (10, 15). We show that the sGC inhibitor ODO effectively blocks calcium-induced nuclear accumulation of NFATc1 and NFAT-dependent transcription. Further-
more, the NO-independent activator of sGC YC-1 induced phosphorylation of GSK-3β, and this effect was not augmented by concurrent treatment with an NO donor. This suggests that NO and sGC are part of the same pathway, rather than exerting separate effects on GSK-3β.

Interestingly, all pharmacological treatments used in this study support our working model (Fig. 10). Two calcium-modifying agents (A23187 and thapsigargin), one calcineurin inhibitor (CsA), one NO donor (NAME), three NO donors (DETA-NO, MAHMA-NO, and PAPA-NO), one sGC inhibitor (ODQ), one sGC enhancer (YC-1), and one GSK-3β inhibitor (LiCl) all provided complementary results, suggesting our findings are not due to nonspecific drug effects. Nevertheless, further studies employing genetic manipulations should confirm these proposed pathways.

Our findings provide insight into the calcium/calcineurin-NFAT-dependent pathway in C2C12 myotubes. NOS activity is essential for NFAT nuclear translocation and transcription. Similarly, mediation of GSK-3β via the NO-cGMP pathway provides a novel mechanism for enhancing NFAT nuclear accumulation. These data improve our understanding of the regulation of skeletal muscle phenotype and open the door for future experiments exploring the mechanisms of cGMP effects on GSK-3β.

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