Nitric Oxide Down-regulates Caveolin-3 Levels through the Interaction with Myogenin, Its Transcription Factor*

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Certain patients suffering from chronic diseases such as AIDS or cancer experience a constant cellular secretion of tumor necrosis factor α and other pro-inflammatory cytokines that results in a continuous release of nitric oxide (·NO) to the bloodstream. One immediate consequence of the deleterious action of ·NO is weight loss and the progressive destruction of muscular mass in a process known as cachexia. We have previously reported that caveolin-3, a specific marker of muscle cells, becomes down-regulated by the action of ·NO on muscular myotubes. We describe herein that the changes observed in caveolin-3 levels are due to the alteration of the DNA binding activity of the muscular transcription factor myogenin. In the presence of ·NO, the binding of transcription factors from cell nuclear extracts of muscular tissues to the E boxes present in the caveolin-3 promoter become substantially reduced. When we purified recombinant myogenin and treated it with ·NO donors, we could detect its S-nitrosylation by three independent methods, suggesting that very likely one of the cysteine residues of the molecule is being modified. Given the role of myogenin as a regulatory protein that determines the level of multiple muscle genes expressed during late myogenesis, our results might represent a novel mode of regulation of muscle development under conditions of nitric oxide-mediated toxicity.

The four members of the myogenic basic helix-loop-helix protein family that orchestrate muscle differentiation are MyoD (Myf3), myogenin (Myf4), Myf5, and Myf6 (Mrf4/herculin). These regulatory factors contain one or two transactivation domains (at N and C termini), a conserved basic DNA-binding domain, and a helix-loop-helix motif required for heterodimerization (1, 2). These transcription factors regulate cell lineage-specific transcription and proliferation and are able to activate the myogenic program in nonmuscle cells when ectopically expressed (3, 4). In addition, the basic helix-loop-helix domain of these myogenic transcription factors can heteroassociate with other helix-loop-helix-containing proteins,

such as E12, E47, HEB, Id, and twist. The heterodimers bind to E box elements (CANNTG motifs) and have been categorized into several "classes" based on tissue distribution, dimerization capabilities, and DNA binding specificities. Expression of these regulatory factors leads to the transcriptional activation of muscle-specific genes, such as muscle creatin kinase, troponin I, α7 integrin, desmin, α-actin, and caveolin-3 (3, 4).

Wasting of skeletal muscle (cachexia) is associated with a variety of chronic or inflammatory disorders, such as AIDS and cancer (5). The detrimental effects of cachexia occur as a consequence of excessive wasting of skeletal muscle tissue resulting in the reduction of many markers of differentiated skeletal muscle cells, as well as the transcription factors responsible for the expression of muscular proteins (6, 7). For instance, TNFα[2] is capable of inducing oxidative stress and nitric-oxide synthase expression in skeletal muscle of cachectic animals (8, 9). The implication of the NOS2-derived ·NO in TNFα-induced muscle wasting was demonstrated by the use of a specific NOS inhibitor, nitro-L-arginine, which was shown to prevent the onset of cachexia in nude mice injected with Chinese hamster ovary cells that secreted TNFα (9). Because phosphorylation of myogenin on Thr[87] within the DNA-binding domain, such as that induced by basic fibroblast growth factor, has an inhibitory effect on the binding to and the transcription from the E box (10), it was conceivable that the effect exerted by ·NO might be due to the activation of a cellular kinase that might use myogenin as a substrate. Nevertheless, this *in vivo* effect of ·NO on the destruction of muscle tissues under high levels of circulating TNFα is not mediated by the direct phosphorylation of the myogenin molecule. This conclusion was reached after observing that phosphatase treatment of muscular nuclear extracts from TNFα mice did not reconstitute the DNA binding activity of myogenin toward the E box of the muscle creatin kinase (9). Consequently, another ·NO-mediated modification of the myogenin polypeptide chain might be responsible for the observed decrease in muscle mass and total weight of the mice injected with TNFα-secreting Chinese hamster ovary cells.

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[2] The abbreviations used are: TNF, tumor necrosis factor; NOS, nitric-oxide synthase; LPS, lipopolysaccharide; IFN, interferon; DETA-NONOate, (Z)-1-[2-aminoethyl]-N-2-(2-ammonioethyl) amino] diazen-1-ium-1,2-diolate; DEA-NONOate, diethylammonium (Z)-1-(N,N-diethylamino) diazen-1-ium-1,2-diolate; GSNO, S-nitrosoglutathione; PBS, phosphate-buffered saline; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; cav, caveolin; Erk, extracellular signal-regulated kinase.

Among the various post-translational modifications induced by ·NO and related reactive nitrogen species, *S*-nitrosylation (the formation of a thionitrite, -S-N=O, in cysteine residues, also called *S*-nitrosation) is emerging as an important effector in cell signaling, involved in several physiological and pathophysiological contexts and could be considered as a potential new paradigm in signal transduction (11–13). Several transcription factors, including NF-κB or AP-1, have been reported to be regulated by *S*-nitrosylation, either by a direct alteration of their DNA binding properties or by other indirect modes of regulation (14–18).

We have previously reported that induction of NOS2 in C2C12 myotubes with a mixture of LPS and IFN-γ resulted in the selective down-regulation not only of caveolin-1 and caveolin-2 but also of the specific muscle cell marker, caveolin-3 (19). Whereas the reduction in the levels of caveolin-1 and -2 was due to the direct activation by the cytokines of a route involving p42/p44 Erk, the down-regulation of caveolin-3 was apparently due to the effect of nitric oxide itself (19). Among the three caveolin isoforms, caveolin-3 is muscle-specific and is found in both cardiac and skeletal muscle. We describe herein that nitric oxide, a molecule present in excess in several diseases that course with inflammation, is able to modify selectively the transcription factor myogenin. This observation rationalizes the observed destruction of muscle tissue and the disappearance of muscle-associated proteins when high levels of ·NO are present.

EXPERIMENTAL PROCEDURES

Materials—Cell culture medium (Dulbecco's modified Eagle's medium), glutamine, antibiotics, transfection reagent Escort-IV, Hoechst, and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase were purchased from Sigma. Trypsin-EDTA and fetal bovine serum were from BioWhittaker Europe. The source of the various antibodies used in this work is as follows: anti-cav-3 monoclonal IgG1 was purchased from BD Transduction Laboratories (610420); anti-β-tubulin I monoclonal clone SAP4G5 was purchased from Sigma-Aldrich (T-7816); myogenin Ab-1 (clone F5D) was purchased from Neomarkers (MS-1113). ECL reagents and streptavidin-peroxidase and Cy2- and Cy3-labeled secondary antibodies were from Amersham Biosciences. DETA-NONOate was from Alexis Biochemicals (catalog number 146724-94-9). DEA-NONOate was from Cayman Chemicals (catalog number 82100). *S*-Nitrosoglutathione (GSNO) was from Calbiochem.

Cell Culture and Materials—C2C12 mouse myoblasts were kindly provided by Dr. Margarita Lorenzo (Universidad Complutense de Madrid). The myoblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine in a 5% CO_2 atmosphere at 37 °C. Differentiation of ~70% confluent myoblasts into myotubes was performed in Dulbecco's modified Eagle's medium supplemented with antibiotics and glutamine plus 50 nM insulin in the absence of serum for 3 days (20). Myotubes (muscular cells) are characterized by the formation of elongated, multinucleated tubules that express cav-3. The differentiation of myoblasts into myotubes has been previously reported (19). H9c2 rat myocytes were a generous gift from Dr. Lisardo Boscá (Instituto de Inves-

tigación Biomédica de Bellvitge Barcelona), whereas mouse L929 fibroblasts were kindly provided by Dr. Portolés (Universidad Complatense de Madrid).

Recombinant Expression of Myogenin and Site-directed Mutagenesis of the Cys Residues—The recombinant expression and purification of mouse myogenin was performed using a pET9 plasmid (generous gift of Dr. Maleki, Arkansas University). We followed the published protocol that included purification using a heparin-agarose resin followed by the elution with increasing concentrations of NaCl (21, 22). Site-directed mutagenesis was performed using the QuikChange method using the wild-type myogenin pET9 as template. The complete sequence of myogenin as well as the desired mutations were confirmed by automated sequencing.

Immunoblot Analysis and Cellular Fractionation—Cellular proteins were resolved by either 7% or 15% acrylamide SDS-PAGE and transferred to nitrocellulose membranes. Western blots were incubated for 2 h in PBS containing 2% powdered skim milk. Subsequently, the nitrocellulose membranes were incubated overnight with the primary antibodies (typically 1:1000 in PBS), washed, and finally incubated for 2 h with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Pierce). Detection was performed using an ECL detection kit (Amersham Biosciences). Quantification of the intensity of the bands was performed using the UVIband V97 software (UVItec St. John's Innovation Centre).

Confocal Fluorescence Microscopy—Cells grown on 0.2% gelatin-coated glass coverslips were washed twice with PBS and fixed for 15 min at room temperature with freshly prepared 2% paraformaldehyde in PBS. The stock paraformaldehyde solution was prepared at 4% in PBS and was centrifuged at 15,000 rpm for 5 min at room temperature in a table-top microcentrifuge to remove insoluble material prior to dilution. After removal of the 2% paraformaldehyde solution, the cells were washed with PBS and incubated with cold methanol at −20 °C for 10 additional min. The methanol was removed, and the coverslips were allowed to dry for 5 min. Then the cells were washed with PBS and incubated with the desired primary antibodies at 37 °C. In general, an overnight incubation with the primary antibodies at a 1:200 dilution in a wet chamber followed by a 2-h incubation with the secondary antibody was performed. Finally, the slides were mounted using Fluoroguard anti-fade reagent (Bio-Rad). The subcellular localization was observed under a Bio-Rad Radiance 2100 confocal microscope, using the excitation wavelength of 405 nm for the Hoechst fluorescence (nuclei staining), 488 nm for the Cy2 fluorescence, and 543 nm for the Cy3 fluorophore. A 60× oil immersion objective was used. Analysis of the pictures was performed with confocal assistant software (Free software by Todd Clark, version 4.02) as well as with Laserpix and Lasersharp software from Bio-Rad. Overlap of green and red labeling is depicted in yellow; overlap of green and blue labeling is depicted in light blue; overlap of red and blue is depicted in violet.

Transient Transfection and Luciferase Assays—H9c2 myocytes and L929 fibroblasts were plated at a 70% density 1 day prior to transfection. The cells were transfected with 5 μg of reporter plasmid using Escort IV as instructed by the manufacturer (Sigma-Aldrich). A detailed description of six pGL3-basic

plasmids (Promega) containing various fragments of the caveolin-3 promoter (L1 to L6) has been reported elsewhere (23). 48 h after transfection, H9c2 cells were washed with PBS and lysed with 50 μl of lysis buffer following the instructions of the luciferase reporter gene assay kit (Roche Applied Science). Afterward, 100 μl of luceferine solution was added, and the luminiscence of the total 150 μl was determined using the Lumat LB9507 luminometer (Berthold Technologies). Addition of the nitric oxide-releasing compound DETA-NONOate was performed when the transfection reagent was removed and maintained for 48 h. 24-Well plates were used in all cases, and every point was performed in triplicate. In the case of the L1 to L5 constructs, a similar amount of total photon count was obtained, and for every case we considered the 100% luciferase signal that was obtained from the untreated samples. The decrease in luciferase expression for each plasmid in the presence of nitric oxide was correlated with that of the untreated sample. When the H9c2 cells were required to be in the presence of ·NO, the donor DETA-NONOate was added during the indicated times.

Isolation of Cell Nuclear Extracts—The nuclear extracts from H9c2 myocytes, C2C12 myoblasts and myotubes, and COS7 cells were isolated by means of a detergent method (24) with the purpose of assaying the binding to the proximal E box of the caveolin-3 promoter. The cells were maintained in culture until confluent and were then washed with PBS and harvested through scraping in an Eppendorf tube. They were subsequently centrifuged at 2000 rpm in a refrigerated table-top microcentrifuge for 5 min at 4 °C, and the pellet was resuspended in one volume of prechilled NAR A buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA) in the presence of 1 mM DTT plus the protease inhibitors 0.2 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin, and phosphatase inhibitors (1 mM β-glycerophosphate, 1 mM NaF, and 1 mM Na$_3$VO$_4$). The mixture was kept in ice for 10 min. Afterward, 10% (v/v) of 1% Nonidet P-40 was added and allowed to sit for 3 min at room temperature. Then the samples were mixed in a vortex for at least 10 s until the appearance of a white pellet. The tubes were then centrifuged at 5,000 rpm for 2 min at 4 °C in a refrigerated table-top microcentrifuge, and the supernatant was kept at −20 °C (cytoplasmic extracts). One volume of NAR C buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA) was then added to the nuclei, and the mixture was vortexed at 4 °C for 30–60 min. The nuclei extracts were then centrifuged at 14,000 rpm for 5 min at 4 °C, and the supernatants (nuclear extracts) were then kept at −80 °C. To quantify the total amount of protein present in the nuclear extracts, a colorimetric Bradford microassay was carried out.

Electrophoretic Mobility Shift Assay (EMSA)—Two synthetic complementary oligonucleotides that included the proximal E box-responsive element of the caveolin-3 promoter (23) were used: myogenin forward, 5′-GTGGGA**CAGCTG**TCAGC-CGC-3′, and myogenin reverse, 5′-GCGGCTGA**CAGCT-G**TCCCAC-3′ (the hexabase motif corresponding to the E box is in bold type). The forward oligonucleotide was labeled with biotin at the 3′ end. Both oligonucleotides were mixed at a 1:1 molar ratio, heated up to 70 °C, and allowed to cool slowly at room temperature. In the experiments in which a mutated E

box was tested, we used the oligomers: 5′-GTGGGA**GCGCG-C**TCAGCCGC-3′ and 5′-GCGGCTGA**GCGCGC**TCCCAC-3′. The H9c2 nuclear extracts, both treated and nontreated with DEA-NONOate as well as the nuclear extracts obtained from DETA-NONOate-treated H9c2 cells were incubated with the dimeric probe for 30 min at 37 °C. Then a nondenaturing electrophoresis was performed in a 4% acrylamide gel which was subsequently transferred to a positively charged nylon membrane (Amersham Biosciences). After the transference, the membrane was incubated with streptavidin-peroxidase overnight at 4 °C and developed by ECL.

Tissue Immunostaining—Mouse hearts were extracted in 30% sucrose in PBS and frozen in liquid N$_2$, and serial 7-μm-thick sections were cut with a Leitz sledge microtome onto gelatinized glass coverslips. The preparations were fixed in a 3.7% paraformaldehyde solution in PBS, pH 7.4, for 45 min at room temperature, washed with PBS, and permeabilized with cold methanol for 15 min at room temperature. After blocking with 3% bovine serum albumin for 1 h at room temperature, the sections were incubated overnight with the indicated antibodies in PBS at 4 °C. The sections were incubated with fluorescent secondary antibodies (labeled with Cy2 or Cy3) and treated with Hoechst 33258 for 30 min at room temperature. Fluorescence was visualized on a MRC 1024 microscope (Bio-Rad) with Lasershap software.

Analysis of Myogenin S-Nitrosylation—The biotin switch assay (25) was performed as previously described (26, 27) on recombinant purified wild-type and Cys mutants of myogenin first incubated with 1 mM GSNO or 1 mM DEA/NO for 15 min at room temperature, precipitated with acetone, and resuspended in HEN buffer. Wild-type myogenin S-nitrosylation was also determined by chemical reduction and chemiluminescence as previously described (28, 29). After treatment with 100 μM GSNO for 15 min, the protein was separated from GSNO by passing the sample through a Sephadex G-25 column. Eluted fractions were injected into an 8-ml anaerobic solution containing 100 μM CuCl, 1 mM cysteine, pH 6, at 50 °C, and purged continuously with nitrogen and analyzed in a Sievers 280 nitric oxide analyzer.

Characterization of S-Nitrosylation by Mass Spectrometry—Recombinant myogenin (10 μg) was incubated with 1 mM GSNO for 15 min at room temperature in the darkness. S-Nitrosylated protein was digested by adding 0.5 μg of modified porcine trypsin (sequencing grade; Promega). In-solution digestion proceeds at 37 °C for 1 h. The resulting tryptic peptides were injected onto a C-18 reversed phase nano-column (Discovery® BIO Wide pore; Supelco, Bellafonte, PA) and eluted at a flow rate of ∼300 nl/min with a continuous acetonitrile gradient to an electrospray ion source coupled to an ion trap mass spectrometer (Esquire HCT Ultra, Bruker Daltonics, Bremen, Germany) for real time ionization and fragmentation. The multiple reaction monitoring mode was used to analyze the masses of interest.

RESULTS

Correlation between Myogenin Expression and Detection of Caveolin-3 in Muscle Cells—Two different systems of muscular cells in culture have been used in this work: mouse C2C12 myo-
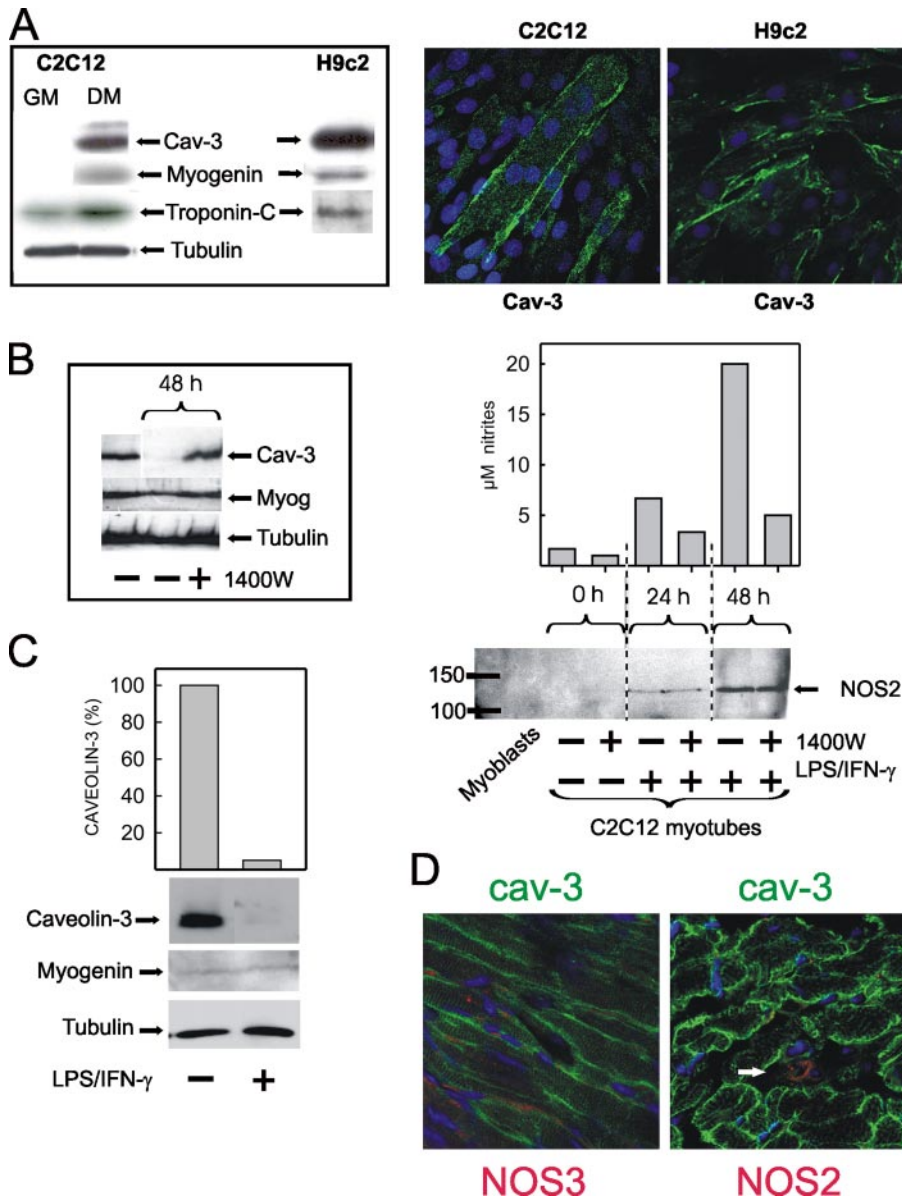
FIGURE 1. **Endogenously produced ·NO results in the down-regulation of the caveolin-3 levels in muscle cells.** C2C12 mouse myoblasts express protein markers characteristic of a muscular phenotype when changed from a growth medium (*GM*) into a differentiation medium (*DM*) for 48 h, whereas rat H9c2 myocytes express muscle protein markers in standard growth medium (*A*). Among the proteins markers expressed in muscle cells, caveolin-3 appears concentrated in the sarcolemma (plasma membrane) in both C2C12 myotubes and in H9c2 myocytes (*green staining*). Induction of NOS2 expression in C2C12 myotubes using a mixture of LPS and IFN-γ results in the release of ·NO to the cellular medium, whereas 1400W inhibit this ·NO release (*B, right panel*). The caveolin-3 protein levels within C2C12 myotubes are significantly reduced when NOS2 releases ·NO into the medium (*B, left panel*). Co-culture of Raw 264.7 macrophages treated with LPS/IFN-γ with H9c2 rat myocytes for 48 h results in the down-regulation of the caveolin-3 protein levels without noticeable changes in the myogenin protein levels (*C*). The co-cultures were done in Transwell chambers. The results are representative of three individual experiments. *D*, immunostaining of caveolin-3 and NOS2 or NOS3 in heart muscle. Mouse hearts were analyzed using immunofluorescence to detect caveolin-3 together with the presence of nitric-oxide synthases. The staining of NOS3 (eNOS) was mostly restricted to the vessels. Mice injected intraperitoneally with a single dose of LPS from *Salmonella typhimurium* (2 mg/kg body weight) were sacrificed after 48 h, and the distribution of caveolin-3 and NOS2 (iNOS) in the hearts was determined. The presence of an infiltrated macrophage is depicted with an *arrow* (*right panel*). The caveolin-3 fluorescence was visualized by confocal microscopy at an excitation wavelength of 488 nm and is shown in *green*, whereas the NOS2 and NOS3 was obtained after excitation at 543 nm and is shown in *red*. The position of the cell nuclei (shown in *blue*) was obtained after staining with Hoescht and excitation at 405 nm.

cytes, where both proteins were present (Fig. 1*A*). Myogenesis of C2C12 myoblasts was induced in a differentiation medium that contained either 50 nM insulin or 2% horse serum. Under these conditions, C2C12 myoblasts exited the cell cycle and fused forming multinucleated myotubes, and caveolin-3, troponin-C, and myogenin expression was observed (Fig. 1*A*). These differentiation conditions did not significantly alter either the caveolin-3 or myogenin levels of H9c2 muscular cells or their shape (data not shown). Caveolin-3 immunostaining was mostly restricted to the sarcolemma (plasma membrane) of both differentiated C2C12 cells and H9c2 myocytes (Fig. 1*A*).

It is well established that muscular cells derived from heart tissues are extremely refractory to respond to a pro-inflammatory stimulus (LPS or cytokine mixtures) and fail to express inducible NOS (30). Hence C2C12 myoblasts were converted into myotubes and then challenged with a mixture of LPS and IFN-γ. With this treatment, expression of NOS2 was apparent after 24 h and even more at 48 h (Fig. 1*B, right panel*). When NOS2 expression was induced in the presence of the inhibitor 1400W, the amount of nitrites that were measured in the extracellular medium diminished considerably (Fig. 1*B*). With that in mind, we determined the expression levels of both caveolin-3 and the transcription factor myogenin when NOS2 expression was induced in C2C12 myotubes in the presence of cytokines (Fig. 1*B, left panel*). Caveolin-3 is a well characterized marker of the conversion of myoblasts into myocytes (myogenesis), and its expression is known to be selectively regulated by the transcription factor myogenin (23). Interestingly, nitric oxide itself is responsible for the decrease in caveolin-3 levels in C2C12 myotubes, whereas the myogenin levels

blasts and rat heart-derived H9c2 myocytes. When grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum, C2C12 myoblasts were unable to express muscle cells protein markers, such as myogenin or caveolin-3, unlike H9c2 myoblasts and rat heart-derived H9c2 myocytes, myo-

remain almost unchanged. Next, we established co-cultures of Raw 264.7 macrophages together with H9c2 myocytes in Transwell chambers. When the macrophages were challenged with a mixture of cytokines, large amounts of ·NO were released into

the medium (data not shown). Analysis of the caveolin-3 protein levels 48 h post-induction of NOS2 expression revealed that only ~5% of the initial caveolin-3 levels remained in the myocytes, whereas the myogenin levels were unchanged (Fig. 1*C*).

We then inspected the distribution of caveolin-3 and of the constitutive endothelial nitric-oxide synthase (NOS3) in ventricular sections of mouse hearts. Caveolin-3 immunofluorescence stained the sarcolemma of the myofibrils as well as the I-bands of the sarcomere repeats (Fig. 1*D*). We were unable to detect a strong NOS3 staining inside cardiac muscular cells but rather only in the endothelial cells of the veins and arteries that irrigate the tissue (Fig. 1*D*, *left panel*, *red staining*). When mice were injected with LPS to induce an inflammatory situation and we performed immunostaining of heart sections, we could observe the presence of infiltrated macrophages within the cardiac tissue (Fig. 1*D*, *right panel*, *arrow*). Consequently, either the ·NO released by the NOS3 present in the vessels that irrigate the heart or the ·NO released by the NOS2 present in infiltrated macrophages might be initiating the destructive effects of this gaseous radical on muscle tissues.

Nitric Oxide Is Able to Down-regulate the Caveolin-3 Levels in Muscle Cells—We have previously shown that when NOS2 is induced in C2C12 muscular myotubes, a concomitant decrease in the protein levels of caveolin-1 and -2 can be observed together with a retarded decrease in caveolin-3 protein levels that responds to the released ·NO (19). With that in mind, we first tested whether nitric oxide could also induce the down-regulation of caveolin-3 levels in H9c2 cells. The addition of increasing concentrations of the ·NO-releasing drug DETA-NONOate to a culture of H9c2 myocytes resulted in the decrease of caveolin-3 levels when determined 48 h post-treatment (Fig. 2*A*). We chose this ·NO-liberating compound because it releases nitric oxide at slow rates, displaying a half-life of ~20 h at 37 °C (31). Next, we maintained a 100 μM constant concentration of DETA-NONOate and determined the changes in caveolin-3 protein levels over a 3-day period. As shown in Fig. 2*A*, the caveolin-3 protein levels decreased after 24 h of treatment reaching 30–40% of the initial levels that remained upon longer exposure times. When the H9c2 were analyzed for caveolin-3 distribution by immunofluorescence of fixed cells, we did not observe a translocation of caveolin-3 from the sarcolemma of the cells but rather a general diminution of the total levels (Fig. 2*B*), whereas myogenin concentrated in the cell nucleus of H9c2 myocytes at both times tested. To examine whether nitric oxide might be affecting caveolin-3 protein levels through the synthesis of cGMP via the activation of soluble guanylate cyclase, we tested the cav-3 levels in the presence of 8-Br-cGMP, a nonhydrolyzable analogue of cGMP. Interestingly, the levels of cav-3 in H9c2 myocytes not only did not decrease, but rather they showed a certain increase both at 2 and 4 μM 8-Br-cGMP (Fig. 2*C*). Hence, the observed decrease in the cav-3 levels caused by ·NO is not mediated by an increase in the cGMP levels.

At this point we wondered whether the mechanism by which ·NO decreased caveolin-3 levels might be due to a decrease in the myogenin protein levels. A precedent for this can be found in the reduction of myogenin protein levels observed under
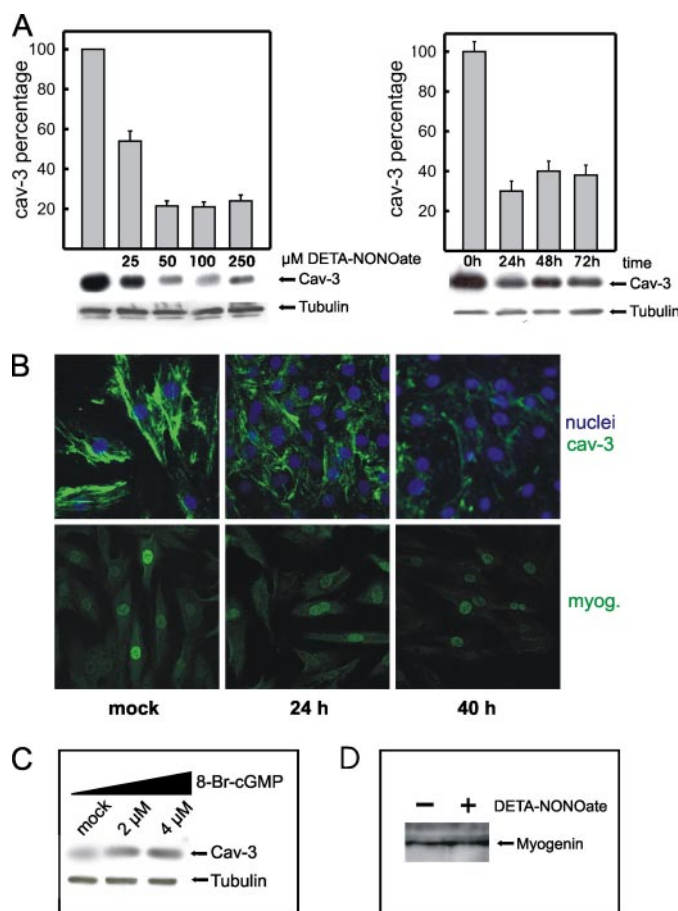
FIGURE 2. **Down-regulation of the caveolin-3 protein levels in H9c2 myocytes in the presence of ·NO donors.** Increasing concentrations of the ·NO donor DETA-NONOate were added to a cell culture of H9c2 myocytes, and the amount of caveolin-3 protein levels was determined by Western blot after 48 h of treatment (*A*, *left panel*). Time dependence changes were observed in the caveolin-3 protein levels in H9c2 myocytes treated with 100 μM DETA-NONOate (*A*, *right panel*). The amount of caveolin-3 protein levels present in the untreated sample was referred to as 100%. Confocal microscopy immunofluorescence of H9c2 myocytes was treated with 100 μM DETA-NONOate for the indicated times (*B*). Caveolin-3 and myogenin staining was determined with a Cy2-labeled secondary antibody after excitation at 488 nm, whereas the cell nuclei were stained with Hoechst with an excitation wavelength of 405 nm. The transcription factor myogenin appears to be concentrated in the cell nucleus in all cases. Rat H9c2 myocytes were treated with 2 or 4 μM 8-Br-cGMP for 40 h, and the changes in caveolin-3 levels were determined by Western-blot (*C*). Finally, rat H9c2 myocytes were incubated in the presence or absence of 100 μM DETA-NONOate for 48 h, and the protein levels of myogenin were determined by Western-Blot (*D*). The results are representative of three independent experiments.

hypoxic conditions. For instance, rabbits raised under hypoxic conditions are known to down-regulate their cav-3 protein levels (32). Remarkably, hypoxia induces the disappearance of numerous muscle proteins through the reduction in the protein levels of their transcription factors, such as myogenin and MyoD (33). Therefore, we inspected whether ·NO was able to induce changes in the myogenin protein levels that might ultimately lead to the decrease in the cav-3 levels aforementioned. As shown in Fig. 2*D*, treatment of H9c2 myocytes with 100 μM DETA-NONOate for 48 h did not result in a diminution in the myogenin protein levels. More importantly, the treatment did not result in the phosphorylation of myogenin, a modification known to abrogate its DNA binding activity (10) because this
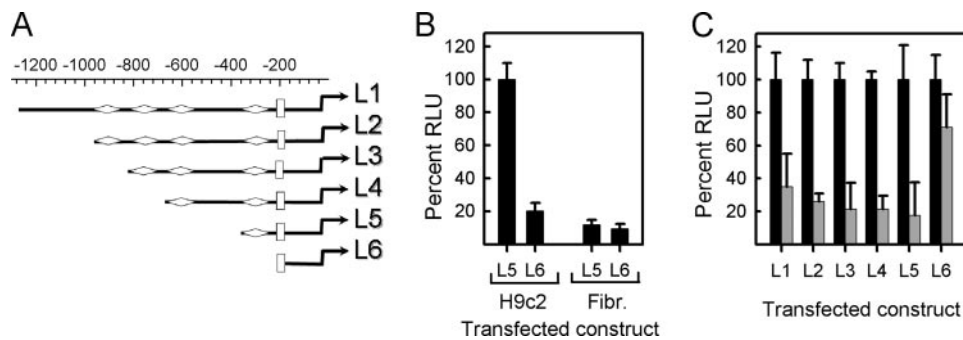
FIGURE 3. **Nitric oxide diminishes the response of the caveolin-3 promoter in a luciferase reporter assay.** The caveolin-3 promoter possesses four consensus E box elements that represent putative binding sites for the myogenic basic helix-loop-helix transcription factors (23). Deletion analysis of the caveolin-3 promoter was used to inspect the individual effects of ·NO donors (*A*). We employed constructs with the complete caveolin-3 promoter that included the four E box elements (L1 and L2), shorter constructs with varying numbers of E boxes (L3 to L5), or a construct without any E boxes (L6). E box elements are depicted as *diamonds*. The L5 construct with a single E box and the L6 construct that lacks any responsive element were transfected in H9c2 myocytes and in L929 fibroblasts (*B*). Constructs L1 to L6 were transfected in rat H9c2 myocytes, and the changes in luciferase signal in the absence (*black bars*) or presence of a 100 μM DETA-NONOate (*gray bars*) were measured (*C*). The number of photons present in the untreated sample is considered 100% in all cases. (*C*). The data are representative of six individual experiments.

post-translational modification would result in a significant decrease in its apparent molecular weight in SDS-PAGE (34).

The Caveolin-3 Promoter Activity Is Down-regulated by the Presence of ·NO—Because myogenin is known to bind to the cav-3 promoter and regulate its expression levels (23), we inspected the effect of nitric oxide in a luciferase-driven transcription assay using six different constructs of the promoter. These progressive deletional mutants of the cav-3 promoter are fused to the promoterless luciferase reporter plasmid pGL3 Basic (Promega). The L1 and L2 constructs possess the four E boxes that are present in the cav-3 promoter. Three E boxes were present in construct L3, and just two were in construct L4. Construct L5 has one E box at position -64, and construct L6 lacks any *cis*-regulatory element that might mediate cav-3 expression (Fig. 3*A*; Ref. 23). When construct L6, which lacks the proximal E box, was transfected into H9c2 cells and its luciferase activity was compared with identical amounts of transfected construct L5, the former only reached 20% of the activity displayed by the latter (Fig. 3*B*). Because L6 lacks any *cis*-regulatory element we could conclude that these muscle cells responded to the muscle-specific hexabase motif, hence increasing significantly the promoter activity. This contrasts with the activity displayed when constructs L5 and L6 were transfected in L929 fibroblasts, where none of them displayed a significant luciferase activity (Fig. 3*B*). Subsequently, identical amounts of each of the six constructs that contained responsive E boxes (L1 to L5) as well as L6 were transfected in H9c2 muscle cells, and the effect of nitric oxide was analyzed 48 h post-transfection. In all cases the amount of the promoter activity was significantly diminished in the presence of 100 μM DETA-NONOate, resulting in 35%, 26, 21, 22, 18, and 71% when compared with their untreated control samples (Fig. 3*C*). This is clearly indicative of the drastic effect that ·NO exerts on the transcriptional activity of the cav-3 promoter. However, the activity of the L6 construct, which is devoid of a functional E box, also displayed a reduction albeit to less degree than the L1–L5 counterparts. Because it has been reported that ·NO itself can destroy the luciferase mRNA selectively (35), our data

reveal that, in the cases of L1–L5, the decrease in promoter activity was larger than that expected if a direct action of ·NO on the messenger was taking place, whereas in the case of L6 this decrease would be explicable considering the direct destruction of the mRNA of the luciferase by the direct action of ·NO.

Treatment of the Nuclear Extracts of H9c2 Cells with Nitric Oxide Results in the Loss of Band Retardation with the Myogenin-specific Enhancer—To analyze DNA-protein complexes interacting with the proximal E box of the caveolin-3 promoter, synthetic oligonucleotides forming a double-stranded E box-binding site were annealed and used for electromobility shift assays (Fig. 4). The E box oligomer formed a single retarded band that was present when we used the purified nuclear extracts from H9c2 myocytes and C2C12 myotubes, partially in COS7 cells and was completely absent when purified nuclear extracts from C2C12 myoblasts (undifferentiated) were used. This result concurs with the appearance of myogenin during myogenesis of C2C12 myotubes (Fig. 1). When cells were treated for various times with the ·NO donor DETA-NONOate and the nuclear extracts were purified and incubated with the annealed oligonucleotides covering the proximal E box of the caveolin-3 promoter, the retarded complex was completely lost (Fig. 4*B*). When this treatment was extended for longer periods (up to 72 h), the H9c2 muscle cells started to recover and somehow regained the ability to retard the DNA complex. This might be due to the increased expression of another myogenic transcription factor that might rescue the loss in myogenin DNA binding activity or perhaps a cellular mechanism that reverts the action of ·NO on myogenin. Alternatively, because the half-time of DETA-NONOate is 20 h at 37 °C, it is conceivable that after 72 h of treatment the effective ·NO levels that reach the cells might be insufficient to induce an effect. The loss in DNA retardation was observed when the C2C12 myotubes were treated with DETA-NONOate for 24 h (Fig. 4*B*). At this point it must be remarked that C2C12 myoblasts express significant amounts of MyoD but fail to express myogenin or caveolin-3 (23). Hence, we can infer that the cav-3 promoter responds exclusively to myogenin and its four E boxes and that despite displaying four consensus CANNTG sequences, it does not respond to other myogenin factors. This is in agreement with the known fact that myogenin is found later than Myf5 and MyoD, coinciding with the initial appearance of transcripts of the cardiac α-actin gene, the first muscle structural gene to be expressed in vertebrates, which is usually associated with muscle differentiation (36).

The Reducing Agent DTT Is Able to Revert the Effect of Nitric Oxide in EMSA Experiments—Next, the purified nuclear extracts were treated with the nitric oxide-releasing compound DEA-NONOate, which releases ·NO with a half-life of 2 min in aqueous solution at 37 °C (31). Afterward, we wanted to inspect
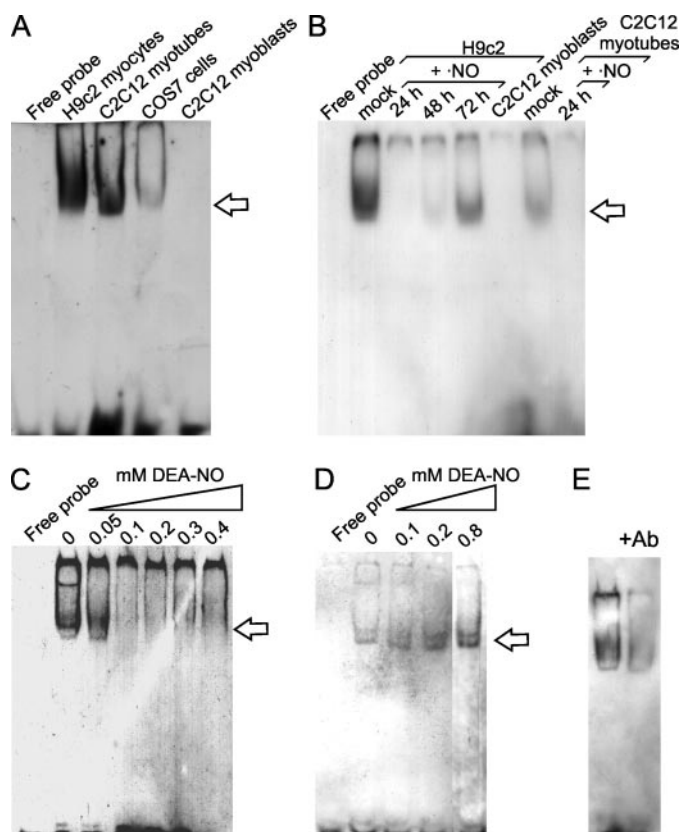
FIGURE 4. **Gel shift assays with nuclear extracts.** Binding of transcription factors to the E box of the caveolin-3 promoter induces a clear retardation band when cellular nuclear extracts isolated from H9c2 myocytes or C2C12 myotubes were used (*A*). Treatment of the live cells with 100 μM DETA-NONO-ate followed by the purification of the nuclear extracts rendered a preparation that partially abolishes the ability to retard the E box from the caveolin-3 promoter both in H9c2 myocytes and in C2C12 myoblasts (*B*). Treatment of the purified nuclear fraction of H9c2 myocytes with increasing concentrations of the ·NO donor DEA-NONOate (up to 0.4 mM) for 1 h at 25 °C resulted in the progressive loss of the retardation band (*C*). This same treatment in the presence of 10 mM DTT resulted in the protection of the DNA-protein complex even at 0.8 mM DEA-NONOate (*D*). An anti-myogenin antibody significantly diminished the intensity of the retarded band obtained when nuclear extracts were used (*E*). The position of the retarded DNA-protein complexes is depicted with a *white arrow* on the *right-hand side* of each gel. The results are representative of four individual experiments.

whether the purified nuclear extracts incubated with ·NO behaved in a similar fashion than the intact cells. The additional advantage of the direct treatment of the purified nuclear extracts with ·NO-releasing compounds is that we can rule out cellular mechanisms that might be responsible for the loss of the retarded band, including the putative phosphorylation of myogenin at Thr[87] by cellular kinases. When the purified H9c2 nuclear extracts were incubated with increasing concentrations of the ·NO donor DEA-NONOate for 1 h, the band shift observed for the E box of the myogenin promoter was lost. Whereas a certain retarded complex could still be observed at 0.05 mM DEA-NONOate, the band retardation observed in EMSA gels was almost completely lost for concentrations of the donor above 0.1 mM (Fig. 4*C*). However, when the nuclear extracts were incubated with 10 mM DTT after their treatment with DEA-NONOate, we were able to observe a partial reversion of the effect of ·NO (Fig. 4*D*). In fact, we needed to add 1 mM DEA-NONOate or larger concentrations to modify the

myogenin molecule and abrogate its interaction with the DNA dimer. Hence, the cysteine reducing agent DTT is very likely protecting the side chains of residues involved directly in DNA binding from a covalent modification by the ·NO released by DEA-NONOate. Finally, to confirm that myogenin was interacting with the proximal responsive element of the caveolin-3 promoter, anti-myogenin antibodies were added to the nuclear extract in the presence of the oligonucleotides. Although no supershift could be observed, the intensity of the retarded band clearly diminished, hence indicating that the anti-myogenin antibody and the oligomer competed for myogenin binding (Fig. 4*E*).

Recombinant Purified Myogenin Is Selectively S-Nitrosylated in the Presence of Nitric Oxide—We next expressed in *Escherichia coli* recombinant myogenin and purified it to homogeneity utilizing affinity chromatography. Pure recombinant myogenin appeared as a single band in a Coomassie Blue-stained SDS-PAGE (Fig. 5*A*, lane 1). This band was strongly recognized by anti-myogenin antibodies (Fig. 5*A*, lane 2). We performed the incubation of recombinant myogenin with a ·NO-releasing agent, DEA-NONOate, and a nitrosothiol, GSNO, followed by the biotin switch methodology (25, 27). These treatments rendered positive bands (Fig. 5*B*), hence indicating that purified recombinant myogenin can be efficiently modified by ·NO *in vitro*. A complementary method to asses *S*-nitrosylation is based on specific reduction of *S*-nitrosothiols by treatment with Cu^+/cysteine at pH 6 to liberate ·NO, which can be measured on a nitric oxide analyzer (28, 29). In this assay, the GSNO-myogenin mixture is passed through a Sephadex G-25 gel filtration column to separate the protein from the low molecular mass compounds, and the nitrosothiol content was determined in each individual fraction. As shown in Fig. 5*C* recombinant myogenin treated with GSNO released protein-bound nitrosothiols, demonstrating that the treatment *S*-nitrosylated the protein. When GSNO was used alone, the nitrosothiol appeared only in the low molecular mass fractions (Fig. 5*D*). The same pattern was obtained when the same amount of *S*-nitrosylated glyceraldehyde-3-phosphate dehydrogenase was analyzed by this approach (not shown).

Identification of S-Nitrosylated Peptides of Myogenin Using Mass Spectrometry—Next we analyzed the tryptic digest of myogenin treated with the ·NO donor GSNO with the intention of confirming *S*-nitrosylation and identify *S*-nitrosylated cysteines. Among the six Cys residues that are present within the mouse myogenin sequence, Cys[162] and Cys[169] are positioned away from the DNA-binding region, and they were included in the C-terminal tryptic peptide, which is extremely long for mass spectrometry analysis (residues 150–224). The peptide containing Cys[73] (Val-Cys-Lys) is too small to be characterized by mass spectrometry. The other three cysteines, Cys[61], Cys[65], and Cys[70], lie in a single tryptic peptide (GLGTPEHCPGQCLP-WACK) that was clearly identified in an ion trap mass spectrometer (not shown). After treatment of the protein with GSNO, we identified this peptide mononitrosylated (Fig. 6*A*) and to a lesser extent trinitrosylated (Fig. 6*B*). The fragmentation of the peptides induces primarily the loss of the NO moiety or moieties (because of the breakage of the labile S-N bond), and the ions corresponding to these loses predominate in the spectra. The rest of ions correspond to peptide fragments with-
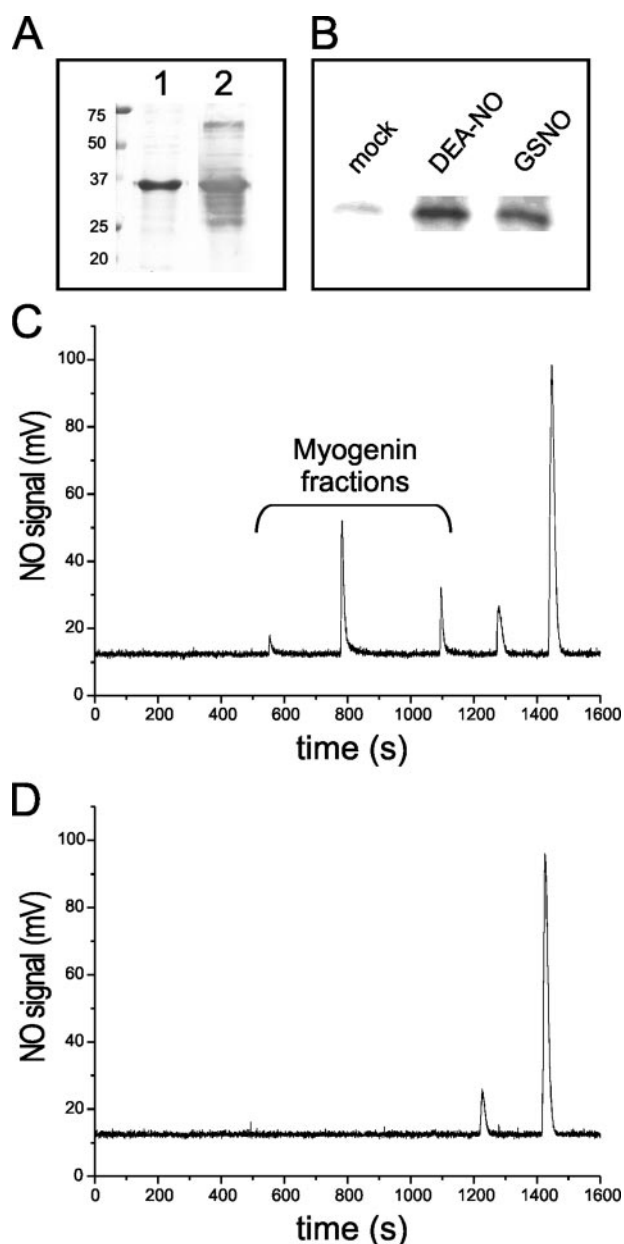
FIGURE 5. **Purification of myogenin and its covalent modification when treated with ·NO donors.** Recombinantly expressed mouse myogenin was purified by chromatography until a homogeneous protein band was obtained (*A, lane 1*) that resulted in a positive detection using commercial anti-myogenin antibodies (*A, lane 2*). Detection of *S*-nitrosylation by the biotin switch technique (*B*). Recombinant myogenin (20 μM) was left untreated (*mock*) or treated with 1 mM DEA-NONOate (DEA/NO) or 1 mM GSNO. The myogenin preparations were then subjected to the biotin switch assay, and biotinylated protein was detected by Western blot with streptavidin. The image shown is representative of three independent experiments. *C* and *D*, recombinant myogenin was reduced with Cu$^+$/cysteine, and ·NO release was detected in a nitric oxide analyzer apparatus. The figures shown are representative of at least three independent experiments. Protein (20 μM) (*C*) or buffer (*D*) was treated with 100 μM GSNO, and protein and low molecular mass fractions were separated in a Sephadex G-25 column before injecting them in the ·NO analyzer at the indicated times. The fractions injected between 500 and 1200 s corresponded to myogenin that released a ·NO signal that could be measured. Free GSNO peaks appear between 1200 and 1600 s.

out NO. Thus, we were unable to identify the exact Cys residue that was modified in this peptide. Nevertheless, our data clearly indicate that at least one of these Cys residues that is close to the

basic DNA-binding region is susceptible to covalent modification by ·NO.

Site-directed Mutagenesis of Cys Residues Positioned in the Proximity of the DNA-binding Domain—Because a putative *S*-nitrosylation occurring on Cys^{73} would be missed using mass spectrometry techniques, we performed a Cys to Ser mutation at this position. Recombinant mutant C73S was purified to homogeneity, and a clear sharp band could be observed in a Coomassie-stained gel (Fig. 6*C*). When we performed the biotin switch assay on the C73S mutant, a clear and reproducible increase in the nitrosylation signal could be obtained when the mutant was treated with GSNO (Fig. 6*D*). This increase was comparable with that obtained in the case of the wild-type myogenin. Consequently, Cys^{73} seemed to be an unlikely candidate for S-nitrosylation. Next, we created a C65S myogenin mutant, expressed it recombinantly, and purified it until homogeneity (Fig. 6*C*). A very subtle increase could be observed when we tested the *S*-nitrosylation of this mutant using the biotin switch methodology (Fig. 6*D*). Because no clear conclusion would be inferred from the mutation at this position, we decided to create a double mutant at positions 61 and 65. Finally, this double mutant C61S,C65S seemed to display no response toward the nitrosylating agent when compared with the untreated sample (Fig. 6*D*). Therefore, we could conclude that Cys^{65} and perhaps Cys^{61} are the targets of the *S*-nitrosylation observed for myogenin.

Characterization of the DNA Binding Properties of Purified Recombinant Myogenin—Next we decided to inspect whether the recombinant myogenin molecule was able to bind to the labeled double-strand 20-mer that presented the proximal E box present in the caveolin-3 promoter that we had used in our EMSA studies. As expected, the myogenin homodimer displayed an increased mobility when compared with the retarded band shown by the nuclear extract (Fig. 7*A*). This is probably due to the presence of myogenin-E protein heterodimers in the nuclear extract that are several orders of magnitude more stable than the myogenin homodimers (37). In addition, neither the nuclear extracts nor the purified recombinant myogenin were able to bind to a mutated E box in which the consensus CANNTG motif had been deleted (Fig. 7*A, right panel*). As expected, binding of recombinant purified myogenin to the proximal E box of the caveolin-3 promoter was sensitive to the presence of ·NO, because a clear diminution could be observed in the retarded band (Fig. 7*B*). However, according to our data, the myogenin heterodimers are more sensitive than the homodimers in terms of sensitivity to ·NO.

DISCUSSION

In striated muscle cells, caveolin-3 is the principal structural protein of the caveolae membrane domains, where it localizes to the sarcolemma (muscle cell plasma membrane). During the differentiation of the C2C12 skeletal myoblasts into myotubes, caveolin-3 becomes expressed coincident with the fusion of cells into multinucleated myotubes (23, 38). The importance of caveolin-3 in the muscle phenotype is further highlighted by the observation that targeted down-regulation of caveolin-3 is sufficient to inhibit myotube formation in differentiating C2C12 myoblasts (38). The late expression of caveolin-3 during
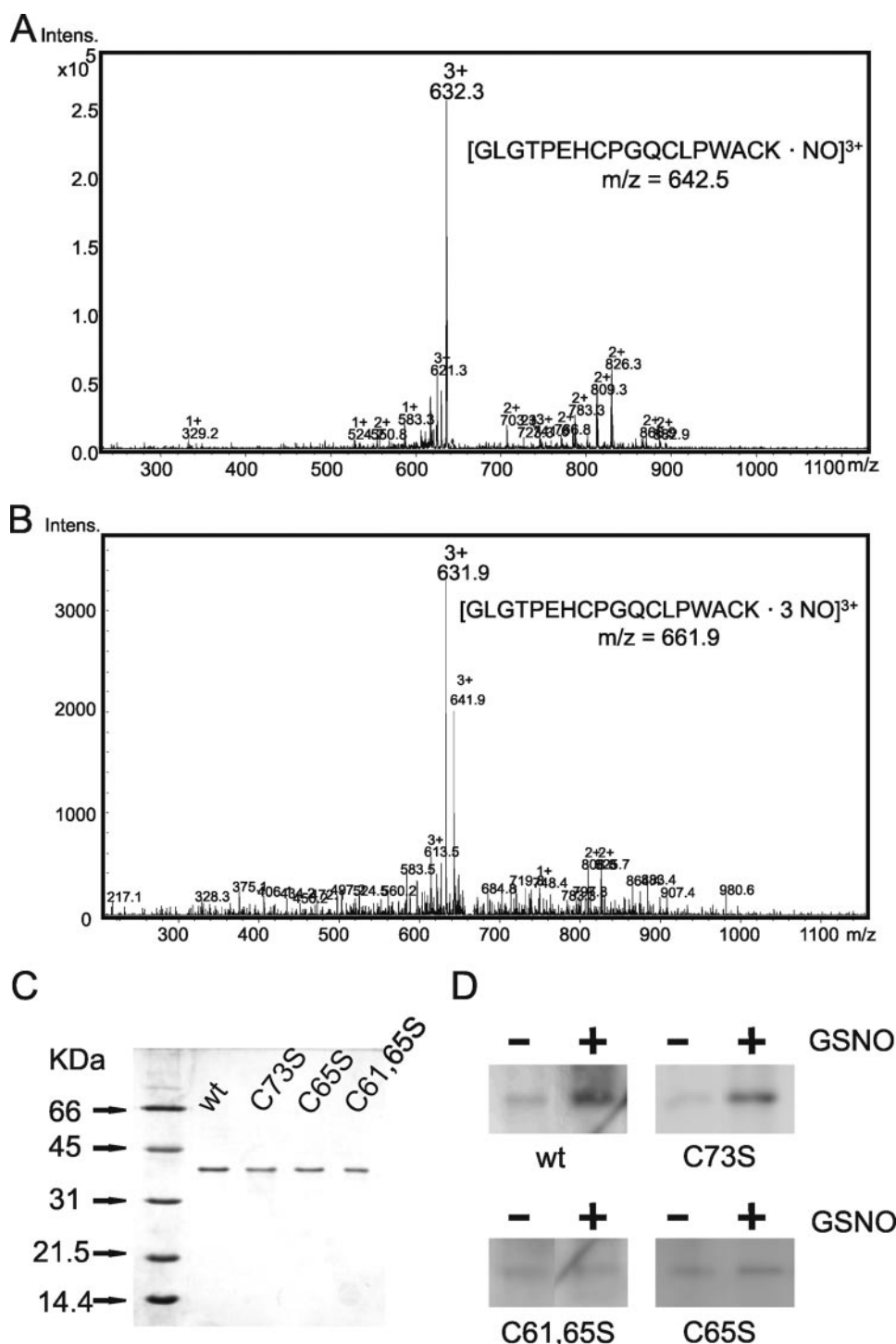
FIGURE 6. **Detection and identification of *S*-nitrosylation by mass spectrometry and site-directed mutagenesis.** Recombinant mouse myogenin was treated with 1 mM GSNO in PBS. The samples were then trypsin digested and analyzed by liquid chromatography-mass spectrometry by using a linear ion trap detector, as described under "Experimental Procedures." Shown is the spectrum of the mono-*S*-nitrosylated peptide (*A*) and tri-*S*-nitrosylated peptide (*B*) corresponding to residues 54–71 of myogenin. Mutants C73S and C65S and the double mutant C61S,C65S were recombinantly expressed and purified to homogeneity. A Coomassie-stained gel of the wild-type (*wt*) protein together with the Cys mutants is shown in (*C*). The biotin switch method was employed to detect the nitrosylation of the purified recombinant wild-type and the Cys mutants of myogenin in the absence and presence of GSNO (*D*).

abundantly in C2C12 myoblasts, the expression of caveolin-3 remains restricted to the differentiated C2C12 myotubes in the presence of 2% horse serum or insulin (20, 23, 33). We previously observed that in mature C2C12, induction of NOS2 expression with the addition of pro-inflammatory cytokines ultimately resulted in the progressive down-regulation of caveolin-1, -2, and -3 (19). We concluded that the protein levels of caveolin-1 and -2 were down-regulated through a p42/p44 Erk-dependent process involving cellular receptors that responded to either bacterial LPS or IFN-γ. Interestingly, the case of caveolin-3 was somehow different, because its down-regulation was retarded in time, and it required the presence of nitric oxide itself because the decrease in its protein levels could be abrogated with the NOS2 inhibitor Nitro-L-Arg (19). In this manuscript we have undertaken the study of the down-regulation of the caveolin-3 protein levels in the presence of both cell-generated ·NO and ·NO donors. The fact that C2C12 myoblasts express MyoD but fail to express caveolin-3 led us to focus on the activity of these transcription-responsive elements with respect to the functional properties of myogenin. Our data indicate that myogenin and not other myogenic transcription factors is the main target of the action of nitric oxide because: (i) the caveolin-3 promoter activity is strictly dependent on the presence of added ·NO, (ii) EMSA experiments indicate that the *cis*-transcriptional activity of the proximal E box of the cav-3 promoter is regulated by ·NO, and (iii) anti-myogenin antibodies selectively inhibit the binding of a nuclear extract to the oligonucleotide dimer covering the proximal E box of the cav-3 promoter. When we inspected five different constructs with one or several myogenin-responsive elements (L1–L5) all of

myogenesis is in agreement with the fact that, among the various myogenic transcription factors, the four E boxes present in the promoter of the caveolin-3 gene seem to respond exclusively to myogenin (23). In fact, although MyoD is present

them displayed comparable diminished activity in a luciferase assay, indicating that ·NO affects the binding of myogenin to DNA, and no other transcription factor within the H9c2 myocytes is binding to the cav-3 promoter.
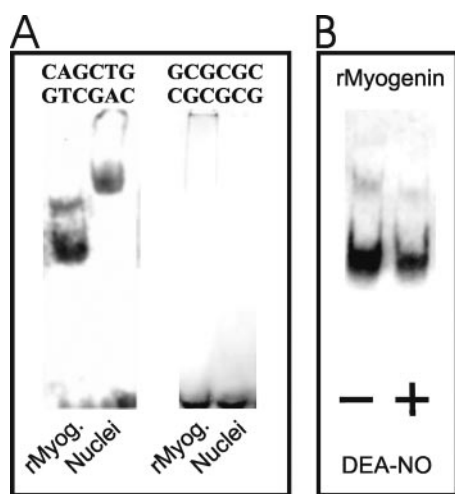
FIGURE 7. **Binding of myogenin to a mutated E box and effect of ·NO on recombinant myogenin.** The binding of both purified recombinant myogenin and a cell nuclear extract was analyzed using 20-base pair labeled oligomers that contained the consensus E box (CANNTG) found in the caveolin-3 promoter or a mutated E box (*A*). The sequences contained at the center of each oligomer are depicted above the gels. The effect of ·NO on purified recombinant myogenin was revealed incubating the protein for 1 h with 200 μM DEA-NONOate followed by an EMSA using annealed oligomers corresponding to the sequence of the caveolin-3 promoter that included the proximal E box (*B*).

We must also consider at this point whether nitric oxide, besides the direct *S*-nitrosylation of myogenin, might be able to activate certain cellular kinases that ultimately phosphorylate myogenin hence altering its DNA binding activity. In fact, it has been reported that treatment of L929 fibroblasts with fibroblast growth factor inhibits myogenesis through the activation of protein kinase C, which in turn phosphorylates Thr[87] of myogenin and abrogates its DNA binding activity (10). However, when H9c2 myocytes were treated with ·NO, in none of our gels were we able to detect the retarded band corresponding to phosphorylated myogenin. In addition, because ·NO abrogates the DNA binding of purified nuclear extracts, it is very unlikely that a kinase activity might be responsible for the observed effect.

Several precedents for the covalent modification of transcription factors by ·NO include NF-κB, hypoxia-inducible factors, c-Jun, and zinc finger transcription factors (see Ref. 13 for a recent review). For instance, NF-κB is involved in the response to many different stimuli, including oxidative stress, and regulates the expression of a wide variety of genes. These include COX-2, NOS2, interleukin 2, and tumor necrosis factor α, all of which participate in crucial biological pathways such as the protection from apoptosis and inflammation. It has been shown that cysteine 62 of p50, which is located in the DNA-binding region, can become nitrosylated. In fact, a role for p50 cysteine 62 has been established in the inhibition by *S*-nitrosothiols of recombinant NF-κB binding to DNA (39).

In this manuscript we describe how the transcription factor myogenin binds to and becomes directly regulated by ·NO both inside the cell as well as when it is expressed as a recombinant protein. In addition, both exogenous ·NO as well as cell-derived ·NO (such as in the case in the co-cultures with macrophages) regulate the ability of myogenin to bind DNA. Our mass spectrometry and site-directed mutagenesis data indicate that Cys[61]

and Cys[65] are the most likely candidates to be the targets of the *S*-nitrosylation of myogenin observed both *in vivo* and *in vitro*. These two cysteine residues are included within a tryptic peptide (GLGTPEHCPGQCLPWACK) in the proximity of the DNA-binding region that was identified as being *S*-nitrosylated. Remarkably, cysteine residues at positions 61, 65, 70, and 73 are positioned in the proximity of the basic DNA-binding region and are completely conserved among the various mammalian myogenin sequences available.

$$\downarrow \quad \downarrow \quad \quad \downarrow \quad \downarrow$$

CPGQCLPWACKVCKRKSVSVDRRRAATLREKRRLKKVNEA

SEQUENCE 1
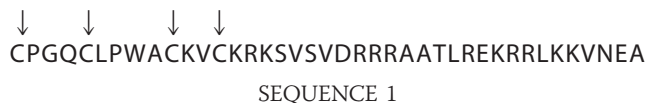
In summary, although there is no atomic structure of myogenin available, the results described in this manuscript clearly indicate that the *S*-nitrosylation of one or several of these cysteine residues alters the interaction of this molecule with DNA. Furthermore, this observation rationalizes previous observations of muscle destruction in human pathologies in which high levels of circulating ·NO are observed.

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