Increased Resting Intracellular Calcium Modulates NF-κB-dependent Inducible Nitric-oxide Synthase Gene Expression in Dystrophic mdx Skeletal Myotubes*‡

Received for publication, January 24, 2012, and in revised form, April 19, 2012. Published, JBC Papers in Press, May 1, 2012, DOI 10.1074/jbc.M112.344929

Francisco Altamirano1, Jose R. López5, Carlos Henríquez3, Tadeusz Molinski4, Paul D. Allen1, and Enrique Jaimovich1†

From the ¹ Centro de Estudios Moleculares de la Célula, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago 8389100, Chile, the ² Department of Anesthesiology Perioperative and Pain Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115, and the ³ Department of Chemistry and Biochemistry and Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California at San Diego, La Jolla, California 92093

Background: The mechanisms by which NF-κB signaling is up-regulated in dystrophic muscles are unclear.

Results: ([Ca²⁺]rest) is elevated in mdx myotubes as a result of both sarcolemmal Ca²⁺ entry and SR release, resulting in NF-κB-induced iNOS expression.

Conclusion: Ca²⁺ alterations at rest modulate NF-κB transcriptional activity and pro-inflammatory gene expression.

Significance: This allows for understanding the mechanism that relates elevated resting calcium and altered gene expression in muscular dystrophy.

Duchenne muscular dystrophy (DMD) is a genetic disorder caused by dystrophin mutations, characterized by chronic inflammation and severe muscle wasting. Dystrophic muscles exhibit activated immune cell infiltrates, up-regulated inflammatory gene expression, and increased NF-κB activity, but the contribution of the skeletal muscle cell to this process has been unclear. The aim of this work was to study the pathways that contribute to the increased resting calcium ([Ca²⁺]rest) observed in mdx myotubes and its possible link with up-regulation of NF-κB and pro-inflammatory gene expression in dystrophic muscle cells. [Ca²⁺]rest was higher in mdx than in WT myotubes (308 ± 6 versus 113 ± 2 nM, p < 0.001). In mdx myotubes, both the inhibition of Ca²⁺ entry (low Ca²⁺ solution, Ca²⁺-free solution, and Gd³⁺) and blockade of either ryanodine receptors or inositol 1,4,5-trisphosphate receptors reduced [Ca²⁺]rest. Basal activity of NF-κB was significantly up-regulated in mdx versus WT myotubes. There was an increased transcriptional activity and p65 nuclear localization, which could be reversed when [Ca²⁺]rest was reduced. Levels of mRNA for TNFα, IL-1β, and IL-6 were similar in WT and mdx myotubes, whereas inducible nitric-oxide synthase (iNOS) expression was increased 5-fold. Reducing [Ca²⁺]rest using different strategies reduced iNOS gene expression presumably as a result of decreased activation of NF-κB. We propose that NF-κB, modulated by increased [Ca²⁺]rest, is constitutively active in mdx myotubes, and this mechanism can account for iNOS overexpression and the increase in reactive nitrogen species that promote damage in dystrophic skeletal muscle cells.

Duchenne muscular dystrophy (DMD) is a lethal human X-linked genetic disorder caused by mutations in the dystrophin gene (1). DMD is a progressive muscle-wasting disease characterized by loss of the ability to walk between 6 and 12 years of age and death, caused by respiratory failure and cardiac dysfunction in their twenties (2). Like humans with DMD, mdx mice lack dystrophin due to an X-linked mutation providing an accepted model to study the human disease (1). In normal skeletal muscle, dystrophin is associated with a complex of glycoproteins known as dystrophin-glycoprotein complex, providing a linkage between the extracellular matrix and cytoskeleton (3). Dystrophin has an important role in stabilizing the sarcolemma, so in muscle fibers that lack this protein, membrane damage is recurrent (4, 5). However, although membrane fragility is an important factor, it does not fully explain the onset and progression of DMD.

The microenvironment of dystrophic muscle consists of activated immune cell infiltrates and up-regulated inflammatory gene expression (6). Nuclear factor-κB (NF-κB) consists of a family of transcription factors that play critical roles in inflammation, immunity, cell proliferation, differentiation, and survival (7). The NF-κB transcription factor family in mammals consists of five proteins, p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1), and p100/52 (NF-κB2), that form homo- and heterodimers.

* This work was supported, in whole or in part, by National Institutes of Health Grants AR492140 and AR053254 (to P. D. A. and J. R. L.). This work was also supported by Grants Fondo Nacional de Investigación Científica y Tecnológica 1110467, Fondo de Financiamiento de Centros de Excelencia en Investigación 15010006, and Asociación Nacional de Investigadores, Universidad de Chile, and Programa de Mejoramiento de la Calidad y Equidad de la Educación travel support UCH 0714, Universidad de Chile (to F. A.).

† To whom correspondence should be addressed: Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Casilla 70005, Santiago 7, Chile. E-mail: ejaimovi@med.uchile.cl.

‡ This article contains supplemental Figs. S1–S3.

1 The abbreviations used are: DMD, Duchenne muscular dystrophy; iNOS, inducible nitric-oxide synthase; IP3, inositol 1,4,5-trisphosphate receptor; Ry, ryanodine; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; XeC, xestospongin C; SOCE, store-operated calcium entry; B5, bastadin 5; IKK, IκB kinase; DAF-FM diacetate, 4-amino-5-methylamino-2′,7′-difluoro-fluorescein diacetate.

2 The abbreviations used are: DMD, Duchenne muscular dystrophy; iNOS, inducible nitric-oxide synthase; IP3, inositol 1,4,5-trisphosphate receptor; Ry, ryanodine; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; XeC, xestospongin C; SOCE, store-operated calcium entry; B5, bastadin 5; IKK, IκB kinase; DAF-FM diacetate, 4-amino-5-methylamino-2′,7′-difluoro-fluorescein diacetate.
Ca²⁺-dependent NF-κB Up-regulation in mdx Myotubes

NF-κB has been implicated in mdx pathology, because blockade of this pathway through pharmacological or genetic approaches improves muscle histology, reduces pro-inflammatory gene expression, and ameliorates damage (8–12). NF-κB activity is increased in muscles from mdx mice and DMD patients (10, 13–15). The p65/p50 heterodimer is the predominant form of NF-κB in most cells and controls the expression of a wide array of genes critical in the immune response and inflammation (16). IkBα retains the p65/p50 heterodimer in the cytoplasm. Upon stimulation, IkBα is quickly phosphorylated by the IKK complex, ubiquinated, and degraded, thus allowing the translocation to the nucleus of the NF-κB complex (7).

IKKα/β or p65 gene ablation in transgenic animals or by adeno-associated virus improves pathology in mdx mouse muscles (8–12). Acharyya et al. (10) have shown that NF-κB activity can be seen in both muscle and immune cells and that mdx muscle pathology was improved in mdx/p65−/− but not mdx/p50−/− mice.

NF-κB gene targets, such as the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and iNOS are up-regulated in muscles from Duchenne patients and mdx mice (10, 17–20). In mdx mice, injections of the nonspecific NF-κB inhibitor curcumin have been shown to reduce NF-κB activation and TNF-α, IL-1β, and iNOS expression (6, 9).

iNOS or NOS2, originally discovered in cytokine-induced macrophages, is a largely calcium-independent NOS, which is expressed at highest levels in immunologically activated cells and is normally absent in resting cells (21). iNOS expression is increased in muscles from mdx mice and can be reversed by curcumin (9, 22, 23). High levels of nitric oxide (NO) production lead to the formation of peroxynitrite, a highly reactive species contributing to muscle oxidative damage (21, 24). In addition, iNOS expression has been associated with S-nitrosylation of type 1 ryanodine receptor (RyR1), calcium dysregulation, and muscle pathology in mdx mice (22).

Although there are many examples in the literature indicating that resting intracellular free Ca²⁺ concentration ([Ca²⁺]₉₉) is higher in skeletal muscle cells from mdx mice and DMD patients compared with normal cells (25–30), others authors have not (31, 32). The mechanism that has been proposed for causing this elevation in [Ca²⁺]₉₉ assumes recurrent membrane damage due to the failure of dystrophin function to stabilize the sarcolemma (5, 33), allowing Ca²⁺ leak into the cell through the damaged membrane. An alternative explanation is an increased Ca²⁺ entry through transient receptor potential channel 1 (TRPC1) and hyperactive store-operated calcium entry (SOCE) in mdx muscle fibers (25, 34–38).

Several studies have shown that NF-κB activity can be modulated by intracellular Ca²⁺ levels (39–42). In skeletal muscle cells, depolarization with high K⁺ or electrical stimulation activates NF-κB through Ca²⁺ signals elicited by the ryanodine (RyR) and inositol 1,4,5-triphosphate (IP₃) receptors (40).

In dystrophic muscle cells, increased [Ca²⁺]₉₉ has been mainly thought to cause necrosis through calpain activation and mitochondrial permeability transition pore (29, 43, 44). Here, we have revisited the issue of elevated [Ca²⁺]₉₉ in dystrophic mdx skeletal muscle cells showing that it is a complex process that involves sarcolemmal Ca²⁺ entry as well as SR Ca²⁺ leak, both through RyRs and IP₃Rs. In addition, we demonstrate that the level of [Ca²⁺]₉₉ modulates the transcription factor NF-κB activity and iNOS expression in mdx myotubes.

MATERIALS AND METHODS

Cell Culture—All procedures for animal use were in accordance with guidelines approved by the Bioethical Committee at the Facultad de Medicina, Universidad de Chile. Primary myotubes from wild type C57BL/6 and mdx mice were isolated according to the method of Rando and Blau (45). The myoblasts were grown and differentiated as described previously (46).

Determination of [Ca²⁺]₉₉ by Ca²⁺-selective Microelectrodes—Double-barreled Ca²⁺-selective microelectrodes were prepared and calibrated as described previously (47). Only those electrodes with a linear relationship between pCa and pCa₈ (Nernstian response, 28.5 mV per pCa unit at 24 °C) were used experimentally. To better mimic the intracellular ionic conditions, all calibration solutions were supplemented with 1 mM Mg²⁺. All electrodes were then re-calibrated after making measurements of [Ca²⁺]₉₉ and if the two calibration curves did not agree within 3 mV from pCa₈ to pCa₈, the data from that microelectrode was discarded. Myotubes were impaled with the double-barreled microelectrode, and potentials were recorded via high impedance amplifier (WPI Duo-773). The potential from the 3 M KCl barrel (V₉₉₉₉) was subtracted electronically from VCa₉₉ to produce a differential Ca²⁺-specific potential (V₉₉₈₈) that represents the [Ca²⁺]₉₉. V₉₉₈₈ and VCa₈ were filtered (30–50 KHz) to improve the signal-to-noise ratio and stored in a computer for further analysis. The experiments were performed in Krebs-Ringer solution (in mM: 125 NaCl, 5 KCl, 2 CaCl₂, 1.2 MgSO₄, 6 glucose, and 25 Hepes/Tris, pH 7.4). The low Ca²⁺ solution was prepared replacing the CaCl₂ with MgCl₂ (~7 μM Ca²⁺). Ca²⁺-free solution was prepared by omitting Ca²⁺ and adding Mg²⁺ (2 mM) and EGTA (2 mM). We avoided measurements of [Ca²⁺]₉₉ after long incubations in both solutions (more than 5 min) because, despite the fact that the solution was supplemented with 2 mM Mg²⁺, all myotubes began to show a significant depolarization (>8 mV) after this interval.

In every experiment, we determined the [Ca²⁺]₉₉ in control conditions in both WT and mdx myotubes, and data were expressed as the total average for basal [Ca²⁺]₉₉ for WT and mdx myotubes (Fig. 1).

Sarcoplasmic Reticulum Ca²⁺ Content—To estimate the total amount of Ca²⁺ stored in the intracellular compartments, primarily from the SR stores, myotubes were loaded with 5 μM Fluo-4-AM or Fluo-5N-AM for 30 min at 37 °C. Cells were placed on the stage of an inverted microscope equipped with epifluorescence illumination (Xcite® Series 120 or Lambda DG4) equipped with a CCD cooled camera (Retiga 2000R or Stanford Photonics 12 bit digital). The cell-containing coverslips or μ-clear 96-well/plates (Greiner Bio-one) were placed in the microscope for fluorescence measurements after excitation with a 488-nm wavelength filter system (Lambda 10–2 or DG4, Sutter Instruments). The emission signal was acquired at a frequency of 10 frames/s. The amount of SR Ca²⁺ was estimated by taking the area under the curve of the signal induced by 5 μM
**Ca^{2+}-dependent NF-κB Up-regulation in mdx Myotubes**

ionomycin in Ca^{2+}-free solution to minimize Ca^{2+} entry. Fluorescence data (F) was analyzed by normalizing with respect to basal fluorescence (F_0) and expressed as (F - F_0)/F_0.

**Resting Ca^{2+} Entry**—Myotubes were loaded with Fura2-AM (5 μM) for 30 min at 37 °C and the cells were perfused with low Ca^{2+} solution for 1 min; then the perfusion system was switched to Mn^{2+}-containing solution (in mM: 125 NaCl, 5 KCl, 0.5 MnCl_2, 2.7 MgSO_4, 6 glucose, and 25 Hepes/Tris, pH 7.4) for 1 min. During the quench, the perfusion system was switched to Mn^{2+} containing solution with gadolinium tri-chloride for an additional 1 min (Gd^{3+}, 20 μM), to study the effect of the later on Ca^{2+} entry. To calculate the fluorescence quench rate, the stable part of the signal was fitted to a linear regression. The derived slope was expressed as fluorescence arbitrary units/s. The excitation wavelength used to measure Mn^{2+} quench of Fura-2 was monitored using a 357/7-nm excitation and 510/80-nm emission filter.

**Immunofluorescence and Confocal Microscopy**—For immuno-fluorescence localization of the NF-κB p65 subunit, differentiated myotubes were fixed in 4% paraformaldehyde for 10 min at RT. Cells were rinsed with PBS, then blocked with PBS, 1% BSA for 1 h at room temperature, and incubated overnight with p65 antibody at 1:200 dilution at 4 °C. Cells were washed and then incubated for 1 h with Alexa Fluor-488 anti-rabbit antibody (Invitrogen). Hoechst was used for nuclear visualization. Immunofluorescence was observed in a confocal microscope (Carl Zeiss, Axiovert 200, LSM 5-Pascal) and images were deconvolved using Iterative Deconvolution software of ImageJ. To determine the nuclear localization of the NF-κB p65 subunit, the fluorescence intensity of nuclear and cytosolic region of interest was calculated for at least 10 different myotubes in three different experiments and averaged to calculate the ratio of nuclear over cytoplasmic intensity using the ImageJ program. z-stack images were reconstructed using the Interactive 3D Surface Plot ImageJ plug-in (rsb.info.nih.gov) that translates the luminance of an image as height for the plot. DAF-FM diacetate (Molecular Probes) fluorescence was detected according to the manufacturer’s instructions, and light detection was carried out in a Berthold F12 luminometer. Results were normalized with total protein, and the relation “luciferase mg^-1 protein” was shown. We studied the response to lipopolysaccharide (LPS), a strong activator of NF-κB as a control (data not shown).

**Real Time PCR**—Total RNA from myotubes cultures was obtained using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. cDNA was prepared by reverse transcription of 1 μg of total RNA, using SuperScript II (Invitrogen). Real time PCR was performed using a Stratagene Mx3000P as follows. Primers were used at a final concentration of 400 nm. Briefly, 1–4 μl of cDNA reaction together with the appropriate primers was added to 10 μl of Brilliant III UltraFast SYBR Green QPCR master mix (Agilent Technologies) to a total volume of 20 μl. No-template control reactions were also prepared for each gene. The cycling parameters for all genes were as follows: 95 °C for 3 min, then 50 cycles of 95 °C for 20 s, and 60 °C for 20 s. Expression values were normalized to GAPDH and are reported in units of 2 -ΔΔC_T ± S.E (49). PCR products were verified by melting-curve analysis, resolved by electrophoresis on 2% agarose gel, and stained with ethidium bromide.

The TNF-α, IL-1β, IL-6, iNOS, and GAPDH mRNA transcripts were quantified using oligonucleotide primers designed based on sequences published in NCBI GenBank™ with the open-source PeriPrimer software (50). The forward and reverse primers sequences used in this study are shown in Table 1.

**Statistics**—All values are expressed as mean ± S.E. from at least three different determinations. Results of luciferase activity, p65 immunofluorescence, DAF-FM fluorescence, and Western blot were transformed with the WT basal average (y = y’/(WT basal average)) to normalize to 1 with S.E. Statistical analysis was performed using an unpaired two-tailed t test or analysis of variance-Bonferroni to determine significance (p < 0.05).

**RESULTS**

[Ca^{2+}]_rest Is Increased in Dystrophic mdx Myotubes—Resting membrane potentials (V_m) and [Ca^{2+}]_rest were measured in dif-
differentiated WT and mdx myotubes with Ca\(^{2+}\)-selective microelectrodes. The \([\text{Ca}^{2+}]_{\text{rest}}\) observed in mdx myotubes was significantly higher than that observed in WT myotubes (308 ± 6 nm, \(n = 38\) versus 113 ± 2 nm, \(n = 20\), \(p < 0.001\)) (Fig. 1A). \(V_{m}\) was significantly increased in mdx myotubes compared with the WT counterpart (−50 ± 0.2 mV, \(n = 38\) versus −62 ± 0.2 mV, \(n = 20\), \(p < 0.001\)) (Fig. 1B).

**Blockade of Ca\(^{2+}\) Entry in mdx Myotubes Reduced but Did Not Normalize \([\text{Ca}^{2+}]_{\text{rest}}\)**—Several studies have suggested that \([\text{Ca}^{2+}]_{\text{rest}}\) is increased in mdx skeletal muscle cells, due to an increased Ca\(^{2+}\) entry from extracellular space through TRPC1 and/or SOCE channels (25, 34–38). To explore the contribution of extracellular Ca\(^{2+}\) to \([\text{Ca}^{2+}]_{\text{rest}}\) in WT and mdx myotubes, we used four different strategies as follows: low Ca\(^{2+}\) solution, Ca\(^{2+}\)-free solution, Krebs-Ringer solution supplemented with gadolinium trichloride (Gd\(^{3+}\), 20 μM), and Ca\(^{2+}\)-free solution with Gd\(^{3+}\) (see under “Materials and Methods”). Cells were incubated for 2 min before \([\text{Ca}^{2+}]_{\text{rest}}\) determinations were made. We observed a nonsignificant reduction in \([\text{Ca}^{2+}]_{\text{rest}}\) in WT myotubes after the addition of low Ca\(^{2+}\) solution, Ca\(^{2+}\)-free solution, and Gd\(^{3+}\)-free solution (92.9 ± 1 nm, \(n = 20\), 91.0 ± 1 nm, \(n = 15\), and 86.3 ± 1 nm, \(n = 20\), all \(p > 0.05\) compared with the WT basal value) (Fig. 2A). In mdx myotubes, there was a significant decrease in \([\text{Ca}^{2+}]_{\text{rest}}\) in all conditions (184 ± 8 nm, \(n = 12\), with low Ca\(^{2+}\), 148 ± 6 nm in Ca\(^{2+}\) free solution, \(n = 10\), 147 ± 1 nm, \(n = 11\), after Gd\(^{3+}\), all of them \(p < 0.001\) compared with the mdx basal value) (Fig. 2A). The addition of Gd\(^{3+}\) to the Ca\(^{2+}\)-free solution did not cause a further reduction of \([\text{Ca}^{2+}]_{\text{rest}}\) suggesting that Gd\(^{3+}\) by itself was able to block the active Ca\(^{2+}\)-entry pathway. In addition, we estimated Ca\(^{2+}\) entry by Mn\(^{2+}\) quench of Fura-2 fluorescence. Rates of Mn\(^{2+}\) quench were significantly higher in mdx myotubes (Fig. 2, B and C), and this was completely blocked by the addition of Gd\(^{3+}\) (20 μM). Although inhibition of Ca\(^{2+}\) entry by either Gd\(^{3+}\) or removal of extracellular Ca\(^{2+}\) reduced \([\text{Ca}^{2+}]_{\text{rest}}\) in mdx myotubes, it did not return it to WT levels, suggesting an additional mechanism(s) causing \([\text{Ca}^{2+}]_{\text{rest}}\) dysregulation in mdx myotubes.

**Inhibition of RyRs and IP\(_3\)Rs Reduced \([\text{Ca}^{2+}]_{\text{rest}}\) in mdx Myotubes**—We have previously reported that \([\text{Ca}^{2+}]_{\text{rest}}\) depends largely on a Ry-insensitive leak by RyR1 (“RyR1 leak”) and was unaffected by Ry treatment (47). Bastadin 5 (B5), a brominated macrocyclic derivative of dityrosine, isolated from the marine sponge *Icanthellabasta* (51), interacts with RyR1, modulating RyR1 gating behavior in an FKBP12-dependent manner. B5 can be used as a pharmacological tool to convert RyR1 from its leak conformation into a gating conformation, restoring the Ry sensitivity (47). We studied the contribution of RyR1 to the \([\text{Ca}^{2+}]_{\text{rest}}\) in mdx myotubes (Fig. 3A). Ry treatment alone did not modify \([\text{Ca}^{2+}]_{\text{rest}}\) in WT myotubes but did cause a significant reduction of \([\text{Ca}^{2+}]_{\text{rest}}\) in mdx myotubes (99 ± 3 nm, \(n = 15\), \(p > 0.05\), 213 ± 5 nm, \(n = 19\), \(p < 0.001\) compared with WT and mdx basal values, respectively). Addition of B5 in the presence of Ry significantly diminished \([\text{Ca}^{2+}]_{\text{rest}}\) in both WT and mdx myotubes (80 ± 1 nm, \(n = 9\), \(p < 0.05\), 166 ± 9 nm, \(n = 9\), \(p < 0.001\) compared with WT and mdx basal values, respectively) but also did not reduce mdx basal values to those seen in WT.

We have previously demonstrated that the expression of *IP\(_3\)R* and *IP\(_3\)R* in both mdx and human DMD-derived cell lines compared with normal cells (52). U-73122 (a PLC inhibitor) and Xestospongin C (an IP\(_3\)R blocker) significantly reduced the \([\text{Ca}^{2+}]_{\text{rest}}\) only in mdx myotubes (241 ± 7 nm, \(n = 24\), and 232 ± 6 nm, \(n = 20\), respectively, both \(p < 0.001\) compared with the mdx basal value), without any significant effect in WT myotubes (113 ± 2 nm, \(n = 21\), and 97 ± 2 nm, \(n = 16\), respectively, \(p > 0.05\) compared with WT basal values) (Fig. 3A), whereas U-73343 (an inactive PLC inhibitor) did not modify \([\text{Ca}^{2+}]_{\text{rest}}\) in either WT or mdx myotubes.

To quantify the level of the SR Ca\(^{2+}\) store, we exposed WT and mdx myotubes loaded with Fluo-4-AM to 5 μM ionomycin in Ca\(^{2+}\)-free solution. Under these conditions, the total Ca\(^{2+}\) released was significantly smaller in mdx myotubes compared with WT myotubes (area under curve = 23.7 ± 2.4 versus 40.4 ± 4.2, \(p < 0.01\)) (Fig. 3, B and C, and representative fluorescence images in supplemental Fig. S1). Similar results were obtained in Fluo-5N-loaded myotubes (supplemental Fig. S2). Moreover, treatment (3 h) with either Ry (30 μM) or XeC (5 μM), partially restored the SR Ca\(^{2+}\) content in mdx myotubes (Fig. 3C), suggesting that the reduction in the SR Ca\(^{2+}\) levels is

---

**TABLE 1**

| mRNA         | Accession no. | Forward primer (5′ → 3′) | Reverse primer (5′ → 3′) | Amplicon (bp) | Source or Ref. |
|--------------|---------------|--------------------------|--------------------------|---------------|----------------|
| GAPDH        | NM_008084.2   | CTCACTGACCAAGGTTGTCAGCA  | TTTCACTGGTGTAGACCCCTTT  | 155           | This study     |
| iNOS         | NM_010927.3   | CAGCTGAAGACCCGAGAAGAACG | TTTAGCTGGTGACTGGTTCC     | 141           | This study     |
| IL-1β        | NM_008361.3   | GTCTAAGAGGATGACCCTCC     | TTTCTGACTGGTGGTTCTC      | 229           | This study     |
| IL-6         | NM_031681.1   | CCAATTTCCACACCTCTCCT     | ACACCAGTGAGAGATGCACCA   | 182           | (83)           |
| TNF-α        | NM_013692.2   | TACACCTCAGCAAGCTCTATCC   | ATCGAGTAAACATGCGCTG     | 261           | This study     |

---

**FIGURE 1.** A, resting intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_{\text{rest}}\)); B, resting membrane potentials (\(V_m\)) measured by double-barreled microelectrodes in WT and mdx primary myotubes. Data are expressed as means ± S.E., \(n = 20\) for WT and \(n = 38\) for mdx, indicated inside the bars. ***, \(p < 0.001\) versus WT.
due to a Ca²⁺ leak from the SR through both RyRs and IP₃Rs that modulates [Ca²⁺]rest.

NF-κB Activity Is Up-regulated in Dystrophic Myotubes and Can Be Reversed with Inhibitors That Reduce [Ca²⁺]rest—We studied the subcellular distribution of the p65 subunit of NF-κB. Fig. 4A shows an increased nuclear localization of p65 in mdx myotubes compared with WT myotubes, measured by immunofluorescence and confocal microscopy. Three-dimensional reconstruction of z-stack images shows that p65 is located primarily in the cytosol, but in mdx myotubes, the distribution is diffuse with both cytoplasmic and nuclear localization. Basal fluorescence ratio of p65 between nucleus and cytosol was increased about 50% in dystrophic myotubes compared with normal myotubes (Fig. 4B). We assessed the transcriptional activity of NF-κB using a reporter that contains five tandems repeats of NF-κB-binding sites cloned upstream of a luciferase gene (see "Materials and Methods"). Luciferase activity was increased 2.5-fold in mdx myotubes compared with WT myotubes (Fig. 4C). To establish a correlation between [Ca²⁺]rest and NF-κB transcriptional activity, we treated myotubes for 6 h with Gd³⁺, Ry, and XeB (53, 54) as described previously at the same concentrations. None of these drugs caused a significant change in the luciferase reporter activity in WT myotubes (p > 0.05) (Fig. 4C). However, blockade of sarcolemmal Ca²⁺ entry with Gd³⁺ reduced the luciferase reporter activity by 19% (p > 0.05), and pretreatment with Ry or XeB reduced it by 58 and 38%, in mdx myotubes, respectively (p < 0.001 and p < 0.01, respectively, compared with the mdx basal value). Furthermore, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA AM, 50 μM) treatment reduced NF-κB transcriptional activity in mdx myotubes by 43% (p < 0.05) without any significant effect in WT myotubes. To confirm the contribution of Ca²⁺ released by SR, we measured the subcellular distribution...
of p65 in the presence of Ry or XeB. Both inhibitors reduced nuclear/cytosol p65 fluorescence by mdx myotubes (Fig. 4B).

TNF-α, IL-1β, and IL-6 Gene Expression Was Similar in WT and mdx Myotubes—To identify gene targets that can be modulated by [Ca$$^{2+}$$]$$_{\text{rest}}$$-dependent NF-κB up-regulation, we studied the levels of mRNA for TNF-α, IL-1β, and IL-6 in both myotube models. We did not observe any significant difference in the mRNA levels of these cytokines between WT and mdx myotubes under resting conditions (supplemental Fig. S3).

iNOS Is Overexpressed in mdx Myotubes and Is Dependent on [Ca$$^{2+}$$]$$_{\text{rest}}$$ and NF-κB Activity—We observed increased iNOS mRNA levels and protein expression in mdx myotubes (p < 0.001 and p < 0.01, respectively, compared with WT myotubes) (Fig. 5, A and B). Moreover, nitric oxide (NO) production, assessed by DAF-FM fluorescence, was ~20% higher in mdx compared with WT myotubes (Fig. 5C). In myotubes transfected with p65 siRNA, the expression of p65 protein, after 48 h, was reduced by 89 and 82% in WT and mdx myotubes, respectively (Fig. 5D). p65 knockdown in mdx myotubes normalized iNOS protein levels to WT values showing that the latter is regulated by the activity level of the former (Fig. 5D). Moreover, treatment with compounds that lower [Ca$$^{2+}$$]$$_{\text{rest}}$$ for 6 h significantly reduced iNOS mRNA levels in mdx myotubes (75% Gd$$^{3+}$, 86% Ry, and 66% XeB), but it had no significant effect in WT myotubes (Fig. 5A).

p38 MAPK Is Involved in NF-κB Up-regulation in mdx Myotubes—Several Ca$$^{2+}$$-sensitive pathways can modulate the activity of the NF-κB signaling pathway (41, 55). To determine the signal transduction pathways involved in the [Ca$$^{2+}$$]$$_{\text{rest}}$$-dependent NF-κB up-regulation, we used specific pharmacological blockers for ERK1/2, JNK, p38 MAPKs, Ca$$^{2+}$$/calmodulin-dependent kinase II, calcineurin A, and protein kinase C (PKC) (Fig. 6). Only p38 MAPK inhibition with SB-203580 (10 μM) significantly reduced the NF-κB luciferase reporter activity in both WT and mdx myotubes by 82 and 73%, respectively (p < 0.001).
Ca\(^{2+}\)-dependent NF-\(\kappa B\) Up-regulation in mdx Myotubes

**DISCUSSION**

In dystrophic skeletal muscle cells, increased \([\text{Ca}^{2+}]_{\text{rest}}\) has been mainly related with calpain activation and mitochondrial permeability transition pore aperture as factors that induce death in normal muscle fibers. Here, we show the first evidence that elevated \([\text{Ca}^{2+}]_{\text{rest}}\) in dystrophic myotubes causes altered function of the transcription factor NF-\(\kappa B\) leading to iNOS expression. In addition, our data show that the increased \([\text{Ca}^{2+}]_{\text{rest}}\) in mdx myotubes is multifactorial, involving both Ca\(^{2+}\) entry and Ca\(^{2+}\) SR leak, through RyRs and IP\(_3\)Rs.

We have previously shown that the \([\text{Ca}^{2+}]_{\text{rest}}\) in DMD muscle fibers was \(~370\ \text{nm}\), although in normal muscle fibers it was \(~100\ \text{nm}\) (27). Other authors have shown similar calcium concentrations in adult mdx fibers compared with the WT fibers (25, 28). A possible explanation to why some authors did not find elevated \([\text{Ca}^{2+}]_{\text{rest}}\) in dystrophic muscle cells may be due, in part, to methodological differences in fluorescent dye calibration, the previous contractile activity, and age of the fibers. Moreover, fluorescent dyes, as 1,2-bis(2-aminophenoxy)-ethane-\(N,N,N',N'\)-tetraacetic acid derivatives, are Ca\(^{2+}\) chelators and can artificially reduce \([\text{Ca}^{2+}]_{\text{rest}}\). Thus in most cases, the \([\text{Ca}^{2+}]_{\text{rest}}\) values that have been reported in muscle cells using this method are significantly lower (range 20–80 nm) than those reported using Ca\(^{2+}\)-selective microelectrodes (100–120 nm).

TRPC1-dependent Ca\(^{2+}\) entry is increased in mdx muscle fibers (25, 34, 36). Both GsMTx4 and streptomycin reduced \([\text{Ca}^{2+}]_{\text{rest}}\) and prevented the rise of the \([\text{Ca}^{2+}]_{\text{rest}}\) following eccentric contractions improving the muscle function and increasing myofiber regeneration in mdx mice (25, 28, 36). In addition to stretch channel activation, SOCE has emerged as another contributor in increased resting Ca\(^{2+}\) entry in mdx fibers (35, 37, 38). We have found that Gd\(^3+\), an unspecific blocker of Ca\(^{2+}\)-entry through SOCE and transient receptor potential channels (56), reduced \([\text{Ca}^{2+}]_{\text{rest}}\) by 52% in mdx myotubes and that long term treatment with Gd\(^3+\) was associated with a reduction in NF-\(\kappa B\) activity and iNOS expression. However, blocking Ca\(^{2+}\) entry did not completely normalize \([\text{Ca}^{2+}]_{\text{rest}}\), suggesting a possible intracellular contribution.

In primary mdx myotubes, treatment with Ry reduced the \([\text{Ca}^{2+}]_{\text{rest}}\) by 31% and adding B5 decreased it further to 46%. We have previously shown that \([\text{Ca}^{2+}]_{\text{rest}}\) depends largely on Ry-insensitive leak of RyR1 channels (RyR1 leak) that can be blocked by Ry + B5 treatment in normal myotubes (47). Bellinger et al. (22) have shown that RyR1 isolated from mdx skeletal muscle shows an age-dependent increase in S-nitrosylation coincident with muscle pathology, which depleted the channel complex of FKBP12, resulting in “leaky channels.” Depletion of FKBP12 from RyR1 channel to nitrosative stress may render it sensitive to Ca\(^{2+}\)-mediated activation (22).
Both IP₃R blockade with XeC (IP₃R blocker) and U-73122 (PLC inhibitor) treatment resulted in 25 and 22% reduction in the [Ca²⁺]ᵣₑˢᵗ in mdx myotubes, respectively. These combined data strongly demonstrate that the SR plays an important role in the dysregulation [Ca²⁺]ᵣₑˢᵗ observed in dystrophic myotubes.

There is a controversy concerning the SR Ca²⁺ levels in mdx skeletal muscle cells. Robert et al. (57) demonstrated an increased SR Ca²⁺ loading capacity after depletion in mdx compared with WT. However, other authors have shown a reduced expression of calsequestrin-like proteins, lower SR Ca²⁺ loading (58), and reduced sarco/endoplasmic reticulum Ca²⁺-ATPase activity in mdx muscles (59). Recently, Robin et al. (60) demonstrated an elevated passive SR Ca²⁺ leak in mdx fibers, using fibers voltage-clamped at -80 mV and exposed to cyclopiazonic acid. Our results have shown that Ry- or XeC-treated mdx myotubes have an increase in SR Ca²⁺ store content suggesting that SR leak occurs through these Ca²⁺ channels.

SERCA1a overexpression in mdx diaphragm muscle by adeno-associated virus gene transfer resulted in a reduction of centrally located nuclei and reduced susceptibility to eccentric contraction-induced damage (61). More recently, sarcoglycan-null and mdx mice animals that overexpress SERCA1 through transgenesis show an improvement in muscle damage and excitation-contraction coupling and restore the [Ca²⁺]ᵣₑˢᵗ and [Ca²⁺]ᵢₐᵲₑₛₜ in both dystrophic models (62). Together, this suggests that the filling state of the SR contributes significantly to the dysregulation [Ca²⁺]ᵣₑˢᵗ observed in mdx muscles.

Several reports indicate that resting membrane potentials are more positive in mdx muscles fibers than WT (27, 63–65). We have found that mdx myotubes showed a partial membrane depolarization compared with WT. None of the drugs used in this study, all of which have a major effect on [Ca²⁺]ᵣₑˢᵗ, induced a significant repolarization in the mdx myotubes. These are not surprising results because none of them have any effect on ion permeability or ion-translocat-
ing enzymes involved in maintaining the resting membrane potential value.

Numerous facts indicate that the dystrophic skeletal muscle cells have impaired excitation-contraction coupling. Comparisons of the cytosolic Ca\(^{2+}\) transients evoked by a single action potential have shown that the Ca\(^{2+}\) transients are reduced in mdx and mdx\(\times\)utr\(-/-\) fibers compared with WT fibers (66–68). Muscle weakness observed in isolated fibers from mdx mice and DMD patients has not been fully explained. The reduction in the Ca\(^{2+}\) transient evoked by single action potential, increased \(V_{\text{m}}\) increased \([Ca^{2+}]_{\text{rest}}\) and a reduced Ca\(^{2+}\) loading capacity of the SR could provide a mechanism for contractile dysfunction and impaired force production in DMD patients.

Several studies have shown that NF-\(\kappa\)B activity is increased in mdx skeletal muscles (8–15), but the mechanisms causing this abnormality have not been previously unveiled. Acharyya et al. (10) reported an increased NF-\(\kappa\)B DNA binding activity and IKK activation, without any change in IkB\(\alpha\) expression and phosphorylation and normal levels of p65 with an increased phosphorylation. The authors proposed direct p65 activation by IKK\(\beta\) (10). On the contrary, Singh et al. (15) have found an increase in the expression of both p65 and IkB\(\alpha\) and increased IkB\(\alpha\) phosphorylation, indicating that NF-\(\kappa\)B activation in mdx muscles is due to a complex mechanism and not only IKK activation. Both examined activation of NF-\(\kappa\)B in whole muscle extracts. Because dystrophic muscles are associated with a large amount of activated immune cell infiltrates, which have increased NF-\(\kappa\)B activity (7, 10), it is possible that this increase was not due to changes in muscle cells. Here, we used the myotube model to determine whether NF-\(\kappa\)B can be activated in dystrophic skeletal muscle cells without contribution from the immune system. We observed that NF-\(\kappa\)B transcrip
tional activity, measured by a luciferase reporter, was increased in mdx myotubes, and we observed a significant increase in p65 nucleus/cytosol fluorescence. Both luciferase activity and p65 nuclear localization could be reduced by agents that modulate \([Ca^{2+}]_{\text{rest}}\) in mdx myotubes but were not changed by these drugs in WT myotubes.

We do not know the exact mechanism that accounts for \([Ca^{2+}]_{\text{rest}}\)-dependent activation of NF-\(\kappa\)B in muscle cells. Several Ca\(^{2+}\)-sensitive pathways can modulate the activity of NF-\(\kappa\)B (41). We have previously shown that membrane depolarization activates NF-\(\kappa\)B through increases in intracellular Ca\(^{2+}\) through RyR and IP\(_3\)R. This Ca\(^{2+}\)-dependent modulation has been attributed to calcineurin A, PKC, and ERK1/2 pathway activation in normal myotubes (40). We have not found any significant effect in the luciferase activity when we preincubated with specific blockers of these signaling pathways in WT and mdx myotubes. Similar results were obtained with Ca\(^{2+}\)/calmodulin-dependent kinase II and JNK inhibitors. Surprisingly, p38 inhibition by SB-203580 dramatically reduced the luciferase activity of the NF-\(\kappa\)B reporter. The p38 MAPK is activated by various stimuli, including exercise, contraction, insulin, environmental stress, and pro-inflammatory cytokines (69). SB-203580 is a specific blocker of the p38 MAPK that inhibits the catalytic activity of this protein (70).

Badger et al. (71) has shown that SB-203580 blocks IL-1-induced p38 kinase activity, NO production, and iNOS expression in chondrocytes. In addition, in C6 glioma cells, the stimulation with LPS increases iNOS mRNA expression, NO production, phosphorylation of p38, and the activation of NF-\(\kappa\)B. Treatment with SB-203580 reduced iNOS expression and NO production; however, it did not modify the NF-\(\kappa\)B DNA binding activity (72).

Nakamura et al. (73) have shown that calcineurin A, JNK1, and p38 signaling pathways were constantly activated in dystrophic mdx\(\times\)utr\(-/-\) hearts, associated with an increased p38 phosphorylation. However, in skeletal muscle, a reduction in p38 phosphorylation has been shown but was accompanied by an increase in p38 protein expression in whole lysates from mdx tibialis anterior muscles (74). Several reports have shown that calcium activates p38 MAPK, but the mechanisms by which it does so are poorly understood. In cerebellar granular cells, glutamate stimulates the activity of p38 through Ca\(^{2+}\)/extracellular space and Rho GTPase activation (75, 76). In myotubes, caffeine increases p38 phosphorylation via Ca\(^{2+}\)/calmodulin-dependent kinase II activation and participates in the expression of PGC-1\(\alpha\) and mitochondrial biogenesis (77). Further studies will be required to clarify this issue in mdx skeletal muscle cells and the precise mechanism involved in the NF-\(\kappa\)B activation.

Finally, we observed that iNOS expression could also be modulated by \([Ca^{2+}]_{\text{rest}}\) through NF-\(\kappa\)B under resting conditions. p65 knockdown normalized the iNOS protein levels in mdx myotubes to WT levels, similar to the effect of the agents that lowered \([Ca^{2+}]_{\text{rest}}\) had on iNOS mRNA expression; iNOS overexpression by this mechanism could be responsible for the oxidative and nitrosative stress observed in mdx muscles (26) and can provide a positive loop for Ca\(^{2+}\) deregulation in dystrophic skeletal muscle cells.

Overexpression of TRPC3 (skeletal muscle-specific transgenic mice) and the associated increase in calcium influx resulted in a phenotype of muscular dystrophy (78). The authors have shown an increase in central nucleation of fibers, increased numbers of smaller myofibers, fibrosis, and infiltration of inflammatory cells. Moreover, sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase overexpression in Sgcg\(-/-\), mdx, and in TRPC3 transgenic mice mitigated biochemical and histological features of muscular dystrophy improving the altered intracellular Ca\(^{2+}\) handling (62). As described above, S-nitrosoylation of RyR induces Ca\(^{2+}\) alterations related with an augmented spontaneous Ca\(^{2+}\) spark frequency (22). In addition, transient receptor potential channels elicited robust elevation of Ca\(^{2+}\) in response to the NO donor S-nitroso-N-acetyl-dl-penicillamine, especially TRPC5 (79), TRPC3, TRPA1, and TRPM1 channels were increased in mdx skeletal muscle at certain stages (80). These modifications induced by NO could exacerbate the pathology in mdx muscles.

We did not find any difference in cytokine expression in mdx myotubes (supplemental Fig. S3). Because macrophages and lymphocytes are specialized immune cells (infiltrated in dystrophic muscle), we think that they may be responsible for the secretion of these cytokines. This hypothesis is reinforced by IKK\(\beta\) (upstream activator of NF-\(\kappa\)B) deletion in myeloid cells from mdx mice, a procedure that reduced inflammation and concomitantly TNF-\(\alpha\) and IL-1\(\beta\) expression (10). In addition,
production of pro-inflammatory cytokines is probably a complex process that requires simultaneous activation of pathways other than NF-κB. iNOS promoter has two bona fide NF-κB-binding sites (reviewed in Ref. 81). TNF-α is often described as one of the classical NF-κB-dependent cytokines. However, there are numerous contradictory data for a role for NF-κB as a classic activator of TNF-α, and it seems that expression of this cytokine requires nuclear factor of activated T-cells activation, as well as other co-activators (reviewed in Ref. 82).

In summary, we have found that increased \([\text{Ca}^{2+}]_{\text{rest}}\) is modulated by Ca²⁺ entry as a result of SR unloading caused by Ca²⁺ leak through RyR and IP₃R in dystrophic myotubes and that this alteration increases NF-κB activity and iNOS expression, likely through p38 activation. These mechanisms can provide several potential therapy targets to improve muscle degeneration observed in DMD patients and explain the progressive damage observed in this pathology (Fig. 7).

Acknowledgments—We thank Dr. Claudio F. Perez and Dr. Karen Westerman for their help in designing our experimental procedures. We also acknowledge Dr. Peter Schupp for the gift of Ianthella basta.

REFERENCES

1. Blake, D. J., Weir, A., Newey, S. E., and Davies, K. E. (2002) Function and genetics of dystrophin and dystrophin-related proteins in muscle. *Physiol. Rev.* 82, 291–329
2. Emery, A. E. (2002) The muscular dystrophies. *Lancet* 359, 687–695
3. Henry, M. D., and Campbell, K. P. (1996) Dystroglycan. An extracellular matrix receptor linked to the cytoskeleton. *Curr. Opin. Cell Biol.* 8, 625–631
4. Pasternak, C., Wong, S., and Elson, E. L. (1995) Mechanical function of dystrophin in muscle cells. *J. Cell Biol.* 128, 355–361
5. Petrof, B. J., Shragger, J. B., Stedman, H. H., Kelly, A. M., and Sweeney, H. L. (1993) Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc. Natl. Acad. Sci. U.S.A.* 90, 3710–3714
6. Evans, N. P., Misyak, S. A., Robertson, J. L., Bassaganya-Riera, J., and Grange, R. W. (2009) Immune-mediated mechanisms potentially regulate the disease time course of Duchenne muscular dystrophy and provide targets for therapeutic intervention. *PM R* 1, 755–768
7. Oeckinghaus, A., and Ghosh, S. (2009) The NF-κB family of transcription factors and its regulation. *Cold Spring Harbor Perspect. Biol.* 1, a000343
8. Siegel, A. L., Bledsoe, C., Lavin, J., Gatti, F., Berge, J., Millman, G., Turin, E., Winders, W. T., Rutter, J., Palmeiri, B., and Carlson, C. G. (2009) Treatment with inhibitors of the NF-κB pathway improves whole body tension development in the mdx mouse. *Neuromuscul. Disord.* 19, 131–139
9. Pan, Y., Chen, C., Shen, Y., Zhu, C. H., Wang, G., Wang, X. C., Chen, H. Q., and Zhu, M. S. (2008) Curcumin alleviates dystrophic muscle pathology in mdx mice. *Mol. Cells* 25, 531–537
10. Acharya, S., Villalta, S. A., Bakkar, N., Bupha-Intr, T., Janssen, P. M., Carathers, M., Li, Z. W., Beg, A. A., Ghosh, S., Sahen, Z., Weinstein, M., Gardner, K. L., Rafael-Fortney, J. A., Kamin, M., Tidball, J. G., Baldwin, A. S., and Guttridge, D. C. (2007) Interplay of IKK/NF-κB signaling in macrophages and myofibers promotes muscle degeneration in Duchenne muscular dystrophy. *J. Clin. Invest.* 117, 889–901
11. Tang, Y., Reay, D. P., Salay, M. N., Mi, M. Y., Clemens, P. R., Guttridge, D. C., Robbins, P. D., Huard, J., and Wang, B. (2010) Inhibition of the IKK/NF-κB pathway by AAV gene transfer improves muscle regeneration in older mdx mice. *Gene Ther.* 17, 1476–1483
12. Messina, S., Bitto, A., Aguinouzou, M., Minutoli, L., Monici, M. C., Al-tavilla, D., Squadrito, F., and Vita, G. (2006) Nuclear factor-κB blockade reduces skeletal muscle degeneration and enhances muscle function in Mdx mice. *Exp. Neurol.* 198, 234–241
13. Dogra, C., Srivastava, D. S., and Kumar, A. (2008) Protein-DNA array-based identification of transcription factor activity differentially regulated in skeletal muscle of normal and dystrophic-deficient mdx mice. *Mol. Cell. Biochem.* 312, 17–24
14. Durham, W. J., Arbogast, S., Gerken, E., Li, Y. P., and Reid, M. B. (2006) Progressive nuclear factor-κB activation resistant to inhibition by con-traction and curcumin in mdx mice. *Muscle Nerve* 34, 298–303
15. Singh, R., Millman, G., Turin, E., Polsiakiewicz, L., Lee, B., Gatti, F., Berge, J., Smith, E., Rutter, J., Sumski, C., Winders, W. T., Samadi, A., and Carl-sohn, C. G. (2009) Increases in nuclear p65 activation in dystrophic skeletal muscle are secondary to increases in the cellular expression of p65 and are not solely produced by increases in IκB-α kinase activity. *J. Neurosci. Sci.* 285, 159–171
16. Tak, P. P., and Firestein, G. S. (2001) NF-κB. A key role in inflammatory diseases. *J. Clin. Invest.* 107, 7–11
17. Hnia, K., Gayraud, J., Hugon, G., Ramonaxo, M., De La Porte, S., Matecki, S., and Mornet, D. (2008) i-Arginine decreases inflammation and modulates the nuclear factor-κB/matrix metalloproteinase cascade in mdx muscle fibers. *Am. J. Pathol.* 172, 1509–1519
18. Kumar, A., and Boriek, A. M. (2003) Mechanical stress activates the nuclear factor-κB pathway in skeletal muscle fibers. A possible role in Duchenne muscular dystrophy. *FASEB J.* 17, 386–396
19. Porreca, E., Guglielmi, M. D., Uncini, A., Di Gregorio, P., Angelini, A., Di Febbo, C., Pierdomenico, S. D., Baccante, G., and Cuccurullo, F. (1999) Hemostatic abnormalities, cardiac involvement, and serum tumor necrosis factor levels in X-linked dystrophic patients. *Thromb. Haemost.* 81, 543–546
20. Porter, J. D., Khanna, S., Kaminski, H. J., Rao, J. S., Merriam, A. P., Richmonds, C. R., Leahy, P., Li, J., Guo, W., and Andrade, F. H. (2002) A chronic inflammatory response dominates the skeletal muscle molecular signature in dystrophin-deficient mdx mice. *Hum. Mol. Genet.* 11, 263–272
21. Iti, L. L. (2008) Modulation of skeletal muscle antioxidant defense by exercise. Role of redox signaling. *Free Radic. Biol. Med.* 44, 142–152
22. Bellinger, A. M., Reiken, S., Carlson, C., Mongillo, M., Liu, X., Rothman, L., Matecki, S., Lacampagne, A., and Marks, A. R. (2009) Hypernitrosylated ryanodine receptor calcium release channels are leaky in dystrophic muscle. *Am. J. Physiol.* 298, 153–161
23. Louboutin, J. P., Rouger, K., Tinsley, J. M., Halliday, J., and Wilson, J. M. (2001) iNOS expression in dystrophinopathies can be reduced by somatic gene transfer of dystrophin or utrophin. *Mol. Med.* 7, 355–364
24. Pacher, P., Beckman, J. S., and Liaudet, L. (2007) Nitric oxide and per-oxynitrite in health and disease. *Physiol. Rev.* 87, 315–424
25. Yeung, E. W., Whitehead, N. P., Suchyna, T. M., Gottlieb, P. A., Sachs, F., and Allen, D. G. (2005) Effects of stretch-activated channel blockers on...
Ca\(^{2+}\)-dependent NF-κB Up-regulation in mdx Myotubes

[Ca\(^{2+}\)], and muscle damage in the mdx mouse. J. Physiol. 562, 367–380

26. Whitehead, N. P., Yeung, E. W., and Allen, D. G. (2006) Muscle damage in mdx (dystrophic) mice. Role of calcium and reactive oxygen species. Clin. Exp. Pharmacol. Physiol. 33, 657–662

27. López, J. R., Briceno, L. E., Sánchez, V., and Horvart, D. (1987) Myoplastic [Ca\(^{2+}\)] in Duchenne muscular dystrophy patients. Acta Cient. Venez. 38, 503–504

28. Allen, D. G., Gervasio, O. L., Yeung, E. W., and Whitehead, N. P. (2010) Calcium and the damage pathways in muscular dystrophy. Can. J. Physiol. Pharmacol. 88, 83–91

29. Turner, P. R., Westwood, T., Regen, C. M., and Steinhardt, R. A. (1988) Increased protein degradation results from elevated free calcium levels found in muscle from mdx mice. Nature 335, 735–738

30. Bakker, A. J., Head, S. L., Williams, D. A., and Stephenson, D. G. (1993) Ca\(^{2+}\) levels in myotubes grown from the skeletal muscle of dystrophic (mdx) and normal mice. J. Physiol. 460, 1–13

31. Gailly, P., Boland, B., Himpens, B., Casteels, R., and Gillis, J. M. (1993) Critical evaluation of cytosolic calcium determination in resting muscle fibers from normal and dystrophic (mdx) mice. Cell Calcium 14, 473–483

32. Pressman, J., Brinkmeier, H., Seewald, M. J., Naumann, T., and Rüdel, R. (2008) TRPC1 binds to caveolin-3 and is regulated by Src kinase. J. Cell Sci. 121, 2246–2255

33. Spencer, M. J., Croall, D. E., and Tidball, J. G. (1995) Calpains are activated in dystrophic muscle during dystrophinopathy. Am. J. Physiol. Cell Physiol. 269, C924–C931

34. Rando, T. A., and Blau, H. M. (1994) Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. J. Cell Biol. 125, 1275–1287

35. Casas, M., Altamirano, F., and Jaimovich, E. (2012) Measurement of calcium release due to inositol trisphosphate receptors in skeletal muscle. Methods Mol. Biol. 798, 383–393

36. Elitt, J. M., Yang, T., Li, H., Molinski, T. F., Pessah, I. N., Allen, P. D., and Lopez, J. R. (2010) Ryr1-mediated Ca\(^{2+}\) leak and Ca\(^{2+}\) entry determine resting intracellular Ca\(^{2+}\) in skeletal myotubes. J. Biol. Chem. 285, 13781–13787

37. Westerman, K. A., Pervose, A., Yang, Z., Allen, P. D., and Vacanti, C. A. (2010) Adult muscle “stem” cells can be sustained in culture as free-floating myospheres. Exp. Cell Res. 316, 1966–1976

38. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real time quantitative PCR and the 2(−ΔΔC\(_{\text{t}}\)) method. Methods 25, 402–408

39. Marshall, O. J. (2004) PeriPrimer. Cross-platform, graphical primer design for standard, bisulfite, and real time PCR. Bioinformatics 20, 2471–2472

40. Mack, M. M., Molinski, T. F., Buck, E. D., and Pessah, I. N. (1994) Novel modulators of skeletal muscle FKBP12/calcium channel complex from Jannichella basta. Role of FKBP12 in channel gating. J. Biol. Chem. 269, 23236–23249

41. Lilienbaum, A., and Israel, A. (2003) From calcium to NF-κB-dependent regulation of the cell cycle via a novel MAPK-NF-κB pathway in neurons. J. Neurosci. 23, 1245–1259

42. Sée, V., Rajala, N. K., Spiller, D. G., and White, M. R. (2004) Calcium-dependent regulation of the cell cycle. J. Cell Biol. 165, 1966–1976

43. Jaimovich, E., Mattei, C., Liberon, J. L., Cardenas, E., Estrada, M., Barbier, J., Debitus, C., Laurent, D., and Molgò, J. (2005) Xestospongin B, a competitive inhibitor of IP\(_3\)-mediated Ca\(^{2+}\) signaling in cultured rat myotubes, isolated myonuclei, and neuroblastoma (NG108-15) cells. FEBS Lett. 579, 2051–2057

44. Ho, R. C., Hirshman, M. F., Li, Y., Cai, D., Farmer, J. R., Aschenbach, W. G., Witzczak, C. A., Shoelson, S. E., and Goodyear, L. J. (2005) Regulation of I\(_{\text{K}+}\) and NF-κB in contracting adult rat skeletal muscle. Am. J. Physiol. Cell Physiol. 289, C794–C801

45. Varmi, P., Hunsardy, L., and Balla, T. (2009) STIM and Orai. The long-awaited constituents of store-operated calcium entry. Trends Pharmacol. Sci. 30, 118–128

46. Robert, V., Massimino, M. L., Tosello, V., Marsault, R., Cantini, M., Sorrentino, V., and Pozzan, T. (2001) Alteration in calcium handling at the subcellular level in mdx myotubes. J. Biol. Chem. 276, 4647–4651

47. Culligan, K., Banville, N., Dowling, P., and Ohlendieck, K. (2002) Drastic reduction of carsequestrin-like proteins and impaired calcium binding in dystrophic mdx mouse muscle. J. Appl. Physiol. 92, 435–445

48. Kargacin, M. E., and Kargacin, G. J. (1996) The sarcoplasmic reticulum calcium pump is functionally altered in dystrophic muscle. J. Gen. Physiol. 109, 4–8

49. Robin, G., Berthier, C., and Allard, B. (2012) Sarcoplastic reticulum Ca\(^{2+}\) permeation emerged from the lumen side in mdx muscle fibers under voltage control. J. Gen. Physiol. 139, 209–218

50. Mori, K. J., Sleeper, M. M., Barton, E. R., and Sweeney, H. L. (2010) Overexpression of SERCA1a in the mdx diaphragm reduces susceptibility to contraction-induced damage. Hum. Gene Ther. 21, 1735–1739

51. Goonasekera, S. A., Lam, C. K., Millay, D. P., Sargent, M. A., Hajjar, R. I., Krainas, E. G., and Molkentin, J. D. (2011) Mitigation of muscular dystrophy in mice by SERCA overexpression in skeletal muscle. J. Clin. Invest. 121, 1044–1052

52. Carlson, C. G., Samadi, A., and Siegel, A. (2005) Chronic treatment with agents that stabilize cytosolic I\(_{\text{K}+}\)-α enhances survival and improves resting membrane potential in MDX muscle fibers subjected to chronic passive stretch. Neurobiol. Dis. 20, 719–730

53. Miles, M. T., Cottey, E., Cottey, A., Stefanski, C., and Carlson, C. G. (2011) Reduced resting potentials in dystrophic (mdx) muscle fibers are secondary to NF-κB-dependent negative modulation of ouabain-sensitive Na\(^{+}\)-K\(^{+}\) pump activity. J. Neurosci. 303, 53–60
65. Carlson, C. G., and Roshek, D. M. (2001) Adult dystrophic (mdx) end plates exhibit reduced quantal size and enhanced quantal variation. *Pflugers Arch.* **442**, 369–375

66. Woods, C. E., Novo, D., DiFranco, M., and Vergara, J. L. (2004) The action potential evoked sarcoplasmic reticulum calcium release is impaired in mdx mouse muscle fibers. *J. Physiol.* **557**, 59–75

67. Hollingworth, S., Zeiger, U., and Baylor, S. M. (2008) Comparison of the myoplasmic calcium transient elicited by an action potential in intact fibers of mdx and normal mice. *J. Physiol.* **586**, 5063–5075

68. Capote, J., DiFranco, M., and Vergara, J. L. (2010) Excitation-contraction coupling alterations in mdx and utrophin/dystrophin double knockout mice. A comparative study. *Am. J. Physiol. Cell Physiol.* **298**, C1077–C1086

69. Röckl, K. S., Witzczak, C. A., and Goodyear, L. J. (2008) Signaling mechanisms in skeletal muscle. Acute responses and chronic adaptations to exercise. *IUBMB Life* **60**, 145–153

70. Kumar, S., Liang, M. S., Adams, J. L., and Lee, J. C. (1999) Pyridinylimidazole compound SB-203580 inhibits the activity but not the activation of p38 mitogen-activated protein kinase. *Biochem. Biophys. Res. Commun.* **263**, 825–831

71. Badger, A. M., Cook, M. N., Lark, M. W., Newman-Tarr, T. M., Swift, B. A., Nelson, A. H., Barone, F. C., and Kumar, S. (1998) SB-203580 inhibits p38 mitogen-activated protein kinase, nitric oxide production, and inducible nitric oxide synthase in bovine cartilage-derived chondrocytes. *J. Immunol.* **161**, 467–473

72. Won, J. S., Lee, J. K., and Suh, H. W. (2001) Forskolin inhibits expression of inducible nitric-oxide synthase mRNA via inhibiting the mitogen-activated protein kinase in C6 cells. *Brain Res. Mol. Brain Res.* **89**, 1–10

73. Nakamura, A., Harrod, G. V., and Davies, K. E. (2001) Activation of calcineurin and stress-activated protein kinase/p38-mitogen-activated protein kinase in hearts of utrophin-dystrophin knockout mice. *Neuromuscul. Disord.* **11**, 251–259

74. Ljubetic, V., Khogali, S., Renaud, J. M., and Jasmin, B. J. (2012) Chronic AMPK stimulation attenuates adaptive signaling in dystrophic skeletal muscle. *Am. J. Physiol. Cell Physiol.* **302**, C110–C121

75. Kawasaki, H., Morooka, T., Shimohama, S., Kimura, J., Hirano, T., Gotoh, Y., and Nishida, E. (1997) Activation and involvement of p38 mitogen-activated protein kinase in glutamate-induced apoptosis in rat cerebellar granule cells. *J. Biol. Chem.* **272**, 18518–18521

76. Semenova, M. M., Mäki-Hokkonen, A. M., Cao, J., Komarovski, V., Forsberg, K. M., Koistinaho, M., Coffey, E. T., and Courtney, M. J. (2007) Rho mediates calcium-dependent activation of p38α and subsequent excitotoxic cell death. *Nat. Neurosci.* **10**, 436–443

77. Wright, D. C., Geiger, P. C., Han, D. H., Jones, T. E., and Holloszy, J. O. (2007) Calcium induces increases in peroxisome proliferator-activated receptor γ coactivator-1α and mitochondrial biogenesis by a pathway leading to p38 mitogen-activated protein kinase activation. *J. Biol. Chem.* **282**, 18793–18799

78. Millay, D. P., Goonasekera, S. A., Sargent, M. A., Maillet, M., Aronow, B. J., and Molkentin, J. D. (2009) Calcium influx is sufficient to induce muscular dystrophy through a TRPC-dependent mechanism. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 19023–19028

79. Yoshida, T., Inoue, R., Morii, T., Takahashi, N., Yamamoto, S., Haru, Y., Tominaga, M., Shimizu, S., Sato, Y., and Mori, Y. (2006) Nitric oxide activates TRP channels by cysteine S-nitrosylation. *Nat. Chem. Biol.* **2**, 596–607

80. Krüger, J., Kunert-Keil, C., Bipping, F., and Brinkmeier, H. (2008) Transient receptor potential cation channels in normal and dystrophic mdx muscle. *Neuromuscul. Disord.* **18**, 501–513

81. Kleinert, H., Schwarz, P. M., and Förstermann, U. (2003) Regulation of the expression of inducible nitric-oxide synthase. *Biol. Chem.* **384**, 1343–1364

82. Falvo, J. V., Tsytzykova, A. V., and Goldfeld, A. E. (2010) Transcriptional control of the TNF gene. *Curr. Dir. Autoimmun.* **11**, 27–60

83. Buvinic, S., Almarza, G., Bustamante, M., Casas, M., López, J., Riquelme, M., Sández, J. C., Huidobro-Toro, J. P., and Jaimovich, E. (2009) ATP released by electrical stimuli elicits calcium transients and gene expression in skeletal muscle. *J. Biol. Chem.* **284**, 34490–34505