Nitric oxide donors improve prednisone effects on muscular dystrophy in the mdx mouse diaphragm

Wataru Mizunoya,1 Ritika Upadhaya,1 Frank J. Burczynski,3 Guqi Wang,4 and Judy E. Anderson 1,2

1Department of Biological Sciences and 2Department of Human Anatomy and Cell Science, and 3Faculty of Pharmacy and Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada; and 4McColl-Lockwood Laboratory for Muscular Dystrophy Research, Cannon Medical Center, Charlotte, North Carolina

Submitted 22 November 2010; accepted in final form 23 January 2011

Mizunoya W, Upadhaya R, Burczynski FJ, Wang G, Anderson JE. Nitric oxide donors improve prednisone effects on muscular dystrophy in the mdx mouse diaphragm. Am J Physiol Cell Physiol 300: C1065–C1077, 2011. First published January 26, 2011; doi:10.1152/ajpcell.00482.2010.—In Duchenne muscular dystrophy (DMD), palliative glucocorticoid therapy can produce myopathy or calcification. Since increased nitric oxide synthase activity in dystrophic mice promotes regeneration, the outcome of two nitric oxide (NO) donor drugs, MyoNovin (M) and isosorbide dinitrate (I), on the effectiveness of the anti-inflammatory drug prednisone (P) in alleviating progression of dystrophy was tested. Dystrophic mdx mice were treated (18 days) as controls or with an NO donor ± P. Fiber permeability and DNA synthesis were labeled by Evans blue dye (EBD) and bromodeoxyuridine uptake, respectively. P decreased body weight gain, M increased quadriceps mass, and I increased heart (EBD) and bromodeoxyuridine uptake, respectively. P decreased %EBD and DNA synthesis were labeled by Evans blue dye treated (18 days) as controls or with an NO donor

Address for reprint requests and other correspondence: J. E. Anderson, Dept. of Biological Sciences, Faculty of Science, Univ. of Manitoba, Winnipeg, MB, R3T 2N2, Canada (e-mail: janders@ms.umanitoba.ca).

MUSCULAR DYSTROPHIES are lethal genetic muscle diseases, and the most common form, Duchenne muscular dystrophy (DMD), is caused by mutation in the dystrophin gene (37). Absence of dystrophin from the cytoskeleton in DMD fibers leads to disruption of the sarcolemma by mechanical stress or exercise and progressive muscle weakness and wasting (20, 27, 56, 69). Investigations of possible therapeutic interventions using the mdx mouse model of DMD have examined gene therapy by viral delivery of full-length dystrophin (28) or microdystrophin (97), stem (satellite) cell transplantation (40), and read-through (91) or exon-skipping approaches (2, 55, 93) to restore dystrophin expression. As these investigations are ongoing, pharmacological treatment with glucocorticoids remains the current “gold standard” therapy in DMD.

Treatment with prednisone promotes a palliative reduction in inflammation and delays disease progression (57, 58) in DMD. In DMD patients and mdx mice, prednisone and deflazacort reduce the secondary inflammation that extends muscular fiber necrosis outside the region of primary damage that is caused by exercise-induced membrane breaks or eccentric contraction (25, 63). In addition, deflazacort also upregulates the calcineurin/nuclear factor of activated T cells (NFAT) pathway activity, which counteracts the muscle-specific activation of JNK1 that damages fibers (49, 78). In mdx mice treated for 3–4 wk after the onset of dystrophic muscle damage, the benefits of deflazacort and prednisone treatment are therefore well established (3, 4, 7, 8, 10, 60).

Although the beneficial effect of glucocorticoid in DMD is well established, glucocorticoids also have detrimental effects linked to myopathy (19), skeletal muscle atrophy (36, 73), and increased proteolysis (26, 30, 38, 88). Furthermore, corticosteroids are associated with reduced skeletal muscle recovery following injury (15, 35). Glucocorticoid treatment also increases calcification in mdx heart (77) and in hindlimb muscles of a canine model of DMD (53). The appearance of such effects from treatment complicates management of respiratory and cardiac complications of DMD in older patients. These complications include respiratory muscle weakness, hypoxemia, hypercapnia, fatigue, reduced coughing ability (forced vital capacity), sleep hypoventilation (often presenting in teen-aged boys with progressive DMD), and dilated cardiomyopathy, which affects up to 90% of DMD patients who reach adulthood (57).

Satellite cells (SCs) are muscle stem or precursor cells; they are inactive in normal adult muscle and are activated to proliferate by fiber damage and stretching and during exercise-induced growth. Their number is critical to determining the outcomes of dystrophy, regeneration, and aging. The process of activation is mediated by nitric oxide (NO) (3), a molecule produced by the enzymatic activity of nitric oxide synthase (NOS) on l-arginine, and ensuing signaling via hepatocyte growth factor (HGF) interaction with c-met receptor on SCs (76, 80–84). In normal skeletal muscle, NOS1 receptor is localized to the membrane cytoskeleton and is downregulated by dystrophic deficiency. Reduced NOS1 is thought to lead to hyperactivation of SCs in mdx muscle and dysregulated activation after injury or stretching (94, 95). Restoration of NOS1 to the membrane is an excellent marker for dystrophin restoration after treatment of mdx mice (92). Previous experiments using cultured muscle cells and whole muscles showed that NO donors and l-arginine activate muscle precursor cells and induce muscle cell division (16, 51). More importantly, treatment with l-arginine in vivo also activates SCs to divide and promotes muscle regeneration from injury and from muscular dystrophy (10, 87). We showed that l-arginine was very effective in increasing the benefit of steroids for treating mdx dystrophy. Combined glucocorticoids and l-arginine spared...
limb muscle from exercise-induced damage (4), increased myotube formation, and increased expression of CAPON (a NOS anchor protein) and utrophin that relate to cytoskeleton stability (75) in mdx mouse muscle.

We developed a new drug molecule, MyoNovin, formulated so it would release NO to muscle. MyoNovin in a safe transdermal application resulting in muscle cell proliferation in vivo: SCs proliferated 170–200% more after 24 h than without treatment (87). The base compound of MyoNovin is guaifenesin, which is the active metabolite of methocarbamol. Guaifenesin is a muscle relaxant with NO-independent effects on vascular and nervous systems and promotion of SC activation. After the addition of two NO groups, the new formulation, MyoNovin (guaifenesin dinitrate), is an NO donor and promotes activation via NO release. Oral MyoNovin administration also increased muscle regulatory genes and follistatin expression, both important in muscle formation, in normal adult muscle (87). The positive effect of MyoNovin on SC proliferation in vivo in normal adult mice is mirrored by results from muscle culture studies in which an NO donor, isosorbide dinitrate, restored the regulation of SC stretch activation in muscle from 8-mo-old mice (51).

We tested whether treatment of NO donors, MyoNovin or isosorbide dinitrate, with or without glucocorticoid (prednisone) will improve the condition of diaphragm in mdx dystrophic mice. Our hypothesis was that the combination of NO donors and an anti-inflammatory glucocorticoid will increase the effectiveness of prednisone alone, in reducing disease progression and increasing muscle function in dystrophic mice by regulating SC activation, reducing fiber damage, and alleviating glucocorticoid detrimental effects.

MATERIALS AND METHODS

Materials. A novel compound, guaifenesin dinitrate (MyoNovin), was synthesized as described previously (87). All other chemicals were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada) unless otherwise stated.

Animal treatment protocol and tissue collection. Animals were housed and treated according to regulations set by the Canadian Council on Animal Care as approved by the University of Manitoba (protocol no. F09-001). Mice were maintained with 12:12-h light-dark cycles and food and water ad libitum. Dystrophic mdx mice at the onset of dystrophy (3.5 wk old) were randomly assigned to six groups: untreated control mice (C group, canola oil, 1.0 μl/g orally; n = 8) and mice treated with MyoNovin (M group, 80 mg/kg orally; n = 8), isosorbide dinitrate (I group, 66 mg/kg orally; n = 8), prednisone (P group, 1.0 mg/kg subcutaneously; n = 9), MyoNovin and isosorbide dinitrate (M+P group; n = 8), and isosorbide dinitrate and prednisone (I+P group; n = 8). MyoNovin and isosorbide dinitrate were suspended in canola oil; prednisone was suspended in 0.5% methylcellulose saline. Mice were treated for 18 days and received an injection of Evans blue dye (EBD, 100 mg/kg ip) 24 h before euthanasia. EBD complexes with albumin and penetrates fibers with permeable membranes (10, 59). Two hours before euthanasia, mice were injected with bromodeoxyuridine (BrdU; 100 μg/kg ip) to label DNA synthesis. Before and during treatment, forelimb grip strength was measured with a calibrated digital force meter (Chatillon, Greensboro, NC) at 0 day, 7 days, and 14 days of treatment and dissection day. The peak force (in pounds) of 10 pulls was recorded for each mouse.

Whole diaphragm plus hindlimb muscles (quadriceps and triceps surae) and heart were rapidly dissected from mice after euthanasia (cervical dislocation under inhalation anesthesia). Right-sided hindlimb muscles and heart were weighed and frozen at −80°C for future study. Only diaphragm muscles were used for analysis in this study. We dissected diaphragms rapidly from mice after opening the anterior abdominal wall at the midline and cutting “flaps” laterally to both sides from the midline; the central tendon was gripped in forceps, and scissors were used to cut quickly around the perimeter of the muscle at anterior and posterior costal and lumbar attachments (5). Diaphragms from four groups (C, P, M, M+P) were photographed for later macroscopic assessment. Each diaphragm was cut in half across the midline, and halves were either frozen for cryosectioning or collected into labeled Eppendorf tubes and snap frozen in liquid nitrogen for protein assays. On the basis of previous findings, the same area of the diaphragm was always collected for sectioning (left with more severe dystrophy) and protein assay (right) in an attempt to minimize variability in pathology among samples (5) and include similar mixtures of spanning (sternal) and nonspanning (mid- and dorsal costal and lateral crural) fibers (18). Samples were coded to prevent observers knowing the source-treatment group during later assays. For cryosections, muscle was immediately snap frozen in CryoMatrix in isopentane at −70°C cooled with dry ice. Sets of two 5-μm sections from each block, cut 100 μm apart, were collected onto slides coated with 3-amino-propyl-triethoxy-silane and stored at −20°C until use. All samples and slides were coded by number to remove experimenter bias.

Muscle histopathology. Macroscopic photographs of whole diaphragm were coded and assigned blindly to one of five groups with a five-point Likert scale for the severity of damage according to the number, size, and density of the EBD-stained blue areas on the muscle. A relative severity score of “1” was recorded when there was no EBD staining; a score of “5” indicated extensive EBD infiltration, with over a quarter of the muscle area stained dark blue. For reference, diaphragm tissue from normal age-matched mice injected with EBD 24 h before euthanasia do not display any red fluorescence within the muscle.

One set of diaphragm sections was stained with the routine histochemical stain hematoxylin and eosin (H & E) (71), and viewed at ×100 under an Olympus BH-2 microscope for assessment of central nucleation and muscle histopathology. Typically, diaphragm muscle sections demonstrate fibers in a range of orientations from longitudinal and transverse to oblique in sectioning, because of the development of a complex adult architecture of thousands of muscle fibers (17, 18, 34). The central nucleation index (CNI) was calculated by observing the proportion of fibers that contained central nuclei and used as an indication of accumulated regeneration following dystrophic damage. All fibers observed along the midregion of the length of each section (80–630 fibers/section) were assessed for the location of the myonuclei (i.e., central or not), and the CNI was calculated as the proportion of fibers containing central nuclei.

The distribution of regions showing mainly degeneration and inflammation, mainly regenerating fibers, and mainly intact fibers was determined. The total area of diaphragm sections was determined from a photograph at low magnification (×20), and regions within the section were photographed at ×190. All areas of degenerating and regenerating muscle were outlined as irregular polygons with NIH ImageJ. Regenerating muscle was defined as clusters of myotubes; degenerating regions were areas ≥ 4,000 μm2 containing inflammatory infiltrates and necrotic or hyalinized fibers. The area of normal, mainly intact muscle included larger centrally nucleated fibers (possibly interspersed with small foci of inflammation or regeneration) and was calculated by subtracting areas of degeneration and regeneration from total section area. Areas of calcification (dark purple fibers in H & E) were outlined as a separate parameter. Calcification was confirmed by histochemical staining with Alizarin red (pH 4.2), which detects calcium deposits in many tissues including postischemic and dystrophic dog skeletal muscle (42, 53, 85). For the assessment of fiber permeability, sections of diaphragm were immunostained for laminin as described below and viewed with...
epifluorescence optics. All fibers were outlined by laminin. In a total of 150 fibers per section (sampled in a line across the longest chord of each section), those fibers containing red fluorescence from the EBD staining were determined as a proportion of total fibers counted. The proportion was used as an indicator of fiber permeability due to damage associated with “active” muscular dystrophy. Fluorescence was viewed and photographed with an Axio Imager.Z1 and AxioCam (Carl Zeiss Canada). Zeiss images were exported as TIFF files for analysis with NIH ImageJ 1.40g software. Parameters were compiled as the distribution or mean ± SE as appropriate.

**Immunohistochemical analysis.** Immunostaining for laminin, BrdU, and developmental myosin heavy chain (including embryonic plus neonatal isoforms; devMyHC) was obtained according to a protocol that employed multiple blocking steps (39). Slides for immunostaining were thawed and air dried at room temperature for 30 min before fixation.

For laminin detection, slides were fixed for 60 s in acetic acid, then air dried and blocked in a normal goat serum solution 2% goat serum (Jackson ImmunoResearch, B159318), 1% bovine serum albumin, 0.1% cold fish skin gelatin, 0.1% Triton X-100, 0.05% Tween 20, and 0.05% sodium azide in 0.01 M phosphate-buffered saline (PBS), rinsed in Tris-buffered saline with Tween (TBST; 0.5 M Tris base and 9% NaCl in water with 0.5% Tween 20), incubated for 30 min in rabbit polyclonal anti-laminin (Sigma L9393, 1:50), rinsed in TBST, and incubated for 20 min with the Fab fragment of Dylight 488-conjugated goat anti-rabbit IgG (Jackson Immunolocal Research Laboratories, 111–487–003, 1:200). After rinsing, sections were mounted with Vectashield and coverslips were sealed with nail polish.

For immune detection of BrdU, sections were fixed for 20 min in 4% paraformaldehyde and washed with PBS, and DNA was denatured by incubation in 2 N HCl (1 h). Slides were rinsed again in PBS and blocked for 30 min in a normal goat serum solution (solution as above with addition of 2 M glycine, mixed 20:1), rinsed in TBST, incubated 1 h in the unconjugated Fab fragment of AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, 0.1 mg/ml or 1:200), rinsed in TBST, incubated 15 min in avidin (0.001% in PBS), rinsed in PBS, and incubated 15 min in biotin (0.001% in PBS). After rinsing in PBS, sections were rinsed in TBST and incubated with mouse monoclonal anti-BrdU (Sigma B2531; 1:1,000) overnight at 4°C. On day 2, sections were washed in TBST, incubated in 3% hydrogen peroxide in PBS (10 min), washed in TBST, and incubated for 1 h in biotinylated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories 115-067-003, 1:200) and then alkaline phosphatase-conjugated ExtrAvidin (Sigma, 1:500). ExtrAvidin was visualized by nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) according to the manufacturer’s instructions (Roche Diagnostics). Slides were washed in PBS and mounted under coverslips as above.

For immunostaining of devMyHC, sections were fixed for 20 min in 4% paraformaldehyde and blocked as reported above for BrdU detection. After blocking, sections were incubated in mouse monoclonal anti-devMyHC (NovoCastra NCL-MHCd, 1:20) overnight at 4°C. On day 2, after rinsing, sections were incubated with secondary antibody as for BrdU.

Fiber diameter was determined from sections immunostained for devMyHC by measuring the shortest diameter of all the nonregenerating and nonnecrotic, intact fibers [including fibers with central (nonregenerated) and peripheral (regenerated) myotubes]. The presence or absence of dark staining from the NBT/BCIP color-detection system enabled separate assessment of newly regenerating (devMyHC-positive) versus mature nonregenerating fiber (devMyHC-negative) segments sampled in the sections. Images of diaphragm were viewed at ×460 for diameter measurement with NIH ImageJ.

The distribution of BrdU-positive mononuclear cells was scored in diaphragm sections with a blinded assessment on a four-point Likert scale as follows: 1+ for few BrdU+ cells; 2+ for small foci and/or scattered BrdU+ cells throughout the section; 3+ for moderate numbers of BrdU+ cells in large foci or across large regions of a section; and 4+ for extensive numbers of BrdU+ cells, widespread (in very large clusters in the section of diaphragm). The frequency distribution of cell proliferation was compared among groups with χ2 statistics. Variability in fiber orientation within sections (particularly notable in diaphragm compared with sections of hindlimb muscle), the focal nature of cells with BrdU+ staining [demonstrating proliferation by cells in situ (including myogenic and endothelial cells and fibroblasts, etc.) and also by infiltrating inflammatory cells], and the very high frequency of BrdU+ cells in some groups precluded taking counts of individual BrdU+ cells that would be comparable within and between groups.

**Muscle precursor cells by in situ hybridization.** The HGF receptor c-met is expressed in both quiescent and activated SCs (3, 23, 43) and is an early-immediate gene in SC activation (94). The in situ hybridization protocol was carried out as reported, to visualize c-met transcript expression in individual SCs in sections with a probe and color detection methods previously reported (8, 96). Sections were counterstained with nuclear fast red. Briefly, templates for c-met were obtained by transforming bacteria with a plBluescript SK+ plasmid containing bases 300–1572 of the met protooncogene cDNA (29, 66), generously supplied by Dr. Carola Ponzetta (Dept. of Biological Sciences and Oncology, University of Torino, Italy). Anti-sense, digoxigenin-labeled riboprobes were synthesized according to Boehringer Mannheim protocols. Labeled probe was confirmed as 1.27 kb. Negative controls included omission of probe or anti-digoxigenin antibody or pretreatment with RNaseA. C-met-positive mononuclear cells were viewed in five randomly chosen, nonoverlapping fields and counted without knowledge of treatment group in regions of intact (nonnecrotic) fibers, including fibers with peripheral nuclei (nonregenerated) and central nuclei (regenerated). Counts excluded regions where very tiny regenerating fibers (still c-met positive) were indistinguishable from c-met-positive mononuclear cells in the myogenic lineage. Counts (number per mm2 of section) were averaged for each group.

**Protein preparation and Western blot.** Proteins were extracted rapidly in ice-cold lysis buffer (63 mM Tris, 4% SDS, 10% sucrose, and protease inhibitors 1.0 μM pepstatin, 0.5 mM dithiothreitol) with lysing matrix beads in single-use tubes mounted in a motorized FastPrep-24 homogenizer (24 samples simultaneously for uniformly timed extractions) fitted with a CryoPrep unit to hold ice (MP Biomedicals, Solon, OH). Total protein concentration was assayed with BCA Protein Assay Reagent (Pierce) and standardized against bovine serum albumin. Samples were handled on ice and were diluted with 2× sample buffer [100 mM dithiothreitol, 4.0% SDS, 0.16 M Tris-HCl (pH 6.8), 43% glycerol, 0.2% bromophenol blue] and lysis buffer to give a final protein concentration of 1.3 mg/ml in 1× sample buffer. Samples were then heated at 97°C for 3 min. Protein (20 μg) was loaded onto 9% polyacrylamide gels for electrophoresis and blotting. The following antibodies were used for Western blotting: goat polyclonal anti-iatrophin (Santa Cruz sc-7459, 1:2,000); rabbit polyclonal anti-NOS1 (Santa Cruz sc-1025, 1:200); anti-MyoD (Santa Cruz sc-760, 1:400); anti-Ki67 (Abcam ab833, 1:200); mouse monoclonal anti-Pax7 (DSHB PAX7, 1:1,000); anti-devMyHC (Novocasta NCL-MHCd, 1:200); anti-β-dystroglycan (NovoCastra NCL-43DAG, 1:500); and anti-actin (Millipore MAB1501, 1:2,000,000).

After overnight incubation of blotted membranes in primary antibody at 4°C (anti-actin incubation was for 1 h at room temperature), membranes were rinsed and probed with the appropriate secondary antibody [one of horseradish peroxidase-conjugated bovine anti-goat IgG (Santa Cruz sc-2350), donkey anti-rabbit IgG (GE Healthcare NA9340V), or goat anti-mouse IgG (Sigma A2304)] at a 1:5,000 dilution for 1 h at room temperature, and bands were visualized by using the standard chemiluminescence method according to the manufacturer’s instructions (Santa Cruz sc-2048). The optical density of bands was normalized to actin as a loading control.
Statistical analysis. One-way ANOVAs were used to determine differences among six groups. Two-way ANOVAs were used to study interdrug differences (prednisone, NO donors). Post hoc analysis was performed where ANOVAs were significant, by Tukey honestly significant difference (HSD) test (comparing multiple groups) or Dunnett’s test (comparison with controls). Nonparametric comparisons of semiquantitative data from a Likert scale were made with the Kruskal-Wallis and Mann-Whitney U-tests. Frequency distributions were tested for significance with $\chi^2$ statistics. A probability $\leq 0.05$ was considered to indicate statistical significance. Statistical tests were performed with SPSS v17.0.

RESULTS

Body and muscle weight. Growth-related weight gain was reduced by all treatments except MyoNovin (Fig. 1). Groups treated with prednisone gained less weight (10–20% less) than other groups ($P < 0.01$). By comparison, male (Fig. 1B) but not female (Fig. 1C) mdx mice treated with I+P showed no change in body weight gain compared with Control and I groups.

There was a muscle-specific change in the ratio of muscle weight to body weight with the various treatments (Table 1). Overall, NO donors increased relative quadriceps weight ($P = 0.02$, 2-way ANOVA); MyoNovin treatment (M and M+P groups) increased relative quadriceps weight compared with groups treated with isosorbide dinitrate (I, I+P) ($P = 0.02$). Relative weight of triceps surae did not change in any group. Relative heart weight was increased by isosorbide dinitrate treatment ($P < 0.01$).

Muscle histopathology. The severity of fiber damage due to the progression of dystrophy was assessed with EBD staining in gross specimens of diaphragm and in tissue sections. From blinded macroscopic observations of whole diaphragms (Fig. 2), there was more EBD staining after prednisone treatment ($P < 0.05$) and the severity score for the P group was higher than for all other groups ($P < 0.05$). The M group had the lowest severity score, and the score in the M+P group was intermediate between M and P groups alone.

The level of active dystrophy was determined by the permeability of fibers to EBD (Fig. 3). Prednisone treatment was accompanied by a three- to fivefold increase in %EBD-positive fibers compared with all other groups ($P < 0.05$). Treatment with NO donors plus prednisone reduced the %EBD+ fibers versus prednisone alone ($P < 0.01$). M and I groups as well as M+P and I+P groups had %EBD+ fibers at control levels. Therefore, NO donors alleviated the detrimental effect of prednisone on fiber permeability.

Dystrophic diaphragm muscle demonstrates a mixture of degenerating muscle infiltrated with inflammatory cells, clusters of small regenerating fibers, and intact normal fibers (peripheral or centralized nuclei) (Fig. 4A; C, I, and M panels, respectively). Intact fibers occupied >80% of the section area in all groups. Treatment with isosorbide dinitrate increased the %area of regenerating fibers in the diaphragm muscle sections ($P = 0.02$). There was no significant change in the areal proportion of regenerating fibers after treatment; values ranged from 9.7 ± 3.5% in C, P, and M groups to 5.6 ± 1.7% in I and IP groups and 3.7 ± 1.4% section area in the MP group.

BrdU incorporation into proliferating cells. The frequency of proliferating cells was significantly lower after treatment with NO donors compared with that in the C and P groups (Fig. 4B). After the blinded observations were decoded, seven of eight Control sections scored 3+ or 4+ on the four-point Likert scale, representing moderate to extensive cell proliferation throughout each section. After prednisone-alone treatment, sections from three of nine mice showed only a few or very dispersed small foci of BrdU+ cells (compared to 1 of 8 sections in the C group). The distribution of BrdU+ cells was further reduced compared with the P group in all groups treated with NO donors (only 1 of 8,
was reduced in I (P < 0.05) and NO donors (P < 0.05), and prednisone did not change the prevalence of diaphragm calcification.

1 of 8, 2 of 8, and 0 of 8 sections scored 3–4+ in M, MP, I, and IP groups, respectively (P < 0.001).

Muscle calcification and central nucleation. Calcified fibers were present in roughly half the sections of diaphragm muscle after prednisone treatment (Fig. 4C) and prednisone increased the %area of calcification versus controls (P < 0.05). Calcification was reduced in I (P < 0.05) versus C and P groups. MyoNovin did not change the prevalence of diaphragm calcification.

CNI (Table 1) was significantly increased by treatment with NO donors (P < 0.001). In combination with prednisone, isosorbide dinitrate increased CNI compared with all groups without isosorbide dinitrate treatment (0.001 < P < 0.01). CNI tended to decrease after prednisone treatment, compared with controls (P = 0.08).

Staining for devMyHC was observed in small to medium-sized myotubes (Fig. 5A) and was absent from SCs, fibers with peripheral nuclei, and larger fibers that were centrally nucleated. Overall, the mean diameter of devMyHC-positive or devMyHC-negative fibers did not change. The distribution of diameter measured from devMyHC-positive fibers from the six groups (Fig. 5B) tended to shift toward larger fibers in isosorbide dinitrate-treated groups compared with P and C groups, and prednisone treatment (alone or combined with MyoNovin or isosorbide dinitrate) increased the distribution of devMyHC-positive fiber diameter (P < 0.05) (Fig. 5C). The diameter (mean or distribution) of devMyHC-negative fibers did not change with treatment (not shown).

Table 1. Measurements of tissue weight, grip strength, and central nucleation index of mdx mice

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 8)</th>
<th>Prednisone (n = 9)</th>
<th>MyoNovin (n = 8)</th>
<th>MyoNovin + Prednisone (n = 8)</th>
<th>ISDN (n = 8)</th>
<th>ISDN + Prednisone (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart weight, % body wt</td>
<td>0.687 ± 0.036bA</td>
<td>0.594 ± 0.027aA</td>
<td>0.683 ± 0.039bA</td>
<td>0.648 ± 0.030bB</td>
<td>0.763 ± 0.028bB†</td>
<td>0.760 ± 0.027bB†</td>
</tr>
<tr>
<td>Quadriceps weight (right), % body wt</td>
<td>0.733 ± 0.020aB</td>
<td>0.772 ± 0.010aB</td>
<td>0.764 ± 0.012a</td>
<td>0.792 ± 0.012a</td>
<td>0.722 ± 0.026b</td>
<td>0.717 ± 0.033b</td>
</tr>
<tr>
<td>Triceps surae weight (right), % body wt</td>
<td>0.597 ± 0.020a</td>
<td>0.600 ± 0.009</td>
<td>0.600 ± 0.015</td>
<td>0.610 ± 0.009</td>
<td>0.618 ± 0.021</td>
<td>0.596 ± 0.021</td>
</tr>
<tr>
<td>Relative grip strength, lb force/g body wt</td>
<td>10.6 ± 0.4</td>
<td>10.8 ± 0.8</td>
<td>10.9 ± 0.4</td>
<td>11.1 ± 0.9</td>
<td>11.0 ± 0.7</td>
<td>11.8 ± 1.1</td>
</tr>
<tr>
<td>CNI of diaphragm, %†</td>
<td>24.1 ± 2.8</td>
<td>21.9 ± 2.4</td>
<td>25.1 ± 2.9</td>
<td>24.4 ± 1.2</td>
<td>16.7 ± 2.3</td>
<td>27.1 ± 3.3‡</td>
</tr>
</tbody>
</table>

Values are means ± SE for n mice [(n = 3 and 4 for grip strength in isosorbide dinitrate (ISDN) and ISDN + Prednisone groups, respectively). CNI, central nucleation index. Values that share different lower case/upper case letters are significantly different by Tukey honestly significant difference test (HSD) (P < 0.05). †Significantly different from Control by Dunnett’s test (P < 0.05). ‡Significantly different from nitric oxide (NO) donor-negative (Control and Prednisone) by Dunnett’s test (P < 0.05). §Significant interaction between prednisone and NO donors (P < 0.05, 2-way ANOVA). ¶Significantly different from all other groups by Tukey HSD (0.001 < P < 0.01).

Muscle precursor cells by in situ hybridization. The number of c-met+ muscle precursors was examined in areas of intact diaphragm muscle (containing fibers with central or peripheral nuclei) to assess treatment effects at some distance from active dystrophy or very recent regeneration. Intense staining for c-met mRNA was localized in SCs, mononuclear cells between fibers, and small myotubes (Fig. 6A). Surprisingly, c-met mRNA persisted around central nuclei in some, but not all, larger myotubes (Fig. 6Ad). There were 54% fewer c-met+ cells per square millimeter after M+P treatment than in the Control group (Fig. 6B, P < 0.05) and after isosorbide dinitrate treatment (I and I+P together) (P < 0.05).

Protein expression. The levels of several key proteins relevant to dystrophy and muscle regeneration were determined by Western blotting relative to actin (Fig. 7). Utrophin concentration increased by one-third in groups treated with prednisone (P = 0.05), consistent with effects of deflazacort (75). Levels of Pax7, Ki67, MyoD, and β-dystroglycan in protein extracts of muscle did not differ among treatment groups (Table 2). There was a trend for isosorbide dinitrate treatment to increase NOS1 (I and I+P) compared with groups without NO donor treatment (C and P) (P = 0.08).

DISCUSSION

These experiments demonstrate two remarkable findings. First, prednisone treatment increased fiber susceptibility to

Fig. 2. Macroscopic evaluation of diaphragm muscle. Representative images are shown above the score for each treatment group. The severity of gross fiber damage was scored on a 5-point Likert scale (1 = mild to 5 = severe) according to the number and density of Evans blue dye (EBD)-derived blue streaks on the muscle. Horizontal lines in the plot for each group mark the group mean. The score for damage severity was lower in the MP group compared with P treatment alone. Values that share different letters are statistically different by Mann-Whitney U-test (P < 0.05).
injury, as evidenced by studies of membrane permeability and calcification in mdx mouse diaphragm muscle. The change in permeability was obvious in macro- and microscopic assessments. Second, combination with NO donors alleviated this adverse effect of prednisone, reducing fiber permeability to levels observed in control diaphragm in untreated mdx mice. Isosorbide dinitrate also reduced calcification during prednisone treatment and improved regeneration, resulting in increased CNI and regenerating area. While the two NO donor drugs overlapped in their effects on calcification and permeability, MyoNovin reduced the number of c-met+ myogenic precursors in areas away from active dystrophy or early regeneration and did not increase relative heart weight as observed after treatment with isosorbide dinitrate. Results showed that the two NO donors had distinct and overlapping benefits with clear implications for

Fig. 3. EBD detection in permeable fibers of dystrophic diaphragm. A: representative merged images of EBD (red fluorescence) and laminin staining (green fluorescence) from each group demonstrate fibers in a range of orientations including longitudinal (f), transverse (d, e), and oblique and mixed-orientation sections. Sections from all animals included this range of fiber orientations because of the complex architecture of the muscle. There were small numbers of EBD+ fibers in groups treated with P + a nitric oxide (NO) donor compared with C and P groups. B: proportionate fiber permeability (EBD+/total fibers) of respective treatment. Proportion of EBD+ fibers displayed a significant increase in P-treated mdx mice. Values with different letters are significantly different (P < 0.05). *Significant difference from C (P < 0.05). †Difference from NO donor− groups (C and P) (P < 0.05).
potential treatment of the longer-term impact of dystrophy on cardiorespiratory function.

An increase in fiber membrane permeability after prednisone contrasts with previous reports for other muscles in steroid-treated dystrophic mice. Deflazacort treatment reduced an exercise-related increase in %EBD+ fibers (10), and the %EBD+ fibers in extensor digitorum longus or soleus muscles of exercised α-sarcoglycan-null mice was unchanged after treatment with prednisolone (21). However, known muscle-specific differences in the susceptibility to exercise-related damage (quadriceps more than other limb muscles) (10) anticipate differential permeability related to the severity of the dystrophic phenotype and changes in membrane strain during contraction of muscle that vary in architecture (18, 79). As
well, increased animal activity after prednisone treatment (5, 7, 61) would itself increase exercise-induced fiber damage, similar to the myoglobinuria reported in DMD despite steroid treatment (32, 41). Altered calcium homeostasis with membrane dysfunction (1) would also raise permeability after prednisone. Membrane damage probably does not lead to immediate cell necrosis, since fibers rapidly repair membrane lesions through dysferlin and a calcium-dependent resealing process (13). Eventually, however, calcium-dependent proteolysis will lead to degeneration (12, 13, 48, 62). Increased utrophin protein after prednisone, also shown previously in vitro (24, 67) and in vivo (75, 78), may help stabilize a dystrophin-deficient cytoskeleton but does not replace the role of dystrophin in localizing NOS1 to the sarcolemma (50, 52). Nonetheless, diaphragm (present report) and limb muscles of mdx (10, 14, 89) and α-sarcoglycan-null mice (21) have a lower fiber permeability after treatment that raises NO levels, indicating that NO likely decreased fiber permeability by improving the resealing process sufficient to prevent injury and/or accelerate membrane repair.

By comparison to permeability, calcification after prednisone treatment is consistent with previous reports. Prednisolone treatment upregulated calcium metabolism genes (14, 31) and increased calcification in mdx mouse heart (77) and the cranial sartorius muscle in golden retriever muscular dystrophy (GRMD) dystrophic dogs (53). Isosorbide dinitrate reduced calcification in the present study, possibly from improved wound healing, angiogenesis, and vascular perfusion during dystrophy. The potent effect of NO to release HGF from damaged fibers (3, 81, 82) and endothelial cells (64) will secondarily promote HGF-mediated angiogenesis and antiapoptosis (64, 98) while stimulating SC activation, and thus coordinate the early regenerative process. NO is also reported to decrease vascular calcification by directly inhibiting TGF-β signaling (44); similar pathways may prevent differentiation to an osteoblastic lineage in skeletal muscle and thereby reduce calcification. Prednisone itself may have antiangiogenic effects similar to those of the anti-inflammatory drug interferon-α (22); notably, antiangiogenic effects are associated with poor wound healing when calcium balance is altered (9). Therefore, NO released from isosorbide dinitrate likely offset effects of prednisone on vascular tissues and prevented osteogenic differentiation in the dystrophic diaphragm muscle.

Regeneration from dystrophy was also enhanced by NO donor treatment with isosorbide dinitrate. CNI increased the most after combined isosorbide dinitrate and prednisone treatment, and the regenerating area was increased by isosorbide

---

**Fig. 5.** Representative micrographs and plots of the frequency distribution of developmental myosin heavy chain (devMyHC)+ fiber diameter. A: typically, devMyHC+ fibers were larger in M and smaller after I treatment compared with C. Note that darkly stained devMyHC+ fibers are regenerating and demonstrate unstained centralized nuclei that mimic freeze artifact within stained fibers. Bar = 20 μm. B and C: plots showing the diameter distribution of fibers that were positive for devMyHC in all groups (B) and in groups with vs. without P treatment (C). P treatment increased the diameter distribution of devMyHC+ fibers to larger values compared with groups without P treatment (P < 0.05). There was no overall effect on distribution from NO donor treatment compared with controls.
This is consistent with previous understanding of NO effects on myogenic differentiation (70, 94). Since c-met receptor is expressed by both activated and quiescent SCs, the reduction in c-met+ cells seen in already-regenerated diaphragm after MyoNovin treatment (both M and M+P groups) is consistent with NO effects on activation. This finding suggests that the new NO donor reduces the need for SC activation as part of a repair response to dystrophy, while it promotes precursor fusion into regenerating fibers. SC activation is higher than normal in muscle fibers deficient in dystrophin and NOS1 because of low NO levels, and exogenous NO from treatment of dystrophy would be anticipated to reduce activation (94). The reduction in number of proliferating cells represented by BrdU+ cells was not detected by Western blotting for Ki67, likely because BrdU was incorporated for 2 h of S phase whereas Ki67 protein is a more general marker of proliferation present in nuclei from G1 to M phases of the cell cycle (90). Therefore NO-donor treatment reduced proliferation by myogenic and nonmyogenic cells and the general level of cell proliferation (frequency of BrdU+ cells), although nonspecific, is consistent with the lower number of c-met+ mononuclear cells in already-regenerated muscle. BrdU uptake during S phase labels myogenic and nonmyogenic cells. However, in our previous study (5), the majority of proliferating cells in mdx mouse diaphragm expressed transcripts for MyoD and/or myf5 (55% and 66%, respectively). Without similar in situ hybridization studies, definitive conclusions about myogenic cell proliferation during treatment with NO donors are pending. The decrease in cell proliferation (frequency of BrdU+ cells), although nonspecific, is consistent with the lower number of c-met+ mononuclear cells in already-regenerated muscle. BrdU uptake during S phase labels myogenic and nonmyogenic cells. However, in our previous study (5), the majority of proliferating cells in mdx mouse diaphragm expressed transcripts for MyoD and/or myf5 (55% and 66%, respectively). Without similar in situ hybridization studies, definitive conclusions about myogenic cell proliferation during treatment with NO donors are pending. The reduction in number of proliferating cells represented by BrdU+ cells was not detected by Western blotting for Ki67, likely because BrdU was incorporated for 2 h of S phase whereas Ki67 protein is a more general marker of proliferation present in nuclei from G1 to M phases of the cell cycle (90). Therefore NO-donor treatment reduced proliferation by myogenic and nonmyogenic cells and the general level of
SC activation in the diaphragm, both with positive effect on the phenotype of dystrophy in that muscle.

Differences in the two NO donor drugs were interesting and likely influenced their net effects on the speed or efficiency of regeneration and reestablishing the balance of satellite cell activation and quiescence. MyoNovin reduced proliferation in dystrophic muscle, consistent with reported effects on activation in normal muscle (87), and reduced the number of c-met+ cells. By comparison, isosorbide dinitrate treatment increased the areal proportion of regeneration and, combined with prednisone, increased CNI, apparently by accelerating the regeneration process since both NO donors prevented the increased fiber permeability related to prednisone treatment.

It is important to note that isosorbide dinitrate increased heart weight relative to body weight. This negative effect may relate to systemic changes in blood pressure, tissue perfusion, or vascular resistance that differ from the effect of MyoNovin treatment, and would tend to exacerbate dystrophic cardiac hypertrophy in mdx mice as the disease progresses (6, 77). The formulation of MyoNovin from a skeletal muscle relaxant may direct its effects to skeletal more than cardiac muscle compared with isosorbide dinitrate, since the NO donor compounds were administered at the same NO release molarity. Differential effects are also likely contributed by variations in distribution and pharmacokinetics. Previous reports showed accelerated regeneration after treatments that increase NO (8, 10, 21, 74, 89) and in the presence of anti-inflammatory drugs (6, 53). Contributions from perfusion, angiogenesis, inflammation, fiber permeability, calcification, proliferation, and regeneration all complicate interpretation of treatment outcomes in muscular dystrophy. Continuous mechanical activity by the diaphragm (5, 54) further modulates disease severity, as fibers are prone to early contraction-induced damage in the absence of dystrophin (68, 79). However, since respiratory insufficiency occurs as a late sequel and is one of the most common causes of death in dystrophy (33), the systemic effects of NO donor formulations in muscular dystrophy, including sildenafil (47), require further study, particularly in relation to the potential to improve vascular perfusion during exercise (11, 72, 86) and the potential to reduce disease progression and offset sequelae of steroid treatment. Similar effects of NO donors on muscle perfusion in old animals merit investigation for potential application to treating age-related atrophy.

Results of this study indicate that combined NO-donor and prednisone treatment could improve the efficacy of steroid therapy in dystrophy and suppress some of its detrimental effects. Interestingly, formulations of steroidal and nonsteroidal anti-inflammatory drugs linked to NO, including NO-prednisolone and NO-ibuprofen (45, 46), have not been tested in DMD or canine and mouse models of muscular dystrophy. Since treatment effects differ between glucocorticoids (7) and anti-inflammatory drugs linked to NO, including NO-prednisolone and NO-ibuprofen (45, 46), have not been tested in DMD or canine and mouse models of muscular dystrophy. Since treatment effects differ between glucocorticoids (7) and also between two NO donor formulations (this study), further elucidation of the mechanisms by which an NO donor improves effects of steroids in muscle would significantly benefit clinical application to DMD and other neuromuscular diseases.

ACKNOWLEDGMENTS

The authors are grateful for collaboration with Dr. Jeff Leiter (Pan Am Clinic, Winnipeg and the Department of Surgery, University of Manitoba) and advice on SPSS from Dr. James Roth (Biological Sciences, University of Manitoba). Present address of W. Mizunoya: Dept. of Bioscience and Biotechnology, Graduate School of Agriculture, Kyushu University, Fukuoka, Japan.

GRANTS

This research was supported by an operating grant from the Manitoba Institute of Child Health (to J. E. Anderson) and a postdoctoral research fellowship from Foreign Affairs and International Trade Canada (to W. Mizunoya).

DISCLOSURES

J. E. Anderson, G. Wang, and F. J. Burczyński are coinventors on a patent application, “Compositions and methods for enhancing nitric oxide delivery,” covering the formulation of MyoNovin used in experiments detailed in this study.

Table 2. Protein levels relative to control (standardized against actin)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Prednisone</th>
<th>MyoNovin</th>
<th>MyoNovin + Prednisone</th>
<th>ISDN</th>
<th>ISDN + Prednisone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pax7</td>
<td>1.00 ± 0.17</td>
<td>0.85 ± 0.09</td>
<td>0.85 ± 0.16</td>
<td>0.85 ± 0.11</td>
<td>0.62 ± 0.05</td>
<td>0.90 ± 0.15</td>
</tr>
<tr>
<td>Ki67</td>
<td>1.00 ± 0.20</td>
<td>0.89 ± 0.14</td>
<td>0.87 ± 0.15</td>
<td>0.86 ± 0.17</td>
<td>0.92 ± 0.15</td>
<td>1.09 ± 0.18</td>
</tr>
<tr>
<td>MyoD</td>
<td>1.00 ± 0.13</td>
<td>1.12 ± 0.09</td>
<td>1.03 ± 0.14</td>
<td>1.06 ± 0.13</td>
<td>0.97 ± 0.06</td>
<td>0.96 ± 0.14</td>
</tr>
<tr>
<td>β-Dystroglycan</td>
<td>1.00 ± 0.10</td>
<td>0.98 ± 0.10</td>
<td>1.01 ± 0.10</td>
<td>0.97 ± 0.09</td>
<td>0.98 ± 0.07</td>
<td>1.02 ± 0.10</td>
</tr>
</tbody>
</table>

Fig. 7. Western blots and graphs showing representative protein levels in mdx mouse diaphragm muscle. Representative Western blots are shown above the respective graphs (normalized to actin) for NOS1 (A) utrophin (B), and devMyHC (C).
REFERENCES


