MUSCULOSKELETAL PATHOLOGY

Mechanical Overloading Increases Maximal Force and Reduces Fragility in Hind Limb Skeletal Muscle from Mdx Mouse

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There is fear that mechanical overloading (OVL; ie, high-force contractions) accelerates Duchenne muscular dystrophy. Herein, we determined whether short-term OVL combined with wheel running, short-term OVL combined with irradiation, and long-term OVL are detrimental for hind limb mdx mouse muscle, a murine model of Duchenne muscular dystrophy exhibiting milder dystrophic features. OVL was induced by the surgical ablation of the synergic muscles of the plantaris muscle, a fast muscle susceptible to contraction-induced muscle damage in mdx mice. We found that short-term OVL combined with wheel and long-term OVL did not worsen the deficit in specific maximal force (ie, absolute maximal force normalized to muscle size) and histological markers of muscle damage (percentage of regenerating fibers and fibrosis) in mdx mice. Moreover, long-term OVL did not increase the alteration in calcium homeostasis and did not deplete muscle cell progenitors expressing Pax 7 in mdx mice. Irradiation before short-term OVL, which is believed to inhibit muscle regeneration, was not more detrimental to mdx than control mice. Interestingly, short-term OVL combined with wheel and long-term OVL markedly improved the susceptibility to contraction-induced damage, increased absolute maximal force, induced hypertrophy, and promoted a slower, more oxidative phenotype. Together, these findings indicate that OVL is beneficial to mdx muscle, and muscle regeneration does not mask the potentially detrimental effect of OVL. (*Am J Pathol* 2015, 185: 2012–2024; <http://dx.doi.org/10.1016/j.ajpath.2015.03.027>)

Dystrophin plays a role in force transmission, sarcolemmal stability and localization, and function of different proteins that initiate a muscle damage process in its absence.^{1–3} The deficiency in dystrophy causes Duchenne muscular dystrophy (DMD), a severe muscle wasting in all skeletal muscles combined with cardiomyopathy.

The mdx mouse, a milder murine model of DMD, exhibits several dystrophic features in hind limb muscle. There is a loss of force-generation capacity in the dystrophin-deficient mdx muscle compared with control muscle. Indeed, the murine dystrophin-deficient muscle exhibits a reduced specific maximal force [ie, the absolute maximal force generated relative to muscle size (cross-sectional area or weight)].^{4–7} Mdx muscle is also more fragile [ie, susceptible to damage

caused by the acute effect of few contractions, especially lengthening (eccentric) contractions producing high forces]. In fact, few lengthening contractions cause an immediate marked reduction in force generation in fast-twitch mdx muscle.^{8,9} Mdx muscle also exhibits regenerating fibers, which are centronucleated, and expressed neonatal (nn) isoform of myosin heavy chain-nn, indicating that the absence of dystrophin causes repeated cycles of degeneration and regeneration. Several studies have reported that calcium

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homeostasis is also altered in mdx muscle.^{10–13} For example, cytosolic calcium content is increased in mdx mice.^{10–12} The physiopathological processes causing these dystrophic features are not well known, but a widely accepted hypothesis is that calcium homeostasis plays a central role in the physiopathology.¹³

Resistance training uses high-force contractions to improve physical performance in healthy subject and combats weakness and atrophy in the cases of disuse and aging. However, studies are needed to determine whether resistance training could also be used to improve the dystrophic phenotype and to confirm that it would not induce muscle damage.^{14,15} Because of the susceptibility of contraction-induced damage, the risk that exercise training using high-force contractions accelerates the muscular dystrophy has been debated.^{14–16} In a recent study, we reported that short-term mechanical overloading (OVL), a model of resistance training, did not worsen dystrophic features because the deficit in maximal specific force and the percentage of centronucleated fibers, a histological marker of muscle damage, were not increased in response to OVL in mdx mice.¹⁷ However, we do not yet know whether it worsens the susceptibility to contraction-induced damage. Moreover, there is a possibility that long-term OVL would aggravate the disease,¹⁸ plausibly because muscle cell progenitors are depleted, or loses its function in a dystrophic environment, resulting in an imperfect muscle repair. OVL that increases both neural activity and loading^{19–23} mimics intensive resistance training, which has been shown to increase absolute maximal force production in animal models by 22% to 37%,^{24–26} whereas OVL induces a threefold higher increase in mice (80% to 97%).^{17,23} OVL also triggers several cellular and molecular adaptations, such as hypertrophy in the skeletal muscle. This aspect of muscle remodeling induced by OVL results from increased fiber diameter and total number of fibers per cross section.^{17,22,27–29} The promotion of a slower and/or oxidative fiber phenotype is another important cellular and molecular muscle adaptation in response to OVL.^{17,22,29–31}

Our aim was to determine whether repeated high-force contractions, as would be induced by OVL, would be detrimental for the mdx plantaris muscle, a fast muscle susceptible to contraction-induced damage. We analyzed maximal specific force, immediate susceptibility to contraction-induced damage, histological markers of muscle damage (regenerating fibers and fibrosis), alteration in calcium homeostasis (resting calcium content), and functional, cellular, and molecular adaptations (gain in maximal force, hypertrophy, and fiber-type transition) in response to OVL. Our general hypothesis was that if repeated high-force contractions would be detrimental, then dystrophic features would be worsened, and functional, cellular, and molecular adaptations in response to OVL would be impaired. More specifically, our first objective was to determine the effect of short-term (1 month) OVL combined with wheel running (OVL+wheel) in mdx mice. OVL is combined with wheel running to increase the use of the overloaded muscle (ie, the level of mechanical stress produced by normal cage activity). The second objective was to examine the effect of long-term OVL

(6 months) in mdx mice. In a previous study, we observed that short-term OVL was not detrimental,¹⁷ but it is possible that the duration was too short to exhaust the muscle repair capacity, because of depletion or loss of function of adult muscle cell progenitors (satellite cells). Our third objective was to determine whether OVL combined with prior irradiation would be more detrimental in mdx mice than in control mice. It is possible that regeneration of fibers may efficiently repair the potential damage induced by OVL in mdx mice. Because muscle regeneration could mask the potential deleterious effect of OVL, muscles from mdx mice were irradiated before OVL; irradiation will destroy satellite cells that are essential for muscle regeneration.³²

Materials and Methods

Animals and Treatments

All procedures were performed in accordance with national and European legislations, under the license 75-1102 from the French government. Mdx mice and age- and sex-matched wild-type (C57BL/10) mice (referred to below as C57 mice) were 2 to 3 months of age at the beginning of the experiments. For mechanical OVL, mice were i.p. anesthetized with 50 mg/kg body weight pentobarbital. The plantaris muscles of both legs were mechanically OVL for 1 and 6 months by surgical removal of soleus muscles and a major portion of the gastrocnemius muscles, as described.¹⁷ Some overloaded mdx mice were placed in separate cages containing a wheel and were allowed to run 1 month ad libitum (referred to below as mdx+OVL+wheel mice). Local irradiation (18 Gy) was performed using an X-ray irradiator to prevent muscle regeneration, by inhibiting the proliferation of muscle cell progenitors.³³ The left hind limbs from some mice were γ -irradiated (0.91 Gy per minute $\times$ 20 minutes), before the surgical removal of soleus and gastrocnemius complex (referred to below as mdx+OVL+irr mice). The irradiated mice were studied after 1-month OVL. In some experiments, to analyze muscle calcium, extensor digitorum longus (EDL) muscles of both legs were mechanically overloaded for 5 to 6 months by the surgical removal of two thirds of distal tibialis anterior muscles.^{18,34,35}

Muscle Force and Susceptibility to Contraction-Induced Damage

Maximal forces of plantaris muscles were evaluated by measuring *in situ* isometric force, as described previously.¹⁷ Mice were anesthetized (i.p., pentobarbital sodium, 50 mg/kg). During physiological experiments, supplemental doses were given as required to maintain deep anesthesia. The knee and foot were fixed with clamps and stainless steel pins. Plantaris muscles were exposed, and the distal tendons of the gastrocnemius and soleus muscle complex were cut. The distal tendons of plantaris muscles were attached to a dual mode servomotor (Aurora Scientific, Aurora, ON, Canada). Sciatic nerves were proximally crushed and distally stimulated using a bipolar silver electrode with supramaximal square wave pulses

of 0.1-millisecond duration. Responses to tetanic stimulation (pulse frequency, 75 to 143 Hz) were successively recorded. At least 1 minute was allowed between contractions. Absolute maximal force was determined at optimal length (length at which maximal tension was obtained during the tetanus). Force was normalized to the muscle weight as an estimate of specific maximal forces. Susceptibility to contraction-induced injury of mdx mice was then estimated from the force decrease resulting from lengthening contraction-induced injury. The sciatic nerves from mdx mice were stimulated for 700 milliseconds (frequency, 150 Hz). A maximal isometric contraction of the plantaris muscle was initiated during the first 500 milliseconds. Then, muscle lengthening (10% optimal length) at a velocity of 5.5 mm per second was imposed during the last 200 milliseconds.^{36,37} All isometric contractions were made at an initial length. Nine lengthening contractions of the muscle were performed, each separated by a 45-second rest period. Maximal isometric force was measured 45 seconds after each lengthening contraction and expressed as a percentage of the initial maximal force. Some mdx mice also performed nine isometric contractions, in which muscle length was not increased. Body temperature was maintained at 37°C using radiant heat. After contractile measurements, the mice were euthanized with an overdose of pentobarbital, and muscles were weighed.

Calcium Content

EDL muscles overloaded for 5 to 6 months were pinned in SYLGARD-coated dishes containing normal physiological solution [148 mmol/L NaCl, 4.5 mmol/L KCl, 2.5 mmol/L CaCl₂, 1 mmol/L MgCl₂, 10 mmol/L HEPES, and 5.5 mmol/L glucose (pH 7.4)]. Small bundles of 10 to 15 fibers, arranged in a single layer, were dissected lengthwise, tendon to tendon, with the use of microscissors. Muscle preparations were loaded for 1 hour with 3 μ mol/L of the fura-2 AM fluorescent calcium probe (Molecular Probes, Life Technologies, Eugene, OR). All experiments were conducted at room temperature. Ratiometric Fura-2 fluorescence measurements were made using optical excitation filters of 380 and 360 nm and an IonOptix micro-Stepper Switch integrated system (IonOptix LLC, Milton, MA). Emitted fluorescence (510 nm) was background subtracted. Cytosolic calcium concentration was calculated at rest, according to a modified method from Fraysse et al.³⁸ A pseudoratiometric approach was used to measure calcium content. Fura-2 fluorescence was first recorded at 1000 Hz under 380-nm excitation. Thereafter, Fura-2 fluorescence was recorded at 360 nm. The 10 records were averaged at each excitation wavelength. Finally, the calcium content was calculated by calculating the ratio of the means (360:380) and transformed in calcium concentration values using the method described for resting cytosolic calcium concentration determination.

Histology

Transverse serial sections (8 μ m thick) of plantaris muscles (collected 1 and 6 months after OVL) were obtained using a

cryostat, in the midbelly region. Some of sections were processed for histological analysis, according to standard protocols (stained for hematoxylin and eosin and Sirius red).¹⁷ Others were used for immunohistochemistry, as described previously.³⁹ For determination of muscle fiber diameter and myosin heavy chain analysis, frozen unfixed sections were blocked for 1 hour in phosphate-buffered saline plus 2% bovine serum albumin and 2% sheep serum and incubated for 30 minutes with mouse Fab (Jackson ImmunoResearch Europe Ltd., Newmarket, UK) 1:100 in phosphate-buffered saline. Sections were then incubated overnight with primary antibodies against laminin (rabbit polyclonal, 1:300; Dako, Les Ulis, France) and myosin heavy chain isoforms (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). After washes in phosphate-buffered saline, sections were incubated for 1 hour with secondary antibodies (Alexa Fluor; Life Technologies, Saint Aubin, France). To evaluate regenerating muscle fibers and the presence of satellite cells, unfixed sections were incubated with primary antibodies against myosin heavy chain isoform neonatal⁴⁰ or Pax-7 (mouse monoclonal, 1:50; Santa Cruz Biotechnology, Heidelberg, Germany), respectively. For morphometric analyses, images were captured using a motorized confocal laser-scanning microscope (LSM 700; Carl Zeiss SAS, Le Pecq, France). Morphometric analyses were made using ImageJ software version 1.49m (NIH, Bethesda, MD) and a homemade macro. The smallest diameter (minimum Ferret) of all of the muscle fibers of the whole muscle section was measured. Herein, only data from pure myosin heavy chain-2b, myosin heavy chain-2x, and myosin heavy chain-2a fiber types were analyzed. Myosin heavy chain-2x fibers were identified as fibers that did not express myosin heavy chain-2b, myosin heavy chain-2a, and myosin heavy chain-1. For muscle fiber diameter and fiber typing analyses, all of the muscle fibers of the muscle section were measured. The extent of fibrosis was assessed by Sirius red staining.

Gel Electrophoresis for the Determination of Myosin Heavy Chain

Plantaris muscles were extracted on ice for 60 minutes in four volumes of extracting buffer (pH 6.5), as previously described.⁴¹ After centrifugation, the supernatants were diluted 1:1 (v/v) with glycerol and stored at -20°C. Myosin heavy chain isoforms were separated onto 8% polyacrylamide gels, which were made in the Bio-Rad (Marnes-la-Coquette, France) mini-Protean II Dual slab cell system, as described previously.⁴²⁻⁴⁴ The gels were migrated for 31 hours at 72 V (constant voltage) at 4°C. For the quantification of the relative concentration of myosin heavy chain and actin, 1 μ g of total proteins was loaded and 12% polyacrylamide gels were used.⁴⁵ The silver-stained gels were scanned using a video acquisition system, and bands were quantified by densitometric software version 381472 (Multi Gauge; Fujifilm, Asnières, France).

Figure 1 Functional dystrophic features in response to short-term overloading (OVL) and short-term OVL combined with wheel running in mdx mice. **A:** Specific maximal force in mdx mice (sP0). **B–F:** Force decrease after lengthening contractions in mdx mice (**B**), lengthening contractions in mdx and C57 mice (**C**), and isometric (Iso.) or lengthening (Length.) contractions in mdx mice (**D**). Utrophin mRNA (**E**) and protein (**F**) levels in mdx and C57 mice. **G:** Representative images of blots concerning utrophin. $n = 9$ to 14 per group (**A** and **B**); $n = 4$ to 12 per group (**C** and **D**); $n = 3$ to 5 per group (**E** and **F**). * $P < 0.05$, OVL mice; $^{\dagger}P < 0.05$, C57 mice; $^{\ddagger}P < 0.05$, isometric contractions. AU, arbitrary unit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Immunoblotting

Plantaris was powdered on dry ice and homogenized in 300 μ L of ice-cold lysis buffer [50 mmol/L Tris·HCl (pH 7.5), 250 mmol/L sucrose, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.25% Nonidet P-40 substitute, 50 mmol/L NaF, 5 mmol/L $\text{Na}_4\text{P}_2\text{O}_7$, 0.1% dithiothreitol, fresh antiprotease, and phosphatase inhibitor mixture]. Then, samples were shaken at 1300 rpm for 30 minutes at 4°C. Samples were subsequently centrifuged at $13,000 \times g$ for 10 minutes at 4°C, and the protein concentration of the supernatant was determined by the Bradford assay (Bio-Rad). Equal aliquots of protein were boiled for 5 minutes in Laemmli sample buffer [250 mmol/L Tris·HCl (pH 6.8), 2% (v/v) SDS, 10% (v/v) glycerol, 0.01% bromophenol blue, and 5% (v/v) β -mercaptoethanol]. Samples were separated onto SDS-polyacrylamide gels and then transferred to nitrocellulose membranes for 1 hour. Membranes were blocked for 1 hour in 3% milk in Tris-buffered saline and 0.1% Tween 20 (TBST) before overnight incubation at 4°C with appropriate primary antibody in TBST (1:1000 dilution). Proteins were detected with

a primary antibody directed to utrophin A (NCL-DRP2; Leica, Newcastle, UK), peroxisome proliferator-activated receptor γ coactivator 1- α (sc13067; Santa Cruz Biotechnology), phospho-AMP activated protein kinase α (mAb2535; Cell Signaling Technology, Leiden, the Netherlands), complex IV subunit IV (A21348; Life Technologies), and glyceraldehyde-3-phosphate dehydrogenase (sc365062; Santa Cruz Biotechnology). After incubation, membranes were washed three times with TBST before incubation with an appropriate peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) in TBST. Antibody binding was detected using an enhanced chemiluminescence horseradish peroxidase substrate detection kit (Thermo Fisher Scientific, Saint Herblain, France) and quantified using ImageJ software.

Quantitative RT-PCR

Total RNA was extracted from TA muscle using TRIzol (Life Technologies) and Ultra Turrax (Ika, Staufen, Germany),

Figure 2 Cellular and molecular dystrophic features in response to short-term overloading (OVL) and short-term OVL combined with wheel running in mdx mice. **A:** Fibrosis in mdx mice. **B:** Fibers expressing the neonatal isoform of myosin heavy chain (MHC-nn) in mdx mice. **C:** Centronucleated fibers in mdx mice. **D:** mRNA levels of different inflammatory cytokines in mdx and C57 mice. $n = 3$ to 5 per group for cellular and molecular measurements. * $P < 0.05$, non-overloaded mice; † $P < 0.05$, overloaded mice. AU, arbitrary unit.

following the manufacturer's instructions. From 500 ng of extracted RNA, the first-strand cDNA was then synthesized using the RevertAid Reverse Transcriptase (Thermo Fisher Scientific, Villebon-sur-Yvette, France) with Oligo(dT)₁₈ primer and according to the manufacturer's instructions. Primer sequences were designed with Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>),⁴⁶ as follows: β -2-microglobulin forward, 5'-CACACTGAATTCACCCAC-3', reverse, 5'-GTCTCGATCCCAGTAGACGG-3'; cytochrome c oxidase subunit IV isoform 1 forward, 5'-CGTCTTGGTCTTCCGGTTG-3', reverse, 5'-TCTGGAAGCCAACATTCGTC-3'; citrate synthase forward, 5'-CATGCCAGTGCTTCTTCCA-3', reverse, 5'-CATGTTGCTGCTTGAAGGTCT-3'; cytochrome c-1 forward, 5'-TTTTCCCTGCTCAC TGGCTA-3', reverse, 5'-TGGGGTGCCATCATCATACT-3'; desmin forward, 5'-GTCCTCACTGCCTCCTGAAG-3', reverse, 5'-AGCATGAAGACCACAAAGGG-3'; hydroxymethylbilane synthase forward, 5'-AGGTCCCTGTT CAGCAAGAA-3', reverse, 5'-TGGGCTCCTCTTGGAATGTT-3'; interferon γ forward, 5'-GTTTGAGGTCAA CAACCCACA-3', reverse, 5'-ACTCCTTTTCCGCTTCC TGA-3'; IL-10 forward, 5'-GGCGCTGTCATCGATTT CTC-3', reverse, 5'-GCCTTGTAGACACCTTGGTC-3'; IL-13 forward, 5'-CATCACACAAGACCAGACTCC-3', reverse, 5'-GGTTACAGAGGCCATGCAAT-3'; IL-1 β forward, 5'-AAGGAGAACCAAGCAACGAC-3', reverse, 5'-CTTGGGATCCACACTCTCCAG-3'; IL-4 forward, 5'-TCTGTAGGGCTTCCAAGGTG-3', reverse, 5'-TCTG CAGCTCCATGAGAACA-3'; IL-6 forward, 5'-TCTC TGCAAGAGACTTCCATCC-3', reverse, 5'-AAGTCTC CTCTCCGGACTTG-3'; myosin, heavy polypeptide 7, cardiac muscle, β forward, 5'-AGGTGTGCTCTCCA GAATGG-3', reverse, 5'-CAGCGGCTTGATCTTGAAGT-3'; Nrf1 forward, 5'-TGGAGTCCAAGATGCTAATGG-3',

reverse, 5'-GCGAGGCTGGTTACCACA-3'; peroxisome proliferator-activated receptor (Ppar) α forward, 5'-CCG AGGGCTCTGTCATCA-3', reverse, 5'-GGGCAGCTG ACTGAGGAA-3'; Ppar δ forward, 5'-ATGGGGGACCA GAACACAC-3', reverse, 5'-GGAGGAATTCTGGGAG AGGT-3'; Ppar γ forward, 5'-ACTCGCATTCCTTTGA CATC-3', reverse, 5'-TCGCACTTTGGTATTCTTGG-3'; Ppar γ 1a forward, 5'-GAAAGGGCCAAACAGAGAGA-3', reverse, 5'-GTAAATCACACGGCGCTCTT-3'; Pprc1 forward, 5'-CTGTGAGTCTGTTGGAGCA-3', reverse, 5'-ACAGCCAAGCTGTCAGCAG-3'; succinate deshydrogenase (Sdh) a forward, 5'-TTACAAAGTGCGGGT CGATG-3', reverse, 5'-GTGTGCTTCTCCAGTGTTC-3'; Sirtuin (Sirt)1 forward, 5'-TCGTGGAGACATTTTAA TCAGG-3', reverse, 5'-GCTTCATGATGGCAAGTGG-3'; Sirt2 forward, 5'-CACTACTTCATCCGCTGCT-3', reverse, 5'-CCAGCGTGTCTATGTTCTGC-3'; Sirt3 forward, 5'-TGCTACTCATTCTTGGGACCTC-3', reverse, 5'-GGGC ACTGATTTCTGTACTGC-3'; transcription factor A forward, 5'-GCAGGCACTACAGCGATACA-3', reverse, 5'-ACTGAGCTCCGAGTCCTTGA-3'. Relative quantification PCR analysis was then performed with green intercalating dye PCR technology using a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Meylan, France). The reaction was performed in duplicate for each sample in a 6- μ L reaction volume made up of 3 μ L of The LightCycler 480 SYBR Green I Master (Roche Diagnostics) containing 1 μ mol/L each of the forward and reverse primer and 3 μ L of diluted (1:25) cDNA. The thermal profile for quantitative PCR was 95°C for 1 minute, followed by 40 cycles at 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 30 seconds. The gene expression of hydroxymethylbilane synthase was used as the reference transcript.

Figure 3 Functional, cellular, and molecular muscle adaptations in response to short-term overloading (OVL) and short-term OVL combined with wheel running in mdx mice. **A:** Absolute maximal force (P0). **B:** Muscle weight. **C:** Fiber diameter. **D:** Percentage of fibers expressing myosin heavy chain (MHC)-2a. The percentage increased but not significantly ($P = 0.09$). **E:** Percentage of highly succinate dehydrogenase (SDH)—stained fibers. **F:** mRNA levels of genes regulating mitochondria biogenesis (*TFAM* and *Nrf1*) and involved in citric cycle and respiratory chain (*CS*, *SDHA*, *Cyc1*, and *Cox4i1*). **G:** Protein level of PGC-1 α . **H:** Protein level of phosphorylated AMP activated protein kinase α . **I:** Protein level of Cox4. $n = 9$ to 14 per group for functional measurements; $n = 3$ to 5 per group for cellular and molecular measurements. * $P < 0.05$, nonoverloaded mice; $^{\dagger}P < 0.05$, C57 mice. AU, arbitrary unit; PGC-1 α , peroxisome proliferator-activated receptor γ coactivator 1- α .

Statistical Analysis

Groups were statistically compared using analysis of variance (one and two ways) and Student's *t*-test, as appropriate. If necessary, subsequent post hoc analysis (Bonferroni) was also performed. Values are given as means $\pm$ SEM.

Results

Effect of Short-Term OVL Combined with Wheel Running

Ablation of synergic plantaris muscles (mechanical OVL and OVL) was performed at the age of 2 to 3 months, and the mice were studied 1 month later. During the OVL period, some of the mdx mice were placed in cages containing a wheel, in which they ran 3.5 ± 0.5 km/day. We first determined whether the functional dystrophic features worsened when OVL was combined with wheel running. By using muscle contractility analysis, we observed that specific maximal force was unchanged in response to OVL in both mdx+OVL+wheel and

mdx+OVL mice (Figure 1A). Moreover, the force decreases after the sixth and ninth lengthening contractions were reduced in response to OVL in both mdx+OVL+wheel and mdx+OVL mice (Figure 1B), indicating that susceptibility to contraction-induced muscle damage was improved in both mdx+OVL+wheel and mdx+OVL mice. The improvement in force decrease was similar in mdx+OVL+wheel and mdx+OVL mice because we observed no difference between mdx+OVL+wheel and mdx+OVL mice. Next, we performed additional experiments to better characterize the reduced force decreases after lengthening contractions. To test whether this improvement induced by OVL was specific to dystrophic muscles, we also analyzed the effect of 1-month OVL in C57 mice expressing dystrophin. We observed that the force decreases after the lengthening contractions in C57+OVL mice were not different from those of C57 mice (Figure 1C). Moreover, we confirmed that OVL improved the force decreases after the third, sixth, and ninth lengthening contractions in mdx+OVL mice because these were reduced compared with those of mdx mice (Figure 1C) and not different from those of C57+OVL mice (Figure 1C). We also

Figure 4 Functional, cellular, and molecular dystrophic features in response to long-term overloading (OVL) in mdx mice. **A:** Specific maximal force (sP0). **B:** Force decrease after lengthening contractions. **C:** Representative image of fibrosis, using Sirius red staining. **D:** Fibrosis. **E:** Centronucleated fibers. **F:** Representative image of the neonatal isoform of myosin heavy chain (MHC-nn)—expressing fibers. $n = 12$ to 14 per group for functional measurements; $n = 6$ per group for cellular and molecular measurements. $*P < 0.05$, nonoverloaded mice; $^{\dagger}P < 0.05$, C57 mice. Scale bar = $30 \mu\text{m}$ (**C** and **F**). RS, sarcoplasmic reticulum.

observed that the force decrease 20 minutes after the ninth lengthening contraction was reduced, but not significantly, in mdx+OVL compared with mdx (Figure 1C). The major portion of the force decrease after lengthening contractions in mdx mice was not because of fatigue (metabolic incompetency) because nine isometric contractions did not result in a similar force decrease after contractions (Figure 1D). Because utrophin is a dystrophin homologue that is up-regulated in mdx mice to compensate dystrophin deficiency, we then determined whether the improvement in the force decrease after lengthening contractions in mdx+OVL was related to increased utrophin expression. Quantitative PCR analysis revealed that utrophin mRNA levels were increased by OVL in both mdx and C57 mice (Figure 1E). However, the utrophin protein level was unchanged in mdx+OVL compared with mdx (Figure 1, F and G).

Cellular and molecular aspects of dystrophic features were evaluated by observing histological markers of muscle damage/regeneration. Results showed that fibrosis (Figure 2A) and the percentage of muscle fibers expressing myosin heavy chain—neonatal (Figure 2B) did not increase in response to OVL in both mdx+OVL+wheel and mdx+OVL mice and were not different between these two groups of mice. Moreover, the percentage of centronucleated muscle fibers was reduced in response to OVL in both mdx+OVL+wheel and mdx+OVL mice (Figure 2C); however, this occurred to a lesser extent in mdx+OVL+wheel compared with mdx+OVL mice.

Additional experiments indicated that the expression of IL-1 β , IL-6, and IL-10, markers of inflammation, was similarly increased in both mdx and C57, as assessed by quantitative PCR analysis (Figure 2D).

Concerning the functional, cellular, and molecular adaptations induced by OVL, we observed that absolute maximal force similarly increased in mdx+OVL+wheel (218%) and mdx+OVL (171%) mice (Figure 3A). We also observed that muscle weight similarly increased in response to OVL in mdx+OVL+wheel (286%) and mdx+OVL (228%) mice (Figure 3B). Next, muscle hypertrophy was characterized by immunohistology. The fiber diameter did not change in response to OVL in both mdx+OVL+wheel and mdx+OVL mice (Figure 3C), indicating that the increased muscle weight induced by OVL was caused by an increased number of fibers per cross section. Regarding the potential fiber-type transition induced by OVL, we observed that both mdx+OVL+wheel and mdx+OVL mice increased the percentage of fibers expressing myosin heavy chain—2a, although this was not significant (Figure 3D). Muscle sections were also treated to show SDH activity, an enzyme of the citric acid cycle/complex II of the respiratory chain. We showed that the percentage of high stained SDH fibers was greater in mdx+OVL mice compared with mdx mice (Figure 3E). Additional experiments were also performed to further analyze the fiber transition induced by OVL. Although gene expression was not markedly increased (Figure 3F), immunoblotting analysis revealed that

Figure 5 Functional, cellular, and molecular muscle adaptations in response to long-term overloading (OVL) in mdx mice. Absolute maximal force (P0; **A**), muscle weight (**B**), fiber diameter (**C**), total fiber number per cross section (**D**), percentage of fibers expressing the neonatal isoform of myosin heavy chain (MHC)-2a, MHC-2b, or no MHC (**E**), representative image of electrophoretic separation of MHC isoforms (**F**), MHC-2a, MHC-2b, MHC-2x, and MHC-1 composition (**G**), and the percentage of highly succinate dehydrogenase (SDH)-stained fibers (**H**). $n = 12$ to 14 per group for functional measurements; $n = 6$ per group for cellular and molecular measurements. * $P < 0.05$, nonoverloaded mice; † $P < 0.05$, C57 mice.

several markers of oxidative energy metabolism increase in mdx+OVL (or C57+OVL) mice (Figure 3, G–I).

In conclusion, we observed no notable difference between mdx+OVL+wheel and mdx+OVL mice. Therefore, our results indicate that there was no notable effect of wheel running in mdx mice during OVL: dystrophic features were not worsened, and functional, cellular, and molecular adaptations in response to OVL were not impaired.

Effect of Long-Term OVL

Synergic plantaris muscle ablation was performed at 2 to 3 months of age, and the mice were studied 6 months later. We observed no effect of 6-month OVL on specific maximal force because there was no difference between mdx+OVL6m and mdx mice (Figure 4A). However, the force decrease after lengthening contractions was reduced in response to 6-month OVL (Figure 4B), indicating an improvement of this functional dystrophic feature. Fibrosis was not increased in response to 6-month OVL (Figure 4, C and D). The percentage of centronucleated fibers was slightly increased by 6-month OVL

(Figure 4E), whereas the percentage of fibers expressing myosin heavy chain—nn was not different between mdx+OVL6m ($16.0\% \pm 2.4\%$) and mdx mice ($16.1\% \pm 2.5\%$). As shown in Figure 4F, the level of staining was reduced in mdx+OVL6m compared with mdx mice. Together, these results indicate that histological markers of muscle damage (ie, cellular and molecular dystrophic features) were not worsened by 6-month OVL.

Functional, cellular, and molecular adaptations in response to 6-month OVL showed that absolute maximal force increased by 151% in mdx+OVL6m compared with mdx (Figure 5A). Moreover, muscle was heavier (188%) in mdx+OVL6m (Figure 5B). This hypertrophy was because of both increased fiber diameter (13%) (Figure 5C) and total fiber number per cross section (74%) in mdx+OVL6m (Figure 5D). We observed no change in the percentage of the different fiber types in response to 6-month OVL (Figure 5E). However, analysis of the myosin heavy chain composition, as assessed by gel electrophoresis, showed that myosin heavy chain—2b expression was decreased in mdx+OVL6m compared with mdx (Figure 5, F and G). Moreover, the percentage of high stained SDH fibers increased in response to 6-month OVL (Figure 5H).

Figure 6 Muscle cell progenitors (satellite cells) and calcium homeostasis in response to long-term overloading (OVL) in mdx. Number of cells expressing Pax 7 in plantaris muscle (A), resting cytosolic calcium content in extensor digitorum longus (EDL) fiber bundles (B), and resting sarcoplasmic reticulum (SR) calcium content in EDL fiber bundles (C). * $P < 0.05$, nonoverloaded mice; † $P < 0.05$, C57. $n = 6$ per group for cellular measurements; $n = 29$ to 101 per group for calcium measurements.

To determine whether 6-month OVL depleted muscle cell progenitors, we counted the number of satellite cells in a muscle section. We observed that 6-month OVL did not change the number of satellite cells expressing Pax 7 (Figure 6A).

In addition, we also studied calcium homeostasis, by using fura-2 microfluorescence analysis, in EDL muscle after long-term OVL (5 to 6 months). Fiber bundles of EDL muscles were used for this analysis because it was not possible to prepare fiber bundles from the plantaris muscles. We observed that calcium homeostasis did not worsen by 6-month OVL because resting cytosolic calcium content did not increase in mdx+OVL6m compared with mdx (Figure 6B). Six-month OVL induced an increase in resting sarcoplasmic reticulum calcium content in mdx+OVL6m and C57+OVL6m mice, indicating that this calcium adaptation in response to OVL was maintained in mdx+OVL6m (Figure 6C).

Together, these results indicate that long-term OVL did not worsen dystrophic features and did not deplete muscle cell progenitors. Moreover, mdx mice exhibited typical functional,

cellular, and molecular adaptations in response to long-term OVL.

Effect of Short-Term OVL Combined with Prior Irradiation

Because muscle regeneration could mask the potential deleterious effect of OVL, the left hind limb was irradiated before ablation of synergic plantaris muscles, to inhibit satellite cell proliferation and thus muscle repair. Plantaris muscles were studied after 1-month OVL. Irradiation similarly decreased specific maximal force in mdx+OVL+irr and C57+OVL+irr mice compared with mdx+OVL (−58%) and C57+OVL (−64%), respectively (Figure 7, A and B). In contrast to C57+OVL+irr mice, specific maximal force was not decreased in mdx+OVL+irr mice compared with mdx, indicating that this dystrophic feature was not worsened by OVL combined with irradiation. Absolute maximal force (Figure 7, C and D) and muscle weight (Figure 7, E and F) were similarly reduced in mdx+OVL+irr (−87% and −69%) and C57+OVL+irr (−90% and −72%) mice compared with mdx+OVL and C57+OVL mice, respectively. Together, these results indicate that OVL did not induce more detrimental effects in mdx than in C57 mice, when plantaris muscles were irradiated before synergic muscle ablation.

Discussion

Dystrophic Features Do Not Worsen but Improve

In the present study, short-term OVL combined with wheel running and long-term OVL have been shown to be not detrimental to mdx mice because they did not worsen the deficit in maximal specific force, histological markers of muscle damage, or calcium homeostasis, confirming and extending our previous study.¹⁷ These results are also supported by the fact that irradiation before short-term OVL did not induce more deleterious effects in mdx than in C57 mice. It is believed that satellite cells are essential for muscle remodeling in response to OVL.^{47,48} Because irradiation blocked similarly the gains in absolute maximal force and muscle weight in response to short-term OVL in mdx and C57 mice, this confirms that satellite cell function was efficiently inhibited in both mice strains, and thus irradiated muscles from overloaded mdx mice were unable to regenerate. Therefore, these latter findings suggest that muscle regeneration did not mask the deleterious effects of OVL in unirradiated mdx muscle.

Interestingly, we demonstrate, for the first time, that both short-term OVL+wheel and long-term OVL improved one of the major dystrophic features: susceptibility to contraction-induced damage. This effect of OVL is specific to mdx muscle because the force decrease after lengthening contractions remains unchanged in C57 mice, suggesting that the mechanism of the force decrease after lengthening contractions differs between mdx and C57 mice. It remains to be determined whether

Figure 7 Functional dystrophic features and muscle adaptations in response to short-term overloading (OVL) combined with prior irradiation in mdx (A, C, and E) and C57 (B, D, and F) mice. Specific maximal force (sP0) in mdx (A) and C57 (B) mice, absolute maximal force in mdx (P0; C) and C57 (D) mice, and muscle weights in mdx (E) and C57 (F) mice. $n = 3$ to 4 per group for functional measurements; $n = 3$ per group for cellular and molecular measurements. * $P < 0.05$, nonoverloaded mice; † $P < 0.05$, overloaded mice.

the reduced immediate force decrease after lengthening contractions in response to OVL in mdx mice could be because of either the increased mechanical load placed on muscle or the increased tonic activation. Removal of synergic muscles imposes forced stimuli because the mice use the remaining plantaris muscle for both movement and maintenance of posture. Thus, because the two types of stimuli coexist after OVL, it is not clearly defined whether the higher intensity of contractions has more impact than the increased duration of the muscle activity.

Because the major portion of the immediate force decrease after lengthening contractions is not related to increased fatigability because of energy metabolism incompetence (the present study^{9,49,50}), it is unlikely that a better fatigue resistance induced by OVL in mdx mice¹⁷ can explain the reduced force decrease in mdx+OVL mice. In view of the crucial role of utrophin in the force decrease after lengthening contractions in mdx mice,^{51,52} it should be reasonable to hypothesize that the increased utrophin protein level induced by OVL could explain the reduced force decrease. This hypothesis was also supported by the findings that OVL increases the expression of utrophin mRNA in mdx mice (the present study).⁵³ However, we observed that utrophin protein level was not increased in mdx+OVL mice. We also examined the possibility that the reduced force decrease could be related to a fast or glycolytic to slow or oxidative fiber-type transition in mdx+OVL, with the beneficial effect of this fiber-type shift in dystrophic muscle receiving much attention recently.⁵⁴ It is well established that soleus muscles from mdx

mice containing fibers expressing myosin heavy chain—2a and myosin heavy chain—1 are less susceptible to contraction-induced damage than EDL muscle containing most fibers expressing myosin heavy chain—2b.^{8,9} In the present study, OVL induced a promotion of fibers expressing myosin heavy chain—2a in mdx mice, as previously reported.¹⁷ Pharmacological and genetic activation of calcineurin, AMP activated protein kinase, PGC-1 α , and PPAR β improves the force decrease after lengthening contractions in mdx mice, although the mechanisms are not yet well understood.^{55–57} Calcineurin, AMP activated protein kinase, PGC-1 α , and PPAR signaling pathways also promote the formation of slow/oxidative fibers, and are likely responsible for the fast/glycolytic to slow/oxidative fiber transition induced by exercise.^{58,59} In the present study, several results suggest an increase in muscle oxidative energy metabolism after OVL in mdx mice, in agreement with previous studies in nondystrophic mice.^{22,31} Finally, an additional explanation is that the OVL-induced activation of Akt—mammalian target of rapamycin signaling in mdx mice¹⁷ can contribute to the reduced force decrease. In fact, genetic activation of Akt has been previously reported to reduce the force decrease after lengthening contractions in mdx mice.⁴⁹

Whatever the exact mechanisms, the reduced fragility to contraction-induced damage of mdx mice in response to OVL could have important consequences because susceptibility to contraction-induced damage is likely the cause of the muscle-

wasting syndrome in DMD (see below). This result suggests that it is not impossible that resistance training with high-force contractions could help to protect the muscle from damage in DMD patients, instead of worsening it, as was commonly thought. Several studies, including the present study, also support the notion that treatments (genetic Akt activation and β -adrenergic administration) making mdx muscles stronger protect them from lengthening contraction-induced damage.^{49,60} Together with a previous study,⁶¹ we have clearly demonstrated that both models of resistance and endurance exercise training can improve susceptibility to contraction-induced muscle damage in mdx mice and can even be as effective as sophisticated genetic therapeutic strategies aiming to restore dystrophin.^{36,62}

Beneficial Functional, Cellular, and Molecular Adaptations in Response to OVL

Short- and long-term OVL also induced adaptations in mdx mice: marked gain in performance, hypertrophy, and a fast/glycolytic to slow/oxidative fiber-type transition, confirming and extending our previous study.¹⁷ Consequently, if the same effects are observed in humans, this approach could be used to improve muscle function in DMD patients. In support of this possibility, a recent study has been shown that resistance training improves muscle strength and endurance in Becker muscular dystrophy patients.⁶³ In the present study, the gain in performance results from muscle hypertrophy because specific maximal force remained unchanged in response to OVL in mdx mice. The hypertrophy might be explained by more fibers per cross section in response to short-term OVL, confirming several studies reporting hyperplasia in response to OVL.^{17,22,27–29} In response to long-term OVL, hypertrophy results from both hyperplasia and fiber hypertrophy in mdx mice. The promotion of slow/oxidative fibers is also beneficial because it not only can improve fragility but also fatigue resistance.¹⁷

We observed no deterioration with time of these functional, cellular, and molecular adaptations. For example, absolute maximal force gain and muscle hypertrophy (increased muscle weight) in response to short- and long-term OVL were similar in mdx+OVL (171% and 228%, respectively) and mdx+OVL6m (151% and 188%, respectively). Because muscle adaptative capacity remained intact in the long-term in mdx mice, these findings also support the idea that OVL is not detrimental.

No Depletion of Muscle Cell Progenitors

Dystrophin deficiency increases the susceptibility to contraction-induced damage, initiates repeated degeneration/regeneration cycles, and leads to muscle weakness and wasting. The progressive loss of function and depletion of muscle cell progenitors is believed to be the cause of the muscle wasting/weakness in DMD patients. Supporting this idea is the fact that depletion or loss of function of muscle

cell progenitors (replicative senescence) results in severe muscular dystrophy and wasting in mouse.^{64,65} Therefore, there is a possibility that repeated high-force contractions and degeneration/regeneration cycles during a long period would deplete muscle cell progenitors in dystrophic muscle. Moreover, this risk might be markedly increased by the fact that these cells are essential not only for muscle repair³² but also for muscle remodeling in response to OVL, as shown in the present and previous studies.^{47,48} However, our results clearly do not support this hypothesis because the number of cells expressing Pax 7 was not reduced by long-term OVL in mdx mice.

Conclusion

Herein, we found that short-term OVL combined with wheel running and long-term OVL, an intensive model of resistance training, did not worsen dystrophic features in mdx mice. We also show that muscle regeneration did not compensate for the potentially deleterious effect of OVL. On the contrary, OVL was beneficial because it reduced a major dystrophic feature (ie, susceptibility to contraction-induced damage), increased absolute maximal force, induced hypertrophy, and triggered a promotion of slower/oxidative fibers. It will now be interesting to see if a similar approach could be used to reduced muscle weakness and protect the muscles of DMD patients from damage.

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