MUSCULOSKELETAL PATHOLOGY

Dietary Phosphorus Overload Aggravates the Phenotype of the Dystrophin-Deficient *mdx* Mouse

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Duchenne muscular dystrophy is a lethal X-linked disease with no effective treatment. Progressive muscle degeneration, increased macrophage infiltration, and ectopic calcification are characteristic features of the *mdx* mouse, a murine model of Duchenne muscular dystrophy. Because dietary phosphorus/phosphate consumption is increasing and adverse effects of phosphate overloading have been reported in several disease conditions, we examined the effects of dietary phosphorus intake in *mdx* mice phenotypes. On weaning, control and *mdx* mice were fed diets containing 0.7, 1.0, or 2.0 g phosphorus per 100 g until they were 90 days old. Dystrophic phenotypes were evaluated in cryosections of quadriceps and tibialis anterior muscles, and maximal forces and voluntary activity were measured. Ectopic calcification was analyzed by electron microscopy to determine the cells initially responsible for calcium deposition in skeletal muscle. Dietary phosphorus overload dramatically exacerbated the dystrophic phenotypes of *mdx* mice by increasing inflammation associated with infiltration of M1 macrophages. In contrast, minimal muscle necrosis and inflammation were observed in exercised *mdx* mice fed a low-phosphorus diet, suggesting potential beneficial therapeutic effects of lowering dietary phosphorus intake on disease progression. To our knowledge, this is the first report showing that dietary phosphorus intake directly affects muscle pathological characteristics of *mdx* mice. Dietary phosphorus overloading promoted dystrophic disease progression in *mdx* mice, whereas restricting dietary phosphorus intake improved muscle pathological characteristics and function. (*Am J Pathol* 2014, 184: 3094–3104; <http://dx.doi.org/10.1016/j.ajpath.2014.07.007>)

Duchenne muscular dystrophy (DMD) is the most common and severe form of muscular dystrophy in children, affecting approximately 1 in 3500 male births.¹ DMD is an X-linked lethal muscular disorder that results in the progressive deterioration of muscles as a result of deficiency of dystrophin protein, which plays an important role in cytoskeletal membrane function.² In the absence of dystrophin, membrane permeability in skeletal muscle is increased, making membranes more susceptible to the stress of contraction and resulting in a continuous muscle degeneration and regeneration cycle. This process is related to chronically increased muscle inflammation.³ Because there is currently no effective treatment for this disease, clinical nutritional approaches have also been investigated in DMD animal models.^{4–9} Low bone density is a major complication of this disorder,¹⁰ and calcium and vitamin D supplementations are beneficial in the control of bone metabolism in patients with

DMD.¹¹ However, there is limited information available on nutritional recommendations for the patients.

Phosphorus (P) is an essential element for living cells and plays important roles in bone development, growth performance, and cellular metabolism. In developed countries, dietary phosphorus/phosphate intake continues to steadily increase as a result of increasing use of additives in a variety of

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processed foods and a general increase in food consumption. Increased intake of a diet high in P/Pi is associated with an increased risk of mortality in patients with chronic kidney disease or cardiovascular disease, and even in members of the general population with a risk of lifestyle-related disease.¹²

The dystrophin-deficient *mdx* mouse was first identified as a mutant strain of C57BL/ScSn10 mice with an X-linked skeletal muscle myopathy mimicking the pathological characteristics of DMD¹³ and was subsequently shown to have a nonsense mutation in exon 23 of the dystrophin gene.¹⁴ Among the pathological features, ectopic calcification in skeletal muscle has been reported to occur in cardiac and skeletal muscles of *mdx* mice.^{15,16} In patients with DMD, a few calcified muscle fibers are also observed in young patients with severe muscle degeneration.¹⁷

In this study, we determined the effects of dietary phosphorus intake on ectopic calcification in skeletal muscle, muscle performance (specific maximal forces and daily running activity), and pathological features during exercise-induced stress in *mdx* mice.

Materials and Methods

Animal Care

Dystrophin-deficient mice (C57BL/10ScSn-*mdx*; *mdx*) and control mice (C57BL/10ScSn; B10) were obtained from the National Center of Neurology and Psychiatry (Tokyo, Japan) and maintained in our laboratory. All mice were housed in cages with pulp bedding (Palmas-μ; Material Research Center, Kanagawa, Japan) in a climate-controlled room with a 12-hour light/dark cycle and a temperature of 25°C. Experimental chow and water were available ad libitum. No phosphoric acid was present in the water given to the mice. Mice were sacrificed with an overdose of diethyl ether. All procedures were performed in accordance with the ethical guidelines of the University of Tokyo (Tokyo, Japan).

Experimental Diets

Weaning (20-day-old) male control and *mdx* mice were each divided into three diet groups that were fed diets containing a phosphorus content of 0.7 g/100 g (low-P diet), 1.0 g/100 g (normal-P diet), or 2.0 g/100 g (high-P diet). The normal-P diet was the same composition as the commercial CE-2 diet (CLEA Japan, Inc., Tokyo, Japan) that was fed to pregnant and nursing mice of both genotypes. All diets were manufactured by the Oriental Yeast Company (Tokyo, Japan). The lowest concentration of a phosphorus diet used in this study contained 0.7 g P in 100 g when all of the diet components of the three types of phosphorus diets were modified on the basis of the open formula of the NIH diet (NIH-07; NIH, Bethesda, MD). Monopotassium phosphate and monosodium phosphate were added to increase the dietary phosphorus levels for the normal- and high-P diets. Other diet components, including Ca²⁺ (1.2 g/100 g), were present at the same concentration in all diets.

Double-Stained Transparent Specimens

To clarify the distribution of Ca²⁺ deposits in skeletal muscle, we used a modified whole-body double-staining method involving Alizarin Red S and Alcian Blue, which stain bones and cartilage, respectively.^{18–20} Ninety-day-old mice were sacrificed and fixed in 95% ethanol for 7 days after removal of the skin and organs. The ethanol was then replaced with acetone, and the samples were further incubated for 3 to 4 days. After partial drying, samples were stained in a solution of 0.3% Alcian Blue 8GX (Buchs, Fluka, Switzerland) in 70% ethanol and 0.1% Alizarin Red S (Wako, Tokyo, Japan) in 95% ethanol, and kept in 2.0% potassium hydrogen phthalate in 70% ethanol for 3 days. Each stained mouse was washed in distilled water and placed in 0.75% potassium hydroxide (KOH) in MilliQ water for 2 days to initiate maceration and clearing (Wako). Clearing was continued by addition of increasing concentrations of glycerol (20%, 50%, 70%, and 100%) in 0.75% KOH to obtain a completely cleared specimen. Calcified regions were stained reddish violet, similar to the appearance of stained bones, whereas the bodies were stained slightly blue because only few Alcian Blue dye molecules were trapped within the skeletal muscle membranes. Images of specimens were captured using a digital camera (Nikon D5000; Nikon, Tokyo, Japan).

X-Ray Micro-CT Observation

To quantify the volume densities of Ca²⁺ deposits in the lower body, whole-body images of the *mdx* mice (*n* = 3 from each group) were taken using a noninvasive animal computed tomography (CT) system [SkyScan-1074 for partial muscle tissues (Bruker, Kontich, Belgium); Latheta LCT-200 X-ray micro CT scanner (Hitachi Aloka Medical, Mitaka, Japan)]. For whole-body scanning, mice were embedded in a 48-mm-diameter holder while under anesthesia induced by constant exposure to 1.5% to 2.0% isoflurane gas. CT scanning was performed at 192-mm intervals four times for each slice, and image acquisition was respiratory gated. The volume densities of calcification in the lower body (from the pelvis to the ankle joint) were calculated using standard analysis software version 3.00 that automatically removed bone volume densities.

Electron Microscopy

Tibialis anterior (TA), extensor digitorum longus, quadriceps, and diaphragm muscles of 30- and 60-day-old *mdx* mice fed a normal- or high-P diet were dissected and fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer for 2 hours. The muscles were cut into rectangular pieces and post-fixed in fixative solution (4% OsO₄, 0.2 mol/L collidine, and 3% lanthanum nitrate) for 2 hours. After dehydration with a graded series of ethanol, muscle segments were embedded in epoxy resin. For light microscopy observations, 1-μm semithin sections were stained

with Toluidine Blue. For observation by electron microscope, 100-nm ultrathin sections were stained with uranyl acetate and lead citrate and were analyzed by a FEI Tecnai Spirit (FEI, Hillsboro, OR) at 120 kV.

Muscle Force Measurements

In situ absolute maximal isometric single force (MSF) and maximal tetanic force (MTF) of right triceps surae muscles (TSM; gastrocnemius and soleus) were recorded using a custom-made isometric force recording system and original experimental protocols on the basis of the design of Dorchie et al.²¹ Sixty-day-old control and *mdx* mice fed the three phosphorus diets ($n = 7$ from each group) were anesthetized by diethyl ether gas and immobilized on a cork board by covering the bodies with Novix-II (Asahi Techno Glass, Shizuoka, Japan). A confined area of skin and myofascia of the right hind limb was cut and exposed, and the sciatic nerve was dissected to induce analgesic conditions. The knee joint was firmly immobilized by a needle that served as the fulcrum, and the Achilles tendon of the leg was severed and connected to a platinum electrode clip of a force transducer gauge (DS2-50N Digital Force Gauge; Imada, Toyohashi, Japan). A second platinum electrode was directly inserted into the TSM. For measurement of MSF, muscles were stimulated with a square wave pulse (0.5-millisecond duration) of stimulation voltage. After the MSF was recorded, the MTF was measured with 200-millisecond bursts of frequency set to 100 Hz. By using manual settings for the optimal muscle length, maximal twitch contractions were measured during trials for up to 20 contractions and all tetanic force measurements were made at locations where the single twitch force was the greatest. The length and weight of the TSM and the density of mammalian skeletal muscle (1.06 mg/mm^3) were measured to calculate the cross-sectional area of the muscle (in mm^2). The specific MSF and MTF were normalized by dividing the measured force by the cross-sectional area.

CTX-Induced Muscle Injury

To investigate the effect of ectopic calcification on muscle function, dystrophic calcification was induced in the TSM by injection of cardiotoxin (CTX) into control mice. CTX is the most abundant protein in cobra venom and causes skeletal muscle injury by blocking the enzymatic activity of phospholipid protein kinase and Na^+ and K^+ -ATPase.²² A single injection of CTX ($10 \mu\text{mol/L}$ in $100 \mu\text{L}$ saline) was performed into the right TSM of 60-day-old control mice that were then fed either a normal-P or a high-P diet ($n = 3$ in each group) during a 14-day recovery period until the injured muscles regenerated. Specific MSF and MTF were measured at the end of the recovery period. Some mice were sacrificed without undergoing force measurements, and the presence of ectopic calcification in the regenerated TSM was observed using an X-ray micro-CT scanner (SkyScan-1074) operated at 40 kV and $1000 \mu\text{A}$. Mice were also given an i.v. injection

of Evans Blue Dye (100 mg/kg), which is incorporated into degenerating myofibers with permeable membranes.²³ In addition, cryosections of TSM were prepared and stained with hematoxylin and eosin (H&E) and Alizarin Red S. CTX was also injected in the right TA muscle of *mdx* mice fed the phosphorus diets at the age of 60 days ($n = 3$ each). After 14 days of recovery from the CTX injection, they were sacrificed and both the left (control) and the right (injected) TA muscles were used to investigate the effects of dietary phosphorus intake on muscle regeneration and ectopic calcification after muscle injury in *mdx* mice.

Voluntary Exercise

To study the combined effects of dietary phosphorus intake and exercise-induced stress after voluntary physical activity, 90-day-old *mdx* mice from the three phosphorus diet groups ($n > 8$ mice) were individually housed in free-spinning running wheel cages designed for mice (SN-450; Shinano,

Figure 1 Body weight and TSM weight of control (cont.) and *mdx* mice. **A:** Body weights of control and *mdx* mice fed the three phosphorus diets at 30, 60, and 90 days of age. **B:** TSM weights of *mdx* mice fed the three phosphorus diets. All groups showed a gradual increase in body weight with age. The muscle mass of TSM was similar among *mdx* mice fed the different phosphorus diets.

Tokyo, Japan) that contained a running wheel (200-mm diameter and 50-mm width) with a counter and a breeding cage (90 × 120 × 90 mm). The experimental chows and water were available ad libitum. Mice were allowed to acclimate to the running wheel cage for 3 days and then data for daily wheel rotations were collected for the following 7 days. The distance traveled for a single turn of the running wheel was 62.8 cm.

Histological Analyses

Rectus femoris muscle (one of the quadriceps) and TA muscle from *mdx* mice of the different phosphorus diet groups were dissected and snap frozen in liquid nitrogen-cooled isopentane. The resulting blocks were stored at -80°C , and transverse serial sections (8 μm thick) were collected. Sections were stained with H&E to evaluate necrotic areas and centrally located myonuclei. Alizarin Red S was used to detect calcium deposits in skeletal muscle. For immunohistochemistry, sections (8 μm thick) of frozen TA muscles were fixed with 10% formalin/PBS. After blocking, samples were incubated with primary antibodies in PBS for 1 hour at 37°C . Primary antibodies used in this experiment were monoclonal antibody against embryonic myosin heavy chain (eMyHC; 1:10; ATCC, Manassas, VA) and polyclonal anti-F4/80 (1:250; AbD Serotec, Kidlington, UK), anti-inducible nitric oxide synthase (iNOS; 1:250; Upstate Biotechnology, Lake Placid, NY), and anti-CD206 (1:250; Serotec) antibodies. Alexa Fluor 488 or

594 secondary antibody (1:1000; Invitrogen, Carlsbad, CA) was used for detection. Fluorescein isothiocyanate-conjugated sheep anti-mouse albumin antibody (Cappel, Cochranville, PA) was directly applied to the sections to detect necrotic myofibers. Images were acquired using an epifluorescence microscope (model Axiophot; Carl Zeiss MicroImaging, Inc., Jena, Germany).

Statistical Analysis

Results were expressed as means $\pm$ SD. In cases where the means of two independent groups were compared, differences were determined by the Student's *t*-test. For multiple comparisons, a one-way analysis of variance with the Tukey's multiple comparison test was performed using Prism 5 (GraphPad, La Jolla, CA). $P \leq 0.05$ was considered statistically significant.

Results

Effects of Dietary Phosphorus Intake on Growth

Body weights of male control mice and *mdx* mice fed the low-P, normal-P, or high-P diet were recorded at age 30, 60, and 90 days. Control and *mdx* mice of all three phosphorus diet groups displayed similar gradual increases in body weight with age (Figure 1A). All mice were alive at 90 days

Figure 2 Quantification of ectopic calcification in skeletal muscle of *mdx* mice. **A:** Lower limbs of control and *mdx* mice fed the three phosphorus diets (transparent specimen). Ectopic calcification was clearly seen in skeletal muscle as bone-like red staining (red arrows). **B:** Density of ectopic calcification. Ectopic calcification of lower limbs of *mdx* mice was quantified using a micro-CT scanner. The volume density of Ca^{2+} deposits was $0.7 \pm 0.2 \text{ mm}^3$ in low-P fed *mdx* mice, $3.1 \pm 0.7 \text{ mm}^3$ in normal-P fed *mdx* mice, and $33.1 \pm 4.7 \text{ mm}^3$ in high-P fed *mdx* mice. Lowering dietary phosphorus intake reduced calcification, whereas dietary phosphorus overloading significantly increased the volume of calcifications in *mdx* mice. **C:** Serial cryosections of quadriceps muscle from high-P fed control, low-P fed *mdx*, normal-P fed *mdx*, and high-P fed *mdx* mice on H&E and Alizarin Red S staining. * $P < 0.05$ compared with other groups.

of age. The TSM of *mdx* mice was weighed at 60 days of age after measurement of muscle forces and was similar among *mdx* mice of the three groups (Figure 1B).

Dietary Phosphorus Overload Increases Ectopic Calcification

The presence of ectopic calcification in skeletal muscle is a characteristic feature of DMD animal models. We used a whole-body double-staining method and X-ray CT micro scanner to detect Ca^{2+} deposits in the skeletal muscle of 90-day-old *mdx* mice fed the three phosphorus diets. During the staining procedure, bone and ectopic calcifications were stained red by Alizarin Red S and cartilage was stained blue by Alcian Blue (Figure 2A). Imaging of the stained and cleared samples revealed no red staining in the skeletal muscles of control mice fed any of the phosphorus diets. In *mdx* mice, stained Ca^{2+} deposits were rarely seen in the whole bodies of animals in the low-P diet group. In addition, relatively few areas of calcification were observed in the gluteus and none were present in the diaphragm of low-P fed *mdx* mice. *Mdx* mice fed the normal-P diet displayed striped and spotty red stained areas, particularly in lower limb muscles, whereas excessive calcification was clearly observed in the samples prepared from high-P fed *mdx* mice. Ectopic calcification in skeletal muscle was quantified using a Latheta LCT-200 CT scanner (Figure 2B). Histological analyses showed that high-P control mice did not have any ectopic calcification or muscle necrosis, whereas calcium deposits were increased in the skeletal muscle of *mdx* mice by dietary phosphorus overload (Figure 2C). These results confirmed that the amount of ectopic calcification in *mdx* skeletal muscle increased with increasing dietary phosphorus in a dose-dependent manner, and suggested that lowering dietary phosphorus intake reduced ectopic calcification.

The Mechanisms of Ca^{2+} Deposition in Dystrophic Skeletal Muscle

Electron microscopic examination revealed irregular highly electron-dense structures in some of the damaged mitochondria and intracellular fragments that were thought to be terminal cisternae of sarcoplasmic reticulum (SR) in degenerating myofibers (Figure 3A). In such cases, the mitochondria appeared to have a swollen and roundish shape. Macrophage infiltration was observed near and within degenerating muscle areas, and these macrophages had engulfed calcified or damaged cells (Figure 3, B and C). Completely or almost completely calcified areas contained clusters of calcified mitochondria and degenerated cells (Figure 3D). A similar process was observed in all muscle areas studied (TA, extensor digitorum longus, quadriceps, and diaphragm) of 30- and 60-day-old *mdx* mice. The fact that this calcification was observed in 30-day-old *mdx* mice on both normal- and high-P diets suggests that it is a

relatively fast process and that calcium deposits are readily formed in dystrophic skeletal muscle. A high-P diet promotes muscle damage and macrophage infiltration in *mdx* muscle.

Albumin immunostaining was evident in degenerating myofibers in *mdx* mice but undetectable in control muscles. *Mdx* mice fed a high-P diet had significantly larger areas of necrotic muscle fibers than *mdx* mice fed a low-P diet (Figure 4, A and B). Histological immunostaining was also used to detect and characterize areas of macrophage infiltration in quadriceps cross sections of 90-day-old *mdx* mice from the three diet groups. The number of inflammatory cells that were labeled with a marker of all macrophages, F4/80, was significantly increased in *mdx* mice fed the high-P diet compared with the other two groups (Figure 5A). Macrophages that are labeled with iNOS are classified as M1 macrophages, whereas CD206 is a marker for M2 macrophages. Serial cross sections stained with F4/80, iNOS, and CD206 showed that high-P fed *mdx* mice had significantly increased numbers of iNOS-positive cells and decreased numbers of CD206-expressing cells among the F4/80-positive cells (Figure 5, B and C). Reducing the phosphorus content in the diet reduced the number of inflammatory macrophages expressing iNOS and increased the number of CD206-positive cells.

Figure 3 Observation of ectopic calcification in skeletal muscle of *mdx* mice by electron microscopy. **A:** Early calcification of cells in skeletal muscle. Inorganic material was mainly observed in mitochondria and fragmented SR. **Arrows** indicate calcified mitochondria; **arrowheads**, fragments of SR. **B:** Macrophage infiltration near the area of necrotic muscle with calcified cells. **Arrow** indicates a macrophage; **arrowhead**, necrotic and calcified cells in a degenerating myofiber. **C:** Macrophage-engulfed necrotic and calcified cells within a degenerating muscle fiber. The dotted lines indicate an infiltrated macrophage. **D:** Typical area of advanced ectopic calcification in skeletal muscle. Numerous mitochondria-like cells were present in the calcified area.

Figure 4 Necrotic myofibers in rectus femoris muscle of *mdx* mice fed with different phosphorus diets. **A:** Immunostaining shows increased clusters of necrotic myofibers in high-P fed *mdx* mice. **B:** Total area of muscle necrosis (anti-albumin positivity) expressed as a percentage of the total muscle area. High-P fed *mdx* mice show significantly greater total areas of necrotic myofibers. * $P < 0.05$.

Dietary Phosphorus Overload Decreases Muscle Function of *mdx* Mice

Absolute single and tetanic muscle forces were measured to determine the effects of dietary phosphorus overloading. The absolute tensions were normalized to muscle-specific forces by applying TSM weight (Figure 1B) and the length and density of mammalian skeletal muscle. Specific maximal single and tetanic muscle forces (MSF and MTF, respectively) are considered to be physiologically more relevant than absolute MSF and MTF.

Measurement of *in situ* lower leg muscle force outputs revealed that high-P fed *mdx* mice had significantly lower specific MSF than *mdx* mice fed the low-P diet ($P < 0.01$), which was higher than that of *mdx* mice fed the normal-P diet (Figure 6A). In addition, *mdx* mice fed a high-P diet produced significantly lower specific MTF than *mdx* mice fed a normal-P and low-P diet ($P < 0.01$ and $P < 0.001$, respectively) (Figure 6B). No significant differences in specific MSF and MTF were detected among control mice of the three phosphorus diet groups (data not shown). Although *mdx* mice fed the low-P diet showed increased muscle forces compared with mice fed a normal-P diet, these forces were lower than those of control mice fed any of the three phosphorus diets.

To investigate the effect of ectopic calcification on muscle function, MSF and MTF were measured after induction of dystrophic calcification in the TSM of control mice. Ectopic calcification in skeletal muscle of control mice after recovery from CTX-induced muscle injury has not been reported previously. Ectopic calcification was

induced by injection of CTX into the skeletal muscle of control mice, which were fed the high-P diet during a 14-day recovery period. Calcium phosphate was deposited in regenerated muscle areas, where muscle fibers of different size and shape with central nuclei were observed (Figure 6C). X-ray micro-CT observations using a SkyScan scanner and cryosections showed that ectopic calcification was present in high-P fed control mice after 14 days of recovery from CTX injection (control + CTX) (Figure 6D), whereas no Ca^{2+} deposition was detected in control + CTX mice fed a normal-P diet (data not shown). Histologically,

Figure 5 Quantification of overall (F4/80-positive areas) (A), M1 (iNOS-positive) (B), and M2 (CD206-positive) (C) macrophage infiltration in rectus femoris muscle from *mdx* mice fed the phosphorus diets. High-P fed *mdx* mice had a significantly larger overall inflammatory area, increased M1 macrophage filtration, and decreased M2-positive area compared with *mdx* mice in the other two groups. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respectively.

ectopic calcification in normal skeletal muscle was similar to that in *mdx* muscle. High-P fed control + CTX mice seemed to exhibit delayed muscle regeneration, especially around fibers with calcification. To clarify the effects of Ca^{2+} precipitation on muscle force outputs, we measured specific muscle forces of high-P fed control + CTX mice (with Ca^{2+} deposits) and normal-P fed control + CTX mice (without Ca^{2+} deposits). Normal-P fed control + CTX mice had similar muscle force values as those of control mice (without CTX treatment) fed the normal-P diet, indicating that the TSM had functionally recovered from CTX-induced injury by 14 days. In contrast, high-P fed control + CTX mice showed a significantly lower specific MTF compared with normal-P fed control + CTX mice ($P < 0.05$) (Figure 6E). The specific MSF of high-P fed control + CTX mice was similar to that of normal-P fed control + CTX mice.

Dietary Phosphorus Overloading Reduces Voluntary Running Wheel Activity

To investigate the effects of phosphorus intake on physical activity, *mdx* mice on the three phosphorus diets ($n > 8$) were individually housed in cages containing a running wheel for 10 days (3 days of acclimation period and 7 days of trials). High-P fed *mdx* mice significantly reduced daily running activity than that of low-P and normal-P fed *mdx* mice ($P < 0.05$) (Figure 7). Therefore, high-P fed *mdx* mice had a significantly lower capacity for exercise in the running wheel. The results for low-P fed *mdx* mice suggest that low phosphorus intake does not adversely affect physical activity levels.

Restricting Dietary Phosphorus Intake Minimizes Exercise-Induced Muscle Necrosis

In addition to increasing the physical activity of mice, running wheels provide a rich environment that promotes

Figure 6 *In situ* measurement of specific maximal lower leg muscle forces of *mdx* mice fed the phosphorus diets, and reduced specific muscle forces after muscle damage in control mice with experimental calcification. Specific MSF (A) and specific MTF (B). Specific muscle forces of *mdx* mice decrease with dietary phosphorus in a dose-dependent manner. High-P fed *mdx* mice have significantly lower specific MSF ($59.6 \pm 5.4 \text{ mN/mm}^2$) and specific MTF ($187.3 \pm 27.5 \text{ mN/mm}^2$) than those of *mdx* mice fed the low-P diet [MSF, $72.4 \pm 7.2 \text{ mN/mm}^2$ ($**P < 0.01$); and MTF, $254.9 \pm 38.7 \text{ mN/mm}^2$ ($***P < 0.001$)] and *mdx* mice fed the normal-P diet [MSF, $65.3 \pm 5.9 \text{ mN/mm}^2$, and MTF, $238.7 \pm 11.2 \text{ mN/mm}^2$ ($**P < 0.01$)]. C: Serial cryosections of high-P fed control mice after recovery from CTX injection. D: Micro-CT images of TSM of high-P fed control mice after recovery from CTX injection. The circle and arrows indicate the presence of calcified areas in skeletal muscle of control mice. E: Measurement of specific MTF. Ectopic calcification present in high-P fed control mice 14 days after CTX injection (high-P fed control + CTX mice) contributed to reduced specific MTF ($290.7 \pm 29.4 \text{ mN/mm}^2$) compared with normal-P fed control + CTX mice ($347.1 \pm 14.2 \text{ mN/mm}^2$; $*P < 0.05$). The specific MSF of high-P fed control + CTX mice ($84.1 \pm 7.7 \text{ mN/mm}^2$) is similar to that of normal-P fed control + CTX mice ($91.4 \pm 1.0 \text{ mN/mm}^2$).

Figure 7 Exercise capacity of *mdx* mice fed the three phosphorus diets. High-P fed *mdx* mice had significantly lower daily running capacity than normal- and low-P fed *mdx* mice. Low-P fed and normal-P fed *mdx* mice ran similar daily averages of 8703 ± 1692 revolutions (5.5 km) and 8432 ± 2037 revolutions (5.3 km), respectively, whereas high-P fed *mdx* mice ran only 6099 ± 1056 revolutions (3.8 km). **P* < 0.05.

general activity. However, the acute physical activity might exacerbate the muscle damage in *mdx* mice. After 10 days of running wheel activities, *mdx* mice of all three phosphorus diet groups were sacrificed and TA muscle samples were collected for histological analyses. TA muscle was selected for analysis because it was the most severely affected by ectopic calcification. Necrosis was macroscopically visible in exercised *mdx* mice fed a high-P diet, whereas few necrotic fibers were observed in the TA muscle of sedentary 90-day-old *mdx* mice on all three phosphorus diets (Table 1).

As a result of increased physical activity, which represents a form of exercise-induced stress, muscle necrosis and inflammation were significantly increased in high-P fed *mdx* mice (Figure 8, A and B). Low-P fed *mdx* mice had relatively few areas of muscle damage compared with *mdx* mice fed normal- or high-P diets. In addition, low-P fed *mdx* mice had few regenerating fibers and centrally nucleated myofibers compared with the marked increase in high-P fed *mdx* mice (Figure 8, A and B). Upper limb muscles, such as the triceps, were also severely affected in high-P fed exercised *mdx* mice and displayed increased precipitation of calcium phosphate. Compared with age- and diet-matched sedentary *mdx* mice, muscle necrosis was dramatically increased in TA muscle from exercised normal-P fed *mdx* mice (147%

increase) (Table 1). Low-P fed exercised *mdx* mice had relatively few areas of necrosis and no ectopic calcification in TA muscles, even though they ran a longer distance daily than mice fed the high-P diet and the degree of muscle necrosis of the exercised mice was similar (only 23% higher) to that of sedentary *mdx* mice fed the same diet. In contrast, severe necrosis and abundant Ca²⁺ deposits were observed in the TA muscle of the high-P fed exercised *mdx* mice, whereas ectopic calcification was minimal in the TA muscles of sedentary *mdx* mice fed the same diet (571% higher in exercised mice). Consistent with differences in the necrotic area, the macrophage population was only 46% higher in low-P fed exercised *mdx* mice than in corresponding sedentary mice, whereas both normal-P and high-P fed *mdx* mice showed a significantly increased macrophage population (250% and 831%, respectively, compared with sedentary conditions). These macrophages were localized in and near the degenerating area. In addition, an increase in nascent regenerative myofibers (eMyHC positive) was detected in exercised *mdx* mice on all phosphorus diets, and the percentage of eMyHC-positive fibers in exercised *mdx* mice increased with increasing dietary phosphorus intake (Table 1). In particular, exercised high-P fed *mdx* mice had clusters of eMyHC-positive myofibers and grouped necrosis was frequently observed. Moreover, the number of centronucleated myofibers was significantly lower in exercised low-P fed *mdx* mice than in those on the other diets. Taken together, these findings indicate that low-P fed *mdx* mice are more tolerant to exercise-induced stress, whereas increasing phosphorus intake exacerbated the muscle pathological characteristics of *mdx* mice under acute exercise stress.

Dietary Phosphorus Overload Does Not Interfere with Muscle Regeneration after Muscle Injury

After recovery from CTX injection to the right TA muscle, the muscle successfully regenerated and all of the myofibers were centronucleated (Figure 8B). The ratio of the right/left TA muscles was similar among *mdx* mice fed the three phosphorus diets. However, fiber size was still smaller than that of the corresponding TA muscle. Ectopic calcification was only seen in high-P fed *mdx* mice that had recovered from the CTX injection (Figure 8B). Interestingly, the presence of calcium deposits was less after the muscle injury

Table 1 TA Muscle Pathological Features of Sedentary and Exercised *mdx* Mice Fed the Three Phosphorus Diets

Type of phosphorus diet	Necrotic area			Macrophage			eMyHC			CNF		
	Sedentary	Exercised	Δ%	Sedentary	Exercised	Δ%	Sedentary	Exercised	Δ%	Sedentary	Exercised	Δ%
Low	1.42 ± 1.2	1.75 ± 1.6	23	1.0 ± 0.7	1.4 ± 0.8	46	0.3 ± 0.4	1.2 ± 1.0	265	77.7 ± 3.8	80.9 ± 1.4	4.0
Normal	1.69 ± 1.1	4.16 ± 4.1	147	1.0 ± 0.7	3.4 ± 3.1	250	0.5 ± 0.4	1.7 ± 1.1	246	80.7 ± 1.8	83.5 ± 1.5	3.5
High	3.21 ± 2.9	21.6 ± 12.0	571	1.5 ± 0.7	14.2 ± 9.2	831	0.7 ± 0.6	3.9 ± 2.9	489	81.5 ± 3.4	84.8 ± 2.4	4.1

Data are given as means ± SD.

CNF, centrally nucleated myofibers.

Figure 8 **A:** Histological analyses of TA muscles from exercised *mdx* mice fed the three phosphorus diets. Quantification of necrotic areas (represented by dotted lines) by H&E staining. Detection of total inflammatory areas by anti-F4/80 antibody. Staining of total regenerating areas with anti-embryonic myosin heavy chain (eMyHC). **B:** High-magnification views for TA muscle sections on H&E staining from sedentary, exercised, and CTX-injected *mdx* mice fed the three phosphorus diets.

compared with those in the left (control) TA muscle of high-P fed *mdx* mice.

Discussion

Dystrophin-deficient muscles are highly susceptible to chronic inflammation and oxidative stress that occurs as of the early onset of muscle degeneration, leading to muscle necrosis and subsequent fibrosis and calcification in muscle fibers.²⁴ Gene therapies for DMD are the ultimate research goal; however, several problems, including the size of the dystrophin gene and the complexity of drug delivery to all skeletal muscles, remain unresolved. In the meanwhile, alternative approaches to slow down disease progression are being actively investigated. Our findings clearly show that dietary phosphorus overload exacerbated dystrophic phenotypes, whereas restricting dietary phosphorus intake alleviated the muscle pathological characteristics of *mdx* mice. The most effective dietary phosphorus intake for improving the muscle pathological characteristics of *mdx* mice remains to be determined and is a focus of future studies. However, because the minimum dietary requirement of phosphorus for growth and reproduction of laboratory mice is estimated to be 0.3 g/100 g,²⁵ a dietary phosphorus intake lower than that of the low-P diet used in this study may be even more beneficial.

The presence of ectopic calcification in skeletal muscle of *mdx* mice was the most obvious change induced by dietary phosphorus overloading. Control mice without muscle damage did not show any abnormal phenotype in skeletal muscle in response to the different phosphorus diets, although experimental ectopic calcification was induced in control animals by CTX injection into skeletal muscle and dietary phosphorus overloading. Macrophage infiltration and delayed regeneration around the experimental calcium deposits suggest that inflammation might be aggravated by dietary phosphorus overloading and/or ectopic calcification might induce continuous macrophage infiltration.

Dietary phosphorus overload also increased the number of necrotic myofibers and the degree of inflammation in skeletal muscle of *mdx* mice. Previous studies clearly demonstrated that dystrophic muscle of 4-week-old *mdx* mice expresses relatively high levels of M1 macrophages in areas with inflammation, and that the population of macrophages shifts to an immunosuppressing M2 population, such that at 12 weeks the ratio of M1/M2 populations is reversed to promote muscle repair.^{26,27} We found that 90-day-old high-P fed *mdx* mice showed increased high overall inflammation involving predominantly M1 macrophages in the rectus femoris muscle. Electron microscopy showed that initial calcium deposits were present mainly in damaged mitochondria and SR of skeletal muscle cells. Digestion of these calcified cells by macrophages ultimately led to death of the macrophages, which were deposited as ectopic calcifications in the skeletal muscle. A previous study

demonstrated that mitochondria are the main triggers of experimental ectopic calcification in cardiac muscle of mice.²⁸ Because calcium deposits are observed in control mice fed a high-P diet and in female *mdx* mice (data not shown), dystrophin gene/protein deficiency per se is not directly related to ectopic calcification. However, muscle fibers with leaky membranes are easily damaged and may deposit calcium salts because of a strong binding capacity for calcium and phosphate, especially when phosphate metabolism is abnormal. Further studies are needed to elucidate how dietary phosphorus overload increases muscle damage and inflammation in dystrophic muscle.

These histological changes were paralleled by reduced muscle function in *mdx* mice. Although *mdx* mice of the three phosphorus diet groups had a larger body weight and TSM mass, they exhibited weaker muscle force than the corresponding control mice, consistent with previous studies.^{21,29} Furthermore, muscle force outputs of *mdx* mice increased with restricted dietary phosphorus intake. Because there were no differences in specific maximal forces among control mice fed the three phosphorus diets and the induction of experimental calcification in skeletal muscle of control mice contributed to a significant reduction in specific muscle force, we conclude that the presence of calcium deposits in skeletal muscle contributed to the decrease in the force outputs in *mdx* mice. This finding explains the decreased muscle force and performance in patients with myositis ossificans, in which ectopic calcification is present in skeletal muscle area with traumatic or non-traumatic damage.³⁰ The decrease in specific muscle forces with phosphorus overloading was reflected in the reduced daily voluntary activity of the *mdx* mice. Moreover, although acute exercise-induced stress is beneficial for training adaptation in control mice, after 10 days of voluntary wheel running, the *mdx* mice fed a normal-P diet showed increased muscle necrosis, macrophage inflammation, regenerating myofibers, and centronucleated myofibers in TA muscles compared with sedentary *mdx* mice fed the same diet. Low-P fed *mdx* mice appeared tolerant to this exercise-induced stress; exercised *mdx* mice fed a low-P diet had similar dystrophic pathological characteristics in TA muscle to that of matched sedentary mice, with no ectopic calcification and only minor muscle necrosis. Although high-P fed *mdx* mice ran significantly less than the mice fed a normal-P or low-P diet, they showed markedly increased necrosis and ectopic calcification after 10 days of physical activity.

Because acute exercise-induced stress exacerbated muscle necrosis and ectopic calcification in high-P fed *mdx* mice, we predicted that recovery from CTX-injected muscle injury is impaired in *mdx* mice with excessive dietary phosphorus intake. After 14 days of recovery, CTX-injected TA muscles of *mdx* mice fed the three phosphorus diets were successfully regenerated, even though fiber size remained smaller than the contralateral muscle. High-P fed *mdx* mice had the ability to regenerate, and ectopic calcification was present, but not exacerbated, by CTX injection.

In conclusion, this study demonstrates that dietary phosphorus overload exacerbates muscle pathological features in *mdx* mice. Although further work is needed to elucidate the underlying molecular and cellular mechanisms, our data support restriction of dietary intake of phosphorus as a therapeutic approach for patients with DMD.

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