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Immunoproteasome in animal models of Duchenne muscular dystrophy

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Abstract

Increased proteasome activity has been implicated in the atrophy and deterioration associated with dystrophic muscles of Duchenne muscular dystrophy (DMD). While proteasome inhibitors show promise in the attenuation of muscle degeneration, proteasome inhibition-induced toxicity was a major drawback of this therapeutic strategy. Inhibitors that selectively target the proteasome subtype that is responsible for the loss in muscle mass and quality would reduce side effects and be less toxic. This study examined proteasome activity and subtype populations, along with muscle function, morphology and damage in wild-type (WT) mice and two murine models of DMD, dystrophin-deficient (MDX) and dystrophin- and utrophin-double-knockout (DKO) mice. We found that immunoproteasome content was increased in dystrophic muscles while the total proteasome content was unchanged among the three genotypes of mice. Proteasome proteolytic activity was elevated in dystrophic muscles, especially in DKO mice. These mice also exhibited more severe muscle atrophy than either WT or MDX mice. Muscle damage and regeneration, characterized by the activity of muscle creatine kinase in the blood and the percentage of central nuclei were equally increased in dystrophic mice. Accordingly, the overall muscle function was similarly reduced in both dystrophic mice compared with WT. These data demonstrated that there was transformation of standard proteasomes to immunoproteasomes in dystrophic muscles. In addition, DKO that showed greatest increase in proteasome activities also demonstrated more severe atrophy compared with MDX and WT. These results suggest a putative role for the immunoproteasome in muscle deterioration associated with DMD and provide a potential target for therapeutic intervention.

Keywords

proteasome; Duchenne muscular dystrophy; immunoproteasome; LMP2; LMP7; LC3

Introduction

Duchenne muscular dystrophy (DMD) is a recessive X-linked muscle wasting disease resulting from one of several mutations in the dystrophin gene that produces the complete loss of the cytoskeletal protein dystrophin in muscles. Boys with DMD are usually wheelchair-dependent in their early teens and die in the early twenties from cardiorespiratory failure (Passamano et al. 2012; Kohler et al. 2009). The first mouse model developed to study the disease mechanism and potential therapeutic treatments is the dystrophin-deficient MDX mouse, which has the dystrophin protein genetically ablated. However, while the MDX mouse is a genetically valid model of DMD, it presents a milder phenotype than the phenotype observed in humans. The milder phenotype in mice is associated with the compensatory up-regulation of utrophin, a homologue of dystrophin. Unfortunately, the utrophin up-regulation is not observed in boys with DMD (Perkins and Davies 2002). To overcome the limitations of the MDX mouse, a second mouse model with both dystrophin and utrophin genetically ablated was developed (Deconinck et al. 1997). These double knockout (DKO) mice have a similar phenotype to that seen in boys with DMD, providing an alternative model for studying the disease where there is significant muscle deterioration and weakness.

Associated with the muscle deterioration found in patients and murine models of DMD are increased proteasome activities, protein aggregates, and a presence of oxidative stress (Kumamoto et al. 2000; Selsby et al. 2010; Niebroj-Dobosz and Hausmanowa-Petrusewicz 2005; Whitehead et al. 2008; Tidball and Wehling-Henricks 2007). The proteasome is a multi-subunit protein complex whose 20S catalytic core is composed of two outer rings of the constitutively expressed α subunits and two inner rings of β subunits. Among the β subunits, three pairs of subunits are catalytically active. Proteasomes can be classified to different subtypes based on the catalytic subunits that are present in the 20S core. The standard proteasome has catalytic subunits composed of $\beta 1$, $\beta 2$, and $\beta 5$, which perform caspase-, trypsin- and chymotrypsin-like protease activities, respectively. The standard subunits can be replaced in nascent proteasomes with the inducible subunits LMP2 ($\beta 1i$), MECL ($\beta 2i$), and LMP7 ($\beta 5i$) to form the core of the immunoproteasome. One of the well-described functions of the immunoproteasome is in the generation of antigenic peptides as part of immune surveillance in immune cells. However, immunoproteasome is also found in non-immune tissues, including skeletal muscles (Husom et al. 2004; Ferrington et al. 2005) and is up-regulated in response to stress and injury (Ferrington and Gregerson 2012). Further evidence suggests the immunoproteasomes possess an enhanced ability to degrade oxidized and mis-folded proteins, thus helping to regulate the homeostasis of cells (Seifert et al. 2010; Kruger and Kloetzel 2012). Collectively, these data support an emerging role for immunoproteasome that involves regulation of cellular homeostasis.

To date, studies investigating the potential role of proteasome inhibitors in DMD focused on general proteasome inhibitors such as MG-132 and Velcade, using MDX mice as an animal model (Bonuccelli et al. 2003; Bonuccelli et al. 2007; Gazzzerro et al. 2010; Assereto et al. 2006). Although these general proteasome inhibitors show some promise in the attenuation of muscle deterioration, two major caveats exist that include lack of specificity for the subtypes of proteasome and proteasome inhibition-induced toxicity (Hollinger and Selsby

2013). Inhibitors that selectively target proteasome subtypes that are responsible for the deterioration in muscle quality would be the logical choice to overcome these caveats. However, the characterization of the proteasome subtypes has not been identified in animal models of DMD. Therefore, in this study, we performed a parallel comparison of two animal models of DMD to gain a more complete understanding of muscle morphology, function, damage, and proteolysis pathways (ubiquitin/proteasome and autophagy/lysosome pathways). Specifically, we focus on proteasome activity and content of both the standard and inducible proteasome subunits.

Materials and Methods

Animal models

Three genotypes of mice [wild type (WT, n=16), dystrophin-deficient (MDX, *mdx*^{-/-}, n=20), and dystrophin/utrophin-deficient double-knockout mice (DKO, *mdx*^{-/-}:*utrn*^{-/-}, n=17)], aged between 45 to 57 days, were used in the current study. The WT mice (C57BL/6) were purchased from NIH, and the MDX and DKO mice, which background strain is also C57BL/6 were purchased from the Dystrophic Mouse Colony at the University of Minnesota. Animals aged between 45-57 days were chosen in this study because both MDX and DKO mice show vigorous muscle degeneration and regeneration at this age. In addition, the up-regulation of utrophin in MDX mice has not reached its peak at this age, thus decreasing the influence of the compensatory up-regulation of utrophin on our outcomes (Rafael et al. 2000; Dangain and Vrbova 1984; Davies and Nowak 2006). The University of Minnesota Institutional Animal Care and Use Committee approved the protocol for this study.

Muscle harvesting

Mice were anesthetized with pentobarbital sodium (50mg/kg body weight) immediately after the overall muscle function test. For the histological experiments, the extensor digitorum longus (EDL) muscles were investigated because this muscle is the most common muscle examined in DMD investigations. The muscles were harvested, placed on corks in embedding medium, and frozen in nitrogen-cooled isopentane. Frozen EDL muscles were stored at -80°C until serial cross-sections were performed. For biochemical experiments (Western blot and proteasome activity assay), hindlimb muscles including quadriceps and hamstrings were dissected, immediately frozen in liquid nitrogen and stored at -80°C. Lastly, blood samples were collected from the heart with uncoated capillary tubes (Chase, Scientific glass, Inc, Rockwood, TN 37854) and centrifuged. After the centrifugation, the supernatant (serum) was collected and stored at -80°C for the determination of the muscle-specific isoform of creatine kinase.

Overall muscle function

The overall muscle function was determined by the inverted grid cling (Mahowald et al. 1988; Graber et al. 2013). This task requires effective grasping ability of both forepaws and hindpaws of the mouse. Initially, the mouse was placed on a grid and was scored on its ability to hold onto the grid in three sequential conditions: hanging sideways (vertical position), hanging upside down, and hanging upside down while the grid is shaken. The

scoring scale was 0-3, where “0” represents failure of the mouse to hold onto the grid when the grid is put into the vertical position; “1” is when the mouse falls off as soon as the grid is turned upside down; “2” is when the mouse falls off as soon as the grid is shaken in the upside down position; and “3” is when the mouse does not fall off the grid after 10 seconds of shaking in the upside down position (Table 1). Incremental scores are based on the time the mouse remains on the grid under each specific condition.

Creatine kinase activity

The muscle-specific isoform of creatine kinase (CK) in the blood is a clinical indicator of muscle damage that occurs when there is damage to the sarcolemma, which allows for leakage of CK from the muscle into the blood. Muscle CK activity was determined by VITROS CK Slides (Vitros, United Kingdom) at a wavelength of 670 nm and 37°C.

Histology

The percentage of muscle fibers with central nuclei and the cross-sectional areas (CSA) of single muscle fibers were determined from histological cross sections. Briefly, frozen EDL muscles were sectioned (10µm thick) from the muscle mid-belly on a cryostat (Leica CM 3050 S, Nussloch, Germany) cooled to -25°C. The sections were stained with Hematoxylin and Eosin and imaged at 40X with a microscope (Nikon Eclipse E400). Muscle fibers with central nuclei were identified and counted from photomicrographs. CSA was determined by circling all the myofibers in each muscle (n=9-10 mice for each genotype of mice, n=500-600 myofibers for each muscle) with ImageJ software (<http://rsb.info.nih.gov/ij>).

Proteasome purification for proteasome activity assay and proteasome subunits determination

Proteasome-enriched muscle homogenates were prepared from hindlimb muscles as described (Husom et al. 2004). Briefly, hindlimb muscles were homogenized in buffer (0.1 M KCl, 20 mM MOPS, pH 7.0) followed by centrifugation at 4000g, 4°C for 20 minutes. The supernatant was collected and centrifuged at 11800g for 20 minutes at 4°C. Next, the supernatant from the second centrifugation was spun at 100,000g for 16 hours at 4°C. The resulting pellet was homogenized in buffer containing 50 mM Tris-HCl, 5 mM MgCl₂, 0.1% CHAPS and 0.4% sucrose (pH 7.5). Proteasome-enriched homogenates were used for the proteasome activity assay and Western blots for proteasome subunits. Protein concentrations of homogenates were determined using BCA protein assay kit (Pierce) with bovine serum albumin as the standard.

Western blots

The expression of utrophin, dystrophin, proteasome subunits, muscle-specific ring finger protein 1 (MuRF1) and microtubule associated protein light chain 3 (LC3) in hindlimb muscles of the three mouse genotypes were determined by Western blot. Briefly, proteins (20µg for utrophin, dystrophin and proteasome subunits β1, β5, LMP2 and LMP7; 7µg for the proteasome subunit α7; 10µg for MuRF1; 16µg for LC3) were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels (5% for utrophin and dystrophin; 13% for proteasome subunits; 4-15% gradient gels for MuRF1; 17% for LC3) using mini-vertical gel

electrophoresis units (BIO-RAD). Proteins were transferred to PVDF membranes (utrophin, dystrophin and proteasome subunits were by Mini Transfer-Blot Cell (BIO-RAD) at 110V for 3 hours; MuRF1 was by Trans-blot SD semidry transfer cell (BIO-RAD) at 12 volts for 25 minutes; LC3 was by Trans-blot SD semidry transfer cell (BIO-RAD) at 11 volts for 23 minutes). Positive controls for dystrophin, utrophin (Santa Cruz Biotechnology, Santa Cruz, CA), proteasome 20S core (isolated by the Ferrington lab) (Ferrington et al. 2005), MuRF1 (Santa Cruz), and LC3 (Novus Biologicals, Littleton, CO, USA) were included on each blot.

The protein bound PVDF membranes were incubated overnight at 4°C with the primary antibody (dystrophin and utrophin primary antibodies were from Santa Cruz Biotechnology, dilution rate 1:1000; proteasome subunit antibodies were from Biomol, Plymouth Meeting, PA, dilution rate 1:1000; MuRF1 were from Santa Cruz, dilution rate 1:500; LC3 were from Novus Biologicals, dilution rate 1:500). Blots probed with the primary antibody were incubated with goat anti-rabbit IgG-HRP (Fisher Scientific with 1:12000 dilutions for utrophin, dystrophin and proteasome subunits; Santa Cruz with 1:2500 dilutions for MuRF1; BioRad with 1:2000 dilutions for LC3) at room temperature for 1 hour. After the incubation, Blots were developed in substrate for 5 minutes (SuperSignal West Dura, Thermo Scientific) and images were captured using a Chemidoc system (BIO-RAD). The optical density (OD) of each sample was obtained using Quantity One software package (BioRad). In order to compare samples across multiple blots, an internal control (standard sample) was loaded on each blot and the intensity of protein-specific bands of all samples were normalized to the intensity of the standard sample.

Proteasome activity

Proteasome activities were determined by measuring the degradation of fluorogenic peptides as described previously (Ferrington et al. 2005). In brief, 3 µg of homogenate was incubated either with or without proteasome inhibitor MG132 (0.2 mM) (Peptide Institute, Louisville, KY) in 100µl of reaction buffer (50mMTris, pH 7.8) at 37°C for 30 minutes. After the incubation, 100µL of fluorogenic peptides (200µM for LLE-AMC, 150µM for VGR-AMC, and 75µM for LLVY-AMC dissolved in buffer (40mMTris (pH 7.5), 20 mM KCL, 10 mM MgCl₂, and 0.5mM ATP) were added into sample wells. Note that the fluorogenic peptides LLE-AMC, VGR-AMC and LLVY-AMC were used as model substrates to test the caspase-, trypsin-, and chymotrypsin-like activities of the proteasome, respectively. The cleavage of substrates was measured (360 nm excitation/460 nm emission) for 2 hours at 5-minute intervals using a fluorescent plate reader (Cytofluor 4000 Multiwell Plate Reader, Applied Biosystems, Foster City, CA). Activity was determined by comparing peptide fluorescence from samples with fluorescence of a standard curve of amino methyl coumarin. The difference between assays with or without MG132, a proteasome inhibitor, represented the proteasome-specific activity.

Statistics

Data are presented as mean ± SEM. One-way analysis of variance (ANOVA) was used to determine the differences among the three genotypes. Tukey-Kramer Multiple Comparison Tests were used as a post-hoc test. Chi-Square test was used to test differences of the percentages of very small fibers (CSA < 500 µm²), normal size fibers (CSA between

500-3000 μm^2) and abnormally large fibers ($\text{CSA} > 3000 \mu\text{m}^2$) among the three genotypes of mice. Significance was set at $p < 0.05$.

Results

Characterization

MDX and DKO mice aged 45-57 days were compared in parallel to characterize the muscle morphology and function, and proteasome activity and subtype content. Age, animal number, and body weight for the three genotypes of mice are shown in Table 2.

Utrophin, a homologue of dystrophin, is up-regulated in the regenerating myofibers in MDX mice and has been proposed to compensate for the loss of dystrophin in these mice (Deconinck et al. 1997). To determine the expression of utrophin in hindlimb skeletal muscles of the three genotypes, utrophin protein content was assessed by Western blots. As shown in Fig 1, MDX mice had an average 6-fold increase in utrophin protein expression compared with WT mice. As predicted from the gene knockout, DKO mice did not express utrophin.

Overall Muscle Function

To evaluate the effect of genotype-associated changes in the overall muscle function, we measured mouse grip performance (Table 1). Fig 2 shows that the overall muscle function of WT mice was better than both MDX mice (70% of that in WT) and DKO mice (61% of that in WT) ($p < 0.05$). There was no significant difference in the overall muscle function between MDX and DKO mice.

Muscle Damage

Creatine kinase activity in the blood was used as a marker of muscle damage. We found that the activity of the muscle-specific isoform of creatine kinase in the blood of MDX and DKO mice was about 3-fold greater than the level in WT mice (Fig 3). Similar levels of creatine kinase were measured in MDX mice and DKO mice. These results suggest significant damage to the sarcolemma has occurred in both models of DMD.

Muscle Morphology

The percentage of central nuclei is an indicator of the extent of regeneration that has occurred in a muscle. The percentages of central nuclei in the EDL muscles were significantly greater ($p < 0.005$) in the MDX mice and DKO mice compared to WT mice. The percentage of central nuclei in the WT mice was $0.4 \pm 0.12\%$, and the percentages were $61 \pm 2.9\%$ and $64 \pm 2.1\%$ in the MDX and DKO mice, respectively (Fig 4). These results indicate significant muscle remodeling is ongoing in both models of DMD.

Myofiber cross sectional area (CSA) reflects the status of muscle atrophy or hypertrophy. We found that the average CSA of individual EDL muscle fibers was significantly smaller in DKO mice compared to WT and MDX mice. The average CSA of the myofibers was $1339 \pm 12 \mu\text{m}^2$, $1280 \pm 12 \mu\text{m}^2$ and $855 \pm 6 \mu\text{m}^2$ in WT, MDX and DKO mice, respectively. Interestingly, when we looked at the variation of fiber size within a muscle by plotting the

distribution of the CSA of muscle cells in EDL, we found that the percentages of very small fibers (CSA < 500 μm^2), normal size fibers (CSA between 500-3000 μm^2) and abnormally large fibers (CSA > 3000 μm^2) among the three genotypes of mice differed significantly. Specifically, DKO mice have a preponderance of smaller muscle fibers. The percentages of very small fibers are 26.35%, 16.33% and 5.54% for DKO, MDX and WT, respectively. In addition, MDX mice have a greater percentage of abnormally large fibers compared to WT and DKO mice. The percentages of abnormally large fibers are 0.48%, 5.3% and 1.82% for DKO, MDX and WT respectively (Fig 5). The abnormally large fibers seen in the MDX may be associated with pseudohypertrophy (muscle hypertrophy related to the increase in the size or number of non-contractile elements) of muscles. Collectively, MDX and DKO mice had greater percentage of small myofibers than WT mice, suggesting muscle atrophy occurs in the two animal models of DMD. In addition, DKO mice showed more severe muscle atrophy than MDX mice.

Protein levels of MuRF1 and LC3

Loss of muscle mass is mainly regulated by the ubiquitin/proteasome and autophagy/lysosome pathways. Specifically, MuRF1 is a protein in the ubiquitin/proteasome pathway and has been found to be up-regulated in multiple models of muscle atrophy (Glass, 2010). LC3 is a marker of the autophagy/lysosome pathway that degrades defective organelles and aggregated proteins (Mizushima and Yoshimori, 2007). The autophagy/lysosome proteolysis pathway has been found to be up-regulated in many models of myopathy (Sandri, 2010). In the current study, we found that MuRF1 protein content was not different among the three genotypes (Fig 6a). However, the protein expression of LC3-I and LC3-II of MDX and DKO mice was greater than the levels in WT mice (Fig 6b, 6c). Protein content of LC3-I and LC3-II in muscles of MDX and DKO mice was the same.

Proteasome Function and Structure

Up-regulation of the proteasome, the major protease responsible for degrading oxidized proteins, has been associated with atrophy of dystrophic muscles (Gazzerro et al. 2010; Briguët et al. 2008). In the current study, we found that the overall proteasome activities of hindlimb muscles were highest in DKO mice. Chymotrypsin-like ($\beta 5/\text{LMP7}$) proteasome activities in DKO mice were 200% greater compared to that in both WT and MDX mice. Caspases-like ($\beta 1/\text{LMP2}$) proteasome activities in DKO mice were 132% and 32% higher than that in WT and MDX mice, respectively. Trypsin-like ($\text{P2}/\text{MECL}$) proteasome activities in DKO mice were 102% and 64% greater compared to that in WT and MDX mice, respectively (Fig 7).

To understand whether the genotype-specific proteasome activities were associated with changes in the proteasome subtypes, the content of the total proteasome ($\alpha 7$), catalytic subunits of the standard proteasome subunits ($\beta 1$, $\beta 5$), and immunoproteasome subunits (LMP2, LMP7) were determined. As shown in Fig 8a, no significant difference was observed in total proteasome ($\alpha 7$) content. Similarly, the content of the standard proteasome subunits, $\beta 5$ and $\beta 1$, were not significant different among the three genotypes of mice (Fig 8b, 8c). However, the content of inducible immunoproteasome subunits, LMP2 and LMP7, were different among genotypes. MDX mice had higher level of LMP2 and

LMP7 compared to WT mice. In addition, the LMP7 content in the DKO mice was greater than WT mice (Fig 8d, 8e).

Discussion

The use of proteasome inhibitors to reduce the rate of muscle loss in dystrophic muscles shows promise (Bonuccelli et al. 2003; Bonuccelli et al. 2007; Gazzero et al. 2010; Assereto et al. 2006). However, toxicity was found to be a major drawback and this toxicity was attributed to the use of proteasome inhibitors that broadly impact all proteasome subtypes (Hollinger and Selsby 2013). Inhibitors that selectively target the proteasome subtype that is responsible for the loss in muscle mass and quality would reduce off-target side effects and be less toxic. However, studies characterizing the proteasome activity and subtypes in muscles from DKO and MDX mice are lacking. Additionally, the proteasome subtype that is responsible for the muscle deterioration observed in DMD has not been defined; yet this information is critical for guiding the development of selective inhibitors. Hence, as the first step, we characterized and compared muscle function, morphology and damage, as well as proteasome activity and subtype populations in three genotypes of mice, WT, MDX and DKO mice. Because of the extensive muscle remodeling in MDX and DKO mice and the onset of up-regulation of utrophin in MDX mice, we selected 45-57 days old mice.

The main findings of this study are (1) dystrophic muscles expressed higher levels of immunoproteasome subunits, (2) greater proteasome activity was observed in dystrophic muscles, especially in muscles of DKO mice, and (3) DKO mice exhibited more severe muscle atrophy than MDX mice.

Proteolysis activity is elevated in dystrophic muscles, especially in muscles of DKO mice

We found that proteasome activities increased significantly in dystrophic muscles, especially in muscles of DKO mice. The finding of the increased proteasome activity in dystrophic muscles is consistent with results of previous studies investigating MDX mice and DMD patients (Gazzerro et al. 2010; Kumamoto et al. 2000). Kumamoto et al. investigated muscle biopsy specimens immunohistochemically and showed concentrated proteasome in necrotic fibers and atrophic fibers among dystrophin-deficient muscle fibers of DMD patients (Kumamoto et al. 2000). Although we did not investigate the localization of proteasome on histological cross sections, we did find the DKO mice had the highest percentage of very small fibers (less than 500 μm^2) among the three genotypes, suggesting that the higher proteasome activity is present in muscle tissue undergoing the most extensive remodeling and atrophy. In contrast to the proteasome activities, the muscles of the three genotypes had similar MuRF1 levels. Although previous findings suggest elevated levels in muscles that are remodeling, this finding is not unexpected because MuRF1 is one out of over 600 E3 ubiquitin ligases and each of the E3 ligases has its specific protein targets (Deshaies and Joazeiro, 2009). Thus, other E3 ubiquitin ligases may contribute more to the increased proteasome activity in the dystrophic muscles. Further examination is needed.

The content of immunoproteasome is increased in dystrophic muscles

The novel finding in the current study is that the inducible subunits of the immunoproteasome are increased in dystrophic muscles. Specifically, we found that while the total proteasome content is not different between normal and dystrophic muscles, the content of LMP2 was significantly greater in muscles of MDX mice and the content of LMP7 was greater in muscles of MDX and DKO mice compared to WT mice. The induction of immunoproteasome subunits has been reported in cardiac muscles of diabetic mice, which the authors suggest as contributing to the reduction of myosin heavy chain protein (Zu et al. 2010). The increase of LMP2 and LMP7 content was also found under conditions of chronic oxidative stress, such as retinal cells from human donors with age-related macular degeneration, and cultured endothelial cells and retinal pigment epithelial cells exposed to chronic oxidative stress (Ethen et al. 2007; Kotamraju et al. 2006; Hussong et al. 2010). Regarding DMD, oxidative stress is one of the proposed mechanisms triggering muscle atrophy (Niebroj-Dobosz and Hausmanowa-Petrusewicz 2005; Whitehead et al. 2008; Tidball and Wehling-Henricks 2007; Buetler et al. 2002). Our finding of increased immunoproteasome subunits is likely a response to the increased oxidative stress of dystrophic muscles. It is not possible to conclude from our study the direct association between the increased proteasome activity and the increased immunoproteasome expression since both the standard proteasome and immunoproteasome cleave the fluorogenic peptide substrates used in the assay of activity. We postulate that the overall elevated activity is likely due to the increased demand for removal of the damaged and oxidized proteins as part of maintaining cellular homeostasis (Kruger and Kloetzel 2012; Ferrington and Gregerson 2012).

The extent of muscle atrophy and the variation of fiber size are greater in DKO

We found that the average cross sectional area (CSA) of EDL muscle fibers is smaller in DKO mice compared to that in WT and MDX mice. This finding is consistent with the result of the study of Cole et al., where the CSA of gastrocnemius fibers were measured (Cole et al. 2002). In addition to the average CSA, we found that the percentage of very small fibers (less than 500 μm^2) is greater in dystrophic muscles and DKO mice had the highest percentage of very small fibers. The findings of the greater percentage of very small fibers and less percentage of normal size fibers in dystrophic muscles were consistent with our finding that LC3 protein content increased in muscles of MDX and DKO mice. Collectively, both ubiquitin/proteasome and autophagy/lysosome pathways play a role in the loss of muscle mass of dystrophic mice. In addition, DKO mice have larger decrease of muscle size compared to that in MDX mice and the DKO mice also have greater proteasome activity than MDX mice. The proteasome is the major proteolytic complex that degrades cytoskeletal proteins, such as actin and myosin, and thus, the elevated activity likely contributes to the significant ongoing muscle atrophy in dystrophic muscle. Indeed, proteasome inhibition has been shown to significantly attenuate the reduction in fiber size in muscles of MDX mice (Briguet et al. 2008; Gazzerri et al. 2010) and thus, may be a viable therapeutic strategy for preserving muscle mass. If the major contributor to muscle fiber atrophy and degeneration is the immunoproteasome, then inhibitors that target this molecule and preserve function of the standard proteasome would be less toxic to general cell

function. However, a more clear understanding of the role that each proteasome subtype contributes to muscle function is required in order to design more effective therapies.

Muscle damage and regeneration occur in dystrophic muscles

Creatine kinase (CK) is an intracellular enzyme that is released into the bloodstream when muscle cells are injured. Indeed, CK activity in the blood is a useful tool used in clinical settings to monitor the disease progression of DMD. In the current study, we found that the activity of CK in the blood of MDX and DKO mice was not different while it was greater than that in WT mice, suggesting muscle injury in these two DMD genotypes. The equivalent CK activity between MDX and DKO mice was consistent with the finding of the study of Deconinck et al, where membrane integrity of muscles was similar between MDX and DKO mice (Deconinck et al. 1997). Collectively, our finding, together with others, suggests that the muscle integrity of MDX and DKO mice aged between 45-57 days is compromised.

The percentage of centrally nucleated fibers in a muscle is an indicator of the amount of regeneration occurred in that muscle. In the current study, we found the percentage of centrally nucleated fibers was greater in dystrophic muscles compared to that in normal muscles. In addition, the percentages of centrally nucleated fibers were not different between muscles of MDX and DKO mice. This finding is consistent with previous studies investigating dystrophic muscles during the period of muscle degeneration and regeneration (Deconinck et al. 1997; Grady et al. 1997; Rafael et al. 2000). In summary, dystrophic muscles possess regenerative capacity during the period when muscles are undergoing degeneration and regeneration; in addition, the regeneration capacity is similar MDX and DKO muscles.

Muscle function

Muscle function, assessed by the inverted grid cling test, was found to be worse in dystrophic mice than in WT mice. However, the differences were not significant between MDX and DKO mice. Based on our findings of less fiber atrophy in MDX mice, one might predict these mice would perform better on the inverted grid cling test compared to the DKO. Our results showed MDX performance was equal to the DKO which we feel may be associated with the effect of animals' body weight. In the functional test (inverted grid cling test), the body weight significantly influences the outcomes since the animals were scored on their ability to hold onto the grid in an antigravity position (hanging sideways, hanging upside down, and hanging upside down while the grid is shaken). Muscle quality is another possible explanation. Even though the muscles from the MDX mice are larger than the muscles from the DKO mice, we did not evaluate contractility; therefore, the increased size may not be associated with an increase in functional contractile elements. Future studies are needed to evaluate the quality of the muscles.

Caveats in the data interpretation

In the current study muscle morphology was determined on EDL muscles, while the determinations of proteasome activity and proteasome subtypes were done in the remaining hindlimb muscles. Thus, the relationship between muscle atrophy and proteasome activity

and subtypes is indirect. The second limitation of this study is that we did not determine proteasome activity and subtypes in different types of muscles. Therefore, it is unknown whether the transformation of proteasome subtypes and the increase of proteasome activity in the dystrophin muscles are similar between fast-twitch muscles and slow-twitch muscles. Our previous work in aged rat muscle, which also exhibits significant muscle atrophy, suggests that proteasome transformation that favors the immunoproteasome is not dependent on fiber type since we observed similar changes in both fast- and slow-twitch muscles (Husom et al. 2004; Ferrington et al. 2005).

Conclusion

DKO mice, the animal model that displays similar clinical features of human DMD, have a greater reduction of muscle fiber size compared to MDX mice. In addition, while proteasome activities increase in both MDX and DKO muscles, the amount of increase is greater in DKO mouse muscles. Notably, we found there was a transformation of standard proteasomes to immunoproteasomes in dystrophic muscles. Findings of the current comparative study among WT, MDX and DKO mice will help the assessment and development of pharmacological strategies for muscular dystrophy.

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a **b** **Fig 1.**

Utrophin content in hindlimb muscles. Representative immunoblot (a) and summary of densitometric analysis (b) are shown from hindlimb muscles of wild type mice (WT), dystrophin-deficient mice (MDX) and dystrophin/utrophin-deficient mice (DKO).

*Significantly different from WT and DKO mice

Fig 2.

Overall muscle function of three genotypes of mice. Scores were determined from a mouse grip test. Values are mean $\pm$ SEM. *Significantly different from WT mice. WT: wild type mice, MDX: dystrophin-deficient mice, DKO: dystrophin/utrophin-deficient mice

Fig 3.

Creatine kinase activities in three genotypes of mice. Values are mean $\pm$ SEM.

*Significantly different from WT mice. WT: wild type mice, MDX: dystrophin-deficient mice, DKO: dystrophin/utrophin-deficient mice

Fig 4.

Central nuclei of EDL muscles of three genotypes of mice. H&E stained cross-sections of EDL muscles of WT mice (a), MDX mice (b), and DKO mice (c). d: Summary of the central nuclei number expressed as mean $\pm$ SEM. *Significantly different from WT mice. WT: wild type mice, MDX: dystrophin-deficient mice, DKO: dystrophin/utrophin-deficient mice

Fig 5.

The distribution of the cross sectional area (CSA) of muscle cells from EDL muscles of WT mice (a), MDX mice (b), and DKO mice (c). The percentages of very small fibers (CSA < 500 μm^2), normal size fibers (CSA between 500-3000 μm^2) and abnormally large fibers (CSA > 3000 μm^2) in three genotypes of mice (d). The cumulative percentage graph showed the shift of fiber size distribution in MDX and DKO mice (e). WT: wild type mice, MDX: dystrophin-deficient mice, DKO: dystrophin/utrophin-deficient mice

Fig 6.

The relative content of MuRF1 (a), LC3-I (b), and LC3-II (c) in the hindlimb skeletal muscles of WT, MDX and DKO mice. Values are mean $\pm$ SEM. *Significantly different from WT mice. MuRF1: muscle-specific ring finger protein 1, LC3: microtubule associated protein light chain 3, WT: wild type mice, MDX: dystrophin-deficient mice, DKO: dystrophin/utrophin-deficient mice

Fig 7.

Proteasome activities of hindlimb skeletal muscles from WT, MDX and DKO mice. Values are mean $\pm$ SEM. *Significantly different from WT mice. [†]Significantly different from MDX mice. WT: wild type mice, MDX: dystrophin-deficient mice, DKO: dystrophin/utrophin-deficient mice

Fig 8.

The relative content of the total proteasome (a), standard proteasome subunit $\beta 5$ (b), $\beta 1$ (c), and immunoproteasome subunit LMP2 (d) and LMP7 (e) in the hindlimb skeletal muscles of WT, MDX and DKO mice. Values are mean $\pm$ SEM. *Significantly different from WT mice. WT: wild type mice, MDX: dystrophin-deficient mice, DKO: dystrophin/utrophin-deficient mice

Table 1
The scoring scale of overall muscle function

Score	Description of the performance
0	The mouse falls as soon as the grid is raised to the vertical position.
0.1-0.9	The mouse holds onto the grid in the vertical position for 1-9 seconds, respectively.
1	The mouse falls off as soon as the grid is turned upside down.
1.1-1.9	The mouse holds onto the grid in the upside down position for 1-9 seconds, respectively.
2	The mouse falls off as soon as the grid is shaken in the upside down position.
2.1-2.9	The mouse holds onto the grid when the grid is shaken in the upside down position for 1-9 seconds, respectively.
3	The mouse does not fall off the grid after 10 seconds of shaking in the upside down position.

Table 2**Three genotypes of mice**

Genotype	Age (days)	Body weight (g)
WT (n=16)	56 ± 0.7	20.8 ± 0.72
MDX (n=20)	54 ± 0.2	26.7 ± 0.70*
DKO (n=17)	45 ± 0.8	12.8 ± 0.73 [†]

Values are mean ± SEM.

* Significantly different from WT and DKO mice.

[†] Significantly different from WT and MDX mice.

WT: wild type mice, MDX: dystrophin-deficient mice, DKO: dystrophin/utrophin-deficient mice.