Proteasome inhibition improves the muscle of laminin α2 chain-deficient mice

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Muscle atrophy, a significant characteristic of congenital muscular dystrophy with laminin α2 chain deficiency (also known as MDC1A), occurs by a change in the normal balance between protein synthesis and protein degradation. The ubiquitin–proteasome system (UPS) plays a key role in protein degradation in skeletal muscle cells. In order to identify new targets for drug therapy against MDC1A, we have investigated whether increased proteasomal degradation is a feature of MDC1A. Using the generated dy3K/dy3K mutant mouse model of MDC1A, we studied the expression of members of the ubiquitin–proteasome pathway in laminin α2 chain-deficient muscle, and we treated dy3K/dy3K mice with the proteasome inhibitor MG-132. We show that members of the UPS are upregulated and that the global ubiquitination of proteins is raised in dystrophic limb muscles. Also, phosphorylation of Akt is diminished in diseased muscles. Importantly, proteasome inhibition significantly improves the dystrophic dy3K/dy3K phenotype. Specifically, treatment with MG-132 increases lifespan, enhances locomotive activity, enlarges muscle fiber diameter, reduces fibrosis, restores Akt phosphorylation and decreases apoptosis. These studies promote better understanding of the disease process in mice and could lead to a drug therapy for MDC1A patients.

INTRODUCTION

Muscle atrophy is defined as a decrease in muscle mass and can result from muscle disuse and aging. It may occur in systemic conditions (diabetes, AIDS, cancer) and is a feature of several muscular dystrophies. Conceivably, it results from the imbalance between protein synthesis and protein degradation. Both the ubiquitin–proteasome system (UPS) and the autophagy–lysosome pathway play key roles in protein degradation in skeletal muscle cells. UPS degrades proteins that are tagged for degradation by several molecules of ubiquitin (1–3). Two skeletal muscle-specific ubiquitin ligases, MAFbx/atrogen-1 and muscle ring finger protein 1 (MuRF1), have been identified and they are both unswerving markers of skeletal muscle atrophy. They are significantly upregulated in various models of atrophy and mice deficient in either gene are resilient to atrophy (1,2). Two major signaling pathways seem to control the activation of MAFbx/atrogen-1 and MuRF1. First, forkhead box O (FoxO) transcription factors induce transcription of MAFbx/atrogen-1 and MuRF1, through inhibition of Akt, a serine–threonine protein kinase that plays an important role in regulating proliferation, size, differentiation and survival of muscle cells (3–7). The other signaling pathway that controls the activation of ubiquitin ligases involves NF-κB, which has been shown to activate MuRF1 (8).

Congenital muscular dystrophy type 1A (MDC1A, OMIM no. 607855) is caused by autosomal recessive mutations in the human LAMA2 gene, encoding the α2 subunit of laminin-211. MDC1A is characterized by severe muscle hypotonia early in life, generalized muscle weakness and joint contractures, and few children achieve the ability to walk. Around 30% of the patients die within their first decade of life (9,10). The histological hallmarks of laminin α2 chain-deficient muscles include degeneration/regeneration cycles, fiber size variability, apoptosis and marked connective tissue proliferation. Also, skeletal muscle atrophy is a prevalent feature of MDC1A (9,10). A number of mouse models for laminin α2 chain deficiency exist, including the generated null mutant dy3K/dy3K. This mouse model presents severe muscular dystrophy and peripheral neuropathy (11,12). Muscle fiber degeneration is evident
from postnatal day 9 in dy3K/dy3K mice and they die at around 4 weeks of age (11).

Laminin α2 chain binds to two major cell surface receptors on skeletal muscle cells: dystroglycan and integrin α7β1 (13–15). Dystroglycan is a member of the dystrophin–glycoprotein complex (DGC). Recently, this complex was shown to interact with phosphatidylinositol-3-kinase, one of the upstream activators of Akt, and Akt phosphorylation was demonstrated to be dependent on laminin binding (16). Integrins transduce important signals, but their cytoplasmic domains lack enzymatic activity. Hence, integrin signals are transduced through accessory molecules, which trigger downstream signaling cascades involving proteins such as Akt (17). Thus, it is possible that laminin α2 chain deficiency leads to diminished Akt phosphorylation (through both dystroglycan and integrin α7β1). Reduced Akt phosphorylation, in turn, may lead to decreased survival of the muscle fiber and increased activity of UPS. Indeed, it has been shown that the UPS is involved in DGC protein degradation in Duchenne muscular dystrophy (DMD, OMIM no. 310200) (23–25).

Using the dy3K/dy3K mouse model of MDC1A, we here show that members of the UPS are upregulated in dystrophic limb muscles and that the global ubiquitination of proteins is raised. We further demonstrate that transient inhibition of the proteasome significantly improves the dystrophic phenotype. These findings suggest a novel pathogenic mechanism whereby increased activity of the UPS contributes to atrophy in MDC1A.

RESULTS

Increased expression of ubiquitin–proteasome-related components in laminin α2 chain-deficient muscle

To elucidate whether proteasome activity is increased in MDC1A muscle, we first analyzed the expression of members of the ubiquitin–proteasome pathway in dy3K/dy3K animals. We detected significantly increased mRNA levels of the key ubiquitin ligases MAFbx/atrogen-1 and MuRF1 in quadriceps muscles from dy3K/dy3K mice (Fig. 1A). We also

Figure 1. Components of the ubiquitin–proteasome pathway are upregulated in laminin α2 chain dy3K/dy3K-deficient muscles. (A) Relative amounts of MAFbx/Atrogin-1 and MuRF1 mRNAs in 3.5-week-old wild-type (n = 7) versus dy3K/dy3K mice (n = 6) and in 3.5-month-old wild-type versus dy3KLNα1TG mice (n = 3 for both). The β-actin gene expression served as a reference. (B) Left panel: Levels of global protein ubiquitination from whole-muscle extracts from hind limbs from dy3K/dy3K and age-matched wild-type mice at 6 days (P6) and 3.5 weeks of age and from dy3KLNα1TG and age-matched wild-type mice at 5.5 weeks of age (n = 6 per genotype). Right panel: Densitometry analysis of global protein ubiquitination and representative western blot image of ubiquitination in quadriceps muscle from wild-type and dy3K/dy3K mice (3.5-week-old; n = 6 per group). The intensity within the entire lane was quantified. Results are expressed in arbitrary units (AU). Labeling of tubulin served as an internal loading control. (C) Co-immunostaining on cross-sections of quadriceps muscles from wild-type (n = 3) and dy3K/dy3K (n = 5) mice: In red, 20S proteasome α1 subunit (a and b), β1 subunit (c and d) and MuRF1 (e and f) and in green, laminin γ1 chain (a–f). Secondary antibody control labeling was performed on cross-sections from dy3K/dy3K quadriceps muscle (g and h). Bar = 40 μm.
assessed muscles from dy3KLNα1TG mice. In these laminin α2 chain-deficient animals, muscular dystrophy and peripheral neuropathy is significantly prevented upon transgenic overexpression of laminin α1 chain (26–28). Most changes in gene and protein expression that occur due to laminin α2 chain deficiency and the subsequent dystrophic processes are expected to be normalized in dy3KLNα1TG muscles and therefore dy3KLNα1TG animals served as an additional control group. Indeed, expression of MAFbx/atrogin-1 and MuRF1 mRNAs was not altered in quadriceps muscles from dy3KLNα1TG mice (Fig. 1A). Next, we discovered an increase in the level of total protein ubiquitination in dy3K/dy3K muscles seen in the protein level. Significantly more MuRF1-positive fibers were seen in dy3K/dy3K quadriceps muscles in comparison to wild-type muscles (Fig. 1C). Also, immunofluorescence analyses revealed that the expression of the core proteasome subunits α1 and β1 was increased within dy3K/dy3K quadriceps muscle fibers in comparison to wild-type muscles (Fig. 1C). Also, the expression of MuRF1 mRNA expression was confirmed at the global muscle level of laminin α2 chain-deficient animals. Indeed, we detected enhanced ubiquitination in pooled dy3KLα1TG limb muscles (at 3.5 weeks of age) (Fig. 1B). Interestingly, total protein ubiquitination appeared normal in dy3K/dy3K muscles at 6 days of age and in dy3KLNα1TG mice, suggesting that this phenomenon is associated with the presence of dystrophic features (Fig. 1B). Also, immunofluorescence analyses revealed that the expression of the core proteasome subunits α1 and β1 was increased within dy3K/dy3K quadriceps muscle fibers in comparison to wild-type muscles (Fig. 1C). Also, the expression of MuRF1 mRNA expression was confirmed at the protein level. Significantly more MuRF1-positive fibers were seen in dy3K/dy3K quadriceps muscle compared with wild-type muscles (P < 0.001) (Fig. 1C).

Finally, the transcription of MAFbx/atrogin-1 and MuRF1 mRNAs is under the control of FoxO transcription factors, and MuRF1 is also activated by NF-κB (7,8). By quantitative real-time PCR, we found that the mRNA levels of transcription factors FoxO1, FoxO3 and NF-κB were all significantly increased in dy3K/dy3K quadriceps muscles but not in corresponding dy3KLNα1TG muscles (Fig. 2). All together, these data indicate a higher activity of the UPS machinery in laminin α2 chain-deficient muscle.

**Inactivation of Akt and reduced expression of Hsp90 in laminin α2 chain-deficient muscle**

Akt controls proteasome protein breakdown in skeletal muscle (7,29–31). Downregulation of its activity leads to atrophy (32), whereas stimulation of Akt leads to skeletal muscle hypertrophy (3). Thus, it is quite likely that Akt activity is reduced in dy3K/dy3K muscle. Consequently, we analyzed phosphorylated Akt levels in laminin α2 chain-deficient muscle. Indeed, phosphorylation on both threonine 308 and serine 473 was diminished in dy3K/dy3K quadriceps muscle whereas the total level of Akt was unchanged (Fig. 3). Hence, Akt appears to be insufficiently activated in laminin α2 chain-deficient muscle.

We next aimed at identifying proteins involved in regulating Akt activity in laminin α2 chain-deficient muscle. Regulation of the kinase activity of Akt is complicated. The two regulatory sites on Akt are phosphorylated by PDK1, which phosphorylates threonine 308 and other kinases, which phosphorylate serine 473 (33). We found that PDK1 phosphorylation was significantly decreased in dy3K/dy3K quadriceps muscle (Supplementary Material, Fig. 1). It has been previously shown that Hsp90 forms a complex with Akt, and that Hsp90 may function to balance the phosphorylation state of Akt during myoblast differentiation (36). Hence, we analyzed Hsp90 expression in dy3K/dy3K lower limb muscles and individual quadriceps and gastrocnemius muscles. The expression of Hsp90 was significantly reduced in pooled limb muscles and in individual quadriceps (Fig. 4A) and gastrocnemius (data not shown) muscles. The expression appeared normal in dy3K/dy3K muscles at 6 days of age and in dy3KLNα1TG muscles (Fig. 4A). Interestingly, immunoprecipitation analyses indicated that Hsp90 from dy3K/dy3K muscle still binds Akt (data not shown). Hence, the capacity of Hsp90 to bind Akt is not modified in dystrophic muscle. These results suggest that the lowered phosphorylation state of Akt in dy3K/dy3K muscle might result from decreased PDK1 phosphorylation and the restricted pool of Hsp90.

**Systemic injection of MG-132 improves laminin α2 chain-deficient muscle morphology**

Since the UPS system seemed to be greatly affected in dy3K/dy3K muscle, we envisaged that inhibition of the proteasome could improve muscle shape and physiology. Thus, we next administered MG-132 into the tail vein of 2.5-week-old dy3K/dy3K mice. At this age, the dy3K/dy3K mice can be...
The dy3K/dy3K mice die at around 4 weeks of age. Therefore, we analyzed mice and muscles 18 days post-injection (a time point when dy3K/dy3K mice should be dead). Remarkably, the systemic injection resulted in considerably improved muscle morphology (Figs 5 and 6). We first evaluated the presence of pathological fibrosis. It has previously been shown that the extracellular matrix protein tenascin-C is upregulated and extends to the interstitium between muscle fibers, especially within focal lesions of laminin α2 chain-deficient muscle (37). Notably, tenascin-C expression was reduced in MG-132-injected dy3K/dy3K mice compared with non-injected dy3K/dy3K mice (Fig. 5B). To further confirm the reduction of fibrosis in MG-132-treated animals, we analyzed collagen III expression (38), which was also significantly decreased in MG-132-injected animals (Fig. 5B). The number of cells with centrally located nuclei was slightly decreased in injected animals (Fig. 6A). We also assessed the muscle fiber number in randomly selected areas. The number of fibers appeared not to be reduced in laminin α2 chain-deficient quadriceps muscle and the number of fibers was not further affected by MG-132 injections (Fig. 6B). The average fiber diameter, on the other hand, was significantly reduced in dy3K/dy3K muscle. Notably, the average fiber diameter was normalized upon MG-132 injection and fiber size distribution in quadriceps muscle was significantly shifted towards larger fibers (Fig. 6C and D). We observed that ~17% of dy3K/dy3K quadriceps fibers have a diameter inferior to 26 μm, whereas the number is about 2 and 7% in wild-type and injected animals, respectively. Furthermore, the ratio of quadriceps muscle wet weight per body weight was significantly reduced in dy3K/dy3K mice, compared with age-matched...
wild-type animals. Interestingly, this ratio was significantly increased in MG-132-injected dy3K/dy3K mice (Fig. 6E).

In order to test whether additional injections of MG-132 would provide additional benefits, we intravenously administered MG-132 to 2.5-week-old dy3K/dy3K mice and 1 week later, mice received a second injection. However, the additional injection did not improve muscle morphology further (Figs 5 and 6, orange panels). Also, double injections did not show any beneficial effects over single injection on the other main outcome measures we used to assess efficacy of treatment (Figs 9 and 10).

Systemic injection of MG-132 restores Akt phosphorylation and normalizes compensatory changes of laminin α4 and β2 chain expression

To determine whether the systemic injection of MG-132 could transiently restore Akt activity in dy3K/dy3K skeletal muscle, we sacrificed injected mice 24 h and 18 days after injection. Twenty-four hours after MG-132 injection, Hsp90 levels were increased and phosphorylation on both threonine 308 and serine 473 was enhanced although phosphorylation on serine 473 was not restored to wild-type levels. (Fig. 7A–C, top histograms). At day 18, Akt phosphorylation on both sites remained augmented in injected mice, but Hsp90 levels were low (Fig. 7A–C, bottom histograms).

We also investigated the expression of laminin α4 and β2 chains in MG-132-treated dy3K/dy3K mice. It has previously been shown that expression of laminin α4 chain is increased at dy3K/dy3K sarcolemma (27,39). Laminin β2 chain expression, on the other hand, is reduced at the sarcolemma of laminin α2 chain-deficient muscle (27,40). Expression of both proteins was near normal in injected mice; laminin α4 chain was significantly decreased even if some weak labeling was still visible in restricted areas and laminin β2 chain was restored at the sarcolemma (Fig. 8).

Apoptosis is decreased after systemic injection of MG-132

As apoptosis contributes to the disease progression, we analyzed the apoptosis rate occurring in skeletal muscle from systemically injected mice. In dy3K/dy3K mice, the number of caspase-3-positive fibers (containing caspase-3 and pro-caspase-3 proteins) was significantly increased when compared with controls (Fig. 9A and B). Eighteen days after injection, we were still able to find caspase-3-positive fibers but in significantly less proportion (Fig. 9A and B). Moreover, we also used the TUNEL enzymatic labeling assay to further determine the proportion of apoptosis in injected mice.
We found that the number of TUNEL-positive myonuclei was significantly diminished in MG-132-treated animals compared with non-treated dy3K/dy3K mice (Fig. 9C).

The lifespan and the locomotion of dy3K/dy3K mice are improved by proteasome inhibition

We also compared the overall health status of the animals (Supplementary Material, Videos 1–3). Accordingly, we investigated whether the systemic injection of MG-132 contributed to better locomotion and longer lifespan. Whereas the dy3K/dy3K mice were less active in an open field test, the systemically injected batch, 18 days after the systemic injection, displayed the same level of activity as control littermates (Fig. 10A). Moreover, dy3K/dy3K mice after treatment survived longer than the non-injected animals (median survival 41 and 22 days, respectively) (Fig. 10B).

Systemic injection with MG-132 is of no apparent help in peripheral nerve

Lastly, although survival and muscle morphology was significantly improved, one hind leg of MG-132-injected dy3K/dy3K mice was often transiently paralyzed (at day 18 after injection). A similar paralysis occurs in uninjected dy3K/dy3K animals (26). Yet, the transient paralysis of MG-132-injected mice had no obvious effect on locomotive behavior (Fig. 10A). Hence, MG-132 did not appear to appreciably improve the pathology of the peripheral nerve. In agreement with this observation, we found neither increased levels of ubiquitination nor decreased levels of Hsp90 in dy3K/dy3K sciatic nerve. Phosphorylation on threonine 308 was diminished in dy3K/dy3K sciatic nerve, whereas phosphorylation on serine 473 and total Akt levels were not changed (Supplementary Material, Fig. S2).

DISCUSSION

MDC1A is a devastating muscle disorder for which there is currently no treatment. Several transgenic approaches aimed at replacing laminin α2 chain have been undertaken. Mini-agrin, laminin α1 and α2 transgenes have been demonstrated to significantly compensate for the absence of laminin α2 chain in muscle (27,41–43). Also, inhibition of apoptosis by genetic means and by pharmacological therapy
ameliorated several pathological symptoms in the \(dy^W/dy^W\) mouse model of MDC1A (20–22, 44). Hence, it is clear that improper induction of apoptosis contributes to the pathogenesis of laminin \(\alpha_2\) deficiency. In muscle cells, it is hypothesized that loss of laminin \(\alpha_2\) chain binding to dystroglycan and integrin \(\alpha_7\beta_1\) leads to abnormal activation of apoptosis, partly through Akt (16, 45, 46). However, other mechanisms are also likely to contribute to reduced muscle mass in MDC1A. We here present data indicating that increased activity of the UPS represents a novel mechanism in the pathogenesis of MDC1A. We found decreased Akt phosphorylation and increased expression of FoxO transcription factors,

Figure 7. Systemic injection of MG-132 modifies the Hsp90 level and Akt phosphorylation in \(dy^{3K}/dy^{3K}\) quadriceps muscle. Densitometric analysis of Hsp90 (A), phospho-Akt 308/Akt (B) and phospho-Akt 473/Akt (C) in quadriceps muscle from wild-type, non-treated \(dy^{3K}/dy^{3K}\) injected \(dy^{3K}/dy^{3K}\) after 24 h (top, \(n = 6, 6\) and 9, respectively) and injected \(dy^{3K}/dy^{3K}\) after 18 days (bottom, \(n = 4\) for each group). Data are expressed in arbitrary units (AU), as Hsp90 is normalized to tubulin and phospho-Akt to Akt. \(*P < 0.05; **P < 0.001.\) Tubulin was used as an internal loading control.

Figure 8. Systemic injection of MG-132 normalizes laminin \(\alpha_4\) and \(\beta_2\) chain expression. (A) Immunofluorescence experiments using antibodies against laminin \(\alpha_4\) (a–c) and laminin \(\beta_2\) (d–f) chain on cross-sections of quadriceps muscle from wild-type (a and d) and \(dy^{3K}/dy^{3K}\) mice without (b and e) or with systemic injection of MG-132 (c and f, 18 days later) (\(n = 3, 5\) and 5, respectively for each genotype). Bar = 40 \(\mu\)m. (B) Densitometric analysis and representative Western blot image of laminin \(\alpha_4\) chain in quadriceps muscle from wild-type, non-treated \(dy^{3K}/dy^{3K}\) and injected \(dy^{3K}/dy^{3K}\) after 18 days (\(n = 6, 4\) and 5, respectively). Samples were run on the same gel but lanes were not contiguous. Data are expressed in arbitrary units (AU), as laminin \(\alpha_4\) was normalized to \(\alpha\)-actinin. \(*P < 0.05; **P < 0.001.\)
MAFbx/atrogin-1, MuRF and proteasome components. Thus, muscle degeneration in MDC1A might result from both increased apoptosis and enhanced UPS-mediated muscle protein breakdown. As a proof of concept, we have shown that inhibition of the proteasome, using the well-characterized proteasome inhibitor MG-132, in the dy3K/dy3K mouse model for MDC1A significantly reduces many of the pathological symptoms. First, MG-132 treatment almost doubled the lifespan of dy3K/dy3K animals. Second, the locomotive activity was significantly increased. Third, the development of muscle fibrosis was inhibited and muscle morphology was improved. Fourth, upon MG-132 treatment, Akt phosphorylation was normalized. Fifth, muscle fiber apoptosis was reduced after treatment. Hence, administration of the proteasome inhibitor is indeed of therapeutic relevance. The neurological dysfunction was not prevented but on the other hand, the partial loss of motor control in dy3K/dy3K mice does not seem to contribute to their premature demise (26). Nevertheless, MG-132 is not suitable as a therapeutic compound in humans, but the proteasome inhibitor Velcade is approved for treatment of patients with multiple myeloma and MDC1A patients may thus benefit from this drug. Still, optimizing dosing regimens to inhibit the UPS will be crucial, considering the dose limitations demonstrated with MG-132. A second treatment failed to further improve muscles and prolong life but it could be that the timing of the second injection was not optimal. Interestingly, Velcade was recently used in dystrophin-deficient mdx animals. Localized treatment with Velcade restored the expression of (truncated) dystrophin and DGC proteins, just like MG-132 reconstituted the expression and localization of dystrophin and DGC proteins in both mdx mice and DMD explants (23,24,47). Hence, it is evident that DMD pathogenesis involves proteasomal degradation of dystrophin and the DGC.

The proteins degraded by the proteasome in laminin α2 chain-deficient muscle cells are probably part of the ‘common atrophy program’ and might to some extent also be specific for lack of laminin α2 chain. The substrates of muscle UPS have only been partly characterized, but the UPS is believed to degrade myofibrillar proteins (48–50). Apart from actin and myosin heavy chain, it is also likely that laminin β2 chain is degraded in the proteasome when
laminin α2 chain is absent since it is reconstituted in the sarcolemma region upon proteasome inhibition. Hsp90 is probably also a specific target of the proteasome system since its expression is upregulated in vitro in the presence of MG-132 (data not shown).

In summary, our study demonstrates for the first time that proteasome inhibition improves the muscle phenotype of laminin α2 chain-deficient mice. Hence, we have generated important pre-clinical data for the development of pharmacological therapies for MDC1A patients.

MATERIALS AND METHODS

Transgenic animals
Laminin α2 chain-deficient dy3K/dy3K and dy3K/LNα1TG mice were used (11,26). Dy3K/dy3K mice are generated null mice that lack laminin α2 chain completely. They develop severe muscular dystrophy and peripheral neuropathy. Dy3K/LNα1TG are also laminin α2 chain-deficient mice but overexpress laminin α1 chain using the β-actin promoter. Muscular dystrophy and peripheral neuropathy are largely prevented in these animals that have a near-normal life span (26–28). Dy3K/LNα1TG mice were compared with their wild-type littermates and dy3K/LNα1TG mice with theirs. All mice were genotyped by PCR as described previously (27). Animals were maintained in the animal facilities of Biomedical Center (Lund) according to animal care guidelines, and permission was given by the regional ethical board.

Protein extraction and western blot analyses
Quadriceps or total skeletal muscles from the hind limbs (n = 6) and sciatic nerves (n = 4) were obtained from wild-type and dy3K/dy3K mice (6 days and 3.5 weeks of age) and from wild-type and dy3K/LNα1TG mice (5.5-week-old). The homogenized samples were heated 15 min at 56°C in SDS buffer [80 mM Tris–HCl, pH 6.8; 10% SDS; 0.12 M sucrose; 5 mM EDTA; protease inhibitors (Complete, Roche) and phosphatase inhibitors (PhosSTOP, Roche)], sonicated and centrifuged for 5 min at 13000 rpm. Protein extracts were quantified using the BCA assay kit (Pierce) following manufacturer’s instructions. Denaturated proteins (30 μg) were loaded on 10–20 or 10% acrylamide SDS gels (Clearpage, CBS Scientific) and blotted (Biorad) onto nitrocellulose membranes (Hybond-C, Amersham). The membranes were blocked for 1 h in PBS, 0.01% Tween-20 and 5% milk and incubated overnight at 4°C with rabbit polyclonal antibodies directed against pAkt (Ser 473, no. 4060 or Thr 308, no. 2965, Cell Signaling Technology), Akt (no. 4685, Cell Signaling Technology), Hsp90 (no. 4874, Cell Signaling Technology) or with mouse monoclonal anti-ubiquitin (clone P4D1-A11, Upstate). Horseradish peroxidase-conjugated polyclonal goat anti-rabbit (sc-2005, Santa Cruz Biotechnology) antibody was applied for 1 h. Membranes were incubated in ECL (Amersham Biosciences), exposed to Hyperfilm (Amersham Biosciences) and developed (AGFA, Curix 60). Each membrane was rehybridized with mouse monoclonal anti-tubulin (clone DM 1A, Sigma) for loading normalization. Quantifications were performed using ImageJ 1.40 (http://rsb.info.nih.gov/ij/download.html).

For laminin α4 chain detection, proteins were isolated from quadriceps muscle from wild-type, dy3K/dy3K and injected mice (n = 6, 4 and 5, respectively) as described previously (51). Rabbit polyclonal anti-laminin α4 chain (clone 1100) was generously provided by Dr T. Sasaki. Mouse monoclonal anti-α-actinin (clone EA-53, Sigma) was used for loading normalization.

Histology and immunofluorescence experiments
Quadriceps muscle from wild-type (n = 3–4), dy3K/dy3K (n = 5–7) and injected mice (n = 5 and 3 for single and double injection, respectively) were dissected and frozen in OCT (Tissue Tek). Sections of 7 μm were either stained with hematoxylin and eosin or processed for immunofluorescence experiments following standard procedures (27) with rabbit polyclonal antibodies directed against 20S proteosome α1 (sc-67046, Santa Cruz Biotechnology) and β1 (sc-67345, Santa Cruz Biotechnology), MuRF1 (sc-32920, Santa Cruz Biotechnology), laminin α4 chain (no. 1100) and laminin β2 chain (no. 1117), both generously provided by Dr T. Sasaki,
rat monoclonal anti-laminin γ1 chain (MAB 1914, Chemicon) and anti-tenascin-C (MTn15), goat polyclonal anti-collagen III (no. 1330, SouthernBiotech) and mouse monoclonal anti-caspase-3 (CPP32, BD Transduction Laboratory). For apoptotic myofiber detection, a TUNEL detection kit was used following instructions of the manufacturer (GenScript). Sections were analyzed using a Zeiss Axioplan fluorescence microscope. Images were captured using an ORCA 1394 ER digital camera with the Openlab 3 software.

RNA extraction, reverse transcription and quantitative real-time PCR

Total RNA was extracted from 10 mg of quadriceps muscle from six dy/3K and seven wild-type (3.5-week-old) and three dy/3K LNeo1TG and three wild-type (3.5-month-old) animals using RNaseasy mini kit (Qiagen), including a step of proteinase K digestion. Complementary DNA was synthesized from 1 μg of total RNA with random primers and SuperScript III reverse transcriptase (Invitrogen) following manufacturer’s instructions. Quantitative PCRs (qPCR) were performed in triplicate with the Maxima SYBR Green qPCR Master Mix (Fermentas). Expression of genes was monitored using a real-time qRT–PCR method (Light Cycler, Roche) with the previously described TaqMan probes (Perkin Elmer) for MAFbx/atrogin-1, MuRF1, FoxO1, FoxO3 and NF-κB-p65 (29,52). The amplification efficiency for each primer pair was evaluated by amplification of serially diluted template cDNAs (E = 10−(1/slope)). RNA levels (in arbitrary units) were calculated by using the formula E−ΔCt. Expression levels were then calculated relative to the endogenous control gene β-actin and relative to wild-type quadriceps muscle.

Systemic injections of MG-132

We administrated a bolus of MG-132 (10 μg/kg in sterile PBS) into the tail vein of dy/3K and control littermates at the age of 2.5 weeks. A second batch of mice was given double injections at 2.5 and 3.5 weeks of age. Mice were sacrificed either 24 h or 18 days after injection and quadriceps muscles were processed for morphometric analysis, immunofluorescence experiments or western blot analysis. Prior to euthanasia, movies were recorded and an exploratory locomotion test was performed. As the injected control littersates did not display any pathological changes after injection, the values added to the morphometric results are from non-injected wild-type littersates.

Exploratory locomotion test

Exploratory locomotion was examined in an open field test. In each experiment, the injected mouse at day 18 (n = 10 for single injection and n = 9 for double injection), wild-type (n = 14) or non-injected dy/3K (n = 7) was placed into a new cage and allowed to explore the cage for 5 min. The time that the mouse spent moving around was measured manually.

Survival curves

Death was monitored in non-injected (n = 24) and MG-132 systemically injected dy/3K mice (n = 7 and 6 for single and double injection, respectively). Survival curves were constructed using the GraphPad Prism 4 software. Statistic logrank test was used for analysis of significance.

Morphometric analysis

Fibrosis quantification (tenascin-C/collagen III-positive areas), proportion of fibers containing centrally located nuclei, evaluation of fiber numbers per area and fiber diameter measurements were performed on whole-quadriceps muscle sections from wild-type (n = 4), dy/3K (n = 7) and dy/3K MG-132-treated animals (n = 5 for single injection and n = 3 for double injection). MuRF1-positive fibers, tenascin-C/collagen III-positive areas, TUNEL-positive myonuclei and fibers diameters were measured using the ImageJ software. Quantification of fibers with centrally located nuclei and minimal Feret’s diameter (53) was measured for at least 2000 fibers for each animal. A χ2-test was calculated for fiber distribution comparison. Wet quadriceps muscle weights were determined from five wild-type, four dy/3K and five dy/3K MG-132-treated animals and correlated to body weight.

Statistics

All tests for analysis of significance were done using the GraphPad Prism 4 software. For qRT–PCR experiments and densitometric analysis of phospho-Akt and Hsp90, Student’s t-test was used for statistical analysis of significance. A non-parametric test (Mann–Whitney) was used for qRT–PCR experiments where n = 3. For protein quantification, morphometric analysis and the exploratory locomotion test, one-way ANOVA followed by a post-multiple comparison test was utilized. A logrank test was used for Kaplan–Meier survival analysis. Data are expressed as mean ± SEM.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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REFERENCES


