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Foxo/Atrogin induction in human and experimental myositis

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Abstract

Skeletal muscle atrophy can occur rapidly in various fasting, cancerous, systemic inflammatory, deranged metabolic or neurogenic states. The ubiquitin ligase Atrogin-1 (MAFbx) is induced in animal models of these conditions, causing excessive myoprotein degradation. It is unknown if Atrogin upregulation also occurs in acquired human myositis. Intracellular β -amyloid ($A\beta$), phosphorylated neurofilaments, scattered in-filtrates and atrophy involving selective muscle groups characterize human sporadic Inclusion Body Myositis (sIBM). In Polymyositis (PM), inflammation is more pronounced and atrophy is symmetric and proximal. IBM and PM share various inflammatory markers. We found that forkhead family transcription factor Foxo3A is directed to the nucleus and Atrogin-1 transcript is increased in both conditions. Expression of $A\beta$ in transgenic mice and differentiated C2C12 myotubes was sufficient to upregulate Atrogin-1 mRNA and cause atrophy. $A\beta$ reduces levels of p-Akt and downstream p-Foxo3A, resulting in Foxo3A translocation and Atrogin-1 induction. In a mouse model of autoimmune myositis, cellular inflammation alone was associated with similar Foxo3A and Atrogin changes. Thus, either $A\beta$ accumulation or cellular immune stimulation may independently drive muscle atrophy in sIBM and PM, respectively, through pathways converging on Foxo and Atrogin-1. In sIBM it is additionally possible that both mechanisms synergize.

Keywords

Inclusion body myositis (IBM); Polymyositis (PM); Experimental allergic myositis (EAM); Intracellular β -amyloid ($A\beta$); Foxo3a; Atrogin (MAFbx); Akt; Insulin

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.nbd.2012.02.011.

Introduction

Skeletal muscle atrophy is common to several conditions and disease states including fasting, cancer, systemic inflammation and metabolic derangements (uremia, diabetes) as well as after denervation and in motor neuron degeneration. More chronic conditions associated with sarcopenia include disuse, aging, drugs (steroids), spaceflight and hypoxia. In the early stages of experimental muscle atrophy, the ubiquitin ligase Atrogin-1 (MAFbx) is strongly induced (Lecker et al., 2004). Atrogin-1 (MAFbx) is a skeletal and a cardiac muscle-specific F-box containing protein that binds to the other components of the SCF (Skp1-Cullin1-F-box protein) complex family of E3 ubiquitin ligases (Gomes et al., 2001; Li et al., 2004). The F-box proteins generally function as adaptors targeting specific substrates for ubiquitin-dependent degradation through protein-protein interaction domains (Li et al., 2004). Upon fasting for instance, the rise in Atrogin-1 expression precedes muscle loss (Gomes et al., 2001). Animals, deficient in Atrogin-1 or the related MuRF1 ligase are resistant to muscle atrophy following denervation (Sacheck et al., 2004; Sandri et al., 2004; Stitt et al., 2004). On the other hand, Atrogin-1 expression inhibits calcineurin-dependent cardiac hypertrophy (Li et al., 2004). In many of these instances, suppression of the IGF-1/PI3-K/Akt signaling pathway leads to the activation of Foxo family transcription factors and enhancement of Atrogin-1/MAFbx expression (Bodine et al., 2001; Sandri et al., 2004; Skurk et al., 2005). Conversely, IGF-1 stimulation reduces Atrogin expression and rescues dexamethasone provoked myofibrillar protein degradation (Sacheck et al., 2004; Stitt et al., 2004). The only degenerative human muscle wasting disorder studied extensively for Atrogin-1 upregulation is amyotrophic lateral sclerosis (ALS), which arises from chronic denervation and sprouting (Leger et al., 2006).

Dystrophic and myositic processes confined to skeletal muscle also activate a muscle wasting program (Lecker et al., 1999), but Atrogin-1 induction has not been investigated. Inclusion body myositis (IBM) is a progressive inflammatory myopathy for which there is no effective treatment. Often sporadic and affecting men, it is the most common acquired muscle disease after age 50. Typical features are asymmetric, slowly progressive muscle weakness and atrophy with predilection for quadriceps and forearm flexors and frequent dysphagia (Griggs et al., 1995; Tawil and Griggs, 2002). Microscopically, skeletal muscle displays focal endomysial infiltrations of inflammatory mononuclear cells, CD8-T cell invasion of non-necrotic myofibers, modest numbers of muscle fibers containing rimmed vacuoles, intracellular protein inclusions (Arnardottir et al., 2004; Dalakas, 2006; Engel and Arahata, 1984). The latter bear similarity to those defining Alzheimer's disease; fibrillar deposits of intracellular β -amyloid ($A\beta$) and various APP proteolytic fragments, ubiquitin, phosphorylated neurofilament proteins including possibly tau (Askanas and Engel, 2001, 2008). Other proteins found in Alzheimer's plaques, can also be identified in IBM deposits (Askanas et al., 1998; Nakano et al., 1999). The weakness in Polymyositis/Dermatomyositis (PM/DM), actually separate disorders, involves the proximal musculature, including neck, more symmetrically. In the latter disorders, atrophy becomes pronounced in later stages. Patients commonly show systemic involvement and have a younger age peak incidence. The pain they often note reflects more pronounced inflammation and muscle necrosis

accompanied by higher serum CPK values. Steroid responsiveness also sets these disorders apart from IBM (Dalakas and Hohlfeld, 2003).

The etiology and precise pathogenesis of the myositis spectrum are unknown. Experimental overexpression of wt (wild type) or mutant β APP (amyloid precursor protein) in vivo (Fukuchi et al., 1998; Moussa et al., 2006; Sugarman et al., 2002) and in vitro (Askanas et al., 1997) can result in A β inclusion formation and recapitulates some IBM pathology. However, A β accumulation seems neither likely to be the proximate cause of the disease or sufficient to drive progression. Amyloid mist metabolism may play an important toxic role in combination with inflammation (Schmidt et al., 2008). However, this is controversial since dyshomeostasis of other marker proteins is prominent (Amato and Barohn, 2009; Weihl and Pestronk, 2010). Regardless of the exact role of A β and inflammation interactions, there are probably additional factors that lead to the more widespread myofibrillar atrophy in IBM. One protein degradation pathway implicated in IBM is the proteasome (Fratta et al., 2004). Endoplasmic reticulum stress dysfunction and defective ERAD may also contribute (Vattemi et al., 2004). We and others found that an E3 ubiquitin ligase, Parkin, is disordered in IBM (Rosen et al., 2006). Alternatively, we reasoned there may exist an ubiquitin ligase having wider spread degradative action and involvement in other muscle wasting states that, if over stimulated, could accelerate myofibrillar loss in the myositis spectrum. Here we examined whether experimental intracellular A β accumulation and/or cellular inflammation could lead to Foxo3A (FKHRL1)-mediated Atrogin induction in cultured myotubes and in vivo and sought evidence in human IBM/PM samples.

Materials and methods

Reagents and antibodies

The following antibodies were used: 6E10 (mouse monoclonal, Signet Laboratories, Dedham, MA), R1282 (rabbit polyclonal, a gift from Dr. Selkoe's lab), anti-Foxo3A (rabbit polyclonal and mouse monoclonal; Sigma), anti-p-Akt (Thr308, rabbit polyclonal, 1:2000; Cell signaling), goat anti-Akt1, anti-MyoD, rabbit anti- α -Tubulin, and rabbit anti-cdk4 (all polyclonal, 1:2000; Santa Cruz), and anti-MAFbx (rabbit polyclonal, 1:2000; a gift from Regeneron Pharmaceuticals Inc., Tarrytown, N.Y.). Anti-CD4 (GK1.5) and anti-CD8 (YTS169.4) rat monoclonals are from ABCAM, San Francisco. Anti- β -Sarcoglycan (mouse monoclonal) is from Vector Lab. Anti-HSP90 from Stressgen and anti-Histone H3 from Upstate are rabbit polyclonals. All other chemicals and reagents were from Sigma.

Cell culture

Mouse C2C12 myoblast cells (ATCC, Manassas, VA) were grown in DMEM, and 20% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), antibiotics, and 2 mM L-glutamine and maintained for passage below 60% confluence. Cultures at or above 90% confluence were differentiated into skeletal myotubes in DMEM, 2% adult horse serum (Differentiation Medium, DM) at 37 °C and 5.5% CO₂ for 2–4 days.

Human biopsy sample and transgenic mice

Human skeletal muscle biopsy specimens were excised from quadriceps and fresh frozen in OCT and kept at -80°C until 10 micron thick sections were cut for histology and immunohistochemistry or $\sim 100\text{ }\mu\text{g}$ blocks were excised for PCR. All IBM (n=9) and PM (n=9) cases were identified using published criteria (Dalakas, 2006; Dalakas and Hohlfeld, 2003; Tawil and Griggs, 2002) by an expert neuropathologist (L.S.A). The IBM cases were all 'sporadic' in type. All IBM samples were positive for Gomori trichrome decorated inclusions and both β -amyloid and phosphorylated neurofilament immunohistochemistry using mAbs 6E10 and SMI31, respectively (data not shown). PM and control cases excluded these features. None of the PM patients were receiving steroid therapy at the time of biopsy. Disease severity was similar in this series of PM and IBM samples judged by reductions in quantified mean myofiber diameter. Comparable assessments were found in our previous case series (Table 1 in (Sugarman et al., 2006)). Control cases underwent biopsy for various symptoms but no individual had atrophy or high CPK values and no abnormalities were found after extensive microscopic review including special histochemistry battery. The degree of histo-pathology was graded 0–4 (Supplemental Table 1) (Arnardottir et al., 2004). Control case sets consisted of age matched normals (n=11) and various dystrophies, inherited metabolic and mitochondrial myopathies (n=13, Supplemental Table 2). Transgenic mice for skeletal muscle directed expression of human wild type β -APP751 under MLC-1 (myosin light chain promoter) were previously reported and characterized (Moussa et al., 2006). Skeletal muscle necropsy specimens were obtained from hamstring and gastrocnemius muscles and treated as above.

Experimental autoimmune myositis

We used an experimental allergic myositis (EAM) model of PM based on skeletal muscle fast-type C protein immunization (CIM) (Sugihara et al., 2007). C57BL/6 mice were given several injections of a recombinant fragment of skeletal muscle C-protein (a myosin binding protein) to induce T-cell mediated injury. Female mice ages 8–10 weeks were immunized with $200\text{ }\mu\text{g}$ C-protein fragment no. 2, emulsified in Freund's complete adjuvant administered intradermal at 4 locations along the back. $2\text{ }\mu\text{g}$ pertussis toxin in PBS was concurrently injected intraperitoneal. Animals were sacrificed at days 14–21 after the last injection. C-protein fragment 2 was amplified from human skeletal muscle cDNA library (pQE) using PCR and recombinant protein made in *E. coli* according to Sugihara et al. (2007).

Immunohistochemistry

Immunohistochemistry was performed on frozen, 15 micron muscle sections and C2C12 myotubes grown on 8-chamber slides. Myotube cultures were infected with Adv TRE- $\text{A}\beta$ and Adv TetOn and induced to express human $\text{A}\beta 42$. Muscle sections or myotube cultures were washed once with ice-cold phosphate-buffered saline (PBS) and fixed with either cold acetone (for R1282 immunohisto-chemistry) or otherwise 4% paraformaldehyde in phosphate buffer for 5 min at 25°C . Slides and culture wells alike were washed three times for 5 min prior to permeabilization with 0.3% Triton X-100 in PBS for 5 min and washed again three times for 5 min. Nonspecific sites were blocked with 10% normal goat serum in

PBS. Samples were incubated overnight at 4 °C with primary antibodies: R1282 1:100, Foxo3A 1:200, CD4 1:200, CD8 1:200, β -Sarcoglycan 1:200 or 6E10 1:100, in PBS containing 10% normal goat serum. The following day and after three washes, the specimens were incubated with secondary antibodies, Cy2-conjugated goat anti-mouse or rabbit IgG, and Cy3-conjugated goat anti-rabbit or mouse IgG (1:200) for 90 min at 37 °C. Sections or chambers were washed and mounted in N-propyl-gallate. Photomicrographs were taken from a Nikon TE200 microscope.

RNA isolation, reverse transcription and real-time (RT) PCR

Atrogin-1 mRNA levels were determined by traditional and confirmed by real-time (RT)-PCR using an Applied Biosystems Model 7300 real-time PCR thermal cycler. Total cellular RNA was isolated from human IBM and transgenic mouse muscle specimens, and cultured C2C12 cells, using the RNeasy mini kit or Trizol, following the manufacturers' instructions (QIAGEN or Invitrogen). RNA was eluted in diethylpyrocarbonate-treated water. RNA (~200 ng) was reverse transcribed using oligo (dT), primers and reverse transcriptase (Multiscribe) in the presence of 2 mM dNTP mixture in a total volume of 50 μ l. First strand cDNA and PCR reactions were started, using equal amounts of starting material, according to the Applied Biosystems TaqMan instructions. Following denaturation at 95 °C for 10 min; RT-PCR was undertaken for 40 cycles of 95 °C for 15 s and 60 °C for 60 s. GAPDH was used as the internal control for the quantification of Atrogin-1 and MuRF1 transcripts. Fluorescent multiplexing allowed for measurement of the relative fluorescence of two signals in a given sample. 30 cycle products were loaded onto 2% agarose gel for electrophoresis and quantification by optical density of the ethidium-stained signals. The sequences of forward, and reverse oligonucleotides were as follows: Atrogin-1, forward 5'-GTG TAT CGG ATG GAG ACG ATT CT-3', reverse 5'-GGC AGG CCT GGT GAT CTG-3'. MuRF1, primers (Applied Biosystems ID no. Rn00590197_m1). TaqMan probe, sequence 5'-FAM-TGC CAT CCT GGA TTC CAG AAG ATT CAA C-TAMRA-3'. GAPDH, forward 5'-GAG AGA TGA TGA CCC TTT TGG C-3', reverse 5'-CCA TCA CCA TCT TCC AGG AGC G-3'.

C2C12 myotube infection with adenovirus

Adv Tet-On, TRE-LacZ and TRE-A β 42 viruses (Magrane et al., 2005) and Adv myrAkt, expressing constitutively active Akt having a c-src myristoylation sequence fused in-frame to the N terminus of the hemagglutinin (HA)-Akt (wild-type) coding sequence, were described previously (Fujio and Walsh, 1999). Adv dn-Foxo3A was constructed to express dominant-negative (dn) Foxo3A, deleted of the C' terminus transactivation domain (Skurk et al., 2004). C2C12 was switched to DM and on day 3 infected with Adv TRE-LacZ/ TetOn or TRE-A β /TetOn (4:1 ratio, total moi~100, 24–36 h) or Adv myrAkt or Adv dnFoxo3A before doxycycline induction (2 μ g/ml) for an additional 18–48 h.

Immunoblot analysis

Whole cell extracts (WCE) were prepared in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 1% NP-40, 10% glycerol, 1 mM Na₄P₂O₇, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A, 1 μ g/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, and pro-tease inhibitor cocktail; Roche). Cell lysates were incubated on ice for 45

min and centrifuged at 14,000 g for 10 min before storage at -80°C . WCE were directly loaded for Western analysis (20–30 μg for cell cultures or 60–80 μg for mouse samples). Crude nuclear fractions were obtained by pelleting at 3000 rpm for 10 min and confirmed by demonstrating histone specificity. Freshly thawed extracts were diluted into Laemmli sample buffer, heated at 95°C for 10 min, cleared by centrifugation, separated on 10–20% SDS-PAGE, and transferred to PVDF membrane (Immobilon-P; Millipore, Bedford, MA). Membranes were blocked in TBS containing 0.3% Tween-20 and 5% (wt/vol) nonfat dry milk. After incubation with primary antibodies (18 h at 4°C in buffer containing 5% BSA and 0.05% NaN_3), blots were washed and incubated in HRP-conjugated secondary antibodies (1:2000 dilution; Cell Signaling). Signals were detected by using ECL reagents and conventional exposure to film (GE Healthcare).

Cell viability and myotube size quantification

Myotube cultures exposed to Adv A β 42 and doxycycline were either pre-treated with insulin for 48 h, before and during A β expression, or post-treated with insulin only in the last 30 min before cell viability was determined by MTT reduction. Cells were washed twice in warm D-PBS and incubated in 1 ml DMEM containing 0.5 mg (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT; Molecular Probes, Eugene, OR) for 2–3 h at 37°C and 5% CO_2 . The medium was aspirated, and the cells were washed twice with warm D-PBS . The formazan salts were dissolved in 1 ml pure ethanol before use. Cells were homogenized by repetitive pipetting and centrifuged for 5 min at 4500 rpm, and the supernatant collected. Absorbance was read against an ethanol blank at 590 nm. Myotube viability was also determined by measuring cellular area. The mean individual myo-tube area was determined for all fiber profiles that crossed photomicrographs made from fields under $40\times$ magnification. For each condition, five fields were semi-randomly sampled (four quadrants and center) per slide chamber (experiment). Myotube areas were determined using ImageJ program algorithm (Collins, 2007) after manually tracing all the sarcolemmal profiles. Measurements of pixel area were converted to μm^2 (1 pixel $\approx$ 1 μm).

2-Deoxyglucose uptake assay

Differentiated myotubes grown in Dulbecco's Modified Eagle Medium (DMEM, 24.75 mM glucose) on 24 well plates were exposed to dual infection with Adv A β 42/TetOn system and induced with doxycycline. Monolayer cultures were switched to glucose-deficient HBSS [5.56 mM glucose] for 2 h and treated with insulin (200 ng/ml; 45 nM) for 30 min at 37°C before adding 50 μl 2-deoxy- $[\text{}^3\text{H}]$ -glucose (0.5 $\mu\text{Ci/ml}$) in the final 4 min of stimulation (Moyers et al., 1996). Some cultures were additionally pre-treated with LY294002 (100 μM) or cytochalasin (10 μM). After 3 rapid rinses, lysates were prepared in 0.5 M NaOH and 0.1% SDS (w/v). The incorporated radioactivity was determined by liquid scintillation counting. Nonspecific counts obtained in the absence of $[\text{}^3\text{H}]$ -glucose were subtracted. Readings in the presence of insulin stimulation were normalized to the absence of insulin. All samples were performed in triplicate.

Statistical analysis

Data are expressed as mean $\pm$ S.E.M or S.D. as indicated in legends. Quantification of experiments utilized independent determinations unless otherwise stated. PCR data reflects

combined results of 3–6 experiments, as indicated in legend. Statistical analysis was performed using Graphpad Prism® Software, and statistical significance was tested using student's *t*-test.

Results

Foxo3A nuclear localization in inflammatory myositis

We first examined skeletal muscle biopsy samples from human inclusion body myositis (IBM) and polymyositis (PM) cases (n=3 each) for Foxo3A nuclear localization (Fig. 1). Foxo3A is a transcription factor and member of the forkhead family which acts to upregulate Atrogin-1 transcription (Sandri et al., 2004). Atrogin is an ubiquitin ligase that controls muscle size (Lecker et al., 1999). Its levels and activity correlate with atrophy in various muscle wasting states in rat (Lecker et al., 2004). Foxo3A immunohistochemistry of human muscle biopsy samples showed nuclear localization in both IBM and PM samples, but not in age matched control cases (Fig. 1). The yellow arrow heads indicate intrafiber nuclei that evidence translocation, the green arrow heads indicate positive nuclei outside of myofibers (endomysium) belonging presumably to inflammatory cells. An indication of the prevalence of Foxo shuttling is shown in the bottom left in a less magnified view of an IBM sample. We confirmed nuclei belonging to inflammatory cells through anti-CD8 marker staining (Fig. 1 bottom, middle), and CD4 and LC3 (not shown). β -Sarcoglycan staining was used to delineate the muscle membrane so that nuclei could be assigned to the endomysium (bottom middle). A β expression (anti-R1282) could be found in some IBM muscle fibers in which Foxo3A was co-localized to a nucleus (bottom right). Myo-fibers with more than two Foxo3A-positive nuclei were manually quantified. Both PM and IBM samples show significant increases in Foxo3A-positive myofiber counts (Fig. 1 graph).

Atrogin-1 mRNA expression in human IBM and PM; real time (RT)-PCR

Atrogin transcript was quantified in human control and PM and IBM samples (n=9 each) by real time (RT)-PCR. Patient characteristics are given in Supplemental Table 1; disease severity was similar in PM and IBM samples judged by reductions in quantified mean myofiber diameter (result not shown). In both PM and IBM, Atrogin-1 levels corrected for control GAPDH signal were significantly increased (Fig. 2A). In separate experiments using conventional PCR and agarose gel electrophoresis, we confirmed increased levels of Atrogin-1 mRNA in IBM (Fig. 2B) and PM (not shown). To determine the specificity of Atrogin-1 induction to inflammatory myositis, bearing in mind the experimental evidence in systemic wasting disorders, we tested a broad group of heritable myopathic and dystrophic conditions presenting in the adult population (n=13: mitochondrial myopathies 3, limb girdle dystrophy 2, central core disease 2, vacuolar and chronic/end stage 3, desmin-McArdle's— β -sarcoglycan disorders 3). We did not find evidence of Atrogin up-regulation in any of these 'non-inflammatory myopathies' (NIM; Supplemental Table 2) (Fig. 2C).

A β -induced myotoxicity is reversed by constitutive Akt and dn Foxo3A

The results of nuclear Foxo translocation and Atrogin mRNA induction consistently show stimulation of this path within the inflammatory myositis spectrum. Perhaps this is not surprising since both IBM and PM have in common muscular atrophy and some

immunologic features. However, different signaling mechanisms converge on Foxo (Skurk et al., 2005; Xie et al., 2009) which IBM and PM do not necessarily share, as these are distinct clinical-pathological conditions. Since inhibition of PI3K-dependent regulation of Akt is a feature of IBM (Lee et al., 2009), we first investigated the effect of intramyofiber A β on this pathway to Atrogin upregulation.

Intracellular A β was expressed in C2C12 myotubes infected with Adv A β /TetOn when induced over 2 days with doxycycline. Myotube area is reduced in cell cultures induced to express A β 42 indicating a direct toxic affect (Fig. 3). In the presence of co-expressed Akt or dominant negative Foxo3A (Adv-myrAkt or Adv-dnFoxo3A), the toxic phenotype is reversed in Fig. 3A. Quantification of myotube area in Fig. 3B demonstrates the maintenance of myotube size when Akt is activated or Foxo3A is suppressed. Interestingly, in the presence of constitutive active myrAkt or dnFoxo alone, myotube size increases over baseline (Ad-Tre-LacZ). This is consistent with a previous report (Skurk et al., 2005).

A β 42 expression and Foxo3A nuclear translocation in myotubes

Intracellular A β 42 expression was induced for short times in cultures of C2C12 myotubes in the presence of doxycycline (Fig. 4A). Expression of A β 42 (detected by 6E10) is associated with decreased levels of both activated p-Akt (Thr308, panel 1) and the transcriptional inactive (phosphorylated) form of Foxo3A (p-Foxo3A, panel 3). Nuclear localization of nonphosphorylated Foxo3A is correspondingly increased (nuclear fraction, panel 4). The purity of the nuclear pellet fraction is shown by a relative concentration of the marker protein histone (inset). Atrogin-1 (MAFbx, Fig. 4B panel 6) expression increases as expected. One downstream target of the ubiquitin ligase Atrogin is the muscle protein MyoD. As expected, levels of MyoD are decreased in the presence of A β 42 (Fig. 4B, panel 7). A β 42 and control protein expression levels are given in panels 8–10. Levels of p-Akt, p-Foxo3A, MAFbx and MyoD are quantified in Fig. 4C.

To further investigate Foxo3A signaling, nuclear translocation was examined in the presence of Akt and Foxo3A depletion. Myotubes infected with Adv LacZ served as a control (Fig. 5A). Accumulation of virally-expressed myocellular A β led to significantly enhanced nuclear localization of Foxo3A (Fig. 5A, lower left panel; nuclei are indicated by yellow arrows). Concomitant expressions of dnFoxo3A or myrAkt greatly reduced the fraction of myofibers with 2 Foxo3A-positive nuclei to control levels. The results are quantified below in Fig. 5B. The ectopic expression of Akt, Foxo and A β 42 in these experiments was confirmed by western blot (Fig. 5C).

Atrogin-1 mRNA upregulation in A β expressing myotubes and transgenic mouse muscle

C2C12 myotubes were infected with Adv Tre-A β 42 and Atrogin-1 mRNA levels were analyzed by qRT-PCR after 24 h of expression. Expression of intracellular A β 42 led to enhanced Atrogin-1 mRNA expression relative to control, an effect that was reversed by co-induction of dnFoxo3A (Fig. 6A, left). Levels of a control ubiquitin ligase, MuRF1 were not significantly altered. Levels of UbcH2, an ubiquitin-conjugating enzyme, also did not change with A β 42 expression (result not shown). In a separate series of experiments, myrAkt expression also reversed Atrogin-1 mRNA upregulation (Fig. 6A, right).

Atrogin-1 mRNA levels were analyzed by PCR in hamstring samples taken from a previously reported IBM-like mice model (MLC1/3- β APP) (Moussa et al., 2006). The Atrogin-1/GAPDH mRNA signal ratio was increased in the transgenic animals relative to control litter-mates (Fig. 6B and quantification). These modest but significant changes were similar in degree to the human samples in Fig. 2B. An RT-PCR data set using the same animal samples showed a more robust increase in Atrogin-1 transcript level in the transgenic mice (Fig. 6B). We hypothesized that Foxo-regulated changes in Atrogin-1 expression in IBM and the β -amyloid-based transgenic model are driven by the activation status of Akt (PKB). This is indicated by Figs. 4 and 5 wherein Akt is deactivated by A β expression and constitutively active (CA) Akt (myrAkt) reverses nuclear Foxo3A localization. To test this in vivo, another Akt substrate, glycogen synthase kinase 3 β (GSK-3 β) was assayed by western blot in the same transgenic muscle samples. MLC-APP mice showed markedly reduced levels of p-GSK (Fig. 6C upper panel), consistent with a relative state of Akt inactivation (Fig. 6C lower panel).

Myocellular A β 42 expression inhibited insulin-mediated glucose uptake

We tested the trophic action of insulin to overcome the A β 42-induced cascade of myotube toxicity (Fig. 7A). C2C12 myotubes were infected for 24 h with Adv Tre-A β 42 followed by 24 h of doxycycline (Dox) induction. In an MTT-based cell viability assay, insulin added to serum-containing tissue culture medium protected or overcame A β 42 toxicity only if added prior to and replenished during the 24 hour A β 42 expression period. Insulin had no effect if added at the end of the Dox-induction (Fig. 7A).

The myocellular uptake of 3 H-glucose was also assayed in C2C12 myotubes to test this action of insulin and the effect of Akt pathway inhibition on muscle metabolism. In control experiments, insulin stimulated uptake to a moderate degree, as noted by others (Iwata et al., 2009; Tulipano et al., 2008). As expected cytochalasin B (a cell trafficking and GLUT-4 translocation inhibitor) and LY294002 (a PI3K inhibitor) reversed insulin mediated uptake (Fig. 7B). Prior A β 42 expression partially prevents the brief (4 min) period of insulin stimulated uptake (Fig. 7B, right). Thus, A β 42 not only antagonizes inactivation of Foxo, but it also antagonizes other insulin/PI3K/Akt mechanisms including inactivation of GSK-3 β and stimulated glucose transport.

Immune-related cellular inflammation also stimulates Foxo/Atrogin

The previous experiments suggest that A β expression and Akt inactivation is one possible mechanism that is sufficient to drive Foxo3A nuclear translocation and Atrogin induction in IBM. However, in PM there is no amyloid stress to explain similar findings. Thus we turned attention to cellular inflammation as an additional independent mechanism to stimulate Atrogin. For this we used an experimental allergic myositis model (CIM) provoked in normal mice immunized with a peptide derived from muscle C protein (Sugihara et al., 2007). Similar to IBM and PM, these mice evidence a cytotoxic lymphocyte response and class I MHC upregulation (Sugihara et al., 2010). In our hands such CIM mice also showed evidence of clinical weakness involving proximal leg function (not shown) and marked endomysial cellular infiltrates involving hamstring muscle (Fig. 8B). A β deposits were not detected (result not shown). We found myofiber nuclei that were decorated by anti-Foxo3A

(Fig. 8C) as well as scattered T cell lymphocytes staining for Foxo3A (Figs. 8D, E and G). Similar prevalence of CD4+ and CD8+ T cells were observed (Fig. 8 and not shown), reminiscent of recent reports in sIBM (Creus et al., 2009; Pandya et al., 2010). Centralized nuclei in both severely affected (Fig. 8F) and non-necrotic myofibers (Fig. 8H) were often bearing Foxo3A. When tested for induction of Atrogin mRNA by PCR, the increase in transcript amount was found similar to the amyloid-based transgenic mice (Fig. 8I).

Discussion

We found that Atrogin -1 expression is upregulated in myositis but not in a group of non-inflammatory conditions which include various dystrophies. Sporadic inclusion body myositis (sIBM) is unique among acquired muscle disorders in that both cell-mediated inflammation and degenerative protein aggregation are likely to play synergistic roles. There is no agreement however, as to their relative contributions to the initiation and progression of disease (Greenberg, 2009). The in-inflammatory component consists of scattered infiltrates of cytotoxic CD8+ (and CD4+) T lymphocytes and macrophages (Creus et al., 2009; Dimitri et al., 2006; Pandya et al., 2010), invasion of MHC-1 bearing myofibers and overexpression of pro-inflammatory chemokines and cytokines (Confalonieri et al., 2000; Raju et al., 2003; Tews and Goebel, 1996). These immune characteristics are shared with polymyositis (PM). The protein inclusions relevant to IBM do not always correlate topographically with the infiltrates suggesting separate processes. However, APP and β -amyloid aggregates within myofibers co-localize with inflammatory molecules CXCL-9, MHC-I and IL-1 β , suggesting at least some molecular relationship between inflammation and degeneration in sIBM (Schmidt et al., 2008).

Very little is known of the mechanisms for myofiber attrition in the inflammatory myositis disorders. Necrosis and inflammation are generally more pronounced in PM. Muscle myofiber apoptosis, whether nonexistent (Nagaraju et al., 2000; Schneider et al., 1996) or present but rare in sIBM and hereditary (h)IBM (Hutchinson, 1998; Querfurth et al., 2001; Yan et al., 2001) cannot account for the loss. Yet, the possibility that the extent of apoptosis in degenerative muscle diseases is underrated has been raised (Amsili et al., 2007; Tews, 2002). Myostatin, a member of the transforming growth factor-beta (TGF- β) superfamily and a principle negative regulator of muscle growth (Carnac et al., 2006), has been implicated in sIBM through cytotoxic aggregation and amyloid formation (Starck and Sutherland-Smith, 2010; Wojcik et al., 2005). Overexpression of myostatin attenuates insulin-like growth factor-1 (IGF-1)-mediated increases in myo-tube diameter, and Akt phosphorylation (Morissette et al., 2009). Similar to our amyloid and inflammation-based experiments, myostatin signaling also leads to Foxo1/Atrogin-1 induction (McFarlane et al., 2006). A common cause of muscle atrophy, steroid use, has been shown to be related to increased levels of Foxo (Waddell et al., 2008). Moreover, steroid treated cultured muscle shows upregulation of atrophy markers Atrogin-1 and MuRF1, both ubiquitin ligases (Stitt et al., 2004). IGF-1 treatment blocked dexamethasone-induced atrophy in C2C12 myotubes, thus implicating either MAPK or Akt pathways in the reversal of Foxo levels. We hypothesized that Foxo3A translocation also occurs in the myositis disorders, leading to stimulated levels and activity of either Atrogin or MuRF ligases.

First, skeletal muscle from patients with either IBM or PM showed increased Foxo3A nuclear localization and Atrogin-1 mRNA expression compared to normal controls. A diverse group of non-inflammatory myopathies showed control levels (Figs. 1 and 2). MuRF1 induction was not observed. To our knowledge, this is the first demonstration of *specific* Atrogin upregulation in the inflammatory myositides. Since Atrogin upregulation is associated with myositis characterized by either amyloid deposition and modest inflammation (IBM) or without protein accumulation but relatively more inflammation (PM), we tested whether either APP/A β expression or immune-based inflammation limited to skeletal muscle could independently drive Foxo/Atrogin. Using differentiated C2C12 myotubes, we show that intracellular A β expression was sufficient to activate Foxo3A nuclear localization and increase Atrogin-1 expression levels (Figs. 5, 6A). Again, MuRF1 induction was not observed. Moreover, A β 42 expression resulted in myotube atrophy, which was reversed by either constitutively active Akt or dominant negative Foxo3A (Fig. 3). A transgenic muscle model of IBM in which holo β APP (and A β 42) expression is muscle-directed also showed Atrogin induction (Fig. 6B). In both animal and cell culture studies, evidence presented that Akt downregulation is proximal to the Foxo/Atrogin-1 response (Figs. 4, 6C). Levels of an Atrogin-1 substrate, MyoD, decrease in A β 42-expressing myotubes (Fig. 4B). The proximal inhibition of the Insulin/PI3K/Akt pathway by A β 42 in this cascade is suggested by the finding that insulin pretreatment rescued cell viability (Fig. 7A) and is supported by our previous study (Lee et al., 2009). Insulin/IGF-1 also protects neurons from A β damage by facilitating exocytosis (Gasparini and Xu, 2003), but whether it does so in skeletal muscle is unknown. A β 42 toxicity also damaged insulin-mediated glucose uptake in C2C12 myotubes indicating additional actions of A β 42 on critical metabolic pathways leading to attrition (Fig. 7B). Interestingly, Foxo3A down-regulates the transcription factor HIF1 α to suppress GLUT-1 mRNA expression (Emerling et al., 2008). Whether A β affects glucose transport through Foxo or Atrogin-1, remains to be tested.

Foxo transcription factor and Atrogin-1 expression are also increased in polymyositis (PM). Using an animal model we show that experimental cellular inflammation apart from A β accumulation is also associated with Foxo3A translocation and Atrogin-1 production (Fig. 8). Thus it is likely that cell or humoral immune based cytokines can also stimulate Atrogin-1, similar perhaps in mechanism to that noted in sepsis (Wray et al., 2003). Consistent with this, we found no Atrogin-1 induction in a group on non-inflammatory myopathies. Whether Atrogin-1 is upregulated in some early onset dystrophies with a cellular inflammatory component such as fascioscapulohumeral (FSH)dystrophy remains to be tested. One possible candidate for such a mechanism is the transcription factor NF κ B. It plays a role in a number of inducible immune gene responses in skeletal muscle (Barnes and Karin, 1997) and expression is increased in PM, IBM, DM and DMD muscle (Creus et al., 2009; Monici et al., 2003). It may however act through MuRF1, not Atrogin-1 (Cai et al., 2004). The inflammatory cytokine, TNF α is also shown to upregulate Atrogin-1 but through p38 MAPK (Li et al., 2005).

The exact mechanisms by which Atrogin-1 mediates atrophy are unclear. One crucial substrate for Atrogin-1 may be the transcription factor MyoD. By targeting MyoD for degradation, Atrogin-1 can inhibit differentiation in myoblasts (Lagirand-Cantaloube et al.,

2009; Tintignac et al., 2005). Another substrate of Atrogin-1 with possible consequence to muscle size is MAP phosphatase-1 (MKP-1 protein) (Xie et al., 2009).

Mechanisms other than by amyloid or inflammation may transduce various signals to degrade myofibrillar proteins via increases in Foxo transcription factors (Emerling et al., 2008; Waddell et al., 2008). Deficiencies in several non-Akt, non-cytokine based negative regulators of Foxo3A such as sirtuin-1, serum and glucocorticoid inducible kinase (SGK), and TGF β -activated kinase (TAK1) might theoretically activate Atrogin production. It was recently reported that HSP70 inhibits Foxo3A-induced promoter activation of Atrogin-1 in skeletal muscle (Senf et al., 2010). On the other hand, MAFbx and MuRF1 expressions are positively regulated by activation of AMPK (Nakashima and Yakabe, 2007). Whether any of these molecular factors are differentially expressed in IBM and PM skeletal muscle is unknown. Conversely, Foxo is surprisingly not involved in Atrogin upregulation in ALS, where it is unclear what Akt-dependent factor is responsible (Leger et al., 2006).

In summary, we have uncovered an additional mechanism of muscle wasting in the myositis spectrum that involves Atrogin-1 (MAFbx) expression and degradation of myofibrillar proteins. Amyloid toxicity via the insulin signaling pathway and cellular inflammatory mediators independently stimulate Atrogin-1 induction and are likely to cooperate in IBM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Foxo3A nuclear localization is enhanced in human IBM and PM, but not in control cases. (Ctrl = normal; PM = polymyositis; IBM; inclusion body myositis; red = Foxo3A; blue = nuclei identified by DAPI stain). In the merged column, yellow arrowheads indicate intrafiber Foxo3A-bearing nuclei (pink), the green arrowheads indicate endomysial Foxo3A-bearing nuclei, mainly belonging to inflammatory cells. Far right, phase contrast, bright-field photomicrographs. Bar=50 μ m. Bottom left, IBM case under lower power. Bottom middle, IBM case showing anti-CD8 decorated inflammatory cells and anti-beta-

Sarcoglycan staining pattern. Bottom right, β -amyloid deposit (low left) and a Foxo3A-laden nucleus (upper right) colocalized to the same muscle fiber (upper left, merged image) (polyclonal R1282, monoclonal Foxo3A). Bar graph, myofibers with two or more Foxo3A-positive nuclei were manually counted, n=3 clinical cases in each condition, results are compiled from 10 random fields per case. Error bars are 1 SE. *p<0.05 compared to control.

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Fig. 2.

Quantification of Atrogin-1 mRNA expression in IBM and PM. (A) Quantitative real time (RT)-PCR. Atrogin-1 mRNA levels in both IBM and PM samples are significantly increased compared to age matched control (n=9 cases each). Data was collected over 6 experiments. Error bar=1 SE. * $p<0.05$. (B) Atrogin-1 mRNA was quantified in control and IBM specimens by conventional PCR and resolved on 2% agarose, detected with ethidium (n=2 patients for each condition are shown). Atrogin-1 band is ~100 bp. Control GAPDH signals are shown below. The densitometric results are quantified right (n=5 control cases, 6 IBM cases). PM cases showed similar results (not shown). Error bars are 1 SD. * $p<0.05$ compared to control. (C) Atrogin-1 mRNA by PCR in non-inflammatory myopathies (NIM) shows no difference compared with control. Cases include various adult onset dystrophies, metabolic or storage disorders, and presumptive mitochondrial myopathies (Table 2). Quantification is shown right, n=13 NIM cases, same controls as in B.

Fig. 3.

Cytotoxicity in myotube cultures induced with doxycycline to express Aβ42. (A) In the presence of co-expressed myrAkt (Adv-myrAkt) with doxycycline or dominant negative Foxo3A (Adv-dnFoxo3A, not shown), the severely toxic phenotype is reversed. 48 h of doxycycline. (B) Quantification of mean individual myotube area in cultures all exposed to doxycycline for 24 h. Scion image software was used to calculate traced myotube profiles from photomicrographs. Note in the presence of constitutive active Akt or dnFoxo alone, myotube size increases over baseline. *and⁺p<0.05 compared to LacZ and TRE-Aβ42,

respectively, $**p < 0.01$ vs. LacZ. Error bars are 1 SE. $n=3$ experiments per condition. Each well (experiment) was sampled using 5 semi-random fields (~80–90 myotubes total).

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Fig. 4.

Experimental β -amyloid expression and signal changes in C2C12 myotubes. (A) Intramyocellular A β accumulates early in C2C12 myotubes infected with Adv A β /TetOn when induced with doxycycline for 18 h. Immunofluorescent detection with 6E10. (B) A β 42 expression (panel 8, 6E10) is associated with decreased levels of activated p-Akt (panel 1, Thr308), and transcriptionally inactive p-Foxo3A (panel 3). Activated Foxo3A is found in nuclear fraction (panel 4), leading to Atrogin-1 (MAFbx) protein expression (panel 6) and decreased levels of MyoD (panel 7). α -Tubulin and cdk4 were used as protein loading

controls. All extracts are whole cell lysates except panel 4. Nuclear fraction purity was assessed by histone concentration and absence of cytosolic marker HSP 90 (inset). (C) Quantification of p-Akt/Akt, p-Foxo 3A/Foxo 3A (nucleus), MAFbx/cdk4, and MyoD/ α -Tubulin ratios. * $p < 0.05$, Error bars are 1 SE. $n = 2-5$ independent experiments with and then again without doxycycline.

Fig. 5.

Foxo3A nuclear localization in C2C12 myotubes. Aβ42 expression was induced with doxycycline (18 h) in C2C12 myotube cultures. (A) Infection with Adv LacZ as control. Nuclear localization of Foxo3A, shown as merged Foxo3A (red) and DAPI (blue) signals (lower left panel, pink) are indicated by yellow arrow heads. Concomitant expressions of myrAkt (middle panel) or dnFoxo3A (right panel) eliminated or decreased nuclear localization. High endogenous cytoplasmic Foxo levels are consistent with previous work (Skurk et al., 2005). (B) Quantification of nuclear signals. The % of myofibers with 2

Foxo-positive nuclei is increased in the presence of A β 42 accumulation and reversed by myrAkt and dnFoxo3A constructs. n=2 experiments each condition, five 40 $\times$ fields were examined in each well, error bars = SD. **p<0.01 vs. control, +p<0.05 vs. bar 4 (A β 42 expression). (C) Expressions of Akt, Foxo, and A β 42 from total lysates were confirmed by western blot.

Fig. 6.

Atrogin-1 mRNA in C2C12 myotubes and transgenic model is increased by Aβ42 expression. (A) C2C12 myotubes were infected with control Adv TRE-LacZ or Adv TRE-Aβ42 and exposed to doxycycline. Atrogin-1 and MuRF1 mRNA levels were analyzed by qRT-PCR after 24 h of expression. Expression of intracellular Aβ enhanced Atrogin-1 mRNA expression, an effect reversed by co-expression of dnFoxo3A (left graph) or myrAkt (right graph). Levels of a control ubiquitin ligase, MuRF1, were not significantly altered. n=3 experiments, each in triplicate. *p<0.05 compared to control, +p<0.05 compared to Adv Aβ42. (B) Left: Atrogin-1 transcript levels were analyzed by conventional PCR in hamstring muscle from an IBM-like muscle model (MLC1/3-βAPP). Atrogin/GAPDH mRNA signal

ratio was increased in the transgenic animals relative to control. $*p<0.05$, $n=6$ controls, 7 Tg animals, $\text{bar}=1$ SD. right: Atrogin-1 transcript levels by real time PCR confirms conventional results. $*p<0.05$ compared to control, $n=6$ controls, 7 Tg animals, $\text{bar}=1$ SD. (C) The protein levels of Akt substrate, GSK were determined by western in Tg mice ($n=3$) hamstring samples. Tg mice had markedly reduced levels of phosphorylated (p)-GSK and moderately reduced activated p-Akt. Actin or Cdk4 levels reflect loading control. $*p<0.05$ compared to control, $n=3$ or 4 controls, 3 or 4 Tg animals, $\text{bar}=1$ SD.

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Fig. 7.

Insulin pretreatment is cytoprotective against A β 42 but inhibition of glucose uptake persists to a partial degree. (A) Insulin rescues cell viability in an MTT assay. C2C12 myo-tubes infected 24 h with Adv TRE-A β 42 and exposed 24 h to doxycycline are less viable (bar 2). Insulin added to the serum-containing DMEM overcame A β 42 toxicity only if added prior to and replenished during the A β 42 expression period (bar 3). Insulin did not protect if added after induction with doxycycline (bar 4). $n=3$ cultures each condition, error bar=1 SD. (B) Glucose uptake in C2C12 myotubes. Left graph; in control experiments, insulin stimulated uptake (bar 2). LY294002 and Cytochalasin B reversed insulin stimulated uptake. $n=3$ experiments, results are normalized to unstimulated control, ** $p<0.01$. (++) $p<0.01$ and + $p<0.05$ compared to insulin alone). Right graph; In a separate group of experiments, all in the presence of 45 nM insulin stimulation (30 min), the expression of A β 42 (24 h doxycycline) partially reduces insulin-stimulated glucose uptake (bar 3) in spite of grossly preserved cell viability. A β 42 expression and inhibition of PI3K/Akt signaling (LY294002) are additive in reducing glucose uptake. Results are normalized to insulin-stimulated control (lane 1).

Foxo3A: red; CD4: green; Nuclei: blue; Foxo3A colocalized nuclei: purple

Fig. 8.

Foxo3A translocation and Atrogin mRNA upregulation in experimental allergic myositis. A. and B. Normal mice immunized with C muscle protein (CIM) develop endomysial infiltrates of inflammatory lymphocytes, indicated by DAPI stained nuclei (blue) in B.), compared to vehicle immunized littermate in A.) Co-localization of nuclear Foxo3A appears as red. Low magnification, bar=50 μm. C. In CIM samples, Foxo3A bearing myonuclei (purple) are indicated with a yellow arrowhead (bar=20 μm). D. and E. In serial sections, scattered CD4+ (green) endomysial T cells in D.) are occasionally also Foxo3A positive in E.) (green arrowheads). A similar number of lymphocytes are identified using anti-CD8 marker (not shown). F. Centralized Foxo3A nuclei (yellow arrowheads) in a severely affected myofiber. G. and H. High magnifications of Foxo3A bearing lymphocyte and myofiber nuclei (purple), respectively. I. Atrogin-1 mRNA was quantified in control (n=5) and CIM (n=8) hamstring muscle relative to GAPDH mRNA **p<0.01.