Heat shock protein 70 overexpression does not attenuate atrophy in botulinum neurotoxin type A-treated skeletal muscle

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Botulinum neurotoxin type A (BoNT/A) is used clinically to induce therapeutic chemical denervation of spastically contracted skeletal muscles. However, BoNT/A administration can also cause atrophy. We sought to determine whether a major proteolytic pathway contributing to atrophy in multiple models of muscle wasting, the ubiquitin proteasome system (UPS), is involved in BoNT/A-induced atrophy. Three and ten days following BoNT/A injection of rat hindlimb, soleus muscle fiber cross-sectional area was reduced 25 and 65%, respectively. The transcriptional activity of NF-κB and Foxo was significantly elevated (1.5- to 2-fold) after 3 days but not 10 days, while atrogin-1 activity was not elevated at any time point. BoNT/A-induced polyubiquitination occurred after 3 days (3-fold increase) but was totally absent after 10 days. Proteasome activity was elevated (1.5- to 2-fold) after 3 and 10 days. We employed the use of heat shock protein 70 (Hsp70) to inhibit NF-κB and Foxo transcriptional activity. Electrotransfer of Hsp70 into rat soleus, before BoNT/A administration, was insufficient to attenuate atrophy. It was also insufficient to decrease BoNT/A-induced Foxo activity at 3 days, although NF-κB activity was abolished. By 10 days both NF-κB and Foxo activation were abolished by Hsp70. Hsp70 overexpression was unable to alter the levels of BoNT/A-induced effects on MuRF1/atrogin-1, polyubiquitination, or proteasome activity. In conclusion, Hsp70 overexpression is insufficient to attenuate BoNT/A-induced atrophy. It remains unclear what proteolytic mechanisms are contributing to BoNT/A-induced atrophy, although a Foxo-MuRF1-ubiquitin-proteasome contribution may exist, at least in early BoNT/A-induced atrophy. Further clarification of UPS involvement in BoNT/A-induced atrophy is warranted.

The ubiquitin-proteasome system (UPS) is known to be a critical regulator of skeletal muscle atrophy in multiple models of disuse (see Refs. 6, 8 for review). Studies have overexpressed select proteases (39), used dominant-negative repression of transcription factors (33), and inhibited the proteasome (9) to illustrate that the UPS contributes significantly toward the muscle atrophy occurring with immobilization (9), unloading (20), and nerve sectioning (3). Furthermore, research shows the nuclear factor-κB (NF-κB) and Forkhead box O (Foxo) transcriptional pathways are key upstream regulators of the UPS (39). Specifically, there is substantial evidence that immobilization and hindlimb unloading-induced skeletal muscle atrophy are regulated via the NF-κB and Foxo transcriptional pathways (33, 34, 38). These signaling pathways can directly regulate the transcription of E3 ligases, which are responsible for the key regulatory steps of protein degradation in the UPS (39). In addition, the atrophy accompanying physically denervated skeletal muscle demonstrates increased proteolytic activity, increased proteasome protein expression, and increased polyubiquitination (15). Collectively, these studies suggest that many models of reduced skeletal muscle activity use the UPS as the major pathway of myofibrillar degradation.

This study investigated whether atrophy induced by chemical denervation could be eliminated via inhibition of the activity of components of the UPS. We used the ubiquitous, cytoprotective protein, 70-kDa heat shock protein (Hsp70), as an UPS intervention, based on its success at abolishing atrophy in other models of disuse (39). There is evidence that Hsp70 levels are unchanged, or downregulated, in multiple models of skeletal muscle atrophy (22, 30, 42). However, Hsp70 has served to protect skeletal muscle from atrophy when it is increased from two- to sevenfold higher than control (10, 27,
One method of inducing such an increase is via whole body heating, which has been shown to increase levels of Hsp70 and attenuate the atrophy accompanying hindlimb unweighting (29, 37). More specifically, overexpression of Hsp70, via plasmid transfection, can completely prevent muscle loss during immobilization (39) and during senescence (10). Furthermore, Hsp70 overexpression has been shown to completely abolish the increase in immobilization-induced transcriptional activity of both NF-κB and Foxo, with the relative mRNA expression of the E3 ligases muscle RING-finger protein-1 (MuRF1) and atrogin-1 significantly attenuated (38, 39).

Given that the atrophy seen in multiple models of disuse is significantly prevented via the overexpression of Hsp70, we hypothesized that Hsp70 overexpression in skeletal muscle injected with BoNT/A would also be completely protected from atrophy, due to an inhibition of the NF-κB and Foxo

**MATERIALS AND METHODS**

**Animals.** Male Sprague-Dawley rats (~225 g starting weight; Charles River Laboratories, Wilmington, MA) were used for all animal experiments. All animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

**Experimental design.** Following a 3-day handling period, animals were assigned to one of four treatments: control (Con) [gelatin phosphate buffer (GBP) hindlimb injection; enhanced green fluorescent protein (EGFP) soleus injection]; Con + Hsp70 (GBP hindlimb injection; Hsp70-EGFP soleus injection); BoNT/A (BoNT/A hindlimb injection; EGFP soleus injection); and BoNT/A + Hsp70 (BoNT/A hindlimb injection; Hsp70-EGFP soleus injection). Because of the contralateral effect of BoNT/A, an individual animal received either control or BoNT/A injection in both their left and right leg. Soleus plasmid injections were performed 3 days before BoNT/A hindlimb injections. Animals were euthanized either 3 or 10 days following hindlimb BoNT/A injections, thus totaling eight total groups. The total number per group was six to eight solei. Solei collected after 3 and 10 days were subsequently analyzed for cross-sectional area (CSA), NF-κB, and Foxo transcriptional activity, MuRF1 and atrogin-1 protein expression, polyubiquitination, and chymotrypsin-like proteasome activity.

**Contractile properties.** For evidence of toxin effect upon force production, twitch force was determined before and after toxin injection, as previously used (11). Animals were placed in a prone position on a specially fabricated Plexiglas apparatus that allowed the animal to be secured in a reproducible position with limited mobility of the lower leg except at the tibiotarsal joint. The animals were kept warm by heating pads and an incandescent light. The core temperature was measured with a thermometer placed in the rectum and maintained between 35 and 38°C. A force/displacement ergometer was calibrated and secured to the forefoot between the first and second footpads by a lightweight metal wire such that the tibiotarsal angle was 90° (Steelon nylon-coated wire; Berkley Pure Fishing America, Spirit Lake, IA). The voltage signal from the force transducer was processed via a computerized data acquisition system (LabView; National Instruments, Austin, TX). A stainless-steel stimulating electrode (Monopolar needle electrode, 15 mm TP; BIOPAC Systems, Goleta, CA) was placed transcutaneously near the sciatic nerve midway between the posterior ischial spine and the greater femoral trochanter. Another stainless-steel stimulating electrode (anode) was inserted 3 mm subdermally in the midline of the lower back. The sciatic nerve was then stimulated proximally with a supramaximal voltage (90–125 V), 0.5 pulses/s, and a stimulus time of 0.5 ms (model S48; Grass Instruments, West Warwick, RI). Force measurements were made immediately before hindlimb injection and immediately before death.

**Expression and reporter plasmids.** The Hsp70-EGFP plasmid was created as previously described (39). The NF-κB/GL3 reporter plasmid was obtained from Dr. Steffan Ho (Beth Israel Deaconess Medical Center, Boston, MA) and has been previously used and described (19). The DAF-16 (Foxo) reporter plasmid has also been previously used and described and was obtained from Dr. Alex Toker (Beth Israel Deaconess Medical Center) (44). The plasmid DNA was prepared and isolated using an Endotoxin-Free Maxi or Mega Prep Kit (Qiagen, Valencia, CA).

**Soleus plasmid injection and electroporation.** Plasmid injection and sequential transfection of skeletal muscle have been described previously (26). In summary, animals were first anesthetized via continuous 2.5% isoflurane gas inhalation, then a small incision was made on the lateral side of the lower leg, and the soleus muscle was isolated. With the use of a 0.3-ml insulin syringe, the plasmid or plasmid combinations were injected evenly along the longitudinal axis of the soleus muscle. Following injection, electric pulses were delivered using an electric pulse generator (Electro Square Porator ECM 830; BTX, Hawthorne, NY) by placing two paddle-like electrodes on each side of the muscle. Five pulses were delivered in 200-ms interpulse intervals, each with an effective charge of 100 V cm and 20-ms duration. Electrotrode of a mixture containing two vectors in skeletal muscle shows cotransduction of a given fiber 75–95% of the time (2, 32); thus a fiber that takes up one vector will most likely also take up the other (39). The plasmid amounts injected were 10 μg of the expression plasmids (Hsp70-EGFP and EGFP control) and/or 40 μg of the reporter plasmids (NF-κB/GL3 and DAF-16) in a total volume of 50 μl × PBS. Verification that EGFP has no effect on muscle fiber CSA has previously been confirmed (21).

**Hindlimb injections.** Animals were administered an intramuscular injection of 0.1 ml of 3 units of BoNT/A (Dysport; Ipsen Pharmaceuticals) or 0.1 ml of GPB (gelatin phosphate buffer) in the triceps surae compartment of both hindlimbs (1 unit Dysport = 1 ng = 1 mouse LD50). Anatomical location of the hindlimb injection site has previously been described (11). Briefly, the rat was placed in a prone position and the length of the hindlimb measured. The injection site was 70% of the length of the hindlimb, proximal to the distal extremity. The injection depth was 20% of the hindlimb length.

**Muscle preparation and analysis.** Soleus muscles were removed and either rapidly frozen in liquid nitrogen and stored at −80°C for subsequent biochemical analyses or fixed in tissue-freezing medium and frozen for muscle cross sectioning and subsequent histochemical analysis. Animals were euthanized via a 150 mg/kg pentobarbital sodium intraperitoneal injection, and manual death was confirmed via pneumothorax.

**Membrane staining and CSA analysis.** Cross sections (10 μm) were cut with a cryostat microtome (Microm HM 550; Microm International, Walldorf, Germany) from the midbelly of the soleus muscle and fixed in 4% paraformaldehyde. The muscle sections were incubated with wheat germ agglutinin Texas Red-X conjugate (Invitrogen, Carlsbad, CA) for visualization of muscle fibers with fluorescence microscopy, and images were captured with an Olympus IX50 camera (Olympus, Tokyo, Japan) at ×20 magnification. The CSA of ~250 fibers from each muscle was traced and measured using Image Pro Discovery software (Media Cybernetics, Bethesda, MD). The selection of fibers for CSA analysis was undertaken as follows. The whole muscle section was divided into four quadrants and an image was captured at ×20 magnification at the center of each of the four quadrants. This yields ~50–100 fibers per image, depending on fiber size. Subsequently, all fibers in each captured image were measured to yield a total of at least 250 analyzed fibers per muscle. This was repeated for six to eight muscles per group. This technique is in accordance with previous studies (33, 39).

**NF-κB and Foxo reporter activity.** Muscles were homogenized in a passive lysis buffer (Promega, Madison, WI) and centrifuged for 20
Protein concentrations were determined using a detergent-compatible luciferase assay (Bio-Rad, Hercules, CA). Samples were diluted in loading buffer (Bio-Rad, Hercules, CA) containing 5% beta-mercaptoethanol to achieve a protein concentration of 2 μg/μl and were heat denatured. Equal amounts of protein were loaded onto 4–15% linear gradient gels and separated using SDS-polyacrylamide gel electrophoresis. Proteins were transferred for 90 min at 100 V onto an Immobilon-FL polyvinylidene fluoride membrane (Millipore, Bedford, MA), blocked in PBS containing 5% BSA for 1 h, and incubated overnight at 4°C with primary antibody diluted in blocking buffer. The following primary antibodies were used: anti-ubiquitin (U-5379; Sigma-Aldrich, St. Louis, MO); anti-Hsp70 (ab6535; Abcam, Cambridge, MA); anti-MuRF1 (MP3401; ECM Biosciences, Versailles, KY); and anti-alpha tubulin (sc-8035; Santa Cruz Biotechnology). Following a series of washes, the membranes were incubated with Alexa Fluor 680 or IRDye800 (LI-COR Biosciences) fluorescent-dye conjugated secondary antibodies and visualized using the Odyssey infrared imaging system (LI-COR Biosciences). Relative quantification of proteins was determined by measuring the fluorescence of each lane at the appropriate molecular fluorescence units was measured using a Spectramax microplate reader (Molecular Devices) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm following 90-min incubation at 37°C. Each assay was conducted in the absence and presence of a specific proteasome inhibitor (Lactacystin; Boston Biochem) to determine proteasome-specific activity. All samples were assayed in triplicate per muscle.

**Statistical analyses.** Hsp70 protein expression (Fig. 1) and overexpression of Hsp70 (Fig. 2) were analyzed using a Student’s t-test. The effect of Hsp70 overexpression on skeletal muscle fiber CSA in control vs. BoNT/A-treated muscle (Fig. 3) was analyzed using a two-way ANOVA followed by a Bonferroni correction. All other data (Figs. 4–7) were analyzed using a one-way ANOVA followed by Bonferroni corrections for multiple comparisons when appropriate (GraphPad Software, San Diego, CA). All data are expressed as means ± SE; significance was established at the P < 0.05 level.

**RESULTS**

Hindlimb force production following 3 units of Dysport injection was reduced 85% compared with control after 3 days and remained reduced to the same extent after 10 days (data not shown), in accordance with our previous study (31).

**Hsp70.** We found BoNT/A-induced atrophy to have no effect on endogenous Hsp70 protein expression compared with

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**Fig. 1.** Endogenous 70-kDa heat shock protein (Hsp70) protein expression. A: representative Western blot of whole cell lysates from solei of hindlimb injected with either control or botulinum neurotoxin type A (BoNT/A) and blotted for endogenous Hsp70. B: quantification of Western Blot analysis reveals BoNT/A does not affect endogenous Hsp70 levels. Bars represent means ± SE from at 6–8 solei per group.

**Fig. 2.** Overexpression of Hsp70 via Hsp70-EGFP plasmid injection. A: representative Western blot of whole cell lysates from solei injected with either an EGFP or Hsp70-EGFP expression plasmid and blotted for Hsp70. B: quantification of Western blot analysis reveals significant Hsp70 overexpression in Hsp70-EGFP plasmid injected solei. Bars represent means ± SE from at 6–8 solei per group. ***Significantly different from control (P < 0.001).
that of control (Fig. 1). To overexpress Hsp70, we injected and electrotransferred an Hsp70-EGFP expression plasmid or a control plasmid into the soleus muscle of rats. Overexpression of the Hsp70-EGFP fusion protein was confirmed via Western blot analysis (Fig. 2) and represents a level of overexpression that has previously been achieved via plasmid transfection (39) and that is normally seen with conditions such as heating (16, 37) or exercise (25, 47). Since Hsp70-EGFP is a fusion protein, the darkened band at 97 kDa in Fig. 2 is representative of Hsp70 (70 kDa) and EGFP (27 kDa) fused.

Muscle fiber atrophy. To determine whether overexpression of Hsp70 attenuates skeletal muscle fiber atrophy, the CSA of green fluorescent fibers (fibers expressing the Hsp70-EGFP fusion protein) was measured and compared with the CSA of nonfluorescing fibers (fibers not expressing Hsp70-EGFP).

Three days following BoNT/A hindlimb injection, soleus fiber CSA was reduced by 25% in both fibers expressing Hsp70-EGFP and not expressing Hsp70-EGFP ($P < 0.05$) (Fig. 3). Ten days following BoNT/A hindlimb injection, soleus fiber CSA was reduced by 65% in both fibers expressing Hsp70-EGFP and not expressing Hsp70-EGFP ($P < 0.05$) (Fig. 3). The transfection efficiency in these experiments was 60%, measured as previously described (39). Verification that EGFP has no effect on muscle fiber CSA has previously been confirmed (21), and therefore, the Con-EGFP group was not included in Figs. 4–7.

Signaling pathways. To determine the activity of the NF-κB and Foxo pathways after BoNT/A administration, we employed the use of either a NF-κB-GL3 or DAF-16 reporter. NF-κB and Foxo signaling has been shown to be required for unloading-induced muscle atrophy (18, 38). Furthermore,
Hsp70 overexpression has been shown to inhibit both NF-κB and Foxo activity and prevent skeletal muscle atrophy (39).

Three days following BoNT/A injection, NF-κB-GL3 luciferase activity was increased twofold in BoNT/A-treated muscle; however, Hsp70 overexpression was able to attenuate NF-κB to that of control (Fig. 4A). DAF-16 luciferase activity increased over fivefold; however, Hsp70 overexpression was unable to reduce the BoNT/A-induced increase in DAF-16 luciferase activity (Fig. 4B).

Ten days following BoNT/A injection, Hsp70 overexpression completely diminished BoNT/A-induced increases in NF-κB and Foxo activity. NF-κB-GL3 luciferase activity was increased over fourfold in BoNT/A-treated muscle not receiving Hsp70, while in BoNT/A-treated muscle overexpressing Hsp70 NF-κB-GL3 luciferase activity was essentially absent (Fig. 4A). DAF-16 luciferase activity was increased approximately sixfold in BoNT/A-treated muscle not receiving Hsp70, while in BoNT/A-treated muscle overexpressing Hsp70, the DAF-16 luciferase activity was abolished (Fig. 4B).

E3 ligases. Three days following BoNT/A injection, protein expression of MuRF1 was significantly elevated in BoNT/A-treated muscle compared with that of control (Fig. 5A), while atrogin-1 mRNA expression was not significantly elevated (Fig. 5B). Ten days following BoNT/A injection, protein expression of MuRF1 (Fig. 5E) and atrogin-1 (Fig. 5F) revealed no difference between control and BoNT/A- and BoNT/A-Hsp70-treated muscle.

Ubiquitinated proteins. Ubiquitinated proteins are representative of proteins targeted for degradation by the UPS. Three days following hindlimb BoNT/A injection, soleus muscle exhibited significant elevation of polyubiquitination, which is not attenuated by Hsp70 overexpression (Fig. 6, A and B). Ten days post-BoNT/A injection, soleus muscle demonstrated no significant elevation of ubiquitinated proteins in either BoNT/A-treated muscle or BoNT/A-Hsp70-treated muscle (Fig. 6, C and D).

Proteasome activity. The chymotrypsin-like activity of 20S and 26S proteasome following BoNT/A injection was assayed. Three days following hindlimb BoNT/A injection, soleus muscle exhibited approximately twofold increase in 20S chymotrypsin-like activity, which was not attenuated by Hsp70 overexpression (Fig. 7A). Similarly, Hsp70 overexpression could not attenuate the BoNT/A induced elevation of the 26S proteasome activity (Fig. 7B). Elevation in 20S and 26S chymotrypsin-like activities was still present 10 days following BoNT/A injection, which Hsp70 overexpression was unable to attenuate (Fig. 7, C and D).

**DISCUSSION**

**Summary of results.** As hypothesized, 3 and 10 days following BoNT/A injection of rat hindlimb, soleus muscle fiber CSA was reduced 25 and 65%, respectively. As also hypothesized, the transcriptional activity of NF-κB and Foxo was significantly elevated by BoNT/A. In contrast, MuRF1 activity was significantly elevated after 3 days but not 10 days, while atrogin-1 activity was not elevated at any time point. Increased polyubiquitination was present at 3 days but not at 10 days.

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Fig. 4. Transcriptional activity 3 and 10 days post-control or BoNT/A injection. A: NF-κB reporter activity from the soleus muscle of control vs. BoNT/A-injected rats, injected with either an EGFP or Hsp70-EGFP plasmid plus the NF-κB reporter plasmid. B: Foxo reporter activity from the soleus muscle of control vs. BoNT/A-injected rats, injected with either a EGFP or Hsp70-EGFP plasmid plus the Foxo reporter plasmid. Bars represent means ± SE from 6–8 solei per group. *Significantly different from control (P < 0.05). **Significantly different from control (P < 0.001). #Significantly different from BoNT/A (P < 0.05). ###Significantly different from BoNT/A (P < 0.05).
However, proteasome activity was significantly elevated after both 3 and 10 days.

Subsequent use of Hsp70 overexpression did not have a prophylactic effect on muscle, as it has done in other models of disuse. Despite Hsp70 overexpression inhibiting NF-κB transcriptional activity, there was no attenuation of atrophy. Hsp70 overexpression was also insufficient to decrease BoNT/A-induced Foxo activity at 3 days. Furthermore, Hsp70 overexpression was unable to alter the levels of any BoNT/A-induced effects on MuRF1/atrogin-1, polyubiquitination, or proteasome.
activity. The reasons for the lack of protective effectiveness of Hsp70 overexpression in our model compared with its protective effect in other disuse models is unclear.

**BoNT/A-induced atrophy.** BoNT/A injection induced an approximate 25 and 65% decrease in CSA of rat soleus muscle fiber at 3 and 10 days, respectively. This is a drastic reduction in muscle fiber area in a relatively short duration. On the basis of other models, we expected an overexpression of Hsp70 in BoNT/A-injected muscle to completely attenuate BoNT/A-induced atrophy via an inhibition of the increase in NF-κB and Foxo transcriptional activity and markers of activation of the UPS. For example, overexpression of Hsp70 during immobilization abolished NF-κB and Foxo transcriptional activity and was sufficient to completely attenuate the loss of muscle fiber CSA (39). Furthermore, Hsp70 overexpression has been shown to inhibit NF-κB activity and attenuate atrophy in multiple models of atrophy (10, 24, 27, 39). However, unlike the phenomenon observed in those models of disuse, Hsp70 overexpression was unable to maintain the CSA of BoNT/A-injected muscle to that of control. Whether this is due to Hsp70 being unable to effect key components of the UPS under these conditions or BoNT/A inducing atrophy via a pathway other than the UPS remains unknown.

**Transcriptional activity.** Hsp70 overexpression was unable to prevent BoNT/A-induced atrophy despite an attenuation of NF-κB activity after 3 days and complete abolishment of both NF-κB and Foxo activation after 10 days. Comparatively, Senf et al. (39) overexpressed Hsp70 during immobilization and completely prevented both NF-κB and Foxo transcriptional activity. Furthermore, Hsp70 overexpression was sufficient to completely attenuate the loss of muscle fiber CSA. In this study, Foxo activity was elevated 3 days following BoNT/A administration but was not attenuated by Hsp70 overexpression. This may represent transcriptional activity that contributes to BoNT/A-induced atrophy. However, both NF-κB activity and Foxo activity were inhibited by Hsp70 overexpres-
sion at 10 days post-BoNT/A injection. We have seen that CSA loss continues between days 3 and 10. Thus, if Hsp70 overexpression is acting to abolish NF-κB and Foxo activation, as seen in other models, it is unclear why Hsp70 overexpression is not also acting to decrease UPS activity and attenuate atrophy.

**E3 ligase activity.** Results revealed a 1.5- to 2-fold elevation of MuRF1 protein expression 3 days after BoNT/A injection but no elevation of atrogin-1. Hsp70 overexpression was unable to attenuate the increased MuRF1 expression. Velders et al. (49) observed an increase in MuRF-1 mRNA (~2-fold) but no elevation in atrogin-1 mRNA, albeit 3 wk following BoNT/A injection in juvenile rat gastrocnemius. Mukund et al. (28) performed global transcriptional analysis of BoNT/A-treated muscle. Seven days following 6 U/kg Botox injection (which equates to ~7 units of Dysport; Ref. 12) in rat tibialis anterior muscle, there were dramatic transcriptional expression changes. Specifically, these authors noted transcriptional alterations in genes responsible for excitation-contraction coupling, sarcomeric contraction, metabolism, mitochondrial biogenesis, and oxidative stress. MuRF1 and atrogin-1 mRNA expression was elevated but not drastically (MuRF1: 0.6-fold; atrogin-1: 1.3-fold), especially considering Mukund’s rats received a neurotoxin dosage twice as potent as the one used in our study. These two studies, taken together with our study, reveal that there may only be minor elevation of expression of these specific E3 ligases following BoNT/A injection. The small degree of E3 ligase elevation seen in BoNT/A studies becomes...
even more apparent when the mRNA expression of atrogin-1 and/or MuRF1 is compared with 7 days of immobilization (3-fold; Ref. 39), 7 days of hindlimb unloading (3-fold; Ref. 42), and 3 days following physical denervation (~3-fold; Ref. 15). Thus it appears the transcriptional activity of these E3 ligases are not elevated to the same extent in BoNT-treated muscle as that seen in immobilized, unloaded, or physically denervated muscle. However, the fact remains that MuRF1 activity was indeed elevated after 3 days. This mirrors the increased transcriptional activity of Foxo, and thus a mechanistic link may exist here. The reason for the differing outcome in NF-κB and Foxo affecting downstream transcription targets in conjunction with BoNT/A administration is unclear.

**Polyubiquitination and proteasome activity.** BoNT/A induced a threefold increase in polyubiquitination and approximately twofold increase in chymotrypsin-like activity of the proteasome after 3 days, neither of which Hsp70 overexpression was able to suppress. Thus, perhaps unsurprisingly, atrophy was not attenuated at this time point. From our results, it appears that at 3 days a Foxo-MuRF1-ubiquitin-proteasome pathway may account for some of the atrophy. Whether this is the sole contributor of the 25% decrease in muscle fiber CSA seen 3 days post-BoNT/A injection seems unlikely. This atrophy represents an extremely swift and drastic phenomenon. Moreover, other disuse models demonstrate highly elevated levels of both NF-κB and Foxo, and several E3 ligases. Of course, MuRF1 and atrogin-1 are not the only E3 ligases known to be involved in atrophy, and the possibility remains that some other unmeasured proteolytic E3 ligases may play a significant role.

At 10 days there is a lack of polyubiquitination. Ten days post-BoNT/A injection may be a time point whereby any rise in ubiquitin levels may have fallen back to that seen in control muscle. However, considering 7–10 days is a time point commonly used to assess polyubiquitinated protein levels in other models of disuse, an absence of polyubiquitination 10 days following BoNT/A injection suggests the UPS may not be active as an atrophic mechanism in the latter stages of BoNT/A-induced atrophy. Indeed, there is continued muscle fiber CSA loss between 3 and 10 days; thus some proteolytic mechanism must be permitting protein degradation. Given the lack of ubiquitination, albeit with increased proteasome activity still observed, there is the possibility that the continued BoNT/A-induced atrophy may occur via an ubiquitin-independent process or via proteolytic mechanisms outside of the UPS. Indeed, limited data investigating BoNT/A-induced atrophy reveal some elevation of a marker of autophagy (45) but an absence of elevated markers of apoptosis (48). However, the inability of Hsp70 overexpression to neither attenuate BoNT/A-induced atrophy nor prevent proteasome activity further supports the notion of an ubiquitin-independent mechanism of atrophy. In immobilized muscle (39), Hsp70 overexpression attenuates atrophy through abolishment of polyubiquitination. If polyubiquitination only presents early in BoNT/A-treated muscle, then it follows suit that Hsp70 will be less able to implement its prophylactic effect upon atrophy, as it has done in other models. However, the extent of the contribution of any potential ubiquitin-independent mechanism is currently unknown.

**Conclusion.** We have demonstrated that transcriptional activity of the Foxo and NF-κB signaling pathways, MuRF1 expression, polyubiquitination, and proteasome activity are all significantly elevated during early BoNT/A-induced atrophy. However, during late BoNT/A-induced atrophy, there is an absence of MuRF1 expression and polyubiquitination. Hsp70 is unable to attenuate BoNT/A-induced atrophy at either time point but is able to abolish late NF-κB and Foxo activity.

It remains unclear what proteolytic mechanism/s are contributing toward BoNT/A-induced atrophy, although our results suggest that a Foxo-MuRF1-ubiquitin-proteasome contribution may exist, at least in early BoNT/A-induced atrophy. However, our results suggest that other mechanisms may be involved at various stages of atrophy, such as ubiquitin-independent proteasome activity. Further clarification of the involvement of the UPS in BoNT/A is warranted, as it is the potential involvement of other proteolytic pathways that are known to cause atrophy in other disuse models.

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