Utility of 17-(allylamino)-17-demethoxygeldanamycin treatment for skeletal muscle injury

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Received: 5 May 2016 / Accepted: 29 June 2016 / Published online: 11 July 2016
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Abstract Repeated eccentric contractions can injure skeletal muscle and result in functional deficits that take several weeks to fully recover. The 70-kDa heat shock protein (Hsp70) is a stress-inducible molecular chaperone that maintains protein quality and plays an integral role in the muscle's repair processes following injury. Here, we attempted to hasten this recovery by pharmacologically inducing Hsp70 expression in mouse skeletal muscle with 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) (40 mg/kg) both prior to and throughout the first 7 days after an injurious bout of 150 maximal eccentric contractions. Hsp70 content in the injured skeletal muscle was strongly induced following the eccentric contractions and remained elevated over the next 7 days as the muscle underwent repair. Treatment with 17-AAG increased Hsp70 content ~five-fold; however, this was significantly less than that induced by the injury. Moreover, 17-AAG treatment did not recover the decrements to *in vivo* isometric torque production following the bout of eccentric contractions. Together, these findings demonstrate that although Hsp70 content was induced in the uninjured skeletal muscle, treatment of 17-AAG (40 mg/kg) was not a preventive measure to either reduce the severity of skeletal muscle damage or enhance functional recovery following a bout of maximal eccentric contractions.

Keywords Chaperones · Damage · Eccentric contractions · Isometric torque · Mouse model

Introduction

Skeletal muscle exhibits a remarkable ability to regenerate and repair from eccentric contraction-induced injury. A common method to quantify functional recovery of skeletal muscle following injury is to measure its ability to generate peak isometric torque at various time points during the repair processes (Warren et al. 1999). For example, we have shown that reductions in isometric torque can exceed 50 % in the days post-injury and take approximately 2 weeks to fully recover (Baumann et al. 2014a, 2016). The majority of strength loss early after the injury results from a failure in voltage-gated sarcoplasmic reticulum (SR) Ca²⁺ release (Ingalls et al. 1998b; Warren et al. 2001), which appears to be partially restored by the return of key excitation-contraction (EC) coupling proteins that are disrupted immediately post-injury (Baumann et al. 2014a; Corona et al. 2010). Beyond this point, strength deficits are primary due to the loss of myofibrillar proteins, such as actin and myosin heavy chain (MHC) (Ingalls et al. 1998a). Therefore, complete recovery of isometric torque is dependent on replacing these damaged or lost contractile proteins through increased protein synthesis rates and satellite cell activity (Baumann et al. 2016; Lowe et al. 1995; Rathbone et al. 2003).

The 70-kDa heat shock protein (Hsp70) is a stress-sensing chaperone responsible for maintaining protein quality control by properly folding newly synthesized or denatured proteins (Kiang and Tsokos 1998; Mayer and Bukau 2005; Morimoto 1991) and is known to play a significant role in skeletal muscle repair processes (Liu et al. 2006; Senf 2013). Importantly, following eccentric contraction-induced injury, Hsp70 content

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increases within hours, remains elevated for several days (Holwerda and Locke 2014; Ingalls et al. 1998a; Paulsen et al. 2007; Thompson et al. 2001), and correlates with strength deficits (Paulsen et al. 2007), all of which suggest Hsp70 is required to preserve and promote protein quality during recovery. In support of this, several investigations have noted beneficial effects after inducing Hsp70 prior to eccentric contraction-induced injury (Kayani et al. 2008a; McArdle et al. 2004). For instance, pharmacological induction of Hsp70 with 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) (Kayani et al. 2008a) and transgenic overexpression of Hsp70 (McArdle et al. 2004) were both able to enhance functional recovery in injured muscle of senescent mice. However, the influence of pre-injury induction of Hsp70 and its effects on subsequent skeletal muscle function after eccentric contraction-induced injury in otherwise healthy adult mice remains unclear. Accordingly, treatment with compounds known to induce Hsp70 may provide a preventive therapeutic strategy to minimize the extent of injury and/or improve the recovery process following exercise-like contractile activity.

To address this contention, we treated 5–7-month-old mice with 17-AAG and assessed *in vivo* isometric torque before and after a bout of 150 maximal eccentric contractions. We hypothesized that 17-AAG treatment would attenuate strength deficits early after eccentric contraction-induced injury, therefore proving to be an effective method of enhancing skeletal muscle function.

Methods

Animals

Male C57BL6 mice 5–7 months old (28.61 ± 0.31 g) were used in this study. Mice were housed in groups of five animals per cage, supplied with food and water *ad libitum*, and maintained in a room at 20–22 °C with a 12-h photoperiod. Mice were euthanized with an overdose of isoflurane followed by cervical dislocation.

Experimental design

Mice were randomly assigned to one of two groups: vehicle-treated or drug-treated. The vehicle-treated (control) group received intraperitoneal (i.p.) injections of dimethyl sulfoxide (DMSO; Sigma, St Louis, MO), while the drug-treated group received i.p. injections of 17-AAG (LC Laboratories, Woburn, MA) at a dose of 40 mg/kg body weight in 100 μ L of DMSO as previously described (Baumann and Otis 2015; Kayani et al. 2008a). This dose of 17-AAG and route of delivery has been shown to result in 99 % bioavailability of the drug in skeletal muscle (Egorin et al. 2001). Treatment of DMSO or

17-AAG began 3 days prior to an eccentric contraction-induced injury to the left anterior crural muscles [tibialis anterior (TA), extensor digitorum longus (EDL), and extensor hallucis muscles] (Fig. 1). Immediately after the injury (~4 h; referred to as “post”) and 2, 5, and 7 days into recovery, the right and left TA muscles were weighed, immediately frozen in liquid nitrogen, and stored at –80 °C for Western blots analysis of Hsp70 protein expression. Mice that were designated to the 5- or 7-day groups received another treatment 4 days post-injury (i.e., 7 days after the first treatment). Using this treatment strategy, we and others have documented significant increases in skeletal muscle Hsp70 content (Baumann and Otis 2015; Kayani et al. 2008a). For each respective treatment, the contralateral uninjured (right) TA muscles from the post and 2-, 5-, and 7-day groups were combined and designated as the uninjured control or uninjured 17-AAG.

To assess if 17-AAG attenuated strength deficits or altered contractile parameters following the eccentric contraction-induced injury, *in vivo* isometric torque produced by the left anterior crural muscles were measured immediately before (pre) and after (post) the eccentric injury and also in subgroups of these mice 2, 5, or 7 days into recovery (Fig. 1). It is important to note that although this *in vivo* model measures isometric torque produced by the left anterior crural muscles, the TA comprises 82 % of the muscle group mass and contributes 89 % of the peak torque produced by the muscle group making it a valid assessment of TA muscle function (Lowe et al. 1995; Warren et al. 2002).

Experimental methodology

In vivo analysis of muscular torque

Contractile function (i.e., torque-frequency relationship) of the left anterior crural muscles was measured *in vivo* as previously described (Baumann and Otis 2015; Baumann et al. 2014a, 2014b, 2016; Lowe et al. 1995; Rogers et al. 2015).

Fig. 1 Experimental timeline for treatment, assessing *in vivo* torque, injury, and tissue collection. *In vivo* torque was measured before (pre), immediately after (post), and 2, 5, or 7 days following a bout of 150 maximal eccentric contractions (i.e., the injury). Treatment was an i.p. injection of 17-AAG or the vehicle

Briefly, mice were anesthetized with isoflurane (1.5 % isoflurane and 400 mL O₂ per minute) and placed on a temperature-controlled platform to maintain core body temperature between 35 and 37 °C. The left knee was clamped and the left foot was secured to an aluminum “shoe” that is attached to the shaft of an Aurora Scientific 300B servomotor (Aurora Scientific, ON, Canada). Sterilized platinum needle electrodes were inserted through the skin for stimulation of the left common peroneal nerve. Stimulation voltage and needle electrode placement were optimized with 5–15 isometric contractions (200 ms train of 0.1 ms pulses at 300 Hz). Following optimization, contractile function of the anterior crural muscles was assessed by measuring isometric torque as a function of stimulation frequency (20–300 Hz), with isometric twitch and peak torque recorded at 20 and 300 Hz, respectively (Fig. 2).

Because the majority of the force loss within the first 3 days following this eccentric injury model is due to impaired voltage-gated SR Ca²⁺ release (Ingalls et al. 1998b; Warren et al. 2001), EC coupling failure was also indirectly assessed.

Fig. 2 Torque-frequency relationship (20–300 Hz) of the anterior crural muscles for vehicle (control) and 17-AAG-treated mice across the study. Measurements were done in vivo before (pre), immediately after (post), and 2, 5, or 7 days post-injury. Control represents mice treated with the vehicle. Torque-frequency data was fit using a Hill, four parameter sigmoidal curve. Pre and post, $n = 18$ –20 per group; days 2, 5, and 7, $n = 4$ –5 per group. Values are mean $\pm$ SEM

In skeletal muscle, EC coupling failure is broadly defined as any disruption in the sequence of events linking the activation of the voltage-sensitive dihydropyridine receptors (DHPR) embedded in the T-tubule membrane to the release of Ca²⁺ through the ryanodine receptor (RyR) located in the SR membrane. To indirectly assess EC coupling failure in the anterior crural muscles, low- and high-frequency (i.e., 20/300 Hz) torques were compared. More pronounced reductions in twitch relative to peak torque have traditionally been used to indicate greater EC coupling failure (Edwards et al. 1977; Ingalls et al. 2004; Jones et al. 1982).

Eccentric contraction-induced skeletal muscle injury

To induce skeletal muscle injury, the left anterior crural muscles performed a single bout of 150 maximal eccentric contractions following the initial torque assessment (i.e., pre-injury torque) as previously described (Baumann et al. 2014a, 2016; Lowe et al. 1995). Briefly, eccentric contractions were performed through a 38° angular movement at 2000° s⁻¹ starting from a 19° dorsiflexed position, which were preceded by a 100 ms isometric stimulation.

Protein extraction, protein quantification, and Western blotting

The tibialis anterior (TA) muscles were homogenized in ice-cold RIPA lysis and extraction buffer (Thermo Scientific, Rockford, IL) supplemented with a protease inhibitor cocktail (Thermo Scientific, Rockford, IL). Total protein content was quantified using the bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL). A portion of the muscle homogenate was then diluted in a loading buffer and boiled for 4 min. Equal amounts of protein (25 µg) were loaded onto a 12 % SDS polyacrylamide gel and separated according to molecular weight (100 V for 80 min). The proteins were then transferred to a nitrocellulose membrane using a trans-blot turbo transfer system at a constant 1.3 A, up to 25 V for 7 min (Bio-Rad Laboratories, Hercules, CA) and blocked overnight at 4 °C in 5 % nonfat dried milk (w/v) dissolved in tris-buffered saline with 0.1 % Tween-20 (TBS-T). Following the block, the membranes were probed with an anti-Hsp70 primary antibody (HSPA1A-72 kDa, 1:8000; R&D Systems, Minneapolis, MN) for 2 h at room temperature on an orbital shaker. Following incubation in the primary antibody, membranes were washed with TBS-T (3 $\times$ 15 min) and then probed with a horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (1:15,000; Sigma-Aldrich, St. Louis, MO) for 1 h at room temperature with shaking and washed as previously stated. Membranes were treated with an enhanced chemiluminescent solution (Thermo Scientific, Rockford, IL) prior to detection using a Bio-Rad ChemiDoc imaging station (Bio-Rad Laboratories, Hercules, CA) and analyzed by

densitometry using QuantityOne software (Bio-Rad Laboratories, Hercules, CA).

Statistics analysis

An independent *t* test or factorial ANOVA (treatment by time) was used for comparisons between treatment groups before or across the study. A Bonferroni's post hoc test was performed in the event of a significant ANOVA. An α level of ≤ 0.05 was used for all analyses. Values are presented in mean $\pm$ SEM. All statistical testing was performed using SigmaPlot version 11.0 (Systat Software, San Jose, CA).

Results

Pre-injury in vivo isometric torque and eccentric contraction-induced muscle injury

As expected, no differences were observed in twitch or peak isometric torque between the control and 17-AAG groups before the injury (Figs. 2 and 3). After this initial torque assessment, the mice performed a single bout of 150 eccentric contractions. Peak eccentric torque was 99 and 102 % greater for the control and 17-AAG groups, respectively, when compared to peak isometric torque ($p < 0.001$) and were not different between groups (Fig. 4). Over the course of the eccentric protocol (i.e., from the first to last contraction), peak eccentric torque was reduced over 60 % ($p < 0.001$), which was also not different between groups.

Recovery of in vivo isometric torque

The single bout of eccentric contractions resulted in significant injury, as evidenced by immediate and prolonged reductions in the torque-generating capacity of the anterior crural

Fig. 3 Twitch (20 Hz) and peak (300 Hz) isometric torque produced by the anterior crural muscles across the study. Measurements were done in vivo before (pre), immediately after (post), and 2, 5, or 7 days post-injury. Control represents mice treated with the vehicle. Values with the

Fig. 4 Peak in vivo torque produced by the anterior crural muscles across 150 maximal eccentric contractions. Control represents mice treated with the vehicle. Values with the same letter are not significantly different from each other. $n = 18$ –20 per group. Values are mean $\pm$ SEM

muscles (Figs. 2 and 3). However, no differences were observed between treatments. Taken together, isometric twitch torque was reduced by 81 % immediately post-injury and reduced by 65, 41, and 36 % at 2, 5, and 7 days into recovery ($p < 0.001$). Similar deficits were also observed as stimulation frequency increased. For example, peak isometric torque (i.e., 300 Hz) was reduced by 55, 43, 34, and 29 % immediately and 2, 5, and 7 days post-injury, respectively ($p < 0.001$).

Low- to high-frequency torque ratio

To indirectly assess if 17-AAG influenced recovery of EC coupling failure, isometric twitch to peak torque ratios were determined at each time point (Fig. 5). The injury depressed twitch to peak torque ratios immediately (59 %) and 2 days (40 %) post-injury ($p < 0.001$), indicative of significantly EC coupling failure. By 5 days post-injury, the ratios had returned to pre-injury levels and remained at baseline through day 7. However, no differences were observed between the control and 17-AAG groups at any time point.

same letter are not significantly different from each other. Pre and post, $n = 18$ –20 per group; days 2, 5, and 7, $n = 4$ –5 per group. Values are mean $\pm$ SEM

Fig. 5 Low- to high-frequency torque ratios of the anterior crural muscles across the study. The twitch (20 Hz) to peak (300 Hz) torque ratios were used to indirectly assess recovery of excitation-contraction (EC) coupling failure. Measurements were done in vivo before (pre), immediately after (post), and 2, 5, or 7 days post-injury. The dashed line indicates the baseline level of uninjured control muscle. Control represents mice treated with the vehicle. Values with the same letter are not significantly different from each other. Pre and post, $n = 18$ –20 per group; days 2, 5, and 7, $n = 4$ –5 per group. Values are mean $\pm$ SEM

Hsp70 content

Treatment of 17-AAG resulted in a ~fivefold increase in Hsp70 content in the uninjured muscle ($p < 0.001$) (Fig. 6). Immediately post-injury, Hsp70 increased in the injured control muscle to levels comparable to the uninjured 17-AAG-treated muscle. By day 2, Hsp70 content in both the injured control and 17-AAG-treated muscle dramatically increased and remained elevated through day 7 ($p < 0.001$). No differences were found in Hsp70 content between the injured control and 17-AAG groups at 2, 5, or 7 days post-injury.

Discussion

The purpose of this study was to determine if treatment with 17-AAG could attenuate torque deficits in adult skeletal muscle after a single bout of 150 maximal eccentric contractions. Here, we report that eccentric contraction-induced injury significantly increased Hsp70 content, which peaked 2 days post-injury and remained elevated through the 7 days measured. Administration of 17-AAG significantly elevated Hsp70 content prior to the injury; however, the injury resulted in a more robust expression of Hsp70. Moreover, the eccentric contractions reduced in vivo isometric torque production capacity to a similar extent in mice treated with 17-AAG or the vehicle (control). These observations clearly demonstrate that treatment of 17-AAG at a dose of 40 mg/kg does not hasten functional recovery in healthy adult mice early after a bout of maximal eccentric contractions.

In the unstressed state, Hsp70 content is virtually undetectable (Baumann and Otis 2015; McArdle et al. 2004). In

Fig. 6 Hsp70 content of the tibialis anterior (TA) muscle across the study. Hsp70 content was determined using Western blot analysis on the contralateral uninjured TA muscles (right legs) and the injured TA muscles (left legs) immediately after (post) and 2, 5, or 7 days post-injury. For each respective treatment, the contralateral uninjured TA muscles across all time points were combined and designated as the uninjured control or uninjured 17-AAG. Control represents mice treated with the vehicle. Values with the same letter are not significantly different from each other. Pre, $n = 15$ per group; post and days 2, 5, and 7, $n = 4$ –5 per group. Values are mean $\pm$ SEM

response to stress, damaged proteins unfold and expose hydrophobic peptide regions that attract Hsp70 (Mayer and Bukau 2005). Cytoplasmic Hsp70 is thought to interact with these denatured proteins and refold them, and possibly prevent their degradation via the proteasome (Marques et al. 2006). The extent and duration of Hsp70 expression is likely dependent on the amount of damage elicited by the injury protocol (Paulsen et al. 2007), with isolated peak eccentric contractions increasing content to a greater extent and for a longer duration when compared to that of less damaging protocols such as downhill running (Ingalls et al. 1998a; Lewis et al. 2013; Thompson et al. 2001; Touchberry et al. 2012). Interestingly, despite the differences between eccentric protocols, Hsp70 content appears to increase around the same time following the injury. For example, we and others have shown that Hsp70 is elevated in the early hours to days post-injury (Holwerda and Locke 2014; Ingalls et al. 1998a; Lewis et al. 2013; Paulsen et al. 2007; Thompson et al. 2001; Touchberry et al. 2012). Together, these findings demonstrate that a clear relationship exists between the magnitude of damage caused by the eccentric protocol and Hsp70 content, and further suggest that Hsp70 is required to mediate the early repair events following skeletal muscle damage.

However, only a handful of investigators have attempted to induce Hsp70 prior to eccentric contraction-induced skeletal muscle injury in order to provide protection from the initial insult and/or hasten recovery. Using an Hsp70 overexpression model, McArdle et al. (2004) found EDL muscle from old mice was protected from secondary damage and recovered at an accelerated rate following a single bout of 450 maximal eccentric contractions in situ. Specifically, old Hsp70 transgenic mice were able to recover in 14 days while old wild-type mice still exhibited force deficit of 44 % at 28 days post-injury (McArdle et al. 2004). Similar findings were also reported with pharmaceutical treatment of 17-AAG at a dose of 40 mg/kg in old mice (Kayani et al. 2008a). This drug is a derivative of the benzoquinone ansamycin antibiotic geldanamycin and is a known Hsp70 inducer in rodent skeletal muscle (Baumann and Otis 2015; Kayani et al. 2008a; Lomonosova et al. 2012; Wagatsuma et al. 2011). Both geldanamycin and 17-AAG drive Hsp70 expression through inhibition of Hsp90 by binding to its ATP-binding pocket thereafter allowing HSF-1 to enter the nucleus (Söti et al. 2005). In 28-month-old mice, Kayani et al. (2008a) reported force deficits of only 16.4 % after 17-AAG treatment while force was still reduced 51.7 % in the controls 28 days after a bout of 450 maximal eccentric contractions in situ.

Interestingly, EDL muscle from Hsp70 transgenic adult mice was also protected from secondary damage and recovered at an enhanced rate following the eccentric contraction-induced injury when compared to their wild-type counterparts (McArdle et al. 2004). In order to mimic these results using a clinically relevant intervention, we treated adult mice with 17-AAG before and after a bout of 150 eccentric contractions and assessed contractile function in vivo up to 7 days post-injury. Contrary to Kayani et al. (2008a), we did not show any protective effects of 17-AAG following injury. However, this was not due to the efficacy of the treatment, as 17-AAG (40 mg/kg) increased Hsp70 content ~fivefold, which is comparable to the 2–4-fold reported by Kayani et al. (2008a). Because of these similarities, it appears the main difference between studies is the age of the mice, 5–7 vs. 28 months old. In old mice, the muscle's ability to express Hsp70 and/or induce its expression is thought to be reduced upon stress (McArdle et al. 2004; Vasilaki et al. 2002), indicating Hsp70 content may be a rate limiting protein, and thus any further increase in Hsp70 content above the physiological stress response in aged muscle would prove beneficial. Additionally, the increase in Hsp70 content after 17-AAG treatment may have been below the threshold needed to influence repair in healthy adult skeletal muscle (Kayani et al. 2008b). As depicted in Fig. 6, Hsp70 content 2 to 7 days following the bout of 150 maximal eccentric contractions was drastically greater (i.e., ~18-fold) than that achieved from 17-AAG treatment. In support of this concept, McArdle et al. (2004) reported Hsp70 content of adult

Hsp70 transgenic mice was 10–20-fold greater than wild-type mice and was sufficient to enhance recovery. In order to achieve these levels of Hsp70 using 17-AAG, a greater dosage would be needed; however, high amounts of 17-AAG are known to be toxic (Page et al. 1997; Solit et al. 2002).

The present findings, along with those previously published (Baumann and Otis 2015; Kayani et al. 2008a), demonstrate the complexity of using pharmaceutical drugs like 17-AAG to induce Hsp70 in attempt to enhance recovery. We recently reported 17-AAG was unable to alter muscular function or morphological recovery following a barium chloride (BaCl₂)-induced injury in the TA of adult mice, even though 17-AAG increased Hsp70 content to a greater extent when compared to the injury (Baumann and Otis 2015). Therefore, we speculated the amount of damage caused by the BaCl₂ was too severe and that Hsp70 induction would be better suited in an eccentric contraction-induced injury model. However, as the present findings show, the dosing strategy was not potent enough to increase Hsp70 content to the levels induced by the bout of 150 maximal eccentric contractions. These data suggest that the efficacy of Hsp70-inducing drugs, like 17-AAG, are not only dependent on age but also the type of injury (i.e., contraction- vs. trauma-induced) and extent of Hsp70 induction. Future studies will need to focus on each of these variables, while considering the drug's toxicity if pharmaceutical induction of Hsp70 is to be a successful method of attenuating functional deficits following skeletal muscle injury.

Compliance with ethical standards All procedures were approved by the Georgia State University Institutional Animal Care and Use Committee.

Funding This study was partially supported by a NIA/NIH training grant (T32-AG029796).

Disclosures No conflicts of interest, financial or otherwise, are declared by the authors.

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