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Regrowth after skeletal muscle atrophy is impaired in aged rats, despite similar responses in signaling pathways

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ABSTRACT

Skeletal muscle regrowth after atrophy is impaired in the aged and in this study we hypothesized that this can be explained by a blunted response of signaling pathways and cellular processes during reloading after hind limb suspension in muscles from old rats. Male Brown Norway Fisher 344 rats at 6 (young) and 32 (old) months of age were subjected to normal ambulatory conditions (amb), hind limb suspension for 14 days (HS), and HS followed by reloading through normal ambulation for 14 days (RE); soleus muscles were used for analysis of intracellular signaling pathways and cellular processes. Soleus muscle regrowth was blunted in old compared to young rats which coincided with a recovery of serum IGF-1 and IGFBP-3 levels in young but not old. However, the response to reloading for p-Akt, p-p70s6k and p-GSK3β protein abundance was similar between muscles from young and old rats, even though main effects for age indicate an increase in activation of this protein synthesis pathway in the aged. Similarly, MAFbx mRNA levels in soleus muscle from old rats recovered to the same extent as in the young, while Murf-1 was unchanged. mRNA abundance of autophagy markers Atg5 and Atg7 showed an identical response in muscle from old compared to young rats, but beclin did not. Autophagic flux was not changed at either age at the measured time point. Apoptosis was elevated in soleus muscle from old rats particularly with HS, but recovered in HSRE and these changes were not associated with differences in caspase-3, -8 or -9 activity in any group. Protein abundance of apoptosis repressor with caspase-recruitment domain (ARC), cytosolic EndoG, as well as cytosolic and nuclear apoptosis inducing factor (AIF) were lower in muscle from old rats, and there was no age-related difference in the response to atrophy or regrowth. Soleus muscles from old rats had a higher number of ED2 positive macrophages in all groups and these decreased with HS, but recovered in HSRE in the old, while no changes were observed in the young. Pro-inflammatory cytokines in serum did not show a differential response with age to different loading conditions. Results indicate that at the measured time point the impaired skeletal muscle regrowth after atrophy in aged animals is not associated with a general lack of responsiveness to changes in loading conditions.

1. Introduction

Sarcopenia, or the loss of skeletal muscle mass with aging (Rosenberg, 1989), has severe negative consequences for health and quality of life in the elderly; muscle mass and strength are highly correlated and predictive of functional performance, morbidity and mortality in older men and women (Bassey et al., 1992; Laukkanen et al., 1995; Metter et al., 2002; Rantanen et al., 1999, 2000; Szulc, 2010). Moreover, the potential to recover muscle mass after an atrophy-inducing event, such as bed rest or malnutrition, is reduced in aged subjects, putting them at a greater risk for falls and subsequent illnesses (Hebuterne et al., 1997; Hvid et al., 2010; Suetta et al., 2009). English and Paddon-Jones have proposed the catabolic crisis model in which the age-related inability to recover muscle mass contributes to the decrease in functional capacity (English and Paddon-Jones, 2010). They further suggested that interventions should be targeted at preventing the loss of muscle mass during disuse as well as aiding in regrowth of muscle in response to a period of inactivity. A thorough understanding of the mechanisms involved in the failed regrowth response of aged individuals is therefore warranted.

Regrowth of skeletal muscle after an atrophy-inducing event is impaired with aging in animals as well as in humans. Muscle size lost due to starvation, glucocorticoid treatment, hind limb suspension, and limb immobilization was not recovered to the same extent in old as in young rats (Dardevet et al., 1995; Hao et al., 2011; Magne et al., 2011; Mosoni et al., 1999; Zarzhevsky et al., 2001a, 2001b) and the growth response to intermittent mobility during atrophy is also inhibited (Gallegly et al., 2004). Similarly, muscle hypertrophy in response to overload is impaired in aged animals (Chale-Rush et al., 2009; Degens and Alway, 2003; Thomson and Gordon, 2006) indicating that aged

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muscle responds differently to a similar growth stimulus than young muscle. However, previous studies have shown contradictory results when investigating cellular mechanisms potentially responsible for the impaired growth response. Some reports indicate that translational signaling, particularly through the Akt-mTOR related pathway, is impaired or delayed in aged animals undergoing hypertrophy (Funai et al., 2006; Haddad and Adams, 2006; Hwee and Bodine, 2009; Thomson and Gordon, 2006), while others show no differences with young (Chale-Rush et al., 2009; Hornberger et al., 2005); also, the potential to sense mechanical activity was not changed with age (Hornberger et al., 2005). Very few studies have directly compared mechanisms in muscles from young and old to identify potential differences during regrowth after atrophy. In a human study, it was concluded that a decrease in the proliferative response of satellite cells in combination with a change in the regulation of myostatin was responsible for the impaired regrowth in the aged, but intracellular pathways involved in protein synthesis and degradation were not studied (Suetta et al., 2013). In rats, data are sparse and contradictory; while the impaired regrowth response after immobilization was associated with a lower level of IGFBP5 and attenuated p70s6k levels in the aged, changes in Akt phosphorylation were not different from young (Morris et al., 2004; Spangenburg et al., 2003). During muscle recovery from starvation, protein synthesis levels in muscles from aged rats increased to the same extent as young rats, while proteolysis was not returned to baseline in the aged, despite similar responses in protein degradation markers after refeeding (Mosoni et al., 1999). Similarly, the lack of muscle recovery after immobilization in aged rats was not associated with a lack of normalization of proteasome-mediated degradation markers and the caspase-dependent apoptosis pathway, but these findings were not compared to young animals (Magne et al., 2011). Thus, it is currently unclear how intracellular mechanisms differ in muscles from aged and young rats during regrowth after hind limb suspension-induced atrophy. In addition, inflammation-related events and autophagy-mediated changes have not been investigated under impaired regrowth conditions. Therefore, the purpose of our current study was to determine if there are differences between young and old muscle in the regrowth response after atrophy. We hypothesized that muscles from aged rats exhibited a blunted recovery in intracellular pathways changed in response to disuse-induced atrophy.

2. Methods

2.1. Animals and experimental procedures

All procedures were performed in accordance with institutional guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. Male Fischer 344 X Brown Norway rats (6 months and 32 months) were purchased from the National Institute on Aging. This strain of rat was chosen because it has increased longevity and decreased cumulative lesion incidence compared with other strains; therefore, aging aspects can be studied in the relative absence of disease (Lipman et al., 1996). The different ages were chosen to reflect a mature rat, post long bone growth (young: 6 months) and an old rat at about 50% mortality (old: 32 months). Rats of both ages were divided into 3 groups (n = 8–10 per group): non-suspended ambulatory controls (amb), hind limb suspended for 14 days (HS), and rats that were hind limb suspended for 14 days and subsequently reloaded and allowed to move around the cage freely for 14 days (HSRE). Rats were allowed free access to food and water at all times and were housed in a 12:12 h light:dark cycle. Hind limb suspension was performed as previously described with minor modifications (Dupont-Versteegden et al., 2006; Hofer et al., 2008). Briefly, a tail device containing a hook was attached with gauze and cynoacrylate glue while the animals were anesthetized with isoflurane (2% by inhalation). After the animal regained consciousness, the tail device was connected via a thin cable to a pulley sliding on a vertically adjustable stainless steel bar running longitudinally above a high-sided cage. The system was designed in such a way that the rats could not rest their hind limbs against any side of the cage. Rats in the HSRE group were released from the tail suspension device after 14 days of unloading and they were allowed to maintain normal ambulation for 14 days (reloading). At the end of the experimental period, rats were anesthetized with sodium pentobarbital and serum was collected through a cardiac puncture; soleus muscles were then dissected, weighed and frozen. Muscles were either frozen in liquid nitrogen and stored at −80 °C for biochemical analyses or were embedded in a freezing medium, frozen in liquid nitrogen-cooled isopentane, and stored at −80 °C for immunohistochemical and histological analyses.

2.2. Serum analysis

Serum IGF-1 was determined using a radioimmunoassay (RIA; ALPCO Diagnostics, Salem, NH) as described previously (Delahunty et al., 2006). The analytical sensitivity of the assay is 0.02 ng/ml and 10 μl serum was used to determine IGF-I concentration (ng/ml). Serum IGFBP-3 was determined using the IGFBP-3 (Mouse/Rat) ELISA kit (ALPCO Diagnostics, Salem, NH) according to the manufacturer's instructions. The analytical sensitivity of the assay is 0.018 ng/ml and 5 μl serum was used to determine IGFBP-3 concentration (ng/ml). Serum insulin levels were determined using the Rat/Mouse Insulin ELISA (Millipore, Saint Charles, MO) according to the manufacturer's instructions. The analytical sensitivity of the assay is 0.2 ng/ml and 10 μl serum was used to determine the insulin concentration (ng/ml). Serum levels of tumor necrosis factor (TNF)-α, interleukin-6 (IL-6) and regulated-on-activation normal T cell-expressed and secreted (RANTES) were measured using a multiplex kit (EMD Millipore, Billerica, MA) according to the manufacturer's recommendation. The analytical linear range of the assay is 4.88–20,000 pg/ml.

2.3. Immunohistochemistry

2.3.1. Cross sectional area determination

Mean fiber cross sectional area (CSA) was determined as described in (Jackson et al., 2012) and adapted for rats. Briefly, cross sections from the mid belly area of soleus muscles were cut on a cryostat (7 μm), air dried, and stored at −20 °C until further analysis. Sections were rehydrated in phosphate buffered saline (PBS) and incubated in dystrophin antibody (1:50, Vector Laboratories, Burlingame, CA) for 1 h at room temperature and overnight at 4 °C. Secondary antibody (1:200, directly conjugated Texas Red goat anti-mouse, Rockland Immunochemicals, Gilbertsville, PA) was applied and sections were coverslipped. Images were captured using a Zeiss AxioImager MI upright fluorescent microscope (Zeiss, Göttingen, Germany) and analysis was performed using AxioVision software (Zeiss). CSA was determined by manually tracing the dystrophin stained sarcolemma of about 200 fibers in 4 different areas of the muscle.

2.3.2. Immune cell detection

Soleus cross sections (7 μm) were fixed in ice-cold acetone and blocked in 3% H_2O_2 in PBS and horse serum, followed by incubation in primary antibody overnight at 4 °C. For detection of immune cells the following antibodies were used: neutrophils (CD43, 1:200, Serotec, Raleigh, NC), ED1 macrophages (CD68, 1:200, Serotec) and ED2 macrophages (CD163, 1:200, Serotec). The Tyramide Signal Amplification (TSA, Invitrogen, Carlsbad, CA) system was used for horse radish peroxidase signal amplification and detection using Cyanine-3 (Cy-3) according to the manufacturer's instructions. Muscle sections were then reacted with DAPI (10 nM: 4′, 6-diamidino-2-phenylindole, Invitrogen) to identify nuclei.

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