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Age-related changes of cell death pathways in rat extraocular muscle

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Abstract

Changes in the structure and function of aging non-locomotor muscles remains understudied, despite their importance for daily living. Extraocular muscles (EOMs) have a high incidence of age-related mitochondrial defects possibly because of the metabolic stress resulting from their fast and constant activity. Apoptosis and autophagy (type I and II cell death, respectively) are lnked to defects in mitochondrial function and contribute to sarcopenia in hind limb muscles. Therefore, we hypothesized that apoptosis and autophagy are alered with age in the EOMs. Muscles from 6-, 18-, and 30-month old male Fisher 344-Brown Norway rats were used to investigate type I cell death, caspase-3, -8, -9, and -12 activity, and Type II cell death. Apoptosis, as measured by TUNEL positive nuclei, and mono- and oligonucleosomal content, did not change with age. Similarly, caspase-3, -8, -9, and -12 activity was not affected by aging. By contrast, autophagy, as estimated by gene expression of Atg5 and Atg7, and protein abundance of LC3 was lower in EOMs of aged rats. Based on these data, we suggest that the decrease in autophagy with age leads to the accumulation of damaged organelles, particularly mitochondria, which resuls in the decrease in function observed in EOM with age.

Keywords

extraocular muscles; aging; apoptosis; autophagy

Introduction

Aging is associated with significant changes in limb skeletal muscles, of which sarcopenia is the most evident. However, the effect of aging on extraocular muscle (EOM) structure and function is not as well studied. Aging increases the latency of eye movement onset and decreases peak velocity, acceleration and range of eye movement (Tian et al., 2002). Eye movement deficits seen in elderly subjects are associated with an increase in head movement tendencies, suggesting a strategy to compensate for EOM dysfunction (Kelders et al., 2003; Kim and Sharpe, 2001). Moreover. EOMs are particularly vulnerable to age-related

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mitochondrial myopathies, such as chronic progressive external ophthalmoplegia (Mitsui et al., 1996; Müller-Höcker et al., 1996).

Mitochondria play a key regulatory role in apoptosis or Type I cell death. We and others have shown that apoptosis is involved in sarcopenia (Alway et al., 2003; Alway et al., 2002; Dirks and Leeuwenburgh, 2004; Dirks and Leeuwenburgh, 2002; Leeuwenburgh, C et al., 2005; Strasser et al., 2000). The increase in apoptosis with skeletal muscle aging has been postulated to result from changes in mitochondrial function (Hiona and Leeuwenburgh, 2008). Mitochondrial oxidative stress and diminished energy production activate apoptotic pathways, which can be executed in eiher a caspase-dependent or caspase-independent manner (for review see Dupont-Versteegden, 2005). Dirks and Leeuwenburgh (2004) report an increase in APAF-1, procaspase-3, and cleaved caspase-3 in gastrocnemius of aging rats, but no change in caspase-9, while Baker and Hepple (2006) report increases in the gene expression of caspase-3, -8 and -9. Other reports have failed to show a difference in caspase-3 activation with aging (Dirks and Leeuwenburgh, 2002; Rice and Blough 2006; Siu et al., 2005) and therefore the involvement of the caspases in sarcopenia-associated apoptosis is stl debatable. All of these studies have been performed on hind limb muscles and the effect of age on apoptosis in EOM remains unknown, despite their high mitochondrial content. One study suggests that apoptosis is prevalent in young EOM. and that it is caspase-3 dependent (McLoon et al., 2004).

Cell death type II, or autophagy, is the intracellular process by which cytoplasmic constituents are sequestered into double-membrane autophagosomes and subsequently delivered to lysosomes for hydrolytic digestion (Mizushima, 2004; Mizushima, 2007). It is responsible for the self-degradative processes implicated in the normal turnover of cellular components as well as in response to such stresses as nutrient starvation and/or growth factor deprivation. One of the functions of autophagy is to remove damaged organelles in order to prevent further injury and cellular dysfunction, a process that may be particularly important with aging (Brunk and Terman, 2002; Terman and Brunk, 2004; Terman et al., 2003). A recent study showed an increase in autophagy with calorie restriction, a life-extending intervention (Wohlgemuth et al., 2007), indicating that autophagy may play a role in the aging process. It is currently unknown whether autophagy is changed with age in EOM The purpose of this study was to determine if the EOMs will display increased Type I (apoptosis) and II (autophagy) cell death with advancing age.

Materials and Methods

Animals

The study was approved by the Institutional Animal Care and Use Committee of the University of Kentucky. Male Fischer 344-Brown Norway Fl hybrid rats (6-, 18- and 30-months (mo) of age) were obtained from the National Institute on Aging Aged Rodent Colony: we used 8 rats/age for histology, immunocytochemistry and biochemical assays, 4 rats/age for electron microscopy and 18 rats/age for real-time quantiative PCR (qPCR) analysis. The age groups were selected to represent 3 points in the life span curve of this strain: 6-mo (early flat portion of low mortality), 18-mo (inial increase in mortality), and 30-mo (linear decrease of survival curve) (Turturro et al., 1999). Upon arrival, the animals were kept in microisolator cages with Harlan Teklad rodent food and water provided *ad libitum*. Prior to the collection of tissues, the rats were anesthetized with ketamine hydrochloride/xylazine hydrochloride (100 mg/8 mg per kg body weight injected i.p.) and euthanized by exsanguination following a medial thoracotomy.

For gene expression and biochemical studies, EOMs were quickly excised, frozen in liquid nitrogen and stored at −80°C until further use. For histology, orbital contents were frozen in 2-methylbutane cooled to its freezing point in liquid nitrogen.

Histology and immunocytochemistry

Ten-μm thick frozen sections were used to examine EOMs. LC3 sections were blocked and incubated with the primary LC3 antibody (Abcam, Cambridge, MA) followed by the corresponding Aexa Fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA). TUNEL staining was performed using the Promega fluorometric TUNEL kit (Promega, Madison, WI). Briefly, slides were incubated in equilibration buffer followed by TDT mediated dUTP-fluorescein reaction mix for sixty minutes in a humidified chamber at 37°C The reaction was terminated by immersion in 2× SSC Slides were then washed with phosphate-buffered saline (PBS). Sections were mounted on gelatin and chromalum coated slides with Vecta™ Shield slow fade. After staining, slides were viewed with a Nikon E600 microscope (Nikon Inc., Melville, NY). Images were captured with a Spot RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and a PowerMac G4 computer (Apple Computer Inc., Cupertino, CA) equipped with Spot RT software version 4.0 (Diagnostic Instruments, Inc.). For analysis, one section from each animal was randomy selected and digital images obtained, as described previously (Leeuwenburgh et al., 2005). The total number of TUNEL positive nuclei were counted from these images and normalized to the total nuclei labeled with DAPI. Therefore, nuclei counted included myofiber as well as interstitial cell nuclei. The observers were blinded to the age of the rats. Twenty percent of histological images used were randomly selected and examined by two raters. Inter-rater reliability was assessed using the intraclass correlation coefficient (two-way, mixed model; single measure). Resuls demonstrated a high degree of agreement between raters (Intraclass Correlaton Coefficient = .978).

Cell death ELISA

Cytosolic fractions of muscles were obtained as previously described (Leeuwenburgh et al., 2005). Briefly, muscles were homogenized using a Polytron in isolation buffer: 220 mM D-mannitol, 75 mM sucrose, 0.1% fatty acid-free bovine serum albumin, 0.5 mM EGTA, and 2 mM HEPES, pH 7.4 (1:10 wt/vol). The homogenate was centrifuged at 700 *g* at 4°C for 10 min, and the supernatant was centrifuged again at 8,000 *g* at 4°C for 10 min. The supernatant was carefully collected, PMSF (1mM) and leupeptine (1μg/ml) were added, and protein concentration was determined according to the Bradford method (Bradford, 1976). Cell death detection ELISA kit (Roche Applied Science, Indianapolis, IN, USA) was used to quantitatively determine DNA fragmentation by measuring the cytosolic histone-associated mono- and oligonucleosomes as described previously (Leeuwenburgh et al., 2005). The amount of peroxidase retained in the immunocomplex was determined photometrically by incubating with 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) as a substrate for 10 min at 20°C. The change in color was measured at a wavelength of 405 nm by using a Molecular Devices plate reader controlled through PC software (Soft Max Pro, Molecular Devices, Toronto, Canada). Measurements were performed in duplicate, with samples from rats at different ages analyzed on the same plate in the same setting. The OD_{405} reading was normalized to the micrograms of protein, and expressed as an apoptotic index.

Caspase activity measurements

Caspase activities for caspase-3, -8, -9, and -12 were measured in the cytosolic fraction of the muscle homogenate using fluorometric substrates as described previously for caspase-3 (Leeuwenburgh et al., 2005). The following substrates were used for caspase-3, -8, and -9 respectively, Ac-DEVD-AMC, Ac-IETD-AMC, Ac-LEHD-AMC (Peptides International, Louisville, KY), and for caspase -12, Ac-ATAD-AFC (MBL International Corporation,

Woburn, MA). The substrates are cleaved proteolyticaly by the corresponding caspases and the fluorescence of free AMC (for caspase-3, -8, and -9) or AFC (for caspase-12) was measured and compared to a standard curve for free AMC (Sigma, St Louis, MO) and AFC (Calbiochem, San Diego, CA), respectively. For determination of caspase-3, -8, and -9 100 μg, and for caspase-12 200 μ*g* of total protein was incubated for 2 hours in caspase buffer (100mM HEPES, 10% sucrose, 10 mM DTT, 0.1% CHAPS, 1 μg/ml leupeptin, 1 mM PMSF) with 100 μM substrate for caspase-3, -8, and -9 and with 50 μM substrate for caspase-12. Fluorescence was determined with an excitation wavelength of 380 nm and an emission wavelength of 460 nm for AMC and an excitation wavelength of 400 nm and an emission wavelength of 505 for AFC using a Spectra Max M2 Fluorescent Microplate reader (Molecular Devices, Sunnyvale, CA). Values were expressed as nmoles AMC or AFC per μg of protein.

RNA isolation and real-time quantitative PCR

Muscles from 4 animals were combined for each independent sample and total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Reverse transcription was carried out using Superscript II RNAse H$^-$Reverse Transcriptase (Invitrogen) with random hexamers. The expression of genes of interest was examined with real-time quantitative PCR (qPCR) with the ABI Prism 7500 Sequence Detection System using β-actin as the housekeeping gene as previously described (Andrade et al., 2004; McMullen et al., 2005). Atg5 and Atg7 were used as markers for autophagy (Mizushima, 2004; Mizushima, 2007). Primers for the mRNAs of interest were designed using the software package Primer Express 2.0 (Applied Biosystems) from GenBank nucleotide sequences as follows: Atg5 accession number AM087012 Forward 5′-AGG CTC AGT GGA GGC AAC AG-3′, Reverse 5′ CCC TAT CTC CCA TGG AAT CTT CT-3′, Atg7 accession number NM_001012097 Forward 5′-GCA GCC AGC AAG CGA AAG-3′, Reverse 5′ TCT CAT GAC AAC AAA GGT GTC AAA-3′, and β-actin accession number NM_031144 Forward 5′-CCC TGG CTC CTA GCA CCA T -3′, Reverse 5′-GAG CCA CCA ATC CAC ACA GA 3′. The relative abundance of target mRNAs was determined with the comparative cycle threshold method (Giulietti et al., 2001; Livak and Schmittgen, 2001).

Electron microscopy

Anesthetized rats were perfused with PBS, followed by 2% paraformaldehyde and 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and 130 mM NaCl. EOMs were postfixed in 1% osmium tetroxide, stained in uranyl acetate, dehydrated in methanol and propylene oxide, and embedded in epoxy resin. Thin (70 nm) sections were stained with uranyl acetate and lead citrate, and photographed with a Philips Tecnai 12 transmission electron microscope. We examined images for autophagy based on the fusion classifications of Eskelinen (2008). Mitochondria size was measured using NIH ImageJ software (Staal et al., 2004) by analyzing the width of the mitochondria based on the methods of Navaratil (2008). For each age group, ten images or more from at least three different occasions were analyzed.

Western blots

50ug of total protein per sample were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked and then incubated with the primary LC3 antibody (Abcam, Cambridge, MA) followed by the corresponding Alexa Fluor 680-conjugated secondary antibody (Invitrogen, Carlsbad, CA.). Blots were scanned with the Odyssey Infrared Imaging System (LiCor, Lincoln, NE); density of resulting bands was quantified using NIH Image J software.

Statistics

One-way analysis of variance was used to determine statistically significant differences between the ages. In case of significant differences Tukey post-hoc test was used. Significance was assumed at $p<0.05$.

Results

Apoptosis and caspase activity in EOMs of aging rats

There was a tendency for increased TUNEL positive nuclei between 6- and 18-mo, remaining elevated at 30- mo, but this did not reach statistical significance indicating that in EOM, aging was not associated with a significant change in apoptotic nuclei (Table 1). To confirm these measurements we performed a biochemical assay for DNA fragmentation. Mono- and oligonucleosomal fragments were quantified in the cytosolic fraction of EOM at all three ages. No statistically significant changes with age were observed. Caspases play an essential role in apoptosis, but are also involved in other processes such as protein degradation (Du et al., 2004;Hasselgren and Fischer, 2001) and skeletal muscle differentiation (Fernando et al., 2002). We therefore examined caspase-3, -8, -9, and -12 activities in EOM. Caspase-8, -9, and -12 are initiator caspases involved in the extrinsic, intrinsic, and endoplasmic reticular initiated apoptosis pathways, respectively, and caspase-3 is an executor caspase (Dupont-Versteegden et al., 2006). Activity was not changed with age for any of the examined caspases (Table 1). Based on these data, we concluded that, in contrast to limb muscles, apoptosis does not increase with age in EOM. Therefore, other mechanisms must be responsible for the observed cellular and functional changes in EOMs with age.

Decreased autophagic capacity with age in EOMs

Electron microscopy showed autophagic vacuoles in EOM, but no obvious visual differences were observed between the 3 age groups (Figure 1). Autophagy is most often measured by quantifying autophagy-related proteins (Atg proteins). Among the 31 identified Atg proteins, Atgl-10 are involved in autophagosome formation and are markers for the isolation membrane (Eskelinen, 2005). We then examined the mRNA levels of Atg5 (necessary for autophagosome formation) and Atg7 (involved in autophagosome induction) at 6-. 18- and 30- mo of age in EOM (Eskelinen, 2005;Mizushima. N. 2007;Uchiyama. Y et al 2008). Using 6-mo EOM as the baseline, Atg5 mRNA abundance significantly decreased ~4 fold at 30-mo with no change at 18-mo. Atg7 significantly decreased ~ 9 fold at 18-mo and ~4 fold at 30-mo (Figure 2).

LC3, the mammalian homologue of yeast Atg8, is essential for the formation of autophagosomes (Eskelinen, 2005). Pre-LC3 is processed from LC3-I by Atg4. LC3-I is then activated by Atg7, transported to Atg3, and changed to its membrane bound form, LC3-II (Maiuri et al., 2007). LC3-I and -II are seen as general markers of autophagic membranes. Immunoreactivity for LC3 was more apparent in EOM from 6- and 18- mo old rats (Figure 3A) while no positive staining was observed in EOM from 30-mo old rats, implying a decrease in total LC3. Immunoblotting confirmed that LC3-I and LC3-II decreased in old age (See Figure 3B and 3C), indicating that autophagy declines in EOM of aged rats. Since a decrease in autophagy correlates with an increase in enlarged and dysfunctional mitochondria, we expected that mitochondrial size would be increased in EOM at old age (Brunk and Terman, 2002; Terman et al., 2003). Contrary to what we anticipated, mitochondrial size was significantly smaller in EOM from 18-mo and 30- mo of age compared to 6-mo (Figure 4).

Discussion

Our initial hypothesis was that apoptosis and autophagy are elevated with aging in EOMs. Instead, we found that apoptosis indices did not change significantly with age in these muscles,

while autophagy was decreased at 30 months. In consequence, damaged organelles are more likely to accumulate and disrupt cellular function in aging EOMs.

No significant changes with age were observed in markers of apoptosis (TUNEL and mono- and oligo-nucleosomal content) or in the activity of caspase-3, -8, -9, and -12, demonstrating that apoptosis and apoptosis-related caspases are not activated in the aging EOMs. Therefore, apoptosis is not likely involved in the age-related functional decline in EOMs. in contrast to its proposed role in sarcopenia for hind limb muscles (Dirks and Leeuwenburgh, 2005; Dirks and Leeuwenburgh, 2002; Dupont-Versteegden, 2005). It is also possible that EOMs are either not subjected or resistant to age-related stresses that activate apoptosis and caspases in other muscles.

Autophagy decreased in EOMs of 30 mo-old rats. The process of autophagy is important for the removal of excess and/or damaged proteins and organelles in order to maintain normal function. For example. Atg5 or Atg7 knockout mice which do not form autophagosomes, have abnormal heart function, and die shortly after birth, illustrating the importance of autophagy (Komatsu et al., 2007; Kuma et al., 2004). Autophagy and apoptosis are triggered by similar mechanisms such as mitochondrial transition pore formation and induction by oxidative stress, and the two types of cell death are likely complementary and co-regulated since deletion or knockdown of autophagy genes decrease apoptosis (Boya et al., 2005; Espert et al., 2007; Gu et al., 2005). We showed that the oldest EOMs have lower levels of three autophagic markers, Atg5, Atg7, and LC- 3, indicating that autophagy actually decreased with old age. The fact that at 18 months there is a larger decrease in Atg7 than at 30 months, no change in Atg5, and an increase in LC3 compared to 6 months, indicates that at this age the EOMs are likely starting to change in response to accumulated damage. Different proteins are likely changing at different rates and some (like LC3) may even be trying to compensate for the decrease in other proteins (like Atg7). We suggest that 18 months is a transitional age and the changes observed at this age might be important indicators for future intervention studies. However, the important findings for this study are that at old age autophagic markers are all decreased. This further results in decreased lysosomal degradation of targeted proteins, leading to accumulation of abnormal or damaged proteins and declining efficiency in protein turnover, all factors likely to affect cellular health. This is not a new concept: decreased autophagy with age can lead to the accumulation of damaged cellular components, particularly mitochondria (Wohlgemuth et al., 2007). For example, the number of enlarged mitochondria increases in the aging heart because they are not removed by autophagy (Brunk and Terman, 2002; Terman et al., 2003). In the present case, EOMs could have an increased number of dysfunctional mitochondria, retained by the failure to activate autophagy. However, EOM mitochondrial size decreased with age, arguing against the retention of enlarged abnormal mitochondria. It has also been reported that mitochondria with low respiratory capacities are less susceptible to autophagy (Kim et al., 2007). We showed recently that EOM mitochondria have lower respiration rates than mitochondria from limb muscles, a characteristic that may result in a lower susceptibility to autophagy (Patel et al., 2009). These intrinsic differences in mitochondrial function between hind limb skeletal muscle and EOMs may influence the differential response of apoptosis and autophagy in these two muscle types with age.

In summary, our data show that apoptosis is not changed with age in EOMs, while the expression of Atg5, Atg7, and LC3 were lower, consistent with a decline in autophagy. The imbalance between apoptosis and autophagy may be responsible for the increase in mitochondria observed in EOMs, potentially altering their metabolic characteristics and leading to the eye movement limitations observed in the elderly.

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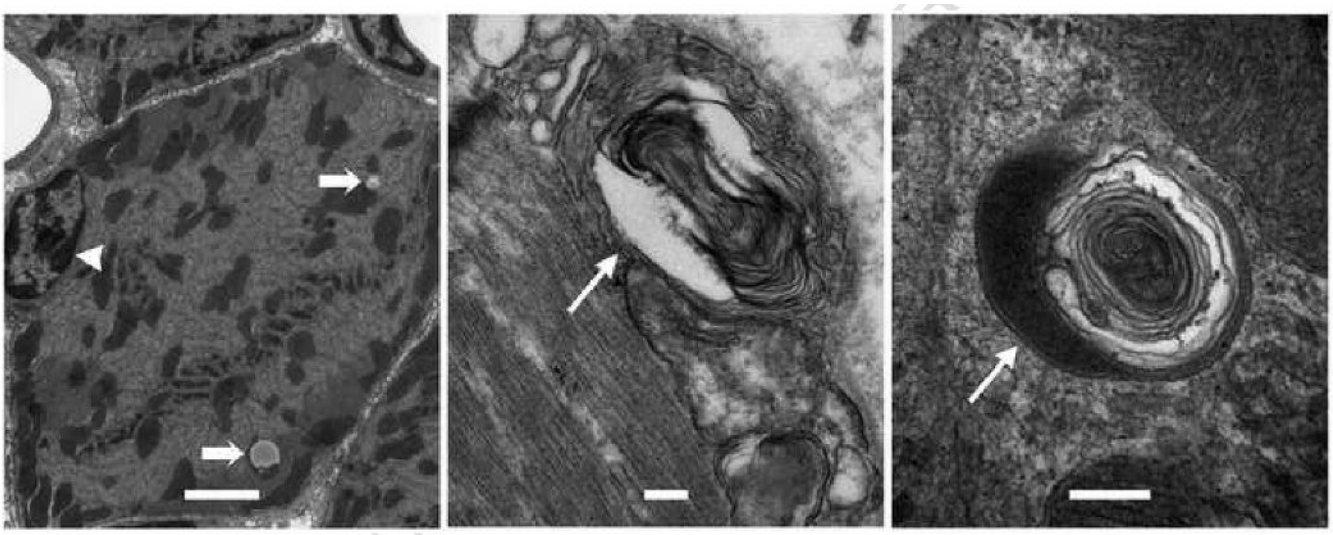

Figure 1. Autophagy is present in EOM at all ages studied

Ultrastructural analyses by means of electron microscopy of ultrathin sections from EOMs of rats at 6- (left), 18-(middle) and 30-(right) months of age. The arrows indicate autophagic vacuoles: 6-mo (left) autophagocytic degradation with sarcolemmal preservation, bold arrows indicate lipid droplets. 18-mo (middle) top arrow indicates a degradative autophagic vacuole that has fused with a lysosome (AVd/Ly) and 30-mo (right) A late autophagic vacuole (AVd). (Scale bars=2μm for 6-mo, 0.2μm for 18 and 30-mo).

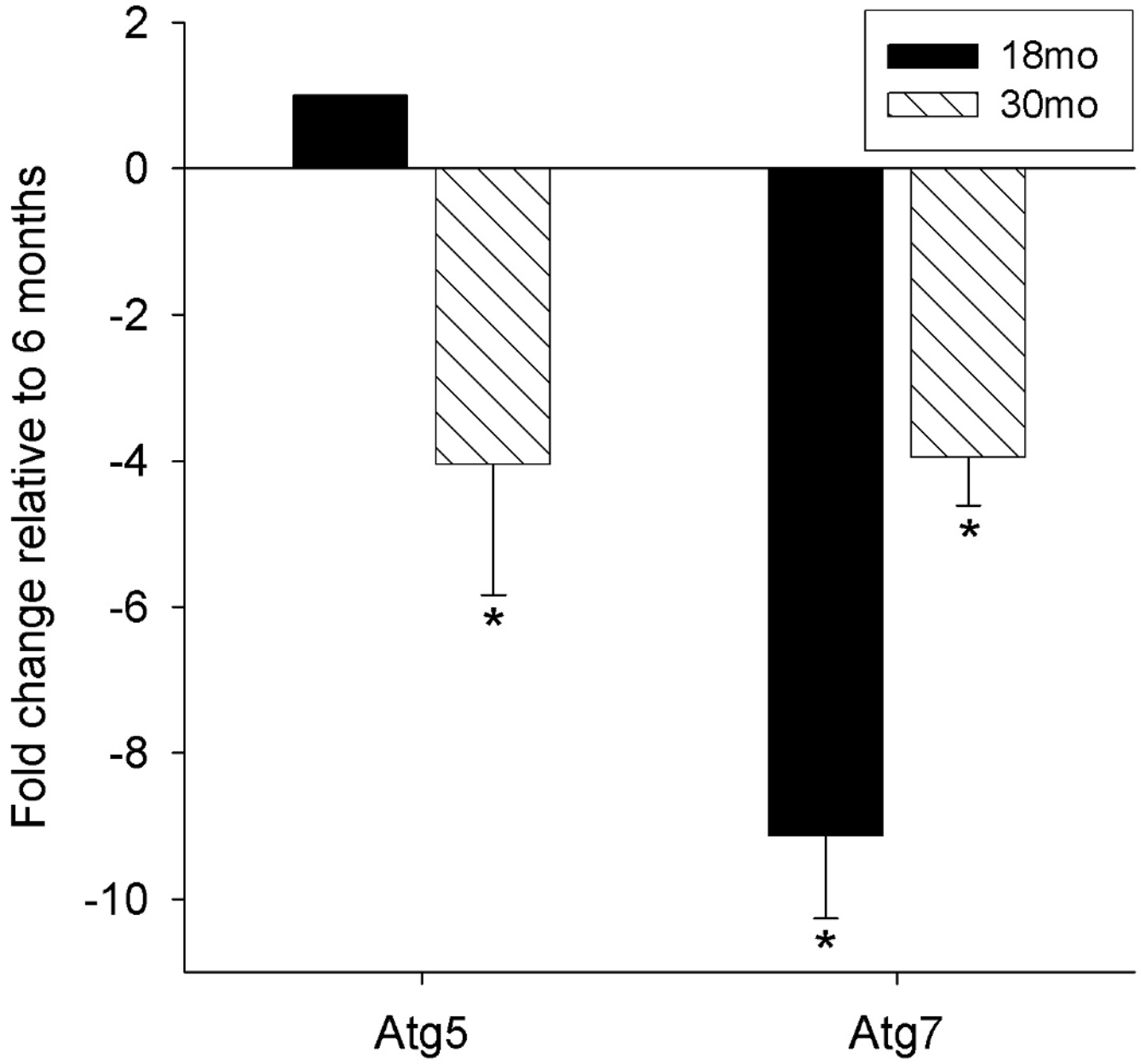

Figure 2. Autophagy markers decrease with age in EOM

Results are -fold changes of the corresponding mRNA in EOM muscles at 18-, and 30-mo compared to 6-mo. * significantly different from 6-mo, $p<0.05$.

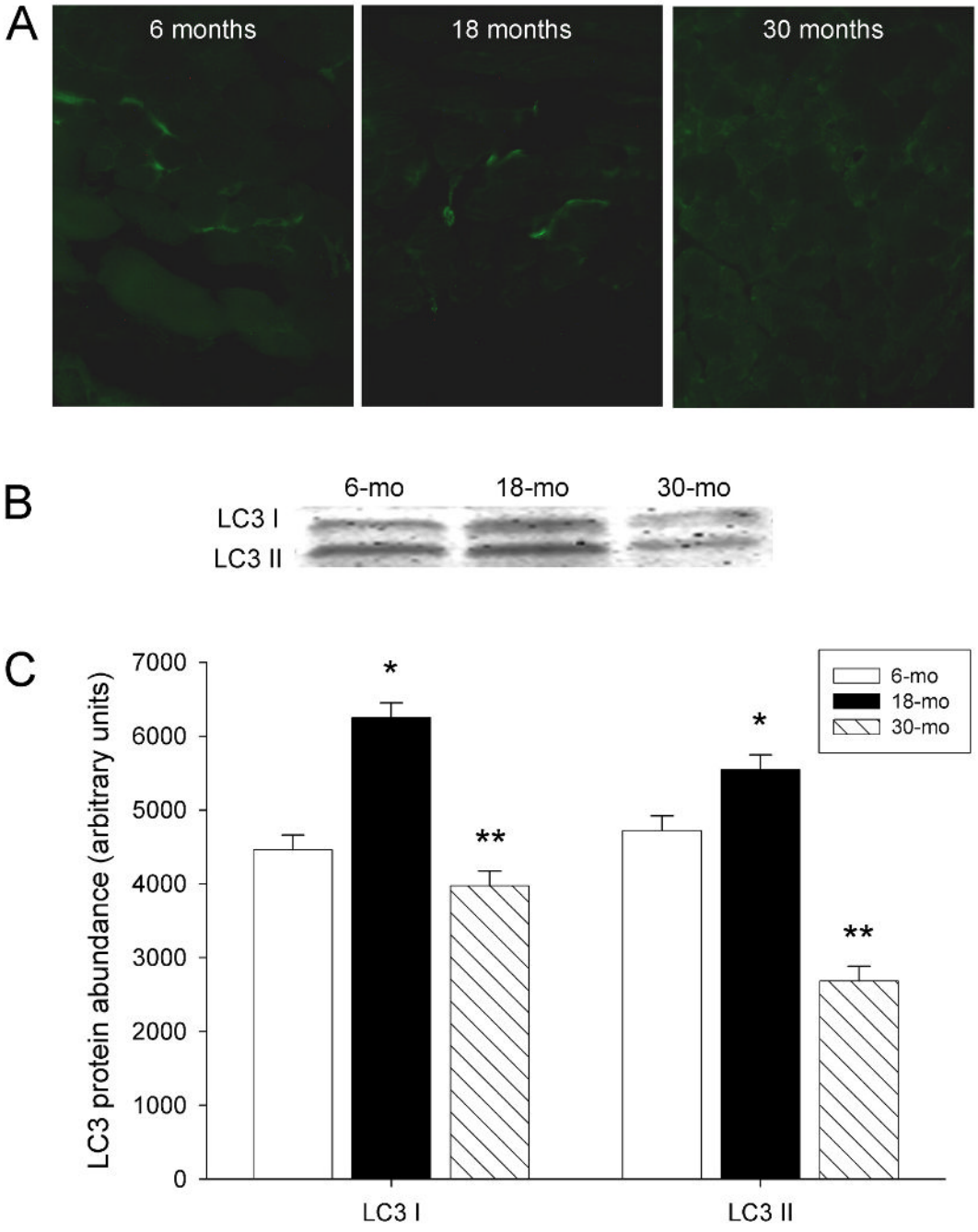

Figure 3. LC3 decreases with age in EOM

A. Muscle cross sections stained for LC3 display a positive staining of autophagic vacuoles in EOM from 6- (left) and 18- (middle) but not in 30- (right) month old rats. Scale bare = 25μm. **B**. Western blot of LC3 I (top band) and II (botom band) showing decrease protein abundance at 30-mo. **C**. Quantificaton of optical density of LC3 western blot. * different from 6-mo, ** different from 18-mo($p<0.05$).

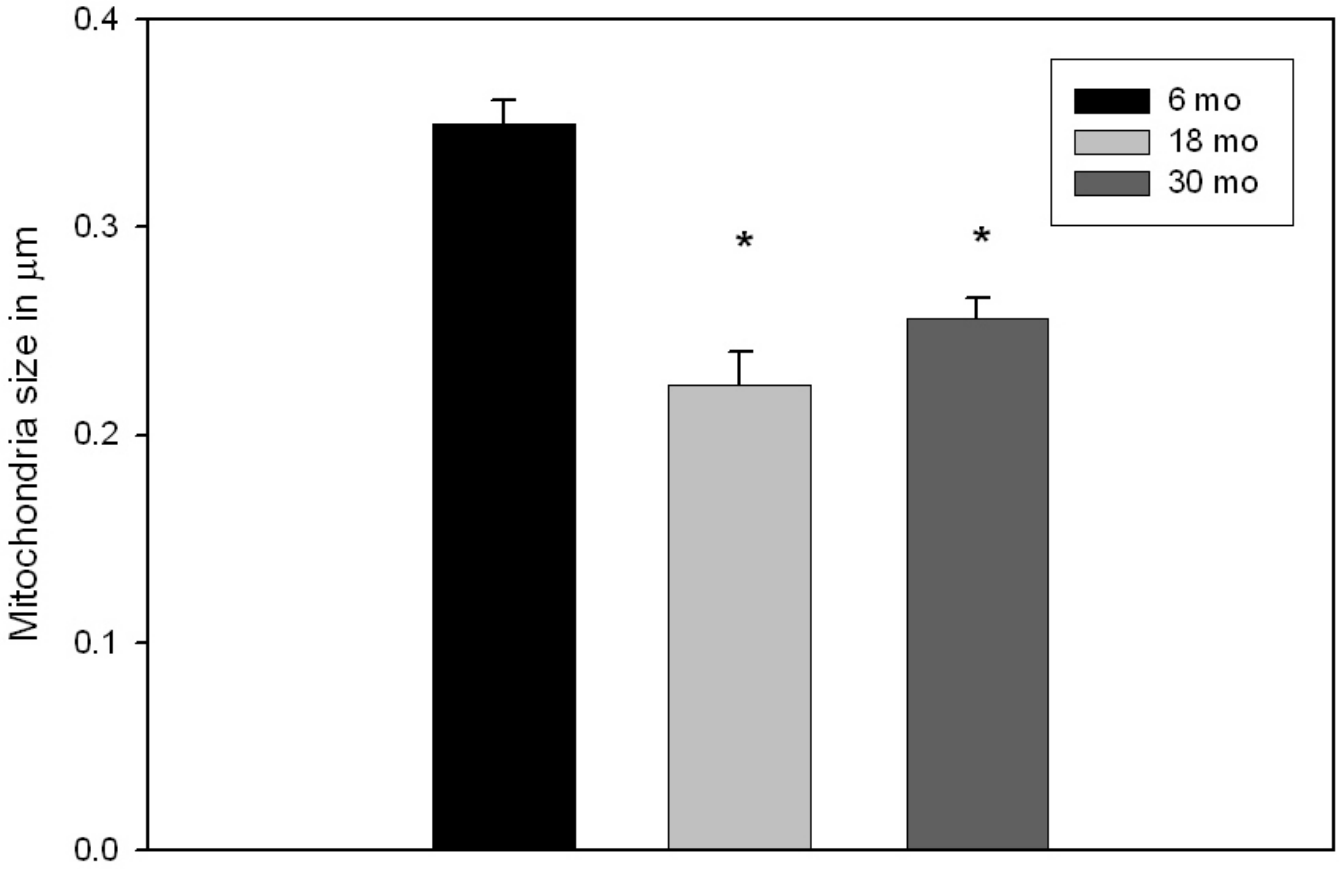

Figure 4. Mitochondrial size in EOM decreases with age

Quantificaton of mitochondrial width in EOM. Width is significantly decreased in 18- and 30-mo compared to 6-mo rats. * different from 6-mo ($p<0.05$). Values are means ± SEM.

Table 1

Apoptosis does not change with age in EOM

TUNEL, apoptotic index, caspase -3, -8, -9, and -12 activies for 6-, 18-, and 30-month old rats are listed. Values are means ± SEM, P > 0.05 for all groups.

	6-month	18-month	30-month
TUNEL (number of nuclei/total nuclei)	29.4±2.7	40.8 ± 3.8	41.3 ± 4.0
Apoptotic Index (absorbance/μg protein)	33.2 ± 1.7	41.1 ± 1.3	21.7±2.1
Caspase 3 activity (**nM**AMC/hour/μgprotein)	3.6±1.5	2.6 ±1.5	2.4± .8
Caspase 8 activity (nM AMC/hour/μgprotein)	28.5 ± 11.5	38.0 ± 21.8	30.4±13.8
Caspase 9 activity (nM AMC/hour/μgprotein)	11.0±5.2	16.3 ± 14.3	13.9 ± 7.5
Caspase 12 activity (nM AFC/2hour/200μg protein)	588.9±124.0	503.4±194.5	520.5 ± 77.2