The Influence of Age, Sex and Exercise on Autophagy, Mitophagy and Lysosome Biogenesis in Skeletal Muscle

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Abstract

Background

Aging decreases skeletal muscle mass and quality. Maintenance of healthy muscle is regulated by a balance between protein and organellar synthesis and their degradation. The autophagy lysosome system is responsible for the selective degradation of protein aggregates and organelles, such as mitochondria (i.e., mitophagy). Little data exist on the independent and combined influence of age, biological sex and exercise on the autophagy system and lysosome biogenesis. The purpose of this study was to characterize sex differences in autophagy and lysosome biogenesis in young and aged muscle, and to determine if acute exercise influences these processes.

Methods

Young (4-6 months) and aged (22-24 months) male and female mice, were assigned to a sedentary, or an acute exercise group. Mitochondrial content, the autophagy-lysosome system and mitophagy were measured via protein analysis. A Tfeb-promoter-construct was utilized to examine Tfeb transcription, and nuclear-cytosolic fractions allowed us to examine Tfeb localization in sedentary and exercised muscle with age and sex.

Results

Our results indicate that female mice, both young and old, had more mitochondrial protein than age-matched males, and mitochondrial content was only reduced with age in the male cohort. Although young female mice had a greater abundance of autophagy, mitophagy and lysosome proteins than young males, we measured increases with age irrespective of sex. Interestingly, young sedentary female mice had indices of greater autophagosomal turnover than male counterparts. Exhaustive exercise was able to stimulate autophagic clearance in young male mice, but not in the other groups. Similarly, nuclear Tfeb protein was enhanced to a greater extent in young male than in young female mice following exercise, but no changes were observed in aged mice. Finally, Tfeb-promoter activity was upregulated following exercise in both young and aged muscle.

Conclusions

The present study demonstrates that biological sex influences mitochondrial homeostasis, the autophagy-lysosome system and mitophagy in skeletal muscle with age. Further, our data suggest that young male mice have a more profound ability to activate these processes with exercise than in the other groups. Ultimately, this may contribute to a greater remodeling of muscle in response to exercise training in males.

Background

The natural aging process is associated with a progressive loss of muscle mass and function [1, 2], commonly referred to as sarcopenia [3]. Since skeletal muscle represents 40% of total body mass and is essential for motor function and whole-body metabolic control [1, 4], these age-related declines are associated with deficits in the quality of life of older individuals, and are related to a higher incidence of falls, hospitalization, and co-morbidities [5]. This is problematic when we consider that physical inactivity rates are greater in those that are older, potentiating the negative effects of age on overall health [6–8]. It is now known that the maintenance of physical activity throughout the lifespan is an essential preventative measure in age-associated loss in mitochondrial volume and function [9, 10]. Thus, there is an evolving need to understand the mechanisms that underly the changes in muscle architecture with age, and how exercise preserves muscle health.

The muscle atrophy observed within aging muscle is achieved by an imbalance protein synthesis and degradation. The autophagy-lysosome system is a proteolytic pathway that is responsible for the breakdown of long-lived, aggregated proteins and organelles [11]. Autophagy is an evolutionary conserved recycling mechanism, whereby damaged or dysfunctional cellular components are engulfed in a double membrane autophagosome and delivered to the lysosomes for digestion. Inhibition of autophagy promotes atrophy, neuromuscular junction decay, sarcomere disarrangement and ultimately, weakness [12–16]. Further a lack of autophagy attenuates the phenotypic remodeling of muscle associated with exercise training [17–19]. Cumulatively, these studies highlight the importance of this proteolytic system in skeletal muscle.

The impact of aging on skeletal muscle autophagy remains controversial [20], but previous reports utilizing “flux” measurements have shown that autophagy is upregulated in aging muscle [21, 22]. These alterations in autophagy have implications for the selective degradation of mitochondria through mitophagy. In fact, in a series of studies, our group has reported enhanced mitophagy in aged muscle [22–24]. We have also shown that muscle from aged rodents displayed an accumulation of lysosomal protein and nondegraded lysosomal content, termed lipofuscin [23, 25]. This would imply lysosomal dysregulation, which may contribute to a reduced capacity to effectively remove damaged intracellular constituents.

In young, healthy muscle, autophagy and lysosome biogenesis are activated following acute endurance activity [26, 27] to assist in the remodeling of muscle. Over time, aerobic training improves the metabolic capacity of the tissue [28–30], enhancing mitochondrial content concomitant with increases in lysosomal content [31, 32]. However, it remains to be seen if these acute-exercise responses occur in aged skeletal muscle, and whether exercise can enhance lysosome capacity to promote the removal of the accumulating damaged constituents.

Recently it was reported that female mice had enhanced catabolic and autophagy signaling in response to hindlimb unloading [33, 34]. These findings highlight the importance of examining biological sex as a variable in muscle physiology. Furthermore, a limited analysis on the autophagy-lysosome markers was conducted in aged male and female mice, investigating the impact of prolonged training in these groups.

No difference between the sexes was reported. [35]. We are unaware of any studies that have examined the influence of biological sex on the autophagy-lysosome system, with a focus on the impact of age and acute exercise.

Thus, the overarching goal of this study was to examine sex differences in autophagic, mitophagic and lysosomal pathways in muscle from young (4-6 mo) and aged (22-24 mo) male and female C57BL6 mice. Based on previous reports [33, 34], we hypothesized that autophagy would be greater in our female cohort. Further, we investigated the utility of acute exercise to activiate these pathways in young and aged muscle, and whether biological sex could influence the exercise response.

Materials And Methods

Animals

All animal procedures were conducted in accordance with the standards set by the Canadian Council on Animal Care, with the approval of York University Animal Care Committee (YUACC). Young (4-6 months) and aged (22-24 months) male and female C57BL/6 mice were obtained from The Jackson Laboratory. Mice used in this study were ordered at ~2 months old and aged in our facility in accordance with YUACC protocols and guidelines. Food and water were provided *ad libitum*. At the appropriate age, mice were assigned to sedentary or acute exhaustive exercise groups so that the final # of animals/group were n=5/male; n=4/female groups.

Acute Exhaustive Exercise Protocol

Animals that were assigned to the acute exhaustive exercise group were acclimatized to the treadmill 48 and 24 hours prior to their exercise date. Acclimatization occurred at 0m/minute, 5m/minute and 10m/min for 5 minutes each. On the day of exercise, prior to protocol, resting blood lactate levels were measured via tail blood. Subsequently, animals were placed on the treadmill at a fixed incline of 10%. The acute exhaustive exercise protocol began with a 5m/minute warmup for 5 minutes and a 10m/min run for 10 minutes, followed by increasing speeds at 1m/minute every 2 minutes until exhaustion was achieved. Exhaustion was defined as the inability of the animal to run on the treadmill despite prodding. Immediately following exercise, post-exercise blood lactate measurements were made, animals were cervically dislocated and tissues were harvested for biochemical analysis.

Luciferase Reporter Assay

The Tfeb promoter containing -1601 bp region of the canonical promoter was subcloned into a pGL3 vector containing a firefly luciferase reporter (rTfeb-pGL3), under control of the constitutively active CMV promoter, as previously described[27]. Ampicillin-resistant bacteria were transformed, and bacterial colonies were then amplified to isolate plasmid DNA using a Maxi Plasmid Isolation Kit (Qiagen). Six

days prior to tissue removal, in both the sedentary and exercised groups mice underwent *in vivo* muscle transfection. Briefly, mice were anesthetized using gaseous isoflurane and the lower hindlimbs were shaved and sterilized. One gastrocnemius muscle of each mouse was injected with 30mg of the rTfeb-pGL3 construct and 50ng of renilla luciferase downstream of the CMV promoter (pRL-CMV), used as a marker of transfection efficiency. The contralateral hindlimb was injected with an empty vector (pGL3) and renilla luciferase, both under the control of the CMV promoter. All injections were conducted using a short 29-gage insulin syringe (BD Canada). Immediately after the injection, trans-continuous electrical pulses were applied using an ECM 380 BTX electroporation system (Harvard Apparatus Saint-Laurent, QC, Canada), whereby the muscle were held on either side of the injection site by forceps-style electrodes, followed by ten 100V/cm^2 pulses. Conductive gel was applied to the electrodes to assist with transfection. The anode and cathode orientation were reversed, and another 10 pulses were delivered. Following tissue extraction, frozen gastrocnemius muscle was pulverized to a fine powder at the temperature of liquid nitrogen. Approximately 30mg of powder was diluted in 1X passive lysis buffer (Promega, cat# E1500) supplemented with protease (Roche Mississauga, ON, Canada) and phosphatase (Sigma Oakville, ON, Canada) inhibitors. The sample was then sonicated on ice (3x3seconds) and spun in a microcentrifuge at 4^{o}C for 10 minutes at 16,000g and the supernatant fraction was collected. Using an EG&G Berthold Luminometer (Lumat LB 7507; Berthold Technologies, Oak Ridge, TN). Following initial background readings of the passive lysis buffer, 20mL of either the rTfeb-PGL3+ pRL-CMV or pGL3+pL-CMV sample tissue were loaded into a test tube and mixed with 100ml of luciferase substrate followed by 100ml of renilla substrate (Promega). Each sample was run in triplicate, and the average was used. The ratio of firefly luciferase reporter (RLU1) to renilla luciferase (RLU2) was taken for both the Tfeb-promoter- and the empty vector-injected limbs. Transcriptional activity was expressed as the Tfeb promoter data divided by the empty vector.

Mitochondrial Isolations

To isolate intermyofibrillar mitochondrial subfractions, muscles from the animal were pooled (one quadricep, hamstrings from both hindlimbs, triceps from both forelimbs and the pectoralis muscles) adding up to ~400mg. Muscle was minced, homogenized mechanically, and underwent differential centrifugation as previously described[36–38]. The final mitochondrial pellets were resuspended in ice-cold buffer (100 mm KCl, 10 mm MOPS, 0.2% BSA). Isolated mitochondria were supplemented with phosphatase inhibitor cocktails as well as protease inhibitors and stored at –80°C for Western blotting procedures.

High Resolution Respiration and ROS-Emission

High-resolution respirometry (Oroboros O2k, Austria) was used to measure oxygen consumption in permeabilized muscle fibers from the lateral portion of the left TA muscle of all mice. Briefly, the muscle was excised, and fibers were mechanically separated in ice cold BIOPS buffer (2.77_{mM} CaK2EGTA,

7.23_{mM} K2EGTA, 7.55_{mM} Na2ATP, 6.56_{mM} MgCl26H2O, 20_{mM} Taurine, 15_{mM} Na2Phosphocreatine, 20_{mM} Imidazole, 0.5_{mM} Dithiothreitol, 50_{mM} MES-Hydrate, pH 7.1). Subsequently, the fibers were permeabilized in BIOPS supplemented with $40_{ug/uL}$ saponin at 4°C for 30 minutes with gentle rocking and washed in Buffer-Z (105_{mM} K-MES, 30_{mM} KCl, 10_{mM} KH2PO4, 5_{mM} MgCl2•6H2O, 1_{mM} EGTA, $5_{mg/ml}$ BSA) with gentle rocking. Fibers were then incubated in the chamber with oxygenated Buffer-Z supplemented with 10µM Amplex-Red to simultaneously measure ROS-production, as well as $1_{µM}$ Blebbistatin to prevent tetanus of the muscle[39], $25_{U/ml}$ Cu/Zn SOD1 to convert O2- to H2O2 and 2_{mM} EGTA. Following oxygenation and measurement of background values, substrates were added to assess respiration and ROS production simultaneously. Substrates were titrated in three separate protocols as follows. In the first protocol, 5_{mM} glutamate + 2_{mM} malate (Complex I – Basal), 5_{mM} ADP (Complex I – Active), and 10_{mM} succinate (Complex I+II – Active) were added to simultaneously measure O_2 consumption and ROS. In the second protocol, O_2 consumption was measured by first titrating 0.5_{mM} rotenone, to prevent electron backflow and slip at Complex I and damage to the fiber. Subsequently, 10_{mM} succinate (Complex-II Basal) and 5_{mM} ADP (Complex-II Active) were added. In the final protocol, ROS emission was measured by titrating 10_{mM} of succinate (Complex-II Basal) and 5_{mM} ADP (Complex-II Active). To test for mitochondrial membrane integrity, cytochrome c was added to the chamber. Respiratory function was determined by oxygen flux rates (pmol/s·ml) minus background rates and corrected to fiber mass (pmol/s/mg). ROS-emission was calculated by dividing the rate of ROS-emission (pmol/s/mg) and correcting it by the corresponding respiration rate (pmol H_2O_2/pmol O_2 consumed).

Cytosolic and Nuclear Fractionation

Nuclear and cytosolic fractions from fresh TA muscles of mice were obtained using the NE-PER extraction reagents (38835, Thermo Scientific Scientific) with minor modifications. Briefly, 50~100 mg of the TA muscle was minced on ice and homogenized using a Douce homogenizer in cytosolic extraction reagent (CER) I. Homogenates were then vortexed and let to stand on ice for 10 minutes. Following the addition of CER II solution, samples were briefly vortexed and centrifuged (16,000g) for 10 min. The cytosolic fractions (supernates) were then collected. The remaining pellets, containing nuclei and cellular debris, were washed 3 times in cold 1×PBS and subsequently resuspended in nuclear extraction buffer (NER). Nuclear fractions were then sonicated for 3 seconds x 3 times, and incubated on ice for 40 min. These samples were vortexed every 10 min during the incubation, and subsequently underwent centrifugation (16,000g) for 10 min. The resulting supernatant nuclear fractions were collected. Both the cytosolic and nuclear fractions were stored at -80°C until further analysis.

Whole Muscle Protein Extracts

One quadricep muscle was snap frozen in liquid nitrogen following excision from the animal and stored at -80°C. The tissue was pulverized to a fine powder at the temperature of liquid nitrogen. Protein extracts

were made by diluting (10x) a small amount of powder (~15-20mg) in Sakamoto buffer (20mM HEPES, 2mM EGTA, 1% Triton X-100, 50% Glycerol, 50 mM ß-Glycerophosphate) containing both phosphatase (Sigma) and protease (Roche) inhibitors and rotated end-over-end for 1 hour at 4^{o}C. Samples were then sonicated on ice (3 seconds x 3 times) and centrifuged (14,000g) for 15 minutes at 4^{o}C. The supernatant fraction was collected and stored at -80^{o}C until further analysis.

Western Blotting

All protein concentrations in isolated mitochondria, nuclear and cytosolic fractions and whole muscle samples were determined using the Bradford method. Equal amounts of protein (~20-30µg) were loaded and separated via SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Mississauga, ON, Canada). Membranes were blocked with wash buffer (0.12% Tris-HCl, 0.585% NaCl, 0.1% Tween, pH 7.5) supplemented with 5% skim milk (w/v) at room temperature for 1 hour with gentle agitation. Membranes were then incubated with primary antibodies overnight at 4^{o}C for OXPHOS Cocktail (Ab110413, Lot 2101000654, Abcam), Beclin1 (3738, Lot 3, Cell Signaling Technologies), ATG7 (A2856, Lot 078M4843V, Sigma), p62 (Ab56416, Lot GR3285986-1, Abcam), LC3-I/II (4108, Lot 3, Cell Signaling Technologies), Bnip3 (Gift from Dr. L.A. Kirshenbaum), Parkin (4211, Lot 7, Cell Signaling Technologies), VDAC (Ab14734, Lot GR3391163-2, Abcam), Lamp1 (Ab24170, Lot GR3235632-1, Abcam), V-ATPase B1/2 (sc-55544 F-6, Lot I1018, SantaCruz), mature Cathepsin B (D1C7Y, Lot 1, Cell Signaling Technologies), mature Cathepsin D (sc-377299, Lot BO419 , SantaCruz), TFEB whole muscle (MBS120432, Lot 319C2a-3, MyBioSource), TFEB nuc/cyto (A303-673A, Lot 7, Bethyl), TFE3 (HPA023881, Lot 000010514, Sigma), GAPDH (ab8254, Lot GR3317834-1, Abcam), a-tubulin (CP06, Lot D00175772, Calbiochem), H2B (2934, Lot 4, Cell Signaling Technologies). The following day, membranes were washed 3x5minutes in wash buffer and incubated for 1 hour at room temperature with the appropriate HRP-conjugated secondary antibody and subsequently washed 3x5 minutes in wash buffer. The protein density was visualized using enhanced chemiluminescence (1705061, Bio-Rad) with an iBright FL1500 Imaging Station (Fischer Scientific, Oakville, ON, Canada). Band densities were quantified by ImageJ software (NIH) and normalized to corresponding loading controls. All gels were run in a sex-specific manner, with each blot containing either male or female samples. For whole muscle western blots, to have sex-comparisons, an identical, arbitrary control sample, was run in the first lane and all values were controlled back to this band. Representative blots with a break between bands denotes that portion of the image was shifted, without alterations in contrast, to show representative data. All data are represented as combined young and old data, and sex-separated data.

Statistical Analysis

Data were analysed using GraphPad Prism Software (Version 9) and values are represented as means ± SEM. Student's unpaired t-tests were utilized to analyze combined, male and female data to investigate an effect of age. Two-way ANOVAs were used to assess the interaction between age and sex where

applicable, and significance was achieved at $p<0.05$. A Bonferroni post-hoc test was used and d represents a significant difference. Due to the limited sample sizes when the sexes were separated, where post-hoc tests failed to uncover significant differences between groups, independent Student's unpaired t-tests were used to assess the differences between male and female, young and old data. Significance is represented by *, and p values are shown for trends in the data. Three-way ANOVAs were used to assess the independent and interaction effects between age, sex and exercise, significance was achieved at $p<0.05$, and p values are reported for trends in the data set where applicable.

Results

Physical characteristics of young and aged male, and female mice.

To determine whether aging differentially impacted muscle mass in male and female mice we first measured body mass (g), muscle mass (mg) and muscle mass corrected for body mass (mg/g) in the predominantly fast tibialis anterior (TA) and predominantly slow-twitch soleus (Sol) muscles (Table 1). Overall, body mass was 1.4-fold greater in aged mice versus young counterparts ($p<0.05$). Main effects of age and sex effects were found. Post-hoc tests revealed that both young and aged male mice were significantly larger than age-matched female counterparts ($p<0.05$). Furthermore, aged male mice were 32% heavier than young males, and aged female mice were 28% heavier than young females ($p<0.05$). Raw TA mass (mg) was lower in female mice (sex effect, $p<0.05$) and TA mass was significantly less in young females versus young males (t-test, $p<0.05$). When corrected for body mass, TA mass was 24% smaller in aged mice ($p<0.05$), and a main effect of sex was observed in our separated analysis ($p<0.05$). On average, TA mass/body mass (mg/g) was 28% lower in female mice (post-hoc, $p<0.05$) and 18% less in male mice (t-test, $p<0.05$). Sol mass (mg) was not different between any groups. When corrected for body mass (mg/g), a significant 30% decrease in Sol mass with age was measured in sex-pooled data ($p<0.05$). In a sex-separated analysis, a main effect of age and sex were observed ($p<0.05$). Young females had a 1.4-fold larger Sol mass/body mass than young males (post-hoc, $p<0.05$). With age, male mice had a 24% decline in Sol mass/body mass (t-test, $p<0.05$), whereas females displayed a 36% decline (post-hoc, $p<0.05$).

Table 1

Animal body weight and muscle mass

		Combined		Male		Female		Statistics
		Young	Aged	Young	Aged	Young	Aged	
Body Mass (mg)		30.73 ± 1.27	41.35 ± 1.91*	34.65 ± 1.01A	45.72 ± 2.45*,B	25.83 ± 0.976C	35.89 ± 1.62*,A	# †
Muscle Mass (mg)	TA	51.41 ± 1.41	53.49 ± 3.95	54.605 ± 1.34A	58.70 ± 3.95A	47.43 ± 1.97A,D	46.98 ± 5.58A	†
	Sol	10.32 ± 0.49	9.60 ± 0.76	10.01 ± 0.42A	9.95 ± 0.916A	10.51 ± 1.00A	9.15 ± 1.32A	
Muscle Mass (mg/g body weight)	TA	1.70 ± 0.06	1.31 ± 0.10*	1.586 ± 0.05A, B	1.295 ± 0.12*, A,B	1.85 ± 0.08A,C	1.34 ± 0.17*, A, B, D	#
	Sol	0.34 ± 0.03	0.24 ± 0.02*	0.29 ± 0.02A	0.22 ± 0.02*, A,	0.409 ± 0.04B	0.26 ± 0.04*,A	# †

Body mass (g), muscle mass (mg) and muscle mass corrected for body weight (mg/g) in combined and sex-separated young and aged muscle. Values are means ± SEM. #, p<0.05 main effect of age; †, p<0.05 main effect of sex; ⍰, p<0.05 interaction of age and sex. Different letters represent post-hoc significance at p<0.05. *p<0.05, t-test between young vs old within the same sex; d p<0.05 t-test between male and females at the same age. N=10/male group, N=8/female group.

Exercise capacity in young and aged; male and female mice.

To determine if age and biological sex impact acute exercise capacity, we exposed a cohort of mice to an exhaustive bout of incremental exercise. In our sex-pooled comparison, aged mice ran for an average of 25 minute less (t-test, p<0.05, Fig.1A) accounting for 645 meters of less distance covered (t-test, p<0.05, Fig. 1B). In sex-separated comparisons, a main effect of age and an interaction of age and sex were found in run time (p<0.05, Fig. 1A). Further analysis revealed 34% and 43% declines in aged male and female mice versus their young counterparts, respectively (post-hoc, p<0.05, Fig. 1A). Effects of age, sex, and an interaction of the two variables was measured in distance to fatigue (p<0.05, Fig. 1B). Run distance was reduced with age in both male and female mice (post-hoc, p<0.05, Fig. 1B). On average young female mice ran 263 meters more than young males (post-hoc, p<0.05, Fig. 1B), whereas aged females ran slightly less (28m) than aged males (post-hoc, p<0.05, Fig. 1B). Blood lactate was similarly increase with exercise in all groups (t-test, p<0.05, Fig. 1C).

Mitochondrial parameters in young and aged, male and female mice.

To understand the divergent endurance capacity with age and sex, we assessed mitochondrial parameters as these organelles are correlated with muscle fatigability. We examined respiration and H_2O_2 emission in permeabilized TA muscle fibers from all groups (Fig. 2A, B). We observed an overall effect of age, whereby aged muscle had lower respiratory capacity (3-way ANOVA, $p<0.05$, Fig. 2A). Independent analyses were performed for each subsequent titration, and we measured a main effect of age for all respiratory measurements (2-way ANOVA, $p<0.05$ Fig. 2A), apart from the Complex I-Basal condition. An interaction between age and sex was found in Complex II-Basal respiration (2-way ANOVA, $p<0.05$ Fig. 2A), however, no post-hoc significance was observed. Overall, no changes were measured in H_2O_2 emission in permeabilized fibers (Fig. 2B), but a trending effect of sex was measured in Complex II-active (2-way ANOVA, $p=0.09$, Fig. 2B), with lower values in female samples.

To determine the effects of age and sex on mitochondrial protein content, we quantified levels of proteins derived from each complex of the electron transport chain (ETC) (Fig. 3). In the sex-grouped data, we found no significant differences in any ETC proteins, and a trending increase in both Complex-V (t-test, $p=0.058$, Fig. 3B) and -II protein (t-test, $p=0.087$, Fig. 3B, E). A main effect of age was observed in both Complex-V (Fig. 3B) and -II (Fig. 3E). Each independent complex (Fig. 3B-F) and total OXPHOS protein (Fig. 3G) exhibited a main effect of sex ($p<0.05$), such that females had more mitochondrial protein. Further, an interaction between age and sex was found in Complex-V ($p<0.05$, Fig. 3B), Complex-II ($p<0.05$, Fig. 3E), Complex-I ($p<0.05$, Fig. 3F), and total OXHOS ($p<0.05$, Fig. 3G) protein, whereby female muscle did not display decrements in mitochondrial protein content with age.

We assessed independent differences between young males and females, and measured 35%, 61%, 38%, and 28% more Complex-V (t-test, $p<0.05$, Fig. 3B), Complex-III (post-hoc, $p<0.05$, Fig. 3C), Complex-II (t-test, $p<0.05$, Fig. 3E), and total OXPHOS (t-test, $p=0.08$, Fig 3G) protein in young females versus young males. The same comparison in aged male and female mice showed that each complex had between 1.8- and 2.1-fold more mitochondrial protein ($p<0.05$, Fig. 3B-F) and 2.1-fold more total OXPHOS in females than in males (post-hoc, $p<0.05$, Fig. 3G).

We then explored independent differences between young and aged muscle from the same-sex mice. In male mice, we observed no change in Complex-V (Fig. 3B) or -II (Fig. 3E) but measured 17% to 39% decreases in all other mitochondrial protein content with age (Fig. 3C, D, F, G). In females, we measured no change in Complex-III (Fig. 3C), -IV (Fig. 3D) or -I (Fig. 3F) protein, but 33% to 47% increases were evident in the remaining mitochondrial proteins (Fig. 3B, E, G) with age in female mice.

Autophagy-related protein expression in aged muscle

To evaluate how aging and biological sex affect the autophagy-lysosome system, we measured upstream autophagy proteins in whole muscle quadriceps samples (Fig. 4 A-C). In combined-sex groups, aging led to a significant 44% increase in Beclin1 protein (t-test, $p<0.05$, Fig. 4B), and a trending 47% increase in Atg-7 protein (t-test, $p=0.09$, Fig. 4C). When the sexes were analyzed separately, no main or interaction effects were measured in Beclin1 protein (Fig. 4B), but a main effect of both age and sex was found in Atg-7 protein (2-way ANOVA, $p<0.05$, Fig. 4C), whereby aging and female muscle displayed increased protein expression.

Independent differences between the groups were then examined for these autophagy proteins. Beclin1 protein was significantly increased by 36% in aged males versus young counterparts (t-test, $p<0.05$, Fig. 4B), whereas female mice displayed no age-effect (Fig. 4B). Atg-7 protein was unchanged in both sexes independently, however both young (t-test, $p<0.05$, Fig. 4C) and aged female mice (post-hoc, $p<0.05$, Fig. 4C) contained ~2-fold more Atg-7 protein in comparison to age-matched male mice.

Autophagosomal protein content in male and female mice with age.

We next wanted to explore how markers of mature autophagosome content are changed in whole muscle samples with age and biological sex in skeletal muscle. Since, these proteins have been shown to change with exercise, we also assessed the impact of exercise in these murine groups. We first measured LC3-II/I as markers of the ratio of mature:immature autophagosomes, respectively. We found a tendency of exercise to reduce LC3-II/I in our combined-group analysis (2-way ANOVA, $p=0.09$, Fig 5.B), with no main effects or post-hoc significance in our sex-separated groups. We observed an overall effect of age on p62 levels in our combined group (2-way ANOVA, $p<0.05$, Fig. 5C) and a trending 37% increase in p62 protein in our young versus aged sedentary animals (t-test, $p=0.085$, Fig. 5C). In the sex-separated data, a significant main effect of age was observed, along with an interaction between age and acute exercise (3-way ANOVA, $p<0.05$, Fig. 5C). When we assessed the influence of age and exercise in independent sexes, a main effect of age was evident in both males and females (2-way ANOVA, $p<0.05$, Fig. 5C). Independent analyses revealed a significant 33% decrease in p62 protein with exercise in young males (t-test, $p<0.05$, Fig. 5C), and a 25% increase with exercise in young females (t-test, $p=0.05$, Fig. 5C).

Mitophagic protein content in whole muscle and isolated mitochondria

To determine if age and sex impact mitophagy in skeletal muscle, we first probed for the mitophagy markers BNIP3 and Parkin in whole muscle samples (Fig. 6A-C). In the sex-combined group, there were 4.8-fold and 3.6-fold increases in aged muscle BNIP3 and Parkin protein, respectively (t-test, $p<0.05$, Fig. 5 B, C). In sex-separated comparisons, a main effect of age was observed in BNIP3 protein (2-way ANOVA, $p<0.05$, Fig. 6B), and post-hoc comparisons revealed similar, significant increases in aged muscle BNIP3

protein vs sex-matched young counterparts (post-hoc, $p<0.05$, Fig. 6B). A main effect of both age and sex were found in Parkin protein, whereby females, both young and old, had more Parkin than their sex-matched, young, counterparts (2-way ANOVA, $p<0.05$, Fig. 6C). Aging in both sexes led to increases in Parkin protein (Male: t-test, $p<0.05$; Female: post-hoc, $p<0.05$; Fig. 6C).

We also explored whether LC3-II protein, a marker of mature autophagosomes was different in isolated mitochondria from young and old male and female mice (Fig. 6D,E). We observed no effect of age in either sex. Since we have previously reported that acute exercise can stimulate mitophagic breakdown, we assessed whether our exercise stimulus altered mitochondrially-localized LC3-II. A main effect of exercise was observed, whereby LC3-II protein was decreased with exercise by an average of 20% overall (3-way ANOVA, $p<0.05$, Fig. 6E).

Lysosomal protein content in young and aged, male and female mice.

To assess the end-stage of the autophagy pathway, we evaluated lysosomal protein content in our groups (Fig. 7A-E). Lysosome-associated membrane protein 1 (Lamp1) levels were unchanged with age in the sex-combined group. Alternatively, vesicular ATPase (V-ATPase), mature Cathepsin B, and mature Cathepsin D were all upregulated by 3.6-, 4.0- and 5.5-fold with age, respectively (t-test, $p<0.05$, Fig.7 C, D, E). When sex was separated, all lysosomal proteins showed a significant main effect of age (2-way ANOVA, $p<0.05$, Fig. 7 B-E). A main effect of sex was found in Lamp1 (2-way ANOVA, $p<0.05$, Fig. 7C), vATPase (2-way ANOVA, $p<0.05$, Fig. 7D) and mature Cathepsin D (2-way ANOVA, $p<0.05$, Fig. 7E), whereby these proteins were higher in the female mice. An interaction between age and sex was found for mature Cathepsin D protein (Two-way ANOVA, $p<0.05$, Fig. 7E). Independent analyses for each protein confirmed significant 1.8-3.9-fold increases in all measured lysosomal proteins with age in the male mice ($p<0.05$, Fig. 7 B-E). In female mice, significant 4.4-6.5-fold increases were found with age in each lysosome protein (post-hoc; $p<0.05$, Fig. 7 C-E), except for Lamp1. We quantified higher Lamp1 (post-hoc, $p<0.05$, Fig 7. B) and mature Cathepsin D (t-test, $p<0.05$, Fig 7. D) in young female mice versus young male mice, and elevated mature Cathepsin D in aged females compared to aged males (post-hoc, $p<0.05$, Fig 7. E).

We measured the protein levels of Tfeb and Tfe3, transcription factors that control the autophagy-lysosome pathway (Fig. 8A-C). Tfeb was 3.8-fold greater with age in the sex-combined analysis (t-test, $p<0.05$, Fig. 8B). In the sex-separated analyses, Tfeb protein exhibited main effects of age and sex, and an interaction existed between these variables (2-way ANOVA, $p<0.05$, Fig. 8B), whereby these proteins were greater in aged, versus young muscle, female muscle versus male, and the increase with age was larger in the female cohort. Specifically, Tfeb protein was 1.8-fold greater in young females (t-test, $p<0.05$, Fig. 8B) and 2.5-fold greater in aged females (t-test, $p<0.05$, Fig. 8B) when compared to age-matched male counterparts. Compared to young, sex-matched animals, Tfeb protein was 3.3-fold greater in aged males (t-test, $p<0.05$, Fig. 8B) and 4.2-fold higher in aged females (post-hoc, $p<0.05$, Fig. 8B). Conversely,

Tfe3 was 1.5-fold greater in our sex-combined analysis (t-test, $p<0.05$, Fig. 8C). In the sex-separated analyses, Tfe3 protein exhibited main effects of age and interaction between age and sex (2-way ANOVA, $p<0.05$, Fig. 8B, C). As such, Tfe3 protein was increased 3.3-fold with age in male mice (post-hoc, $p<0.05$, Fig. 8C), an effect not seen in females. A trending increase was also measured in Tfe3 protein, whereby young females contained 66% more than young males (t-test, $p=0.057$, Fig. 8C).

Influence of exercise on lysosome biosynthetic pathway

We also wished to explore whether exercise can activate lysosome biosynthesis pathways in both young and aged, male and female mice (Fig 9. A-D). Thus, we measured levels of nuclear Tfeb protein in all groups. Sedentary aged, sex-combined muscle exhibited 18% more nuclear Tfeb (post-hoc, $p<0.05$, Fig. 9A). In this sex-combined analysis, there was an interaction between age and exercise (2-way ANOVA, $p<0.05$, Fig. 9B). Following cessation of exercise, nuclear Tfeb was increased by 30%, whereas this was not evident in aged male or female muscle (post-hoc, $p<0.05$, Fig 9B). However, aged male and female muscle appeared to possess approximately 20% higher basal pre-exercise levels of Tfeb in the nucleus, compared to young counterparts (t-test, $p=0.075$ and $p=0.077$ respectively, Fig. 9B). In response to exercise, young male mice enhanced nuclear Tfeb by 40% (post-hoc, $p<0.05$, Fig. 9B), whereas females only upregulated nuclear content by 16% (t-test, $p=0.064$, Fig. 9B). Thus, the fold-change in nuclear Tfeb with exercise was greater in males, compared to females (t-test, $p<0.05$, Fig 9C).

We also utilized a Tfeb-luciferase promoter activity assay to determine whether exercise stimulates Tfeb transcriptional activity. This analysis could only be completed in sex-combined groups. Overall, there was a trending main effect of increase promoter activity with exercise (2-way ANOVA, $p=0.09$, Fig. 9D). There was also a main effect of age (2-way ANOVA, $p<0.05$, Fig. 9D), whereby Tfeb promoter activity was reduced with age. Exercise enhanced Tfeb promoter activity in both young (t-test, $p=0.069$, Fig. 9D) and aged (t-test, $p<0.05$, Fig. 9D) muscle by 1.9-and 2.5-fold, respectively.

Discussion

With advancing age there is a progressive loss of muscle mass and function, ultimately resulting in sarcopenia [3, 40]. This has global implications, as the aging population is continually rising. Understanding the molecular underpinnings that govern muscle loss with age will guide future therapeutic interventions. It is well established that regular exercise can counteract these deficits, partially through upregulating the production of new healthy mitochondria and stimulating the removal dysfunctional ones through mitophagy [20, 41]. Mitophagy is the mitochondrial-specific degradation through the autophagy lysosome system. Previous reports from our group have indicated that acute exercise stimulates lysosome biogenesis [27] and that chronic muscle activity elevates lysosome protein content [42, 43] in young, healthy muscle. These changes are likely to support an enhanced capacity for proteolysis. No studies to date have examined the regulation of lysosome biosynthesis in sedentary or exercised aged muscle, and whether similar mechanisms exist in males and females. The influence of

biological sex is an important factor to consider, as therapeutic targets in males and females may differ. To this end, the present study explores how biological sex influences the autophagy-lysosome system in young and aged skeletal muscle. Furthermore, we wanted to examine whether acute exercise can upregulate lysosomal synthetic pathways in aged muscle, and whether the effect was sex-dependent.

To address these aims, we utilized young (4-6 mo) and aged (22-24 mo), male and female C57BL/6 mice, which were either sedentary or exercised acutely. In our analyses, we performed measures in both combined and sex-separated groups, which allowed us to determine if changes we observed were age- and/or sex- dependent.

We confirmed the observation that female mice have a smaller muscle mass, as previously reported [44], and that they also display greater reductions in muscle mass with age. This may be due to a higher abundance of atrophy susceptible Type I muscle fibers in females [45–48]. It has been reported that female rodents have greater loss of muscle mass with hindlimb unloading due to elevated catabolic signaling [33, 49, 50]. Based on our findings, this seems to be true with age as well. A balance between protein synthesis and degradation is required to maintain muscle mass. Our observation that female mice undergo a greater degree of atrophy with age may be due to greater anabolic resistance, and/or accelerated protein degradation. Here, we have explored the latter pathway, with a focus on the autophagy-lysosome system.

Aging leads to decrements in endurance capacity [20, 41], which we reproduce in the current study. We also report that young female mice have greater exercise capacity than their male counterparts. We can discount the possibility that males and females where not similarly exhausted, as post-exercise lactate levels were similar amongst all groups. Thus, the longer distance to exhaustion in young female mice may be due to the greater abundance of mitochondrial proteins that we measured in our female cohort. Supportively, others have found that young females have higher mitochondrial content [35, 51] and have more fatigue-resistant muscle [52–54]. However, we cannot discount the possibility that these differences in mitochondrial content and exercise capacity are simply a product of young female mice being more active in their cage behavior [55, 56]. Furthermore, the aging-induced reduction in exercise tolerance was greater in our female cohort. This result was surprising as only our male animals showed declines in mitochondrial content with age. This could also be explained by an age-related deterioration of mitochondrial function in females; however our data did not indicate any sex differences in respiratory capacity, or reactive oxygen species production, between our males and females. Thus, it is clear that other mechanisms may influence the divergence in exercise capacity with age in male and female rodents.

Protein aggregates and mitochondria are degraded by the autophagy-lysosome system. Since aberrations in this proteolytic system are associated with muscle dysfunction [12–16], we wanted to investigate whether age and biological sex similarly, or differentially, influenced this system. Like previous studies [22, 23, 57], our results support an increase in autophagy-related proteins such as the nucleation-inducing protein Beclin1 [58, 59], and the LC3 maturation protein ATG7 [59] with age. However, sex

influences this response, since both young and old females had a greater abundance of ATG7 protein in comparison to age-matched counterparts, a finding that has been reported previously in avian muscle [57]. These data would imply that females have a greater capacity to form mature autophagosomes via the conversion of LC3-I to LC3-II.

To delineate whether these upstream markers influence autophagosomal turnover, we measured LC3-I as well as its downstream lipidated form LC3-II, which is a marker of mature autophagosomes [60]. In addition, we assessed the levels of p62, an adaptor protein responsible for tethering the autophagosome to removable cellular constituents [60–62]. Importantly, these proteins are degraded by lysosome proteolysis. Thus, declines in p62 protein, without change and/or deficits in LC3-II/I would indirectly suggest higher levels of autophagic breakdown. It should be noted that these measures do not directly indicate true autophagic flux. Thus, future research investigating gene expression changes and utilizing "flux" measures will be useful in determining whether age, biological sex or acute exercise impact autophagy and mitophagy similarly or differentially. Our finding that young female mice have less p62 protein than young male mice, with greater levels of ATG7 and no difference in LC3-II/I would suggest that females have greater basal autophagy in muscle, similar to a previous report [63]. We also found that aged muscle contains a higher abundance of p62 and total LC3 (data not shown), indicative of greater autophagic signaling with age, regardless of sex. Thus, the sex-difference in basal autophagy was not observed with age, and overall, these results suggest that young females have greater basal autophagy than young males.

Acute endurance exercise is a stimulus for autophagosomal turnover in young muscle [26]. Thus, we wanted to examine if sex and/or age influence this response. In young male mice we measured reductions in p62 protein in the exercised group. LC3-I was similarly changed (data not shown). These findings, in addition to our main effect of exercise in decreasing whole muscle LC3-II protein imply that exhaustive exercise is a stimulus for autophagic clearance in young male muscle. In contrast, increases in p62 protein were observed in age-matched females. Thus, autophagosomal breakdown is not similarly induced between the sexes with acute exercise. Since female muscle appears to have accelerated basal autophagy, exercise may be insufficient to further augment this pathway. In aged muscle, these exercise effects were not apparent, regardless of sex. Overall, these findings suggest that acute exhaustive exercise stimulates autophagy predominantly in young male muscle.

With an interest in how these changes may translate to mitochondrial degradation through mitophagy, we assessed levels of proteins involved in targeting these organelles for digestion at the lysosomes. In whole muscle samples, we found an overall increase in both BNIP3 and Parkin with age. However, the increase with age was much more prominent in female mice. This may suggest divergent regulation of mitophagy-targeting mechanisms in male and female skeletal muscle. To assess autophagosome-bound mitochondria directly, we probed for LC3-II in isolated mitochondria, as done previously [23–25, 42, 64–66]. Contrary to our hypothesis, we failed to measure any increase in mitochondrial LC3-II with age. This contrasts to a previous report from Chen et al., however, in their study aged mice were considerably younger (18 months), compared to those used here (22-24 months) [24]. Further, we measured an overall

effect of exercise in reducing mitochondrial LC3-II protein, suggesting that mitophagic clearance is occurring universally. When these acute exercise-induced changes are repeated in a chronic manner, they likely contribute to the training-induced enhancement in mitochondrial quality observed as a result of training [22]. Ultimately, these observations indicate that mitophagy signaling is elevated in the muscle from aged mice, and that exercise can stimulate the degradation of mitochondria regardless of age, or sex.

Lysosomes act as the terminal step of the autophagic pathway whereby they degrade substrates via their low pH and the presence of digestive enzymes. These organelles play an integral role not just in autophagy, but also in mitochondrial quality control through mitophagy [67]. In muscle from aged rodents and humans the presence of non-digestible lysosomal content, termed lipofuscin is apparent [23, 68]. The presence of such structures is indicative of lysosome dysfunction, which may limit the capacity for autophagic and mitophagic breakdown. Our group and others have reported that aging leads to an accumulation of lysosomal proteins [22, 69], which we recapitulate in the present study. This could be explained by the greater levels of TFEB and TFE3 protein, transcription factors that mediate lysosome biogenesis, in the muscle from aged animals in this study. Uniquely, we also uncovered that young and aged female mice have a greater abundance of lysosomal protein in whole muscle samples as compared to age-matched males. This may in part be explained by the higher levels of TFEB and TFE3 in female muscle. This abundance of lysosome protein in female muscle may serve to support the observed increase in autophagy in females, but more research utilizing direct flux measurements, along with assessments of lysosomal function, are required as a function of age and sex.

TFEB is considered the master transcriptional regulator of lysosome biogenesis [70, 71]. Our work in the past has uncovered that chronic contractile activity, as a model of endurance exercise training, upregulates lysosome content in young healthy muscle [22, 42, 43]. This is due to acute-exercise inductions in the transcriptional activation of TFEB, as well as enhanced nuclear localization of the protein [27]. We also observed this nuclear localization and transcriptional activation of the TFEB promoter construct in young mice. When we analyzed this response in males versus females, the relative shift of TFEB to the nucleus was greater in the male cohort. This finding matches that of the exercise autophagy data. Cumulatively these findings imply that the autophagy-lysosome system is more responsive to exercise in young male, compared to female muscle, and future work should look at mechanisms that underly such changes.

Finally, we wanted to examine these mechanisms in aged muscle, as increasing lysosomal content though exercise could in theory enhance the capacity for autophagic/mitophagic degradation. We hypothesized that the muscle from aged animals would exhibit a blunted lysosome biosynthetic pathway due to attenuated kinase signaling [24, 25, 72, 73]. Supportively, an upregulation of nuclear TFEB protein post-exercise was only found in young animals. However, although aging led to an overall decrease in TFEB transcription as measured by promoter-reporter activity level, exercise was able to upregulate it to the level observed in young muscle. Thus, although aging blunts aspects of

autophagosomal turnover and lysosome biosynthesis with acute exercise, it enhances TFEB expression, which, if repeated over time, may serve to re-establish lysosome function and homeostasis.

Conclusions

Collectively, our studies reveal that aging and exercise have differential effects on the autophagy-lysosome system in skeletal muscle, which is dependent on biological sex. First, we show that in female mice, there is a greater abundance of mitochondrial, autophagy and lysosome proteins. Second, we observed that female mice have a greater index of skeletal muscle autophagosome clearance than male mice. We also demonstrate that acute exercise stimulates autophagosome turnover and lysosome biogenesis in young males, but not in young females or in aged animals from either sex. Finally, and most importantly, exercise was able to activate TFEB promoter activity regardless of age, which can promote lysosome biogenesis. This finding provides merit to further explore the vitality of exercise in re-establishing autophagy-lysosome homeostasis in aged muscle. More work is required to delineate the impact of biological sex on mechanisms that contribute to sarcopenia, as future therapies must be targeted accordingly.

Abbreviations

RLU Relative light units
OXPHOS Oxidative Phosphorylation
ATG7 Autophagy-related gene 7
p62 Sequestosome 1
LC3 Microtubule-associated protein 1A/1B-light chain 3
Lamp 1 Lysosome associated membrane protein 1
V-ATPase B1/2 Vesicular ATPase B1/2
TFEB Transcription factor EB
TFE3 Transcription factor E3
H2B Histone 2 B
TA Tibialis anterior
Sol Soleus
ETC Electron transport chain

Declarations

Ethics Approval

All animal protocols were submitted and approved by the York University Animal Care Committee. Animals were treated in accordance with the Canadian Council of Animal Care guidelines.

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Not applicable.

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All raw data used to generate the figures are available upon request from Dr. David A. Hood (dhood@yorku.ca).

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Author Contributions:

D.A.H. and M.T. conceived the experiment design, analyzed the data, and wrote the manuscript. M.T performed all experiments apart from isolation of mitochondria and Western blots in Figure 5Awhich were performed by A.N.O and R.K, respectively.

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Figures

Figure 1

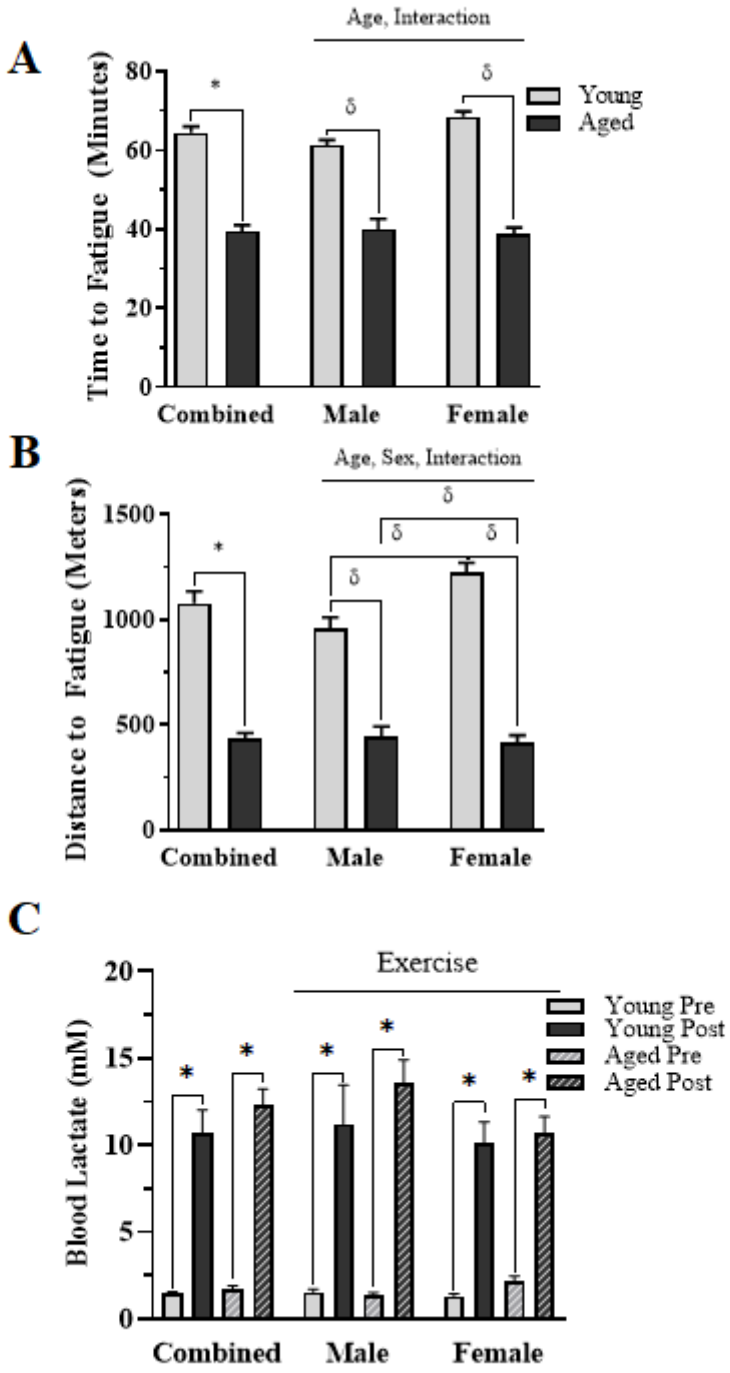

Figure 1

Exercise capacity in young and aged, male and female mice. A. Time to fatigue in minutes. B. Distance to fatigue in meters. C. Blood lactate (mM). Values are means ± SEM. Main effects are represented on graph at p<0.05. *p< 0.05, t-test between indicated groups. □□p<0.05□□post-hoc significance. N=5/male group, N=4/female group.

Figure 2

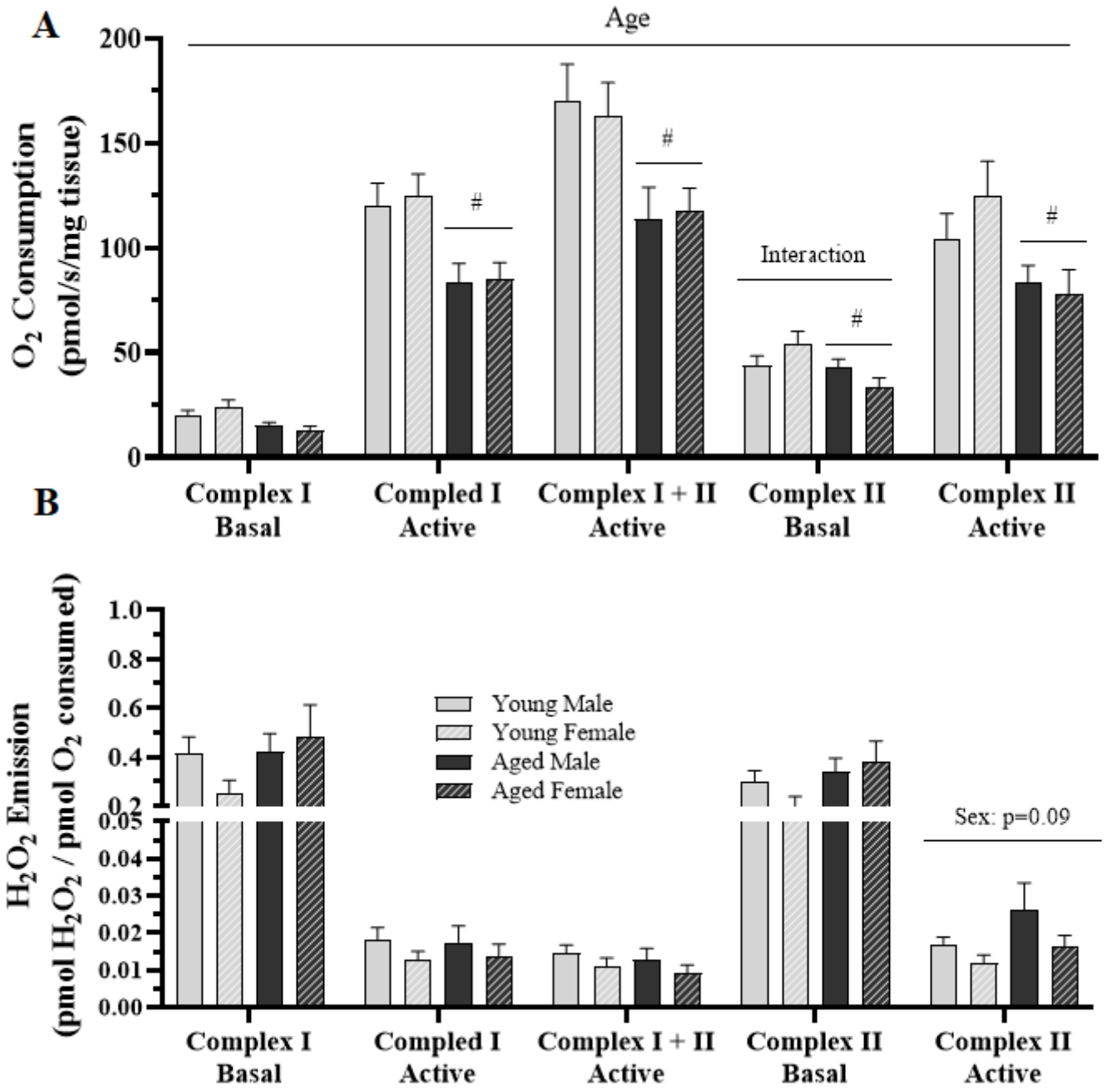

Figure 2

Mitochondrial respiration and reactive oxygen species in young and age, male and female mice. A. Oxygen consumption rates and B. H2O2 emission in the indicated respiratory states. All values are reported as means ± SEM. Main effects of a 3-way ANOVA are represented on the graph at $p<0.05$. Main effects of 2-way ANOVA are represented on graph at $p<0.05$. # $p<0.05$, main effect age. N=10/male group, N=8/female group

Figure 3

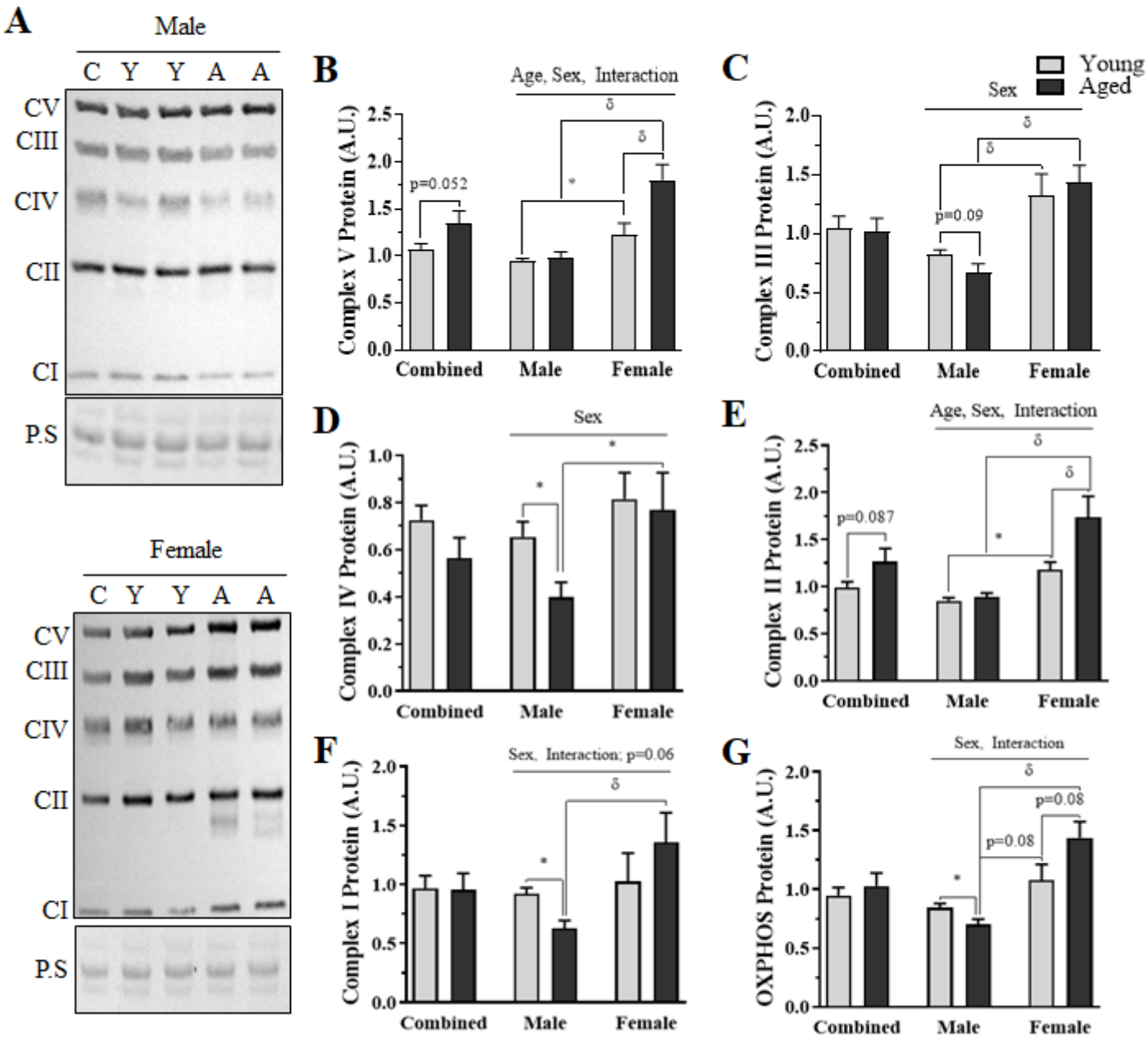

Figure 3

Mitochondrial protein content in young and age, male and female mice. A. Representative western blot from male (top panel) and female (bottom panel) mice for OXPHOS protein. B-F. Quantification of each independent mitochondrial protein. B. Complex-V protein (ATP5A) protein, C. Complex-III (UQCRC2) protein, D. Complex-IV (MTCO1) protein, E. Complex-II (SDH8), and F. Complex I (NDUFB8) protein. G. Quantification of total OXPHOS. All values were corrected to Ponceau stain (P.S), and values are reported as means ± SEM, in A.U. Main effects of 2-way ANOVA are represented on graph at $p<0.05$.

□□p<0.05□□post-hoc significance. *p< 0.05, t-test between indicated groups. N=10/male group, 8/female group.

Figure 4

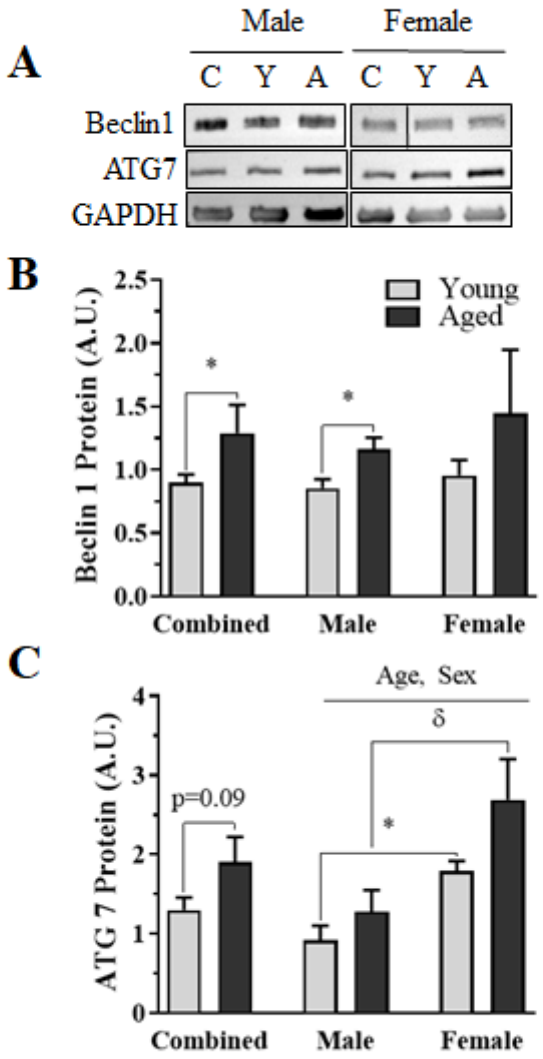

Figure 4

Upstream autophagic proteins in young and age, male and female mice. A. Representative western blots for Beclin1 and ATG7. B. Quantification of Beclin1 protein in combined and sex-separated groups. C. Quantification of ATG7 protein in combined and sex-separated groups. All values were corrected to GAPDH and are reported as means ± SEM, in A.U. Main effects of 2-way ANOVA are represented on graph at p<0.05. □□p<0.05□□post-hoc significance. *p< 0.05, t-test between indicated groups. N=10/male group, 8/female group. Dashed line break in representative blot are different sections from the same blot.

Figure 5

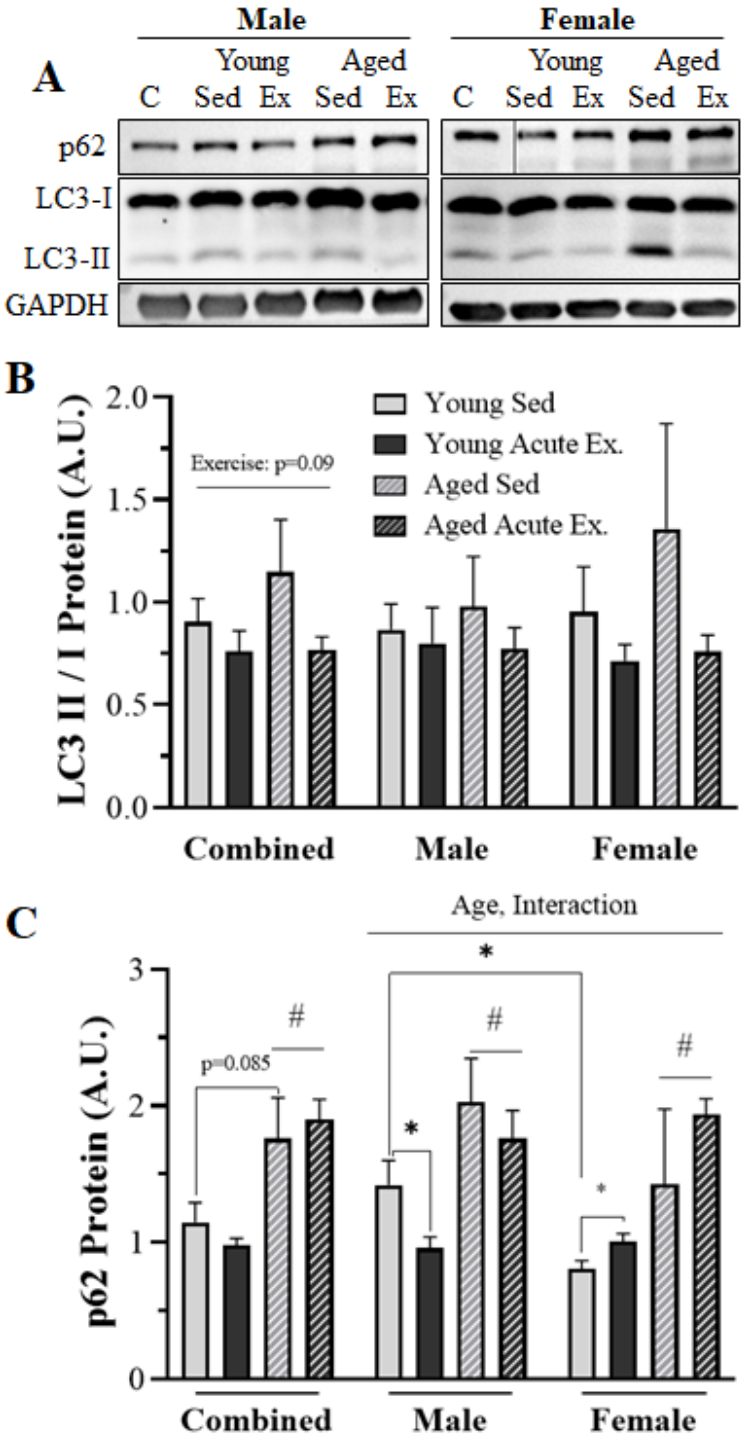

Figure 5

Autophagosomal proteins in sedentary and acute-exercised young and age, male and female mice. A. Representative western blots for p62, LC3-I and LC3-II. B. Quantification of LC3-II/I protein in combined and sex-separated groups. C. Quantification of p62 protein in combined and sex-separated groups. All values were corrected to GAPDH and are reported as means ± SEM, in A.U. Main effects of a 3-way ANOVA are represented on the graph at $p<0.05$. Main effects of 2-way ANOVA are represented on graph at

p<0.05. # p<0.05, main effect age. □□p<0.05□□post-hoc significance. *p< 0.05, t-test between indicated groups. N=5/male group, 4/female group.

Figure 6:

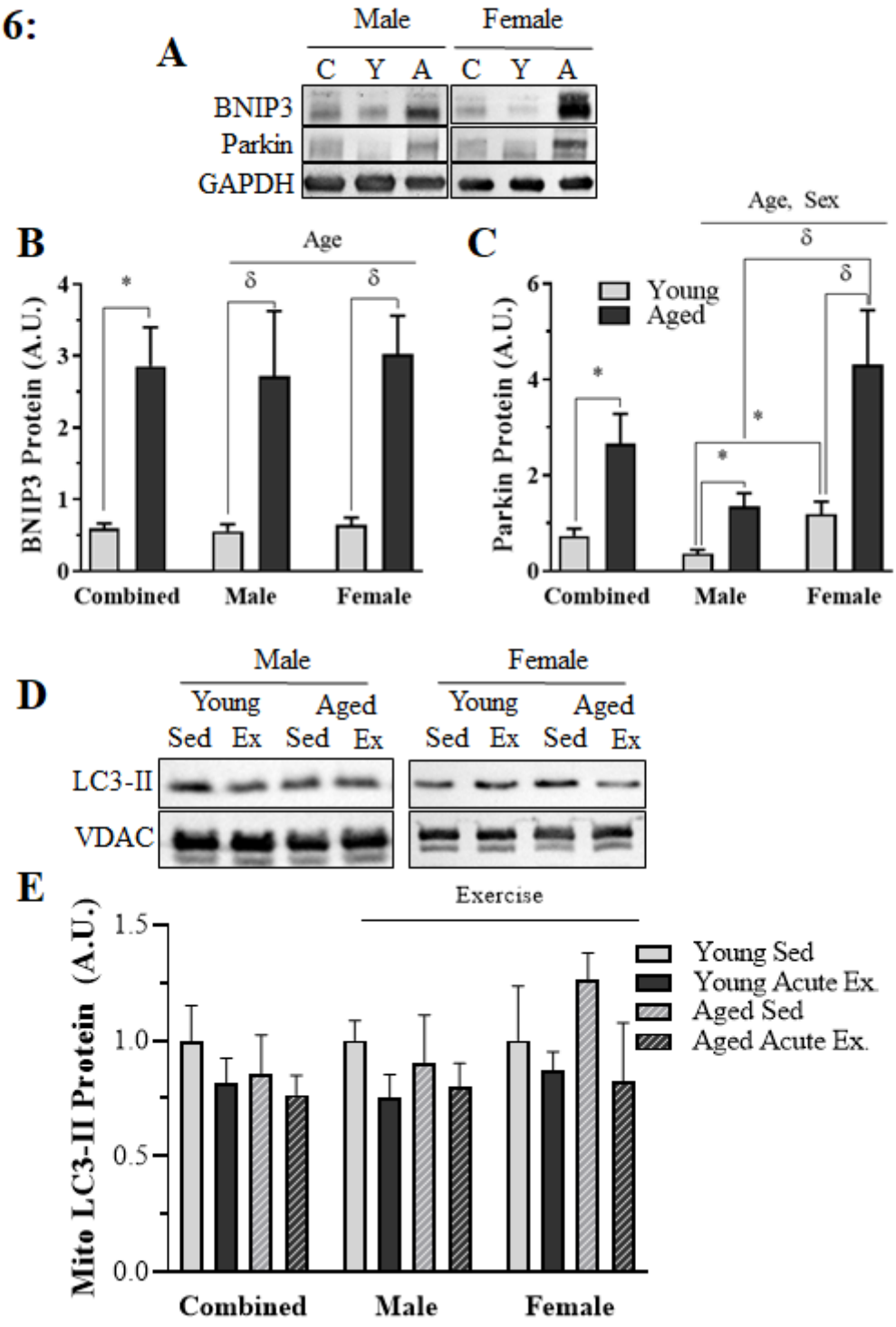

Figure 6

Mitophagy protein content in the muscle and mitochondria in young and aged, male and female mice. A. Representative western blots for BNIP3 and Parkin in whole muscle samples. B. Quantification of BNIP3 protein in combined and sex-separated groups. C. Quantification of Parkin protein in combined and sex-

separated groups. D. Representative western blots for LC3-II in isolated mitochondria with or without exercise in young and aged, male and female mice. E. Quantification of LC3-II protein in combined and sex-separated groups. Whole muscle values were corrected to GAPDH. Isolated mitochondria values were corrected to VDAC. Values are reported as means ± SEM, in A.U. Main effects of a 3-way ANOVA are represented on the graph at p<0.05. Main effects of 2-way ANOVA are represented on graph at p<0.05. p<0.05post-hoc significance. *p< 0.05, t-test between indicated groups. N=10/male group, 8/female group in A-C, and N=5/male group, 4/female group in D-E. Line break in representative blot are different sections from the same blot.

Figure 7:

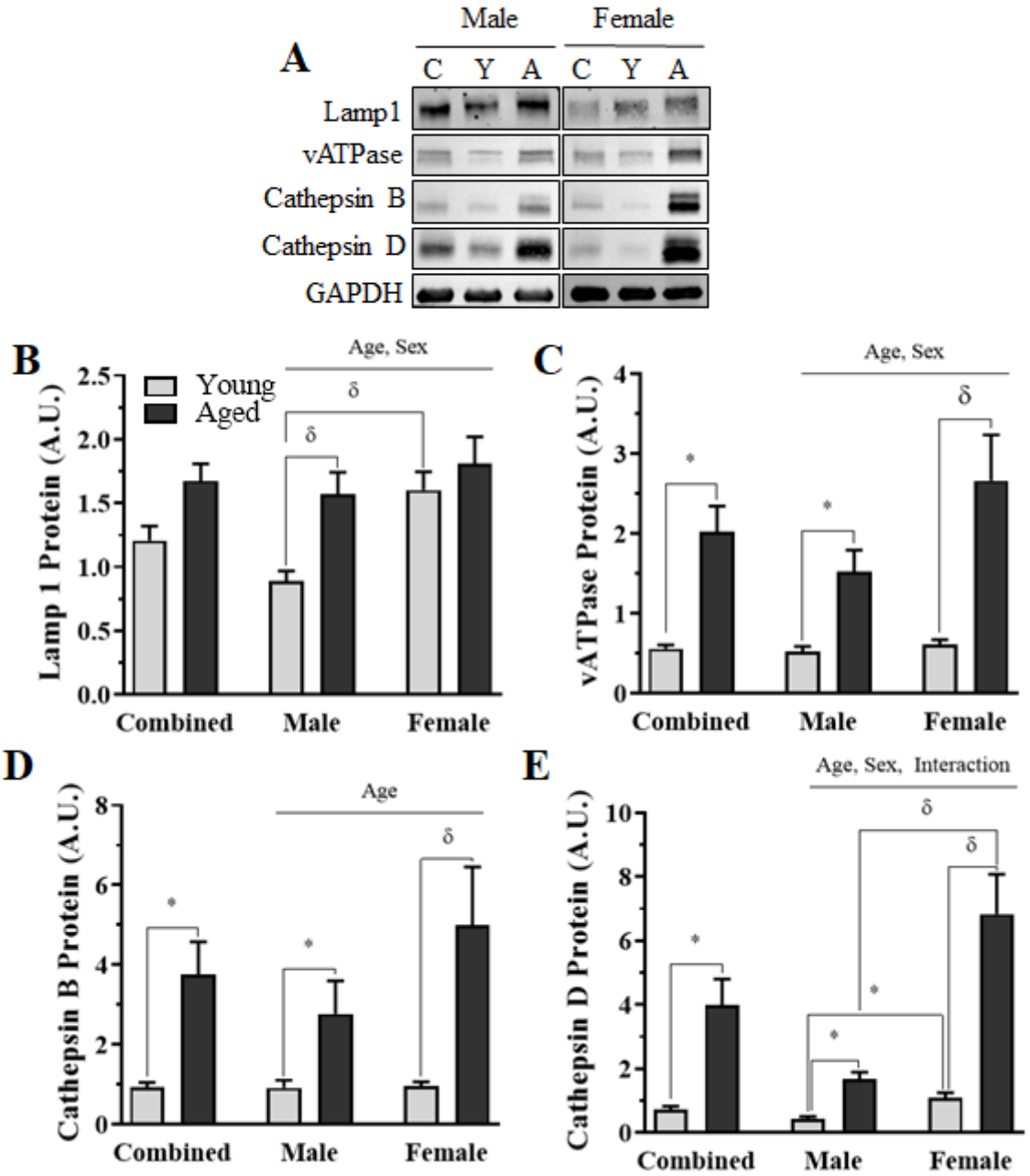

Figure 7

Lysosome proteins in young and age, male and female mice. A. Representative western blots for Lamp1, vATPase, mature Cathepsin B and mature Cathepsin D. B. Quantification of Lamp1 protein in combined and sex-separated groups. C. Quantification of vATPase protein in combined and sex-separated groups. D. Quantification of mature Cathepsin B protein in combined and sex-separated groups. E. Quantification of mature Cathepsin D protein in combined and sex-separated groups. All values were corrected to GAPDH and are reported as means ± SEM, in A.U. Main effects of 2-way ANOVA are represented on graph at p<0.05. □□p<0.05□□post-hoc significance. *p< 0.05, t-test between indicated groups. N=10/male group, 8/female group.

Figure 8:

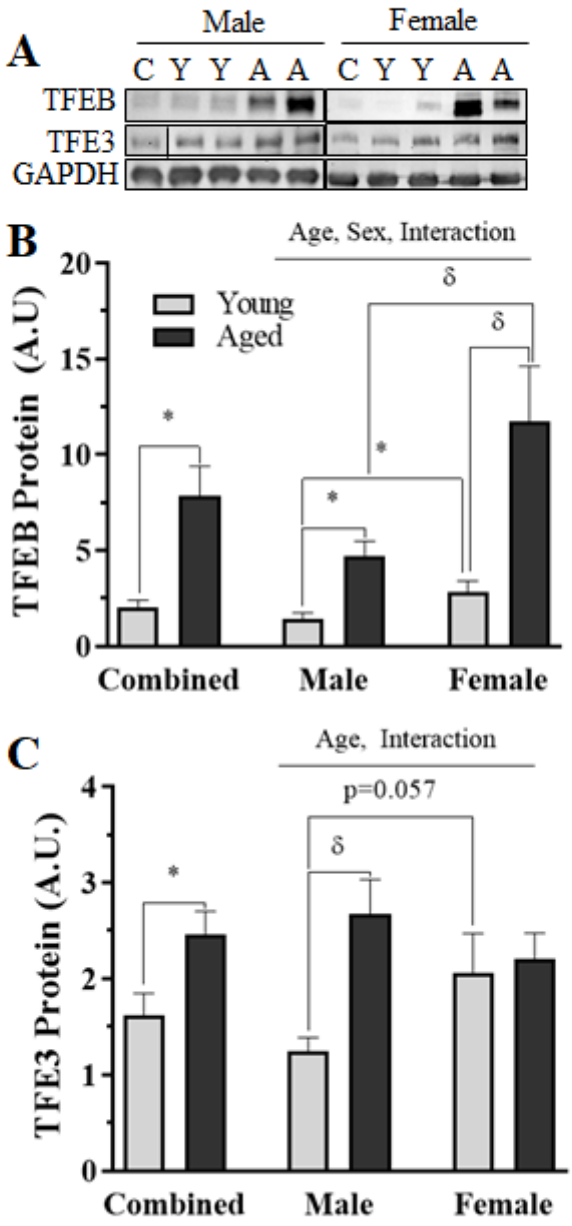

Figure 8

Lysosome transcription factor proteins in young and age, male and female mice. A. Representative western blots for TFEB and TFE3 protein. B. Quantification of TFEB protein in combined and sex-separated groups. C. Quantification of TFE3 protein in combined and sex-separated groups. All values were corrected to GAPDH and are reported as means ± SEM. Main effects of 2-way ANOVA are represented on graph at $p<0.05$. □□$p<0.05$□□post-hoc significance. *$p< 0.05$, t-test between indicated groups. N=10/male group, 8/female group. Line break in representative blot are different sections from the same blot.

Figure 9:

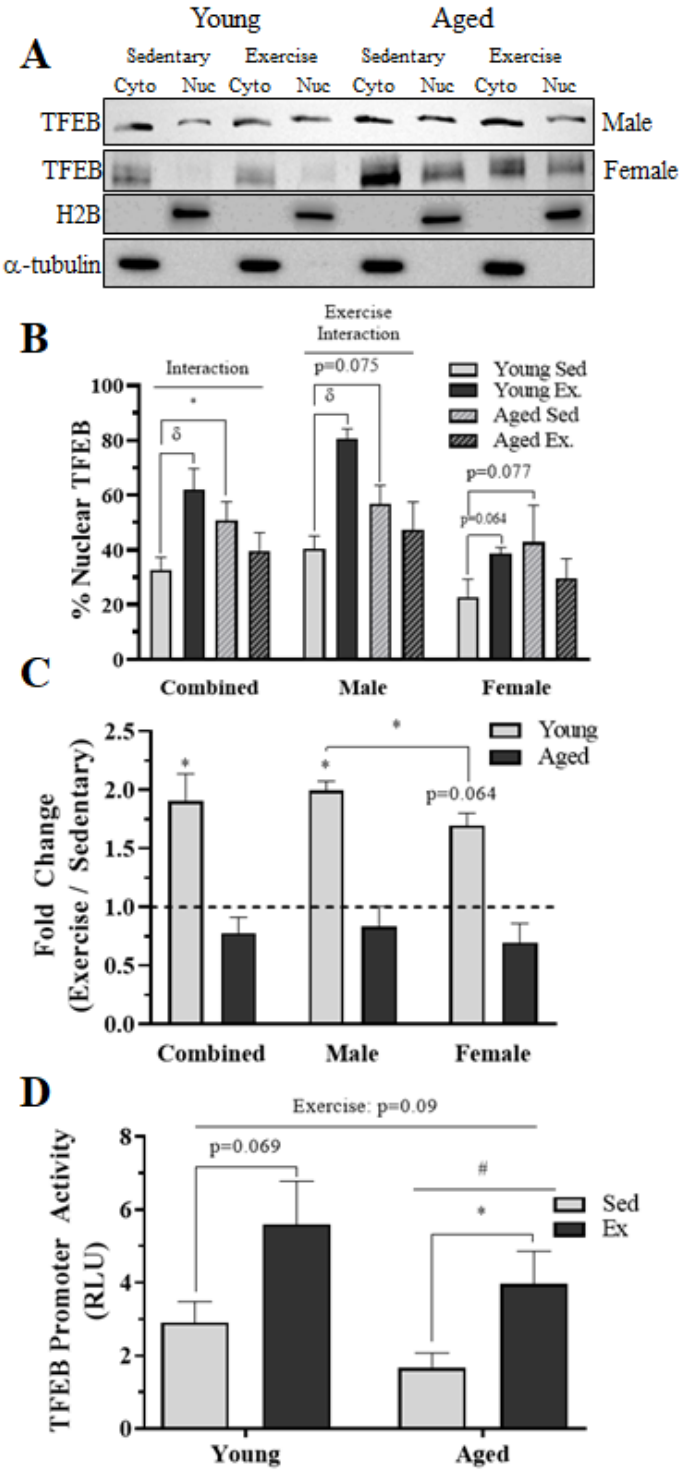

Figure 9

TFEB protein localization and promoter activity in sedentary and acute-exercised young and age, male and female mice. A. Representative western blots for TFEB protein in nuclear and cytosolic fractions in sedentary and exercised male and female mice. B. % nuclear TFEB protein in combined and sex-separated male and female mice. C. Fold-change in nuclear TFEB protein in each group examined. D. TFEB promoter activity (luciferase; RLU) in young and aged, sedentary, and exercised mice. In A-C,

cytosolic values were corrected to a-tubulin, nuclear values were corrected to H2B, and reported as mean ± SEM, in A.U. Main effects of 2-way ANOVA are represented on graph at $p<0.05$. # $p<0.05$, main effect age. □□$p<0.05$□□post-hoc significance. *$p< 0.05$, t-test between indicated groups. N=5/male group, 4/female group in B-C. N=6/group in D.