

**β_2 -agonist administration increases sarcoplasmic reticulum Ca^{2+} -ATPase
activity in aged rat skeletal muscle**

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Running title: Fenoterol increases SERCA activity in aged rats

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ABSTRACT

Aging is associated with a slowing of skeletal muscle contractile properties, including a decreased rate of relaxation. In rats, the age-related decrease in the maximal rate of relaxation is reversed after 4 weeks' administration with the β_2 -adrenoceptor agonist (β_2 -agonist), fenoterol. Given the critical role of the sarcoplasmic reticulum (SR) in regulating intracellular Ca^{2+} transients and ultimately the time course of muscle contraction and relaxation, we tested the hypothesis that the mechanisms of action of fenoterol are mediated by alterations in SR proteins. Sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) kinetic properties were assessed in muscle homogenates and enriched SR membranes isolated from the red (RG) and white (WG) portions of the gastrocnemius muscle in adult (16 month) and aged (28 month) F344 rats that had been administered fenoterol for 4 weeks (1.4 mg/kg/day; *i.p.* in saline) or vehicle only. Aging was associated with a 29% decrease in the maximal activity (V_{\max}) of SERCA in the RG, but not in the WG muscles. Fenoterol treatment increased the V_{\max} of SERCA and SERCA1 protein levels in RG and WG. In the RG, fenoterol administration reversed an age-related selective nitration of the SERCA2a isoform. Our findings demonstrate that the mechanisms underlying age-related changes in contractile properties are fiber type dependent, whereas the effects of fenoterol administration are independent of age and fiber type.

Keywords: aging, calcium, sarcoplasmic reticulum, skeletal muscle, beta-agonist

INTRODUCTION

Sarcopenia, the progressive loss of skeletal muscle mass with advancing age, is associated with a decline in muscle strength, which leads to a loss of functional independence and a reduced quality of life (17). Aging is also associated with a slowing of the time course of skeletal muscle contraction and relaxation (16). These changes can reduce the accuracy and precision of movements, impair the ability to perform simple tasks, and increase the risk of sudden falls and related injuries. Ideally, therapies to prevent or reverse the age-related changes in skeletal muscle should also target the slowing of contraction and relaxation, in addition to preserving or increasing muscle mass and strength.

Neurogenic factors, such as motor unit remodelling and denervation have been implicated in the age-related changes to skeletal muscle (11). However, myogenic factors are also involved, since the slowing of contraction and relaxation occurs before the onset of muscle wasting (18, 20, 21). Intrinsic changes to skeletal muscle fibers appear to be involved in the deterioration of function with age. Specifically, age-related changes in the proteins regulating Ca^{2+} -handling during excitation-contraction coupling and relaxation have been described (16).

Relaxation of skeletal muscle is thought to involve at least three processes: dissociation of Ca^{2+} from troponin, detachment of cross bridges, and uptake of Ca^{2+} by the sarcoplasmic reticulum and myoplasmic Ca^{2+} buffers (33). The factors regulating intracellular $[\text{Ca}^{2+}]$ during skeletal muscle relaxation are fiber type specific. In fast-twitch muscle it has been shown that parvalbumin contributes at least 50% to the final rate constant of decay of $[\text{Ca}^{2+}]$ after a single action potential (2). In slow-twitch muscle (where parvalbumin levels are very low) the sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA) makes the major contribution to the decay of $[\text{Ca}^{2+}]$ (2). Aging has been associated with a decrease in the amount of Ca^{2+} available for triggering contraction (20) due to dihydropyridine receptor (DHPR) uncoupling from the SR Ca^{2+}

release channel (CRC) (5). However, the effects of aging on the kinetic properties of the SERCA are not clear. The reductions in SR Ca^{2+} -resequestration from chemically skinned fast-twitch muscle fibers from aged rats, were hypothesized to be caused by an inactivation of SERCA (13). However, predominantly fast-twitch muscles show no age-related alteration in SERCA protein levels, isoform composition, or kinetic properties (4, 7).

To date, the accumulated data has only excluded an effect of aging on the intrinsic properties of SERCA from predominantly fast-twitch muscle. However, SR vesicles isolated from rat slow-twitch muscles (soleus, adductor longus and vastus intermedius) do show an age-dependent reduction in both SERCA activity and Ca^{2+} -resequestration (32). Furthermore, the age-related inactivation of SERCA activity was associated with a selective nitration of the SERCA2a isoform predominantly at Tyr²⁹⁴-Tyr²⁹⁵ (32), which is close to the Ca^{2+} translocation sites, suggesting that an age-related decrease in SERCA activity could be related to a decreased binding affinity for Ca^{2+} . It has also been shown that the conformation of the nucleotide binding domain of SERCA is altered in aged skeletal muscle (3). Therefore, current evidence suggests that an age-related reduction in SERCA activity in slow-twitch muscle may be associated with changes in the environment of nucleotide and/or Ca^{2+} -binding domains.

We have demonstrated previously that 4 weeks' daily administration of the β_2 -adrenoceptor agonist (β_2 -agonist), fenoterol, can attenuate the age-related decrease in rat skeletal muscle mass and strength and decrease the time course of the isometric twitch (22). However, the mechanisms responsible for this change in contractility have not been elucidated. It has been demonstrated that a 6 week administration of the β_2 -agonist, salbutamol, can reduce contraction time in canine skeletal muscle (36). Salbutamol treatment also increased the rate of SR Ca^{2+} -resequestration, which suggested that changes in skeletal muscle contractile parameters were mediated by alterations in SR proteins, namely a β_2 -agonist-mediated induction of SERCA1 protein levels

(36). In addition, salbutamol treatment attenuated the fast-twitch to slow-twitch transformation of myosin heavy chain (MHC) isoforms, SR protein characteristics and muscle mechanics that accompany chronic low-frequency stimulation (CLFS) (10). Therefore, it is possible that β_2 -agonist administration could attenuate an age-related decrease in skeletal muscle relaxation rate by reversing age-related modifications of SR proteins.

We tested the hypothesis that the age-related decrease and the β_2 -agonist induced increase in the rate of skeletal muscle relaxation are mediated through alterations in the properties of the SR. We also hypothesized that fenoterol treatment in rats would attenuate age-related changes in the maximal activity (V_{\max}) of SERCA, and determined the underlying mechanisms that could account for changes in SERCA activity, including assessments of SERCA isoform protein levels, SERCA2a nitration levels, and indirect measures of the Ca^{2+} and ATP binding affinities of SERCA.

MATERIALS AND METHODS

Adult (16 month old) and aged (28 month old) male Fischer 344 rats (460-500g) obtained from Harlan Sprague Dawley (Indianapolis, Indiana, US), were allocated at random to a control or fenoterol-treated group (n = 8, per group). Fenoterol-treated rats received 1.4 mg/kg of fenoterol (Sigma-Aldrich, Castle Hill, NSW, Australia), administered via intraperitoneal (*i.p.*) injections in 0.5 ml of isotonic saline every day for 4 weeks and control rats received a daily injection of 0.5 ml saline vehicle, as described previously (22). All experiments were approved by the Animal Experimentation Ethics Committee of The University of Melbourne, and adhered to the code of practice for the care and use of animals for scientific purposes as described by the National Health and Medical Research Council of Australia.

Contractile properties: Maximal rate of twitch and tetanus relaxation

At the completion of the treatment period, rats were anesthetized with pentobarbital sodium (Nembutal, Rhone Merieux, Pinkenba, QLD, Australia: 60 mg/kg, *i.p.*). The extensor digitorum longus (EDL) and soleus muscles were surgically excised and isometric contractile properties measured *in vitro*, as reported previously (22). In that study, we reported that although absolute $\frac{1}{2}$ RT of the twitch was not different after fenoterol treatment, twitch force was significantly greater. Hence, compared with untreated control rats, the actual rate of relaxation of the twitch response was significantly faster in treated rats (22). Although twitch force of either EDL or soleus muscles was not different between adult and aged rats (22) the ability of fenoterol treatment to increase twitch force warranted closer investigation. Therefore, to further examine the maximal rate of relaxation of the twitch and tetanic responses, we have studied the maximum negative derivative of a linear-fit curve calculated over 5 ms for the EDL and 25 ms for soleus muscle using custom-written software applications (D.R. Stom Software Solutions, Ann Arbor, MI, USA) and LabView software (National Instruments, Austin, TX, USA). The maximal rate of relaxation was determined during supramaximal twitches and fused tetani (150 Hz EDL, 120 Hz soleus). Although the EDL and soleus muscles from both hind limbs were used previously for examination of the effects of fenoterol on contractile properties and β -adrenoceptor densities (22), the gastrocnemius muscles were also excised and stored for later biochemical analysis, and these muscles serve as the basis for the present study investigating fenoterol's effects on SERCA kinetic properties.

Sample preparation: SR characteristics

Whilst anesthetized, the gastrocnemius muscles were excised from both hindlimbs. The deep red portion of the gastrocnemius muscle (RG) was separated from the predominantly fast-twitch superficial white portion (WG). The rats were killed by opening of the thoracic cavity and

immediate cardiac excision. Crude muscle homogenates were prepared, as described previously (26, 27). SR vesicles were prepared utilizing a combination of two SR isolation protocols (6, 9), as described previously (26). During the entire homogenization and SR vesicle isolation procedure samples were immersed in ice. It is critical to keep the samples on ice to avoid temperature-dependent reductions in SERCA activity (24). SR isolation was carried out on the same day as muscle homogenization and was accomplished by sucrose gradient differential centrifugation using a Beckman ultracentrifuge with a 70.1 Ti fixed angle rotor. Protein determination of homogenates and SR vesicles was made by the method of Bradford and analyzed in triplicate.

SR Ca²⁺-ATPase activity

Ca²⁺-induced SERCA activity in enriched SR membranes was analyzed according to the methods by Leberer and colleagues (14), described previously (26). Ca²⁺-induced SERCA activity in crude muscle homogenates was analyzed by the modifications according to Simonides and van Hardeveld (29), as described previously (27). The reaction buffer for crude muscle homogenates (SR vesicles in parentheses) contained (in mM) 200 (100) KCl, 20 HEPES, 15 (10) MgCl₂, 10 NaN₃, 10 phosphoenolpyruvate (PEP), 5 ATP, and 1 EGTA. The pH of the reaction buffer was adjusted to 7.0 at 37°C. Immediately before the reaction was started, 18 U/ml lactate dehydrogenase (LDH), 18 U/ml pyruvate kinase (PK), 0.3 mM NADH, 1 μM Ca²⁺ ionophore A23187 (Sigma-Aldrich, Castle Hill, NSW, Australia), and 15-30 μL of muscle homogenate or 1-5 μL of enriched SR membrane were added to 1 ml of reaction buffer. The markers of SERCA enzyme kinetics in muscle homogenates included the maximal activity (V_{\max}), cytosolic free Ca²⁺ concentration ($[Ca^{2+}]_f$) required for half-maximal activity (pCa_{50}), and the Hill coefficient (n_H), which is a measure of the cooperative Ca²⁺-binding affinity of the SERCA enzyme. In SR vesicles, only the V_{\max} was assessed. In order to assess changes in SR membrane permeability to

Ca^{2+} , the assay was also performed without the Ca^{2+} ionophore A23187 in a sub-set of samples for each condition (muscle homogenates only; $n=3$). Assays were performed at 37°C and 340 nm (Multiscan Spectrum; Thermo Electron, Waltham, MA, USA). In all cases, the V_{max} of SERCA activity was established by progressively raising the $[\text{Ca}^{2+}]_f$ until a plateau and subsequent decline in SERCA activity occurred. Basal (or Mg^{2+} -ATPase) activity was determined by adding cyclopiazonic acid (CPA), a specific inhibitor of the SERCA enzyme, to a final concentration of $40\ \mu\text{M}$. The $[\text{Ca}^{2+}]_f$ for a given day of analysis were measured in triplicate on separate aliquots of reaction buffer with a calcium electrode (Model 97-20; Orion, Beverly, MA, USA). SERCA activity was then plotted against the negative logarithm of $[\text{Ca}^{2+}]_f$ (pCa), as described previously (31). Nonlinear regression analysis was performed with GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA) using the following sigmoidal dose-response (variable-slope) relationship,

$$Y = Y_{\text{bot}} + \frac{Y_{\text{top}} - Y_{\text{bot}}}{1 + 10^{(\text{LogCa}_{50} - X) \cdot n_H}}$$

where Y is the plateau, Y_{bot} is the value at the bottom of the plateau, and Y_{top} is the value at the top of the plateau. The Hill coefficient was calculated using values ranging between 10% and 90% of the V_{max} .

Fluorescence measurements

The fluorescent probes, fluorescein 5-isothiocyanate (FITC; Sigma-Aldrich, Castle Hill, NSW, Australia) and *N*-cyclohexyl-*N'*-(dimethylamino- α -naphthyl)carbodiimide (NCD-4; Molecular Probes, Eugene OR, USA) were used to indirectly assess the nucleotide and Ca^{2+} -binding affinities of SERCA, respectively, as previously described (26, 31). An identical amount of enriched SR vesicle protein ($500\ \mu\text{g}$) was labeled with FITC ($25\ \mu\text{M}$; pH 8.8) in the dark for 30 min at 25°C . Fluorescence measurements using the FITC probe were made on a spectrofluorometer (Wallac 1420, PerkinElmer, Wellesley MA, USA), based on previous

methods (11). Samples were excited at 495 nm and the maximum emission at 525 nm was recorded. Similarly, samples were labeled with NCD-4 (150 μ M; pH 6.2) in the dark for 4 hours at 25°C. NCD-4 fluorescence measures were made by exciting samples at 340 nm and recording the maximum emission intensity at 420 nm. The maximum fluorescence intensity for both FITC and NCD-4 in each sample was recorded in triplicate.

SDS-PAGE for myosin heavy chain determination and western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate and isolate SERCA isoforms according to molecular mass. Immunoblotting was performed using primary monoclonal antibodies specific for rat SERCA1 (110 kDa; I1H11, Affinity Bioreagents, Golden, CO, USA) and SERCA2a (95 kDa; 7E6, Affinity Bioreagents, Golden, CO, USA), which were diluted 1: 2500 and 1:1000 in 10% nonfat milk, respectively. In addition, immunoblotting was performed using a polyclonal antibody specific for nitrotyrosine (A-21285; Molecular Probes, Eugene OR, USA), which was diluted 1:1000 in 10% nonfat milk. Immunoblotting was done in enriched SR vesicles, as described previously (26), with the following minor modifications. After application of the appropriate secondary antibody, each was conjugated with biotinylated-streptavidin alkaline phosphatase. Blots were developed using an alkaline phosphatase conjugate substrate kit with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT) (170-6412-1, Bio-Rad, Hercules, CA, USA). Blots were digitized and relative protein levels determined by scanning densitometry. Relative protein levels for SERCA1 were quantified using the single band detected at 110kDa. Immunoblotting revealed several lower molecular weight SERCA2a products, in addition to the predicted band at 95 kDa. Therefore, for both SERCA2a and nitrotyrosine, bands migrating at an apparent molecular weight of 95 kDa and ~70 kDa were summed for quantification. Values ($n \geq 5$ for each condition) were expressed as a percentage of the muscles from untreated adult rats.

Myosin heavy chain (MHC) isoform composition was determined in whole muscle homogenates using SDS-PAGE, as reported previously (30), with minor modifications. 2-mercaptoethanol (0.08%) was added to the running buffer to improve MHC isoform resolution and proteins were visualized using a commercially available silver stain kit (LC6100, InVitrogen, Carlsbad, CA, USA). Gels were digitized and relative protein levels were determined by scanning densitometry. Values ($n \geq 5$ for each condition) were expressed as a percentage of total MHC protein.

Statistical analyses

Individual variables were compared between groups using a one-way or two-way analysis of variance (ANOVA) as appropriate. Bonferroni's post hoc multiple comparison procedure was used to detect differences between specific means. Significance was set at $P < 0.05$. All values are expressed as mean \pm SE unless specified otherwise.

RESULTS

Contractile properties: Maximal rate of twitch and tetanus relaxation

In soleus muscles, the maximal rate of relaxation of the twitch and tetanus was decreased by 27% and 28% in untreated aged rats than untreated adult rats, respectively ($P < 0.05$, Table 1, Figure 1A). Fenoterol treatment increased the maximal rate of relaxation of the tetanus in soleus muscles of adult and aged rats by 38% and 51%, respectively, and a similar effect was observed for the twitch response ($P < 0.05$, Table 1, Figure 1A). In EDL muscles, the maximal rate of relaxation of the twitch and tetanus was decreased by 21% and 24% in untreated aged rats compared to untreated adult rats, respectively ($P < 0.05$, Table 1, Figure 1B). Fenoterol treatment increased the maximal rate of relaxation of the tetanus in EDL muscles of adult and aged rats by

24% and 33%, respectively, and a similar effect was observed for the twitch response ($P < 0.05$, Table 1, Figure 1B).

SR Ca^{2+} -ATPase activity

The V_{max} of SERCA activity measured in RG muscle homogenates was 29% lower ($P < 0.05$) in untreated aged rats compared to untreated adult rats (Table 2; Figure 2A). Treatment with fenoterol increased the V_{max} in RG muscle homogenates from adult and aged rats by 24% and 34%, respectively ($P < 0.05$; Table 2; Figure 2A). In WG muscle homogenates no age-related alteration in the V_{max} was detected. However, fenoterol treatment increased the V_{max} in WG muscle homogenates from adult and aged rats by 29% and 35%, respectively ($P < 0.05$ Table 2; Figure 2B).

The V_{max} in SR vesicles enriched from RG muscles was 26% lower in untreated aged rats compared to untreated adult rats ($P < 0.05$; Figure 3C). In fenoterol treated adult and aged rats the V_{max} in SR vesicles enriched from RG muscle was 19% and 27% higher than control rats, respectively ($P < 0.05$; Figure 3C). The V_{max} of SR vesicles enriched from WG muscles of fenoterol treated adult and aged rats was 25% higher than rats administered vehicle alone, but no age-dependent alteration was detected ($P < 0.05$ data not shown).

In muscle homogenates, the sensitivity of the SERCA enzyme to Ca^{2+} was reduced in RG and WG muscles from aged rats, as shown by lower pCa_{50} values and the right shift of the activity- pCa relationship ($P < 0.05$, main effect, Table 2, Figure 2). Fenoterol treatment did not alter pCa_{50} values in either age group of rats (Table 2, Figure 2). The Hill coefficient (n_H) of the SERCA activity- pCa relationship was not altered by age or treatment. In crude homogenates, fenoterol treatment or aging did not alter stimulation due to A23187, indicating that SR

membrane permeability to Ca^{2+} was not altered. However, WG demonstrated a generally higher stimulation by the Ca^{2+} ionophore A23187 compared to RG (main effect; $P < 0.05$).

Fluorescence measurements

The maximum fluorescence intensity of SR vesicles labelled with FITC was 12%, 13% and 19% lower in samples from RG of untreated aged rats compared to those from treated aged rats, untreated adult rats and treated adult rats, respectively ($P < 0.05$, Figure 3A, 3C). FITC fluorescence was not altered in SR vesicles isolated from WG muscle (Figure 3A). The maximum emission intensity was unchanged in all conditions for NCD-4 (Figure 3B).

SDS-PAGE and western blotting

SERCA1 protein levels in SR vesicles isolated from RG and WG muscle were higher in fenoterol treated rats. Compared to values for untreated adult rats, RG SERCA1 protein levels were 46% and 42% higher in treated adult and aged rats, respectively ($P < 0.05$; Figure 4A). No age-dependent changes in SERCA1 protein levels were apparent in RG or WG. SERCA2a protein levels were not different in WG or RG (Figure 4B). The SERCA1 to SERCA2a ratio in RG and WG muscles was greater in fenoterol treated rats compared with untreated rats ($P < 0.05$; data not shown).

In both RG and WG muscle, western blotting detected a nitrated protein migrating at an apparent molecular weight of 95 kDa, which corresponded to the molecular weight of SERCA2a (Figure 4C). Several nitrated proteins were also evident at ~70 kDa (Figure 4C), which align with the lower molecular weight bands detected in SERCA2a immunoblotting (Figure 4B). There was no evidence of age-related SERCA1 (110 kDa) tyrosine nitration. In SR vesicles isolated from RG muscle, an age-dependent increase in nitration of SERCA2a was evident, with nitrotyrosine levels in RG from untreated aged rats being 74% higher than values for untreated

adult rats. Importantly, RG muscles from treated aged rats had equivalent SERCA2a nitrotyrosine levels to those from untreated adult rats. Unlike RG, no alterations in nitrotyrosine levels were observed in WG (Figure 4C).

Determination of the relative MHC composition confirmed that RG muscle contained a different MHC isoform profile compared to WG muscle. Specifically, RG muscle contained 8-28% of MHC Type Iib and 20-30% of MHC Type I. In contrast, WG muscle contained 45-60% MHC Type Iib. No MHC Type I was detected in WG muscle. In general, aging was associated with a shift toward a higher proportion of slow MHC isoforms (IIB \rightarrow Iia/x \rightarrow I), whereas fenoterol treatment was associated with a shift toward fast MHC isoforms (I \rightarrow Iia/x \rightarrow Iib) (Figure 5).

Data correlation

The V_{\max} of SERCA activity in enriched SR vesicles was statistically correlated ($P < 0.05$) to measurements in crude muscle homogenates for both RG ($r^2 = 0.68$) and WG muscles ($r^2 = 0.36$), indicating no selective yield of SR vesicles during the isolation protocol ($P < 0.05$). In RG, maximum FITC fluorescence was statistically correlated ($r^2 = 0.62$) with the V_{\max} of SERCA activity in enriched SR vesicles ($P < 0.05$; Figure 3C).

DISCUSSION

Aging is associated with deleterious changes in skeletal muscle contractility, including factors that contribute to an overall slowing of movement. The findings of this study indicate that treatment of aged rats with the β_2 -agonist, fenoterol, can reverse the slowing of relaxation in slow- and fast-twitch skeletal muscle due to increased SERCA activity and SERCA protein levels. We also provide evidence that there is an age-related alteration in the environment of the

nucleotide binding domain and/or a selective nitration of the SERCA2a isoform, which is associated with the depression in SERCA activity. Treatment with fenoterol ameliorated the age-related decrease in nucleotide binding affinity and reversed the age-related accumulation of nitrotyrosine residues on the SERCA2a isoform. These changes, in combination with increases in SERCA1 protein levels, appear to be the underlying mechanisms of fenoterol treatment in reversing age-related decreases in the V_{\max} of SERCA. Our results demonstrate that the underlying mechanisms resulting in age-related changes in contractile properties are fiber type specific. An age-related decrease in SERCA activity occurred in muscles with a slow or mixed fibre type (RG) but not in predominantly fast-twitch muscles (WG). In slow-twitch muscle (where parvalbumin levels are low or negligible), the age-related slowing of relaxation was associated with a decrease in SERCA V_{\max} . Conversely, in fast-twitch muscle (where parvalbumin levels are high), SERCA V_{\max} was unchanged despite the age-related slowing of relaxation. The effects of fenoterol are clearly age-independent, since its administration increased SERCA activity, SERCA1 protein levels and increased the maximal rate of relaxation in muscles from both adult and aged rats. These results demonstrate that muscles from aged rats retain their capacity to adapt to different stimuli including anabolic agents.

Based on the literature to date, the effects of aging on SERCA activity are controversial. It has been argued that aging has no effect on the intrinsic properties of SERCA (16). However, the studies that failed to demonstrate age-related changes in SERCA activity were performed on muscles comprised of fibers with predominantly fast-twitch characteristics (4, 7), with one exception. Narayanan and colleagues (18) showed that SERCA activity in rat soleus muscle was unchanged despite an age-related depression in Ca^{2+} -resequestration. However, since they assessed SERCA activity at only a single $[\text{Ca}^{2+}]_f$ (8.21 μM), it cannot be said definitively that the

activity measured was the V_{\max} . Differences in SERCA activity could be masked by possible age-related changes in pCa_{50} and/or the Hill coefficient.

Our findings are consistent with more recent studies investigating the dependence of muscle fibre type on age-related changes in the kinetic properties of SERCA. Viner and colleagues (32) showed an age-dependent loss of SERCA activity in SR preparations isolated from muscles with mixed fiber composition but not from preparations containing predominantly fast-twitch muscle fibers.

It has been shown previously that treatment with the β_2 -agonist, salbutamol, for 6 weeks, increased SERCA1 protein levels in canine latissimus dorsi and vastus intermedius muscles (36). Similarly, our findings show that fenoterol treatment increased SERCA1 protein levels in the RG and WG muscle of both adult and aged rats. Furthermore, we have previously shown that fenoterol treatment can reverse age-related muscle wasting and weakness (22), a finding that is similar in some respects to another study, where salbutamol treatment attenuated the muscle atrophy usually associated with CLFS (10). The results of the present study show that fenoterol treatment reverses the age-related decrease in both the rate of relaxation and the V_{\max} of SERCA activity, a finding similar to salbutamol treatment attenuating the prolongation of contraction and the depression in SR Ca^{2+} -resequestration concomitant with CLFS (10).

SERCA has a relatively long half-life (~14 days), which is extended by ~27% in aged muscle (8). Therefore, SERCA has a high potential for post-translational modification, particularly in aged muscle. The ATP binding domain of SERCA appears to be particularly susceptible to post-translational modification, which may result in altered enzymatic activity. For example, hydroxyl radicals have been shown to inhibit SERCA function in cardiac and skeletal muscle by altering the ATP binding site (35). Aged skeletal muscle shows an altered ATP binding site conformation (3). CLFS has been shown to decrease FITC binding, which

corresponded with an increase in the number of inactive SERCA enzymes (15). It should be noted that FITC labels Lys₅₁₅ in the nucleotide-binding domain of SERCA and, as such, is sensitive to changes in the environment around that residue. Our results demonstrate an age-related reduction in FITC binding affinity, indicating that a similar mechanism may mediate this effect during both aging and CLFS. We have also shown that fenoterol treatment can attenuate the age-related decreases in FITC binding affinity. This measure could reflect SERCA E2 to SERCA E1 transitions and/or alterations in the environment of the nucleotide binding domain (19).

Our findings are consistent with previous reports demonstrating a selective age-related nitration of the SERCA2a isoform (32). It appears that nitration of SERCA2a is, in part, due to a peroxynitrite mediated process (28). However, the mechanisms linking nitration of SERCA2a to reductions in enzyme activity have not been elucidated. To our knowledge, this is the first report to demonstrate that treatment with a β_2 -agonist reverses the age-related accumulation of nitrotyrosine residues on the SERCA2a isoform. The mechanism underlying this effect is not known, however, we have shown that fenoterol increases SERCA1 protein levels, and it is possible that the synthesis of SERCA1 protein and/or the consequent increase in the ratio of SERCA1 to SERCA2a could reduce the amount of SERCA2a available for nitration.

Age-related alterations in the CRC, such as DHPR-CRC uncoupling (4), may be associated with skeletal muscle twitch prolongation (4). We have demonstrated that the underlying mechanisms of age-related alterations in the rate of skeletal muscle relaxation are clearly fiber type specific. Our results demonstrate no age-related changes in SERCA activity in fast-twitch muscle; hence alterations in excitation-contraction coupling remain a likely explanation of age-related changes in contractile properties in fast muscle (20). However, our results demonstrate that in slow-twitch muscle uncoupling of the DHPR-CRC complex is not the only age-related

alteration to the proteins involved in excitation contraction and relaxation. Although SERCA activity is not the only factor controlling the rate of skeletal muscle relaxation, it is reasonable to associate decreased SERCA activity with the reduced rate of relaxation in muscles of aged rats. In addition, given the coordination between SR Ca^{2+} -resequestration and Ca^{2+} release (23), it is possible that alterations in SERCA activity in slow-twitch muscle may manifest as changes in SR release kinetics and ultimately as changes in contractile parameters, such as force production (20). We have provided evidence that an age-related reduction in SERCA activity may be mediated through SERCA2a nitration and/or alterations in the environment of the nucleotide binding domain of SERCA. Several other mechanisms may also be important. Phosphorylation of SR-bound calmodulin kinase II (CaMKII) increases the V_{\max} of SERCA2a (34). The protein levels of γ and δ CaMKII subunits are conserved through aging of rat muscle (16). However, the enzymatic activity of CaMKII has not been assessed during aging and this assessment could prove useful. It is unlikely that fenoterol's actions are mediated through CaMKII phosphorylation since this pathway is only activated to by β_1 -adrenoceptor stimulation in rat cardiac muscle (1).

In summary, we demonstrate that aging was associated with a decrease in the rate relaxation of both slow- and fast-twitch muscles. Aging was also associated with depression in the V_{\max} of rat SERCA in muscle with a mixed fiber type, but not in predominately fast-twitch muscle. The mechanism underlying the depression in the V_{\max} of SERCA may be alterations in the environment of the nucleotide binding domain and/or an age-related selective nitration of SERCA2a. We also demonstrated that treatment with the β_2 -agonist, fenoterol, reversed the depression in the V_{\max} of SERCA. We provide evidence that the changes in SERCA activity are related to alterations in SERCA1 protein levels, alterations in the environment of the nucleotide binding domain, and the accumulation of nitrotyrosine residues on SERCA2a. Taken together,

these findings show that the underlying mechanisms responsible for age-related changes in skeletal muscle contractile properties are fiber type specific, whereas the effects of fenoterol are independent of age and fiber type.

GRANTS

Supported by research grants from the Muscular Dystrophy Association (USA), the Rebecca L. Cooper Medical Research Foundation, and the Perpetual Philanthropic Trust (J & R McGauran Charitable Trust).

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Table 1. Maximum rate of relaxation of twitch and tetanus soleus and EDL muscles from adult and aged rats after treatment with fenoterol (1.4 mg/kg/day, *i.p.*) or saline for 4 weeks.

		Adult (16 months)		Aged (28 months)	
		<i>Control</i>	<i>Fenoterol</i>	<i>Control</i>	<i>Fenoterol</i>
Soleus	$-dF_{\text{twitch}}/dt$	1.7 ± 0.1	2.2 ± 0.2*	1.2 ± 0.1†	1.7 ± 0.1‡
	$-dF_{\text{tetanus}}/dt$	9.5 ± 0.3	13.1 ± 1.3*	6.8 ± 0.3†	10.3 ± 0.9‡
EDL	$-dF_{\text{twitch}}/dt$	33.7 ± 1.7	50.1 ± 1.6*	26.5 ± 2.1†	45.0 ± 1.2‡
	$-dF_{\text{tetanus}}/dt$	101.1 ± 4.1	125.2 ± 3.8*	77.1 ± 4.4†	102.4 ± 5.7‡

Maximum rate of relaxation (dF/dt) is reported in mN/ms. EDL; extensor digitorum longus. * $P < 0.05$ adult control *versus* adult treated; † $P < 0.05$ adult control *versus* aged control; ‡ $P < 0.05$ aged control *versus* aged treated. Values are means ± SEM; $n = 8$ per group.

Table 2. SR Ca²⁺-ATPase enzyme activity kinetic parameters of crude homogenates from RG and WG muscles from adult and aged rats after treatment with fenoterol (1.4 mg/kg/day, *i.p.*) or saline for 4 weeks.

		Adult (16 months)		Aged (28 months)	
		<i>Control</i>	<i>Fenoterol</i>	<i>Control</i>	<i>Fenoterol</i>
RG	Basal-ATPase	38.3 ± 3.7	32.9 ± 4.7	31.5 ± 3.0	32.3 ± 5.3
	V _{max}	335 ± 15	438 ± 15*	236 ± 18†	358 ± 21‡
	n _H	1.91 ± 0.17	1.79 ± 0.08	1.84 ± 0.12	1.91 ± 0.15
	pCa ₅₀	6.02 ± 0.07	6.01 ± 0.10	5.70 ± 0.08 [#]	5.62 ± 0.08 [#]
	Fold increase due to A23187	1.52 ± 0.14	1.58 ± 0.24	1.44 ± 0.20	1.61 ± 0.14
WG	Basal-ATPase	72.8 ± 8.0	66.2 ± 17	67.3 ± 9.6	75.6 ± 7.6
	V _{max}	742 ± 81	1044 ± 55*	688 ± 74	1066 ± 57‡
	n _H	1.69 ± 0.07	1.76 ± 0.17	1.83 ± 0.12	1.82 ± 0.10
	pCa ₅₀	5.80 ± 0.08	5.75 ± 0.05	5.54 ± 0.05 [#]	5.50 ± 0.05 [#]
	Fold increase due to A23187	2.51 ± 0.79	2.29 ± 0.13	2.27 ± 0.42	2.29 ± 0.35

Basal-ATPase and V_{max} are reported in nmol•mg protein⁻¹•min⁻¹. Ca²⁺-ATPase activity-pCa curves allowed determination of kinetic parameters. Hill coefficient was determined based on the Ca²⁺-ATPase activity between 10% and 90% of maximal value. SR, sarcoplasmic reticulum; n_H, Hill coefficient; pCa₅₀, the [Ca²⁺]_f required to elicit 50% of the maximal Ca²⁺-ATPase activity; V_{max}, maximal velocity; RG, red gastrocnemius; WG, white gastrocnemius; A23187, a Ca²⁺ ionophore. * P < 0.05 adult control *versus* adult treated; † P < 0.05 adult control *versus* aged control; ‡ P < 0.05 aged control *versus* aged treated; [#] P < 0.05 adult *versus* aged (main effect). Compared to RG, WG generally demonstrated a higher V_{max}, basal ATPase activity and fold stimulation by A23187 and a lower pCa₅₀ (main effect, p < 0.05). Values are means ± SEM; n = 8 per group, except for fold increase due to A23187, n = 3.

Legends for Figures

Fig. 1. Sample recordings of muscle twitch characteristics measured in the slow-twitch soleus (A) and predominately fast-twitch extensor digitorum longus muscles (B) of adult (16 month) and aged (28 month) Fischer 344 rats that were treated with fenoterol (---; Fen) or saline vehicle only (—; Con). Tracings based on data from Ryall and colleagues (22) which showed that fenoterol treatment increased muscle force producing capacity in proportion to the increase in muscle mass.

Fig. 2. Mean data for SR Ca^{2+} -ATPase activity at various free Ca^{2+} concentrations. Activity-pCa curves were determined in red gastrocnemius (RG) (A) and white gastrocnemius (WG) (B) crude muscle homogenates from adult (16 month; ■) and aged rats (28 month; ▲) that were administered fenoterol (---) or saline vehicle only (—). Refer to Table 2 for the description of kinetic parameters of the enzyme that were obtained from individual activity-pCa curves for each condition. Values are means \pm SE; $n = 8$.

Fig. 3. Maximum fluorescein 5-isothiocyanate (FITC) fluorescence (A) and *N*-cyclohexyl-*N'*-(dimethylamino- α -naphthyl)carbodiimide (NCD-4) fluorescence (B) measured in enriched SR vesicles isolated from RG and WG muscles. The V_{\max} of Ca^{2+} -ATPase activity is statistically correlated with the maximum FITC fluorescence in enriched SR vesicles from the RG muscle (C) ($P < 0.05$; $r^2 = 0.62$). Linear regression was performed on raw data. † $P < 0.05$ adult control *versus* aged control; ‡ $P < 0.05$ aged control *versus* aged treated; Values are reported in relative fluorescence units (RFUs) and are means \pm SE; $n = 8$.

Fig. 4. Western blotting analysis and relative concentration of sarco-endoplasmic reticulum the (SERCA)1 isoform (A), SERCA2a isoform (B) and protein nitrosylation (C) in enriched SR vesicles isolated from RG and WG muscles. SERCA1 migrates at an apparent molecular weight of 110 kDa. SERCA2a migrates at an apparent molecular weight of 95 kDa. Several smaller molecular weight (~70 kDa) SERCA2a products are evident. Levels of protein nitrosylation were detected by a polyclonal antibody specific for nitrotyrosine residues. Blots represent typical measurements from each condition. Relative protein concentrations were determined using SDS-PAGE, western blot analysis and densitometry. * $P < 0.05$ treated *versus* control; † $P < 0.05$ adult control *versus* aged control. Values are means \pm SE; $n \geq 5$.

Fig. 5. Myosin heavy chain (MHC) isoform profile as determined by SDS-PAGE and silver staining of protein. A typical gel and relative MHC concentrations are shown for RG muscle homogenates and WG muscle homogenates. * $P < 0.05$ adult fenoterol MHC Iib *versus* aged control MHC Iib. Values are means \pm SE; $n \geq 5$.

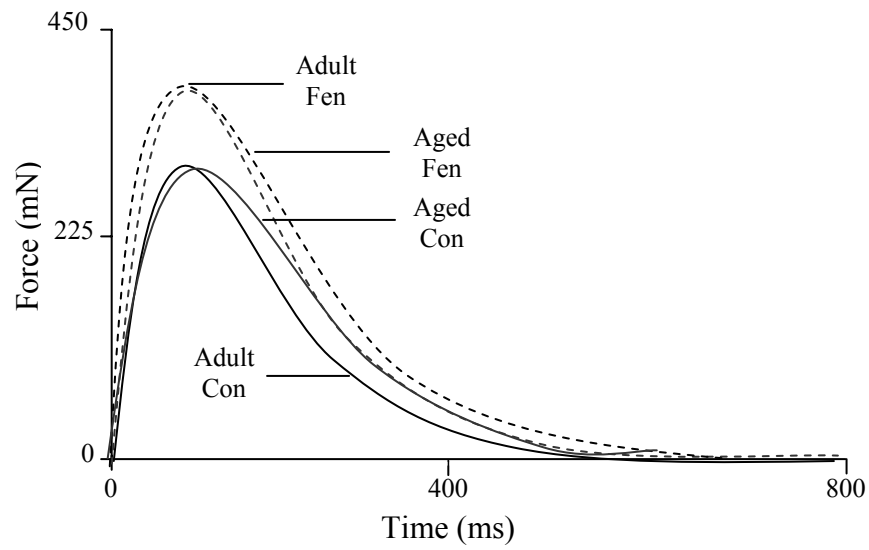
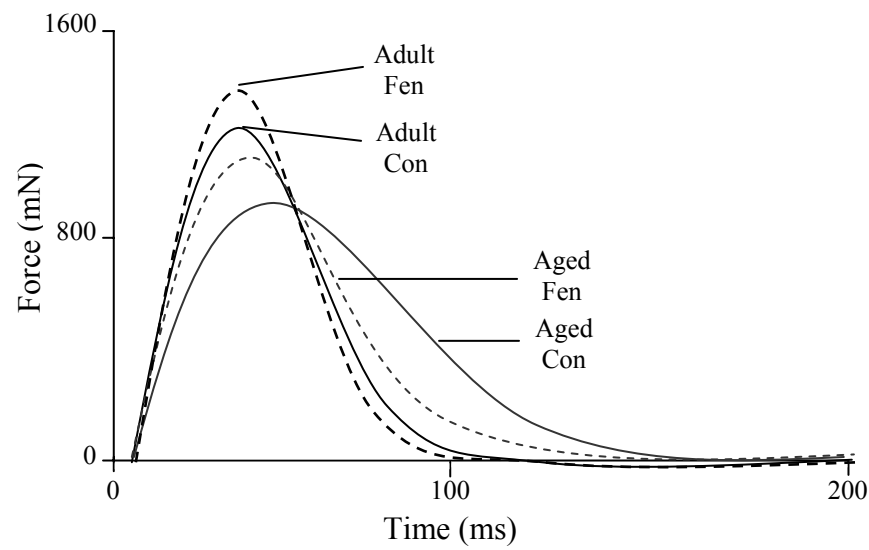
A**B**

Figure 1

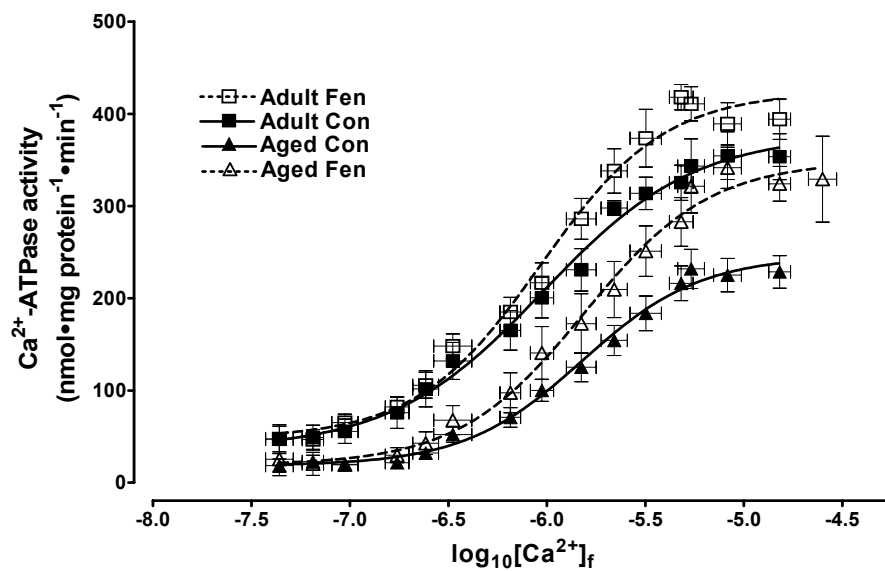
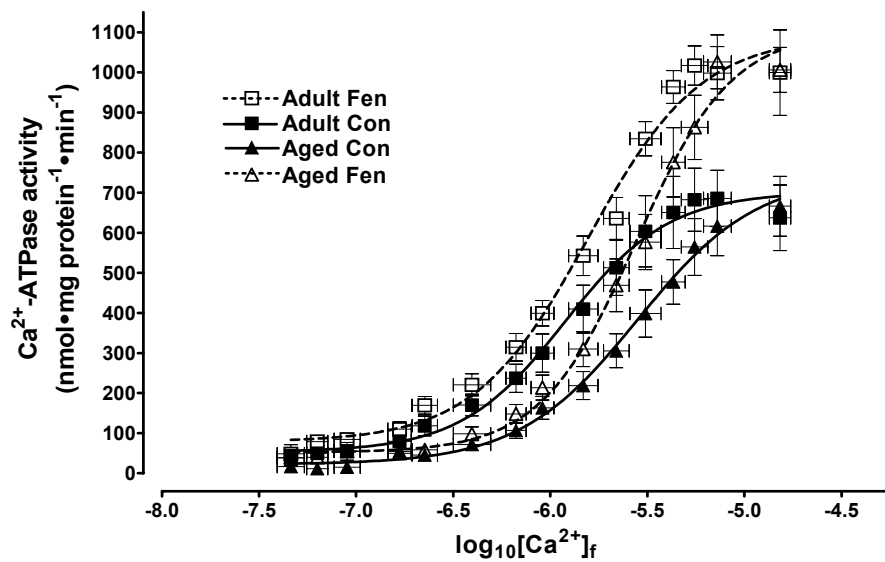
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Figure 2

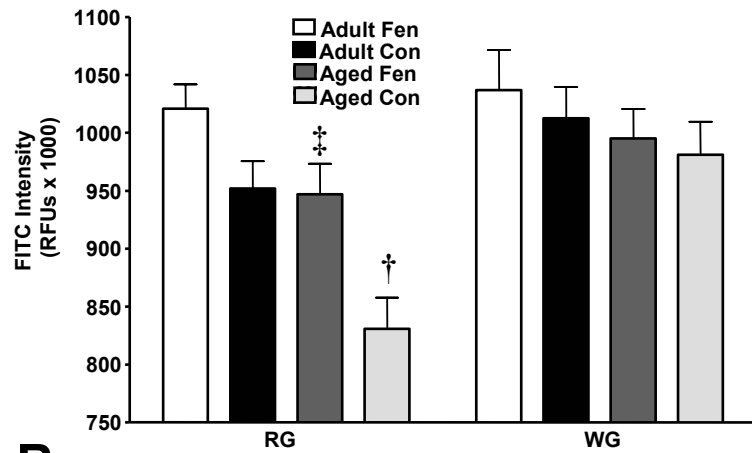
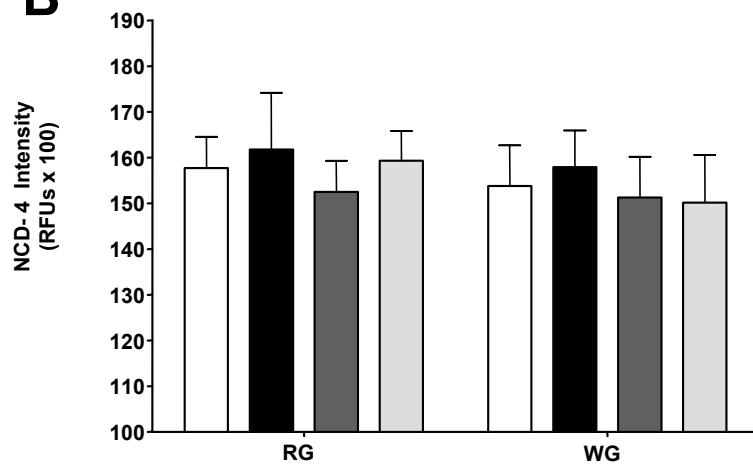
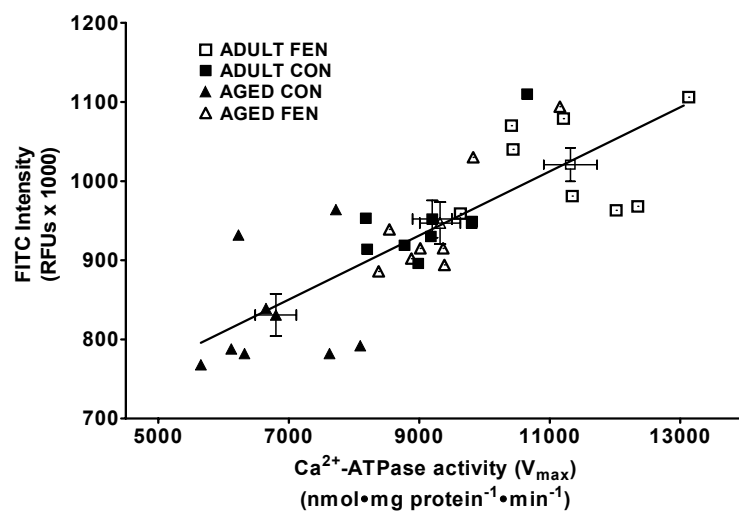
A**B****C**

Figure 3

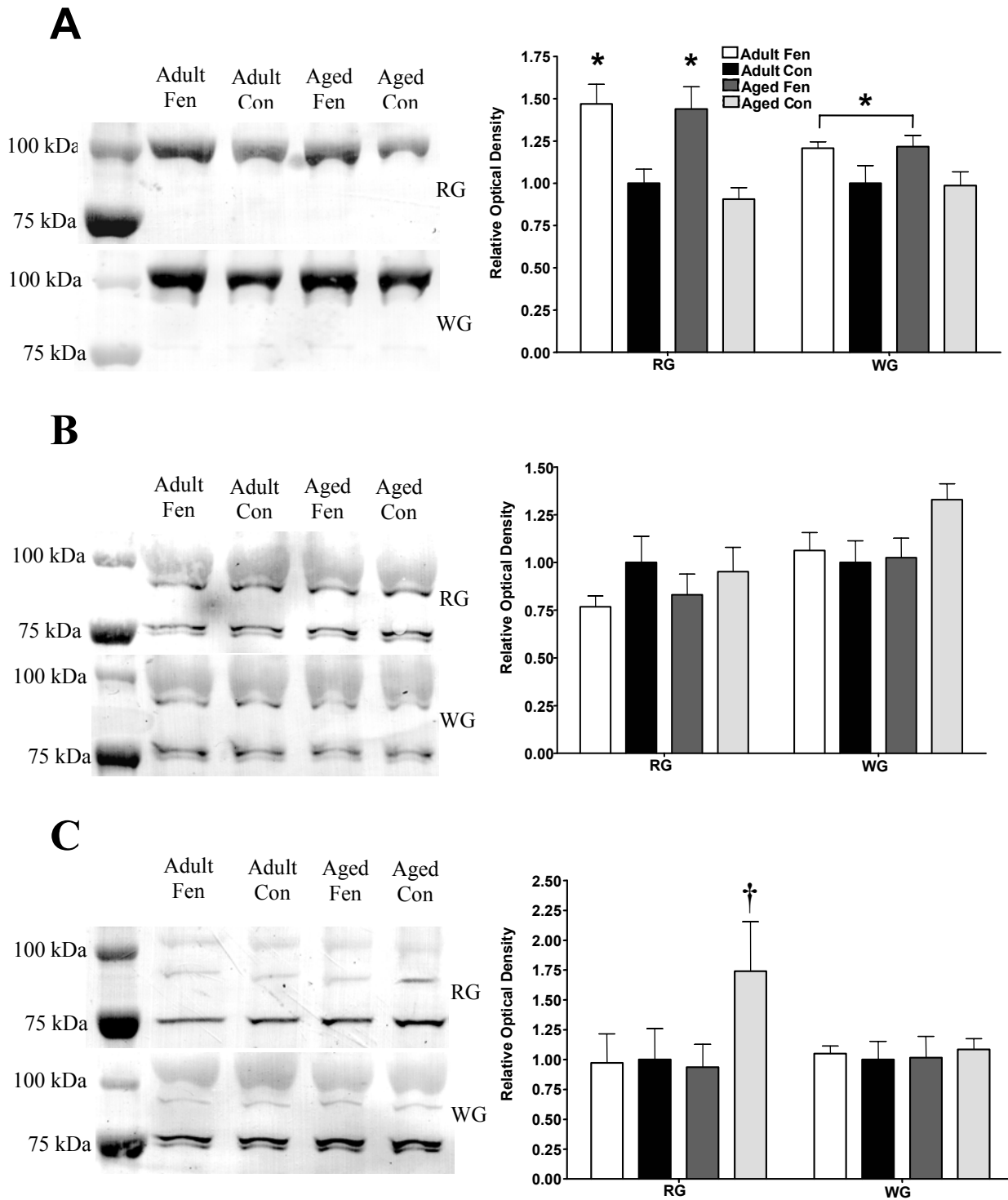


Figure 4

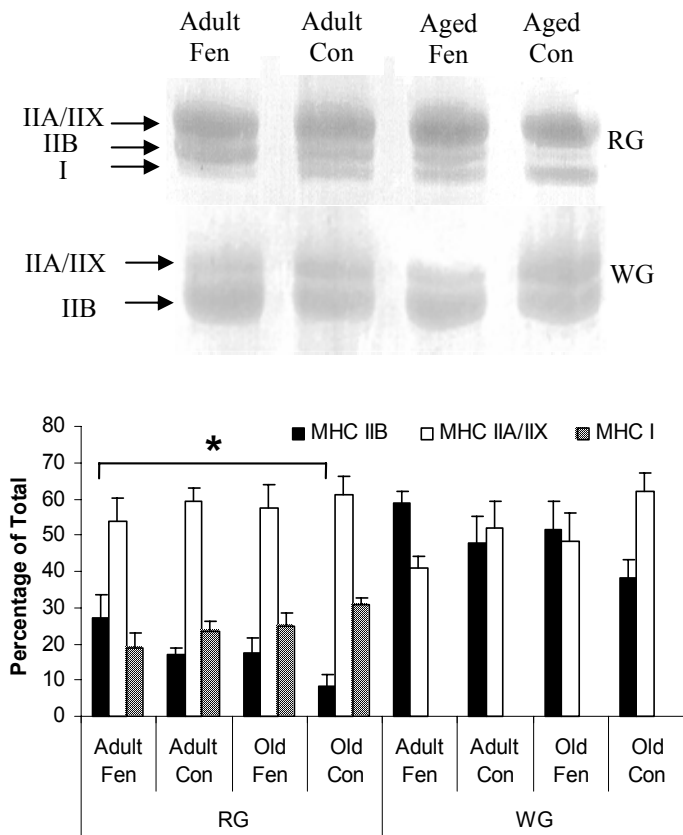


Figure 5