Exercise training differentially modifies age-associated alteration in expression of Na\textsuperscript{+},K\textsuperscript{+}-ATPase subunit isoforms in rat skeletal muscles

Yuk-Chow Ng\textsuperscript{1}, Murali Nagarajan\textsuperscript{1}, Korinne N. Jew\textsuperscript{2}, and Lisa C. Mace\textsuperscript{2}, Russell L. Moore\textsuperscript{2}

\textsuperscript{1}Department of Pharmacology, The Milton S. Hershey Medical Center, College of Medicine, The Pennsylvania State University, Hershey, Pennsylvania 7033-0850; and \textsuperscript{2}Department of Integrative Physiology, University of Colorado, Boulder, Colorado 80309

Correspondence:

Yuk-Chow Ng, Ph.D.
Dept of Pharmacology
College of Medicine
The Pennsylvania State University
Milton S. Hershey Medical Center
Hershey, PA 17033

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Our previous study demonstrated age-associated alterations in expression of the Na⁺,K⁺-ATPase subunit isoforms in rat skeletal muscles (J. Appl. Physiol. 87(3): 1132-1140, 1999). The present study tests the hypothesis that endurance exercise training (ETr) reverses these age-related changes. Expression of the subunit isoforms (α₁, α₂, β₁, β₂, β₃) in skeletal muscles of F-344/BN rats was examined by western blotting in three groups of rats: 16-month-old sedentary middle-aged rats (Ms), 29-month-old sedentary senescent rats (Ss), and 29-month-old treadmill exercise trained senescent rats (St). Levels of α₁ increased with age in red gastrocnemius (GR), white gastrocnemius (GW), and extensor digitorum longus (EDL), and ETr further increased its levels. Levels of α₂ were unchanged in GR, had a strong trend for a decrease in GW, while that of EDL decreased significantly. ETr increased the expression of α₂ in all three muscle groups in senescent rats. There was no significant increase in β₁ levels in GR, GW, or EDL with age, while ETr markedly increased levels of β₁ in the three muscle types in senescent rats. There was a marked decrease with age in levels of β₂ in the three muscle types, which was not reversed by ETr. By contrast, levels of β₃ increased with age in GR and GW, and ETr was able to reverse this increase in senescent rats. Na⁺,K⁺-ATPase enzyme activity was unchanged with age in GR and GW and slightly, but significantly, increased in EDL. ETr increased enzyme activity in GR and GW, and did not change in EDL. Myosin heavy chain isoforms in the three muscle groups did not change significantly with age; ETr caused a general shift toward more oxidative fibers. Thus, ETr differentially modifies age-associated alterations in expression of the Na⁺,K⁺-ATPase subunit isoforms. Since ETr was able to reverse only some of the age-associated
changes, mechanism(s) other than physical inactivity appears to play significant role in those changes.
**Introduction**

Skeletal muscle dysfunction during aging is a syndrome of profound clinical importance, although the underlying mechanisms remain incompletely understood (9,27,46). In skeletal muscle, Na\(^+\),K\(^+\)-ATPase plays a central role in the maintenance of Na/K homeostasis (8,10,32), which in turn modulates muscle contractile function (15,38,39). Thus, a change in the expression and/or function of the Na,K-pump with advancing age may alter physiological function of aging skeletal muscle. Indeed, a recent report demonstrated that in gene-targeted mice skeletal muscles expressing different ratios of the Na\(^+\),K\(^+\)-ATPase isoforms exhibited distinct contractile properties (18).

Na\(^+\),K\(^+\)-ATPase consists of a transmembrane catalytic \(\alpha\)-subunit and a \(\beta\)-subunit. Multiple isoforms of the \(\alpha\)- and \(\beta\)-subunit have been cloned and sequenced (29-31,40,42). Skeletal muscle of mature rats expresses the \(\alpha1\)- and \(\alpha2\)-subunit isoforms, and the three \(\beta\)-subunit isoforms (1-3,21,28). Fast and slow oxidative rich fibers express more \(\alpha1\) and \(\beta1\) than fast glycolytic fibers, whereas the opposite is true for \(\beta2\) (22,44). Relative expression of \(\beta3\) in the different fibers has not been determined.

We have demonstrated previously that in F344xBN rats, between 6- and 30-months of age, advancing age is associated with increased levels of \(\alpha1\) and \(\beta1\) subunit isoform and decreased levels of \(\alpha2\) and \(\beta2\) in red and white gastrocnemius (41). Our data demonstrated, for the first time, dynamic regulation of skeletal muscle Na\(^+\),K\(^+\)-ATPase
isozymes during aging. Mechanism(s) underlying the age-associated differential expression of the Na\(^+\),K\(^+\)-ATPase subunit isoforms is unclear. One of the important adaptations associated with aging, common in humans and in animal models, is reduced spontaneous physical activity (20,48). Furthermore, it has been well established that exercise training alters the abundance of the Na\(^+\),K\(^+\)-ATPase (11,17,33). Therefore, in the present study we tested the hypothesis that increased physical activity, by endurance exercise training, attenuates or reverses age-associated changes in the Na\(^+\),K\(^+\)-ATPase. The corollary to this hypothesis is that physical inactivity contributes to altered expression of the Na\(^+\),K\(^+\)-ATPase subunit isoform associated with advancing age. Our result shows that endurance exercise training differentially modifies expression of the isoforms in aged skeletal muscle; the training paradigm reversed the expression of some, but not all, of the subunit isoforms.
Methods

Endurance exercise training. Male F-344/BN rats at 13- and 26-months were housed in a 12:12-h light-dark cycle and given standard rat chow and water ad libitum. 26-month-old rats were arbitrarily assigned to either the sedentary or the exercise trained group. Animals in the trained group underwent 13-14 weeks of running on a motorized treadmill using a protocol similar to what we have described previously (23). During the first 2 weeks, running duration and speed were progressively increased. From the start of week 3 until the sacrifice date the final training protocol consisted of running 5 days/wk up a 10% grade at 14 m/min for 5 min and 17 m/min for 40 minutes for a total of 45 minutes. Animals in the sedentary group underwent similar procedure but without running. About twenty-four hours (± 4 hrs) after the last bout of exercise training, the rats were anesthetized with intraperitoneal sodium pentobarbital (35 mg/kg) at least 15 min following heparin injection (250 U). Skeletal muscles were dissected after hearts were removed. Thus, the three experimental groups were: 16-month-old middle-aged sedentary (Ms); 29-month-old senescent sedentary (Ss); and 29-month-old senescent exercise trained (St), and the number of animals in each group ranges from 12 to 15. All the following measurements were performed using tissues from a group of 5 to 6 animals randomly picked from each experimental group, except the citrate synthase activity assay, which was performed in tissues from the entire groups of rats. All animal use protocols were approved by the institutional animal care committee.

Preparation of tissue homogenates. Total tissue homogenates were prepared as described previously (41). Briefly, skeletal muscles (~200-300 mg) were pulverized and
homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) at a speed of 6.5 (11.0 full scale) for three 20-s periods at 4°C in a buffer containing Tris-HCl (10 mM, pH 7.5), EDTA (1 mM), protease inhibitors (phenylmethylsulfonyl fluoride; 500 µM), leupeptin (1 µM), pepstatin (1 µM), and E-64 (10 µM). Protein concentrations were determined by BioRad protein assay (Bio-Rad, Melville, NY).

**Western blotting.** Subunits of the Na⁺,K⁺-ATPase were resolved by SDS-PAGE and immunoblotted as previously described (41). For analysis of the β-subunits, equal amounts of homogenates (80 µg) were first deglycosylated with N-glycosidase F (Glyko, Novato, CA) for 18-20 h at 37°C according to manufacturer’s instructions. Antibodies for α1 and α2 were kindly provided by K. Sweadner (Harvard University). β1(SpEtB1) and β2 antibody (GP50) was kindly provided by P. Martin-Vasallo (Tenerife, Spain) and P. Beesley (Royal Holloway and Bedform New College, Egham, Surrey, U.K.), respectively, and β3 was purchased from Upstate Biotechnology (Lake Placid, NY). Bound monoclonal antibodies were detected with rabbit anti-mouse-IgG antibody followed by ¹²⁵I-labeled Protein A (ICN, Costa Mesa, CA), whereas bound polyclonal antibodies were detected with ¹²⁵I-Protein A alone. The blots were subjected to autoradiography for the purpose of displaying the images. Subsequently, band signal intensities were quantitated by a phosphor imager (Molecular Dynamics, Sunnyvale, CA). Because of the relatively low abundance of the β-subunit isoforms, a chemiluminescent detection method was used (Pierce SuperSignal West Pico, Rockford, IL). Blots were exposed to multiple films to ensure that signals were within the linear range of the film, and relative intensity of the bands were quantitated by densitometry.
(Molecular Dynamics Laser densitometer, Sunnyvale, CA). Since the anti-β subunit antibodies generate significant number of non-specific bands, we verified the bands of interest by running skeletal muscle controls to observe band shifts at appropriate molecular sizes before and after N-glycosidase F treatment, as demonstrated previously (37) (data not shown). A N-glycosidase F digested brain microsomal preparation, which has much higher levels of the β-subunits than in skeletal muscle, was run in the reference lane of the blots to identify the appropriate bands (data not shown). All core β-subunit isoforms have apparent molecular size around 35 kDa as revealed by molecular mass markers. The transferred gels and portions of some of the membranes were stained with Coomassie blue to verify, respectively, efficient transfer of proteins and equal loading (data not shown).

**Enzyme activity.** Citrate synthase activity in plantaris muscles was determined as described previously (34) in the entire group of exercise trained and control rats. Ouabain sensitive, Na⁺ and K⁺ stimulated hydrolysis of γ-32P-ATP was determined as described by Feschenko and Sweadner (14) with slight modifications. Briefly, tissue homogenates were diluted in 10mM Tris with 1mM EDTA (pH 6.8, room temp) to 150μg/40μl. The homogenates underwent freeze/thaw 10X in dry ice/acetone. The homogenates were then added to a buffer (0.16 ml total) containing the following in mM concentrations: 100 NaCl, 15 KCl, 5 MgCl₂, 5 NaN₃, 50 Tris (pH 7.8 room temp), and 1 EGTA. After a 5-min preincubation, the reaction was started by the addition of γ-32P-ATP (5.5 mM). Non-specific enzyme activity was assayed in the presence of 3 mM ouabain. The reaction was allowed to proceed for 25 min and stopped with 0.5 ml of
quenching solution (1 N sulfuric acid, 0.5% ammonium molybdate). After one ml of isobutanol was added, the phosphomolybdate complex was extracted into the organic phase by vigorous vortexing, separated by centrifugation, and 0.5 ml of the extract was counted with a scintillation counter. For each sample the assay was performed in triplicates. Specific activity is calculated as the difference between total and non-specific activity.

**Separation of myosin isoforms.** The method is basically identical to that described by Yu et al (49), with only slight modifications. Briefly, total tissue homogenates (0.075 µg/15µl) were separated on 6% SDS-gel (0.75mm thick) at 15°C. Electrophoresis was performed at a constant voltage of 120 V for 24 hours, and the gels were stained with silver stain (Amersham Pharmacia Biotech, Piscataway, NJ) immediately after electrophoresis. Relative intensity of the bands was quantitated by densitometry (Molecular Dynamics Laser densitometer, Sunnyvale, CA).

**Statistical analysis of data.** Results are expressed as mean ± SE. One-way ANOVA was used to compare group means, and the Duncan test was used for post hoc analysis. Data were examined at p < 0.05 and p < 0.10 to indicate statistical significance and trends, respectively.
Results

Effects of exercise training on muscle weight and citrate synthase enzyme activity: Red and white gastrocnemius muscle weight decreased by 25.97% and 28.02%, respectively, in 29-month-old senescent sedentary rats (Ss) compared to 16-month-old middle-aged sedentary rats (Ms) (red gastrocnemius: Ms = 1.286±0.071 g (n=6); Ss = 0.952±0.044g (n=6); White gastrocnemius: Ms = 0.811±0.046g (n=6), Ss = 0.583±0.027g (n=6)).

After endurance exercise training, the weight of gastrocnemius muscle in senescent animals (St) was not significantly different from that of the senescent sedentary animals (red gastrocnemius: St = 0.937±0.045g (n=5), white gastrocnemius: St = 0.563±0.040g (n=5)). On the other hand, the training paradigm significantly increased citrate synthase enzyme activity in the treadmill-trained rats (in µmol x min⁻¹ x g wet wt⁻¹) (Ms = 21.7±0.8 (n=15), Ss = 21.4±1.7 (n=12), St = 26.1±2.0 (n=13)) (p< 0.05 between Ss and St).

Effect of exercise training on subunit isoform expression: Expression of the subunit isoforms in red gastrocnemius, white gastrocnemius, and EDL was examined. Total tissue homogenates were used in the present study in order to avoid unintentional selection of subcellular pools. In red gastrocnemius, levels of α1 isoform increased in senescent sedentary rats compared to middle-aged sedentary rats (Fig. 1). Expression of α1 in senescent rats further increased after exercise training (Ms = 1.00±0.06, Ss = 1.25±0.08, St = 1.44±0.06, arbitrary densitometry units). With age, there was a statistically insignificant decrease in expression of α2. Exercise training increased
expression of \(\alpha_2\) isoform in senescent rats to levels significantly higher than that of middle-aged and senescent sedentary rats (\(M_s = 1.00\pm0.05, S_s = 0.88\pm0.06, St = 1.53\pm0.07\)). Similar changes were observed in white gastrocnemius (Fig. 1), except that there was a strong trend for a decrease in \(\alpha_2\) in senescent sedentary rats, and that exercise training failed to further increase the already elevated levels of \(\alpha_1\) in senescent sedentary rats (\(\alpha_1: M_s = 1.00\pm0.11, S_s = 1.77\pm0.15, St = 1.83\pm0.26; \alpha_2: M_s = 1.00\pm0.12, S_s = 0.74\pm0.07, St = 1.40\pm0.05\)). In EDL, the changes are also similar to that of the gastrocnemius: there was a strong trend for an increase in \(\alpha_1\) in senescent sedentary rats, and exercise training significantly increased the levels (\(M_s = 1.00\pm0.26, S_s = 2.01\pm0.39, St = 2.70\pm0.36\)); there was a small, but significant, decrease in \(\alpha_2\) in senescent sedentary rats, and exercise training reversed that decrease (\(M_s = 1.00\pm0.06, S_s = 0.78\pm0.05, St = 0.98\pm0.06\)).

Levels of \(\beta_1\) isoform in red gastrocnemius were slightly increased in senescent sedentary rats compared to middle-aged sedentary rats, although the difference was not statistically significant (Fig. 2). Exercise training markedly increased its expression in the senescent trained rats, compared to middle-aged and senescent sedentary rats (\(M_s = 1.00\pm0.22, S_s = 1.51\pm0.19, St = 4.69\pm0.45\)). Expression of \(\beta_2\), by contrast, was markedly decreased in senescent sedentary rats compared to middle-aged rats, and exercise training failed to reverse the decrease (\(M_s = 1.00\pm0.10, S_s = 0.23\pm0.07, St = 0.23\pm0.05\)). Expression of \(\beta_3\) is very different than that of \(\beta_1\) and \(\beta_2\): its levels significantly increased
in senescent sedentary rats and exercise training reversed that increase (Ms = 1.00±0.16, Ss = 2.16±0.17, St = 1.44±0.17), though not completely.

In white gastrocnemius, expression of the β-subunit isoforms is almost identical to that of the red gastrocnemius (Fig. 2) (β1: Ms = 1.00±0.35, Ss = 1.04±0.36, St = 3.22±0.37; β2: Ms = 1.00±0.19, Ss = 0.39±0.18, St = 0.25±0.08; β3: Ms = 1.00±0.30, Ss = 3.30±0.29, St = 1.63±0.51). In EDL, expression of β1 and β2 is also similar to that of the gastrocnemius (β1: Ms = 1.00±0.18, Ss = 1.34±0.30, St = 3.51±0.55, β2: Ms = 1.00±0.16, Ss = 0.41±0.13, St = 0.43±0.15). Because of insufficient amount of tissue samples, expression of β3 in EDL was not examined. Table 1 summarizes the above data regarding expression of the subunit isoforms.

**Na⁺,K⁺-ATPase enzyme activity**: Ouabain sensitive, Na⁺ and K⁺ stimulated ATPase enzyme activity was determined in total tissue homogenates. In red and white gastrocnemius, there were no detectable changes in enzyme activity between middle-aged and senescent sedentary rats; however, enzyme activity increased significantly after exercise training (Fig. 3) (red gastrocnemius: Ms = 21.63±0.97, Ss = 22.51±1.52, St = 29.13±0.37; white gastrocnemius: Ms = 44.93±4.21, Ss = 45.14±3.16, St = 57.50±3.30 µmol/mg prot/h). In EDL, enzyme activity was significantly higher in senescent sedentary rats compared to middle-aged sedentary rats, and exercise training failed to further increase this elevated level of enzyme activity (Ms = 46.07±2.41, Ss = 55.35±3.04, St = 58.45±1.36 µmol/mg prot/h).
Expression of the myosin heavy chain isoforms: Previous studies suggested a fiber-type dependent differential expression of the Na\(^+\),K\(^+\)-ATPase subunit isoforms (22,44). In order to examine whether the observed alterations in expression of subunit isoforms with age and exercise training can be correlated with muscle fiber-type changes, relative abundance of myosin heavy chain isoforms was determined by SDS-PAGE. Overall, there was no statistically significant change in the level of the myosin heavy chain isoforms in red gastrocnemius, white gastrocnemius, and EDL between middle-aged and senescent sedentary animals (Fig. 4). After exercise training, there was increase in I and IIX myosin isoforms in red gastrocnemius, and a statistically insignificant decrease in IIB in senescent trained rats compared to senescent sedentary rats. In white gastrocnemius, exercise training increased levels of IIX in senescent trained rats with no significant change in IIB. In EDL, the IIA and IIX isoform appear to migrate close together, and could not be resolved in the gel. Exercise training caused a significant decrease in the level of IIB, with no detectable change in IIA/X in senescent trained rats.
Discussions

The present study was undertaken to test the hypothesis that increasing physical activity, through endurance exercise training, reverses age-associated changes in expression of the Na\(^{+}\),K\(^{+}\)-ATPase subunit isoforms. The corollary to this hypothesis is that physical inactivity contributes to the age-related changes. The data demonstrated that endurance exercise training differentially modifies age-associated alterations in expression of the subunit isoforms, and suggest that some of the age-associated alterations in the expression of Na\(^{+}\),K\(^{+}\)-ATPase subunit isoforms involve a mechanism(s) that is unrelated to physical inactivity.

Our study shows that senescent F-344/BN rats can be trained to undergo a fairly vigorous endurance running paradigm. Because of the physical condition of the senescent rats, the animals were not trained at a higher intensity in order to avoid unreasonable loss of animals due to injury. It is important to note that the paradigm significantly increased citrate synthase enzyme activity in plantaris muscle of the senescent rats. In recruited skeletal muscle citrate synthase activity is known to increase with endurance training. Therefore, increases in this activity provide a peripheral marker of skeletal muscle adaptation to exercise. Furthermore, our result showed a significant shift toward slower myosin isoforms in both red and white gastrocnemius muscles after exercise training. These data strongly suggest that the senescent rats have received exercise training of sufficient intensity. Along with significant changes in expression of the Na\(^{+}\),K\(^{+}\)-ATPase isoforms in white gastrocnemius muscle, our results suggest
recruitment of this skeletal muscle under our training paradigm. This is somewhat unexpected since previous studies suggested recruitment of white gastrocnemius muscle occurs only when exercise training is at very high intensity (35,36). Whether the advanced age of the rats is responsible for this apparent difference is unclear at present. On the other hand, we cannot completely eliminate the possibility that some general training effect was producing the training-induced changes. In order to limit the number of exercise trained animals to a more manageable size, we chose not to include exercise trained young or matured rats in this study. We feel the simpler experimental design is justified because in this study we specifically are interested in whether exercise training reverses the changes that occur during senescence, not during growth or maturation of the rats. Some important questions, such as whether exercise training has similar affects in young vs. old rats, cannot yet be answered by the present study, and will be the subject of future investigations.

In the present study red gastrocnemius, white gastrocnemius, and EDL were examined because they represent different muscle types. Red gastrocnemius muscle of rat consists of predominately fast oxidative glycolytic fibers mixed with oxidative fibers, white gastrocnemius muscle consists of predominately fast glycolytic fibers, and EDL consists of roughly equal amounts of fast oxidative glycolytic and fast glycolytic fibers. We have detected qualitatively very similar, but less dramatic age-associated changes in expression of the Na⁺,K⁺-ATPase, compared to what we have reported previously (41). In addition, the previously observed age-associated increase in enzyme activity between middle-aged and senescent rats is not apparent in this study, at least in the red and white
gastrocnemius (a significant increase was detected in EDL). The reason for these apparent differences is not clear. A careful review of the history of the rat colonies reveals that our previous experiments were performed using animals before the 1999 rederivation of the Harlan colony. We speculate that the difference could be due to subtle variations between the colonies. Life-span data for the current colony is not presently available (NIA Office of Biological Resources and Resource Development, personal communication). It is possible that a greater magnitude of changes may be observed if older rats were used. In addition, because the rats were housed and trained in Boulder, Colorado (altitude ~1,650 m), an unexpected influence of high altitude on expression and activity of the subunit isoforms cannot be excluded. Interestingly, Green et al. (16) showed that the number of Na\(^+\),K\(^+\)-ATPase in human skeletal muscle decreased following a 21-day expedition at high altitude. It must be emphasized, however, qualitatively, data from the present study and our previous study are in excellent agreement. Furthermore, the small differences in no way obscure the conclusions of this study.

As mentioned above, ouabain sensitive Na\(^+\) and K\(^+\) stimulated ATPase enzyme activity in red and white gastrocnemius was unchanged between middle-aged and senescent sedentary rats. By contrast, enzyme activity in EDL increased with age, similar to what we have reported previously. Exercise training increased Na\(^+\),K\(^+\)-ATPase activity in both red and white gastrocnemius. Thus, it seems unlikely that physical inactivity plays an important role in the increased enzyme activity that was detected in the present and previous studies.
Expression of the two α-subunit isoforms in skeletal muscle responded very differently to aging and exercise training. Since increasing physical activity did not reverse the increased levels of α1, we conclude that the age-associated increase in α1 is unlikely to be due to physical inactivity. Furthermore, endurance exercise training appears to elicit tissue-specific differential effects in expression of α1. Mechanism(s) underlying this tissue specific regulation of α1 is not clear at present. It is worth noting that the age-associated changes occurred without significant switches in myosin isoforms, and that despite a switch toward slower fibers in both tissues, exercise training affects red and white gastrocnemius differently. Thus, the tissue-specific effect of exercise training does not appear to be primarily due to fiber type changes.

With regard to the α2 isoform, its levels in both red and white gastrocnemius were not significantly different between middle-aged and senescent sedentary rats, in accordance with what we have reported previously, even though a trend toward decreased levels is evident. Indeed, the decrease in α2 in EDL of senescent sedentary rats reached statistical significance. It is worth noting that previously we have observed decreased expression of α2 between young (6-month-old) and middle-aged (18-month-old) rats (41). Thus, collectively, advancing age is associated with decreased levels of α2. Exercise training increased levels of α2 in senescent rats in all three muscle groups examined. The reversal could be due to a switch of fiber types since the present study shows that exercise training increase relative amount of oxidative fiber and that expression of α2 is higher in this fiber type. However, it appears unlikely that is the
primary, or sole reason since data from our previous study and the present study (α2 in EDL) showed that decreased expression of α2 with advancing age is not dependent on fiber type changes.

How these changes in the Na⁺,K⁺-ATPase α-subunit isoforms ultimately affect function of the skeletal muscle in aging and after endurance exercise training remains to be elucidated. The α subunit isoforms appear to have different affinities for Na⁺ and K⁺ (6,13,24), and the α1 isozyme is a better substrate for phosphorylation by kinase (4,14). Insulin and exercise appear to selectively translocate α2-isozyme from the intracellular site to the plasma membrane (21,25). Importantly, Lingrel and co-workers recently demonstrated that mouse skeletal muscle lacking one copy of the α1 isoform gene showed lower force compared to wild type mouse (18). Conversely, mouse skeletal muscle lacking one copy of the α2 isoform gene showed greater force. Although it is difficult to extrapolate data from a genetically manipulated animal model, it may be speculated that an increase in α1 and a decrease in α2 levels in skeletal muscle with advancing age may result in altered contractile function. Future studies will try to elucidate this important question.

Similar to the α-subunits, the three β-subunit isoforms also exhibited unique adaptations in their expression during aging and in response to exercise training. Our data demonstrated that expression of β1 is highly sensitive to exercise training. Whether the increase is primarily due to an increase in oxidative fiber after exercise training cannot be definitively determined in the present study, although our previous study
demonstrated that increased expression of \( \beta_1 \) in aged skeletal muscle is not associated with a significant switch in expression of myosin isoforms.

Levels of \( \beta_2 \) decreased dramatically with age and exercise training failed to reverse or modify such a decrease, making it the only subunit isoform examined in the present study whose expression is not modified by exercise training. The data suggest that factors other than physical inactivity are important for its altered expression with age, or that in aged skeletal muscle expression of \( \beta_2 \) lost its responsiveness to increased physical activity. Levels of \( \beta_3 \) increased in aged skeletal muscles and exercise training substantially reversed that increase. Thus, physical inactivity may be responsible, at least in part, for increased expression of \( \beta_3 \) with aging. It is interesting to note that expression of \( \beta_3 \) in skeletal muscle is among the highest in the various tissues examined (1).

Whether the \( \beta_3 \) isoform performs special function in skeletal muscle is an interesting question to be explored in future studies. It remains to be determined in skeletal muscle whether the \( \beta \)-subunit isoforms play different roles in the assembly of the \( \alpha-\beta \) complex or during differentiation of muscle cells. The age-associated changes in levels of \( \beta_2 \) and \( \beta_3 \) may be a compensatory response to aging of skeletal muscle, or signify a pathological maladaptation process. However, the fact that levels of \( \beta_3 \) are lower in younger rats, and that increased levels of \( \beta_3 \) in aged skeletal muscle was reversed by exercise training, seem to suggest that in rat skeletal muscle a low level of \( \beta_3 \) is preferred.

In skeletal muscle of exercise trained senescent rats, as the result of a marked increase in \( \beta_1 \), a decrease in \( \beta_3 \), and unchanged levels of \( \beta_2 \), a significant shift toward
more $\beta_1$-type isozyme may be expected, if the elevated levels of $\beta$-subunits indeed combine with $\alpha$-subunits to form functional units. Interestingly, Klip and co-workers demonstrated that intracellular membranes of red skeletal muscles contain primarily $\alpha_2$ and $\beta_1$ subunits (28), and insulin stimulates their translocation from the internal membrane to the plasma membrane (21). Thus, one may speculate that levels of $\alpha_2$-$\beta_1$ type Na-pump in the internal membrane may be elevated in aged skeletal muscle following exercise training. Such an elevated pool of intracellular Na-pump may boost availability of the plasma membrane Na-pump when there is acute demand. In addition, it appears that isozymes with $\beta_2$ isoform have lower apparent $K^+$ affinity (12) and higher affinity for $Na^+$ (5,7). Thus, the age-associated decrease in $\beta_2$ isoform could significantly affect the kinetics of ion transport in aging skeletal muscle.

A recent report by Helwig et al. (19) demonstrated exercise-induced tissue- and isoform-specific alterations in expression of the Na$^+$,K$^+$-ATPase subunit isoforms in rats with myocardial infarction. In their study, exercise training failed to alter expression of all the subunit isoforms examined, except the $\beta_2$ isoform. The reason(s) for these apparent differences between their study and ours is not clear at present, but could be due to difference in exercise training paradigm, or, perhaps more interestingly, suggest some fundamental differences in responses of the subunit isoforms to exercise training in aged rats vs. heart failure rats.

Cellular mechanisms responsible for the exercise-induced altered expression of the subunit isoforms in senescent rats remain unclear. Previous study in young rats
demonstrated that an acute bout of exercise increased mRNA content of the α1 and β2 subunit isoforms, but not that of α2 and β1 (45). It was speculated that increase in intracellular Na⁺ content could have played a role. Indeed, we and others have reported that elevation of intracellular Na⁺ in various cell types in culture increased expression of the subunit isoforms and/or pump units (26,43,47). Nevertheless, it is interesting to note that in our study using the skeletal muscle C2C12 cell line, increased Na⁺ transport is associated with increased expression of the α2, but not the α1 subunit. These data suggest that either skeletal muscle cells in culture respond to change in Na⁺ differently than intact skeletal muscle, or mechanism(s) other than changes in intracellular Na⁺ play an important role in expression of the subunit isoforms. Cellular mechanisms responsible for the divergent responses of the three β-subunit isoforms clearly cannot be explained by changes in intracellular Na⁺ alone, and remain to be elucidated.

In conclusion, aging of skeletal muscle is associated with distinct patterns of alterations in expression of the Na⁺,K⁺-ATPase subunit isoforms, and exercise training differentially modifies these age-associated alterations. Since endurance exercise training was able to reverse only some of the age-associated changes, physical inactivity appears to play at most only a partial role in the aged-associated changes. Future investigations will explore the role of other well known aging related factors, including hormonal changes and oxidative stress, in altering expression of the Na⁺,K⁺-ATPase subunit isoforms during aging.
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Table 1: Alterations in expression of the isoforms

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The table summarizes changes in the expression of the subunit isoforms during aging and after exercise training. The first arrow in each cell depicts changes associated with aging (between Ms and Ss), the second arrow depicts effects of exercise training on senescent animals (between Ss and St). ↑: increased expression, ↓: decreased expression, →: no change in expression.
**Figure legend**

**Fig. 1** Levels of α-subunit isoforms in gastrocnemius and EDL muscles of rats with age.

Skeletal muscle homogenate (100 µg) of mature sedentary (Ms) (n=6), senescent sedentary (Ss) (n=6) and senescent exercise trained (St) (n=5) rats was resolved by SDS-PAGE. Transferred blots were immunoblotted with either α1- or α2-specific antibodies. A: typical autoradiograms of the blots. B: radioactivity of specific bands was quantitated by a phosphor imager, and data were normalized to those of mature sedentary rats. Open bar: α1; Solid bar: α2. \(^a\) : Significantly different from Ms, p < 0.05; \(^b\) : significantly different from Ss, p < 0.05. Data in bar graphs expressed as mean ± SEM.

**Fig. 2** Levels of β-subunit isoforms in gastrocnemius and EDL muscles of rats with age.

Equal amounts of tissue homogenates (80 µg) from mature sedentary (Ms) (n=6), senescent sedentary (Ss) (n=6) and senescent exercise trained (St) (n=5) rats were deglycosylated with N-glycosidase F, resolved by SDS-PAGE, transferred, and immunoblotted with β1-, β2-, or β3-specific antibodies. Bound antibodies were detected by chemiluminescent method. A: typical image of the bands; B: intensity of specific bands was quantitated by densitometry, and data were normalized to those of Ms rats. Open bar: β1; Solid bar: β2; Hatched bar: β3. \(^a\) : Significantly different from Ms, p < 0.05; \(^b\) : significantly different from Ss, p < 0.05. Data in bar graphs expressed as mean ± SEM.
**Fig. 3** Na,K-ATPase enzyme activity in skeletal muscle of rats with age. Tissue homogenates (150 µg/40 µl) from red and white gastrocnemius and EDL were used. Total enzyme activity in mature sedentary (Ms) (n=6), senescent sedentary (Ss) (n=5) and senescent exercise trained (St) (n=5) rats was measured as the hydrolysis of γ-32P-ATP in the presence of 100 mM NaCl and 15 mM KCl, as described in Methods. Non-specific activity was assayed in the presence of 3 mM ouabain and specific activity was calculated as the difference between total and non-specific activity. Enzyme activity from five samples in each age group was assayed and the assay was performed in triplicates. Open bar: red gastrocnemius; Solid bar: white gastrocnemius; Hatched bar: EDL. 

Significantly different from Ms, p < 0.05; \( ^{b} \) significantly different from Ss, p < 0.05.

Data in bar graphs expressed as mean ± SEM.

**Fig. 4** Expression of myosin heavy chain in gastrocnemius and EDL muscle of rats with age. Tissue homogenates (0.075 µg) from mature sedentary (Ms) (n=6), senescent sedentary (Ss) (n=5) and senescent exercise trained (St) (n=5) rats were subjected to SDS-PAGE in 6% gel containing 30% glycerol. Gels were stained with silver to visualize the myosin heavy chain isoforms. A: Typical silver stained bands. B: Intensity of the bands was quantitated by densitometry. Open bar: IIX; Solid bar: IIB; Hatched bar: I; Double hatched bar: IIA/IIX. \( ^{a} \) Significantly different from Ms, p < 0.05; \( ^{b} \) significantly different from Ss, p < 0.05. Data in bar graphs expressed as mean ± SEM.
Reference


Fig. 1

Red Gast

White Gast

EDL
Fig. 2

Red Gast

White Gast

EDL
Fig. 3

![Graph showing enzyme activity.](image)
Fig. 4