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Exercise Training Attenuates Age-Induced Changes in Apoptotic Signaling in Rat Skeletal Muscle

WOOK SONG,[1,2] HYO-BUM KWAK,[1] and JOHN M. LAWLER[1]

ABSTRACT

The aging process in skeletal muscle is characterized by a loss of myocytes and reduction in cross-sectional area of the remaining myocytes, particularly in Type II (fast-twitch) muscle fibers. In multinucleated skeletal muscle, apoptosis may contribute to both fiber atrophy and loss of muscle fibers. Recent evidence suggests that the mitochondrial Bcl-2 family pathway may be a target of aging. Here the authors demonstrated that aging increased DNA fragmentation, cleaved caspase-3, and pro-apoptotic Bax in rat skeletal muscle. Twelve weeks of treadmill exercise training increased anti-apoptotic Bcl-2, while markedly reducing DNA fragmentation, and cleaved caspase-3, Bax, and Bax/Bcl-2 ratio in the white gastrocnemius and soleus muscles of old rats. Upstream anti-apoptotic NF-κB activity decreased in aging skeletal muscle, and increased with exercise training. Regulation of NF-κB activity with aging and exercise was not related to changes in NF-κB subunit protein levels. Instead, changes in post-translational activation of NF-κB occurred as a function of altered phosphorylation of IκB. These results indicate that treadmill exercise training attenuates fiber atrophy and pro-apoptotic signaling in aging skeletal muscle. *Antioxid. Redox Signal.* 8, 517–528.

INTRODUCTION

Principal characteristics of aging related to skeletal muscle are the progressive decline in physical capacity, contractile function, and skeletal muscle mass (sarcopenia) (26). Sarcopenia adversely affects the health and quality of life of the elderly. There is increasing evidence that skeletal muscle fiber atrophy and myocyte loss are directly related to the reduction in skeletal muscle function observed with aging (6). In fact, 30% or more of skeletal muscle myocytes, particularly in fast-twitch fibers, may be lost with age due to apoptosis or necrosis (6, 18, 25). In concert, there is a 30–40% reduction in strength by age 80 (46). Recently, Phillips and Leeuwenburgh (34) demonstrated that life-long caloric restriction reduced markers of apoptosis in aging rat skeletal muscle.

Apoptosis is an evolutionary-conserved genetic program important in regulating growth, differentiation, protein turnover, tissue remodeling, and reducing cancer risk through removal of cells or "cell suicide" (11, 25, 55). Characteristics of cellular apoptosis include cell rounding, shrinkage, DNA fragmentation, plasma membrane blebbing, and formation of apoptotic bodies (14, 55). In addition to cell death, signaling pathways involved in apoptosis also can affect striated muscle contractility and protein degradation (13, 37). Although unresolved, apoptosis of myonuclei may lead to reduced fiber cross-sectional area as well as myocyte removal, since skeletal muscle is multinucleated (2).

Although apoptosis is important in regulating cell proliferation and removal of precancerous cells in adult mitotic tissues (e.g., liver and kidney), dysregulation of apoptosis is now recognized as a mechanism fundamental to numerous pathologies (39, 54). Excessive apoptosis in adult skeletal muscle can be dire, because muscle fiber loss appears irreversible in this predominantly postmitotic tissue (25, 28). For example, apoptosis is now recognized as a potential mechanism contributing to skeletal muscle wasting and weakness from chronic heart failure (9), muscular dystrophy (23, 40),

[1]Texas A & M University, Redox Biology and Cell Signaling Laboratory, College Station, Texas, USA.
[2]University of Texas Health Science Center at San Antonio, Department of Cellular and Structural Biology, San Antonio, Texas, USA.

and models of extreme disuse (2, 20). Indeed, therapeutics for heart failure, such as L-carnitine and thalidomide, which ameliorate myonuclei apoptosis also reduce skeletal muscle atrophy (49).

Aging in skeletal muscle is associated with increased apoptosis (6, 11, 12) as well as increased susceptibility to injury, inflammation, and oxidative stress (27, 53). Oxidative stress, inflammatory cytokines, and impaired stress/cell protection are proposed mechanisms contributing to increased apoptosis in aging tissues (5, 25, 55). Recently, Welle *et al.* (51) reported that aging altered mRNA levels of apoptotic pathway proteins in human skeletal muscle, including Bcl-2 as well as cell protective stress proteins. Upregulation of the mitochondrial Bcl-2 family pathway may be a potential mechanism leading to apoptosis in aging skeletal muscle (25). The balance between competing (a) anti-apoptotic "gatekeepers" including Bcl-2 and Bcl-X_L and (b) pro-apoptotic "gatecrashers" including Bax, Bak, and Bik (16). The ratio of pro- to anti-apoptotic proteins (e.g., Bax/Bcl-2) regulates myonuclei and cell survival by controlling mitochondrial membrane stability (16, 32, 52). Decreased mitochondrial membrane stability and increased pore formation initiate the release of cytochrome c, formation of the apoptosome catalyzed by Apaf-1 (**a**poptotic **p**rotease **a**ctivating **f**actor-1), and followed by subsequent cleavage and activation of caspase-9 and caspase-3 (11).

In contrast, nondamaging habitual exercise using resistive or endurance regimens provides some protection against age-related sarcopenia, impaired contractile function, and risk of muscle injury (5, 40, 45). Interestingly, caloric restriction, which prolongs lifespan and decreases oxidative stress, may also ameliorate age-induced mitochondrial dysfunction, muscle fiber atrophy, and apoptotic signaling (12, 33). Recently, Siu *et al.* (42) found that exercise training resulted in adaptations in Bcl-2 family apoptotic signaling and related stress proteins in skeletal muscle of young rats. However, cellular adaptation to exercise does appear to be reduced in aged muscles (46), and the mechanisms by which exercise protects aging muscle cells remain elusive. Thus we hypothesized that 12 weeks of treadmill exercise training would ameliorate age-induced changes in Bax/Bcl-2 ratio, caspase-3 cleavage, apoptosis, and fiber morphology in rat skeletal muscle.

MATERIALS AND METHODS

Three-month- and 24-month-old Fischer-344 rats were used as our young adult and old groups, respectively. Animals were purchased from the NIH colony and cared for at the Comparative Biology facility at Texas A & M University in accordance with NIH and ULACC (The University Laboratory Animal Care Committee) standards. Rats were housed in a temperature-controlled (23° ± 2°C) room with a 12:12-h light:dark cycle with water and rat chow provided *ad libitum*. One-half of the rats in each group ran for 12 weeks (5 days/week) on a motorized treadmill for 60 min/day, and the other half served as sedentary age-matched controls (n = 10 in each group): young sedentary controls (YS); young exercise trained (YE); old sedentary controls (OS), and old exercise trained (OE).

Exercise training protocol

Rats in the exercise groups ran on a motor-driven treadmill at 15 m/min up a 15° incline, 1 h/day, and 5 days/week for 12 weeks. Rats in the exercise groups were gradually conditioned to perform this level of exercise over the first 5 weeks of the 12-week training program. The intensity was designed to correspond to 75% of VO_{2max} in the old group (24). At the time of sacrifice rats were 6- and 27-months of age. The effectiveness of the exercise training protocol was confirmed by increased heart mass/body mass ratios and increased citrate synthase activity in skeletal muscle. Specifically, heart mass/body mass increased by 18.7% with exercise training in young adult rats (2.81 ± 0.61 g/kg *vs*. 2.37 ± 0.22 g/kg mean ± SD) and by 8.4% with exercise training in old rats (2.61 ± 0.09 g/kg *vs*. 2.41 ± 0.06 g/kg mean ± SD). Exercise training significantly increased citrate synthase activity in the gastrocnemius in both young (+27.1%) (16.9 to 19.7 μmol/gww/min) and old (+57.2%) (13.7 to 21.5 μmol/gww/min) groups, indicative of effectiveness in exercise training.

Experimental design

Skeletal muscle morphology including mass, mass per body mass, fiber cross-sectional area, and % connective tissue was assessed via histochemistry. Histone-associated DNA fragmentation and caspase-3 cleavage were used as markers of nuclear apoptosis. Caspase-3 is downstream of the Bcl-2 family apoptotic cascade. Age and exercise-induced changes in the ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2 was assessed using Western immunoblot analysis. Upstream regulation of apoptosis and Bcl-2 family proteins by HSP70 and transcription factor nuclear factor-kappaB (NF-κB) may be affected by aging and exercise training. Thus we assessed HSP70 protein expression, NF-κB DNA binding activity using a highly sensitive ELISA technique (36), and p65 subunit protein expression. As phosphorylation of the inhibitory protein IκB (IkappaB) initiates translocation to the nucleus and activation NF-κB, we also determined IκB phosphorylation in skeletal muscle.

Histochemistry

Cross-sections of the white (superficial) tibialis anterior were cut (10 μm thick) in a cryostat (−20°C) at resting length, placed on slides, and air-dried for 30 min. For assessment of fiber cross-sectional area, geometry, and extracellular space, cross-sections were stained with 2 drops hematoxylin, incubated for 1 min at room temperature. Hematoxylin was chosen to highlight nuclei and mitochondria. Stained sections were then rinsed with PBS and air dried before mounting in Vectamount medium (Vector Laboratories, Burlingham, CA, USA). Muscle cell area and connective tissue area were calibrated against a stage micrometer.

Tissue preparation

Rats were anesthetized with sodium pentobarbital (50 $mg \cdot kg^{-1}$ i.p.) following the exercise training period in young and old, exercise trained, and sedentary groups. Animals in the exercise training groups were anesthetized 48 h following the last bout of exercise training to avoid influence of the last

acute exercise bout. The soleus, gastrocnemius, and tibialis anterior muscles were quickly extracted, weighed, and placed in ice-cold phosphate-buffered saline (PBS, pH 7.4). Exercise results in a large increase in blood flow in all three muscles (4). The white and red portions of the gastrocnemius were separated. The white gastrocnemius (92% Type IIb fibers) and soleus (90% Type I fibers) muscles (10) were then frozen in liquid nitrogen and stored at −80°C until analyses.

Homogenization procedure

Gastrocnemius and soleus samples were minced into fine pieces and homogenized (20:1 w/v) in ice-cold (4°C) lysis buffer solution (pH 7.40) containing the following: 20 m*M* HEPES, 350 m*M* NaCl, 20% glycerol, 1% Igepal-CA630, 1 m*M* $MgCl_2$, 0.1 m*M* DTT, 0.5 m*M* EDTA, 0.1 m*M* EGTA, and protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany). Minced muscle samples were homogenized (20:1 w/v) using a ground glass on ground glass homogenizer (Bellco Biotechnology, Vineland, NJ, USA) at 4°C, and then twice centrifuged (4°C) for 10 min at 10,000 *g* with the supernatant extracted each time. The cytosolic fraction was removed each time and the pellet discarded to remove debris and nuclear contamination. Total protein was determined using a BCA assay kit (Pierce Biotechnology, Rockford, IL, USA).

DNA fragmentation

Histone-associated DNA fragmentation was assessed using a cell death detection ELISA (Roche). Twenty µl supernatant was transferred into a microplate; 80 µl of the immunoreagent was added to each well, followed by gentle shaking for 2 h at 25°C. Then 100 µl ABTS solution was pipetted to each well, and incubated on a plated shaker at 250 rpm until the color development was sufficient for a photometric analysis (approx. after 10–20 min). Finally, we measured at 405 nm against ABTS solution alone as a blank (reference wavelength of 490 nm).

Western immunoblot analysis

Protein content for cleaved caspase-3, Bax, Bcl-2, p65, and IκB was determined by Western immunoblot analysis. Separating gel (375 m*M* Tris-HCl; pH 8.8; 0.4% SDS; 10% acrylamide) and stacking gel (125 m*M* Tris-HCl; pH 6.8; 0.4% SDS; 10% acrylamide monomer) solutions were made, and polymerization then initiated by TEMED and ammonium persulfate. Separating and stacking gels were then quickly poured into a Bio-Rad Protein III gel-box (Bio-Rad; Hercules, CA, USA). Eighty micrograms of protein from skeletal muscle homogenates in sample buffer (Tris pH 6.8 with 2% SDS, 30 m*M* DTT, 25% glycerol) were then loaded into the wells of the 10% polyacrylamide gels, and electrophoresed at 150 V. The gels were then transferred at 30 V overnight onto a nitrocellulose membrane (Bio-Rad). Membranes were blocked in 5% nonfat milk in PBS with 0.1% Tween-20 at room temperature for 6 h. After blocking, membranes were incubated at room temperature in PBS and the appropriate primary antibodies for 12 h: rabbit polyclonal Bax (1:200, Santa Cruz Biotechnology: Santa Cruz, CA, USA), mouse monoclonal Bcl-2 (1:250, BD Transduction Laboratories: Lexington, KY, USA), rabbit polyclonal cleaved caspase-3 (1:500, Cell Signaling Technology: Beverly, MA, USA), mouse monoclonal p65 (1:500, Santa Cruz Biotechnology), and rabbit polyclonal phosphorylated IκB (1:200, Santa Cruz Biotechnology). Following three washings in PBS with 0.4% Tween-20, membranes were incubated with horseradish peroxidase (HRP)–conjugated secondary antibodies (Santa Cruz Biotechnology) in PBS at room temperature for 90 min. Following three washes in PBS with 0.4% Tween-20, an enhanced chemiluminescence (ECL) detection system (Amersham: Piscataway, NJ, USA) was used for visualization. Densitometry (as area times grayscale relative to background) was performed using a Kodak film cartridge and film, a scanner interfaced with a microcomputer, and the NIH Image J Analysis program. Consistent loading of wells was confirmed with Ponceau-S-staining and protein expression was quantified as area times grayscale relative to background per mg. protein.

ELISA for NF-κB DNA binding activity

We used a sensitive ELISA technique that is specific for activated NF-κB (36). This ELISA kit (Active Motif: Carlsbad, CA) contains a 96-well plate with the immobilized oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′). The active form of NF-κB contained in sample homogenates specifically binds to this oligonucleotide. The primary antibodies used to detect NF-κB recognize an epitope on p65 that is accessible only when NF-κB is activated and bound to its target DNA. Biotinylated double-strand probes containing the consensus NF-κB binding sequence were fixated on streptavidin-coated microplate wells. Muscle homogenate samples were added (in triplicate) and incubated at 25°C for 60 min. Next, wells were incubated with rabbit anti-NF-κB antibodies for 60 min. After three washings with PBS, a horseradish peroxidase-conjugated anti-rabbit secondary antibody was incubated in the wells for 60 min. Colorimetric development was quantified via a microplate reader (Molecular Devices, Sunnyvale, CA, USA) set at an absorbance wavelength of 450 nm with a reference wavelength of 655 nm.

Statistics

Two-way ANOVAs with Fisher's-LSD post-hoc were conducted to determine the existence of mean differences for age and exercise effects. The level of significance was set at $p < 0.05$.

RESULTS

Skeletal muscle morphology

Mean (±SD) body mass was significantly reduced by exercise training in both young (366.6 ± 8.2 *vs*. 344.9 ± 23.5) and old (403.5 ± 28.7 *vs*. 367.5 ± 11.86) age groups. Muscle mass was higher in the old exercised groups than the old sedentary animals for both soleus (+13.3%) and gastrocnemius (14.4%) muscles. Both gastrocnemius mass/body mass and soleus

mass/body mass ratios significantly decreased with age by 22.8% and 14.7%, respectively. Muscle mass/body mass ratio for the hindlimb muscles was 24.5% greater in the old exercised group than the old sedentary group. Histological analysis revealed that exercise training resulted in less rounding of cells (14), decreased fiber cross-sectional area, and increased connective tissue in white skeletal muscle (tibialis anterior) from old rats (Figs. 1A–1C). Mean fiber cross-sectional area was 35.4% lower in old sedentary rats versus young sedentary controls. Conversely, exercise training provided significant protection of mean fiber cross-sectional area in the old group (+27.6%) (Fig. 1B). Exercise had no effect on muscle fiber cross-sectional area in young rats. Percent extramyocyte space increased from 4.5% in young sedentary controls to 13.7% in old sedentary rats, but was only 7.4% in the old exercise group (Fig. 1C).

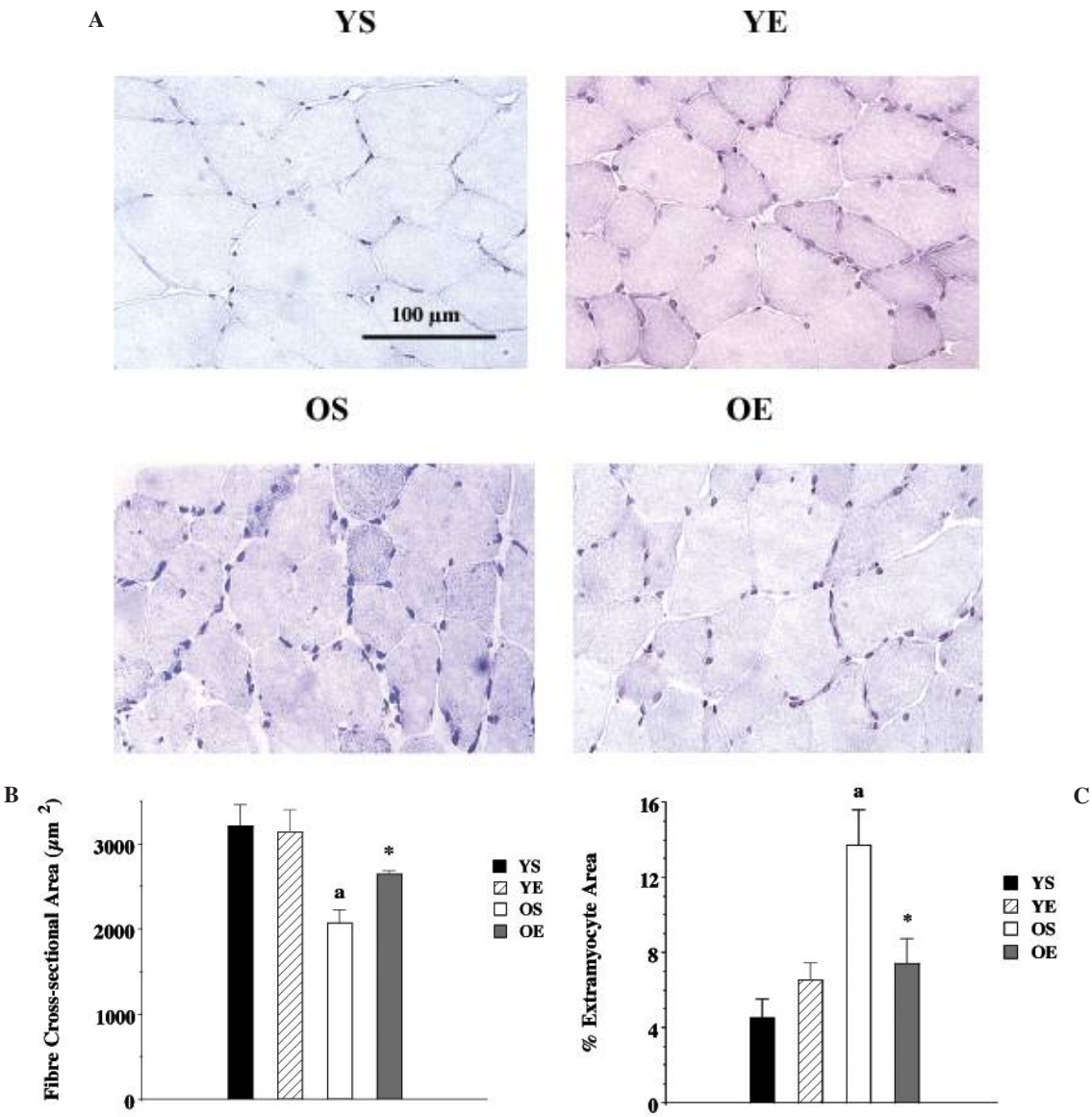

FIG. 1. (For interpretation of the references to color in the figure legend, the reader is referred to the web version of this article). (**A**) **Histological cross-sections of the white tibialis anterior muscle** illustrating changes in extracellular matrix (ECM), cell shape, and fiber size in the following groups: young sedentary controls (YS), young exercise trained (YE), old sedentary controls (OS), and old exercise trained (OE). Aging increased relative extracellular matrix volume and decreased fiber size. Cell rounding and ECM volume were partially attenuated by exercise training in the old age group. (**B**) **Mean white tibialis anterior fiber cross-sectional area** in the following groups: young sedentary controls (YS), young exercise trained (YE), old sedentary controls (OS), and old exercise trained (OE). (**a**) indicates a significant aging effect. (*) indicates a significant exercise effect. (**C**) **Quantification of percent (%) extramyocyte area of the white tibialis anterior fibers** in the following groups: young sedentary controls (YS), young exercise trained (YE), old sedentary controls (OS), and old exercise trained (OE). (**a**) indicates a significant aging effect. (*) indicates a significant exercise effect.

Apoptosis markers

Aging significantly increased histone-associated DNA fragmentation (Fig. 2A) in the white gastrocnemius by 62.7%. Our findings confirm elevated mono- and oligonucleosome fragments in aging skeletal muscle observed by Dirks and Leeuwenburgh (11). Conversely, exercise training drastically attenuated DNA fragmentation (−45.3%) when compared with sedentary controls in the old age group (Fig. 2A). These novel data indicate that exercise training ameliorates the apoptotic process in aging skeletal muscle protecting myofiber nuclei and possibly satellite cells. In contrast, there was little change in DNA fragmentation in the young group. Given that occurrence of apoptosis in undamaged skeletal muscle from healthy young adults is thought to be low, added protection of exercise training against myofiber loss in young adults is uncertain.

Very low levels of cleaved caspase-3 were detected in white gastrocnemius muscles of young sedentary animals (Fig. 2B). In contrast, cleaved caspase-3 protein expression was dramatically higher (717 fold) in the white gastrocnemius of the old sedentary group when compared with the young sedentary group (Fig. 2B). Remarkably, exercise training resulted in vast diminishment (−95.5%) of cleaved caspase-3 protein expression.

Bax and Bcl-2 protein expression

Increased apoptosis and impaired function in skeletal muscle with aging may be related to changes in Bcl-2 family proteins (6, 25). We found that aging significantly increased Bax protein expression (+59.8%) in the white gastrocnemius (Fig. 3A). Conversely, 12 weeks of treadmill exercise training resulted in 91.5% lower protein expression of Bax in the old exercise group compared with the old sedentary group. Exercise training also decreased Bax protein expression in the soleus muscle (−60%) (Fig. 3B). Aging resulted in a downward trend (−20%) in Bcl-2 protein expression ($p = 0.10$) in the white gastrocnemius (Fig. 3C). Bcl-2 protein expression was significantly lower (−36%) due to aging in the soleus (Fig. 3D). In contrast, exercise training upregulated Bcl-2 protein levels in the white gastrocnemius by 166% in the old group. Exercise also increased Bcl-2 protein levels in the soleus muscle (+86%) (Fig. 3D). Thus Bax/Bcl-2 ratio in the white gastrocnemius increased by 98% with age (Fig. 3E), but decreased dramatically (−96.8%) by exercise training in old rats. These data indicate that regular exercise reverses age-induced increases in pro-apoptotic Bcl-2 family signaling, upstream of cleaved caspase-3 and DNA fragmentation.

Age-associated changes in inducibility of cell protective pathways with exercise

Exercise could ameliorate age-induced elevation of Bax/Bcl-2 ratio, caspase-3 cleavage, DNA fragmentation, fiber atrophy through upstream cell protective proteins including heat shock proteins and activation of NF-κB. However, HSP70 protein expression was not altered by age in the white gastrocnemius or soleus (data not shown). In addition, exercise training upregulated muscle HSP70 levels in the young, but not the old group. This is consistent with an impaired response of HSP70 and its upstream transcription factor heat shock factor-1 (HSF-1) to an acute bout of muscle contractions observed by Vasilaki *et al.* (47).

NF-κB DNA binding activity and regulation

We found that NF-κB DNA binding activity was significantly lower (−27%) in with aging in the white gastrocnemius (Fig. 4A), consistent with recent aging data from

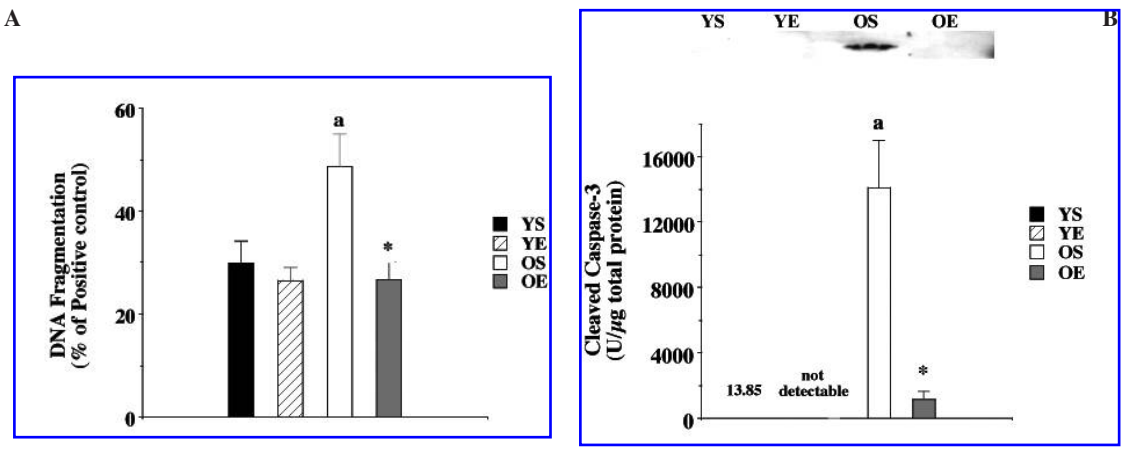

FIG. 2. (**A**) **DNA fragmentation** was used as a marker of apoptosis through quantification of mono- and oligonucleosomes via ELISA in the white gastrocnemius muscles from the following age and exercise groups: young (6-month-old) sedentary controls (YS), young exercise trained (YE), old (27-month-old) sedentary controls (OS), and old exercise trained (OE). Fischer-344 rats ran for 12 weeks on a treadmill, 5 days/week, 60 min/day. (**a**) indicates a significant aging effect. (*****) indicates a significant exercise effect. (**B) Effect of age and 12 weeks of exercise training on cleaved caspase-3 protein expression in the white gastrocnemius**. Western blots and mean (± SEM) data are presented from young sedentary controls (YS), young exercise trained (YE), old sedentary controls (OS), and old exercise trained (OE). Data are expressed in densitometry units (area × pixel density) per mg muscle protein. (**a**) indicates a significant aging effect. (*****) indicates a significant exercise effect.

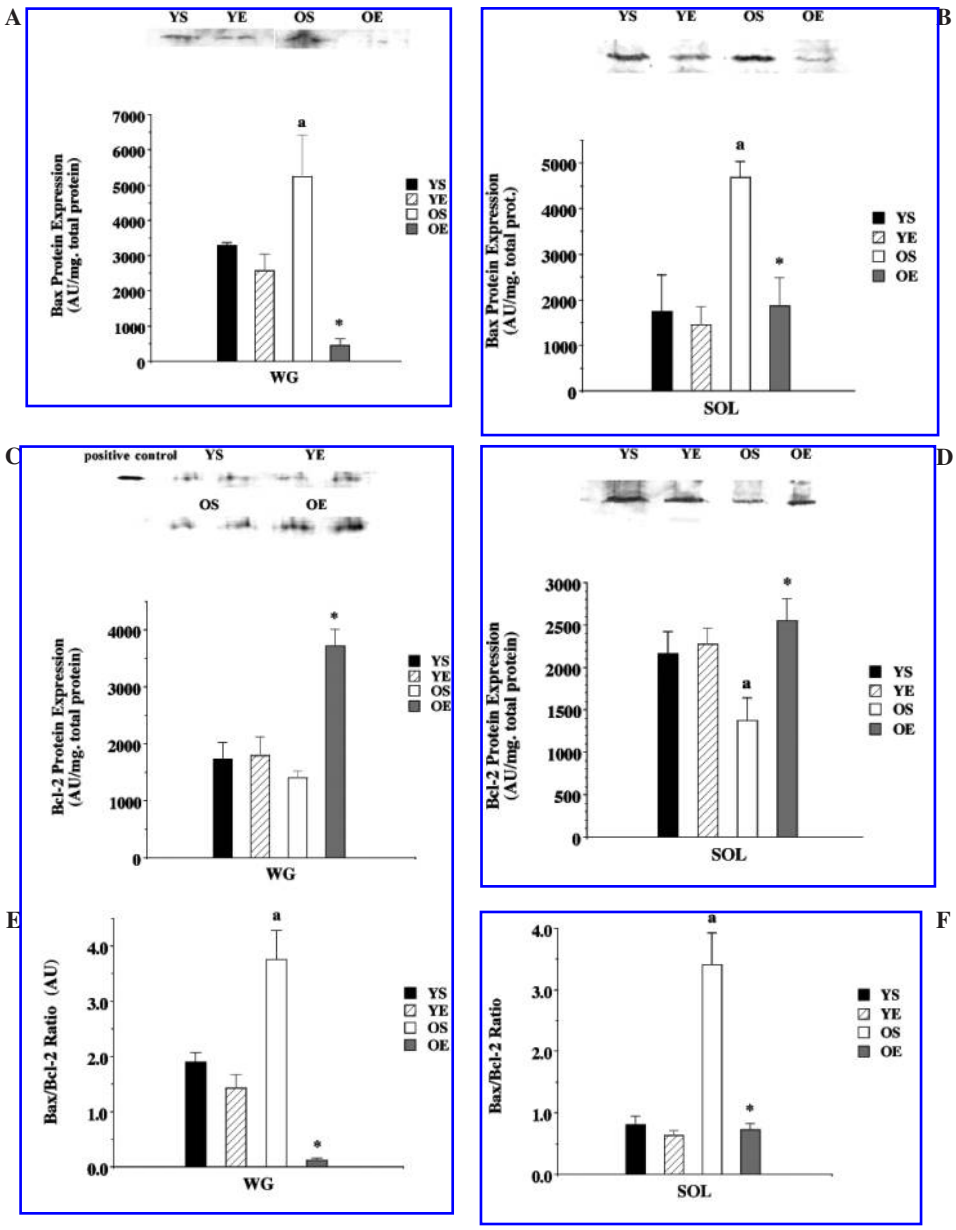

FIG. 3. (**A**) **Effect of age and 12 weeks of exercise training on Bax protein expression in the white gastrocnemius.** Representative Western blots and mean (± SEM) data are presented for young sedentary controls (YS), young exercise trained (YE), old sedentary controls (OS), and old exercise trained (OE). (**a**) indicates a significant aging effect. (*) indicates a significant exercise effect. (**B**) **Effect of age and 12 weeks of exercise training on Bax protein expression in the soleus.** Representative Western blots and mean (± SEM) data are presented for young sedentary controls (YS), young exercise trained (YE), old sedentary controls (OS), and old exercise trained (OE). (**a**) indicates a significant aging effect. (*) indicates a significant exercise effect. (**C**) **Effect of age and 12 weeks of exercise training on Bcl-2 protein expression in the white gastrocnemius.** Representative Western blots and mean (± SEM) data are presented for positive control (PC), young sedentary controls (YS), young exercise trained (YE), old sedentary controls (OS), and old exercise trained (OE). (*) indicates a significant exercise effect. (**D**) **Effect of age and 12 weeks of exercise training on Bcl-2 protein expression in the soleus.** Representative Western blots and mean (± SEM) data are presented for young sedentary controls (YS), young exercise trained (YE), old sedentary controls (OS), and old exercise trained (OE). (**a**) indicates a significant aging effect. (*) indicates a significant exercise effect. (**E**) **Effect of age and 12 weeks of exercise training on Bax/Bcl-2 ratio in the white gastrocnemius.** Mean (± SEM) data are presented for young sedentary controls (YS), young exercise trained (YE), old sedentary controls (OS), and old exercise trained (OE). (**a**) indicates a significant aging effect. (*) indicates a significant exercise effect. (**F**) **Effect of age and 12 weeks of exercise training on Bax/Bcl-2 ratio in the soleus.** Mean (± SEM) data are presented for young sedentary controls (YS), young exercise trained (YE), old sedentary controls (OS), and old exercise trained (OE). (**a**) indicates a significant aging effect. (*) indicates a significant exercise effect.

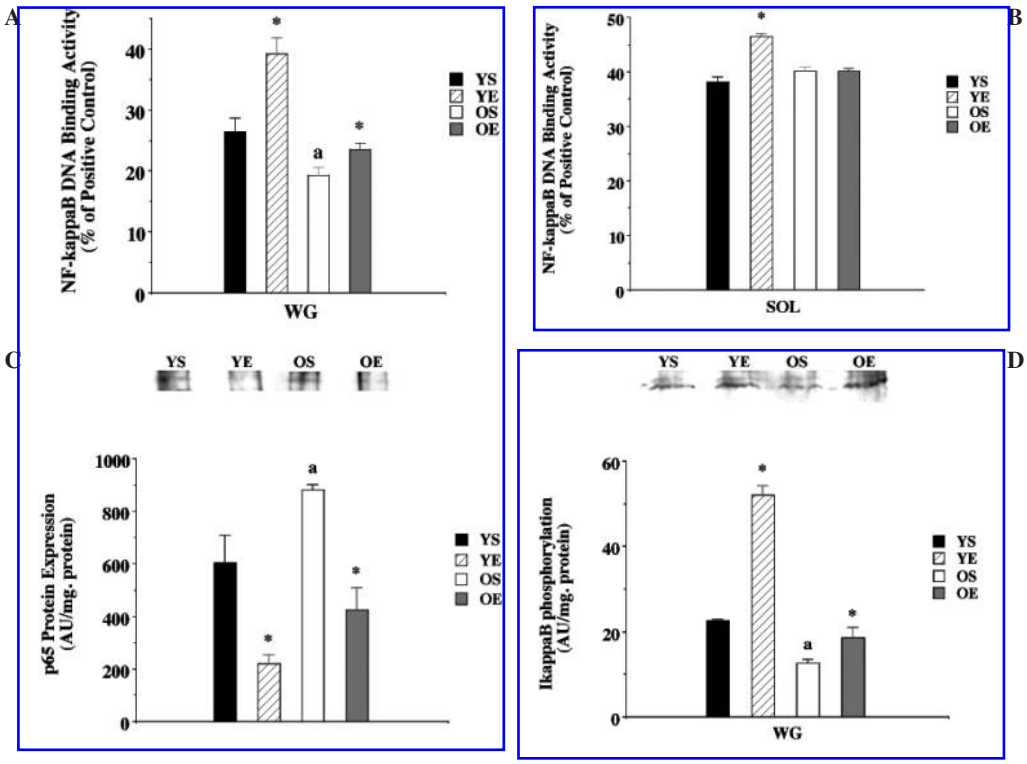

FIG. 4. (**A**) **Age and exercise training effects on NF-kappaB (NF-κB) regulation and activation in the white gastrocnemius** from the following groups: young sedentary controls (YS), young exercise trained (YE), old sedentary controls (OS), and old exercise trained (OE). NF-κB DNA binding activity as a percent of positive controls was quantified via a sensitive ELISA using the consensus NF-κB binding sequence (5′-GGGACTTTCC-3′). (**a**) indicates a significant aging effect. (*****) indicates a significant exercise effect. (**B**) **Age and exercise training effects on NF-κB regulation and activation in the soleus** from the following groups: young sedentary controls (YS), young exercise trained (YE), old sedentary controls (OS), and old exercise trained (OE). NF-κB DNA binding activity as a percent of positive controls was quantified via a sensitive ELISA using the consensus NF-κB binding sequence (5′-GGGACTTTCC-3′). (*****) indicates a significant exercise effect. (**C**) **Age and exercise training effect on protein expression of the p65 subunit of NF-κB in the white gastrocnemius.** Representative Western blots and mean (± SEM) data are presented for young sedentary controls (YS), young exercise trained (YE), old sedentary controls (OS), and old exercise trained (OE). (**a**) indicates a significant aging effect. (*****) indicates a significant exercise effect. (**D**) **Age and exercise training effect on IκB phosphorylatioin in the white gastrocnemius.** Representative Western blots and mean (± SEM) data are presented for young sedentary controls (YS), young exercise trained (YE), old sedentary controls (OS), and old exercise trained (OE). (**a**) indicates a significant aging effect. (*****) indicates a significant exercise effect.

Phillips and Leeuwenburgh (34) identifying a depression in NF-κB DNA binding activity in white skeletal muscle. Exercise training significantly increased NF-κB DNA binding activity of the white gastrocnemius by 49% in the young group and by 23% in the old group. In contrast, aging had no effect on soleus NF-κB DNA binding activity (Fig. 4B). Moreover, exercise training increased soleus NF-κB DNA binding activity in the young group (+18%), but not the old group.

Next we tested the hypothesis that the age-related decline and exercise-induced elevation of NF-κB DNA binding activity were a function of altered NF-κB protein levels. The protein expression of the p65 subunit was used as a marker of NF-κB dimer protein levels. Aging increased p65 protein expression by 41% in the white gastrocnemius (Fig. 4B). Exercise training elicited the opposite effect, decreasing p65 protein expression by 63% in the young age group and by 52% in the old age group (Fig. 4C). Interestingly, we observed similar results in the soleus. Therefore, the decrease in NF-κB DNA binding activity of the white gastrocnemius with aging and increase in NF-κB DNA binding activity from exercise training were not simply a result of altered protein levels.

Given that phosphorylation of the inhibitory protein IκB triggers release of inactive NF-κB and subsequent activation of NF-κB binding to DNA, the depression of NF-κB from aging and elevation with exercise training could be directly related to changes in IκB phosphorylation. Therefore, we tested the hypotheses that (a) age would decrease IκB phosphorylation, and (b) exercise training would increase IκB phosphorylation in the white gastrocnemius. We found that phosphorylated IκB decreased by 44% with aging (Fig. 4D). Exercise increased phosphorylated IκB by 130% in the white gastrocnemius of young rats. In addition, exercise training resulted in 47% higher levels of phosphorylated IκB in the white gastrocnemius of old rats as well. Thus these data

clearly indicate that age and exercise alterations in NF-κB activity were a function of impaired phosphorylation of the inhibitory protein IκB, not altered NF-κB protein levels.

DISCUSSION

Hindlimb muscles from aging rats that exercise regularly had larger muscle mass and greater mean fiber cross-sectional area than sedentary old rats. Exercise training also protected against the age-induced increase in connective tissue. Regular exercise also ameliorated markers of apoptosis (DNA fragmentation, caspase-3 cleavage) as well as elevated Bax/Bcl-2 ratio by decreasing Bax and increasing Bcl-2 protein expression. To our knowledge, these are the first data to indicate that exercise provides a protective effect against apoptotic signaling in aging skeletal muscle, associated with improved muscle morphology.

Data from aging human populations also indicate that exercise training can provide protection against muscle sarcopenia. In older patients, both resistive exercise and endurance exercise appear to lessen loss of muscle mass and atrophy (5, 41, 43). Our results suggest that a contributing mechanism by which regular exercise may protect against muscle wasting in aging populations is through modulation of apoptotic signaling pathways. Interestingly, our findings mimic the protective effects of caloric restriction on aging skeletal muscle morphology detailed recently by Payne *et al.* (33) and McKiernan *et al.* (29). Caloric restriction attenuated the reduction in muscle fiber number and cross-sectional area observed with aging (29, 33). An alternative explanation is that endurance training resulted in muscle hypertrophy during the 12-week program. While the aging literature in humans and rats does not provide support the hypothesis that treadmill endurance training causes hypertrophy in healthy older subjects (5, 38, 41, 45) reported that high-frequency treadmill running in senescence-accelerated mice resulted in hindlimb muscle hypertrophy. A definitive conclusion therefore cannot be drawn without direct comparison with a group of old (24 mo) sedentary rats sacrificed at beginning of the exercise training program.

Caloric restriction also reduced age-induced accumulation of muscle connective tissue (33). Aging increases collagen content in skeletal muscle (33). Increased collagen and connective tissue from aging occurs primarily in white muscle, increasing material stiffness and internal work (33). Therefore, exercise training may reduce stiffness and internal work in aging skeletal muscle, which would thus protect muscle contractile function.

Exercise training reversed age-induced elevation of histone-associated DNA fragmentation indicating that exercise training attenuates age-induced susceptibility to myonuclear and satellite cell apoptosis (42, 44). A critical step in the execution of the apoptotic program eliciting DNA fragmentation is cleavage of caspase-3 (54). Caspases are **c**ysteine-dependent **asp**artate-specific prote**ases** functioning as endonucleases integral in the final execution of nuclei and cell death (16, 54). Remarkably, exercise diminished cleaved caspase-3 levels by over 95%, indicative of a robust effect of regular exercise on key integrative regulator of apoptosis. Given that skeletal muscle is multinucleated, exercise training in the elderly may provide protection against loss of muscle fibers as well as reduction in fiber cross-sectional area by reducing the rate of myonuclei and satellite cell apoptosis (2, 6, 42). Amelioration of apoptotic signaling and caspase activation by regular exercise may also provide protection against protein degradation and reduced contractile function independent of apoptosis of nuclei (13, 37). This area of study is literally in its infancy.

The large effects of age and exercise training on cleaved caspase-3 indicate that exercise training may regulate apoptosis through upstream Bcl-2 family signaling, with potential contributions of the cytokine/fas pathway or the Ca^{2+}/ER pathway (25, 34). Increased pro-apoptotic signaling through the mitochondrial Bcl-2 family has been implicated as an important mechanism leading towards muscle cell loss and atrophy with aging (6, 25). A critical event in mitochondrial-driven apoptosis is the formation of permeable membrane pores (16, 33, 53), regulated by the balance between competing anti-apoptotic Bcl-2 family proteins such as Bcl-2 and pro-apoptotic proteins including Bax (16).

We found that aging increased Bax and Bax/Bcl-2 ratio in skeletal muscle. These findings are consistent with the hypothesis that aging results in dysregulation of Bcl-2 pathway signaling leading to caspase-3 activation and apoptosis in skeletal muscle. Previously, elevation of Bax and caspase-9 protein expression in aging skeletal muscle had been reported by Alway *et al.* (3). In contrast, exercise training resulted in a marked reduction of pro-apoptotic Bax and elevated Bcl-2 protein expression in skeletal muscle from old rats. Exercise-induced decrease in Bax and upregulation of Bcl-2 followed similar patterns in the white gastrocnemius and soleus. This is illustrated by a large reduction of the Bax/Bcl-2 ratio by exercise training in both the white gastrocnemius and soleus muscle soleus. Exercise training dramatically attenuates the age-induced elevation of Bax/Bcl-2 ratio by both decreasing Bax and increasing Bcl-2 protein expression. This is consistent with conference of protection mitochondrial pore formation, caspase cleavage, myonuclei removal, and cell survival by reducing the ratio of pro- to anti-apoptotic proteins (e.g., Bax/Bcl-2) (16, 32).

Our results also indicate that with inducibility of Bcl-2, pathway proteins occur in both fast-twitch and slow twitch muscle. Recently, Siu *et al.* (42) reported that exercise reduced DNA fragmentation and increased Bcl-2 in young skeletal muscle and heart. Large attenuation of Bax/Bcl-2 is consistent with a role of the Bax/Bcl-2 ratio in regulation of downstream cleavage of caspase-3 and apoptosis. Thus aging skeletal muscle did retain some ability to adapt to the stresses of exercise by increasing protection against pro-apoptotic pathways.

Besides Bcl-2 signaling, other apoptotic proteins and pathways appear to be altered with aging, including XIAP, ARC, FLIP, caspase-12, and ID2 (11, 25, 42). It is possible that exercise training may confer muscle cell protection against caspase activation in old rats by upregulating anti-apoptotic proteins Bcl-X_L, XIAP, and ARC (43). In addition, XIAP may be upstream of NF-κB activation (17). While the effect of exercise on modulating these alternate signaling proteins of apop-

tosis in aging skeletal muscle is unknown, it is likely they may be integrated into exercise-induced attenuation of the decline muscle mass and alterations in muscle morphology and a focus of future research.

A number of cell-protective signaling pathways regulate apoptosis upstream of the Bcl-2 family and caspases (30, 31). Upstream stress proteins that play a role in reducing Bax/Bcl-2 ratio and activation of caspase-9 and caspase-3 in skeletal muscle include attenuation of inducible nitric oxide synthase (iNOS), heat shock protein 70 (HSP70), the transcription factor NF-κB, IGF-1, and Mn-isoform of superoxide dismutase (Mn-SOD) (15, 19, 27). Aging is characterized by a general impairment of stress response and cell survival signaling, leading toward disrupted regulation of apoptosis (6). Recently, we demonstrated that exercise training resulted in a reversal of age-induced upregulation of iNOS activity and protein expression in skeletal muscle (44). Here we found that HSP70 protein expression was not inducible by age or exercise, except in the young age group. This is consistent with an impaired response of HSP70 and its upstream transcription factor heat shock factor-1 (HSF-1) to an acute bout of muscle contractions observed by Vasilaki *et al.* (48).

NF-κB is a transcription factor involved in response to mechanical and oxidative stress (15, 22, 23). We found that NF-κB DNA binding activity did not increase with age in the white gastrocnemius as in the liver (35), but in fact was significantly lower in OS than YS (Fig. 4A), consistent with recent data from Phillips and Leeuwenburgh (34) in the superficial (white) vastus lateralis. In contrast, exercise training increased NF-κB DNA activation of the white gastrocnemius in both old and young rats. Moreover, NF-κB DNA binding activity in the old exercise group was similar to young sedentary controls. The reduced response of NF-κB DNA binding activity in the soleus to aging and exercise compared with the white gastrocnemius indicates a fiber type difference in regulation and protection against apoptosis in aging muscle, with white or Type IIb fibers more inducible than red or Type I. While both Type I and Type II fibers experience atrophy, cell loss is predominantly characteristic of Type II muscle fibers (6, 18, 25).

We postulate that elevation of NF-κB DNA binding activity in white skeletal muscle by habitual exercise is a stress adaptation that is cell protective. NF-κB activation has been shown recently to be responsive to mechanical strain in a diaphragm preparation (22). In another recent study, caloric restriction induced an increase in NF-κB DNA binding activity (34) in a manner similar to exercise training found in the current study.

Upregulation of NF-κB DNA binding activity stimulates cellular protection against apoptosis through the Bcl-2 family, via upregulation of the anti-apoptotic protein Bcl-2 and reduction of the pro-apoptotic protein Bax and downstream caspases (1, 15, 19, 55). Both caloric restriction and regular exercise thus protected against the ravages of aging on white muscle morphology.

In contrast, Radak *et al.* (35) recently reported that aging increased NF-κB DNA binding activity in the rat liver, which was reversed by exercise training. We postulate that differences between predominantly mitotic (e.g., liver) and postmitotic (e.g., skeletal muscle) tissues could account for the divergent findings in NF-κB DNA binding activity from aging. NF-κB DNA binding activity is increased in mitotic tissues such as the colon and liver with aging, leading toward an increased risk of cancer (35, 50). In other words, downregulation of apoptosis in mitotic tissues, in which the rate of cell removal by apoptosis is reduced, elevates the risk of cancer. In contrast, upregulation of apoptosis in skeletal muscle, in which cancer risk is minimal, irreversible loss of myocytes poses a danger to healthy tissue function. Aging is associated with chronic elevation of iNOS, cytokines, and other inflammatory proteins with an age-related decline in function of mitotic tissues such as kidney, liver, and brain (8, 29). NF-κB activation increases expression of genes involved in inflammation and repair including adhesion factors (e.g., ICAM-1, VCAM-1), iNOS (inducible nitric oxide synthase), COX-2 (cyclooxygenase-2), HO-1 (heme oxygenase-1), and cytokines tumor-necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) (15, 23). In contrast, caloric restriction, a model that consistently increases lifespan, reversed age-induced upregulation of iNOS, NF-κB, TNF-α, and IL-1β in the liver (8). Our results indicate that inflammatory and apoptotic signaling in skeletal muscle are affected in a complex, interactive manner.

NF-κB is a redox-sensitive dimer comprised of subunits p65 and p50 as heterodimer or homodimer pairs, is inactive in the cytosol when bound to the inhibitory protein IκB. Phosphorylation of IκB releases inactive NF-κB, which then translocates from the cytosol into the nucleus where NF-κB binds to DNA, resulting in activation of NF-κB-regulated transcription (15, 19). Age and exercise effects on NF-κB DNA binding activity were a function of phosphorylation state of its inhibitory protein IκB rather than NF-κB protein subunit levels. The ability of exercise training to reverse age-induced changes in IκB phosphorylation and thus NF-κB DNA binding activity has important physiological and clinical implications as well. Impaired or altered protein phosphorylation and consequent changes in cell signaling regulation have been noted in aging tissues (49). Disrupted phosphorylation of key signaling proteins such as IκB or Akt/PKB may indeed be critical targets of aging in skeletal muscle, reducing cell protection (26, 52). Thus a mechanism by which exercise training provides cell protection is through relief of impaired phosphorylation of key cytoprotective pathway proteins. As treadmill exercise results in cyclic mechanical strain and oxidant production in skeletal muscle, upregulation of NF-κB DNA binding activity through increased IκB phosphorylation is consistent with an adaptive response by aging muscle in order to increase cell protection.

Kayo *et al.* (21) reported selective, age-related upregulation of skeletal muscle transcripts (mRNA) involved in oxidative stress and inflammation including NF-κB, cytokines, and cytokine receptors. However, protein levels and activity were not assessed and the effects of exercise training on NF-κB DNA binding activity, especially in aging skeletal muscle, were not determined. Given that age increases NF-κB activation in skeletal muscle in the current study (Fig. 4A) in a recent publication (34), these findings are consistent with post-translational but not transcriptional regulation of NF-κB activation with aging and exercise in skeletal muscle. However, as other inflammatory proteins, such as iNOS (44), may

be upregulated in skeletal muscle with aging, the complex muscle-specific effects of aging and exercise on skeletal muscle inflammatory signaling need further investigation.

In summary, our results support the ability of exercise to affect key regulatory mechanisms involved in apoptosis of aging skeletal muscle. Combined with DNA fragmentation data and muscle morphology, our data indicate that exercise training increases protection against the pro-apoptotic process in skeletal muscle, and attenuates changes in muscle morphology with advancing age. Future investigations will continue to explore to complex role of exercise in cell protection in aging skeletal muscle.

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ABBREVIATIONS

apaf-1, apoptotic protease activating factor-1; ARC, apoptosis repressor with caspase recruitment domain (CARD); Bak, Bcl-2 homologous antagonist/killer; Bax, Bcl-2-associated X protein; Bcl-2, proto-oncogene anti-apoptotic protein; Bik, Bcl-2 interacting killer; COX-2, cyclooxygenase-2; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol-bis-(2-aminoethyl)-*N*,*N*,*N*′, *N*′-tetraacetic acid; FLIP, FLICE-inhibitory protein; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HO-1, heme oxygenase-1; HRP, horseradish peroxidase; ICAM-1, intercellular adhesion molecule-1; ID2, inhibitor of differentiation-2; IκB, IkappaB; INOS, inducible nitric oxide synthase; IL-1β, interleukin-1 beta; NADPH, reduced nicotinamide diphosphate; NF-κB, nuclear factor-kappaB; PBS, phosphate-buffered saline; PKB, protein kinase B; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylene diamine; TNF-α, tumor-necrosis factor-alpha; VCAM-1, vascular cell adhesion molecule 1; VO_{2max}, maximal oxygen consumption; XIAP, inhibitor of apoptosis protein X.

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Address reprint requests to:
John M. Lawler, Ph.D.
Director: Redox Biology and Cell Signaling Laboratory
Department of Health and Kinesiology
Texas A & M University
College Station, TX 77843-4243

E-mail: jml2621@neo.tamu.edu

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