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Involvement of oxidative stress and caspase 2-mediated intrinsic pathway signaling in age-related increase in muscle cell apoptosis in mice

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Abstract

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Apoptosis has been implicated as a mechanism of loss of muscle cells in normal aging and plays an important role in age-related sarcopenia. To test the hypothesis that caspase 2 and c-Jun NH_2-terminal kinase (JNK)-mediated intrinsic pathway signaling contribute to skeletal muscle cell apoptosis in aging, we compared activation of caspase 2 and JNK and the in vivo expression of 4-hydroxynonenal protein adducts (4-HNE), inducible nitric oxide synthase (iNOS), glucose-6-phosphate dehydrogenase (G6PDH), B-cell lymphoma-2 (BCL-2), BAX, and phospho-BCL-2 in gastrocnemius muscles of young (5 months old) and old (25 months old) mice. A distinct age-related increase in 4-HNE and iNOS expression was readily detected in mice. Increased oxidative stress and iNOS induction were further accompanied by a decrease in G6PDH expression, activation of caspase 2 and JNK, and inactivation of BCL-2 through phosphorylation at serine 70, and caspase 9 activation. Regression analysis further revealed that increased muscle cell death in aging was significantly correlated with changes in the levels of these molecules. Taken together, our data indicate that caspase 2 and JNK-mediated intrinsic pathway signaling is one of the mechanisms involved in age-related increase in muscle cell apoptosis.

Keywords

Oxidative stress; Caspase 2; JNK; BCL-2 phosphorylation; Muscle cell apoptosis; Aging; Mice

Introduction

Skeletal muscle mass declines with advancing age, such that by 60–70 years of age, skeletal muscle mass in humans has decreased by 25–30%, resulting in a significant decrease of muscle strength [reviewed in 1]. Such age-related loss of muscle mass (sarcopenia) has far reaching consequences for the elderly, including impaired physical function, increased risk of falls, fractures, dependency, and death. Apoptosis has been implicated as a mechanism of loss of muscle cells in normal aging [2] and plays an important role in age-related sarcopenia [1–4]. Involvement of apoptosis, characterized by caspase 3 activation, in normal age-related loss of skeletal muscle mass in mice, has also been highlighted in a Science article [5]. However, the key signaling pathway that leads to muscle cell death in aging is not well understood.

The amount of oxidative stress increases as an organism ages and is postulated to be a major causal factor of senescence [6]. Oxidative stress has also been implicated in apoptotic signaling of a variety of cell types during aging and in the pathogenesis of various age-related neurodegenerative diseases [7–9]. There are many possible targets of the oxidative stress, including the c-Jun NH2-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) signaling, which constitute a critical component of apoptotic signaling in various cell systems [10–13]. Activation of JNK or MAPK has been reported in various cell systems during aging [7, 14, 15].

A role for p38 MAPK-mediated signaling in the transcription control of inducible nitric oxide synthase (iNOS) gene expression has been suggested in glial cells [16]. NO-production through up-regulation of iNOS could also be a potential target of JNK signaling [17]. A growing body of evidence indicates that increased NO synthesis, through up-

regulation of iNOS, promotes apoptosis in various cell systems via activation of the mitochondria-dependent intrinsic pathway signaling [12, 18–20]. Indeed, in a recent study, we have demonstrated the involvement of JNK and NO-mediated signaling pathway that promotes muscle cell apoptosis during injury by provoking B-cell lymphoma-2 (BCL-2) phosphorylation, leading to its inactivation, and subsequent activation of the intrinsic pathway signaling [21]. We, thus, hypothesize that increased oxidative stress and iNOS could, through perturbation of the BAX/BCL-2 rheostat, trigger the mitochondria-dependent death pathway and promote muscle cell death in aging.

Of all caspases discovered to date, caspase 2 is the most evolutionarily conserved and plays an important role in inducing apoptosis in various cell systems [22–28]. Caspase 2 appears to be an initiator caspase working upstream of the mitochondria-dependent apoptotic pathway and can be activated by a variety of cellular stresses, including oxidative stress [22–28]. Available evidence further suggests that caspase 2 is an upstream activator of JNK-mediated apoptotic signaling in doxorubicin-induced apoptosis [29]. The role of caspase 2 in muscle cell apoptosis is, however, not known. One possibility is that increased oxidative stress in aging could trigger muscle cell apoptosis through activation of caspase 2, which, in turn triggers JNK-mediated intrinsic pathway signaling.

Aging in mice is further characterized by a decrease in glucose-6-phosphate dehydrogenase (G6PDH) activity in oocytes [30], the first and rate-limiting enzyme of the pentose phosphate pathway, which catalyzes the oxidation of glucose-6-phosphate to 6-phosphoglunolactone and 6-phospho-gluconate, produces NADPH and ribulose 5-phosphate [31]. In an earlier study, Nutt et al. [23] have shown that depletion of G6PDH activates caspase 2 and promote oocyte apoptosis. Given that G6PDH also regulate caspase 2 activation and apoptosis, we further speculate an age-dependent decrease in G6PDH levels in skeletal muscles.

To test these possibilities, in the present study, we analyzed activation of caspase 2 and JNK and in vivo expression of 4-hydroxynonenal protein adducts (4-HNE), iNOS, G6PDH, BCL-2, BAX, and phospho-BCL-2 in gastrocnemius muscles between young and old mice. Our data indicate that the increased oxidative stress coupled with activation of caspase 2 and JNK and serine phosphorylation of BCL-2 leading to its inactivation likely contribute to muscle cell apoptosis in aging.

Materials and methods

Animals

Six young (5 months old) and six old (25 months old) male C57B1/6 mice were obtained from the Jackson Laboratory. Animals were housed in a standard animal facility under controlled temperature (22°C) and photoperiod (12 h of light, 12 h of darkness) with food and water ad libitum. Animal handling and experimentation were in accordance with the recommendation of the American Veterinary Medical Association and were approved by the Charles R. Drew University School of Medicine and Science animal care and use review committee.

Tissue preparation

All mice were euthanized with a lethal injection of sodium pentobarbital (200 mg/kg BW). The gastrocnemius muscles from young and old mice were quickly removed and weighed. Portions of the tissue were quickly frozen in liquid N_2 and stored frozen for subsequent analysis by Western blotting. Additional portions in each group were fixed in 4% paraformaldehyde for in situ detection of muscle cell apoptosis or immunohistochemistry.

Assessment of apoptosis

In situ detection of cells with DNA strand breaks was performed in paraformaldehyde-fixed, paraffin-embedded muscle sections by the terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling (TUNEL) technique [12, 21, 32] using an ApopTag-peroxidase kit (Chemicon International, Inc., San Francisco, CA). Enumeration of TUNEL-positive nuclei was carried out in muscle sections stained with ApopTag peroxidase kit using an American Optical Microscope with an ×40 objective and a pair of ×10 eyepieces. Methyl green was used as a counterstain to detect non-apoptotic nuclei. A square grid fitted within one eyepiece provided a reference of 62,500 μm^2. The rate of muscle cell apoptosis was expressed as the percentage of the TUNEL-positive apoptotic nuclei per total nuclei (apoptotic plus non apoptotic) present within the reference area [21].

Immunohistochemical analyses

Paraformaldehyde-fixed, paraffin-embedded muscle sections were immunostained as described previously [12, 21, 33]. Primary antibodies included rabbit polyclonal G6PDH (1:50; Abcam, Cambridge, UK), iNOS (1:100; BD Transduction Laboratories, San Diego, CA), BAX (1:200), and BCL-2 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), antibodies, and a mouse monoclonal 4-HNE antibody (25 mg/ml; Oxis International Inc., Foster City, CA). Immunoreactivity was detected using biotinylated anti-rabbit or anti-mouse IgG secondary antibody followed by avidin-biotinylated horseradish peroxidase complex, and visualized with diaminobenzidine tetrahydrochloride (DAB) as per the manufacturer's instructions (VECTA-STAIN Elile ABC Rabbit or Mouse IgG kit, Burlingame, CA). Slides were counterstained with hematoxylin. Negative control was run for every assay and was processed in an identical manner, except the primary antibody was substituted by the rabbit or mouse IgG.

In certain cases, immunoreactivity was quantified by computerized densitometry using the ImagePro Plus, version 5.1 software (Media Cybernetics, Silver Spring, MD) coupled to an Olympus BHS microscope equipped with a VCC video camera as described previously [34]. In brief, after images are properly calibrated for background lighting, optical density per fiber area (OD/AREA) results are proportional to the unweighted average optical density which is used to determine the concentration of immunoreactive antigen. For each animal at least 100 fibers were measured.

Western blotting

Western blotting was performed using muscle lysates as described previously [12, 21]. In brief, proteins (50–80 μg) were separated on a 4–12% SDS-polyacrylamide gel with MES or MOPS buffer purchased from Invitrogen (Carlsbad, CA, USA) at 200 V. Gel was

transferred on an Immuno-blot PVDF Membrane (Bio-Rad, Hercules, CA) overnight at 4°C. Membranes were blocked in blocking solution (0.3% Tween 20 in Tris-buffered saline and 10% non-fat dry milk) for 1 h at room temperature then probed using rabbit polyclonal cleaved caspase 2 (1:500) and cleaved caspase 9 (1:500; Santa Cruz Biotechnology), and phospho (serine 70)-BCL-2, which detects endogenous levels of BCL-2 only when phosphorylated at serine 70 (1: 500, Cell Signaling Technology), and G6PDH (1:500) and mouse monoclonal phospho-JNK, which detects JNK only when phosphorylated at threonine 183 and tyrosine 185 (1:200; Santa Cruz Biotechnology), 4-HNE and iNOS (1:1000; BD Transduction Laboratories) antibodies for 1 h at room temperature or overnight at 4°C with constant shaking. Following three 10-min washes in TBS-T buffer, membranes were then incubated in anti-rabbit (Amersham Biosciences, Piscataway, NJ), anti-goat, or anti-mouse IgG-HRP (Santa Cruz Biotechnology) secondary antibodies at a 1:2000 dilution. All antibodies were diluted in blocking buffer. For immunodetection, membranes were washed three times in TBS-T wash buffer, incubated with ECL solutions per the manufacturer's specifications (Amersham Biosciences), and exposed to Hyper film ECL. The membranes were stripped and reprobed with a rabbit polyclonal GAPDH (1:2000) for normalization of the loading. Band intensities were determined using Quantity One software from Bio-Rad (Hercules, CA). All measurements were replicated at least three times.

Muscle fiber cross-sectional area

The immunohistochemical slides were used for determination muscle fiber cross-sectional area (CSA) using the ImagePro Plus, version 5.1 software (Media Cybernetics, Silver Spring, MD) coupled to an Olympus BHS microscope equipped with a VCC video camera [34]. For each animal at least 100 fibers were measured.

Statistical analysis

Statistical analyses were performed using the SigmaStat 2.0 Program (Jandel Cooperation, San Rafael, CA). Results were tested for statistical significance using the *t*-test. Differences were considered significant if $P < 0.05$. Pearson product-moment correlation coefficients were computed to evaluate the relationship between variables.

Results

Normal aging in mice is associated with loss of muscle mass and muscle fiber CSA

Compared to young animals, the relative gastrocnemius muscle weight normalized to body weight were decreased significantly ($P = 0.001$) by 22.9% in the old mice. There was also a significant ($P < 0.001$) reduction (by ∼60%) in individual fiber CSA in old mice (726 ± 32 μm^2) compared to that of young animals (1,801 ± 35 μm^2).

Age-related increase in oxidative stress, iNOS expression, and muscle cell apoptosis in mice

To determine whether induction of oxidative stress and iNOS is a feature of normal aging, we compared the in vivo expression of 4-HNE, a biomarker of oxidative stress [35, 36] and iNOS, which is critical for NO-mediated toxicity, in gastrocnemius muscles of young and old mice. A distinct age-related increase in 4-HNE and iNOS expression, as evidenced by

immunohistochemistry (figures not shown) as well as by immunoblotting (Fig. 1a), was noted in skeletal muscles of the old mice. Such age-related increase in 4-HNE and iNOS levels, as detected by Western blotting, was further corroborated by densitometric evaluation (Fig. 1b).

Given that oxidative stress and increased NO (through up regulation of iNOS) constitute a critical component of apoptotic signaling in various cell systems during aging [7, 9, 34], we then compared the incidence of apoptosis, detected by TUNEL assay, in the gastrocnemius muscles between young and old mice. Morphometric analysis revealed a significant ($P < 0.0001$) increase in the percent of muscle cell apoptosis in the old mice, when compared to that of young animals (Fig. 2). This is consistent with the earlier reports in murine models [4, 5, 37] showing age-related increase in muscle cell apoptosis.

Expression of G6PDH is decreased in skeletal muscle in aging

Given that G6PDH levels are decreased in aging and this can trigger apoptosis in various cell systems [23, 38, 39], here we compared the expression of G6PDH in skeletal muscles of young and old mice. We found a substantial decrease in G6PDH in skeletal muscles of the old mice, as evidenced by both immunohistochemistry (Fig. 3a) as well as by immunoblotting (Fig. 3b) when compared to those of young mice. Image analysis of muscle sections confirmed the visual inspection and showed a significant decrease in G6PDH expression (by 23.7%; $P < 0.001$) with aging. Likewise, densitometric analysis of band intensities in the immunoblot also revealed a significant ($P < 0.05$) decrease in G6PDH levels in skeletal muscles of the old animals compared to that of young mice (Fig. 3c).

Age-related increase in oxidative stress and muscle cell apoptosis is associated with activation of caspase 2 and JNK in skeletal muscles

Given that increased oxidative stress [24, 28] and a decrease in G6PDH activity [23] can trigger caspase 2 activation and apoptosis in some cell lines, we then examined the activation of caspase 2 by immunoblotting, using an antibody that specifically detects cleaved caspase 2. Cleaved caspase 2 levels were increased in skeletal muscles of old mice compared to that of young animals, where no expression of cleaved caspase 2 was detected (Fig. 4a). Such age-related increase in cleaved caspase 2 levels was further substantiated by densitometric evaluation (Fig. 4b). Because caspase 2 is an upstream activator of JNK [29], which constitutes a critical component of apoptotic signaling in skeletal muscles [21], we further examined the activation of JNK in skeletal muscles during aging by Western blotting. We found a substantial increase in phospho-JNK levels in skeletal muscles of the old mice, when compared to those of young mice (Fig. 4c). Such age-related increase in phospho-JNK levels was further corroborated by densitometric evaluation (Fig. 4d). Thus, normal aging is associated with activation of caspase 2 and JNK in skeletal muscles.

Aging induces BCL-2 phosphorylation and activation of caspase 9 in skeletal muscle

Because the relative ratio of antiapoptotic and proapoptotic BCL-2 family members such as BCL-2 and BAX has been hypothesized to be a major determinant of cellular vulnerability to apoptosis in the intrinsic pathway signaling [40, 41], we further compared the expression profiles of these proteins in skeletal muscles between young and old mice.

Immunohistochemical analysis revealed little or no increase in BAX levels whereas the expression of total BCL-2 levels appeared to be markedly increased in old animals compared to young ones (Fig. 5). Image analysis further confirmed the visual inspection. Compared to young mice, we found a significant ($P < 0.001$) increase by ∼50% in BCL-2 expression with no change in BAX immunoreactivity (data not shown) in gastrocnemius muscles of aged mice.

Because the phosphorylation status of BCL-2 plays an important role in its prosurvival activity [42, 43], we further examined whether the increased muscle cell apoptosis in aging is associated with BCL-2 phosphorylation. Levels of serine-phosphorylated form of inactive BCL-2 were increased in skeletal muscles of old mice compared with young animals, where no expression was detected (Fig. 6a) Densitometric analysis further revealed a significant ($P < 0.001$) increase in phospho-BCL-2 levels in muscle lysates in the old mice compared to that of young animals (Fig. 6b). Together, these data indicate a perturbation of BAX/BCL-2 rheostat during normal aging process.

To further explore the involvement of the mitochondria-dependent pathway for induction of muscle cell apoptosis during normal aging, we examined the activation of caspase 9, the key initiator caspase in this pathway, by immunoblotting. Levels of cleaved caspase 9 were increased in skeletal muscles of old mice compared to that of young animals, where no expression was detected (Fig. 7a) Densitometric analysis further revealed a significant ($P < 0.001$) increase in active caspase 9 levels in muscle lysates in the old mice compared to that of young animals (Fig. 7b).

Correlation of changes in the incidence of muscle cell apoptosis with the changes in various stress or apoptotic markers in aging

The relationships between changes in the incidence of apoptosis with the changes in other variables associated with aging are summarized in Table 1. As expected, age-related increase in muscle cell apoptosis was negatively correlated ($r = –0.87$; $P = 0.02$) with the changes in the fiber area. Changes in the incidence of muscle cell apop-tosis in aging were positively and significantly correlated with the changes in levels of 4-HNE, iNOS, active caspase 2, phospho -JNK, and phospho-BCL2 in gastrocnemius muscles between young and old mice. A negative correlation was, however, observed between the changes in the incidence of apoptosis with the changes in the levels of G6PDH between young and old animals.

Discussion

In this study, we have confirmed and extended previous observations [5, 37, 44] by demonstrating age-related loss of muscle mass (sarcopenia) in mice. Compared to young animals, the relative gastrocnemius muscle weight normalized to body weight was decreased by 22.9% in the old mice. Emerging evidence now suggests that muscle cell apoptosis play a considerable role in sarcopenia [1–5, 37, 45]. Indeed, in the present study, we found a marked increase ∼20%; $P < 000.1$) in the TUNEL-positive nuclei in gastrocnemius muscle with aging in mice. It is pertinent to note here that we counted all TUNEL-positive nuclei as muscle cell nuclei. Thus, we do not know whether the TUNEL-positive nuclei are all

myonuclei and not satellite cell nuclei. We also do not know how fast this process occurs in vivo, specifically in multinucleated skeletal muscle fibers in mice during aging. Generally, there is an intimate relationship between nuclear number and muscle fiber CSA and the loss of muscle cell nuclei through increased apoptosis likely contributes to fiber atrophy and, in turn, sarcopenia [reviewed in 45]. Indeed, in the present study, we found a significant (P <0.001) reduction (by 63.1%) in the individual fiber size in old mice compared to that of young animals. Regression analysis further demonstrated an inverse relationship (r = –0.87; P = 0.002) between age-related increase in muscle cell apoptosis and changes in the fiber CSA. Collectively, these data further underscores the importance of increased muscle cell apoptosis in age-related loss of muscle mass.

We next evaluated the signaling pathway in age-related activation of muscle cell apoptosis. Two major pathways, intrinsic and extrinsic, are involved in the process of caspase activation and apoptosis in various cell systems [41]. In an earlier study, Siu et al. [37] have demonstrated the involvement of mitochondria-dependent intrinsic pathway signaling, characterized by cytochrome c and DIABLO release from mitochondria and activation of caspase 9 in age-related increase in muscle cell apoptosis in gastrocnemius muscles in rats [37]. The results of the present study confirm and extend those findings by demonstrating that such age-related increase in muscle cell apoptosis in mice is associated with a distinct increase in oxidative stress but a decrease in G6PDH expression, activation of caspase 2 and JNK, and inactivation of BCL-2 through phosphorylation at serine 70. The observed changes in the incidence of muscle cell apoptosis in aging were significantly correlated with the changes in levels of these molecules in gastrocnemius muscles between young and old mice.

The amount of oxidative stress increases as an organism ages and is postulated to be a major causal factor of senescence [6]. Oxidative stress has also been implicated in apoptotic signaling of a variety of cell types during aging and in the pathogenesis of various age-related neurodegenerative diseases [7–9]. Emerging evidence now suggests that oxidative stress can also promote caspase 2 activation in various cell systems [24, 28]. Thus, the signal for activation of caspase 2 most likely emanates from age-related increase in oxidative stress. It is also pertinent to note here that caspase 2 may also be activated by a decrease in G6PDH levels. For example, Nutt et al. [23] have demonstrated that inhibition of G6PDH promotes caspase 2 activation and promote oocyte apoptosis. Thus, it is possible that increased oxidative stress in normal aging could promote caspase 2 activation and muscle cell death through a defective cellular metabolism. The observed age-related decrease in G6PDH levels in skeletal muscles is consistent with the later possibility. Both these possibilities merits further investigations.

An apoptotic role of caspase 2 has been found in a variety of cell systems, including neurons, Jurkat cells, hepatocytes, lymphocytes, and male and female germ cells [25, 46–50]. However, the regulation of apoptosis by caspase 2 is more complex and varies depending upon tissue type, cell lineage, developmental stage, and nature of the apoptotic stimulus. For example, caspase 2-deficient are resistant to developmental and doxorubicin-induced oocyte apoptosis [46]. B lymhoblasts lacking caspase 2 are deficient for granzyme B but not Fas- or drug-induced apoptosis [46]. Also, while caspase 2-deficient and wild-type

splenocytes underwent almost equivalent levels of apoptosis after treatment with anti-Fas or UV irradiation, caspase 2-deficient splenocytes were substantially resistant to heat shock-induced apoptosis [25]. It is possible that in instances where apoptosis is not blocked by caspase 2 deficiency, its function can be compensated by other caspases in the caspase 2-deficient mice. Indeed, there have been studies indicating activation of initiator caspases 8, 9, and 12 [25, 51, 52]. Thus, consistent with a role for caspase 2 in apoptosis, here we show for the first time an age-related activation of caspase 2 in gastrocnemius muscles of mice. The present finding is in agreement with that of earlier studies which showed activation of caspase 2 in various tissues, including liver, spleen, lung, and brain during aging [53, 54]. Decisive evidence that caspase 2 plays an important in apoptosis in aging derives from a recent study using caspase 2 knockout mice [55]. These mice had a more severe bone loss at advanced ages due to enhanced bone resorption mediated by increased survival of osteoclasts [55]. Together, these data indicate that caspase 2 may play a role in skeletal muscle cell apoptosis during aging.

Interestingly, depending on experimental models, caspase 2 may act early [23, 25–27, 56] or as a down stream executioner in inducing apoptosis [50, 57]. We believe that its role as an executioner or an initiator caspase depends once again upon tissue type, cell lineage, nature of the apoptotic stimulus. A large number of recent studies using specific inhibitor and/or small interfering RNA knockdown of caspase 2 indicate that caspase 2 is an upstream regulator of mitochondrial membrane permeabilization, which through activation of the mitochondria-dependent intrinsic pathway signaling promotes apoptosis in various cell systems other than skeletal muscles [23, 25–27, 56]. It is conceivable that a similar mechanism may also operate in the skeletal muscle. Indeed, in the present study, we found activation of caspase 9, indicating that the mitochondria-dependent pathway is activated during age-related increase in muscle cell death.

The BCL-2 family of proteins governs the mitochondria-dependent pathway for apoptosis [40, 41]. A seemingly paradoxical finding of this study is that the levels of BCL-2 increased while that of BAX remained unchanged in aging skeletal muscles with increased cell death. However, the present finding is consistent with an earlier study showing increased myocyte apoptosis in the decompensated human heart in spite of the enhanced expression of BCL-2 and no change in the expression of BAX [58]. Notably, Siu et al. [37] have also found increased expression of BCL-2 with aging in the gastrocnemius muscles of Fischer 344× Brown-Norway rats. These authors speculated that the elevated levels of BCL-2 could be a compensatory mechanism in the aged gastrocnemius muscles in an attempt to prevent muscle loss and, consequently, muscle mass. Alternatively, one could speculate that the observed increase in muscle cell apoptosis in spite of the enhanced expression of BCL-2 may result from loss of its antiapoptotic function. The obvious question raised by these observations is why these muscles cells are dying in spite of the enhanced expression of BCL-2? A growing body of evidence indicates that serine phosphorylation of BCL-2 leads to its inactivation and its ability to form dimers with BAX, and therefore, results in the loss of its anti-apoptotic function [42, 43, 59]. Indeed, in the present study, we found increased levels of serine-phosphorylated form of the inactive BCL-2 in skeletal muscles of old mice compared to that of young animals, where no expression was detected. The question remains: what triggers BCL-2 phosphorylation? One possibility is that this could be due to

activation of JNK signaling in aging, which is also the potential target of the oxidative stress [11, 60, 61]. Indeed, activation of JNK has been reported in various cell systems during aging [7, 15, 62, 63] and that has know to induce BCL-2 phosphorylation leading to it activation [64–66]. In this context, it is interesting to note that, in a recent study, we showed that activation of JNK promotes cardiotoxin-induced muscle cell death by provoking BCL-2 phosphorylation leading to its inactivation, and the subsequent activation of the mitochondria-dependent apoptotic pathway [21]. It is thus conceivable that the signal for activating mitochondria-dependent pathway during age-related increase in muscle cell apoptosis emanates from JNK-mediated inactivation of BCL-2 through phosphorylation. Together, these data further suggest an important corollary for apoptotic regulation of skeletal muscle cells by BCL-2 family members.

In summary, the present study emphasizes the role of oxidative stress coupled with activation of caspase 2 and JNK and serine phosphorylation of BCL-2 for muscle cell apoptosis in aging. Identifying the signaling pathways leading to increased muscle cell apoptosis paves the way to design therapeutic remedies to prevent loss of muscle mass (sarcopenia) in aging or in neuro-muscular disorders associated with old age.

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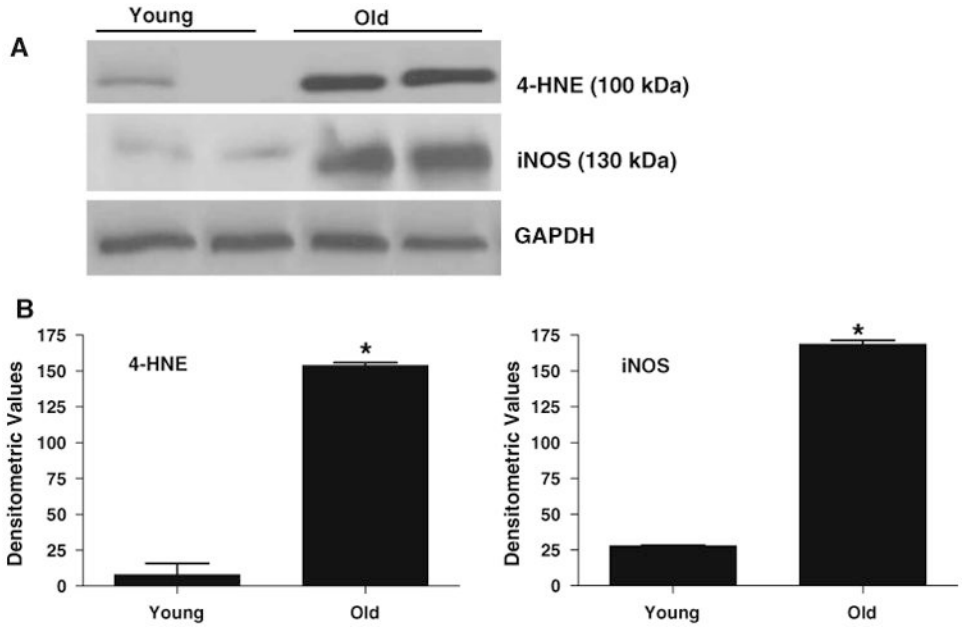

Fig. 1.

(**a**) Western blot (WB) analysis of muscle lysates show increased levels of both 4-HNE and iNOS in gastrocnemius muscles of the old animals. The gels are representative of two animals in each age group from one of three separate experiments. GAPDH in the immunoblot is shown as a loading control. (**b**) Densitometric analysis shows a significant increase in both 4-HNE and iNOS levels in muscle lysates from the old animals compared to that of young animals. Values are mean ± SEM. **P* < 0.001 (*P*-value refers to comparison with young animals)

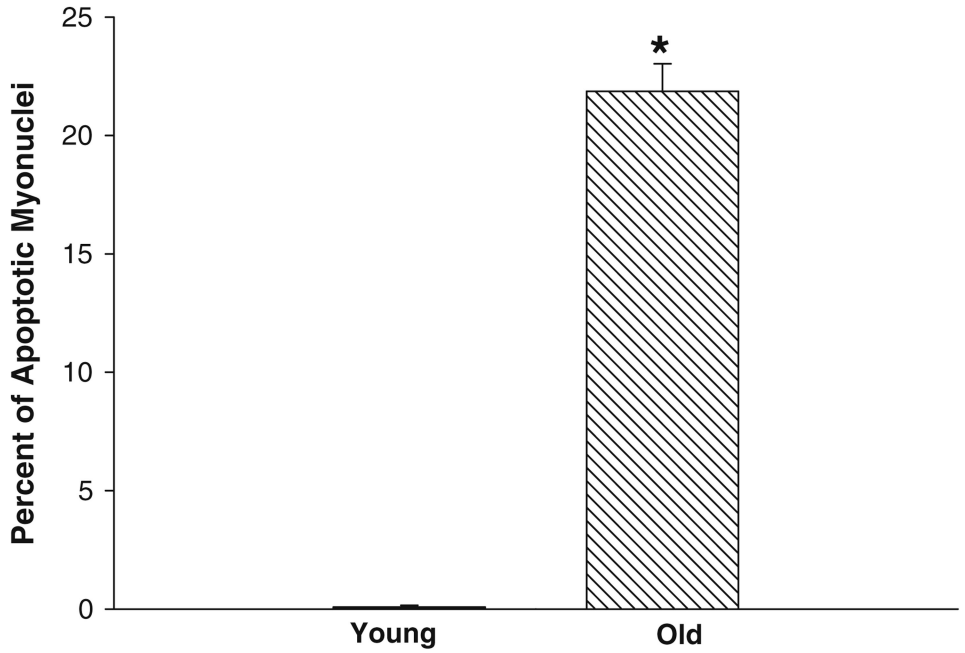

Fig. 2.

Quantitative changes in the incidence of muscle apoptosis between young and old mice. Apoptotic rate was expressed as the percentage of TUNEL positive nuclei per total nuclei (apoptotic plus nonapoptotic nuclei) counted in a unit reference area. Values are the mean ± SEM. *Significantly ($P < 0.0001$) different from young mice

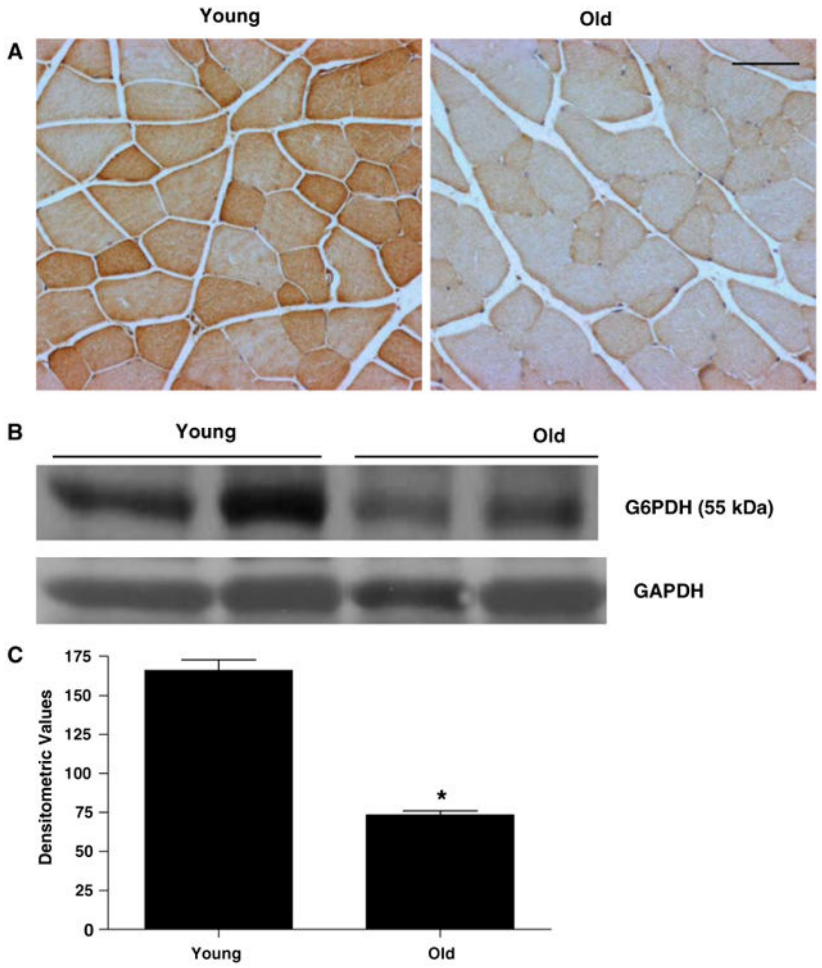

Fig. 3.

(**a**) In vivo expression of G6PDH in gastrocnemius muscles of young and old mice. Hematoxylin was used as a counterstain. Note a marked decrease in G6PDH immunoreactivity in old mice. (**b**) WB analysis of muscle lysates show decreased levels of G6PDH in gastrocnemius muscles in the old animals. The gels are representative of two animals in each age group from one of three separate experiments. (**c**) Densitometric analysis shows a significant ($P<0.05$) decrease in G6PDH levels in muscle lysates from the old animals compared to that of young animals. Values are mean ± SEM. *$P < 0.05$ (P-value refers to comparison with young animals)

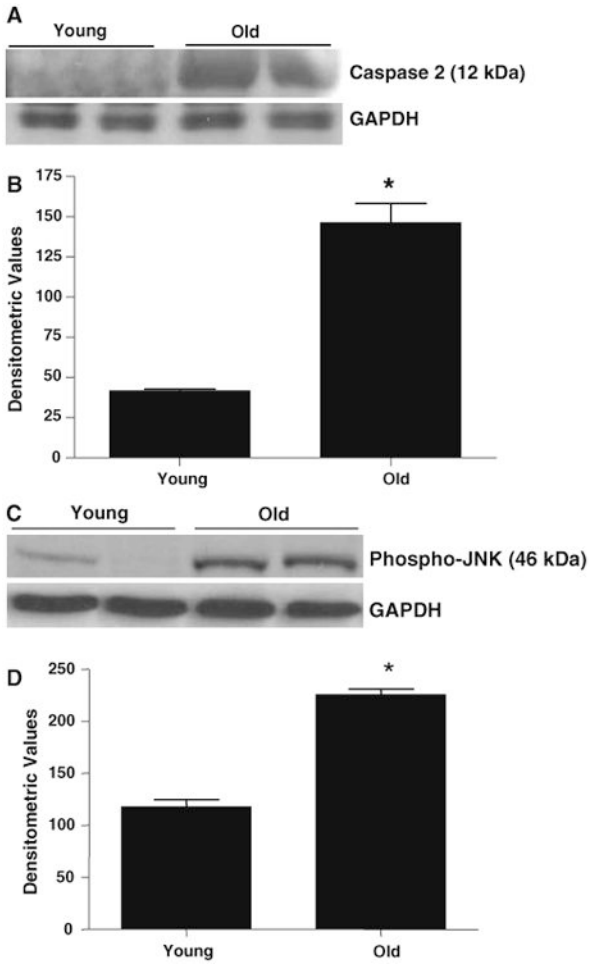

Fig. 4.

(**a**) WB analysis of muscle lysates shows that normal aging in mice is associated with activation of caspase 2, as evidenced by an increase in cleaved caspase 2 levels, in skeletal muscles. The gels are representative of two animals in each age group from one of three separate experiments. GAPDH in the immunoblot is shown as a loading control. (**b**) Densitometric analysis shows a significant ($P < 0.05$) increase in cleaved caspase 2 levels in muscle lysates from the old animals compared to that of young animals. Values are mean ± SEM. *$P < 0.05$ (*P*-value refers to comparison with young animals). (**c**) WB analysis of muscle lysates show increased levels of phospho-JNK in gastrocnemius muscles in the old animals. The gels are representative of two animals in each age group from one of three separate experiments. (**d**) Densitometric analysis shows a significant ($P < 0.05$) increase in phospho-JNK levels in muscle lysates from the old animals compared to that of young animals. Values are mean ± SEM. *$P < 0.05$ (*P*-value refers to comparison with young animals)

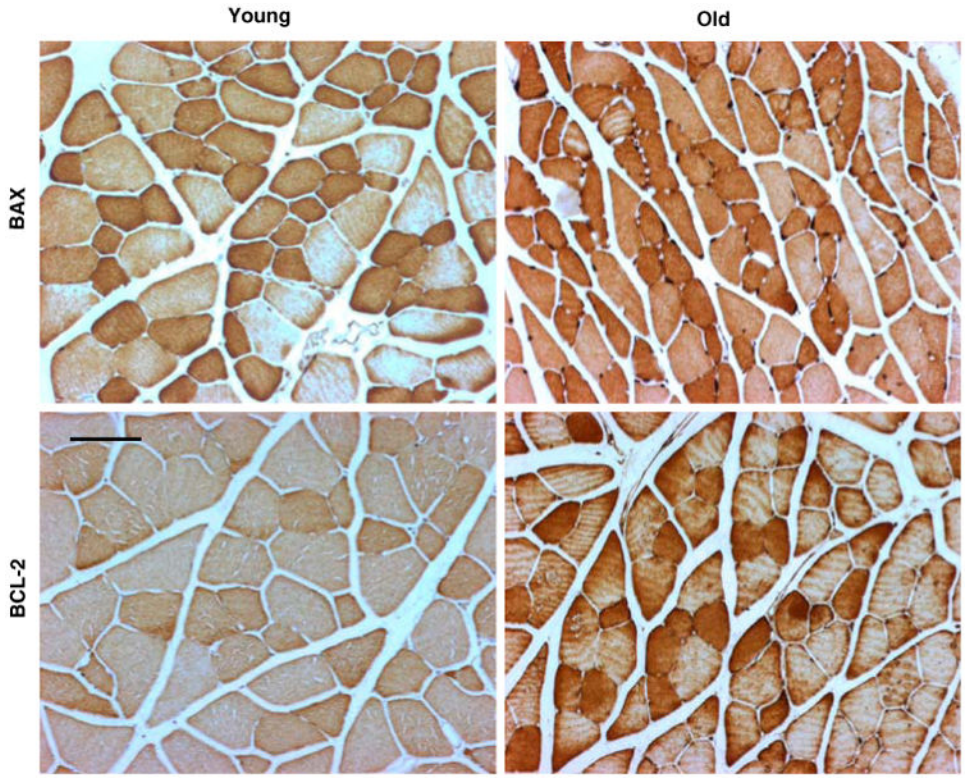

Fig. 5.

In vivo expression of BAX and BCL-2 levels in gastrocnemius muscles in young and old mice. Immunocytochemical analysis reveals little or no increase in BAX expression whereas the expression of BCL-2 appears to be markedly increased in old animals compared to young ones. Scale bar = 50 μm

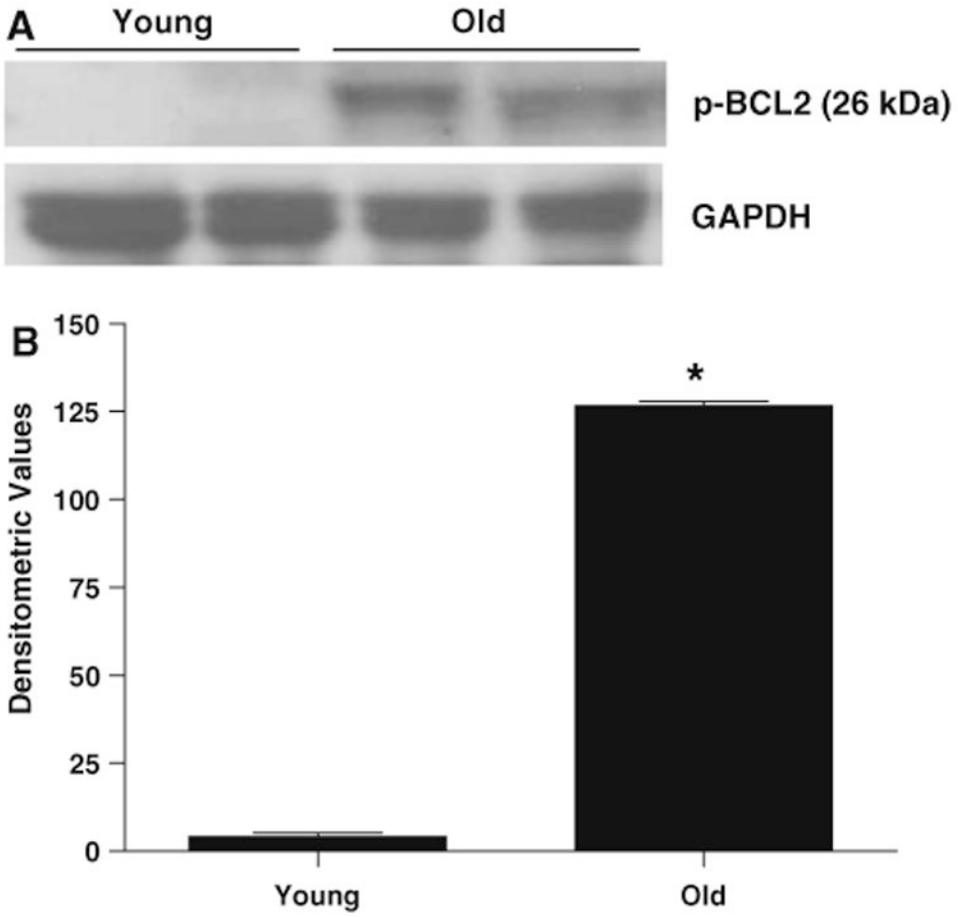

Fig. 6.

(**a**) WB analysis shows increased levels of serine-phosphorylated form of inactive BCL-2 in skeletal muscles of old mice compared to that of young animals, where no expression is detected. The gels are representative of two animals in each age group from one of three separate experiments. GAPDH in the immunoblot is shown as a loading control. (**b**) Densitometric analysis shows a significant increase in phospho-BCL-2 levels in muscle lysates from the old animals compared to that of young animals. Values are mean ± SEM. **P* <0.001 (*P*-value refers to comparison with young animals)

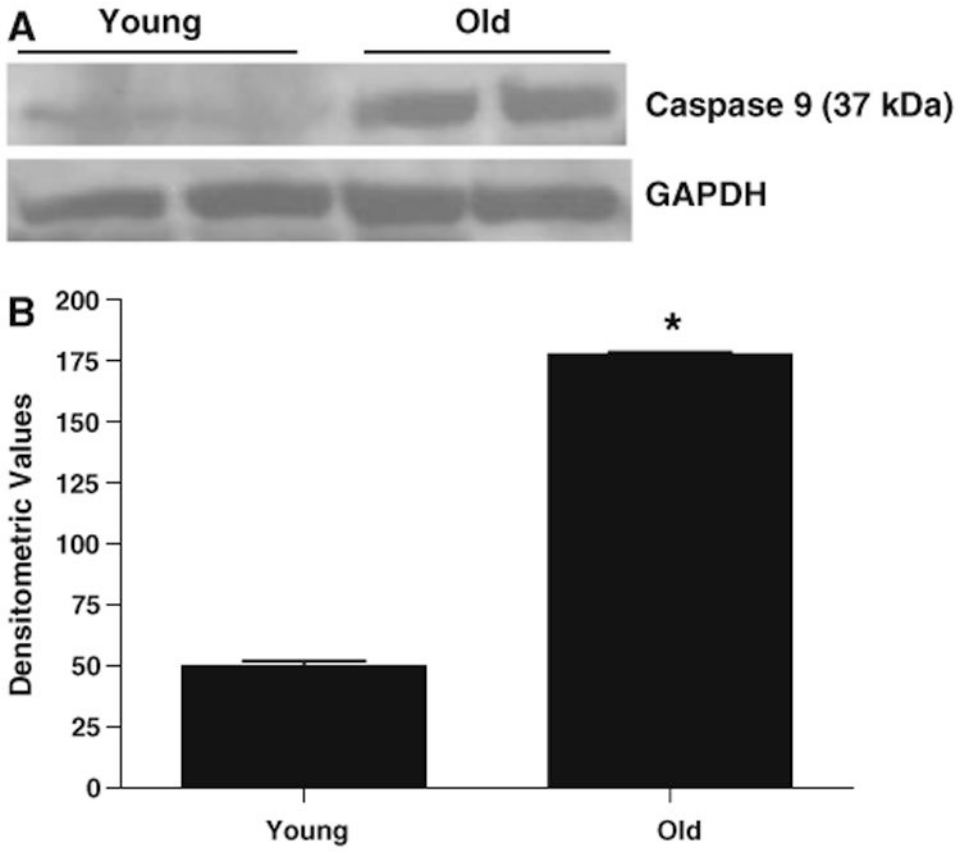

Fig. 7.

(**a**) WB analysis shows age-related activation of caspase 9, as evidenced by an increase in cleaved caspase 9 levels, in skeletal muscles. The gels are representative of two animals in each age group from one of three separate experiments. GAPDH in the immunoblot is shown as a loading control. (**b**) Densitometric analysis shows a significant ($P < 0.001$) increase in cleaved caspase 9 levels in muscle lysates from the old animals compared to that of young animals. Values are mean ± SEM. *P <0.001 (P-value refers to comparison with young animals)

Table 1

Correlation of changes in the incidence of muscle cell apoptosis with the changes in the fiber area and various stress or apoptotic markers in aging (*N* = 10; five young and five old mice)

Variable	Correlation coefficient (*r*)	*P*-value
Fiber area	–0.87	0.02
4-HNE	0.96	0.001
iNOS	0.97	0.001
G6PDH	–0.95	0.003
Active caspase 2	0.99	<0.001
Phospho-JNK	0.97	<0.001
Phospho-BCL 2	0.97	<0.001