Age-related alterations in expression of apoptosis regulatory proteins and heat shock proteins in rat skeletal muscle

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Abstract

Aging of skeletal muscle is often accompanied by muscle atrophy and it appears that apoptosis plays an important role in this process. The detailed mechanism(s) is not completely understood, however. In this study, we examined expression of the apoptosis regulatory proteins as well as the heat shock proteins, which have been shown to modulate the apoptotic process in certain cell types, in order to more completely elucidate apoptotic signaling in aged skeletal muscle. To more specifically identify alterations that are likely to be the result of aging, we compared 16-month-old middle-aged (MD) and 29-month-old senescent (SE) male Fischer 344 × Brown Norway rats in our study. Our results show that the degree of DNA laddering was higher in SE compared to MD rats. Using total tissue homogenates we examined the level of expression of several apoptosis-related proteins in two categories: mitochondria-associated proteins and caspases. Of the mitochondria-associated proteins, the levels of p53 showed a significant increase in SE compared to MD rats. There was also a significant increase in the expression of Bax, Bcl-2 and Apaf-1 in SE rats over that of MD rats; cytochrome c and AIF levels remained unchanged, however. Regarding the caspases, there were increases in the levels of pro-caspases-12 and -7 and cleaved caspase-9, although the levels of pro- and cleaved caspase-3 as well as cleaved caspase-12 remained unchanged. Furthermore, our results showed significant increases in HSP27, HSP60, and the inducible HSP70. These data show that in rat skeletal muscle increased apoptosis occurs between middle-age and senescence, indicating an aging-related increase in apoptosis in skeletal muscle. The involvement of different apoptotic pathways in the aging process is suggested by the selective alterations in the apoptosis regulatory proteins. The increased expression of the HSPs suggests a relationship between HSPs and the aging-related apoptotic process.

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1. Introduction

Aging, either in humans or animal models, is often accompanied by atrophy of the skeletal muscle and this is a major contributor to the muscle weakness associated with the aging process [1,2]. Muscle atrophy is the result of both a decrease in fiber size as well as in number. Even though multiple mechanisms undoubtedly contribute to the loss of muscle fibers [2–4], programmed cell death, or apoptosis, may play an important role. Indeed, recent reports have demonstrated in rats an increase in apoptosis in aged skeletal muscle [5–8].

Apoptosis is mediated either by the death receptor pathway or the mitochondria-dependent pathway [9–11], although recent evidence also suggests a role for endoplasmic reticulum (ER) in the process [12–14]. In the death receptor pathway, binding of ligands such as Fas or TNFα trigger the activation of caspase-8 and subsequent cascades [15,16]. In the mitochondria-mediated pathway, in response to cellular stress or DNA damage, p53 can induce apoptosis by both regulating the

Abbreviations: AIF, Apoptosis inducing factor; Apaf-1, Apoptotic protease activation factor-1; ARC, Apoptosis repressor with caspase recruitment domain; ELISA, Enzyme-Linked Immunosorbent Assay; HSP, Heat shock protein; PARP, poly (ADP-ribose) polymerase; TNFα, Tumor necrosis factor α; XIAP, x chromosome linked inhibitor of apoptosis protein

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proteins of the Bcl-2 family [17–20] and by translocating to the mitochondria and directly activating the apoptotic signaling process [21,22]. In turn, the Bcl-2 family of proteins modulates the release of apoptotic factors such as cytochrome c, HSP60, apoptosis inducing factor (AIF) or endonuclease G [19,23].

The caspases are the other major players in apoptosis. The initiator caspases (caspases-8, -9) activate the effector caspases (caspases-3, -6 and -7) to begin the cascade to cell disassembly [24,25]. For example, pro-caspase-3, which resides in the cytoplasm can be activated by several proteins, including caspase-8, caspase-9 [15] and caspase-12 [12,14]. Caspase-3 activation can then lead to cleavage of the DNA repair protein poly (ADP-ribose) polymerase (PARP) and eventually DNA fragmentation and cell death [26].

Heat shock proteins (HSPs) are a family of proteins induced in the cell as a result of heat shock or other stresses [27,28]. Recent evidence suggests that the HSPs can associate with members of the apoptotic pathway to either prevent or promote apoptosis [29–33]. For example, HSP27 and HSP70 have been shown to prevent apoptosis [29,30,34–36], whereas HSP60 has been shown to both prevent and promote apoptosis [31,32,37,38]. Therefore, a change in expression of the HSPs has the potential of modulating apoptosis in aged skeletal muscle. Expression of HSPs in aged skeletal muscle has not been well studied, however. The study by Vasilaki et al. [39] demonstrated a decreased expression of HSP70 in skeletal muscle of aged rats compared to adult rat. By contrast, the study by Locke failed to detect significant age-related differences in expression of HSP72 [40].

In this study, we examined the expression of apoptosis-associated proteins and the HSPs in order to more completely elucidate the key players associated with the aging-related process. We chose to examine and compare apoptosis in middle-aged and senescent rats in our study because changes that are observed between these age groups are more likely to reflect aging-related alterations rather than changes that may occur as a result of normal development and maturation.

2. Methods

2.1. Animals

Male 16-month-old (MD) and 29-month-old (SE) Fischer 344 x Brown Norway rats (Harlan, Indianapolis, IN) were housed in a 12:12-h light–dark cycle and given standard rat chow and water ad libitum. They were anesthetized with pentobarbital sodium (35 mg/kg i.p.). Mixed red and white gastrocnemius muscles were dissected after hearts were removed and the tissues were immediately frozen in liquid nitrogen and stored at −80 °C. All animal use protocols were approved by the institutional animal care committee.

2.2. DNA laddering

Radioactive labeling of DNA fragments was performed according to the method of Eldadah et al. [41]. powdered gastrocnemius muscle (100 mg) from MD and SE rats (n=4 and 3, respectively) were lysed in 2 ml DNAzol (MRC, Cincinnati, OH). The DNA was ethanol precipitated then dissolved in 8 mM NaOH and the pH was adjusted to pH 7.2 with HEPES buffer. The DNA was labeled by the addition of Taq DNA polymerase (Sigma, St. Louis, MO) and dATP α-32P (ICN, Costa Mesa, CA). The reaction products were separated on a 2% agarose gel, dried, exposed to X-ray film and intensity of the bands was quantitated by densitometry.

2.3. Western blots

Powdered mixed gastrocnemius muscle (200 mg) from MD and SE rats (n=6) was homogenized on ice in 1.5 ml of 10 mM Tris–HCl buffer (pH 6.8 at room temperature) containing 1 mM EDTA and protease inhibitors (500 μM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin and 10 mM E-64) using a Polytron homogenizer at a speed of 5.5. Homogenate was then filtered through wire mesh. Protein concentrations were determined by Bradford protein assay (BioRad, Melville, NY). Samples (50 μg) were run on a 4–20% SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked in TBS-Tween 20 containing 5% dry milk and incubated with primary antibody for various times. Primary antibodies were obtained from the following sources: rabbit anti-Bax (#2772), rabbit anti-caspase-7 (#9492) and rabbit anti-caspase-6 (#9762) were from Cell Signaling (Beverly, MA); goat anti-p53 (C-19), rabbit anti-Bak (G-23), rabbit anti-AIF (H-300), rabbit anti-caspase-3 (H-277), rat anti-caspase-12 (1611), rabbit anti-caspase-9 p53 (H-170) and goat anti-HSP27 (C-20) were from Santa Cruz (Santa Cruz, CA); mouse anti-Bcl-2 (Ab-4) (#AM43T) was from Oncogene (San Diego, CA); mouse anti-cytochrome c (#556433) was from BD Pharmingen (San Jose, CA); rabbit anti-Apaf-1 (#AB16503) was from Chemicon (Temecula, CA); and mouse anti-inducible HSP70 (SPA-810) were from StressGen (Victoria, BC, Canada). Blots were incubated with the appropriate HRP-conjugated secondary antibody. Detection was performed with a chemiluminescent method (SuperSignal West Pico or West Femto, Pierce, Rockford, IL) and imaging analysis was performed with a Fujifilm Luminescent Imagier (Fuji Photo Film Co., Ltd., Japan) using the ImageGuage analysis software. When intensities of the bands were compared between samples they were all run on the same gel. The blot for cleaved caspase-3, because of its weak signal, was exposed to X-ray film (Denville Scientific). The image was captured using the Image Gauge program and quantitated using the ImageGel program downloaded from the NIH website. All quantitated bands were within linear range of detection. The blots frequently were stained for total protein to verify equal loading of the lanes.

2.4. Statistical analysis of data

Values are means±S.E. and a Student’s t-test was used to compare group means. Data were examined at P<0.05 to indicate statistical significance.

3. Results

3.1. DNA laddering in adult and aged rats

DNA laddering, a marker for apoptosis, was examined in the gastrocnemius muscle of 16-month-old rats (MD) and 29-month-old rats (SE). The results show a clear increase in DNA laddering in SE rats compared to MD rats (Fig. 1A). The intensity of the DNA fragment at 400 bp was quantitated and there was a significant increase of 42.1% in SE rats (n=3) compared to MD rats (n=4) (Fig. 1B).

3.2. Mitochondria-associated proteins

The mitochondrial pathway, which contains both pro- and anti-apoptotic members, is one of the major players in apoptosis. Using total tissue homogenates, we examined, by Western blot analysis, the level of proteins within this pathway in skeletal muscle (Fig. 2A and B). There was a 90.3% increase in the level of p53 in SE rats over MD rats (P=0.01, n=6). The Bcl-2 family is made up of several
mitochondria-associated proteins that help control the release of apoptotic factors from the mitochondria. Of this family, the pro-apoptotic protein Bax showed a marked increase (226.0%) in SE rats compared to MD rats (n=6). We also detected an increase of 68.3% in the level of the anti-apoptotic protein Bcl-2 in SE rats over MD rats (n=6). Overall, the Bax/Bcl-2 ratio increased from 1.02 in MD rats to 1.96 in SE rats (n=6). The pro-apoptotic protein Bak, another member of the Bcl-2 family, showed no significant change (Fig. 2).

In response to the influence of the above proteins, the mitochondria can release factors that modulate apoptosis. Two of these factors, cytochrome c and AIF, showed no significant change in SE rats compared to MD rats (Fig. 3A and B). By contrast, Apaf-1, which forms the apoptosome with cytochrome c and caspase-9, increased markedly by 803.8% in SE rats (n=6).

3.3. Proteins of the caspase cascade

Since the caspases can play a pivotal role in apoptosis, we examined some of these proteins in the skeletal muscles (Fig. 4A and B). The level of pro-caspase-9 did not change, whereas the level of cleaved caspase-9 increased 209.8% in SE rats compared to MD rats (n=6). While the level of pro-caspase-12 increased 134.8% in SE rats compared with MD rats (n=6), the level of cleaved caspase-12 showed no significant change. There was no significant change in the level of pro- and cleaved caspase-3 in MD and SE rats. The level of pro-caspase-7 increased 55.4% in SE rats compared to MD rats (n=6). We were unable to detect a band of the correct size for cleaved caspase-7 as well as pro- and cleaved caspase-6 (data not shown).

3.4. Heat shock proteins

The level of the heat shock proteins HSP27, HSP60 and inducible HSP70 were measured (Fig. 5A and B). Western blots showed an increase of 105.5% in the level of HSP27 (n=6) as well as a 61.3% increase in HSP60 (n=6) in SE rats compared to MD rats. In addition, the inducible HSP70...
showed a 29.5% increase in SE rats compared to MD rats (n = 6).

4. Discussion

In the present study, we found significant aging-related changes in the levels of apoptosis regulatory proteins, namely, p53, Bcl-2, Bax, Apaf-1, cleaved caspase-9, pro-caspase-12, pro-caspase-7, as well as the heat shock proteins HSP27, HSP60 and inducible HSP70. Recent studies by two other groups have identified some of the apoptotic proteins that may contribute to the increased apoptosis in aged skeletal muscle [5,6,8]. These include the pro-apoptotic proteins caspase-3, Bax, Bcl-2, Apaf-1, pro-caspase-12, total AIF, XIAP and ARC. Some of our results are in agreement, while others apparently diverge from those earlier findings. The details and possible explanations for the differences will be discussed. While it does not provide explanation for discrepancies between different studies, by examining the changes that occurred between middle-age and senescence, we minimized the possibility that interpretations of the results may be confounded by the effects of normal developmental maturation.

One of the unique findings of our study is that by using Taq DNA polymerase to label the DNA fragments, we believe, for the first time, a clear increase in DNA laddering was detected in aged skeletal muscle. It is worth mentioning that we have attempted and failed to detect DNA laddering by the commonly used ethidium bromide method, most likely due to the lesser sensitivity of that method [41]. Dirks and Leeuwenburgh [5] have shown evidence of apoptosis in aged skeletal muscle using ELISA. Since DNA fragmentation due to cleavage at internucleosomal sites is one of the most important biochemical hallmarks of programmed cell death [42], our data uniquely compliments those earlier findings.

The mitochondria are the main regulatory centers of apoptosis due to the influence of p53 and the Bcl-2 family of proteins, including Bax and Bcl-2 [11,43,44]. Indeed, mitochondrial damage as a result of aging can be one of the initiating steps of apoptosis [45]. The tumor suppressor p53 can be turned on by oxidative stress as well as by DNA damage, which in turn affects apoptosis by either increasing the expression of Bax or decreasing the expression of Bcl-2 [17,46], also by directly translocating to the mitochondria to activate the apoptotic process [21,22]. We observed increased levels of p53 in aged skeletal muscle compared to middle-aged skeletal muscle, thus suggesting a potential role for p53 in the aging-related apoptotic process. Information regarding the role of p53 in the apoptotic cascade in skeletal muscle is very limited. In a recent study by Siu and Alway [47], p53 was shown to be involved in apoptosis during unloading-induced

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Fig. 4. The level of caspases in skeletal muscle. (A) Representative Western blots showing the levels of pro- and cleaved caspases-9, -12 and -3 and procaspase-7 in the skeletal muscle of middle-aged (MD) (n = 6) and senescent (SE) (n = 6) rats. (B) Bands were quantitated and values were normalized to those of the MD samples (*P = 0.0003, **P = 0.0003 and ***P = 0.02 compared to MD rats). Light color bar: MD. Solid bar: SE.

Fig. 5. The level of heat shock proteins in skeletal muscle. (A) Representative Western blots showing the levels of HSP27, HSP60 and inducible HSP70 in the skeletal muscle of middle-aged (MD) (n = 6) and senescent (SE) (n = 6) rats. (B) Bands were quantitated and values were normalized to those of the MD samples (*P = 0.00006, **P = 0.0002 and ***P = 0.02 compared to MD rats).
muscle atrophy in the wing muscle of young quail but not in aged quail muscle. Because the level of expression of p53 was not directly compared between young and aged quail muscle, it is unclear from that study whether the level of expression actually changed with age.

A large body of evidence suggests that it is the ratio between the pro-apoptotic Bax and the anti-apoptotic Bcl-2 that determines the mitochondrial release of apoptotic factors such as cytochrome c and AIF [48,49]. We observed increased levels of both Bax and Bcl-2 in aged skeletal muscle. Our results are in partial agreement with the work of Alway and co-workers [7,50] who have reported an increase in Bax and a decrease in Bcl-2 levels in the skeletal muscle of aged rats. Importantly, their study and ours both showed an increase in the Bax/Bcl-2 ratio with aging, suggesting a possible role for the mitochondrial Bax/Bcl-2 apoptotic signaling pathway in the overall increase in pro-apoptotic tendency in the aged skeletal muscle. It should be noted that Alway and co-workers compared expression of the proteins between young adult (9 months old) and very old (37 months old) rats [7] which may be a factor in the different findings in Bcl-2 levels between the two studies. It may be speculated that the increase in Bcl-2 in our study may signify a compensatory response in the aged skeletal muscle, and that compensatory response ultimately diminishes at very old age. By contrast, Dirks and Leeuwenburgh [5] did not detect a change in Bax or Bcl-2 levels in the skeletal muscle of aged Fischer 344 rats. Whether this discrepancy can be explained by the differences in the strain of rats being used is not clear. However, there appear little growth or maturation differences between Fischer 344 and the Fischer 344 x Brown Norway rats since previous reports by us [51] and others [6] have shown that muscle mass in both strain of rats decreases between middle age and senescence. In addition, their study measured Bax and Bcl-2 in the mitochondrial fraction of gastrocnemius muscle. By contrast, Dirks and Leeuwenburgh [6] demonstrated increased caspase-9 activity and increased pro-caspase-12 levels in aged skeletal muscle, respectively. Together, the results argue for a role of caspase-9 in the apoptosis process during aging. The role of caspase-12 is less clear because an increase in the cleaved caspase-12 was not detected in either study. Nevertheless, the data suggest a possible role for ER stress as a component of the age-related apoptotic process because of the central role of caspase-12 in that process [12,14,58]. Of the downstream effector caspases, namely caspases-3, -6 and -7, we were able to detect an increase in pro-caspase-7, but have not been able to detect any increase in the cleaved forms. It is worth mentioning that we also have examined caspase-3 activity in the skeletal muscle of 4-month-old and 29-month-old rats using fluorescence assay and found no change in the activity (data not shown). Interestingly, a recent report by Siu et al. [59] showed an increase in caspase-9, but not caspase-3, activity in aged gastrocnemius muscle. By contrast, Dirks and Leeuwenburgh [6] showed an increase of both pro- and cleaved caspase-3, even though caspase-3 activity again remained unchanged. The inability of the three studies to detect changes in caspase-3 activity put into question a role of caspase-3 in the age-related apoptosis in skeletal muscle. Whether these data suggest in skeletal muscle an alternative bifurcation of the apoptotic pathway downstream of caspase-9 will be the subject of future investigation.

Heat shock proteins play an important role in helping cells cope with a number of stresses such as heat shock and oxidative stress [27,28]. Importantly, the HSPs have been shown to have the ability to regulate apoptosis by interacting, directly or indirectly, with a number of apoptotic proteins. For example, in transfected U937 cells HSP27 associates with both cytochrome c [29,60] and caspase-3 [34] to prevent apoptosis. Similarly, HSP70 has anti-apoptotic effects in transfected MEF cells by its ability to bind to AIF and Apaf-1 [30]. On the other hand, HSP60 is pro-apoptotic by promoting activation of procaspase-3 [31,38] but also anti-apoptotic by preventing Bax and Bak translocation to the mitochondria [32,37]. In this study, we demonstrated, for the first time, in aged skeletal muscle an apparent selective increase in the level of HSP27, HSP60 and inducible HSP70. This finding is in contrast to a previous study showing decreased levels of HSP70 in aged female Wistar rats (28 months old) compared to young adult rats (6 months old) [61]. The discrepancy between the studies
could be the result of species, gender, and/or age group differences. In our study, the increases in HSPs may suggest the aged skeletal muscle has mounted a compensatory stress response but yet it was insufficient to prevent the apoptotic process. On the other hand, the increase in HSP60 may suggest a deficit in the aged skeletal muscle, which may result in increased muscle degenerations due to its ability to promote apoptosis. It is worth mentioning that in addition to the HSPs, proteins that regulate production of free radicals, such as superoxide dismutase and catalase, have also been shown, directly and indirectly, to modulate apoptosis in various tissues and cells [62,63].

In summary, our data show that in rat skeletal muscle increased apoptosis occurs between middle-age and senescence, confirming an aging-related increase in apoptosis in skeletal muscle. The involvement of both mitochondria dependent and independent apoptotic pathways in the aging process is suggested by the selective alterations in the apoptosis regulatory proteins, namely, Bax, Bel2, Apat-1 and caspases 7, 9 and 12. The increased expression of the HSPs suggests a potential role of HSPs in the aging-related apoptotic process. We are currently investigating whether, and if so, how these HSPs interact with the apoptosis regulatory proteins in the young and aged skeletal muscle.

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References


