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Differential apoptosis-related protein expression, mitochondrial properties, proteolytic enzyme activity, and DNA fragmentation between skeletal muscles

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McMillan EM, Quadrilatero J. Differential apoptosis-related protein expression, mitochondrial properties, proteolytic enzyme activity, and DNA fragmentation between skeletal muscles. Am J Physiol Regul Integr Comp Physiol 300: R531–R543, 2011. First published December 9, 2010; doi:10.1152/ajpregu.00488.2010.—Increased skeletal muscle apoptosis has been associated with a number of conditions including aging, disease, and cardiovascular disease. Skeletal muscle is a complex tissue comprised of several fiber types with unique properties. To date, no report has specifically examined apoptotic differences across muscles or fiber types. Therefore, we measured several apoptotic indices in healthy rat red (RG) and white gastrocnemius (WG) muscle, as well as examined the expression of several key proteins across fiber types in a mixed muscle (mixed gastrocnemius). The protein content of apoptosis-inducing factor (AIF), apoptosis repressor with caspase recruitment domain (ARC), Bax, Bcl-2, cytochrome c, heat shock protein 70 (Hsp70), and second mitochondria-derived activator of caspases (Smac) were significantly (P < 0.05) higher in RG vs. WG muscle. Cytosolic AIF, cytochrome c, and Smac as well as nuclear AIF were also significantly (P < 0.05) higher in RG compared with WG muscle. In addition, ARC protein expression was related to muscle fiber type and found to be highest (P < 0.001) in type I fibers. Similarly, AIF protein expression was differentially expressed across fibers; however, AIF was correlated to oxidative potential (P < 0.001). Caspase-3, -8, and -9 activity, calpain activity, and DNA fragmentation (a hallmark of apoptosis) were also significantly higher (P < 0.05) in RG compared with WG muscle. Furthermore, total muscle reactive oxygen species generation, as well as Ca2⁺-induced permeability transition pore opening and loss of membrane potential in isolated mitochondria were greater in RG muscle. Collectively, these data suggest that a number of apoptosis-related indices differ between muscles and fiber types. Given these findings, muscle and fiber-type differences in apoptotic protein expression, signaling, and susceptibility should be considered when studying cell death processes in skeletal muscle.

skeletal muscle; fiber type; mitochondria; apoptosis repressor with caspase recruitment domain; cell death

APOPTOSIS IS A REGULATED FORM of cell death that plays an important role in tissue development and disease (24). Apoptotic signaling cascades can be triggered through several pathways. The death receptor pathway is initiated upon binding of death ligands to their corresponding death receptor, leading to the formation of a signaling complex and subsequent activation of proteolytic enzymes (i.e., caspase-8) (36). The mitochondrial pathway can be initiated by intracellular damage or stress signals, resulting in the release of cytochrome c from the mitochondria into the cytosol, apoptosome formation, and caspase-9 activation (39). A number of other apoptogenic factors, such as apoptosis-inducing factor (AIF) and endonuclease G (EndoG), can also be released from the mitochondria and participate in caspase-independent apoptosis (38, 66, 69). The endoplasmic reticulum (ER) stress pathway can be initiated by an accumulation of misfolded and/or unfolded proteins in the ER that can lead to ER stress, Ca2⁺ release, calpain activation, and subsequent caspase-12 and caspase-9 activation (60). Caspase-8 and caspase-9 can promote the activation of caspase-3 that can cleave a number of substrates in the cell and eventually lead to cell death (24, 67). Members of the Bcl-2 family of proteins, such as Bcl-2 and Bax also play a central role in apoptosis by regulating events at the mitochondria and ER (45, 47). In addition, several other factors, such as X-linked inhibitor of apoptosis protein (XIAP) and heat shock protein 70 (Hsp70), are also important in apoptotic signaling through their inhibitory roles (7, 19).

The signaling pathways, molecular machinery, and morphological changes that occur during apoptotic cell death are highly conserved in most cells and tissues; however, there are several important considerations when studying apoptosis in skeletal muscle. First, skeletal muscle fibers are generally considered to be long-lived, terminally differentiated cells (1, 35, 44). Cell culture studies indicate that in contrast to the high rate of apoptosis and cell turnover observed during stages leading to myogenic differentiation, terminally differentiated myotubes are more resistant to apoptosis (70). Although the mechanisms that attenuate apoptosis in terminally differentiated and mature skeletal myocytes are not fully elucidated, this may be related, in part, to the expression of apoptosis repressor with caspase recruitment domain (ARC). ARC is an antiapoptotic protein capable of inhibiting apoptosis mediated by both the death receptor and mitochondrial pathway (35, 46). Interestingly, ARC is highly expressed in long-lived cells, such as cardiac and skeletal myocytes (1, 35). In addition, it has been demonstrated that ARC is undetectable in undifferentiated H9c2 cells, but levels rise upon differentiation (33). Therefore, the unique expression of antiapoptotic proteins, such as ARC, may contribute to the low rate of cell death and turnover observed in skeletal muscle. Second, skeletal muscle fibers have a multinucleated morphology. It has been suggested that nuclei within the myofiber regulate and sustain the necessary gene and protein expression for a specific and local region known as the myonuclear domain (3, 25). It has also been proposed that apoptosis in skeletal muscle results in the selective loss of individual nuclei through a process termed “nuclear apoptosis” rather than complete fiber death (3, 5).
mitochondrial content can vary between muscles and fibers (42, 64, 72). It is well established that the mitochondria and a number of mitochondria-associated proteins [i.e., AIF, EndoG, cytochrome c, second mitochondria-derived activator of caspases (Smac)] play an important role in apoptosis (20, 38, 39, 66, 69). In addition, reactive oxygen species (ROS) can be produced at the mitochondria (15, 50) and play a critical role in apoptotic signaling (12, 50). Therefore, it is possible that differences in mitochondrial content between muscles and fibers may influence apoptotic signaling. Furthermore, the mitochondrial population within skeletal muscle is composed of two distinct subpopulations, subsarcolemmal and intermyo-fibrillar mitochondria (2). Interestingly, Adhihetty et al. (2) found a differential susceptibility to apoptotic stimuli between subsarcolemmal and intermyofibrillar mitochondria.

To date, apoptosis-related differences across muscles and fiber types have not been well defined. Riva et al. (61) found that basal Bax protein content was higher, whereas Bcl-2 protein content was lower, in rat soleus compared with white gastrocnemius (WG) muscle. Immunohistochemical data from Braga et al. (9) showed a differential staining pattern for Bax and Bcl-2 protein across fibers in mouse gastrocnemius muscle; however, fiber type was not determined. It has also been demonstrated that a key cytokine involved in death receptor signaling, tumor necrosis factor-α (TNF-α), is predominantly expressed in type II fibers of human muscle (56). Interestingly, apoptotic susceptibility may also be different across muscles. For example, the number of caspase-3-positive fibers was higher in rat soleus compared with tibialis anterior muscle following administration of clenbuterol (10) or angiotensin II (11). Koçtürk et al. (34) also found that the percentage of apoptotic nuclei increased rapidly in rat soleus muscle following strenuous exercise, but only gradually in gastrocnemius muscle.

Despite a number of unique morphological, functional, and metabolic characteristics that have been well defined in different muscles and fiber types (18, 42, 64, 72), little is known regarding apoptotic differences. We suggest that it is important to identify whether differences in apoptosis-specific protein expression and signaling exist between muscles and fiber types for several reasons. First, an apoptotic response observed in one muscle/fiber type may not be comparable across muscles or fiber types. Second, the expression pattern of apoptotic factors within a muscle or fiber type may differentially influence apoptotic susceptibility to a particular stress or disease state. Third, it would be important to determine the presence and participation of a particular molecule(s) and apoptotic pathway(s) during apoptosis in specific muscles or fiber types. This would be particularly important when identifying a potential target (and therapeutic strategy) to alter apoptotic signaling in muscle disorders. Fourth, basal differences in apoptotic signaling and protein expression could impact data analysis and interpretation, particularly in conditions that cause fiber-type changes and/or alterations in mitochondrial content. Therefore, the purpose of the present study was to examine apoptosis-related protein expression, enzyme activity, mitochondrial properties, and DNA fragmentation in several muscles in healthy rats. In addition, we investigated the fiber-type expression of ARC and AIF as well as the subcellular localization of these proteins. We hypothesized that there would be a number of apoptosis-related differences between muscles and fibers.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats (16–18 wks) were obtained from Harlan (Indianapolis, IN) and group housed on a 12:12-h reverse light-dark cycle in a temperature and humidity-controlled environment. Rats were provided standard rodent lab chow and tap water ad libitum. All animal procedures were approved and performed in accordance with the guidelines established by the University of Waterloo Animal Care Committee.

Tissue isolation. Rats were anesthetized with pentobarbital sodium and euthanized by removing the heart. The gastrocnemius muscle was removed, immediately placed on ice, and divided into WG, red (RG), and mixed (MG) gastrocnemius muscle portions using the scheme outlined by Armstrong and Phelps (6). In addition, the whole soleus and plantaris muscles were removed for confirmatory experiments. A portion of each muscle was quickly frozen in liquid nitrogen and stored at −80°C for subsequent analysis. A small section of each muscle/portion was also embedded in OCT compound (Tissue-Tek), frozen in liquid nitrogen-cooled isopentane, and stored at −80°C for subsequent histochemical analysis. For subcellular fractionations, ROS generation, and isolated mitochondrial experiments, freshly isolated muscle was used as described below.

Preparation of whole-muscle lysates and muscle subcellular fractions. For preparation of whole-muscle lysates, muscle was homogenized in ice-cold lysis buffer (20 mM HEPES, 10 mM NaCl, 1.5 mM MgCl₂, 1 mM DTTP, 20% glycerol and 0.1% Triton X-100; pH 7.4) containing protease inhibitors (Complete Cocktail; Roche Diagnostics) by using a glass homogenizer. Homogenates were then centrifuged at 1,000 g for 10 min at 4°C, the supernatant collected, and total protein concentration determined by the BCA protein assay. Subcellular fractions were prepared as previously performed (63). Briefly, fresh muscle was minced and gently homogenized on ice in subcellular fractionation buffer (in mM: 250 sucrose, 20 HEPES, 10 KCl, 1 EDTA, 1 EGTA, 1 DTTP; pH 7.4) with protease inhibitors (Complete Cocktail; Roche Diagnostics). Homogenates were centrifuged at 800 g at 4°C for 10 min, yielding a pellet (P1) and supernatant (S1). The S1 fraction was centrifuged at 800 g at 4°C for an additional 10 min to remove any residual debris, and the resulting supernatant was transferred to a new tube (S2). The S2 fraction was centrifuged at 16,000 g at 4°C for 20 min, yielding a pellet containing mitochondria (M1) and a supernatant consisting of the cytosol (C1). The M1 pellet was washed again with subcellular fractionation buffer and centrifugation at 16,000 g at 4°C for 20 min. The resulting pellet was retained and considered the mitochondrial-enriched fraction. The C1 supernatant was centrifuged at 16,000 g at 4°C for 20 min to remove any residual mitochondria, which resulted in a cytosolic-enriched fraction. The P1 pellet was washed three additional times, with centrifugation at 800 g at 4°C for 10 min. Lysis buffer (200 µl) and 5 M NaCl (27.7 µl) was added to the pellet and rotated for 1 h at 4°C. The samples were then centrifuged at 20,800 g at 4°C for 15 min, and the supernatant was recovered as the nuclear-enriched fraction. The protein concentration was determined in each subcellular fraction by the BCA protein assay.

Immunoblot analyses. Equal amounts of protein were loaded and separated on a 12% SDS-PAGE gel, transferred onto PVDF membrane (Bio-Rad Laboratories, Hercules, CA), and blocked overnight at 4°C with 5% milk-Tris-buffered saline-Tween 20 (milk-TBST). Membranes were then incubated either overnight at 4°C or for 1 h at room temperature with primary antibodies against AIF, ARC, adenosine nucleotide translocase (ANT), Bcl-2, Bax, cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA), Hsp70, MnSOD, XIAP (Stressgen Bioreagents, Enzo Life Sciences, Plymouth Meeting, PA), and Smac (Assay Designs, Enzo Life Sciences). Membranes were then washed with TBST, incubated with the appropriate horseradish peroxidase...
(HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature, washed with TBST, and bands visualized using enhanced chemiluminescence western blotting detection reagents (GE Healthcare) and the ChemiGenius 2 Bio-Imaging System (Syngene, Frederick, MD). The approximate molecular weight for each protein was estimated using Precision Plus Protein WesternC Standards and Precision Protein Strep-Tactic HRP Conjugate (Bio-Rad Laboratories). Equal loading and quality of transfer were confirmed by staining membranes with Ponceau S (Sigma-Aldrich, St. Louis, MO).

For subcellular fractionation analyses, additional immunoblots were performed to verify the enrichment of each fraction using primary antibodies against histone H2B (Upstate Cell Signaling Solutions, Millipore, Billerica, MA) for the nuclear fraction, CuZnSOD (Stressgen Bioreagents) for the cytosolic fraction, and ANT (Santa Cruz Biotechnology) for the mitochondrial fraction. All immunoblot analyses were performed in duplicate. Relative protein levels were expressed as optical density in arbitrary units.

**Caspase and calpain enzyme activity.** Enzymatic activity of caspase-3, caspase-8, and caspase-9 were determined in muscle homogenates using the substrates, Ac-DEVD-AMC (Alexis Biochemicals, Enzo Life Sciences), Ac-IETD-AMC (Sigma-Aldrich), and Ac-LEHD-AMC (Alexis Biochemicals, Enzo Life Sciences), respectively (58). These fluorogenic substrates are weakly fluorescent but yield highly fluorescent products following proteolytic cleavage by their respective active caspase enzyme. Muscle was homogenized in ice-cold lysis buffer (not containing protease inhibitors) and centrifuged at 1,000 g at 4°C for 10 min. Supernatants were then incubated (in duplicate) with either Ac-DEVD-AMC, Ac-IETD-AMC, or Ac-LEHD-AMC at room temperature. Fluorescence was measured using a SPECTRAmax Gemini XS microplate spectrophotometer (Molecular Devices). Mitochondria were then treated with 50 μM, 100 μM, or 200 μM CaCl2, and absorbance was measured for an additional 30 min at 37°C. A decrease in absorbance is indicative of PTP opening (mitochondrial swelling), an event that can occur during mitochondrial-mediated apoptotic signaling (31). Data were expressed as the percent decrease in absorbance relative to the initial absorbance (before the addition of CaCl2).

Mitochondrial membrane potential was determined in isolated mitochondria using rhodamine 123. Uptake and retention of rhodamine 123 is dependent on mitochondrial membrane potential and has been examined previously in isolated mitochondria (48). A decrease in rhodamine 123 fluorescence is indicative of membrane depolarization; an event that can occur during mitochondrial-mediated apoptotic signaling (31). Mitochondria at a concentration of 200 μg/ml were incubated in the dark in duplicate (in swelling buffer) with 5 μM rhodamine 123 for 5 min at 37°C. Mitochondria were then incubated with 50 μM, 100 μM, 200 μM or no CaCl2 for an additional 30 min at 37°C and then washed and resuspended in swelling buffer. Fluorescence was determined using a SPECTRAmax GEMINI XS microplate spectrophotometer (Molecular Devices) with excitation and emission wavelengths of 500 nm and 535 nm, respectively. Data were expressed as the percent decrease in fluorescence relative to the no CaCl2 condition.

**Histochemical, immunohistochemical, and immunofluorescence analyses.** For all histochemical, immunohistochemical, and immunofluorescence analyses, frozen skeletal muscle samples embedded in OCT compound were cut into 10-μm serial cross sections with a cryostat (Thermo Electronic) maintained at −20°C. NADH-tetrazolium reductase (NADH-TR) activity was determined in muscle cross sections as a measure of oxidative potential (32). Cross sections were incubated in a reaction buffer (20 mM MOPS, 1.0 mM nitroblue tetrazolium, and 1.5 mM NADH, pH 7.2) for 30 min at 37°C as previously performed (32). The resulting staining was visualized with a brightfield Nikon microscope linked to a PixeLink digital camera and quantified with Image-Pro PLUS analysis software. Data were expressed as optical density in arbitrary units.

**Mitochondrial permeability transition pore opening and membrane potential in isolated mitochondria.** Mitochondria were isolated from fresh muscle as previously described, with slight modifications (51). Briefly, muscle was minced and gently homogenized in mitochondrial isolation buffer (in mM: 220 mannitol, 70 sucrose, 20 HEPES, 2 Tris, 1 EDTA, pH 7.2), containing 0.4% BSA and 0.15 mg/ml Nagarse (Sigma-Aldrich) by using a glass homogenizer on ice. The homogenate was centrifuged for 5 min at 500 g at 4°C. The pellet was briefly homogenized and centrifuged for 5 min at 500 g at 4°C. The supernatants were combined and centrifuged at 17,000 g for 3 min at 4°C. The pellet was washed and briefly resuspended in isolation buffer (not containing BSA or Nagarse), and a small aliquot was removed to perform protein quantification via the BCA protein assay. The sample was then centrifuged (17,000 g for 3 min at 4°C), and the mitochondrial pellet resuspended in mitochondrial isolation buffer containing BSA.

For determination of mitochondrial permeability transition pore (PTP) opening (mitochondrial swelling), mitochondria were plated at a concentration of 400 μg/ml in duplicate in swelling buffer (in mM: 215 mannitol, 71 sucrose, 3 HEPES, 5 succinate, pH 7.4) and 540 nm absorbance, initially measured over a 5-min period at 37°C using a SPECTRAmax Plus spectrophotometer (Molecular Devices). Mitochondria were then treated with 50 μM, 100 μM, or 200 μM CaCl2, and absorbance was measured for an additional 30 min at 37°C. A decrease in absorbance is indicative of PTP opening (mitochondrial swelling), an event that can occur during mitochondrial-mediated apoptotic signaling (31). Data were expressed as the percent decrease in absorbance relative to the initial absorbance (before the addition of CaCl2).
sections were then washed in PBS and incubated with goat anti-mouse IgG-rhodamine (Santa Cruz Biotechnology) for 1 h in the dark at room temperature. Sections were counterstained with DAPI, washed 3×5 min in PBS, and coverslips were mounted with Prolong Gold antifade reagent. Positive control sections (pretreated for 30 min with 50 µg/ml DNase I in 1 mM MgSO₄), which showed TUNEL-positive staining of nearly all nuclei were also included (data not shown). Slides were visualized with the fluorescent microscopy system above. Apoptotic nuclei were counted only if colocalization of TUNEL and DAPI staining was observed. Nuclei residing within the sarcolemma were identified as myonuclei, whereas nuclei located outside the sarcolemma were considered to be located to interstitial cells. All TUNEL-positive nuclei were counted and expressed per whole-muscle section as previously described (22, 37).

Cytoplasmic histone-associated mono- and oligonucleosomes were determined using the Cell Death Detection ELISA Plus Kit (Roche Diagnostics) as previously reported (58, 59). Briefly, muscle was homogenized in the supplied lysis buffer and centrifuged at 200 g for 10 min at room temperature. Supernatant (20 µl) was incubated with 80 µl of anti-histone-biotin/anti-DNA-POD reagent in a streptavidin-coated microplate for 2 h at room temperature under gentle shaking. The solution was then removed, wells were washed several times, and 100 µl of ABTS substrate solution was added to each well. Absorbance was measured at 405 nm and 490 nm using a SPECTRAMax Plus spectrophotometer (Molecular Devices). As a control for DNA fragmentation, a sample containing a DNA-histone-complex was included and produced a strong positive signal (data not shown). Absorbance was normalized to total protein content and expressed as arbitrary units per mg protein.

Statistical analysis. Immunoblot, proteolytic enzyme activity, DNA fragmentation, TUNEL, ROS generation, mitochondrial PTP, and mitochondrial membrane potential data were analyzed by Student’s t-tests, whereas fiber-type-specific data were analyzed with a one-way ANOVA. In the case of a significant ANOVA, Tukey’s honestly significant difference post hoc test was used to determine differences between means. Correlation analysis was performed by calculating the Pearson coefficient. All statistical analyses were performed using SPSS analysis software. In all cases, $P < 0.05$ was considered statistically significant. All results are given as means ± SE.

RESULTS

Whole-muscle and subcellular apoptotic protein expression. As an initial step to examine apoptotic differences between muscles of different fiber-type composition, we utilized the gastrocnemius muscle and separated the RG and WG portions. As can be seen in Fig. 1, immunofluorescence analysis of MG can easily identify type I, IIA, IIX, and IIB fibers (Fig. 1, A and B). Also shown are representative images of the RG, which is composed of type I, IIA, and IIX fibers (Fig. 1, C), as well as the WG, which is composed primarily of type IIB fibers (Fig. 1D). Fiber-type analysis confirmed that the RG was composed of 40.2 ± 2.0% type I, 31.5 ± 1.7% type IIA, and 28.3 ± 3.0% type IIX fibers. In contrast, the WG was composed of 88.3 ± 2.5% type IIB and 11.7 ± 2.5% type IIX fibers. Immunoblot analyses were then performed on the RG and WG to determine the expression of several key apoptosis-related proteins. The protein content of ARC, Bax, Bcl-2, and Hsp70 protein was 68% ($P < 0.001$), 12% ($P < 0.05$), 44% ($P < 0.05$), and 153% ($P < 0.001$) higher, respectively, in RG compared with WG muscle. The level of XIAP protein was not different between RG and WG muscle (Fig. 2, A and B). The Bcl-2-to-Bax ratio was significantly ($P < 0.05$) higher in RG compared with WG muscle (1.28 ± 0.11 and 1.0 ± 0.03 arbitrary units, respec-
Similarly, higher levels of the mitochondrial-related apoptotic proteins, AIF (2.3-fold; \( P < 0.001 \)), Smac (10.2-fold; \( P < 0.001 \)), and cytochrome c (4.9-fold; \( P < 0.001 \)) in RG compared with WG muscle (Fig. 3, A and B). This was somewhat expected given the higher mitochondrial content of the RG muscle (as indicated by higher levels of the mitochondrial markers ANT and MnSOD) (Fig. 3A).

Subcellular distribution studies were conducted in mitochondrial-enriched, cytosolic-enriched, and nuclear-enriched fractions (Fig. 4). Cytosolic levels of AIF, Smac, and cytochrome c are commonly used as indicators of apoptogenic protein release, whereas nuclear AIF levels are used as an indicator of AIF-nuclear translocation. The protein levels of cytosolic AIF, Smac, and cytochrome c, as well as nuclear AIF, were significantly (\( P < 0.05 \)) higher in RG compared with WG muscle.

Caspase and calpain activity, DNA fragmentation, and TUNEL staining. Caspase and calpain enzyme activity was evaluated by fluorescent spectroscopy in skeletal muscle homogenates. The activity of the initiator caspases, caspase-8 and caspase-9, were 16% (\( P < 0.05 \)) and 24% (\( P < 0.001 \)) higher, respectively, in RG compared with WG muscle (Fig. 5A). Similarly, the activity of the executioner caspase, caspase-3, was 37% (\( P < 0.001 \)) higher in RG compared with WG muscle (Fig. 5A). In addition, calpain activity was found to be 36%...
Higher in RG compared with WG muscle (Fig. 5A). The level of DNA fragmentation (a hallmark of apoptosis), as determined by ELISA in muscle homogenate, was approximately threefold ($P < 0.01$) higher in RG compared with WG muscle (Fig. 5B). TUNEL staining was also performed to identify apoptotic nuclei (Fig. 5C). Across eight cross sections, the average number of TUNEL-positive nuclei was $2.63 \pm 0.96$ (0.63 ± 0.26 myonuclei and 2.00 ± 0.76 interstitial nuclei) in RG muscle and $1.63 \pm 0.71$ (0.13 ± 0.13 myonuclei and 1.50 ± 0.60 interstitial nuclei) in WG muscle; however, these differences were not statistically significant.

Total muscle ROS generation. Skeletal muscle tissue homogenate steadily generated ROS over time, as indicated by DCFH oxidation (data not shown). ROS generation was 55% ($P < 0.05$) higher in RG compared with WG muscle (Fig. 6A). Incubation of samples with the ROS scavenger Tiron significantly ($P < 0.005$) reduced DCFH oxidation by ~80% across both muscles; confirming that the fluorescent signal obtained was due to ROS generation (Fig. 6A).

$Ca^{2+}$-induced loss of membrane potential and PTP opening in isolated mitochondria. The membrane potential of freshly isolated mitochondria from RG and WG muscle was evaluated by rhodamine 123 fluorescence. Membrane potential significantly ($P < 0.05$) decreased in response to $CaCl_2$ in mitochondria from both the RG and WG muscle. Interestingly, the percent decrease in membrane potential was significantly ($P < 0.05$) greater in mitochondria isolated from RG compared with WG muscle at several $CaCl_2$ concentrations (Fig. 6B). Similarly, PTP opening (demonstrated by a decrease in 540 nm absorbance) was significantly ($P < 0.05$) induced in response to $CaCl_2$ in mitochondrial from both RG and WG. However, the percent decrease in absorbance was significantly ($P < 0.05$) greater in mitochondria isolated from RG compared with WG muscle at several $CaCl_2$ concentrations (Fig. 6C).

Distribution of ARC and AIF. Given the important role of ARC in regulating apoptosis and that we found higher ARC levels in RG compared with WG muscle (see Fig. 2), we were interested in performing additional analyses to examine ARC distribution. Therefore, we examined whether ARC expression was influenced by fiber-type and/or mitochondrial content by performing histochemical analysis on MG muscle (due to the presence of all 4 major fiber types). ARC protein was significantly ($P < 0.001$) different across fiber types, with the highest levels in type I fibers and a rank order of type I > IIA > IIX > IIB fibers. In addition, NADH-TR staining revealed that ARC was not directly dependent on oxidative potential (Fig. 7, A–D). This finding was consistent with our fiber-type data since ARC levels were lower in type IIA fibers compared with type I fibers, even though type IIA fibers are more oxidative in rat muscle (62). Additional immunohistochemical experiments revealed a strong uniform staining pattern across fibers in soleus (primarily composed of type I fibers),
whereas a weaker and mixed staining pattern across fibers was observed in plantaris (primarily composed of type II fibers) (data not shown). As an additional measure of the subcellular localization of ARC, mitochondrial-enriched, cytosolic-enriched, and nuclear-enriched fractions were prepared and revealed that ARC was predominantly localized to the cytosol in healthy muscle (Fig. 7E).

We performed similar experiments to examine the distribution of AIF in muscle. As shown previously (see Fig. 4), AIF was predominantly localized to the mitochondria. In agreement with this finding, immunofluorescence analysis revealed that AIF was found to be highest in type IIA fibers and lowest in type IIB fibers, and was correlated to NADH-TR staining (Fig. 8, A–D). This was confirmed on four different muscle samples ($r_1 = 0.80, r_2 = 0.83, r_3 = 0.86, r_4 = 0.87; \forall P < 0.001$). In addition, immunofluorescence imaging revealed a punctate cytoplasmic and strong subsarcolemmal staining pattern in muscle fibers, consistent with intermyofibrillar and subsarcolemmal mitochondrial localization (Fig. 8E).

We also performed whole-section imaging of MG stained for ARC and AIF to examine the regional expression of these two proteins. As can be seen, there are clear differences in the expression of both ARC (Fig. 9A) and AIF (Fig. 9B) depending on the muscle region investigated.

**DISCUSSION**

In the present study, we examined several apoptosis-related indices in muscles with distinct fiber-type, functional, and metabolic characteristics. We found significantly higher levels of ARC, AIF, Bax, Bcl-2, cytochrome c, Hsp70, and Smac, but not XIAP in RG compared with WG muscle. Similar observations were noted when comparing soleus (primarily composed of type I fibers) to plantaris (primarily composed of type II fibers) muscle (data not shown). There was also a greater expression of AIF, Smac, and cytochrome c in cytosolic-enriched fractions, as well as higher AIF protein in nuclear-enriched fractions from RG compared with WG muscle. Furthermore, the expression of the antiapoptotic protein ARC was dependent on fiber type and found to be highly expressed in type I fibers. Similarly, the expression of the proapoptotic protein, AIF, was differentially expressed across fibers and highly related to oxidative potential. In addition, caspase -3, -8, -9 and calpain activity, as well as the level of DNA fragmentation (a hallmark of apoptosis), were significantly higher in RG compared with WG muscle. Moreover, total muscle ROS generation, as well as Ca$^{2+}$-induced PTP opening and loss of membrane potential in isolated mitochondria, were greater in RG muscle.
Skeletal muscle is a complex tissue comprised of several fiber types, each characterized by a particular expression of proteins related to metabolic, contractile, and structural parameters (18, 42, 64, 72). No report to date has comprehensively examined or discussed the significance of differences in key apoptotic indices between muscles or fiber types. However, there are a few reports that indicate that differences may exist between muscles. For example, basal Bax protein content was...
found to be higher in rat soleus compared with WG muscle, whereas the opposite trend was observed for Bcl-2 protein (61). These findings are in partial agreement with the present findings, as we found a significantly higher level of Bax (albeit small) in RG compared with WG muscle; however, the previous findings are in contrast to our Bcl-2 data. It should be noted that although the Bax levels were only slightly higher in RG compared with WG, much higher levels were observed in soleus compared with plantaris muscle (data not shown). In a report by Pistilli and Alway (54), the level of DNA fragmentation per milligram protein was higher in rat soleus relative to plantaris muscle; however, a direct comparison was not performed. This is consistent with our data showing significantly elevated levels of DNA fragmentation in RG compared with WG muscle. Caspases and calpains are important proteolytic enzymes involved in apoptotic signaling (24, 60, 67). Consistent with our DNA fragmentation data, caspase-3, -8, and -9, and calpain activity were higher in RG compared with WG muscle. Overall, these data demonstrate that apoptosis-related protein expression, proteolytic enzyme activity, and DNA fragmentation differ between muscles.

It is well established that mitochondrial content can vary between muscles and fibers (42, 64, 72). Given these differences and the essential role of mitochondria on apoptotic signaling (20, 38, 39, 66, 69), it would be important to consider mitochondrial content when studying muscle apoptosis. In this

Fig. 8. Relationship between AIF expression and oxidative potential in MG muscle. A: representative cross section of MG muscle showing AIF protein expression. B: serial cross section of A showing type I (blue), IIA (green), IIB (red), and IIX (unstained) fibers. C: serial cross section of A and B showing NADH-TR staining. D: representative Pearson’s correlation analysis of AIF by NADH-TR staining in MG muscle. E: a magnified microscopy image of AIF staining in MG muscle illustrating punctate cytoplasmic staining and strong subsarcolemmal staining.

Fig. 9. Distribution of apoptosis-related proteins in muscle. A: ARC protein expression across a full cross section of MG muscle. B: AIF protein expression across a full cross section of MG muscle.
study, we found higher levels of AIF, cytochrome c, and Smac protein in RG compared with WG muscle, which were, at least in part, related to the higher mitochondrial content in RG muscle. Of particular interest was the magnitude of these differences (AIF: 2.3-fold, cytochrome c: 4.9-fold, and Smac: 10.2-fold), suggesting that there are differences in the expression of these proteins that are likely independent of mitochondrial content. It is important to note that these data alone do not imply higher levels of apoptotic signaling, as these factors have little immediate influence on apoptotic signaling when residing in the mitochondria. Several mitochondrial apoptotic factors have dual roles, participating in both vital cellular functions and apoptotic signaling. For example, cytochrome c and AIF play important roles in electron transport and oxidative phosphorylation (reviewed in Ref. 27). Overall, these results point to the fact that there may be differences in the involvement of particular signaling molecules/pathways between muscles during apoptosis. For example, it is possible that a muscle with more mitochondria may show different responses or kinetics to a mitochondria-mediated apoptotic stimulus or disease state compared with a muscle with less mitochondria; however, this remains to be determined.

For these mitochondrial proteins to participate in cell death signaling they must be released from the mitochondria and interact with their respective cytosolic and nuclear targets (20, 27, 38, 39, 66, 69). In this study, we found higher protein levels of AIF, cytochrome c, and Smac in cytosolic-enriched fractions, as well as higher AIF levels in nuclear-enriched fractions of RG compared with WG muscle. This state would tend to increase basal apoptotic signaling and is consistent with our caspase and DNA fragmentation data. Specifically, higher cytosolic cytochrome c and Smac levels would amplify caspase-9 and -3 activity as well as DNA fragmentation (20, 39, 69), whereas higher cytosolic and nuclear AIF levels would also contribute to elevated DNA fragmentation (66, 69). Activation of the PTP and loss of mitochondrial membrane potential are important events that occur during mitochondrial-mediated apoptosis and are linked to the release of mitochondrial housed proteins. PTP and loss of membrane potential can be triggered by a number of factors including Bax, ROS, and elevated cytosolic Ca\(^{2+}\) levels (2, 31, 53). We found that Ca\(^{2+}\)-induced PTP opening and loss of membrane potential were higher in isolated mitochondria from the RG compared with WG. This is consistent with a previous report demonstrating a greater sensitivity to Ca\(^{2+}\)-induced PTP opening in mitochondria isolated from the soleus compared with the WG muscle (53). It has also been reported that resting cytosolic Ca\(^{2+}\) (13, 26), as well as mitochondrial matrix Ca\(^{2+}\) levels (53) are higher in slow compared with fast muscles/fibers. Furthermore, our data also demonstrate that total muscle ROS generation is higher in RG compared with WG muscle. Given the current and previously reported differences in PTP opening, loss of membrane potential, Ca\(^{2+}\) concentrations, ROS generation, and Bax levels between muscles, it is likely that mitochondrial-derived apoptogenic factors would be higher in the cytosol of slow/oxidative muscle; effects that would influence basal caspase activity and DNA fragmentation.

We also demonstrate that several apoptosis-related proteins are differentially expressed across fibers. In particular, we examined the fiber type and subcellular distribution of ARC protein. A number of cytoprotective proteins that regulate apoptosis are present in cells (i.e., ARC, Bcl-2, XIAP, FLIP, Hsp70) (7, 19, 36, 45, 46, 68). Unlike most antiapoptotic proteins, which mediate their action on one of the main apoptotic signaling pathways, ARC is able to regulate both the death receptor and mitochondrial pathway (35, 46). ARC also has a unique tissue distribution, being highly expressed in cardiac and skeletal muscle but absent or low in other tissues (1, 35). These unique characteristics suggest that ARC is likely a key regulator of apoptotic signaling in skeletal muscle. In the present study, we found that ARC was significantly elevated in RG compared with WG muscle. It has been shown that ARC can be localized to the mitochondria, cytosol, and nucleus (23, 40, 44, 71). Given that ARC can be localized to the mitochondria, we examined whether ARC expression in muscle was related to fiber type or oxidative potential. Our data demonstrate that ARC protein is considerably higher in type I fibers and is not directly related to oxidative potential. We have recently reported that ARC protein is differentially expressed across fibers in rat muscle (57), and higher in type I compared with type IIA and type IIX fibers in human muscle (58). Abmayr et al. (1) reported that ARC staining colocalized with cytochrome oxidase subunit V staining in mouse muscle, indicating that ARC is mainly expressed in oxidative fibers and localized with mitochondria; however, muscle fiber type was not determined. According to this previous investigation, it would be expected that ARC levels would be highest in type IIA fibers [the most oxidative fibers in rat (62)] and predominantly localized to the mitochondrial fraction; however, this was not found. Although it is currently unclear what accounts for these discrepancies, this may be related to differences in the species utilized or muscle/muscle area analyzed. For example, if analysis is performed on a muscle/region with little to no type I fibers, the general relationship reported by Abmayr et al. (1) would hold true as both oxidative potential and ARC expression would be highest in type IIA fibers followed by type IIX and type IIB fibers.

There are a few reports that show differential expression of additional apoptosis-related proteins across fibers. Plomgaard et al. (56) found that TNF-α protein was predominantly expressed in type II fibers of human muscle. Immunohistochemical staining images from Braga et al. (9) show a nonuniform expression pattern for Bax and Bcl-2 protein across fibers in mouse gastrocnemius muscle; however, fiber-type analysis was not performed. We found that AIF protein was differentially expressed across fibers and was strongly related to oxidative potential. Furthermore, punctate cytoplasmic staining along with strong subsarcolemmal staining was observed in fibers, indicative of mitochondria localization. This is in agreement with our subcellular distribution data showing that AIF is predominantly localized to the mitochondria. Leeuwenburgh et al. (37) reported a similar fiber staining pattern for EndoG (another mitochondrial-derived apoptosis factor) in rat muscle. Taken together, these data suggest that both fiber type and oxidative potential are important considerations when studying apoptosis in skeletal muscle. It remains to be determined whether several other apoptotic proteins (i.e., Bcl-2, Bax, caspases) are differently expressed across fibers. It also remains to be determined whether and how distinct expression patterns of apoptosis-related factors influence signaling and susceptibility in specific fiber types during stress-induced or disease states.
The higher content of apoptotic factors found in RG compared with WG may reflect differences in basal degradation, damage, and apoptotic signaling. In support of this, caspase-3 is known as an important proteolytic enzyme (67) that can cleave actin and increase muscle protein degradation (21). Interestingly, the rate of protein degradation and synthesis (turnover) are higher in slow/oxidative muscle compared with fast/glycolytic muscle (28, 30). A recent report has also found that a number of factors involved in autophagy (an important degradation system) are also expressed at higher levels in soleus compared with plantaris muscle (43). Satellite cells play an important role in the repair and regeneration of damaged muscle fibers (reviewed in Ref. 14). Interestingly, the density of satellite cells is higher in slow compared with fast muscles/fibers (14, 29). Therefore, it is possible that the greater proportion of satellite cells may be a mechanism to ensure sufficient muscle repair and/or replacement of lost nuclei in response to increased muscle degradation and apoptotic signaling; however, this remains to be determined. Heat shock proteins such as Hsp70 are rapidly synthesized in response to protein-damaging stress (49) and can alter apoptotic signaling by influencing a number of factors (68). Under basal conditions Hsp70 is highly expressed in slow compared with fast muscle and is restricted to type I and type IIA fibers (41, 49), which is consistent with our findings. It has been suggested that the higher level of Hsp70 in these fibers may be due to a continual exposure to a more stressful environment (41). Considering the important antiapoptotic roles of Hsp70 (7, 68), Bcl-2 (24, 45), and ARC (35, 46), higher levels of these factors in RG may suggest that this muscle (and possibly type I and IIA fibers) is under higher levels of apoptotic stress. In agreement with this, previous work has shown that several antiapoptotic proteins (i.e., Bcl-2, FLIP, Hsp70) are elevated in aged and denervated skeletal muscle; possibly as a mechanism to counteract higher apoptotic signaling (16, 55, 63).

It is currently unclear whether basal differences in apoptosis-related indices would influence muscle susceptibility to cell death stimuli in vivo. Previous investigators found that the number of caspase-3 positive fibers is higher in rat soleus compared with tibialis anterior muscle following administration of clenbuterol (10) or angiotensin II (11). The percentage of apoptotic nuclei also increases rapidly in rat soleus muscle following strenuous exercise, but only gradually in gastrocnemius muscle (34). Bax levels also increase to a greater extent in soleus compared with gastrocnemius muscle following denervation; an effect suggested to be related to the higher mitochondrial content in soleus (4). Furthermore, Degens et al. (17) observed elevated levels of DNA fragmentation in soleus but not plantaris and gastrocnemius muscle of the emphysematous hamsters; an effect they speculated may be a consequence of an increased susceptibility to mitochondrial-mediated apoptosis in soleus due to a greater mitochondrial content. It has also been reported that TNF-α-related apoptotic events and DNA fragmentation increase with age and are more prevalent in type II- than in type I-containing muscle (52). Similarly, Pistilli et al. (55) found that muscles made up of type II fibers may be more susceptible to mass losses via death receptor signaling during aging. Collectively, these data suggest that apoptotic susceptibility may differ across muscles and fiber types, and that different pathways (i.e., death receptor vs. mitochondrial) may be preferentially activated in distinct muscles/fibers during particular stress or disease states.

Considering the significant differences in several apoptotic factors between muscles and fibers observed in this study, careful consideration should be taken from both a methodological and interpretation perspective when studying muscle apoptosis. For example, as demonstrated in Fig. 9, regional differences in ARC and AIF expression are evident in a mixed muscle. Depending on the region analyzed or area isolated, marked differences in the protein content obtained can be expected. Therefore, careful and consistent isolation/analysis of muscle with mixed fiber types and varying mitochondrial content is essential. In addition, this is of particular importance when studying conditions that show alterations in fiber-type composition and/or mitochondrial content (i.e., chronic heart failure, aging, chronic obstructive pulmonary disease). For example, a condition that causes a preferential atrophy or loss of type I fibers may display a decrease in ARC protein (and possibly other apoptotic markers), which may be due to changes in fiber-type composition and not apoptosis/apoptotic signaling per se. Similarly, conditions that alter mitochondrial content will likely influence total AIF expression (and other mitochondrial-associated apoptotic proteins), which will not necessarily imply alterations in mitochondrial-mediated apoptotic signaling.

Perspectives and Significance

In the present study, we found that a number of apoptotic indices differ across muscles and fibers. Our data show significantly higher levels of several pro- and antiapoptotic factors, ROS generation, and apoptosis-related mitochondrial events in RG compared with WG muscle, possibly indicating a greater basal level of apoptotic signaling/stress. Independent of the direct implications of these findings on basal apoptosis rates, the present data suggest that the participation of specific proteins and signaling pathways in particular muscles and/or fiber types should be considered when studying skeletal muscle apoptosis. In addition, these data demonstrate that researchers studying skeletal muscle apoptosis should consider muscle region, mitochondrial content, and fiber type when performing biochemical and molecular analyses.

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DISCLOSURES

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