Effect of chronic contractile activity on SS and IMF mitochondrial apoptotic susceptibility in skeletal muscle

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Adhihetty PJ, Ljubicic V, Hood DA. Effect of chronic contractile activity on SS and IMF mitochondrial apoptotic susceptibility in skeletal muscle. Am J Physiol Endocrinol Metab 292: E748–E755, 2007. First published November 14, 2006; doi:10.1152/ajpendo.00311.2006.—Chronic contractile activity of skeletal muscle induces an increase in mitochondria located in proximity to the sarcolemma [subsarcommaal (SS)] and in mitochondria interspersed between the myofibrils [intermyofibrellar (IMF)]. These are energetically favorable metabolic adaptations, but because mitochondria are also involved in apoptosis, we investigated the effect of chronic contractile activity on mitochondrially mediated apoptotic signaling in muscle. We hypothesized that chronic contractile activity would provide protection against mitochondrially mediated apoptosis despite an elevation in the expression of proapoptotic proteins. To induce mitochondrial biogenesis, we chronically stimulated (10 Hz; 3 h/day) rat muscle for 7 days. Chronic contractile activity did not alter the Bax/Bcl-2 ratio, an index of apoptotic susceptibility, and did not affect manganese superoxide dismutase levels. However, contractile activity increased antiapoptotic 70-kDa heat shock protein and apoptosis repressor with a caspase recruitment domain (ARC) protein by 1.3- and 1.4-fold (P < 0.05), respectively. Contractile activity elevated SS mitochondrial reactive oxygen species (ROS) production 1.4- and 1.9-fold (P < 0.05) during states IV and III respiration, respectively, whereas IMF mitochondrial state IV ROS production was suppressed by 28% (P < 0.05) and was unaffected during state III respiration. Following stimulation, exogenous ROS treatment produced less cytochrome c release (25–40%) from SS and IMF mitochondria, and also reduced apoptosis-inducing factor release (∼30%) from IMF mitochondria, despite higher inherent cytochrome c and apoptosis-inducing factor expression. Chronic contractile activity did not alter mitochondrial permeability transition pore (mtPTP) components in either subfraction. However, SS mitochondria exhibited a significant increase in the time to Vmax of mtPTP opening. Thus, chronic contractile activity induces predominantly antiapoptotic adaptations in both mitochondrial subfractions. Our data suggest the possibility that chronic contractile activity can exert a protective effect on mitochondrially mediated apoptosis in muscle.

Exercise; respiration; reactive oxygen species; apoptosis; mitochondrial permeability transition pore

Programmed cell death (apoptosis) is characterized by distinct morphological features such as cell shrinkage, DNA fragmentation, plasma membrane blebbing, and the formation of apoptotic bodies (2, 24). The dismantled cell is then removed via phagocytosis (2, 4). Apoptosis within skeletal muscle is unique compared with other cells, since muscle cells are multinucleated. Thus, when apoptosis strikes a myonucleus, the remaining nuclei, and the rest of the muscle fiber, may remain intact. Thus, programmed cell death within muscle is more appropriately described as “myonuclear apoptosis,” and this process contributes to muscle atrophy during periods of chronic muscle disuse, sarcopenia, and specific pathological muscle disorders (2, 4, 16, 36).

Mitochondria are intimately affiliated with apoptosis since they contain the proteins apoptosis-inducing factor (AIF) and cytochrome c, which can activate cell death through alternative pathways once released into the cytosol (27, 47). Cytochrome c-mediated cell death involves a complex series of proteolytic cleavages known as the caspase cascade, whereas AIF directly translocates to the nucleus to induce DNA fragmentation and nuclear degradation (14, 27). Mitochondrial proapoptotic protein release is contingent on the formation of a specialized mitochondrial permeability transition pore (mtPTP), composed of the voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT), and cyclophilin D. The mtPTP is regulated by Bcl-2 family members, such as proapoptotic Bax, which facilitates pore opening, and antiapoptotic Bcl-2, which inhibits pore opening (40). An increase in the ratio of Bax to Bcl-2 is typically indicative of an increased susceptibility to mitochondrially mediated apoptosis (40). However, a triggering stimulus causing mitochondrial proapoptotic protein release does not necessarily foreshadow cell death, since inhibitory cytosolic factors can impede the progression of apoptotic pathways. For example, 70-kDa heat shock protein (HSP70) has a primary function as a cytosolic chaperone (30), but it is also capable of inhibiting both the cytochrome c- and AIF-mediated apoptotic signaling pathway (9, 38). Furthermore, the apoptosis repressor with a caspase recruitment domain (ARC) protein has also been shown to specifically inhibit the cytochrome c-mediated pathway within the cytosol (17, 25).

Mitochondria are also a primary source of reactive oxygen species (ROS), which can either directly or indirectly evoke apoptosis (11). ROS can interact with mtPTP components to induce pore opening, causing proapoptotic protein release, and can also evoke cytochrome c dissociation from the inner membrane (8, 32). In addition, ROS can indirectly influence the apoptotic pathway by activating various redox-sensitive transcription factors involved in the expression of both anti- and proapoptotic genes (13, 22, 34). Whether elevations in ROS directly or indirectly influence the apoptotic pathway seems to be dependent on the concentration of ROS attained. ROS levels that exceed a certain threshold appear to promote the activation of apoptosis, whereas ROS levels below the threshold initiate signaling pathways leading to changes in gene expression. Although ROS are primarily produced by mitochondria and can induce apoptotic and/or signaling events, there are also several mitochondrial antioxidants, such as...
manganese superoxide dismutase (MnSOD), that can serve to neutralize ROS.

Chronic contractile activity of skeletal muscle induces a wide variety of biochemical and physiological adaptations. It is well established that the metabolic perturbations produced by electrically induced chronic contractile activity using low-frequency stimulation and/or endurance training lead to the activation of multiple signaling pathways to ultimately produce an increase in mitochondrial density known as mitochondrial biogenesis (1, 18, 29). In addition to increasing mitochondrial content, chronic contractile activity-induced biogenesis has also been shown to alter organelle composition and functional properties (48). These adaptive changes affect mitochondria that are in proximity to the sarcolemma [subsarcolemmal (SS) mitochondria], as well as those interspersed among the myofibrils [intermyofibrillar (IMF) mitochondria (3, 12, 49)]. Although mitochondrial biogenesis is clearly an advantageous metabolic adaptation, the effect of chronic contractile activity on mitochondrially mediated apoptotic signaling is not established. It is known that mitochondrially mediated apoptosis is involved in inducing muscle atrophy following chronic muscle disuse, sarcopenia, and various other pathological muscle disorders (2, 5, 16, 36, 43). Since the chronic contractile activity induction of mitochondrial biogenesis could produce an obligatory increase in a number of proapoptotic mitochondrial proteins, this could potentially result in an exacerbated apoptotic response following a triggering stimulus. We hypothesized that, despite the possible upregulation of mitochondrial proteins involved in apoptosis, mitochondrial apoptotic susceptibility would be reduced following chronic muscle use. Thus, the purpose of this study was to determine the impact of chronic contractile activity-induced mitochondrial biogenesis on mitochondrial apoptotic susceptibility in skeletal muscle.

**METHODS**

In vivo chronic contractile activity protocol. Male Sprague-Dawley rats (n = 40; Charles River, St Constant, QC, Canada) weighing between 300 and 325 g were individually housed and given food and water ad libitum. All procedures involving animals were approved by the York University Animal Care Committee, in accordance with the Canadian Council on Animal Care. Surgical implantation of electrodes and chronic low-frequency electrical stimulation of animals was performed as previously described (48). Briefly, rats were anesthetized using pentobarbital sodium (60 mg/kg). Under sterile conditions, a small incision (1 cm) was made on the left hindlimb penetrating through the superficial hamstring muscle group, and two platinum electrodes were sutured on either side of the common peroneal nerve. Electrode wires were passed subcutaneously from the hindlimb to the back of the neck where they were exteriorized and secured to an external portable stimulation unit fastened to the back of the animal. Stimulation intensity was adjusted to produce maximal contractions of the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles. The overlying tissue was sutured and the skin stapled. Animals recovered for 1 wk and were then subjected to 7 days of chronic stimulation (10 Hz, 0.1 ms duration, 3 h/day) with the contralateral limb serving as a nonstimulated, intra-animal control. Tissues were extracted from anesthetized (60 mg/kg pentobarbital sodium) animals 21 h after the last stimulation to avoid any acute contractile activity effects and to allow for the typical recovery period adaptations (18) to occur following the final day of stimulation. Animals were subsequently killed by removal of the heart.

**Immunoblotting.** Whole muscle protein extracts were separated by 10–15% SDS-PAGE and subsequently transferred to nitrocellulose membranes by means of a semidy electrotransfer apparatus. Nitrocellulose membranes were blocked (1 h) with 5% skim milk in 1× TBS-T (Tris-buffered saline-Tween-20, 25 mM Tris-HCl, pH 7.5, 1 mM NaCl, and 0.1% Tween-20) solution. Membranes were then incubated with primary antibodies directed against AIF (1:1,500), cytochrome c (1:750), MnSOD (1:2,000), Bax (1:500), Bcl-2 (1:1,000), HSP70 (1:500), calpain (1:1,000), or ARC (1:1,000) overnight at 4°C. Membranes were subsequently washed (3 × 5 min) using TBS-T to remove excess primary antibody and incubated with the corresponding secondary antibody at room temperature (45 min). Antibodies were obtained from Santa Cruz Biotechnology (AIF, sc-9416; Bax, sc-526; Bcl-2, sc-492), Stresgen (HSP70, SP-810), Cayman (ARC, 160737), Upstate (MnSOD, 06-984) and Calbiochem (calpain, 208751). The cytochrome c antibody was produced in our laboratory (44). Membranes were washed (3 × 5 min) in TBS-T to eliminate unbound secondary antibody, and detection was revealed using the ECL method. Films were scanned and analyzed using ImageJ (v. 1.35S software). Enzymatic reactions were performed in triplicate and normalized to the protein content of each sample.

**Mitochondrial isolation.** TA muscles were excised from anesthetized animals, placed into ice-cold buffer, briefly miniced, and then homogenized. Differential centrifugation was used to fractionate SS and IMF mitochondria, as described previously in detail (12, 28, 49). SS and IMF mitochondria were resuspended in medium 1 (100 mM KCl, 10 mM MOPS, 0.2% BSA) for mitochondrial respiration, ROS production, and the mitochondrial release assay or in medium 2 (215 mM mammal, 71 mM succrose, 3 mM Hepes, 5 mM succinate) for the mtPTP assay.

**Mitochondrial respiration.** Rates of respiration in isolated SS and IMF mitochondria were measured in oxygen consumption (V\textsubscript{O2}) buffer (250 mM sucrose, 50 mM KCl, 25 mM Tris-HCl, 10 mM K2HPO\textsubscript{4} with continuous stirring at 30°C. V\textsubscript{O2} (natoms O\textsubscript{2} min\textsuperscript{-1} mg\textsuperscript{-1}) was assessed in the presence of 11 mM glutamate (state IV respiration) or glutamate plus 0.4 mM ADP (State III respiration) by use of a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH), as done previously (3, 12, 28).

**Cytochrome c oxidase enzyme activity.** Whole muscle powdered tissues were diluted in a buffer (0.1M KH\textsubscript{2}PO\textsubscript{4} + 2 mM EDTA, pH 7.2) and sonicated (3 × 5 s) on ice as previously described in detail (12). Enzyme activity was determined by the maximal oxidation rate of completely reduced cytochrome c, evaluated as a change in absorbance at 550 nm using a Beckman DU-64 spectrophotometer.

**Protein release assay.** Isolated SS and IMF mitochondrial fractions (150 μg) from control and stimulated muscle tissue were incubated with 250 μM H\textsubscript{2}O\textsubscript{2} and 50 mM FeSO\textsubscript{4} for 60 min at 30°C, as described previously (3). Reaction mixtures were subsequently centrifuged at 14 000 g (4°C) to pellet mitochondria, and the supernatants were analyzed for AIF and cytochrome c released from mitochondria by means of Western blotting. Mitochondrial protein release supernatants obtained from control and stimulated muscle (both nontreated and treated with H\textsubscript{2}O\textsubscript{2}) were analyzed on the same gels. SS and IMF mitochondrial supernatant fractions were analyzed on separate gels.

**mtPTP assessment.** mtPTP opening was measured by assessing the reduction of light scattering associated with mitochondrial swelling at 540 nm, as described previously (3). SS and IMF mitochondria from control and stimulated muscle were treated with 400 μM Ca\textsubscript{2+} and 75 μM t-BuOOH in the presence of 10 mM succinate. The decrease in absorbance was measured spectrophotometrically for 15 min at 30°C (Beckman DU-64).

**ROS production.** ROS were measured as described previously (3). In brief, SS and IMF mitochondria (50 μg) from control and stimulated muscle were incubated with 50 μM dichlorodihydrofluorescein diacetate (H\textsubscript{2}DCFDA) and V\textsubscript{O2} buffer at 37°C for 80 min on a 96-well plate. The fluorescence emission between 480 and 520 nm measured with a multidetection microplate reader (Synergy HT, Biotek Instruments, Winooski, VT) is directly related to ROS production. Data
were acquired and interpreted using KC4 (v. 3.0) software. ROS production was assessed during simulated states IV and III respiration by adding 11 mM glutamate or glutamate plus 0.4 mM ADP, respectively, immediately prior to the addition of H$_2$DCFDA. ROS production measured in absolute fluorescence units was linear over the entire measurement period, a time frame used to ensure a high level of fluorescence units that were ≥10-fold above background. ROS levels were expressed per nanomol of O$_2$ consumed measured during mitochondrial respiration.

Statistical analyses. Data are expressed as means ± SE. Paired Student’s t-tests were used for comparison of data between control and stimulated whole muscle tissue or mitochondrial subfraction. Two-way analyses of variance were performed when control and stimulated whole muscle tissue or mitochondrial subfraction. Expression measured in absolute fluorescence units was linear over the entire measurement period, a time frame used to ensure a high level of fluorescence units that were ≥10-fold above background. ROS levels were expressed per nanomol of O$_2$ consumed measured during mitochondrial respiration.

RESULTS

Effect of chronic contractile activity on SS and IMF mitochondrial biogenesis and pro- and antiapoptotic protein expression. Our chronic-stimulation protocol was effective at inducing mitochondrial biogenesis in both subfractions, as indicated by significant 27 and 30% increases in the yield of isolated SS and IMF mitochondria, respectively, compared with control (P < 0.05; Fig. 1A). In addition, whole muscle cytochrome c oxidase (COX) activity, a measure of mitochondrial content, was significantly elevated by 37% following chronic contractile activity (Fig. 1A). Contractile activity significantly elevated the expression of proapoptotic AIF and cytochrome c by 2.2- and 1.4-fold, respectively (Fig. 1B). The levels of antiapoptotic ARC and HSP70 were also significantly increased by 1.4- and 1.3-fold, respectively (Fig. 1B). Chronic contractile activity induced parallel, modest 20% elevations of proapoptotic Bax and antiapoptotic Bcl-2 compared with control (Fig. 1B). MnSOD levels were not significantly increased following chronic contractile activity.

Effect of chronic contractile activity on SS and IMF mitochondrial respiration and ROS production. In accord with our previous results, IMF mitochondrial state III respiration rates were approximately threefold greater than SS rates of respiration (P < 0.05; Fig. 1A) (12). SS and IMF state III respiration rates were unaffected by chronic contractile activity (Fig. 2A). In contrast, our previous results showed that chronic stimulation induced a significant 40–45% elevation in state IV (passive) IMF respiration but did not alter state IV respiration rates in SS mitochondria (Fig. 2A, inset) (28). ROS production was measured under stimulated state III and IV respiration conditions. In both mitochondrial subfractions, ROS production was significantly greater during state IV respiration compared with maximally active respiration (state III; Fig. 2, B and C), in agreement with our previous results (3). In addition, ROS production was 5- to 12-fold greater in SS compared with IMF mitochondria under all conditions measured. Following chronic contractile activity, SS mitochondrial ROS production per nanomol of oxygen consumed was elevated 1.4- and 1.9-fold during state IV and state III respiration, respectively (P < 0.05; Fig. 2B). In contrast, IMF mitochondrial ROS production during state IV respiration was significantly suppressed by 28% (P < 0.05), whereas IMF ROS production during state III respiration was not altered by chronic contractile activity (Fig. 2C).

Effect of H$_2$O$_2$ on AIF and cytochrome c release from isolated SS and IMF mitochondria following chronic contractile activity. SS and IMF mitochondria from control and stimulated muscle were compared for their ability to release cytochrome c and AIF under basal, nontreated (NT), and H$_2$O$_2$-treated (T) conditions within the same assay. The treatment of SS and IMF mitochondria with H$_2$O$_2$ caused a significant increase in the release of cytochrome c and AIF compared with nontreated mitochondria (P < 0.05; Fig. 3). In SS mitochondria, chronic contractile activity did not affect cytochrome c
A chronic low-frequency stimulation of muscle is an experimental paradigm that induces a variety of biochemical and physiological adaptations (29). One of the most prominent adaptations is the rapid induction of mitochondrial biogenesis. Although mitochondrial biogenesis is a favorable adaptation from an energy standpoint, mitochondria are also intimately involved in apoptosis. Since a greater mitochondrial content in stimulated muscle also produces an obligatory increase in proapoptotic factors contained within these additional mitochondria, it is possible that a cell death stimulus may result in release under non-H₂O₂-treated conditions, whereas H₂O₂-induced cytochrome c release from SS mitochondria tended to decrease (25%; 0.1 > P > 0.05) compared with control (Fig. 3A). Chronic contractile activity also resulted in a significant reduction in cytochrome c release from IMF mitochondria by 40 and 30% under basal and H₂O₂-treated conditions, respectively (P < 0.05; Fig. 3B). Chronic contractile activity increased basal and H₂O₂-induced AIF release from SS mitochondria 2.2- and 1.3-fold, respectively (Fig. 3C). In contrast, contractile activity reduced the basal and H₂O₂-induced AIF release from IMF mitochondria by ~30% compared with control (Fig. 3D). Thus, a differential response to contractile activity was observed within the two mitochondrial subfractions with respect to AIF release.

**Calpain expression and mtPTP function in SS and IMF mitochondria following chronic stimulation.** Calpain, a calcium-activated protease, has been reported to induce cleavage of AIF from the inner mitochondrial membrane, and this represents the initial step leading to mitochondrial AIF release (35). Thus, we evaluated mitochondrial calpain expression to specifically address whether our enhanced AIF release (basal and H₂O₂ induced) from SS mitochondria following chronic stimulation might be attributable to altered calpain expression. Chronic contractile activity tended to produce an increase (2-fold) in the expression of calpain within the SS subfraction (P = 0.07) but did not alter the level calpain in IMF mitochondria. In addition, basal SS calpain expression was 50% lower in SS compared with IMF mitochondria (Fig. 4A). As calpain is typically considered a cytosolic protease, we measured the cytosolic protein GAPDH in our mitochondrial samples and confirmed that our mitochondrial fractions contain minimal cytosolic contamination (≈2%; data not shown).

Because the mitochondrial release of proapoptotic proteins into the cytosol is ultimately dependent on the formation of the mtPTP, we also measured mtPTP kinetics. Following the chronic contractile activity, SS mitochondria exhibited a modest but significant elevation in the time to Vₘₐₓ of pore opening (P < 0.05; Fig. 4B), whereas IMF mitochondria remained unchanged compared with control. Chronic contractile activity did not alter the Vₘₐₓ of pore opening in either SS or IMF mitochondria (Fig. 4C). In agreement with our previous work (3), Vₘₐₓ and time to Vₘₐₓ were significantly greater in IMF compared with SS mitochondria (P < 0.05; Fig. 4C). This can be accounted for by the different protein composition of the mtPTP (ANT, VDAC, and cyclophilin D) in SS and IMF mitochondria (3). However, chronic contractile activity had no significant effect on the levels of these proteins within either of these mitochondrial subfractions (data not shown).

**DISCUSSION**

Chronic low-frequency stimulation of muscle is an experimental paradigm that induces a variety of biochemical and physiological adaptations (29). One of the most prominent adaptations is the rapid induction of mitochondrial biogenesis. Although mitochondrial biogenesis is a favorable adaptation from an energy standpoint, mitochondria are also intimately involved in apoptosis. Since a greater mitochondrial content in stimulated muscle also produces an obligatory increase in proapoptotic factors contained within these additional mitochondria, it is possible that a cell death stimulus may result in
Given the involvement of mitochondria in apoptosis, and the fact that chronic stimulation increases both mitochondrial content and composition, we investigated whether chronic contractile activity would alter muscle apoptotic susceptibility.

Our data clearly illustrate that our model of chronic contractile activity significantly increased muscle SS and IMF mitochondrial content, as found previously (28, 48, 50). A number of mitochondrial proteins were elevated by chronic contractile activity, including cytochrome c, which has a primary function in oxidative phosphorylation and energy provision. Thus, the chronic contractile activity-induced elevation in cytochrome c is an adaptation that has the potential of exerting a beneficial effect on mitochondrial electron transport chain function. However, this adaptation also raises the possibility of a greater apoptotic potential, since cytochrome c can initiate cell death if its release from the organelle is triggered by an apoptotic stimulus. Another protein that was markedly elevated by chronic contractile activity was AIF. This was somewhat unexpected, because the principal role of AIF appears to be the induction of cell death. Taken together, these results suggest that stimulated muscle might be more vulnerable to apoptosis upon a triggering death stimulus. However, there is evidence to suggest that AIF may serve multiple purposes within the mitochondria, as it has been proposed to potentially have antioxidant properties and has also been shown to facilitate oxidative phosphorylation (23, 51). Thus, increased AIF expression following chronic contractile activity might be a compensatory response to neutralize ROS, particularly as a result of the elevated ROS production within the SS mitochondria. In addition, chronic contractile activity also increased the expression of the antiapoptotic HSP70, which inhibits both the cytochrome c- and AIF-mediated cell death signaling pathways (9, 38). This is in agreement with previous results showing the chronic contractile activity induction of HSP70 to support important roles as a molecular chaperone, in protein folding, and in cell survival (9, 30, 33, 38). In addition, the expression of antiapoptotic ARC, a known inhibitor of the cytochrome c apoptotic death pathway, was also enhanced following chronic contractile activity. Other studies have shown that ARC levels in muscle are enhanced following endurance training, and also during long-term caloric restriction, to effectively reduce the activity of the cytochrome c-mediated cell death pathway (15, 42).

Two other proteins that are intimately involved in apoptosis are Bax and Bcl-2. An elevation in the Bax/Bcl-2 ratio is commonly used as an indicator of greater apoptotic susceptibility (40). In response to chronic contractile activity, whole muscle levels of Bax and Bcl-2 were increased in parallel. Thus, the Bax/Bcl-2 ratio was unchanged, suggesting that the impact of these proteins on the regulation of the mPTP remained unaffected by chronic contractile activity. When taken together, the changes in protein expression suggest that, despite higher levels of cytochrome c and AIF, the adaptive response to chronic contractile activity appears to favour an antiapoptotic protein environment, potentially resulting in an attenuated apoptotic response to a death-evoking stimulus.

Given the important role of ROS in mediating apoptotic cell death, we next investigated the effect of chronic contractile...
activity on mitochondrial ROS production. As we and others have shown that ROS production is inversely related to VO₂, we measured mitochondrial respiration and expressed ROS production as a function of the oxygen consumed during maximal (state III) and basal (state IV) conditions (3, 41). Our data show that ROS production in the SS mitochondrial subfraction is significantly elevated whereas ROS production in IMF mitochondria is suppressed by chronic contractile activity. These changes in ROS cannot be accounted for by the lack of change in whole muscle MnSOD observed. However, this does not preclude the possibility that other antioxidant enzymes, such as catalase and glutathione peroxidase, may be altered following contractile activity and influence ROS production. Thus, either 1) a differential expression of other antioxidant enzymes within the mitochondrial subfractions occurred, or 2) the accelerated synthesis and assembly of electron transport chain (ETC) components during contractile activity-induced mitochondrial biogenesis favored the production of additional ROS in the SS subfraction. This could arise from the nonsteady-state nature of the adaptive mitochondrial response during the 7-day stimulation period, when nuclear and mitochondrial ETC subunits are assembled according to their individual synthesis patterns. This would likely be most evident in the more labile SS mitochondrial subfraction. In addition, given the proximity of SS mitochondria to the peripherally located myonuclei, chronic stimulation-induced elevations in SS mitochondrial ROS production may exert pronounced effects on local muscle gene regulation. ROS are known to be important signaling molecules that can activate redox-sensitive transcription factors involved in gene expression (13, 22, 34). Whether the elevated ROS induces damage and/or apoptotic events compared with activating signal transduction pathways appears to depend on a certain threshold level of ROS (22).

We speculate that the modest elevations in ROS production in stimulated SS mitochondria might be involved in the activation of redox-sensitive transcription factors but that ROS production is unlikely to be of adequate intensity to initiate direct apoptotic signaling events (i.e., cytochrome c release, mtPTP opening), but this remains to be proven.

Because chronic contractile activity increased the expression of AIF and cytochrome c, we specifically determined whether high exogenous ROS exposure would induce a greater release of these proteins from mitochondria. Despite the greater cytochrome c levels in stimulated muscle, ROS-induced release of cytochrome c from mitochondria was reduced compared with...
mitochondria from control muscle. The ROS-induced release of AIF from IMF mitochondria followed a similar pattern. Thus, the upregulation of both cytochrome c and AIF in response to chronic contractile activity appears to serve only as a beneficial adaptation to muscle, without invoking a secondary, proapoptotic function. Indeed, these results suggest that chronic contractile activity induced a protective adaptation within mitochondria, resulting in a reduced proapoptotic mitochondrial release, despite a greater potential for the release of these proteins.

It has been reported that AIF release from mitochondria occurs via a two-step process, with calpain cleavage initiating AIF release from the inner mitochondrial membrane followed by release through the mtPTP (35). Calpain is a Ca$^{2+}$-sensitive cytosolic protease typically involved in the degradation of muscle proteins in response to damage and/or chronic disuse (7, 21). However, calpain activation has also been shown to occur with exercise and also with chronic low-frequency muscle stimulation that does not elicit any damage and/or fiber injury (6, 10, 37, 45, 46). Instead, contractile activity-mediated calpain activation appears to be involved in remodeling muscle from fast-twitch to slow-twitch phenotypic properties (45). Although calpain functions primarily in the cytosol, some studies have shown that calpain activation promotes its translocation to other cellular compartments (6, 45). Thus, we investigated whether contractile activity altered calpain levels within the mitochondrial subfractions as a potential cause for the elevated AIF release from SS mitochondria. Indeed, chronic stimulation led to an increased level of calpain in the SS mitochondrial subfraction but had no effect on the calpain level in IMF mitochondria. Thus, the greater level of induced AIF release in SS mitochondria as a result of chronic contractile activity could be attributable to increased calpain cleavage of AIF, to liberate it from the inner mitochondrial membrane and allow it to be released upon opening of the mtPTP. Calpain could also serve a broader function within mitochondrial subfractions, associated with steady-state mitochondrial turnover in muscle. Since chronic stimulation caused a preferential elevation of calpain in the SS subfraction, this suggests that calpain levels prior to contractile activity were unable to accommodate alterations in protein turnover within SS mitochondria but sufficient within the IMF subfraction. This is supportive of the idea that SS mitochondria are more adaptive than IMF mitochondria following periods of use and disuse (19, 20, 26, 31, 39).

Because chronic stimulation altered the proapoptotic release rates from isolated mitochondria, we wanted to address mtPTP function specifically. We (3) have previously shown that IMF mitochondria have a greater expression of VDAC and cyclophilin D but similar levels of ANT compared with SS mitochondria. Our additional results indicate that chronic contractile activity has no effect on any of the three mtPTP components, ANT, VDAC, or cyclophilin D (data not shown). Despite this, the time to $V_{\text{max}}$ of mtPTP was modestly lengthened by chronic contractile activity in the SS mitochondrial subfraction. Although the reason for this remains to be determined, this resilience to pore opening likely serves an antiapoptotic role in contributing to less protein release at the onset of an apoptotic stimulus.

Figure 5 summarizes the possible protective effect of chronic contractile activity on mitochondrionally mediated apoptosis, based on the results of our study. Chronic contractile activity attenuates cytochrome c and AIF release when subjected to a maximal ROS stimulus despite induced increases in the mitochondrial levels of both of these proteins. In addition, chronic contractile activity also caused a protective upregulation of HSP70 and ARC, which would impede the progression of the cytochrome c and AIF apoptotic pathways upon release by a triggering stimulus. Following contractile activity, ROS production was significantly suppressed in the IMF subfraction but elevated in the SS subfraction. We speculate that the modest elevations in ROS production in the SS subfraction likely contribute to the activation of redox-sensitive transcription factors that contribute to muscle fiber plasticity rather than the initiation of apoptotic pathways. In any event, the overall level of ROS within the muscle cell as a whole is likely reflective of the production by IMF mitochondria, which are in much greater abundance (~80%) compared with SS mitochondria (~20%) within the myocyte (19).

In conclusion, given that the majority of contractile activity-induced antiapoptotic adaptations occurred within the more apoptotic-sensitive IMF mitochondrial subfraction, and that IMF mitochondria comprise a much larger fraction of the total mitochondrial content, our data suggest a protective effect of chronic contractile activity. It will be important in future work to relate these alterations in mitochondrial susceptibility to apoptosis, with the propensity for apoptosis within the framework of the whole muscle. Assuming that these beneficial effects of contractile activity on mitochondria have physiological consequences at that level, our results have implications for the onset of degenerative processes in skeletal muscle. The data suggest that the muscle mitochondrial adaptations produced by regular exercise not only improve the metabolic environment within muscle, leading to enhanced work performance, but also may retard the onset of apoptotic events evident during aging-induced sarcopenia, in muscle diseases, or during periods of chronic muscle disuse.

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