Morphological and Apoptotic Alterations in Skeletal Muscle of Mice Deficient in Apoptosis Repressor with Caspase Recruitment Domain

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Altered apoptotic signaling in skeletal muscle has been observed in a number of disease states associated with skeletal muscle atrophy. Therefore, understanding the mechanisms that lead to increased skeletal muscle apoptosis may help to prevent the atrophy associated with various diseases. Apoptosis repressor with caspase recruitment domain (ARC) is a potent anti-apoptotic protein that is able to inhibit apoptosis mediated by both the death-receptor and mitochondrial pathways. In addition, ARC has a unique distribution pattern and is highly expressed in terminally differentiated tissue such as skeletal muscle. To characterize the role of ARC in skeletal muscle morphology and apoptosis, soleus and plantaris muscles of 18 week-old ARC-deficient mice were excised and compared to those of age-matched wild-type littermates. While no differences were seen in muscle weights between genotypes, in the ARC KO animals, the cross-sectional area (CSA) of the soleus was smaller, while the CSA of the plantaris was larger. With respect to fiber type distribution, both muscles demonstrated a shift towards a faster myosin heavy chain expression pattern. For example, soleus muscles of ARC KO animals had significantly less type I fibers and more IIa fibers, while plantaris muscles had significantly less type IIa fibers, and more IIb fibers. In ARC KO animals, type I and IIa fibers were significantly smaller in the soleus, while type IIb fibers were larger in the plantaris. DNA fragmentation (a hallmark of apoptosis) was increased in the soleus, but not plantaris muscles of ARC KO animals. Surprisingly, activity of the proteolytic enzymes caspase-2, -3, -8, and -9, as well as calpains, was not different in either soleus or plantaris muscles. To determine whether a lack of ARC protein affects apoptotic signaling in skeletal muscle, the total expression of pro- and anti-

apoptotic proteins were also assessed. In the soleus, no changes were observed in whole tissue AIF, cytochrome c, EndoG, and Smac. In the plantaris, there was no change in total muscle AIF; however, there were trends towards decreased cytochrome c, and increased Smac, as well as a significant decrease in EndoG ARC KO animals. No changes were observed in Bcl-2 and XIAP in the soleus; however, there were significant reductions in FLIP(s) and HSP70 content. In the plantaris, no changes were observed in anti-apoptotic protein content. Subcellular fractionation of red quadriceps for ARC KO mice revealed an increased Bax:Bcl-2 ratio in the isolated mitochondrial fractions. Furthermore, in cytosolic fractions of red quadriceps, AIF protein content was significantly increased in ARC KO animals. Conversely, no changes in apoptotic-related protein content were observed in any fractions from white quadriceps between groups. In agreement with these findings, isolated mitochondria from ARC-deficient animals were more susceptible to calcium induced swelling, as well as membrane potential loss compared to controls. Taken together, these results suggest that in slow-oxidative skeletal muscle of ARC-deficient mice there is increased apoptosis due to caspase-independent, mitochondrial-mediated apoptotic signaling. Furthermore, this study is the first to show ARC plays an important role in skeletal muscle morphology, as ARC KO mice have an altered skeletal muscle phenotype and morphology.

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Introduction

Apoptosis

Understanding the mechanisms that influence the growth and maintenance of tissue is critical in prolonging and enhancing tissue function. An important aspect of tissue maintenance and function is apoptosis. Apoptosis is a highly conserved physiological process that is implicated in the proliferation, differentiation, and remodelling of tissue [1]. Unlike necrosis, apoptosis is a highly regulated process whereby unnecessary, aberrant, damaged or mutated cells are specifically eliminated with limited damage to surrounding tissue. In typical mononucleated cells, this process is characterized by cell shrinkage, chromatin condensation, nuclear fragmentation and membrane blebbing [2, 3]. These blebs, which contain intracellular components, are engulfed by phagocytic immune cells and subsequently degraded. Deregulated apoptosis has been implicated in several diseases such as cancer, AIDS, and autoimmune disorders, to name a few [4, 5].

Apoptotic Signaling

The induction of apoptosis occurs through three main signaling pathways; the death receptor, mitochondrial, and endoplasmic reticulum (ER) stress. In general, apoptosis is carried out by groups of proteases known as caspases. Caspases can divided into two distinct groups; initiator and effector caspases [6]. The upstream initiator caspases are activated directly by with signals from the main signaling stimuli. Specifically, caspase-8 is activated by the death receptor pathway, caspase-9 by the mitochondrial mediated pathway, and caspase-12 by the ER stress pathway. Once activated, these upstream caspases converge on

downstream effector caspases. Effector caspases, in particular caspase-3, initiate the disassembly of cells by cleaving cellular structures, DNA, and anti-apoptotic proteins, as well as promoting the activation of other apoptotic processes [7, 8].

The initiation of the death receptor pathway, also known as the extrinsic pathway, requires the binding of an extracellular ligand to a membrane bound receptor. The main extracellular death ligands and their corresponding receptors belong to the tumour necrosis factor (TNF) and TNF receptor super-families [9]. In skeletal muscle, major death ligands (and their receptors) include TNF-α (TNFR) and Fas ligand (Fas) [10-12]. Binding of a ligand to their respective receptor causes a conformational change on the intracellular domain of the receptor. This exposes the binding sites of the death receptors' death domains. Once exposed, the death domains can then bind to intracellular adaptor proteins such as tumour necrosis factor receptor type 1-associated death domain (TRADD) and Fas associated protein with a death domain (FADD). Adaptor proteins, such as TRADD and FADD, facilitate the binding and dimerization of initiator caspase-8 [9]. This intracellular signaling complex, consisting of the receptor, adaptor protein and caspase, leads to the activation of caspase-8 which in turn leads to activation of caspase-3.

Mitochondria-mediated apoptosis occurs when cells are exposed to any of a variety of intra or extra-cellular stressors [13]. This pathway is initiated by the activation of pro-apoptotic proteins from the B-cell lymphoma 2 (Bcl-2) family, such as Bcl-2-associated X protein (Bax) [14]. Bax has been shown to become activated in response to DNA damage; a process likely regulated by the tumour suppressor p53 [15, 16]. Activated Bax oligomerizes

in the cytosol, then translocates and inserts into the outer mitochondrial membrane where it forms pores. This Bax pore is a major factor in mitochondria outer membrane permeabilization (MOMP) [17]. MOMP allows for the entry of proteases into the mitochondria to cleave their respective substrates and allows for the release of soluble proteins within the inter-mitochondrial membrane space [18, 19].

Another mechanism by which mitochondria regulate apoptosis is through induction of the permeability transition pore (PTP). Under basal conditions, several outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) proteins regulate the flux of solutes through the mitochondrion. Following exposure to high levels of Ca^{2+}, proteins such as the voltage-dependent anion channel (VDAC), adenine nucleotide translocator (ANT), and cyclophilin D, interact with each other to form a pore complex [13, 20-22]. Pore formation is also regulated by Bax and Bcl-2, as high levels of mitochondrial Bax has been shown to promote MOMP [23]. Sustained stress enhances the pore, allowing solutes and water to rapidly enter the mitochondria. Consequently, the mitochondrial matrix swells, and the OMM and IMM rupture [13]. Once ruptured, membrane potential is dissipated and mitochondrial-housed pro-apoptotic proteins are released into the cytosol.

Generally, four main apoptotic related proteins are released from the mitochondria following MOMP and PTP opening [19]. These include cytochrome c and second mitochondrial-derived activator of caspases (Smac), as well as apoptosis inducing factor (AIF) and endonuclease G (EndoG). Once released cytochrome c interacts with dATP and apoptotic protease activating factor-1 (Apaf-1) to form the apoptosome. The apoptosome

proceeds to activate caspase-9, and thus initiates the caspase signaling cascade [24]. Although Smac does not directly activate caspases, it facilitates their activation by blocking other proteins such as X-linked inhibitor of apoptosis (XIAP), which can inhibit caspase-9 and caspase-3 activity [25]. Unlike cytochrome c and Smac, AIF and EndoG release causes caspase independent apoptosis. Upon their release from the mitochondria, AIF and EndoG translocate to the nucleus and promote DNA fragmentation [26, 27].

Finally, the ER-stress pathway regulates apoptosis through the release of calcium. With respect to cellular damage, the ER acts as a barometer for cellular stressors. In particular, it monitors levels of unfolded or misfolded proteins and consequently responds via the unfolded protein response (UPR) [28-30]. This type of cellular stress can occur as a result of redox imbalance, glucose deprivation, and viral infection [31-33]. The UPR mediates the accumulation of unfolded proteins by promoting genes that enhance protein folding, inhibiting the entry of proteins into the ER to be translated, and exporting damaged proteins into the cytosol for degradation [34]. If unsuccessful, the UPR up-regulates mitogen-activated protein kinase (MAPK) signaling pathways that result in a cellular stress response. If the stress on the ER is not alleviated, apoptosis is induced by the release of calcium into the cytosol [35]. In skeletal muscle, high cytosolic calcium levels have been shown to activate a group of apoptotic related proteases known as calpains. Calpains themselves can cleave structural/contractile proteins such as titin, nebulin, myosin, tropomyosin, and troponin; but can also cleave and activate caspase-12, leading to downstream executioner caspase activation [8, 36, 37].

Inhibitors of Apoptosis

While pro-apoptotic factors are constantly present within the myofiber, under basal conditions their actions are inhibited by anti-apoptotic proteins. Heat shock protein 70 (HSP70) is a molecular chaperone known to act at several points along the apoptotic signaling cascade. In particular, HSP70 attenuates caspase-8 activation, inhibits the translocation of Bax to the mitochondria, prevents AIF from translocating to the nucleus, and assists in the folding of proteins to alleviate ER-stress [38]. FLICE-like inhibitory protein (FLIP) is another anti-apoptotic protein which is highly expressed in skeletal muscle. FLIP has two isoforms, FLIP-L and –S, both of which have been shown to inhibit DISC assembly, and prevent caspase activation [39]. B-cell lymphoma 2 (Bcl-2) is an anti-apoptotic protein which prevents Bax pore formation and thus mitochondrial outer membrane permeabilization [40]. Overexpressing Bcl-2 in cells causes the cells to become resistant to various apoptotic stimuli such as ROS and Ca^{2+} [41]. Also, Bcl-2 allows Ca^{2+} to leak from the SR by interacting with ryanodine receptors. The slow leak of Ca^{2+} from the mitochondria decreases SR calcium stores, which reduces the calcium insult following SR-stress mediated apoptosis [42]. Finally, the inhibitor of apoptosis (IAP) group of proteins are downstream inhibitors of apoptosis. In particular, XIAP is able to prevent caspase-3 activation by inhibiting apoptosome formation, as well as directly binding to the active sites on caspase-3 and caspase-9 [43].

Unique Considerations when Studying Apoptosis in Skeletal Muscle

Skeletal muscle is morphologically unique compared to other tissues; as such, special considerations must be taken into account when studying apoptotic signaling in skeletal

muscle. For example, skeletal muscle is made up of a heterogeneous distribution of fiber types. Muscle fiber types have been shown to differ in morphology, function, metabolism and mitochondrial content [44-46]. With respect to apoptosis, fiber types also have a differential expression pattern of various apoptotic related proteins and proteases [47]. For example, red gastrocnemius in rat, which is composed mostly of type I and IIa fibers, has higher expression of pro-apoptotic AIF, Bax, cytochrome c and Smac, as well as higher expression of anti-apoptotic Bcl-2 and HSP70 compared to white gastrocnemius. Similarly, red muscle has increased activity of caspase-3, -8 and -9, as well as calpains, and have increased ROS production [47].

Another important apoptotic consideration is that mitochondrial content differs across muscle fibers. Generally, oxidative fibers will have more mitochondria compared to glycolytic fibers. Since mitochondria house several pro-apoptotic proteins, differences in the total amount of mitochondria and the proteins may influence apoptotic signaling.

Apoptosis in Skeletal Muscle

Skeletal muscle fibers are long lived, terminally differentiated tissue cells [48-51]. Each fiber contains thousands of nuclei that support a volume of cytoplasm known as the myonuclear domain [52]. Following a hypertrophic stimulus, quiescent muscle stem cells, or satellite cells, become incorporated into the muscle fiber, increasing the myonuclear number [53]. By increasing the DNA content within a fiber, the muscle fiber's ability to synthesize new proteins is improved. Therefore, the newly incorporated myonuclei can increase the total cytosolic volume of the myofiber and lead to hypertrophy [54, 55]. Skeletal muscle atrophy

has been associated with the elimination of these myonuclei through apoptotic signaling mechanisms. Unlike apoptosis in mononucleated cells where the entire cell is eliminated, apoptosis in skeletal muscle targets individual nuclei within a fiber. This process has been coined myonuclear apoptosis [56, 57]. Since skeletal muscle is comprised of irreplaceable, post-mitotic cells, removing fibers via apoptosis would have significant consequences to its function. Not surprisingly, increased or aberrant apoptotic signaling has been implicated in muscle wasting associated with a plethora of diseases and injuries. These conditions include cancer, sepsis, chronic obstructive pulmonary disease, chronic heart failure, diabetes, burn injuries, renal failure, denervation, and sarcopenia [56, 58].

ARC is a Unique Inhibitor of Apoptosis

ARC is a 30 kDa protein that is highly expressed in long-lived, terminally differentiated tissue such as cardiac and skeletal muscle, neurons, as well as in a variety of cancers[49, 51, 59-63]. ARC is unique in that it has been shown to inhibit the death receptor, mitochondrial, and ER-stress signaling pathways. Its potent ability to inhibit apoptosis is due to ARC's structure, which consists of an n-terminal caspase recruitment domain (CARD) as well as an acidic proline/glutamine (P/E) rich c-terminus. The CARD domain is similar to domains on several other proteins involved in apoptotic signaling, allowing ARC to selectively bind to and inhibit the activation of certain molecules [49, 64]. Similarly, ARC's P/E rich domain allows for the binding of cytosolic free Ca^{2+} [65, 66]. Therefore, not only is ARC able to influence the proteins involved in apoptosis, but also buffer potentially harmful signaling ions.

Anti-Apoptotic Mechanisms of Action

As mentioned previously, ARC is a potent inhibitor the of death receptor pathway of apoptosis [49]. Transfecting non-ARC expressing cells with ARC blocks apoptosis following treatment with death-inducing cytokines [49]. In overexpression models, ARC inhibits apoptosis by binding directly to procaspase-8 and inhibiting its activation by preventing DISC assembly [49, 67, 68]. Its ability to bind to procaspase-8 is dependent on ARC's CARD domain, as mutating this domain renders ARC unable to inhibit Fas-induced apoptosis [67]. Interestingly, ARC's death domain interactions are quite specific. Although ARC directly interacts with caspase-2 and -8, it does not bind other death domain containing proteins such as FLIP, TRADD, or Bcl-2 [49, 67]. ARC also does not bind directly to downstream effector caspases; however, overexpression does ultimately prevent caspase-3 activation; a mechanism most likely regulated by inhibiting upstream initiator caspases [49, 69]. Taken together, ARC is a potent inhibitor of caspase-dependent, death receptor induced apoptosis.

During mitochondrial-mediated apoptosis, ARC has been shown to prevent the initiation of apoptosis by stabilizing the mitochondria [67]. This is achieved primarily through ARC's interaction with Bax. By binding directly to Bax via its CARD domain, ARC prevents Bax oligomerization and translocation to the mitochondria [64, 67]. Furthermore, ARC inhibits Bax pore formation by binding upstream Bax activators, such as PUMA and Bad [70].

Another mechanism by which ARC stabilizes the mitochondria is by preventing mitochondrial fission. Mitochondrial fission is an important step in the progression of mitochondrial-mediated apoptosis, as it contributes to MOMP and allows for the release of mitochondrial-housed pro-apoptotic proteins [71]. Overexpressing ARC in cardiomyocytes prevented mitochondrial fission in H_2O_2-induced apoptosis [72]. Subsequent studies have found that ARC prevents mitochondrial fission by specifically interacting with PUMA [73]. PUMA allows for the accumulation of the fission protein Drp-1 in the mitochondria. There, Drp-1 facilitates the remodelling of the mitochondrial membranes which allows the mitochondria to split in two. By inhibiting this PUMA-Drp-1 interaction, ARC ultimately prevents fission. By protecting the integrity of the mitochondria, ARC attenuates mitochondrial membrane depolarization and prevents the release of several pro-apoptotic proteins [72, 74]. Therefore, preventing mitochondrial fission may be another mechanism by which ARC prevents mitochondrial-mediated apoptosis [72, 74].

Evidence suggests ARC also inhibits ER-stress-induced cell death. In ARC overexpression models, cells show decreased Ca^{2+} transients as well as resistance to A23187- and thapsigargin-induced cell death [66, 75]. Conversely, cells that do not express ARC are sensitized to Ca^{2+}-induced apoptosis [66]. This is likely due to ARC's ability to bind cytosolic free Ca^{2+}, therefore influencing Ca2+ sensitive proteolytic enzymes and mitochondrial stability.

ARC may also play a role in the development of some cancers. In healthy cells, ARC is found primarily in the cytosol and mitochondria. In cancerous cells, ARC resides in the

nucleus where it inhibits p53-mediated apoptosis [50, 59, 63]. p53 is a well characterized transcription factor that participates in cell cycle arrest, apoptosis, and particularly tumour suppression [76]. In healthy cells, an apoptotic stimuli activates p53, causing it to self-tetramerize and transcribe various pro-apoptotic proteins such as Bax and PUMA [76]. When ARC is present in the nucleus, it binds p53. ARC binding prevents p53 tetramerization and promotes the export of p53 from the nucleus [59]. Thus, p53's ability to promote apoptosis is lost. ARC expression is dramatically increased in cancers that are highly resistant to drug and radiation treatment [50, 63]. One example of this is ARC in melanoma. In healthy melanocytes, ARC is not basally expressed. When melanocytes become cancerous, the melanoma cell lines that express high levels of ARC are highly resistant to ER-stress-induced apoptosis. Conversely, melanoma cells with low ARC expression are sensitized to apoptosis [61]. Therefore, ARC has a strong anti-apoptotic function when highly expressed in a variety of cells.

Factors that Influence ARC's Anti-Apoptotic Activity

There are several factors that influence ARC's regulation of apoptosis. First and foremost, a cell's resistance to apoptosis coincides with the abundance of ARC present within that tissue. This observation may explain why ARC is highly expressed in long lived, terminally differentiated tissue types that generally have a low cellular turnover. In cell types that do not express ARC, it has been shown that forced expression causes those cells to become resistant to apoptosis [49, 66, 68, 69, 77-79]. In rat heart, a 1.5-fold increase in ARC protein expression reduced infarct size and prevented cardiomyocyte apoptosis following ischemia-reperfusion (I/R) [80].

Conversely, down-regulating ARC sensitizes cardiomyocytes to H_2O_2-induced apoptosis [77]. While hearts of ARC deficient mice show no morphology or function changes compared to wild-type animals, following pressure overload, these mice are prone to cardiac decompensation and cardiomyocyte apoptosis [81]. After I/R injury, ARC deficient mice have increased Bax activation and DNA fragmentation, as well as significantly greater infarct sizes [81]. Not surprisingly, patients with heart failure show a 36.7% decrease in ARC protein content compared to healthy controls [81]. Whether these patients are genetically predisposed to expressing less ARC, or ARC degradation is secondary to another factor has yet to be determined. Interestingly, women, who are generally more resistant to myocardial I/R injury compared to men, have a higher constitutive ARC expression [82]. In general, increased ARC content seems to have a robust cardioprotective effect.

The anti-apoptotic effects of ARC are significantly affected by ROS. In particular, H_2O_2 has been shown to decrease ARC transcription and promote its degradation [65, 67, 77]. Knockdown of p53 prevented decreases in ARC mRNA and protein content after H_2O_2 treatment [70]. Low levels of H_2O_2 have been shown to activate p53, causing it to bind to the promoter region on the ARC gene and prevent its transcription. This process is regulated primarily through the phosphorylation of the tumour-suppressor p53 [83]. If levels of H_2O_2 are sustained, p53 promotes the transcription of mouse double minute-2 (MDM2) [84-86]. MDM2 is a ubiquitin E3 ligase that poly-ubiquitates ARC protein, and marks it for 20s proteosome degradation [87, 88]. Therefore, p53 can influence ARC protein content by not only suppressing its transcription, but by promoting its degradation [70, 88].

ARC in Skeletal Muscle

As myoblasts differentiate into myotubes, ARC mRNA and protein content increase significantly [89]. The increases in ARC content may explain why differentiated myotubes are more resistant to apoptotic stimuli compared to undifferentiated myoblasts [89]. In mature skeletal muscle, ARC expression is different across fibers. Initial work with cross sections stained for cytochrome oxidase revealed that ARC protein content was highest in oxidative fibers [51]. Recent work from our lab has demonstrated that ARC is related to MHC expression rather than mitochondrial content [47, 90]. Specifically, ARC content is highest in slow twitch type I fibres and stepwise lower across the faster myosin heavy chain isoforms [47, 90]. As such, ARC protein content is much higher in red compared to white gastrocnemius [47]. Despite the strong correlation between ARC content and myosin heavy chain expression, studies have yet to determine whether ARC content influences fiber type.

Several studies have shown that increases in ARC expression are associated with decreased apoptosis in skeletal muscle. For example, ARC protein content increased in rat soleus after 8 weeks of training, which corresponded to a decrease in DNA fragmentation [91, 92]. Similarly, chronic stimulation over 1 week increased ARC protein content by approximately 40% in tibialis anterior and extensor digitorum longus muscles [93]. This effect is associated with decreased cytochrome c release in IMF mitochondria, as well as an increased resistance to Ca^{2+} induced mtPTP opening in isolated mitochondria from both the IMF and SS [93]. Conversely, a decrease in ARC protein content corresponds with increased DNA fragmentation, nuclear AIF, and caspase-3 activity in the soleus of hypertensive rats

[94, 95] . Together, these data suggest that, ARC may play an important role in preventing apoptosis in skeletal muscle.

Purpose

Currently, the majority of research on ARC has focused primarily on cardiac tissue and cancer. In these tissues, ARC has proven to be an important factor in promoting tissue viability following an apoptotic insult. While there is some work looking at changes in ARC content in skeletal muscle, the function of ARC in skeletal muscle has yet to be fully elucidated. Therefore, the purpose of this thesis is to examine the role of ARC in the maintenance of skeletal muscle. Experiments were performed to examine basal differences in skeletal muscle morphology and apoptotic signaling between wild-type and ARC-deficient mice. Since ARC expression is fiber type specific, comparisons were made between soleus (composed primarily of type I and IIa fibers) and plantaris (composed primarily of type IIx and IIb fibers) muscles. Specifically, morphological measures include muscle weights, muscle cross-sectional area, fiber type distribution, and fiber cross-sectional area. Apoptotic signaling mechanisms include proteins and proteases involved in death-receptor, mitochondrial-mediated, and ER-stress pathways, mitochondrial susceptibility measures, as well as markers of DNA fragmentation.

Hypothesis

The hypotheses of the current work are as follows:

- Skeletal muscle of ARC KO mice will be morphologically different than WT mice

    - Since ARC protein expression is higher in slow twitch fibers, ARC content may influence fiber type. Therefore, we hypothesize that ARC KO mice will have a shift in fiber type distribution towards type II fibers
    - Individual fibers of ARC KO mice will have a smaller cross-sectional area compared to WT animals
    - Whole muscle cross-sectional areas and muscle weights will be decreased in ARC KO animals
- Skeletal muscle of ARC KO mice will have altered apoptotic protein expression and protease activity
    - Since ARC is highly anti-apoptotic, pro-apoptotic protein expression as well as protease activity will be increased in KO animals
    - To compensate for the lack of ARC, anti-apoptotic protein expression will be increased in KO animals
    - Since ARC is highly expressed in skeletal muscle, we hypothesize that despite a compensatory increase in anti-apoptotic proteins, KO animals will have more DNA fragmentation
- Isolated mitochondria of ARC KO mice will be more susceptible to an apoptotic insult
    - Addition of calcium to isolated mitochondria of ARC KO mice will cause more swelling, and greater loss of membrane potential

Methods

Animals

ARC KO mice (kindly provided by Dr. Rudiger VonHarsdorf and Dr. Stefan Donath) were derived from C57BL/6 mice. C57BL/6 mice (Charles River) were crossbred with ARC knockout (KO) mice to produce mice heterozygous for wild-type (WT) and disrupted ARC alleles. Heterozygous breeding pairs were set up and male pups homozygous for the WT or KO allele were used for analysis. Mice from the F1 generation were used in all experiments. Littermates were used whenever possible. Mice were housed without access to running wheels in a temperature (20-21˚C) and humidity (~50%) controlled environment and on a 12:12hr reversed light/dark cycle. Standard rodent chow was provided ad libitum. All animal procedures were approved by the University of Waterloo Animal Care Committee.

Genotyping

To determine genotype, ear notches from 4 week old mice were snap frozen in liquid nitrogen. DNA was extracted and purified from the ear notches using the Purelink DNA extraction kit (Invitrogen), and DNA samples were stored at 4^{o}C for no longer than 48 hours. DNA samples were added to a mixture of RedTaq Polymerase (Sigma-Aldridge), H_2O, and the appropriate forward and reverse primers. The sequences of the WT ARC allele forward and reverse primers were 5'GATACCAGGAGATCTCTCAAAATT3' and 5'CAGCGCATCCAA GGCTTCGTACTC3', respectively. Forward and reverse primers for the disrupted ARC allele were 5'GATACCAGGAG ATCTCTCAAAATT3' and 5'GATTGGGAAGACAATAGCAGGCATGC3', respectively. Samples were placed in a

thermal cycler (BIO-RAD) and denatured for 2 minutes at 93^{o}C followed by 1 minute of annealing at 55^{o}C and 5 minutes of extension. Next, samples underwent 28 cycles of denaturing for 30 seconds at 93^{o}C, annealing for 30 seconds at 55^{o}C, and extension for 3 minutes at 72^{o}C. Samples then underwent a final extension at 72^{o}C for 7 minutes. After the amplification, samples were separated on a 1% agrose gel containing 0.01% ethidium bromide (BioShop), and then imaged using the ChemiGenius 2 Bio-Imaging System (Syngene). Genotyping was confirmed with subsequent western blot analysis for ARC protein expression.

Determination of Metabolic Rate, Activity Levels and Food Intake

Whole body metabolic rate, food consumption, and cage activity were measured using a 12-chamber Comprehensive Lab Animal Monitoring System (CLAMS) (Oxymax series; Columbus Instruments, Columbus, OH). Prior to the CLAMS trial, mice were acclimated to a single housed clear mesh bottom cage for one week. Mice were subjected to three, 48 hour trials in the CLAMS, whereby only data collected within the final 24 hours of the trial was used for analysis. Any VO_2 values over the three trials that were considered erratic were discarded, and then remaining trails were averaged.

Isolation of Skeletal Muscle

At 18 weeks of age, mice were sacrificed using cervical-dislocation. The soleus and plantaris muscles were quickly weighed, and either snap frozen in liquid nitrogen and stored at -80^{o}C for subsequent analyses or frozen in embedding media (OTC) for sectioning and subsequent immunohistochemistry. Quadriceps were also removed and either snap frozen in

liquid nitrogen and processed immediately for isolated mitochondrial preparations. For fractionation analysis, whole quadriceps were divided into red (red quadriceps) and white (white quadriceps). Total body weight, as well as heart and kidney weights were recorded immediately after sacrifice.

Isolation of Mitochondria

Fresh mitochondria were isolated from quadriceps as previously described [47]. Muscle was immediately placed in mitochondrial isolation buffer (220 mM mannitol, 70 mM sucrose, 20 mM HEPES, 2 mM Tris, 1 mM EDTA, pH 7.2), containing 0.4% BSA and 0.15 mg/ml Nagarse (Sigma-Aldrich), then minced and homogenized using a glass mortar and pestle on ice. Homogenate was then centrifuged at 500g for 5 min, the supernatant collected, the pellet further homogenized in mitochondrial isolation buffer, and centrifuged at 500g for 5 min. The supernatant was then combined and centrifuged at 17,000g for 3 minutes. 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.

Immunofluorescence Analyses of Myosin Heavy Chain Expression

Soleus and plantaris skeletal muscle samples embedded in OCT were cut into 10 μm serial cross sections with a cryostat (Thermo Electronic) maintained at -20°C. For immunofluorescence analysis of myosin heavy chain (MHC) expression, cross-sections were blocked with 10% goat serum, then incubated with primary antibodies against MHCI (BA-

F8), MHCIIa (SC-71), and MHCIIb (BF-F3) (Developmental Studies Hybridoma Bank). Sections were washed 3 x 5 minutes in PBS and incubated with anti-mouse isotype-specific Alexa Fluor 350, Alexa Fluor 488, and Alexa Fluor 555 secondary antibodies (Molecular Probes). Sections were washed 3 x 5 minutes in PBS, and coverslips were mounted with Prolong Gold antifade reagent (Molecular Probes). This method allowed for the identification of type I (blue), type IIA (green), type IIB (red), and type IIX (unstained) fibers. Fiber type composition analysis was performed on composites of 10X magnification pictures by counting all fibers across the entire cross section. Slides were visualized with an Axio Observer Z1 structured-illumination fluorescent microscope equipped with an AxioCam HRm camera and associated AxioVision software (CarlZeiss). Cross-sectional area of the individual fibres was determined by counting 20-50 fibers per fiber type per section using Image Pro-Plus imaging software.

Preparation of Whole Muscle Lysates and Muscle Subcellular Fractions

Whole muscle lysates of soleus and plantaris were prepared by homogenizing muscle with a glass pestle and mortar in ice-cold lysis buffer (20 mM HEPES, 10 mM NaCl, 1.5 mM MgCl, 1 mM DTT, 20% glycerol and 0.1% Triton X100; pH 7.4) and protease inhibitors (Complete Cocktail; Roche Diagnostics). Samples were centrifuged at 1000 x g for 10 min at 4°C and the supernatant was collected. Protein content of each sample was determined by the BCA protein assay.

Subcellular fractions were obtained by gently hand homogenizing red and white quadriceps samples using a glass pestle and mortar as previously described [47, 96]. This

resulted in the isolation of mitochondria-enriched, nuclear-enriched, and cytosolic-enriched fractions. The protein content of each fraction was determined using the BCA protein assay. The content of each fraction was verified by immunoblots using antibodies against histone H2B (Cell Signaling Technology) for the nuclear fraction, copper zinc superoxide dismutase (CuZnSOD) (Stressgen Bioreagents) for the cytosolic fraction, and adenine nucleotide translocase (ANT) (Santa Cruz Biotechnology) for the mitochondrial fraction.

Immunoblot Analyses

Equal amounts of protein were loaded and separated on 12% or 15% SDS-PAGE gels, transferred onto PVDF membranes (Bio-Rab Laboratories), and blocked overnight at 4^{o}C with 5% milk-TBST. Membranes were incubated either overnight at 4^{o}C or for 1 hour at room temperature with primary antibodies against: apoptosis inducing factor (AIF), apoptosis repressor with caspase recruitment domain (ARC), Bcl-2 associated X protein (Bax), B-cell lymphoma 2 (Bcl-2), cytochrome c, FLICE-like inhibitory protein (FLIP), (Santa Cruz Biotechnology); endonuclease G (EndoG) (Abcam); and second mitochondrial activator of caspase (Smac) (Assay Designs); heat shock protein 70 (HSP70), and X-linked inhibitor of apoptosis (XIAP) (Stressgen). Membranes were washed with TBST and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 hour at room temperature. Proteins were visualized using the Amersham Enhanced Chemiluminescence Western Blotting detection reagents (GE Healthcare) and the ChemiGenius 2 Bio-Imaging System (Syngene). Following detections, membranes were stained with Ponceau S (Sigma-Aldrich) to ensure equal loading and quality of protein transfer.

Caspase and Calpain Activity

Enzymatic activity of caspase -2, caspase-3, caspase-8, and caspase-9 were determined in duplicate in muscle homogenates using the substrates, Ac-VDVAD-AFC (Alexis Biochemicals), Ac-DEVD-AMC (Alexis Biochemicals), Ac-IETD-AMC (Sigma-Aldrich), and Ac-LEHD-AMC (Alexis Biochemicals), respectively [47, 97]. Muscle was homogenized in ice-cold lysis buffer without protease inhibitors and centrifuged at 1000 x *g* at 4°C for 10 min. Supernatants were then incubated in duplicate in the appropriate substrate at room temperature and fluorescence measured using a SPECTRAmax Gemini XS microplate spectrofluorometer (Molecular Devices) with excitation and emission wavelengths of 360 nm and 440 nm, respectively. Samples containing purified active enzymes and specific caspase inhibitors (caspase-2, Ac-VDVAD-CHO; caspase-3, Ac-DEVD-CHO; caspase-8, Ac-IETD-CHO; caspase-9, Ac-LEHD-CHO) were also included for control experiments. Incubation of each caspase substrate with purified enzyme resulted in a strong fluorescent signal, whereas inhibitors almost completely blocked the fluorescent signal (*data not shown*). Protease activity was normalized to total protein content and expressed as mean fluorescence intensity in AU per mg protein.

To determine calpain activity, muscle homogenates (processed as above) were incubated in duplicate at 37^{o}C using the substrate, Suc-LLVY-AMC (Enzo Life Sciences) with or without the specific calpain inhibitor, Z-LL-CHO (Enzo Life Sciences). Fluorescence was measured using a SPECTRAmax Gemini XS microplate spectrofluorometer (Molecular Devices) with excitation and emission wavelengths of 380nm and 460nm, respectively. Calpain activity was determined by subtracting the fluorescence obtained from the sample

with calpain inhibitor from the fluorescence obtained from the sample without calpain inhibitor as previously described [47, 98].

Detection of DNA Fragmentation

DNA fragmentation was determined by assessing cytoplasmic histone associated mono-and oligonucleosomes in whole muscle homogenate using the Cell Death Detection ELISA^{PLUS} Kit (Roche Diagnostics). Soleus and plantaris muscles were homogenized in 100 volumes of the supplied lysis buffer and centrifuged at 200 x *g* for 10 minutes at room temperature. For each sample, 20 µl of supernatant was incubated with 80 µl of anti-histone-biotin/anti-DNA-POD reagent in a streptavin coated microplate at room temperature for 2 hours under gentle shaking. Wells were washed several times, and 100 µl of ABTS substrate solution added, and absorbance measured at 405 nm and 490 nm using a SPECTRAmax Plus spectrophotometer (Molecular Devices). Data was normalized to total protein content, and expressed as AU per mg protein. A sample containing a DNA-histone-complex was included as a positive control for DNA fragmentation control.

Analysis of Mitochondrial Permeability Transition Pore Opening and Membrane Potential

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 (215 mM mannitol, 71 mM sucrose, 3 mM HEPES, 5 mM succinate, pH 7.4) and 540 nm absorbance initially measured over a 5 minute period at 37°C using a SPECTRAmax Plus spectrophotometer (Molecular Devices). Previous work from our

lab has demonstrated that concentrations of 100 μM and 200 μM of $CaCl_2$ are sufficient to elicit an apoptotic response in isolated mitochondria from rodents [47]. Mitochondria were then treated with 100 μM, or 200 μM $CaCl_2$, and absorbance was monitored for an additional 30 minutes at 37°C. A decrease in absorbance is indicative of PTP opening (mitochondrial swelling), an event that can occur during mitochondrial-mediated apoptotic signaling [17]. Data were expressed as the percent decrease in absorbance relative to the initial absorbance (before the addition of $CaCl_2$).

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 [99, 100]. A decrease in rhodamine 123 fluorescence is indicative of membrane depolarization; an event that can occur during mitochondrial-mediated apoptotic signaling. 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 minutes at 37°C. Mitochondria were then incubated with 100 μM, or 200 μM or no $CaCl_2$ for an additional 30 minutes at 37°C, then washed and resuspended in swelling buffer. Fluorescence was determined using a SPECTRAmax GEMINI XS microplate spectrofluorometer (Molecular Devices) with excitation and emission wavelengths of 490 nm and 535 nm, respectively. Data were expressed as the percent decrease in fluorescence relative to the no $CaCl_2$ condition.

Statistical Analysis

All results are given as means ± SEM. All data is analyzed by a Student's t-tests with $p<0.05$ considered statistically significant and $p<0.10$ considered a trend. All statistical analyses were performed using Microsoft Excel.

Results

ARC KO Mouse Model

To determine the role of ARC in skeletal muscle, transgenic mice were generated with a mutated ARC gene. PCR analysis demonstrates that KO animals do not express the wild type ARC allele (Figure 1A). This was confirmed by western blotting for the ARC protein, as KO animals have undetectable expression of ARC protein in either soleus or plantaris muscles (Figure 1B). Previous work has shown that after normalizing for total protein content, ARC protein expression is higher in the soleus compared to the plantaris [100].

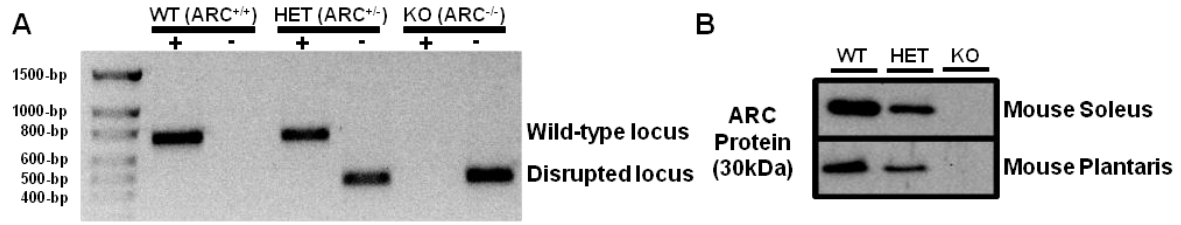

Figure 1. Panel A – PCR analysis showing expression of the WT (750bp) and KO (450bp) allele. Panel B - Western blot analysis of ARC protein content in WT and KO animals, as well as animals heterozygous for both alleles.

		WT	KO
Age (weeks)		18.63 ± 0.14	18.51 ± 0.16
Body Weight (g)		28.67 ± 0.53	28.37 ± 0.38
Heart Weight (g)/Body Weight (kg)		4.53 ± 0.07	4.72 ± 0.06*
Heart Weight (g)/Kidney Weight (g)		0.73 ± 0.01	0.75 ± 0.01
Muscle Weight (g)	Soleus	8.36 ± 0.23	8.33 ± 0.19
	Plantaris	17.58 ± 0.36	16.78 ± 0.36
Muscle Weight (g)/Body Weight (kg)	Soleus	0.28 ± 0.004	0.28 ± 0.005
	Plantaris	0.58 ± 0.009	0.56 ± 0.009*
Muscle Weight (g)/Kidney Weight (kg)	Soleus	45.26 ± 1.04	44.38 ± 0.94
	Plantaris	92.96 ± 1.68	88.86 ± 2.17

Table 1. Table displaying basic anthropometric data of relative heart and muscle weights (n=23-33). Data are expressed as means ± SEM (*p<0.05).

Morphological Characteristics

In 18 week old ARC KO mice, there were no differences in body weight and heart weight relative to kidney weight; however, relative to body weight the heart weight of KO animals was increased by 4.2% ($p<0.05$) compared to controls. With respect to muscle weights, there were no differences in soleus weight relative to body weight or kidney weight. Interestingly, there was no difference in plantaris weights relative to kidney weight; however,

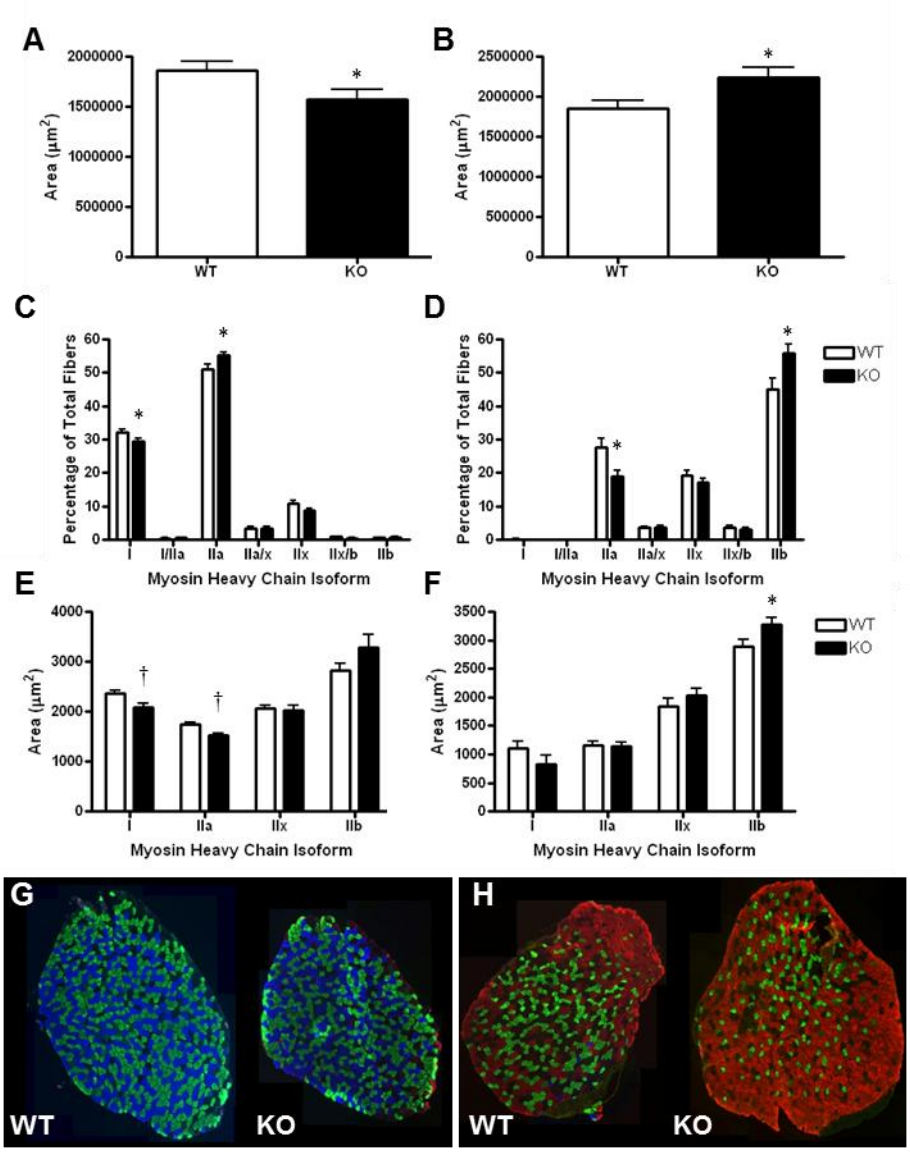

Figure 2. Morphological Characteristics in Skeletal Muscle of WT and KO animals. Total muscle cross-sectional area for soleus (Panel A) and plantaris (Panel B) muscles (n=12). Fiber type distribution (percentage of total fibers) in soleus (Panel C) and plantaris (Panel D) muscles (n=12). Fiber type specific cross-sectional areas in soleus (Panel E) and plantaris (Panel F) muscles (n=11-12). Representative composite images of soleus (Panel G) and plantaris (Panel H) whole muscle cross-sections treated with antibodies specific for the individual myosin heavy chains. Type I (blue), type IIa fibers (green), type IIb fibers (red), and IIx fibers (unstained). Data are expressed as means ± SEM (*p<0.05, †p<0.01).

relative to body weight, KO animals have a decreased plantaris weight (-4.8%) ($p<0.05$) (Table 1). Histological examination of the total cross-sectional area (CSA) of soleus and plantaris muscles revealed KO animals had an 18.4% decrease in soleus CSA ($p=0.05$), and an 18.9% increase in plantaris CSA ($p<0.05$) compared to controls (Figures 2A and 2B).

To explain the changes in total CSA between genotypes, total fiber number, fiber type specific CSA, and fiber type distribution were assessed. While the total fiber number was not different between genotypes (*data not shown*), KO animals demonstrate a fiber type shift towards type II fibers. In the soleus, KO animals had a decrease in type I fibers (32.2% to 29.4%; $p<0.05$) and an increase in type IIa fibers (51.0% to 55.2%; $p<0.05$) (Figure 2E). Likewise, in the plantaris, KO animals show a reduction in type IIa fibers (27.7% to19.0%; $p<0.05$) and an increase in type IIb fibers (45.1% to 55.0%; $p<0.05$) (Figure 2F). The average type I and type IIa cros-sectional area in the soleus of KO animals were decreased by 13.2% ($p<0.01$) and 14.8% ($p<0.005$), respectively (Figure 2C). In the plantaris, the cross-sectional area of type IIb fibers was increased 13.3% ($p<0.05$) in KO animals compared to controls (Figure 2D). Since physical activity can influence fiber type distribution, animals

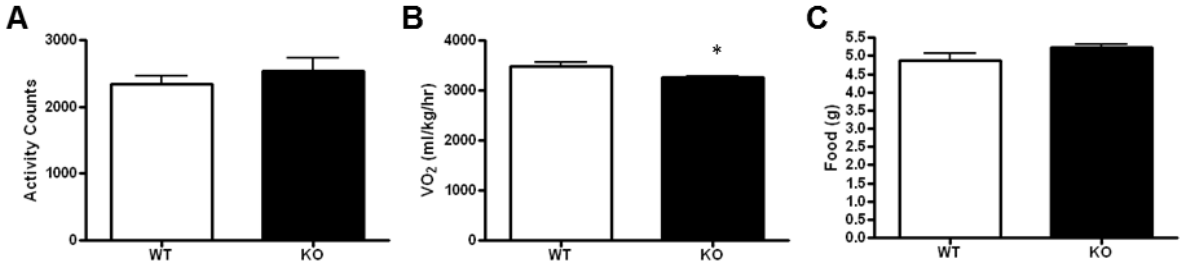

Figure 3. Metabolic data showing dual beam activity counts (Panel A), average daily VO2 (Panel B), and total food intake (Panel C) averaged over three, 24 hour bouts in the CLAMS (n=8). Data are expressed as means ± SEM (*p<0.05).

were subjected to three, 48 hour sessions in the comprehensive lab animal monitoring system (CLAMS) to monitor ambulation. No differences in total activity were seen between genotypes (Figure 3A); suggesting that activity levels are not responsible for the observed fiber type changes. Interestingly, animals deficient in ARC had a 6.4% decreased in waking energy expenditure (as represented by waking VO_2) (Figure 3B) ($p<0.05$). No differences in food intake were observed between groups (Figure 3C).

Apoptotic Signaling and Protease Activity

Since increased apoptotic signaling has been shown to cause fiber atrophy, we next measured various markers of apoptosis, as well as the several proteolytic enzymes involved in apoptotic signaling. DNA fragmentation (a hallmark of apoptosis) was 16.0% higher ($p<0.05$) in soleus of ARC KO compared to WT mice. No difference in DNA fragmentation was observed in plantaris muscles between groups (Figure 4A). These results compliment the morphological measures nicely, suggesting the atrophy and differential fiber type composition observed in KO animals may be due to increased apoptosis.

To assess if the increased apoptosis was mediated by proteases, activity of caspases -2, -3, -8, and -9, as well as calpains were measured. In both muscle types, no differences

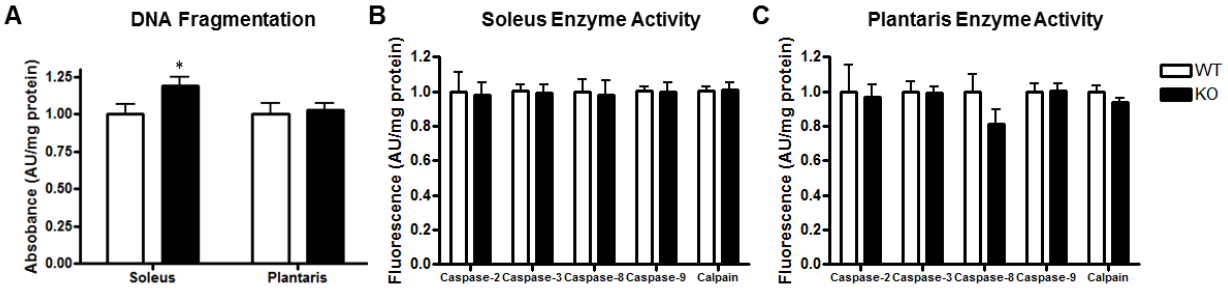

Figure 4. Panel A - DNA fragmentation in soleus and plantaris muscles (n=11-12). Panel B and C - Maximal activity of apoptosis-related proteases in soleus and plantaris (n=12). Data are expressed as means ± SEM (*p<0.05).

were observed in total enzymatic activity (area under the curve; AUC) (*data not shown*) or maximal enzymatic activity (Vmax) of any protease (Figure 4B and C).

Apoptotic Protein Expression

To determine whether a lack of ARC protein affects apoptotic signaling in skeletal muscle, the total expression of several pro- and anti-apoptotic proteins were assessed. The total muscle content of the mitochondrial housed proteins AIF, cytochrome c, EndoG, and Smac were not different between groups in soleus muscle (Figure 5A). In the plantaris, there was no change in total muscle AIF; however, there were trends towards decreased cytochrome c (-11.9%; p=0.06), increased Smac (+20.2%; p=0.09); as well as significantly decreased EndoG (-22.6%; p<0.05) (Figure 5C).

There was no change in the expression of the anti-apoptotic protein XIAP in the soleus; however expression of FLIP(s) (p<0.01) and HSP70 (p<0.05) was decreased 18.9% and 22.1%, respectively (Figure 5B). In the plantaris, there were no changes in FLIP(s), HSP70, or XIAP protein content (Figure 5D).

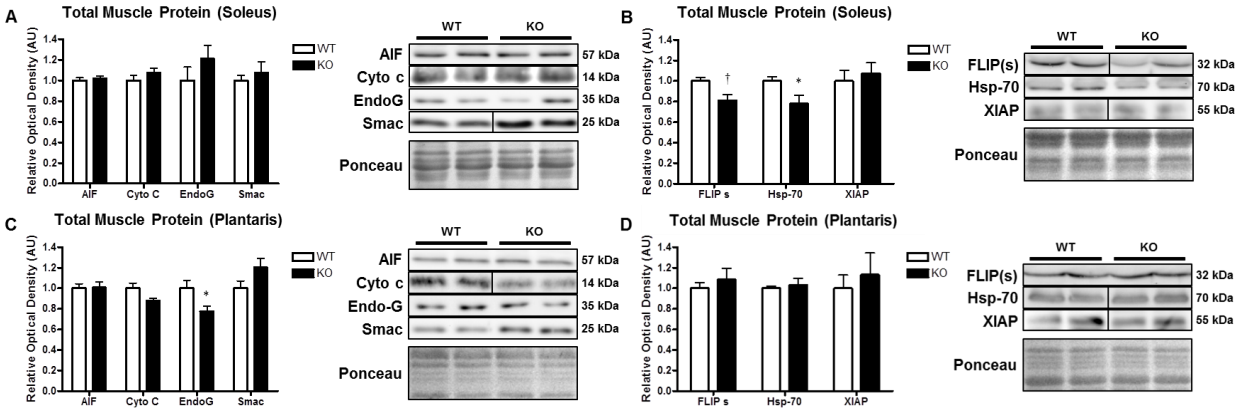

Figure 5. Panel A and C - Expression of whole tissue mitochondrial housed pro-apoptotic proteins in the soleus and plantaris (n=9-12). Panel B and D - Anti-apoptotic protein expression in soleus and plantaris (n=9-12). Representative ponceau stained membranes are also shown as loading control. Data are expressed as means ± SEM (*p<0.05, †p<0.01).

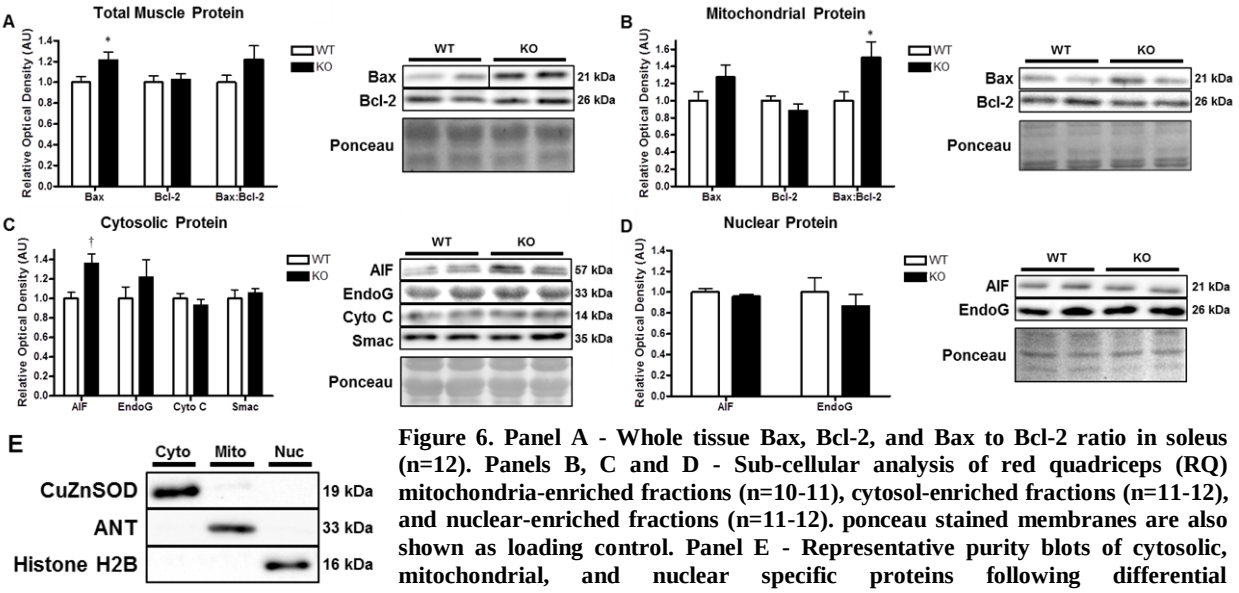

Figure 6. Panel A - Whole tissue Bax, Bcl-2, and Bax to Bcl-2 ratio in soleus (n=12). Panels B, C and D - Sub-cellular analysis of red quadriceps (RQ) mitochondria-enriched fractions (n=10-11), cytosol-enriched fractions (n=11-12), and nuclear-enriched fractions (n=11-12). ponceau stained membranes are also shown as loading control. Panel E - Representative purity blots of cytosolic, mitochondrial, and nuclear specific proteins following differential centrifugation. Data are expressed as means ± SEM (*p<0.05, †p<0.01).

In the soleus of KO animals, Bax expression was increased 21.1% ($p<0.05$); however, the whole tissue Bcl-2 and Bax:Bcl-2 ratio was not different between genotypes (Figure 6A). Due to tissue limitations further subfractionation analyses were performed in red (RQ) and white (WQ) quadriceps muscle. In mitochondrial-enriched fractions of RQ, the Bax to Bcl-2 ratio was higher in KO animals (+50.5%; $p<0.05$) (Figure 6B). Cytosolic-enriched fractions of RQ from KO animals also had a 35.8% increase in AIF ($p<0.01$), but no change in EndoG, cytochrome c, or Smac protein (Figure 6C). No differences were observed in nuclear localization of the pro-apoptotic proteins AIF and EndoG (Figure 6D).

In the plantaris there were no changes in Bax, Bcl-2, or the Bax:Bcl-2 ratio (Figure 7A). Similarly, no changes were observed in Bax, Bcl-2, or the Bax to Bcl-2 ratio in mitochondrial-enriched fraction of WQ (Figure 7B). In accordance with these findings, there were no changes in WQ cytosolic levels of AIF, EndoG, cytochrome c, or Smac (Figure 7C). Similarly, no differences were observed in nuclear levels of AIF or EndoG (Figure 7D).

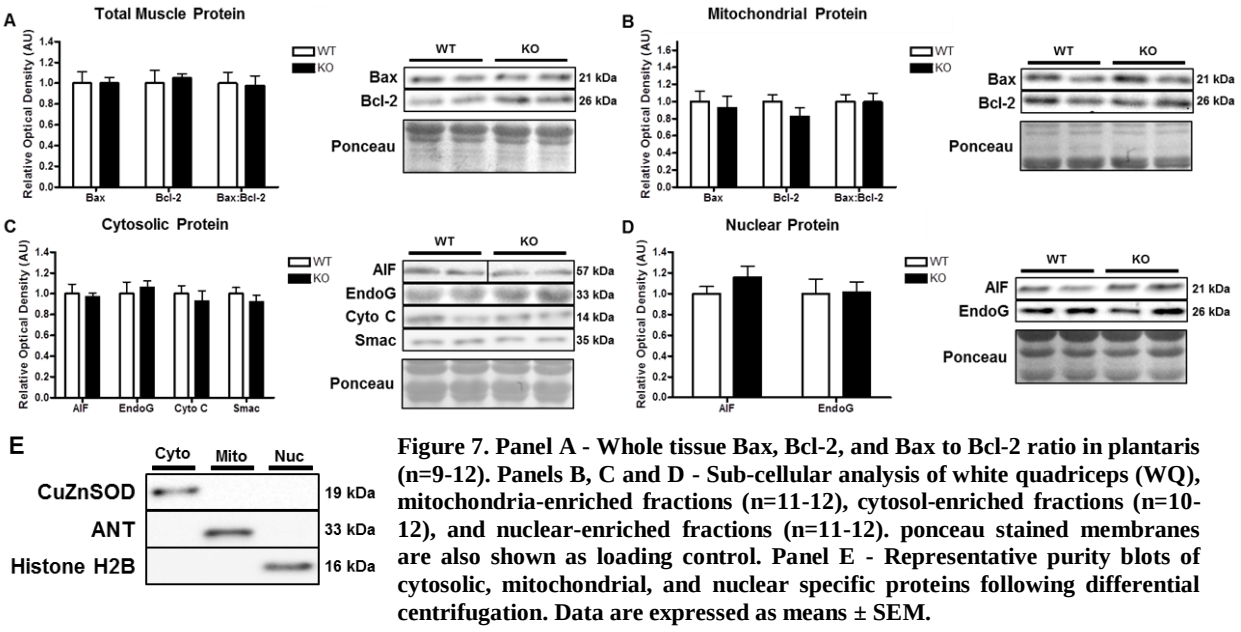

Figure 7. Panel A - Whole tissue Bax, Bcl-2, and Bax to Bcl-2 ratio in plantaris (n=9-12). Panels B, C and D - Sub-cellular analysis of white quadriceps (WQ), mitochondria-enriched fractions (n=11-12), cytosol-enriched fractions (n=10-12), and nuclear-enriched fractions (n=11-12). ponceau stained membranes are also shown as loading control. Panel E - Representative purity blots of cytosolic, mitochondrial, and nuclear specific proteins following differential centrifugation. Data are expressed as means ± SEM.

Mitochondrial Susceptibility Measures

Since the data suggested increased mitochondrial mediated apoptotic signaling in skeletal muscle of ARC KO animals, experiments on isolated mitochondrial from quadriceps were performed to examine susceptibility to apoptotic stimuli. Following the addition of 100μm $CaCl_2$, isolated mitochondria from KO animals demonstrated increased PTP (swelling) kinetics (+19%; $p<0.05$) and formation (+7.3%; $p<0.05$) than mitochondria from control animals (Figures 8A and 8B, respectively). Furthermore, isolated mitochondria from ARC KO mice demonstrated a 22.5% larger decrease ($p<0.05$) in membrane potential compared to controls following the addition of 100μm $CaCl_2$ (Figure 8C). No differences were observed in either swelling or loss of membrane potential following the addition of 200μm $CaCl_2$. Taken together, these results show that without ARC, mitochondria are more susceptible to

calcium-induced swelling and loss of membrane potential, two early events in apoptotic signaling.

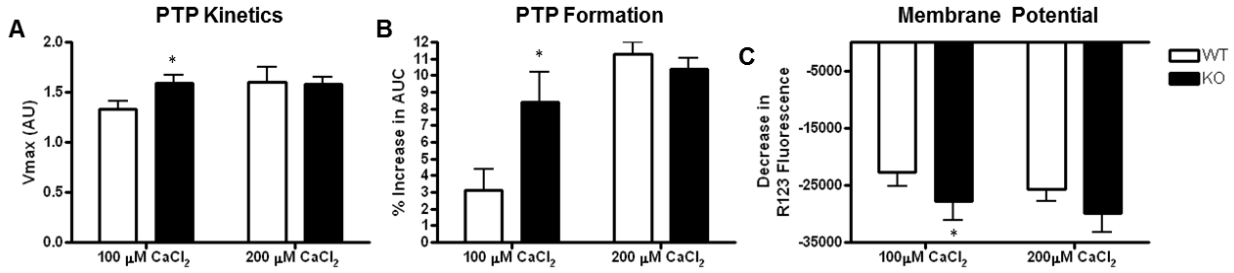

Figure 8. Experiments showing differences in the susceptibility of isolated mitochondria to apoptotic events following exposure to an apoptotic stimulus. Panels A and B - Permeability transition pore (swelling) kinetics (Vmax) and formation (percent increase in AUC) in isolated mitochondria treated with 100 and 200μm $CaCl_2$ (n=7-8). Panel C - Loss of membrane potential in isolated mitochondria treated with 100 and 200μm $CaCl_2$ (n=15). Data is expressed as means ± SEM (*p<0.05).

Discussion

The goal of this study was to determine the role of the anti-apoptotic protein apoptosis repressor with caspase recruitment domain (ARC) in skeletal muscle. To determine this, morphological characteristics of hindlimb skeletal muscles, as well as molecules involved in apoptotic signaling were assessed and compared between wild type and ARC-deficient (ARC KO) mice. Since ARC is a potent anti-apoptotic protein highly expressed in skeletal muscle, it was hypothesized that ARC KO mice would have an increased level of basal apoptotic signaling. Given that increased apoptotic signaling has been implicated in muscle atrophy, we also hypothesized that the ARC KO animals would have some degree of atrophy. In addition, this atrophy would be particularly evident in muscles and fibers which typically express higher levels of ARC protein.

Since soleus muscle has higher constitutive ARC expression, we suspected that any morphological or apoptotic signaling changes would be exacerbated specifically in this tissue. While there were no changes in average soleus weights, assessment of total and fiber type-specific area demonstrated overall smaller muscle and type I and IIa fibers CSA in ARC KO mice. Furthermore, there was a shift in fiber type distribution with ARC KO animals having a decreased percentage of type I fibers, and a higher percentage type IIa fibers. With respect to apoptotic signaling, DNA fragmentation was significantly increased in the soleus of ARC KO mice compared to controls. Interestingly, this increased apoptosis could not be explained by elevated proteolytic enzyme activity, as no differences were observed in caspase-2, -3, -8, -9, or calpains between groups. Analysis of whole tissue pro-apoptotic proteins revealed increases in Bax. In contrast to our hypothesis that ARC KO mice would

show a compensatory increase in anti-apoptotic factors, FLIP(s) and HSP70 total protein content were decreased, with no detectable differences in Bcl-2 or XIAP protein content. Subcellular fractionation of red quadriceps muscle demonstrated ARC KO mice have an increased mitochondrial Bax:Bcl-2 ratio, as well as increased cytosolic AIF. However, no changes were observed in nuclear anti-apoptotic protein content. Regardless, evidence of mitochondrial-mediated apoptotic signaling was strengthened by experiments with isolated mitochondria. When exposed to high levels of Ca^{2+}, isolated mitochondria from ARC KO animals experienced a greater loss of membrane potential, and were more susceptible to permeability transition pore formation.

We also examined morphological and apoptotic signaling changes in the plantaris muscles of WT and ARC KO animals to determine if a differential response occurred in type II-glycolytic muscle. Since the plantaris is made up of predominately type II fibers, basal ARC expression is relatively less compared to soleus. Thus, we hypothesized that any morphological and apoptotic changes induced in skeletal muscle due to a lack of ARC protein would be relatively less in the plantaris muscle. Interestingly, while we observed no differences in soleus weights, the average plantaris weight (relative to body weight) in ARC KO animals was approximately 3.4% less compared to WT controls. However, we observed a significant increase in plantaris overall muscle CSA. Also, we observed a shift in fiber type distribution, with ARC KO mice having less type IIa and more type IIb fibers, as well as significantly larger type IIb fiber cross-sectional area. In agreement with the lack of fiber type-specific atrophy, no changes were observed in DNA fragmentation between WT and ARC KO animals. Similar to the soleus, there were no changes in the activity of several

proteolytic enzymes. Analysis of whole tissue pro-apoptotic proteins revealed decreased levels of the mitochondrial-housed protein, EndoG, as well as a trend towards decreased cytochrome c protein content. Given that type IIa fibers have the highest mitochondrial content in mice, whereas type IIb fibers have the lowest content, these changes most likely reflect decreased mitochondrial content at the whole muscle level due to the fiber type redistribution pattern rather than alterations in apoptotic signaling [101]. In further agreement with this is the finding of lower ANT protein (a mitochondrial marker) in plantaris of ARC KO mice (*Data not shown*). With respect to anti-apoptotic factors, no changes were observed in FLIP(s), HSP70, or XIAP protein expression. Assessment of mitochondrial-mediated apoptotic signaling revealed no change in Bcl-2 or Bax protein, as well as the Bax:Bcl-2 ratio in whole plantaris lysates, or mitochondrial fractions from white quadriceps. In agreement with these findings, no differences in pro-apoptotic cytosolic or nuclear protein content were observed between WT and ARC KO animals in white muscle tissue.

Apoptotic Signaling in Skeletal Muscle

ARC is a potent regulator of apoptosis through its role on multiple signaling pathways. We found that DNA fragmentation (a hallmark of apoptosis) was elevated in the soleus muscle of ARC KO mice. Additional experiments employing subcellular fractionation analysis and isolated mitochondrial preparations suggest the altered apoptotic signaling in slow-oxidative muscle seems to be mitochondrial-mediated. Our data also suggest that death-receptor signaling (as demonstrated by no changes in caspase-8 activity), as well as SR-mediated signaling (as demonstrated by no changes in calpain activity) were not responsible for the elevated DNA fragmentation observed in the soleus of ARC KO mice. Furthermore,

the mitochondrial-mediated apoptotic signaling is likely specific to caspase-independent mechanisms, since we observed elevated cytosolic AIF protein levels but not cytosolic cytochrome c and Smac. In agreement with this, the activity of caspase-3 and -9, (which are both positively influenced by cytosolic cytochrome c and Smac content) were not different between groups. Several recent papers have shown an important role for caspase-independent mechanisms in skeletal muscle apoptosis. For example, Dam et al. showed that increased DNA fragmentation in muscle of glutathione depleted rats was associated with increased cytosolic and nuclear AIF, but not cytosolic cytochrome c and Smac, and was caspase-independent [102]. Similarly, increases in nuclear AIF and EndoG, but not cytosolic cytochrome c or caspase-3 activity were associated with increased DNA fragmentation in skeletal muscle of aged rats [103]. Caspase-independent apoptosis was also observed in skeletal muscle following hindlimb suspension, which was associated with increased EndoG translocation to the nucleus [104].

Surprisingly, no changes in caspase activity, particularly that of caspases-2 and -8, were observed in either muscle of the ARC KO animals. In the seminal study describing its anti-apoptotic mechanisms, ARC was shown to bind directly to caspase-2 and -8 and prevent their activation [49]. Subsequent cell culture experiments demonstrated that knocking down or mutating ARC lead to increased caspase-8 activation when those cells were treated with an apoptotic stimulus [67, 68, 105]. While the findings in this study seem to contradict previous work, it is important to note that these studies were conducted in cell culture models using non-myogenic cell types. Furthermore, in these studies the extrinsic pathway was specifically stimulated by exongenous administration of molecules known to interact with the TNF-

family of death receptors [67, 68, 105]. In contrast, our study was intended to examine basal apoptotic characteristics and signaling in skeletal muscle of ARC-deficient animals with no effort to stimulate this apoptotic signaling pathway. Furthermore, we are unaware of any evidence to suggest that these animals would have increased circulating or skeletal muscle death-receptor-related cytokines. Therefore, it is reasonable to expect that with no provocation to death receptor mediated signaling, no increase in caspase-2 or -8 activities would be observed in the absence of ARC protein.

Another mechanism by which ARC has been shown to inhibit apoptosis is by suppressing the ER-stress-mediated pathway. Specifically, this inhibition is due to ARC's high affinity for Ca^{2+}, as studies have shown overexpressing ARC decreases calcium transients as well as suppresses A23187 and thapsigargin induced cell death [66]. ER-stress can lead to Ca^{2+} release, which would activate proteolytic enzymes such as caspase-12 and calpains [36]. However, we found no evidence *in vivo* of increased calpain activity providing little evidence for a role of ER-stress-mediated signaling. This measure of ER-stress is by no means a comprehensive assessment of this signaling pathway, and in order to fully rule out ER-signaling, specific ER-stress proteins would need to be measured. For example, following ER-stress, activation of CCAAT/enhancer-binding protein homologous protein (CHOP) and eukaryotic initiation factor 2α (eIF-2α) can lead to apoptosis in skeletal muscle [106]. Much like the death-receptor mediated pathway, the lack of ER-stress-mediated apoptotic signaling may be due to the fact that no effort was made to perturb this mechanism either chemically or physically. Thus, it remains to be determined if induction of ER-stress or

events associated with ER-stress (accumulation of misfolded proteins) would augment skeletal muscle apoptosis in the ARC depleted state.

Through its ability to bind cytosolic Ca^{2+}, ARC has been shown to be protective against Ca^{2+} mediated apoptotic signaling [65, 66]. Ca^{2+} has a well established role signaling apoptosis at the mitochondria. Specifically, high levels of Ca^{2+} have been shown to stimulate permeability transition pore (PTP) formation. Sustained PTP formation causes the mitochondria to swell, consequently rupturing the outer and inner mitochondrial membranes, which leads to membrane potential loss and release of pro-apoptotic proteins into the cytosol [13]. Interestingly, isolated mitochondria from knockout animals were more susceptible to mitochondrial swelling and a loss in membrane potential following the addition of Ca^{2+}. Thus, although ARC KO animals do not show signs of elevated ER-stress-related signaling at a basal state, they are more susceptible to a Ca^{2+} challenge at the level of the mitochondria.

Our data indicate that the increased DNA fragmentation seen in the red muscle of ARC-KO animals is due to mitochondrial-mediated apoptotic signaling. Previous studies have shown that ARC can directly bind Bax and prevent its activation and translocation to the mitochondria [64, 67]. Thus, a lack of ARC would presumably influence cellular/mitochondrial Bax levels and/or signaling. Furthermore, ARC has been shown to bind and inhibit Bad and PUMA, both of which inhibit Bcl-2 [70]. In support of this, the soleus muscle had significantly higher Bax protein expression. As a more specific measure of the influence of these apoptosis proteins on mitochondrial signaling, we determined their expression in isolated mitochondrial preparations. Similar to the soleus data, the red

quadriceps had significantly higher Bax protein as well as an elevated Bax:Bcl-2 ratio. Interestingly, Nam et al. demonstrated that even without the addition of an apoptotic stimulus, knockdown of ARC in H9c2 cells was sufficient to cause spontaneous Bax activation and apoptosis [67]. While this was indeed the case in our red muscle, this effect was not observed in the plantaris or white quadriceps of ARC KO mice. This discrepancy may reflect differences in tissue/cell type, as well as inherent differences between cell culture and in vivo models. One possible explanation for this difference between our red and white muscle may be due to inherent fiber type specific differences in apoptotic signaling and susceptibility [100]. For example, McMillan et al. reported significantly higher levels of many pro- and anti-apoptotic proteins, and apoptosis-related mitochondrial events in red compared with white gastrocnemius muscles [100]. Furthermore, compared to white, red gastrocnemius had significantly higher ROS generation and DNA fragmentation, possibly suggesting a greater basal level of apoptotic signaling/stress. In addition, Degens et al reported increased DNA fragmentation in the soleus, but not plantaris or gastrocnemius of emphysematous hamsters [107]. The investigators speculated that the differences in DNA fragmentation were due to an increased susceptibility to mitochondrial mediated signaling in fibers with higher mitochondrial content. Similarly, exogenous administration of angiotensin II or clenbuterol induced more apoptosis in soleus muscles, than in tibialis anterior of the same animal [108, 109]. Interestingly, following strenuous exercise, apoptotic signaling in the soleus involved the intrinsic pathway and was triggered by oxidative stress, whereas apoptotic signaling in the gastrocnemius involved the extrinsic pathway and was stimulated by cytokines [110]. Taken together, it is likely that basal levels of stress/damage are higher in

slow-oxidative than in fast-glycolytic muscles. Therefore, since red-oxidative muscles are under more stress, they would likely be more affected by a lack of ARC protein.

The increased Bax:Bcl-2 ratio in our red mitochondrial fractions, along with results from our isolated mitochondria experiments, suggest mitochondria from ARC KO animals are more susceptible to mitochondrial outer membrane permeabilization (MOMP). It is well documented that the permeabilization of the outer mitochondrial membrane is associated with the release of several pro-apoptotic proteins such as AIF, EndoG, cytochrome c, and Smac [19]. Through its interaction with Bax, ARC has been shown to prevent the release of EndoG, cytochrome c, and Smac [64, 69, 72, 74]. For the first time, we show that a lack of ARC protein in skeletal muscle results in increased basal cytosolic AIF. Surprisingly, the increased AIF release occurred without increased release of EndoG, Smac, or cytochrome c; however, previous research has demonstrated this selective release of mitochondrial-housed pro-apoptotic factor in other models. Yu et al. demonstrated after an apoptotic insult, AIF release occurred alongside DNA condensation, both of which happened before cytochrome c had been released into the cytosol [111]. In T-lymphocytes, Bidère et al. showed that Bax activation and mild outer membrane permeabilization caused AIF to be released independent of EndoG, Smac or cytochrome c [112]. Similarly, treating leukemia cells with HLA-DR caused AIF to be released but not cytochrome c [113]. Furthermore, results from our lab demonstrated that in skeletal muscle depleted of glutathione, DNA fragmentation was associated with AIF release independent of any EndoG, Smac, and cytochrome c [102]. Thus, under basal conditions, ARC may be a novel regulator of caspase-independent signaling events in skeletal muscle mediated through the release of AIF.

Another interesting finding in this study was that there were no increases in other anti-apoptotic proteins to accommodate for the lack of ARC in our KO animals. Furthermore, not only was there no increase in Bcl-2 and XIAP, but levels of FLIP(s) and HSP70 were significantly decreased. In particular, HSP70 can also influence Bax activation and translocation [38]. Thus, the increased apoptotic signaling observed in red muscle may likely be mediated by several proteins/factors that are directly and indirectly influenced by ARC expression. Interestingly, the tumor suppressor p53 has been shown to promote the degradation FLIP(s) and inhibit the transcription HSP70 [114, 115]. Foo et al. demonstrated that endogenous ARC expression in MCF7 breast cancer cells prevents p53-dependent transcription of pro-apoptotic factors by exporting p53 from the nucleus [59]. Since ARC has been shown to inhibit p53-mediated apoptosis, it is possible the increased apoptosis in our ARC KO animals may be, in part, be due to uninhibited p53 signaling. Similar to our results, overexpressing p53 in HeLa cells caused apoptosis, which was associated with increased Bax expression, disruption of membrane potential, and occurred independent of cytochrome c release [116]. Similarly, p53 has been shown to increase Bax transcription as well as directly activate Bax and promote mitochondrial membrane permeabilization [117, 118]. Since ARC has been shown to interact with p53, total p53 protein content, as well as the subcellular localization of p53 should be examined in subsequent studies in ARC-deficient animals.

Skeletal Muscle Morphological and Phenotypic Changes

As hypothesized, we found that a lack of ARC was associated with morphological (alterations in fiber CSA) as well as phenotypic (changes in fiber type composition) alterations in both the soleus and plantaris muscles. A number of other reports have

examined the effect of apoptotic regulatory proteins on muscle morphology, phenotype, and function. For example, Bcl-2 expression increases early during myogenic differentiation; an effect which is important for the formation of healthy myotubes, and their resistance to apoptotic stimuli [119]. Similarly, Fernando et al reported that caspase-3 signaling may be an important component in myocyte differentiation, since caspase-$3^{-/-}$ myoblasts demonstrated impaired differentiation [120]. Furthermore, although caspase-3 KO mice showed no change in type II fiber cross-sectional area, only half of these mice survive to be healthy adults [121]. Conversely, p53-KO mice exhibit increased muscle weights and produce higher titanic force; however, muscle from p53-KO mice show greater fatigue and these mice run significantly less compared to WT controls [122]. These detrimental effects associated with a lack of p53 are presumably due to less mitochondrial content, as well as impaired mitochondrial function [122]. A recent study by Armand et al. reported that AIF deficient mice have decreased cross-sectional areas in soleus and EDL muscles, as well as a shift towards slower fiber types [123]. Interestingly, muscle specific overexpression of the anti-apoptotic protein HSP70 resulted in a 10% reduction in body weight, and a 20% reduction in muscle mass [124]. Collectively, these papers suggest that apoptosis-specific regulatory proteins have a significant role in the development and function of skeletal muscle.

Changes in Cross-Sectional Area

One of our major findings was that the total and fiber type-specific CSA was decreased in type I and IIa fibers of the soleus of ARC KO animals. Several studies have reported atrophy of specific fiber types. For example, age-related muscle loss is associated with atrophy in type II fibers while type I fibers remain relatively unaffected [125, 126].

Similarly, inactivity has been shown to preferentially decrease CSA of type I and IIa fibers in soleus muscles [127]. Although there were no differences in activity levels between genotypes, our animals were relatively "inactive" as they were cage bound without access to a running wheel. It has been previously shown that given access to a running wheel, C57BL/6 mice will voluntarily run approximately 4km a day [128]. While our cage-bound mice were not as "inactive" as some forced disuse models (i.e., hind-limb suspension, and denervation), the inability to meet those activity demands could result in mild disuse stress. Therefore, the changes in CSA in ARC KO animals may reflect a greater sensitivity to this mild stress.

Despite having a differential fiber type distribution, ARC is still present in all fiber types. Therefore, although it was expected that a lack of ARC would affect the soleus to a greater extent than the plantaris, it is somewhat surprising that a lack of ARC had no detrimental effects on fiber CSA in the plantaris. It is likely that the differential response reflects the fiber type composition of these muscles. A decrease in CSA of type I and IIa fibers in the soleus would have major effects on overall CSA given that these fibers make up approximately 80% of the total number of fibers. In contrast, even though a non-significant decrease in type I CSA was observed in plantaris, a significant increase was seen in type IIb fibers. This would tend to increase overall CSA since IIb fibers are approximately three times larger than type I fibers and make up over 40% of the total population. Also of interest was the differential CSA response in the type IIa fiber across soleus and plantairs. ARC is highest in type I fibers but is also highly expressed in type IIa fibers [100]. What is currently unknown is if the level of ARC is similar in type IIa fibers across muscles, as this could

influence the currently observed responses. Therefore, the consequences of a lack of ARC are fiber type specific but not universal across all muscles.

It is possible that recruitment patterns of particular muscles could account for some of these discrepancies. In rodents, the soleus acts as a postural muscle and is highly recruited [129]. Conversely, the plantaris becomes active during locomotion and thus is recruited less than the soleus [130]. Therefore, over the lifespan of these animals, the soleus would be exposed to significantly more muscle contractions compared to the plantaris. Contracting skeletal muscle has been shown to signal many intracellular processes, including apoptosis [131]. Consequently, the absence of ARC may leave highly recruited myofibers more susceptible to contraction-associated apoptotic signaling than less recruited ones

Changes in Skeletal Muscle Fiber Type

The other morphological change in the ARC KO animals was a shift in fiber type distribution. In the soleus, there were less type I fibers, and a greater percentage of type IIa fibers. This shift towards a faster phenotype was also seen in the plantaris, as there were less type IIa fibers, and more type IIb fibers. Inactivity has been shown to cause shift towards a faster phenotype [132-134]; however, our data suggest that the differences in fiber type between genotypes are not due to activity differences. Regardless, as stated previously, our animals were cage-bound without access to a running, thus representing a relatively "inactive" animal model. Therefore, even though there were no differences in activity levels between mice, ARC-KO animals may be more sensitive to "inactivity" which may explain the phenotype.

Fiber type shifts similar to those observed in this study, have also been observed in various disease states. For example, Green et al. reported atrophy in type I fibers, as well as a fiber type shift towards faster type II fiber in a case study of two human male patients with COPD [135]. In a larger cohort of COPD patients of both sexes, myosin ATPase staining revealed a lower percentage of type I fibers, and a greater percentage of type IIx fibers [136]. Similarly, patients with congestive heart failure demonstrate a shift towards less type I fibers, and more type II fibers [137-139]. While many thought that these changes were consequences of inactivity associated with the disease, it was later shown that the shifts in fiber type composition were independent of activity levels [137, 140]. Another disease state associated with a slow to fast fiber type shift in skeletal muscle is hypertension. [141, 142]. Interestingly, hypertension is associated with increased apoptotic signaling in skeletal muscle, as well as a dramatic reduction in ARC protein levels [107, 143-145]. Taken together, these reports show that the skeletal muscle phenotype exhibited by ARC KO mice is similar to that of pathological conditions. While decreased skeletal muscle ARC content has only been observed in hypertension, it will be of interest to determine if ARC is lower and plays a role in the phenotypic changes observed in skeletal muscle during COPD and heart failure as well.

Finally, while most studies have demonstrated ARC's role in preserving tissue, previous studies have shown ARC may actually play an important role in the development of tissue as well. Work from our lab and by other has demonstrated that ARC protein content is relatively low in myoblasts. However, as myoblasts differentiate into myotubes ARC content increases dramatically [146]. Work by Fernando et al. found an association between

differentiation and apoptosis, as caspase-3 activity was shown to increase dramatically after one day of differentiation, after which caspase-3 activity quickly returned to levels slightly higher than found in undifferentiated myoblasts [120]. Although counterintuitive, the importance of caspase-3 during differentiation was evident as inhibiting caspase-3 lead to impaired myocyte proliferation and differentiation [120]. This seminal study showed for the first time that mechanisms which are intimately involved in cell death processes are also critical for cell growth and development of skeletal muscle. Unfortunately, little is known about how these cell death processes are regulated to promote development, rather than initiating apoptosis. Seeing as ARC protein content increases in concert with caspase-3 activation during differentiation, ARC may be a candidate for such a regulatory protein. If so, a lack of ARC during myogenic development may have contributed to the altered skeletal muscle phenotype observed in ARC KO animals. Thus, through its ability to alter apoptotic signaling, ARC may play an important role in the development and maintenance of healthy skeletal muscle morphology and phenotype.

Conclusion

Muscle fiber composition and size are highly predictive of muscle strength in elderly individuals [147]. Understanding the mechanisms that regulate these factors will no doubt lead to interventions to preserve muscle function as we age, and during various disease states. The results from this study show that ARC plays an important role in caspase-independent signaling, mitochondria apoptotic susceptibility, and DNA fragmentation. In addition, ARC protein influences fiber type distribution, and muscle size. Whether these morphological changes are strictly a result of altered apoptotic signaling due to a lack of ARC, or due to other mechanisms by which ARC participates in during the development of skeletal muscle has yet to be determined. Regardless, this study demonstrates that a lack of ARC can cause increased apoptotic signaling along with several phenotypic and morphological alterations in skeletal muscle of healthy animals.

Limitations

A major limitation of this study is the fact our transgenic mouse model is not a muscle specific KO. Since ARC is present in other tissues, it is possible that changes in these tissues may have influenced our results. Nevertheless, ARC expression is specific to only skeletal, cardiac and smooth muscles, as well as the brain. Therefore, it is unlikely that the changes we observed were due to secondary effects from other tissues.

Another limitation associated with this study is the fact that whole muscles are heterogeneous mixtures of several different fiber types. As such, measuring a specific protein in a whole muscle homogenate does not elucidate the changes that are happening in individual fiber types. For example, we reported altered apoptotic signaling in the soleus; however, only type I and IIa fibers were atrophied, while no changes were observed in the CSA of type IIx fibers. Therefore, the altered apoptotic signaling may not be occurring in all fiber types within the soleus. To determine whether or not these changes in apoptotic signaling are fiber type specific, immunohistochemistry could be used to co-localize changes in protein content/protease activity with myosin heavy chain expression. Moreover, this technique would rule out any apoptotic signaling in other cell types within the muscle.

Future Directions

Although the results from this study indicate that ARC does in fact play an important role in regulating apoptotic signaling and morphology in skeletal muscle, further studies are needed to elucidate the exact mechanisms by which these changes occur. For example, we observed changes in fiber type distribution in ARC KO animals. To determine whether or not these changes in fiber type distribution are due to alterations in the development of skeletal muscle without ARC, or whether these changes occur over time, a time course analysis following the development and aging of ARC KO animals would be of interest. If the changes in fiber type distribution are due to developmental changes, this phenotype should be present throughout the healthy life-span of ARC KO animals. However, if the changes are due to altered-apoptotic signaling, the fiber type changes may be exacerbated as the mice age.

To further determine ARC's role in the development of skeletal muscle, current work is underway to investigate the effect of knocking down ARC expression in differentiating myoblasts. This will allow us to measure other apoptotic-related proteins and proteases that have been previously shown to be involved in differentiation (i.e., caspases). Furthermore, these experiments will allow us to determine whether ARC contributes to the apoptotic resistance observed in differentiated myotubes.

Skeletal muscle fiber type and size are associated with various metabolic and mechanical properties specific to that phenotype. Since our ARC KO animals had changes in

fiber type and size, it would be important to examine the functional consequences of the lack of ARC, as well as the increase in apoptosis on muscle contractile and fatigue properties.

As stated in the discussion, the fiber type changes may have been the result of a mild stress due to the animals being cage-bound and relative inactive. To determine whether that hypothesis is correct, a more dramatic model of inactivity (such as hindlimb suspension) could be implemented to see if phenotypic changes in muscle fibers are exacerbated. Conversely, giving the mice access to a running wheel may alleviate any inactivity-related stress and prevent any morphological and phenotypic changes.

Exercise has previously been shown to be effective in preventing skeletal muscle apoptosis [148]. Similarly, ARC content has also been shown to increase following exercise training [91, 93]. It would be of interest to definitively determine if ARC is a major mediator of exercise-induced protection against apoptosis through exercise training studies with ARC-deficient mice.

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