The Effect of Mitochondrial Biogenesis on Apoptotic Susceptibility
in L6 Myoblasts

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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Aaron Dam

ABSTRACT

Mitochondria play an essential role in cell metabolism as well as apoptotic signaling. Chronic endurance exercise has been shown to increase mitochondrial content in skeletal muscle. Interestingly, endurance exercise has also been associated with decreased skeletal muscle apoptosis; however, the direct effect of increased skeletal muscle mitochondrial content on apoptotic signaling has not been examined. The purpose of this study was to induce mitochondrial biogenesis in L6 myoblasts and examine the susceptibility of these cells to stress- induced apoptosis. Mitochondrial biogenesis was accomplished using 5-Aminoimidazole-4-carboxamide-ribonucleoside (AICAR) and S-nitroso-N-acetylpenicillamine (SNAP), which activate AMPK and donate nitric oxide, respectively. Successful induction of mitochondrial biogenesis was determined by western blot analysis for mitochondrial specific markers. Following SNAP and AICAR treatment, the average increase in the mitochondrial markers was 24% and 38%, respectively. Subsequent exposure of cells to several apoptosis-inducing agents increased apoptosis. Interestingly, SNAP- and AICAR- treated cells had a lower percentage of apoptotic cells as determined by AnnexinV-FITC/PI fluorescent staining, cell cycle analysis, and cell counting/size analysis. In addition, it was shown that SNAP- and AICAR-treated cells had reduced caspase-3 activity following exposure to apoptotic stimuli. Furthermore, treatment with SNAP and AICAR resulted in increased protein content of the antioxidants MnSOD and catalase. Interestingly, mitochondrial ROS production was not significantly altered between groups with total cellular ROS production being increased in the SNAP- and AICAR-treated groups. In summary, this work demonstrates that increasing mitochondrial content in L6 myoblasts provides protection against stress-induced apoptosis. The mechanism for this protective effect remains to be determined; however, it may be

mediated by a combination of increased antioxidant capacity and improved mitochondrial calcium buffering capacity.

ACKNOWLEDGEMENTS

DEDICATION

This thesis is dedicated to my family – My parents: Don and Tilda; my brother Mike, his wife Annie and my niece Elly; my sister Selena, her husband Mike and my nephew Logan; and my brother Ken and his wife Claire. The love and encouragement they have provided throughout the years has undoubtedly brought me to where I am today.

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Introduction

Overview

The purpose of this literature review is to examine apoptotic signaling and mitochondrial biogenesis. An in-depth description of the apoptotic signaling pathways and their regulation will be given. Furthermore, a review of the current literature in regards to apoptotic signaling and physical activity in skeletal muscle will be described. The processes regulating mitochondrial biogenesis both *in vivo* and *in vitro* will also be reviewed. Upon reading this literature review it will become evident that the relationship between endurance training and apoptosis has been thoroughly examined in rodent models; however, the specific effect of mitochondrial biogenesis on apoptotic signaling has not been established. The review will conclude with some possible linkages between mitochondrial biogenesis and apoptotic signaling indicated in the literature.

Apoptosis in Health and Disease

Apoptosis is a fundamental process for normal development as well as for physiological cell turnover. Physiologically, the role of apoptosis is vast. For example, apoptosis is required for the formation of body cavities [1], is involved in homeostatic regulation of cell number [1,2], embryogenesis, the morphogenesis of fingers and toes [1], and in cellular injury [1]. In addition to these important physiological roles, dysregulation of this tightly regulated process can have severe consequences. It is well established that a balance between an appropriate amount of apoptosis and too much apoptosis is important in the pathogenesis of disease as an upregulation can lead to tissue damage and atrophy whereas down-regulation can lead to cellular overgrowth. Hence, apoptosis is implicated in

pathogenesis of many disease states including acquired immunodeficiency syndrome (AIDS), where the HIV virus causes death of T-helper cells which are required for proper immune function [3]. Excess apoptotic cell death is also implicated in other autoimmune diseases such as Hashimoto's thyroiditis [4]as well as cardiovascular diseases[5] and in neuronal diseases like Alzheimer's and Parkinson's[6]. On the other hand, an inhibition of apoptosis in a number of tissues is the basis of cancerous tumor formation [1,2,7]. Due to its involvement in numerous disease states, a great deal of research is examining apoptotic regulators as potential therapeutic treatments.

Regulation of Apoptosis

Apoptosis is a tightly regulated process controlled by the activation of a pre-existing program within the genome and can be distinguished from other types of cell death such as necrosis. Necrosis tends to occur in response to injury by toxins, physical trauma, or ischemia [1,8]. This type of cell death is characterized by cellular swelling, disruption of membrane integrity, cellular leakage, and lysis of nuclear chromatin [1,2,8]. Necrosis also often involves large groups of cells and since large volumes of cellular contents leak into the extracellular space, it often elicits an intense inflammatory response [8]. Apoptosis, on the other hand, is a tightly controlled, energy dependent mechanism characterized by cell shrinkage, nuclear condensation, DNA fragmentation, and membrane blebbing that eventually lead to phagocytosis of the dying cell with little or no inflammatory response [1,8,9]. Although apoptosis and necrosis can be considered separate entities, certain circumstances involving cellular stress imposed by toxins, physical trauma, or ischemia, may cause cell death via necrosis as well as apoptosis [1]. The ultimate death pathway a cell follows (apoptotic or

necrotic) is often determined by the type, intensity, and duration of death stimuli as well as the energy capacity remaining within the cell. In addition, *in vitro* models lack the phagocytes that are normally required for clearance of apoptotic bodies. When clearance of the apoptotic cell does not occur, the cell moves through the apoptotic process until a transition is made to necrosis [10]. This transition to necrosis is known as secondary necrosis [10]. These cells that once showed characteristics of apoptosis begin to show necrotic characteristics such as ATP depletion, swelling, membrane rupture, and release of cellular contents [10].

Apoptosis can be triggered by many different stimuli but execution occurs through two main pathways; the extrinsic and intrinsic pathways. Both pathways have overlapping characteristics in the sense that there is an initiation phase (receipt of death inducing signals) and an execution phase (nuclear and cellular breakdown). The execution phase is initiated by activation of specific proteolytic enzymes known as caspases [11] (cysteine-aspartic proteases). Caspases are produced and stored within the cytosol as inactive proenzymes and as procaspases [11]. In order to perform their proteolytic function, they must first be cleaved at specific aspartic residues into their active form. Caspases involved in the apoptotic pathways can be divided into two groups, initiator caspases (caspases-8, -9, -10) and effector caspases (caspases-3, -6, -7) [11,12]. Upon activation of the initiator caspases, they begin breaking down intracellular proteins important for regular cell function, as well as cleaving and thereby activating effector caspases [11,12,13]. The effector caspases then continue to break down important cellular and nuclear proteins such as the nuclear enzyme poly ADP- ribose polymerase (PARP) (an important DNA repair enzyme) as well as cleaving more procaspases [11,12]. This caspase signaling cascade ultimately results in massive cellular degradation and cell death [11].

In the intrinsic apoptotic pathway, death can be triggered in response to a variety of signals such as UV irradiation, oxidative stress, growth factor withdrawal, or cytotoxic drugs [1,2,14]. These stress signals result in the activation of Bax, a cytosolic protein which translocates to the mitochondria. At the mitochondria, Bax participates in the formation of pores in the outer membrane which ultimately allows for the release of cytochrome c into the cytosol [14,15,16,17]. Upon release into the cytosol, cytochrome c binds to the Apoptotic Protease Activating Factor-1 (Apaf-1) along with procaspase-9 and dATP forming the apoptosome [1,11]. Formation of the apoptosome activates self cleavage of procaspase-9 to caspase-9. Subsequently, caspase-9 activates downstream effector caspases (eg. caspase -3, -6, -7), ultimately leading to breakdown of cellular substrates and eventual cell death [1,11,14] (*see figure 1*).

In addition to caspase-mediated cell death, there are also caspase-independent mechanisms that can mediate apoptotic cell death through the intrinsic pathway. Upon receiving a pro-apoptotic signal, the mitochondria can release apoptosis-inducing factor (AIF) and endonuclease G (EndoG), both of which translocate to the nucleus and induce DNA fragmentation and chromatin condensation directly without caspase activation[1,18] (*see figure 1*).
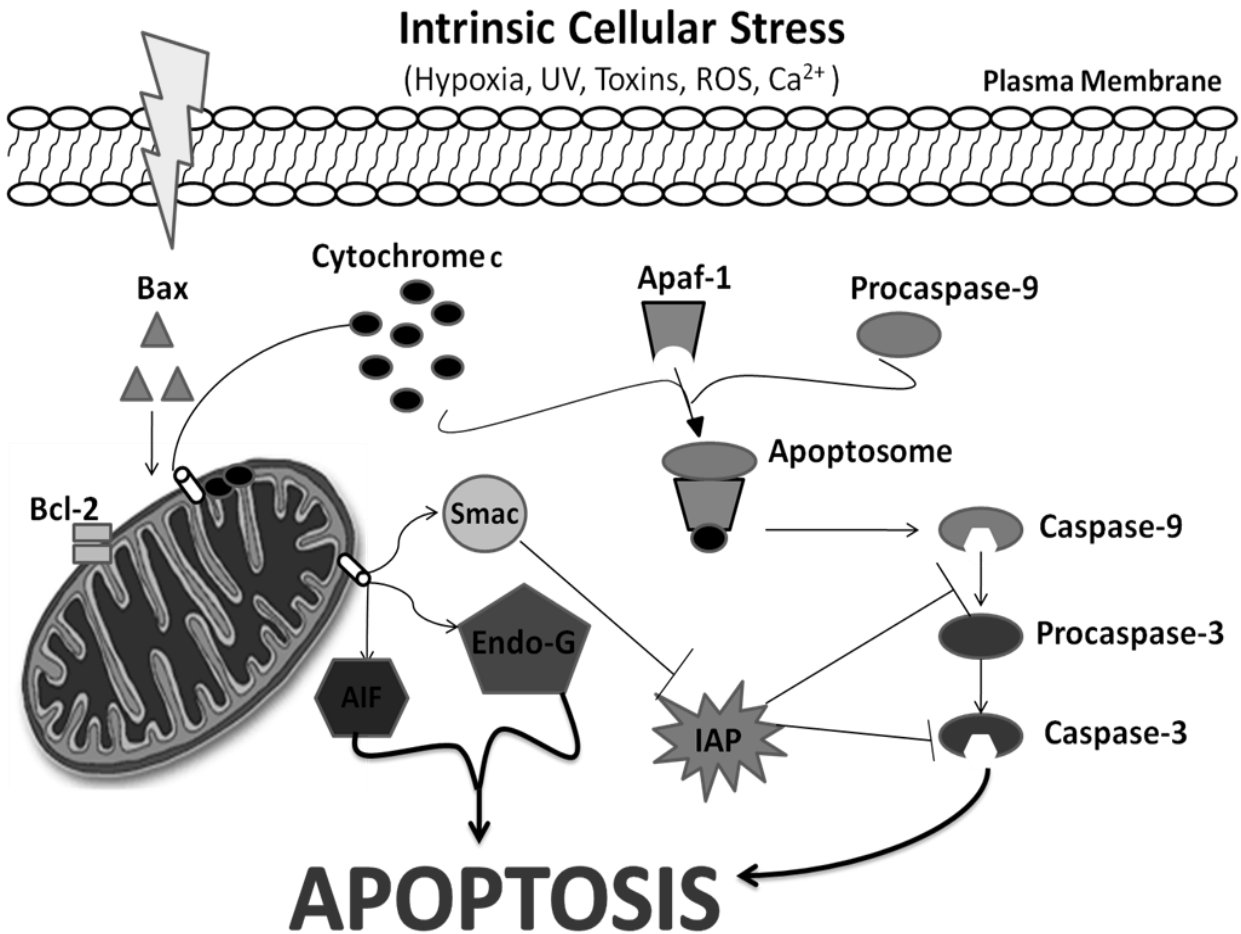
Figure 1. Intrinsic (mitochondrial) pathway of apoptosis. Intrinsic cellular stresses result in Bax activation and translocation to the mitochondria membrane and subsequent pore formation. Mitochondria release cytochrome c which leads to the formation of the apoptosome and subsequent activation of the caspase cascade ultimately leading to cell death. Mitochondria can also release caspase independent factors AIF and Endo G, which translocate to the nucleus and initiate cell death by cleavage of nuclear factors.

In the extrinsic pathway, the decision for cell survival or death is determined though signals received extracellularly. The extracellular signals are often a combination of cessation of anti-apoptotic cell survival signals and receipt of positive apoptotic signals binding to what are commonly known as the 'death receptors' [1,18]. The two main death receptors are known as the tumor necrosis factor receptor-1 (TNFR1), and the Fas (CD-95) receptor [1,2,19]. Each receptor has an amino acid sequence that projects into the cell which is known as the death

domain (DD) [1,2]. Apoptotic cell death is initiated by the binding of tumor necrosis factor-α

(TNF-α), or Fas ligand (FasL) to their respective receptors. Upon binding of ligands to

receptors, intracellular signals recruit the adaptor proteins known as TNFR-associated death

domain protein (TRADD) and Fas-associated death domain protein (FADD) which bind to the

death domain to form the death inducing signaling complex (DISC) [1,2,7,19]. Pro-caspase-8

then binds to the death effector domain (DED) on the DISC resulting in its cleavage to the

active form caspase-8 which sequentially activates the effector caspases (eg. caspase-3, -6, -7)

[1,2,12]. Activation of effector caspases then results in the breakdown of cellular and nuclear

substrates much like the intrinsic pathway [1,19,20,21]. Activation of caspase-8 can also lead

to cleavage and activation of BID, a cytosolic protein which translocates to the mitochondria

and causes cytochrome c release, ultimately linking the extrinsic (death receptor) and intrinsic

(mitochondrial) apoptotic pathways [1,2,15,16] (*see figure 2*).
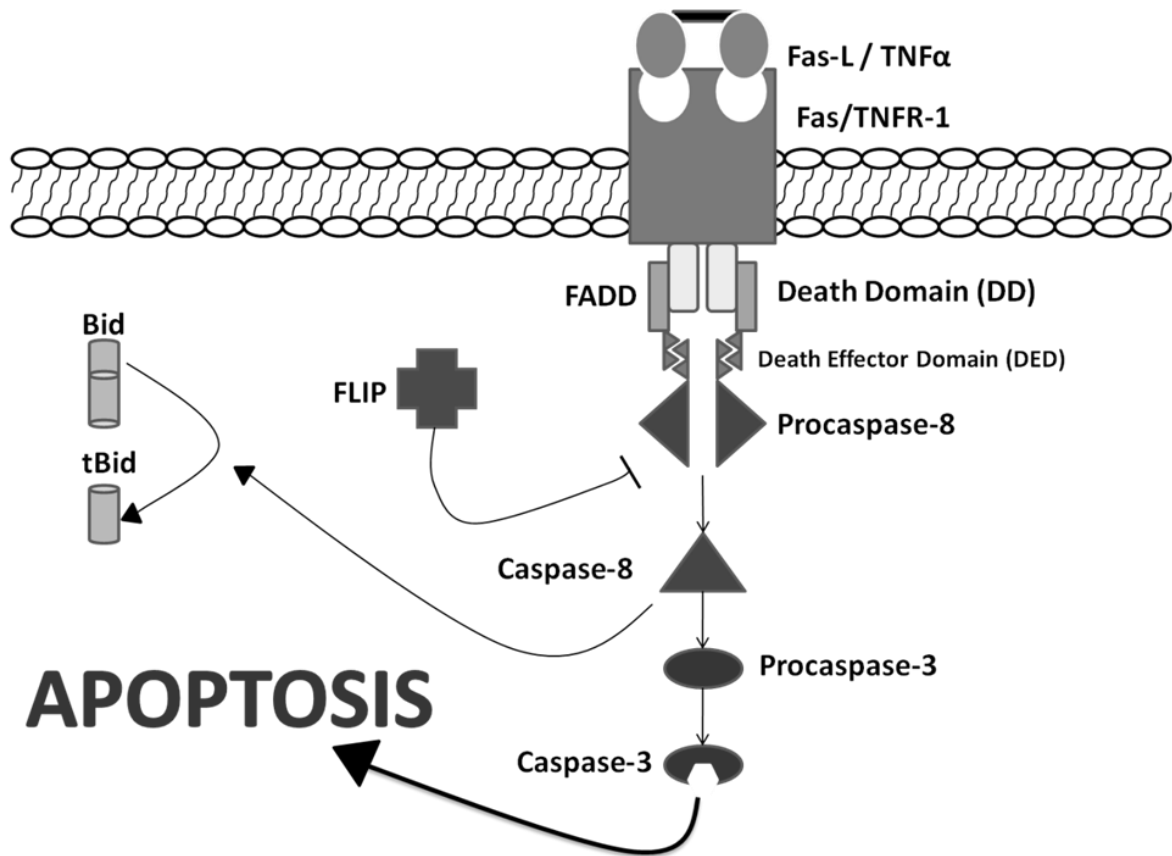
Figure 2. Extrinsic (Death Receptor) pathway of apoptosis. Biding of the appropriate ligand to the death receptor on the cell membrane results in formation of the death inducing signaling complex (DISC) consisting of pro-caspase 8 and bound with FADD or TRADD to the death domain of the death receptor. Formation of the DISC results in activation of caspase-8 with can then cleave caspase-3 which ultimately leads to cleavage of more apoptotic substrates and cell death. Caspase-8 can also cleave and activate Bid which then translocates to the mitochondrial membrane causing pore formation and activation of the intrinsic pathway of apoptosis.

Another important apoptotic pathway is the calcium-dependent calpain-mediated pathway. Calcium plays a large role in intracellular signaling and a number of effects on numerous processes. For example, in skeletal muscle cytosolic calcium is involved in metabolism, muscle contraction, transcription activation, and proliferation [21]. Given that calcium signaling plays such a vital role in normal cellular function, it is not surprising that it is

also a player in apoptotic signaling. Calcium's role in apoptosis can vary depending on tissue type but its execution pathways are consistent between tissues [22]. In this pathway, calcium-dependent proteases known as calpains are activated in response to imbalances in calcium regulation. Calpains are structurally similar to caspases and function in a very similar fashion, cleaving many of the same apoptotic proteins as caspases [22,23,24]. Calpains can cleave and activate the Bcl-2 family proteins Bid and Bax which can then translocate to the mitochondria and induce pore formation at the membrane [22]. Additionally, calpains can activate caspase-12, an effector caspase which can act on mitochondria and induce them to release pro-apoptotic factors [22,23,25]. Calpains can also cleave a number of proteins required for DNA repair and cell cycle regulation, thereby disrupting normal cell function and promoting cell death [22].

Apoptosis Regulatory Proteins

There are many proteins that effect the regulation of apoptosis which can be either pro- or anti-apoptotic and can act at different points within the apoptotic pathway. For example, in the extrinsic pathway, the protein TRAF2 (TNF receptor-associated factor 2) interacts with the TNFR-1 and TRADD to prevent binding and subsequent caspase-8 activation [1,19,20]. TRAF2, along with the binding of other inhibitors of apoptosis proteins (IAPs), activates NF-κB, which mediates up-regulation of anti-apoptotic genes such as FLIPs [19,20]. FLIPs (FLICE inhibitory proteins) can bind to the death domain of Fas and TNFR1 and prevent DISC formation and subsequent activation of caspase-8, thereby inhibiting apoptosis [1,26].

In the intrinsic pathway, the Bcl-2 family of proteins comprises a large portion of these apoptotic regulatory proteins. Pro-survival members of the Bcl-2 family include Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bim, and EGL- 1[15,16]. These proteins tend to be found in membranes such as

those of the mitochondria, endoplasmic reticulum, and the nucleus [16]. One of the main

functions of these proteins is to inhibit mitochondrial pore formation and release of cytochrome

c and other pro-apoptotic proteins from the mitochondria [15,16,17]. Pro-apoptotic members of

the Bcl-2 family can be subdivided into two subfamilies: the Bax family, which includes Bax,

Bak, Bok, Bcl-rambo, and Bfk, and the BH-3 family, which includes Bid, Bad, Bik, Bim, Bmf,

Noxa, Puma, and Hrk [15,16]. These proteins are found mainly within cytosolic portions of

the cell but upon receiving the appropriate death stimuli, they migrate to the mitochondria to

induce pore formation [16]. Hence, the balance of the two major pro-apoptotic and anti-

apoptotic Bcl-2 family proteins (the Bax:Bcl-2 ratio) is a useful indictor to determine a cells

fate or susceptibility to apoptotic death [16].

Another protein that has a critical role in apoptotic signaling is apoptosis repressor with

caspase recruitment domain (ARC). ARC is a unique protein in that it has only been shown to

be expressed in heart, skeletal muscle, vascular muscle, and the brain [5,27,28]. It is also

unique in that unlike other anti-apoptotic proteins it has the ability to inhibit apoptosis by

interacting in both the intrinsic and extrinsic death pathways. In the extrinsic pathway, ARC

successfully inhibits apoptosis by binding to the death domains of both the Fas receptor and

FADD thereby inhibiting caspase-8 activation [27,28]. In the intrinsic pathway, ARC can

prevent apoptosis by binding to the C-terminus of Bax, which inhibits the conformational

change that results in Bax activation and subsequent translocation to the mitochondrial

membrane [27]. Cellular ARC content has also been shown to be altered in disease states such

as cardiovascular diseases [5,29] and cancer [30], which suggest it may be a key player in

regulating apoptosis in disease states. ARC has also been shown to be rapidly degraded upon

apoptotic stimuli such as reactive oxygen species [31], therefore observations of ARC levels may be important when studying muscle apoptosis.

Apoptotic Signaling in Skeletal Muscle

Apoptosis is a biological process conserved across many tissues and species; however, the mechanisms by which different types of tissue undergo apoptosis can vary. Skeletal muscle is particularly unique from most other tissues due to its multi-nucleated morphology. In most tissues, initiation of apoptosis results in removal of an entire cell, but skeletal muscle can undergo what has been termed myonuclear apoptosis, a process where only individual nuclei and their associated cytoplasmic portions are lost [32,33,34]. Although the fate of the cell differs, the traditional characteristics of apoptosis, such as elevated Bax/Bcl-2 ratio, elevated AIF release, elevated caspase-3, -8, -9, cytochrome-c release, Smac/Diablo release, nuclear condensation and degradation, all occur in skeletal muscle just as it would in many other tissues [32,35].

Upon apoptotic removal of specific nuclei, specialized progenitor cells located between the sarcolemma and the basal membrane known as satellite cells become activated [36,37]. Once activated, satellite cells migrate to the site of damage or nuclear removal, and can fuse to fiber, forming new myonuclei and cytoplasmic regions, thereby repairing the cell [34,36]. The satellite cells; however, do not have infinite availability and the satellite cell 'pool' can become depleted over time [38,39]. In degenerative skeletal muscle states, including motor nerve denervation, aging, atrophy due to immobilization, and Duchenne muscular dystrophy, satellite cell numbers and subsequent proliferative and repair potential significantly decreases [40]. Hence, the limited capacity for muscle regeneration plays a large role in many disease states in

which increased susceptibility to apoptosis in skeletal muscle is implicated and plays a large role in the atrophy of muscle in disuse and aging [38,39].

Mitochondria play a very important role in cell metabolism, not only for energy generation, but also for being a major site for production of reactive oxygen species (ROS) and apoptotic signaling [41]. In skeletal muscle, there are a number of fiber types characterized by their myosin heavy chain expression and their oxidative capacity. Fibers that are more oxidative have greater mitochondrial content [42]. Most skeletal muscle contains a mixed population of fiber type; however some muscles have varying degrees of expression of certain fiber types. For example, the slow twitch soleus muscle of rats is highly oxidative and is composed mostly of type I fibers whereas extensor digitorum longus (EDL) muscle of rat is glycolytic and expresses mainly type IIb and IIx fibers[43]. Studies have found that content of apoptotic proteins can vary between muscle groups in response to exercise [44,45,46,47], suggesting that individual fiber types may differ in their susceptibility to apoptosis. Due to the fact that mitochondria play such a vital role in apoptotic signaling, that mitochondrial content can vary between muscle groups and fiber types, and that mitochondrial content can be altered in skeletal muscle in response to various stimuli, mitochondria have become a main area of focus in the study of apoptosis in skeletal muscle.

Mitochondrial Biogenesis - Introduction and Basic Mechanisms

Given the importance of mitochondria in apoptotic signaling, it is understandable that altering mitochondrial content could have a drastic effect on apoptotic signaling within skeletal muscle. Mitochondrial biogenesis refers to the growth of existing mitochondria or to the growth and development of new functional mitochondria [48]. Mitochondrial biogenesis is a

complex process that can be activated through many mechanisms. Peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α) is a transcriptional coactivator, meaning that it increases the rate of transcription of certain genes by interacting with transcription factors but does not itself bind to DNA [49]. PGC-1α has been shown to be one of the main regulators of mitochondrial biogenesis and does this by increasing many nuclear transcription factors involved in the expression of both nuclear and mitochondrial encoded genes required for mitochondrial proliferation [50,51,52]. PGC-1α's main target in inducing mitochondrial biogenesis is nuclear respiratory factor-1 (NRF-1) and -2 (NRF-2)[52]. NRF-1 and -2 proteins form homodimers and function as transcription factors that are responsible for activating genes that encode for mitochondrial transcription factor A (mtTFA) (also known as Tfam) as well as other mitochondrial oxidative enzyme proteins such as cytochrome c oxidase and delta-aminolevulinate synthase (ALAS)[53].   mtTFA then activates the mitochondrial genes involved in mitochondrial DNA transcription and replication such as mitochondrial DNA polymerase [49].

The importance of PGC-1α in muscle mitochondrial biogenesis has been clearly demonstrated by gene overexpression models *in vitro*. In C2C12 muscle cells, over-expression of PGC-1α led to significantly increased gene expression of cytochrome c, COXIV, COXII, elevated mitochondrial DNA copy number, and increased mitochondrial volume [54]. Similarly, Bianchi et al. [55] found that L6 muscle cells transfected with PGC-1α had significantly increased NRF-1 expression as well as significantly increased mitochondrial volume. In addition, transgenic mice over-expressing PGC-1α show similar increased RNA levels of COXII and COXIV, as well as increased protein expression of cytochrome c [56]. Interestingly, complete knockout of the PGC-1α gene in rats resulted in significant decreases in

total mitochondrial content in skeletal muscle [52]. In addition, complete knockout of PGC-1α in mice has been shown to result in a high fetal mortality rate [57], highlighting the importance of PGC-1α's role in survival.

Activity and Mitochondrial Biogenesis

Exercise has been demonstrated to increase mitochondrial volume whereas physical inactivity has been shown to have the opposite effect [50,51]. Terada et al. [58] have demonstrated that a single bout of prolonged exercise in rats can lead to a significant increase in PGC-1α mRNA expression and can also increase NRF-1 and NRF-2 binding activity [59] signifying that exercise activates the mitochondrial biogenesis signaling pathway. In addition, five daily bouts of endurance exercise in rats results in significant increases in ALAS, citrate synthase, cytochrome c oxidase I (COXI), and cytochrome c in comparison to control rats. Similar results have also been found in human studies. For example, 24 weeks of endurance exercise led to significantly higher levels of mitochondria in quadriceps muscle of young females compared to that of controls [60].

In contrast, prolonged physical inactivity is has been shown to be related to decreased mitochondrial content. In a study using hindlimb unweighting as a model of inactivity in rats, Desplanches et al. [61] found that the absolute volume of subsarcolemmal and inter-myofibrillar mitochondria significantly decreased by 65% and 35% respectively compared to control animals. Similarly, following 21 days of skeletal muscle denervation in rats, it was found that PGC-1α and cytochrome c protein levels were significantly reduced [62]. Additionally, Timmons et al. [63] found that following unilateral unloading of the lower limb in humans, mRNA expression of PGC-1α, citrate synthase, COXIV, and Tfam were all

significantly reduced compared to the control limb. Taken together, these data suggest a strong

link between physical activity levels and mitochondrial content in skeletal muscle.

Factors Regulating Mitochondrial Biogenesis

     Calcium

     The mechanisms by which physical activity may modulate mitochondrial content

within muscle are vast given that contractile activity can lead to a number of biochemical and

signaling events. As such, several pathways in which the initial cellular perturbations

associated with muscle activity leading mitochondrial biogenesis have been described. Calcium

release from the sarcoplasmic reticulum is an important signal that initiates force production

but it is also an important second messenger within the cell that has been implicated in the

activation of transcription factors regulating mitochondrial biogenesis [64]. Increases in

calcium lead to the activation of calcium-calmodulin dependent kinases (CaMK), protein

kinase C (PKC), calcineurin, and p38 mitogen-activated protein kinase (p38 MAPK) [50,51].

All together, these factors can initiate mitochondrial biogenesis through the activation of PGC-

1α [52,65,66]. For example, overexpression of CaMKIV in mouse muscle was demonstrated to

significantly increase PGC-1α RNA levels, cytochrome c protein expression, and

subsarcolemmal mitochondrial content [67] .

     In addition, several cell culture experiments using L6 myoblasts or differentiated L6

myotubes have shown that calcium administration results in mitochondrial biogenesis. For

example, Ojuka et al. [68,69,70] demonstrated that intermittent treatments with caffeine or the

calcium ionophore ionomycin, both of which stimulates calcium release from the sarcoplasmic

reticulum, resulted in significant increases in mitochondrial enzymes. They found significant

increases in the protein content of ALAS, COX1, COXIV, cytochrome c, and citrate synthase [69,70,71,72]. In addition, caffeine administration to L6 myotubes which stimulates intracellular calcium release from the sarcoplasmic reticulum has been shown to up-regulate a number of the transcription factors/co-factors including PGC-1, NRF-1, and NRF-2 [70,71]. Interestingly, administration of a CaMK inhibitor (KN93) along with caffeine administration completely blocks the calcium induced increases in the mitochondrial marker proteins, suggesting calcium works through a CaMK mediated pathway to induce mitochondrial biogenesis [70]. Ultimately this research provides strong evidence for an important role of calcium in the process of mitochondrial biogenesis.

AMPK

AMPK has been established as an important signaling molecule within the cell and acts as a major sensor for AMP level in cells [72]. This enzyme is activated by cellular stresses that result in ATP depletion. Stimuli of AMPK are generally either processes that inhibit ATP production (e.g. metabolites, hypoxia, or glucose deprivation) or increase ATP consumption (e.g. muscle contraction) [73]. AMPK is also activated by the adipokines leptin and adiponectin, important regulators of whole-body energy metabolism [73]. Additionally, AMPK plays a large role in the signaling pathway regulating mitochondrial biogenesis. Exercise and cell culture studies suggest that AMPK works through PGC-1α to induce mitochondrial biogenesis [72]. Terada et al. [58] showed that 6 hours of low intensity swimming resulted in increased AMPK activation and PGC-1α mRNA expression. Electrical stimulation designed to mimic endurance exercise also activated AMPK and increased PGC-1α protein expression [74]. Similarly, this effect can also be observed *in vivo* following exposure of L6 myotubes to

the AMPK activating drug, 5-Aminoimidazole-4-car- boxamide-β-D-ribofuranoside (AICAR), as shown by elevated AMPK and CaMK phosphorylation [69]. In addition, it was shown that exposure of L6 myoblasts to AICAR, resulted in significant increases in ALAS, cytochrome c, PGC-1α, COXI and COXIV protein expression compared with control cells [69,70,71]. Interestingly, administering the nitric oxide synthase (NOS) inhibitor, L-NAME, results in attenuation of the AICAR-induced increases in mitochondrial biogenesis markers, signifying that there is also a role for nitric oxide in mitochondrial biogenesis [71].

Nitric Oxide

Nitric oxide (NO) is a simple diatomic molecule with complex pleiotropic implications in cell signaling [75]. NO has an unpaired electron which allows it to react rapidly with other molecules and at the same time, can diffuse easily through plasma membranes to reach target cells due its lipophillic nature [76]. NO is produced endogenously by nitric oxide synthase (NOS) which catalyzes the oxidation of L-arginine to L-citruline[76,77]. In skeletal muscle, NO has also been shown to modulate mitochondrial biogenesis in response to exercise and in calorie restriction. For example, Civitarese et al. [77] showed that calorie restriction of 25% or calorie restriction of 15% with increased daily exercise by 15% results in significant increases in mitochondrial content within skeletal muscle. It was also shown that caloric restriction led to significantly increased activity of NOS, indicating a role for NO in mitochondrial biogenesis [48,77,78]. Interestingly, eNOS knockout animals have been shown to demonstrate a reduced exercise capacity [79] and have a reduced mitochondrial content within skeletal muscle [77,80,81]. Cell culture studies in human, rat, and mouse myoblasts and myotubes have shown that intermittent administration of NO donors results in increased PGC-1α and NRF-1 mRNA

content and protein expression, as well as increased expression of other mitochondrial markers

such as cytochrome C, COXI, COXIV, and mtTFA [71,77,80]. Furthermore, it has been shown

that NO works through cGMP mediated pathway, as administration of the cGMP analog 8 Br-

cGMP results in similar increases in PGC-1α, NRF-1, COXIV and cytochrome c expression

[80]. In addition, administration of the cGMP inhibitor ODQ abolishes the effects of the NO

donor and 8- Br-cGMP on mitochondrial content in cultured myoblasts [80]. Mitochondrial

biogenesis within skeletal muscle is a complicated process that involves the coordinated

signaling of many different molecules. A simplified summary of the process is shown figure 3.
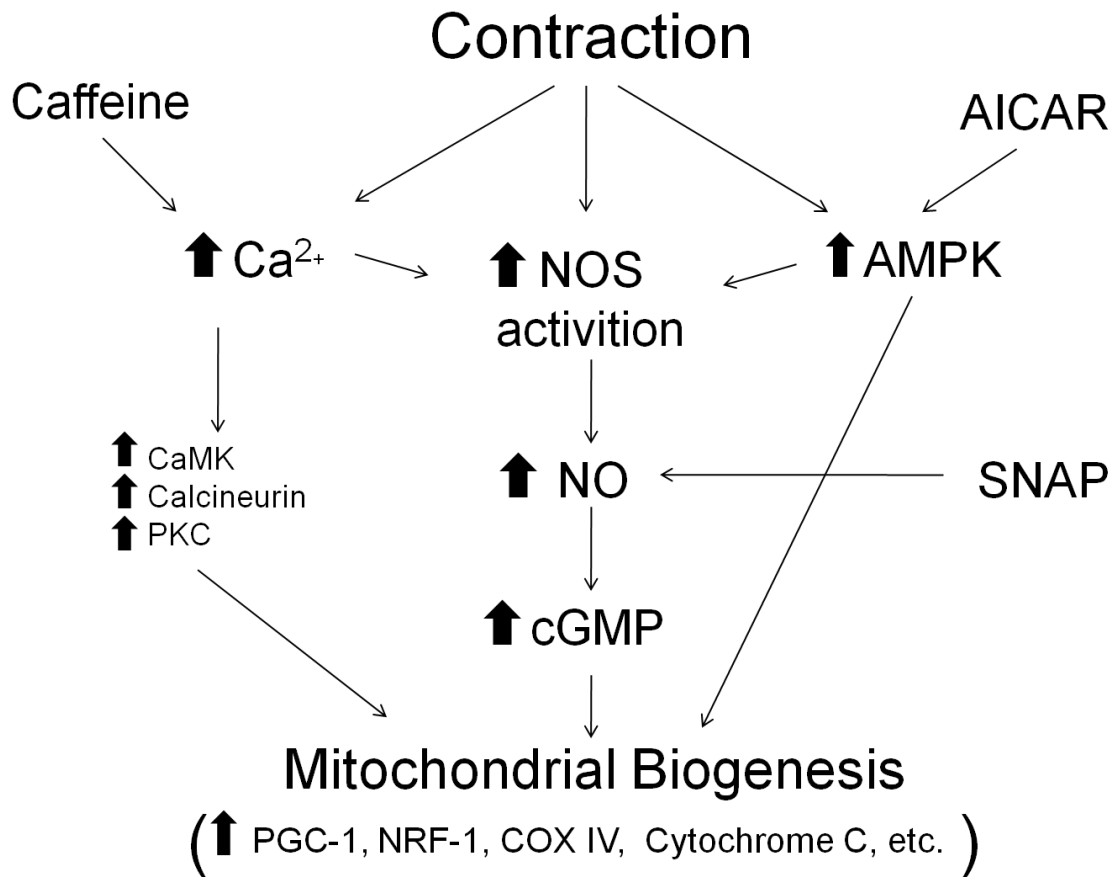
Figure 3. Schematic of mitochondrial biogenesis pathways in skeletal muscle. Skeletal muscle contraction leads to increased in intracellular calcium, increase NOS activity and increased AMPK activity which can lead to increase PGC-1α content. Increased PGC-1α leads to activation of nuclear and mitochondrial genes that ultimately results in mitochondrial biogenesis.

Activity and Apoptosis

Acute Exercise

Exercise has well been established as a physiological stimulus that alters a number of biochemical and cellular processes. Recently it has been demonstrated that exercise can alter apoptotic signaling in a number of tissues including skeletal muscle. For example, acute strenuous exercise has been shown to alter several apoptotic proteins and signaling events. In healthy mice and rats, single bouts of spontaneous wheel running has shown altered Bcl-2 family protein expression, increases in myonuclei apoptosis, and increases in caspase activity [44,82,83]. Similarly, a single bout of treadmill running in healthy rats has been shown to increase reactive oxygen species (ROS) generation which may mediate the corresponding increases seen in muscle caspase activity and appearance of apoptotic nuclei [84,85]. In addition, it has been demonstrated that acute exercise in healthy humans alters skeletal muscle Bcl-2 family gene expression [87,88] as well as caspase-3 mRNA levels [87], protein content, and proteolytic activity [86,87,88].

Regular Exercise

Regular treadmill exercise or endurance training has also been shown to positively influence apoptosis-specific protein and mRNA expression and decreases DNA fragmentation in skeletal muscle [46,89,90]. For example, Siu and colleagues [89] demonstrated that in response to endurance training, mRNA levels and protein content of XIAP increased significantly compared to controls. Additionally, it was shown that ARC and FLIP protein content are significantly elevated in skeletal muscle of exercised animals compared to controls [89]. It has also been demonstrated that endurance trained rats express increased levels of the

anti-apoptotic proteins Bcl-2 [45,90], HSP70 [90,91], and MnSOD [90] while expressing

reduced levels of the pro-apoptotic proteins Bax [45] and cleaved caspase-3 [45]. In addition,

Bax mRNA content and Apaf-1 protein content in the skeletal muscle of the trained animals is

significantly lower than that of controls [90]. Collectively, these data suggest that endurance

training has a significant anti-apoptotic effect on skeletal muscle.

Inactivity and Aging

Interestingly, inactivity and aging, have been shown to be associated with increased

apoptotic signaling [45,92]. For example, Leeuwenburgh et al. [93] showed that there was a

significant increase in the level of apoptosis, by TdT- mediated dUTP nick end labeling

(TUNEL) and DNA fragmentation, in aged rats compared to young controls. They also found

that in response to hindlimb unweighting, there were significant increases in apoptosis in both

young in old rats [93]. It has also been demonstrated that the anti-apoptotic proteins XIAP and

ARC both significantly decrease in aging [94] while endoG localization to the nucleus has also

been shown to be increased in hindlimb unweighting [93] as well as aging [93,95].

Links Between Mitochondrial Biogenesis and Apoptosis

Introduction

Given the important role of mitochondria in cell function as well as apoptosis, it is not

unexpected to hypothesize that changes in mitochondrial content could have a strong affect on

apoptotic signaling. Currently literature suggests exercise training can increase mitochondrial

biogenesis [52,59,61] and decreased apoptosis [45,89,90] whereas inactivity leads to decreased

mitochondrial content [61,62,63] and is associated with increased apoptotic signaling [93,95].

Similarly, caloric restriction can lead to increased mitochondrial biogenesis [48,77,78] and decreased apoptosis [94] whereas aging is associated with decreased mitochondrial content [96] and increased levels of apoptosis [45,92]. Collectively this suggests that there may be a relationship between mitochondrial content and muscle apoptosis. Currently, this relationship has not been systematically been studied, however, potential mechanisms for this relationship can be deduced from the literature. A review of the current literature suggests that altering mitochondrial content may function to mediate apoptotic signaling by two main pathways; by reducing ROS production and by maintaining calcium homeostasis.

Reactive Oxygen Species

Increasing ROS content within muscle cells can have a significant effect on the apoptotic signaling within skeletal muscle. Mitochondrial ROS have been proposed to initiate early apoptotic signaling events. For example, ROS can directly interact with and facilitate mtPTP opening [97] resulting in release of cytochrome c or AIF from the mitochondria [1,14]. Reactive oxygen species also indirectly influence the apoptotic pathway by activating mitogen-activated protein kinases and various redox-sensitive transcription factors involved in the expression of both anti-apoptotic and pro-apoptotic genes [98].

It has been demonstrated that endurance training results in reduced ROS production and increased NF-κB activation [99]. Decreased ROS production could help reduce activation of the intrinsic pathway of apoptosis and increased NF-κB activation could result in increased transcriptional activation of anti-apoptotic proteins thereby further protecting the muscle from apoptotic cell death. The decreases in ROS production could possibly be mediated by increased mitochondrial content within the cell that results from endurance training. Caloric restriction

models have been shown to increase mitochondrial content [48,77,78] as well as reduce ROS production and enhance metabolic efficiency in liver [78] and in skeletal muscle [100]. Collectively, these data suggest that increased mitochondrial content may enhance cellular energy efficiency resulting in the production of less ROS.

Calcium Homeostasis

Mitochondrial biogenesis may also mediate apoptotic signaling by mediating calcium homeostasis within the cell. Mitochondria act as a 'calcium sink' to buffer intracellular concentrations by up-taking free calcium from the cytosol. Too much calcium intake can lead to mitochondrial swelling and pore formation or bursting of the mitochondria leading to apoptosis through the release of pro-apoptotic proteins such as cytochrome c and AIF. Bianchi et al. [101] determined that increasing mitochondrial content resulted in a significant decrease in calcium induced apoptosis. Therefore, increased mitochondrial content may allow for increased calcium buffering capacity resulting in a decreased susceptibility to calcium induced apoptosis.

Project Objectives

Examination of the literature shows that the muscle degeneration observed in some of these states can be attenuated with exercise training and caloric restriction [95,103]. A common adaptation seen in skeletal muscle following both exercise training and caloric restriction is the upregulation of mitochondrial content [48,51,58,77]. Though the relationship between endurance training and apoptosis has been examined in rodent models, the specific effect of mitochondrial biogenesis on apoptotic signaling has not been examined. Thus, the

purpose of this project is to specifically examine the effect of mitochondrial biogenesis using a cell culture approach. To accomplish this, L6 myocytes will be cultured under a number of conditions to induce mitochondrial biogenesis. Cells will then be administered several apoptotic stimuli and be examined for changes in apoptotic markers and signaling events.

Hypotheses

- Examination of the literature on regulation of mitochondrial biogenesis revealed that both AMPK and NO play significant roles in this process. Given that studies have reported that both the AMPK activator, AICAR, and the NO donor, SNAP, induce mitochondrial biogenesis in L6 myoblasts and myotubes, it is expected that mitochondrial biogenesis will be successfully induced with both agents. We expect to see increases in all four of the mitochondrial biogenesis/content markers PGC-1α, cytochrome c, AIF, and MnSOD.

- Upon review of the literature, it can be shown a relationship between mitochondrial content and skeletal muscle apoptosis may exist. Therefore, it is expected that increasing mitochondrial content by intermittent SNAP and AICAR treatment will result in decreased apoptotic cell death in response to apoptotic stimuli as determined AnnexinV-FITC/PI staining, cell cycle analysis, and cell size/counting analysis.

- Similarly, it is hypothesized that caspase-3 and caspase-9 activity will be reduced in the biogenesis groups following exposure to apoptotic stimuli

- Increasing mitochondrial content has been associated with decreased ROS production. Therefore it is hypothesized that following mitochondrial biogenesis mitochondrial

specific ROS generation as well as total cellular ROS generation will be reduced in comparison to control cells.

- Staurosporine induces apoptosis through it actions as a global cellular kinase inhibitor and the exact pathway by which it induces apoptosis is currently unknown. However, one study using a PGC-1α overexpression model to increase mitochondrial content found that the PGC-1α overexpressing cells were not protected against staurosporine induced cell death but were protected against C_2-ceramide induced cell death. Therefore, it is hypothesized, that increasing mitochondrial content in L6 myoblast by SNAP and AICAR treatment will protect against hydrogen peroxide- and C_2-ceramide-induced cell death but not staurosporine-induced death.

Methods

Cell Culture and Treatments

L6 rat myoblast were cultured in low glucose Delbecco's Modified Eagles Medium (DMEM - ThermoScientific) containing 10% fetal bovine serum (ThermoScientific) and 1% penicillin and streptomycin (ThermoScientific) on 60 mm polystyrine cell culture dishes (BD biosciences). Cells were kept in continuous passage by trypsinization (0.25% trypsin - ThermoScientfic) of subconfluent cultures. Cells used were between passages 3 to 10. For experiments, cells were seeded at a density of 150,000 cells per dish. Cells were allowed to adhere to the dish surface and grow for 24 hours at which point cells were washed twice with calcium and magnesium free phosphate buffered saline (PBS) and media replaced. To induce mitochondrial biogenesis, two different treatments were then administered as described by Wadley et al. [71]: No treatment (control), 100μM of the NO donor, S-nitroso-N-acetylpenicillamine (SNAP - Invitrogen) diluted in ethanol, and 2mM of the AMPK activator, 5-Aminoimidazole-4-carboxamide-ribonucleoside (AICAR -Toronto Research Chemicals) diluted in culture medium. Treatments were applied for 5 hours per day for 5 days in the same growth media. After each 5 hour treatment media was aspirated and cells were washed twice with PBS to remove any remaining drugs and fresh pre-warmed media was then added to each dish. Each experiment was repeated on 5 separate occasions in duplicate.

Induction of Apoptosis

To evaluate the affect of increased mitochondrial content on apoptotic susceptibility, apoptosis was induced by treating cells with three different apoptotic stimuli: Staurosporine and C_2-ceramide as described by Bianchi et al. [101], and Hydrogen Peroxide (H_2O_2) as

described by Alway et al. [103]. To determine appropriate concentration of apoptotic stimuli, a concentration curve was produced. Staurosporine was administered at a concentration 2µM for 4 hours while H_2O_2 and C_2-ceramide were administered at a concentration of 2mM and 50µM for 24 hours. Apoptosis was induced approximately 18 hours following the last 5 hour treatment. After administration of the apoptosis inducing agents the media was collected and the remaining adherent cells were harvested by using 0.25% trypsin.

Cell Counting/Size Analysis

Cell counts were performed on each treatment group as well as apoptosis induced groups to determine the concentration of cells. Briefly, isolated cells were resuspended in 4mL of growth media. Using the Z2 Coulter Counter (Beckman-Coulter), cells between 10-18µm were counted (as viable cells) and cell concentration was determined. A second count was then made of cells between 5-10um as representation of apoptotic cells. The quantity of apoptotic cells was then expressed as a percentage of the total cell population and normalized to control cells.

Flow cytometry

Mitochondrial ROS Production

MitoSOX Red is a highly selective mitochondrial superoxide indicator and was used to evaluate mitochondrial ROS production in the treatment groups. Briefly, 500,000 cells of each treatment group were aliquoted into separate tubes. Cells were then centrifuged at 1000g for 4 minutes. Supernatant was removed and cell pellet was resuspended in 500µL of PBS. MitoSOX Red was then added to each sample to a final concentration of 5µM and incubated in

the dark at 37°C for 30 minutes. Cells were then centrifuged and washed twice with PBS.

MitoSOX fluorescence was then measured by flow cytometry (FACSCalibur, BD Bioscience)

and analyzed using Cell Quest Pro software (BD Bioscience).

Total Cell ROS Production

Total cell ROS generation was determined using 2',7'dichlorohydrofluorescein-diacetate

(DCFH-DA). DCFH-DA is hydrolyzed by intracellular esterases to yield the nonfluorescent

DCFH. DCFH is then oxidized by exposure to cellular ROS to the highly fluorescent

compound DCF. Briefly, 500000 cells were resuspended in PBS and DCFH-DA (Invitrogen)

was added to a final concentration of 10μM and then incubated in the dark for 15 minutes at

37°C. Samples were then washed twice with PBS and DCF fluorescence was measured by flow

cytometry.

Annexin V-FITC and PI Apoptosis Assay

Annexin V-FITC is a fluorescent protein that binds to phosphotidylserines that appear on the

membrane at the initiation of apoptosis while propidium iodide (PI) is a fluorescent dye that

binds to nuclear DNA following rupture of the cell membrane. Isolated cells were washed in

PBS and then resuspended and in Annexin Binding Buffer (Hepes buffer: 10 mM

HEPES/NaOH, pH 7.4, 150 mM NaCl, 1.8 mM CaCl$_2$). Cells were then counted and

concentration was adjusted so that cells were suspended at 1×10^6 cells/mL. 1×10^5 cells were

then aloquoted into to tubes. 5μL of Annexin V-FITC and 10μL of 50μg/mL PI were then

added to the tubes. Cells were incubated in the dark for 15 minutes at room temperature after

which 400μL of Annexin Binding Buffer was added. AnnexinV-FITC and PI fluorescence was

then measured by flow cytometry.

Cell Cycle Assay

Cells were harvested by 0.25% trypsinization as described above. Cells were then centrifuged at 1000g for 5 minutes and then washed twice with PBS and resuspended in PBS. Cells were then counted and 2 x 10^6 cells were aliquoted into new tubes. Cells were then centrifuged at 1000g for 5 minutes and supernatant was removed (approximately 100µL of supernatant was left in the bottom of the tube with cells to allow for easier suspension in 70% ethanol). Cells were gently vortexed and while vortexing, 1mL of ice cold 70% ethanol was slowly added to the tube to fix the cells. Cells were fixed for at least 24 hours. Following fixation, cells were centrifuged at 1000g for 5 minutes. The supernatant was removed and cells were washed twice with PBS. 100µL of RNAase was then added along with 400µL of propidium iodide solution (50µg/mL in PBS containing 0.1% TritonX). Cells were then incubated in the dark at room temperature for 30 minutes. PI fluorescence was then measured using flow cytometry.

Analysis of Total Cellular Protein Content

Isolated cells were centrifuged at 1000g for 4 minutes. Cells were then washed with ice cold PBS and centrifuged one more time at 1000g for 4 minutes. Supernatant was removed and cells were lysed using a buffer containing 20mM HEPES, 10mM NaCl, 1.5mM MgCl, 1 mM DTT, 20% glycerol and 0.1% Triton X100; pH 7.4 with protease inhibitors (Complete Cocktail; Roche Diagnostics) and homogenized by hand using a plastic homogenizer. Homogenates were sonicated for 20 seconds. Total protein was determined by the BCA protein assay method.

Western Blot Analysis

Aliquots of homogenate were loaded in duplicate on 10-15% SDS-PAGE gels such that

each sample contained an equal amount of protein (concentration curves were detected to

determine appropriate amount of protein to load for detections). Gels were transferred onto

PVDF membrane (Bio-Rad Laboratories) and blocked with 5% milk in Tris-buffered Saline

with 0.1% Tween (TBS-T) for 1 hour at room temperature or overnight at 4˚C. The blots were

probed with primary antibodies at room temperature for 1 hour or overnight at 4°C. To verify

increased mitochondrial content, the following primary antibodies were used: mouse

monoclonal anti-cytochrome c, rabbit polyclonal anti-PGC-1, mouse monoclonal anti-AIF

(Santa Cruz Biotechnology)rabbit polyclonal anti-Manganese Superoxide Dismutase

(MnSOD)(Stressgen Bioreagents). To evaluate apoptotic characteristics following treatment of

apoptotic stimuli the following primary antibodies were used: rabbit polyclonal anti-ARC,

mouse monoclonal anti-Heat Shock Protein 70 (Hsp70) (Stressgen Bioreagents); rabbit

polyclonal anti-Second Mitochondrial Activator of Caspases (Smac) (Assay Designs), rabbit

polyclonal anti-Bax (Santa Cruz), mouse monoclonal anti-Bcl-2 (Santa Cruz), mouse

monoclonal anti-XIAP (Stressgen), rabbit polyclonal anti-catalase (Chemicon), and rabbit

polyclonal anti-Copper Zinc Superoxide Dismutase (CuZnSOD)(Stressgen). Following

incubation of primary antibody, membranes were washed with TBS-T and incubated with the

corresponding horseradish peroxidase (HRP)-conjugated secondary antibody for 1-2 hours at

room temperature. Membranes were then washed with TBS-T and visualized using Amersham

Enhanced Chemiluminescence Western Blotting detection reagents (GE Healthcare) and the

ChemiGenius 2 Bio-Imaging System (Syngene). Protein levels are expressed as mean optical

density with arbitrary units and the molecular weights of the proteins was determined using

Precision Plus Protein WesternC Standards in conjunction with Precision Protein Streptactin HRP Conjugate (BioRad Laboratories). For verification of equal protein loading, membranes were evaluated with Ponceau S staining (Sigma-Aldrich).

Fluorometric Caspase Activity Assay:

To determine the enzyme activity for caspase-3, caspase-8, and caspase-9, a spectrofluorometric assay of cell homogenate was performed. Isolated cells were homogenized in ice cold muscle lysis buffer without protease inhibitor cocktail. The homogenate was then centrifuged at 1000 g for 4 minutes at 4^oC and the remaining pellet was discarded. To determine caspase-3 and -9 activities, supernatants were then incubated in duplicate on a 96 well plate for two hours using the following fluorescent substrates Ac-DEVD-AMC (Alexis Biochemicals) for caspase-3 and Ac-LEHD-AMC (Alexis Biochemicals) for caspase-9. These substrates are weakly fluorescent but fluorescence increases dramatically following proteolytic cleavage by their corresponding active caspase. Fluorescence was measured using a SPECTRAmax Gemini XS microplate spectrofluorometer (Molecular Devices) with an excitation wavelength of 360nm and an emission wavelength of 440nm. Human recombinant active caspase-3 and -9 (Alexis Biochemicals) was loaded as a positive control for caspase activity. Inhibitors for caspase-3(Ac-DEVD-CHO, Alexis Biochemicals), and caspase-9 (Ac-LEHD-AMC, Alexis Biochemicals) were also run to verify substrate specificity. Caspase activity was then normalized to total protein content and expressed as mean fluorescence intensity in AU per mg protein.

Statistical Analysis

Comparisons were made only between control cells and SNAP-treated cells or controls cells and AICAR-treated cells. No comparisons were made between SNAP-treated cells and AICAR-treated cells. Additionally no comparisons were made between the apoptotic-inducing agents staurosporine, H_2O_2, or C_2-ceramide. Values are expressed as means $\pm$ SE relative to control cells. Statistically significant differences were determined using paired Student's t-tests. Differences were considered significant when $p < 0.05$.

Results

Mitochondrial Biogenesis

For induction of mitochondrial biogenesis, L6 myoblasts were left untreated or treated for 5 hours/day over 5 days with either 2mM AICAR or 100μM SNAP. Pilot studies performed using these dosages on L6 myoblast resulted in increased mitochondrial biogenesis (see appendix). In the present study, the levels of several mitochondrial content/biogenesis markers were increased following acute treatment with AICAR and SNAP. AICAR increased the protein content of peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α) by 36%, cytochrome c by 71%, apoptosis inducing factor (AIF) by 19%, and manganese superoxide dismutase (MnSOD) by 26% (Fig. 4). Similarly, SNAP increased the protein content of PGC-1α by 34%, cytochrome c by 20%, AIF by 19%, and MnSOD by 22% (Fig. 4). In addition, to verify that SNAP and AICAR successfully induced mitochondrial biogenesis, cells were stained with the mitochondrial specific dye Mitotracker Green *(see appendix).*
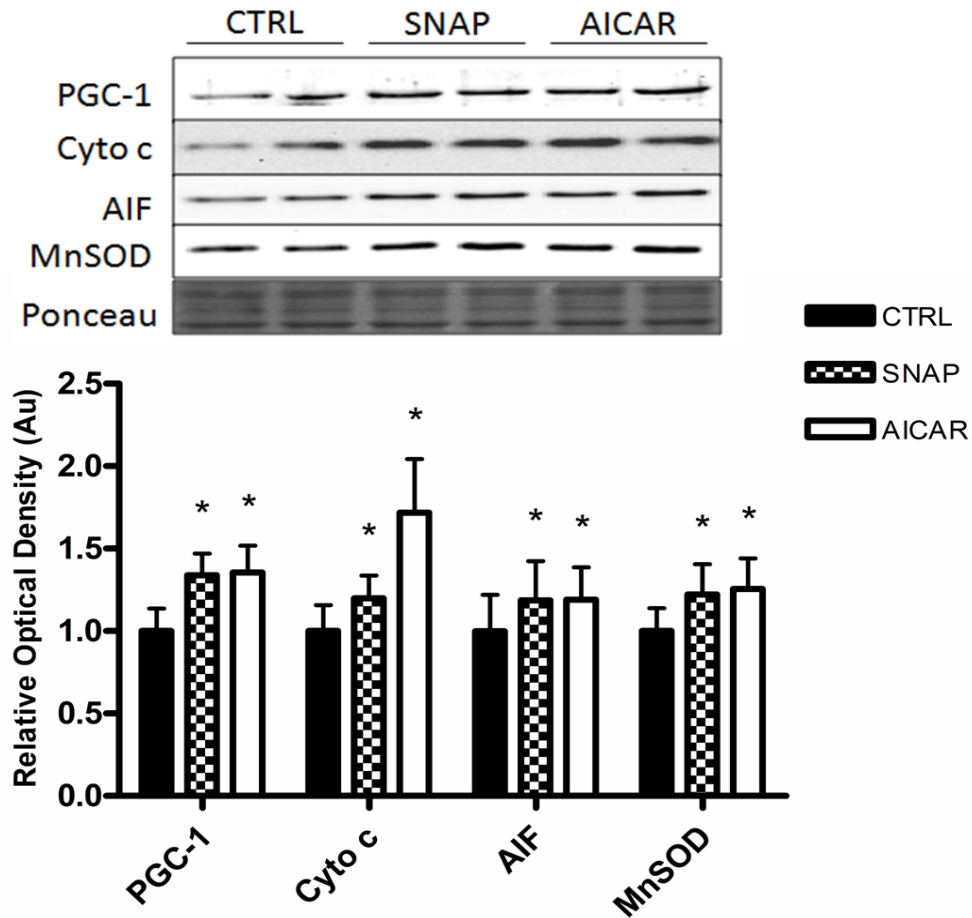
Figure 4. Mitochondrial Biogenesis Markers. Representative western blot and corresponding analysis of the mitochondrial biogenesis proteins/markers PGC-1, cytochrome c, AIF, and MnSOD following intermittent exposure (5 hrs/day for 5 days) to the nitric oxide donor SNAP (100μM) and the AMPK activator AICAR (2mM). * *p<0.05 relative to CTRL cells. (n=5)*

Mitochondrial ROS Production

Mitochondrial specific ROS production was evaluated using a highly selective

mitochondrial superoxide indicator dye, MitoSOX red. MitoSOX red fluorescence in all cells

was then measured by flow cytometry and values were expressed relative to control cells.

Under basal conditions (not treated with apoptotic inducing agent), MitoSOX fluorescence was

not significantly different between controls, SNAP, or AICAR treated cells (Fig. 5). In cells

induced to undergo apoptosis with staurosporine and C_2-ceramide, there was no significant

difference between control cells and SNAP- or AICAR-treated cells. Interestingly, when cells

were induced to undergo apoptosis using H_2O_2, MitoSOX fluorescence was significantly

increased ($p < 0.05$) by 23% in AICAR-treated cells compared to control cells, whereas

MitoSOX fluorescence in SNAP-treated cells was not significantly different from control cells

(Fig. 5).
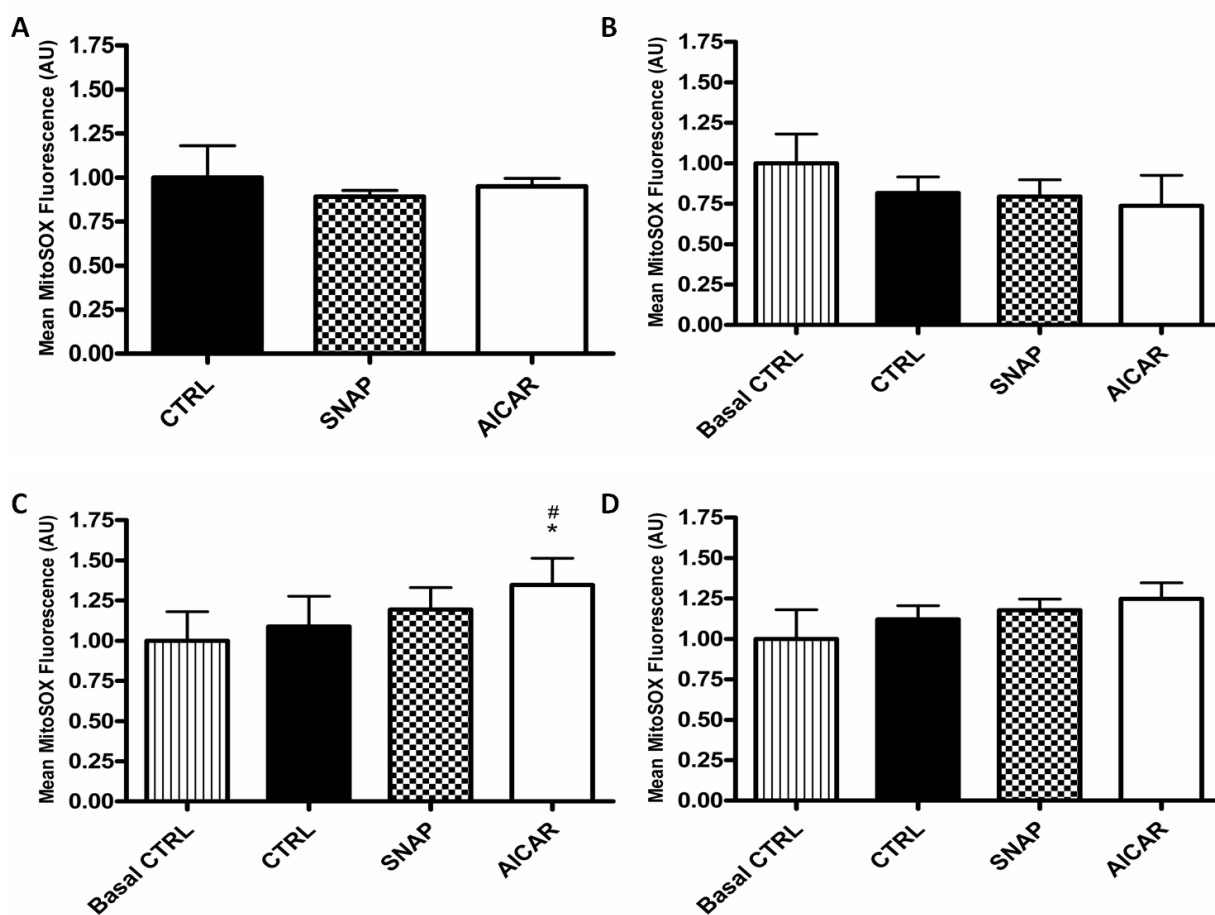
Figure 5. MitoSOX Fluorescence - Mitochondrial ROS Production Following Mitochondrial Biogenesis and Exposure to Apoptotic Stimuli. Mitochondrial ROS production as determined by MitoSOX fluorescence under basal conditions (A), and following 4 hours exposure to 2μM staurosporine (B), 24 hours exposure to 2mM H_2O_2 (C), or 24 hours exposure to 50μM C_2-ceramide. *p<0.05 relative to CTRL cells within a particular apoptosis-inducing condition. # p<0.05 relative to Basal CTRL. (n=3)*

Total ROS Production

To determine if induction of mitochondrial biogenesis alters total cellular ROS, the ROS-reactive dye, DCFH-DA was utilized and fluorescence was measured by flow cytometry. Interestingly, under basal conditions AICAR-treated cells showed a trend towards increased ROS production with a 23% increase in DCF fluorescence, however, this did not reach statistical significance ($p=0.06$) (Fig. 6). DCF fluorescence was significantly elevated ($p<0.05$) by 39% in AICAR-treated cells compared to controls treated with staurosporine (Fig. 6). Although not statistically significant ($p=0.075$), DCF fluorescence in SNAP-treated cells was increased by 24% compared to control cells following exposure to staurosporine (Fig 6.). DCF fluorescence was not found to be significantly different between control cells and SNAP- or AICAR-treated cells following exposure to H_2O_2 and C_2-ceramide. Interestingly, following exposure to staurosporine and H_2O_2, DCF fluorescence was significantly lower in control cells and SNAP-treated cells compared to basal controls.
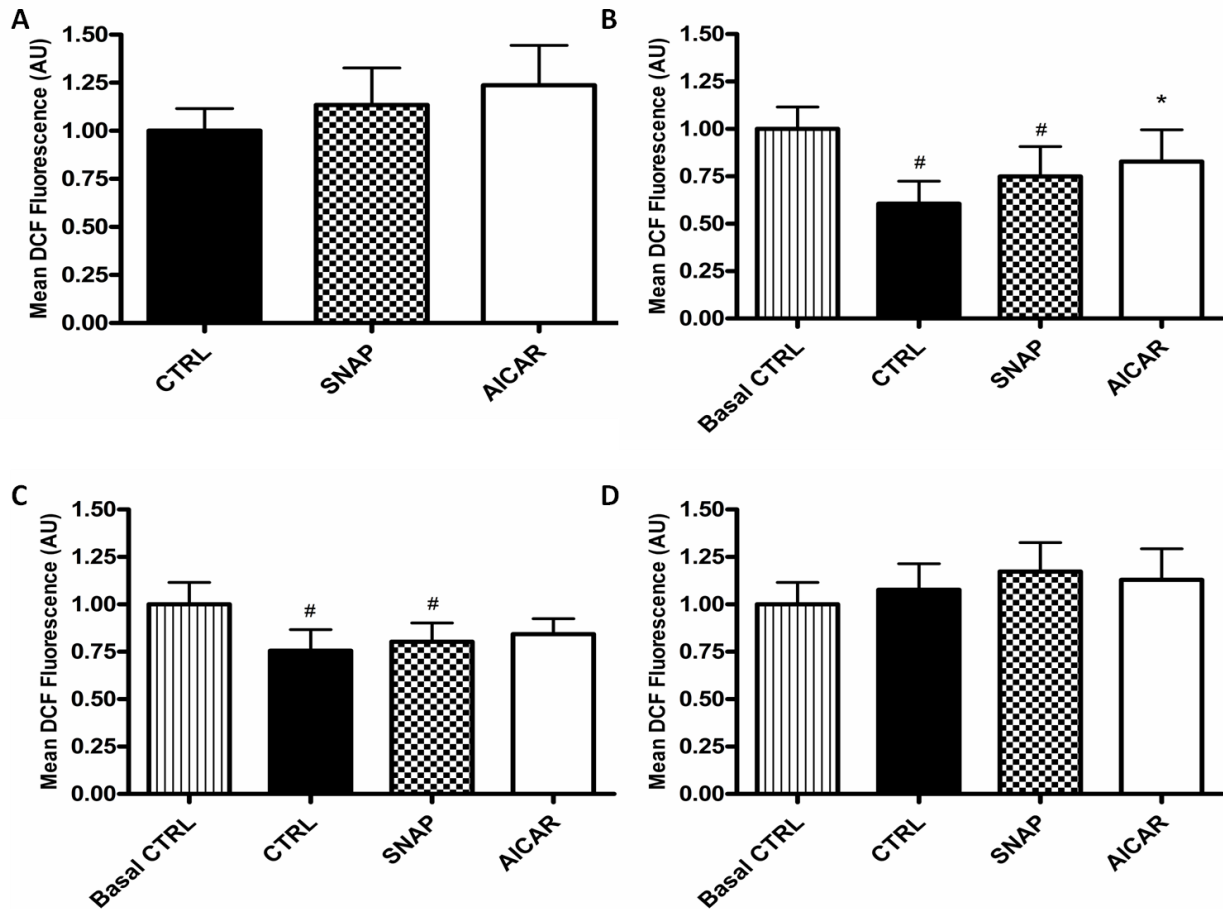
Figure 6. DCF Fluorescence - Total Cellular ROS Production Following Mitochondrial Biogenesis and Exposure to Apoptotic Stimuli. Total cell ROS generation as determined by mean DCF fluorescence under basal conditions (A) and following 4 hours exposure to 2μM staurosporine (B), 24 hours exposure to 2mM H_2O_2 (C), or 24 hours exposure to 50μM C_2-ceramide. *p<0.05 relative to CTRL within a particular apoptosis-inducing condition. # p<0.05 relative to Basal CTRL. (n=5)*

AnnexinV- FITC and Propidium Iodide Staining

To determine the proportion of apoptotic cells, the L6 myoblasts were stained with AnnexinV-FITC and propidium iodide. Cells expressing no fluorescence (AnnexinV-/PI-) were categorized as healthy. In the early stages of apoptosis, cells begin to express phosphatidylserine on their membrane. AnnexinV-FITC will then bind to the phosphatidylserines (AnnexinV+/PI-) and are categorized as early apoptotic. As apoptosis progresses, the cell membrane ruptures and allows propidium iodide to bind to the DNA, as

such, these cells will emit fluorescence. These cells which express both AnnexinV and propidium iodide fluorescence (AnnexinV+/PI+) were categorized as late apoptotic (*see appendix B*). Pilot studies using L6 myoblasts showed that treatment with increasing concentrations of staurosporine, H_2O_2, and C_2-ceramide resulted in increasing proportion of early and late apoptotic cells (*see appendix A*). These data were used to find the appropriate concentrations of staurosporine, H_2O_2, and C_2-ceramide to induce apoptosis. The percentage of healthy cells in AICAR-treated cells following staurosporine, H_2O_2, and C_2-ceramide exposure were 70%, 77%, and 78%, respectively, and was significantly higher ($p<0.05$) compared to the 54%, 71%, and 72% observed in control cells within a particular apoptosis-inducing condition, respectively (Fig. 7). Additionally, the percentage of early apoptotic cells in AICAR-treated cells following staurosporine, H_2O_2, and C_2-ceramide exposure were 18%, 10%, and 10%, respectively, and was significantly lower ($p<0.05$) compared to the 31%, 16%, and 13% observed in the control cells within a particular apoptosis-inducing condition, respectively (Fig. 7). Similarly, the percentage of late apoptotic cells (AnnexinV+/PI+) were 6% and 10% following staurosporine and C_2-ceramide exposure, respectively, which was significantly lower ($p<0.05$) than the percentage observed in the control cells (7%, and 11%, respectively) within a particular apoptosis-inducing condition, respectively (Fig. 7). SNAP-treated cells were also protected against staurosporine-, H_2O_2-, and C_2-ceramide-induced apoptosis with the percentage of early apoptotic cells (24%, 12%, and 12%, respectively) being significantly lower ($p<0.05$) than the percentage of early apoptotic cells (31%, 16%, and 13%, respectively) in control cells within a particular apoptosis-inducing condition (Fig. 7). SNAP-treated cells also had significantly lower ($p<0.05$) percentage of late apoptotic cells (6%) compared to the percentage of late apoptotic cells in control cells (7%) following exposure to staurosporine

(Fig.7). The percentage of late apoptotic cells following exposure to C_2-ceramide also tended to be lower (p=0.062) in SNAP-treated cells compared to control cells. Additionally, the percentage of healthy cells following staurosporine, H_2O_2, and C2-ceramide (64%, 75%, and 77%, respectively) was significantly higher (p<0.05) in SNAP-treated cells when compared to the percentage of healthy cells (54%, 71%, and 72%, respectively) in the control cells within a particular apoptosis-inducing condition (Fig. 7).
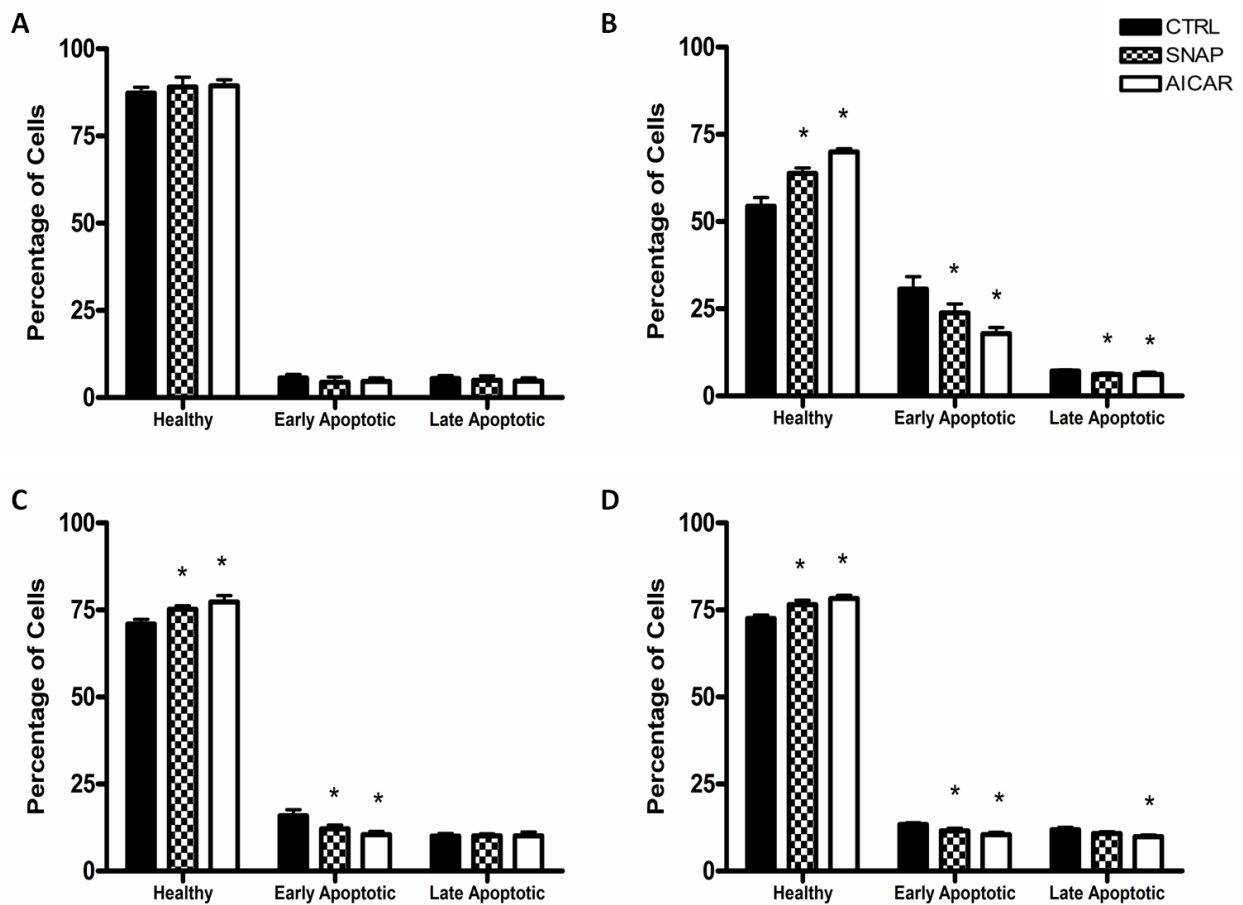
Figure 7. AnnexinV-FITC/PI Staining Following Apoptotic Stimuli. Proportion of healthy (AnnexinV-/PI-), early apoptotic (AnnexinV+/PI-), and late apoptotic (AnnexinV+/PI+). Cells were analyzed under basal conditions (A), and following exposure to 2μM staurosporine for 4 hours (B), 2mM H_2O_2 for 24 hours (C), or 50μM C_2-ceramide for 24 hours (D). *p<0.05 relative to CTRL within a particular apoptosis-inducing condition. (n=5)

Cell Counting/Size Analysis

Cell counts were performed to determine the concentration of suspended cells. Cells between 10-18μm were counted (as viable cells) and cell concentration was determined. A second count was then made of cells between 5-10μm likely representing apoptotic cells. SNAP treatment significantly lowered ($p<0.05$) the percentage of apoptotic cells compared to controls under basal conditions. Additionally, following exposure to staurosporine, H_2O_2, and C_2-ceramide, the percentage of apoptotic cells (50.7%, 24.3%, and 27.3%, respectively) was significantly lower ($p<0.05$) in SNAP-treated cells compared to the percentage of apoptotic cells (52.6%, 30.3%, and 32%, respectively) in control cells within a particular apoptosis-inducing condition (Fig. 8). Similarly, AICAR treatment resulted in significantly lower ($p<0.05$) percentage of apoptotic cells (25.5% and 27%) following H_2O_2 and C_2-ceramide exposure, respectively, compared to the percentage of apoptotic cells (30.3% and 32%, respectively) in control cells within a particular apoptosis-inducing condition (Fig. 8). There were no significant differences in the percent of apoptotic cells between control cells and AICAR-treated cells under basal conditions and following staurosporine exposure (Fig. 8).
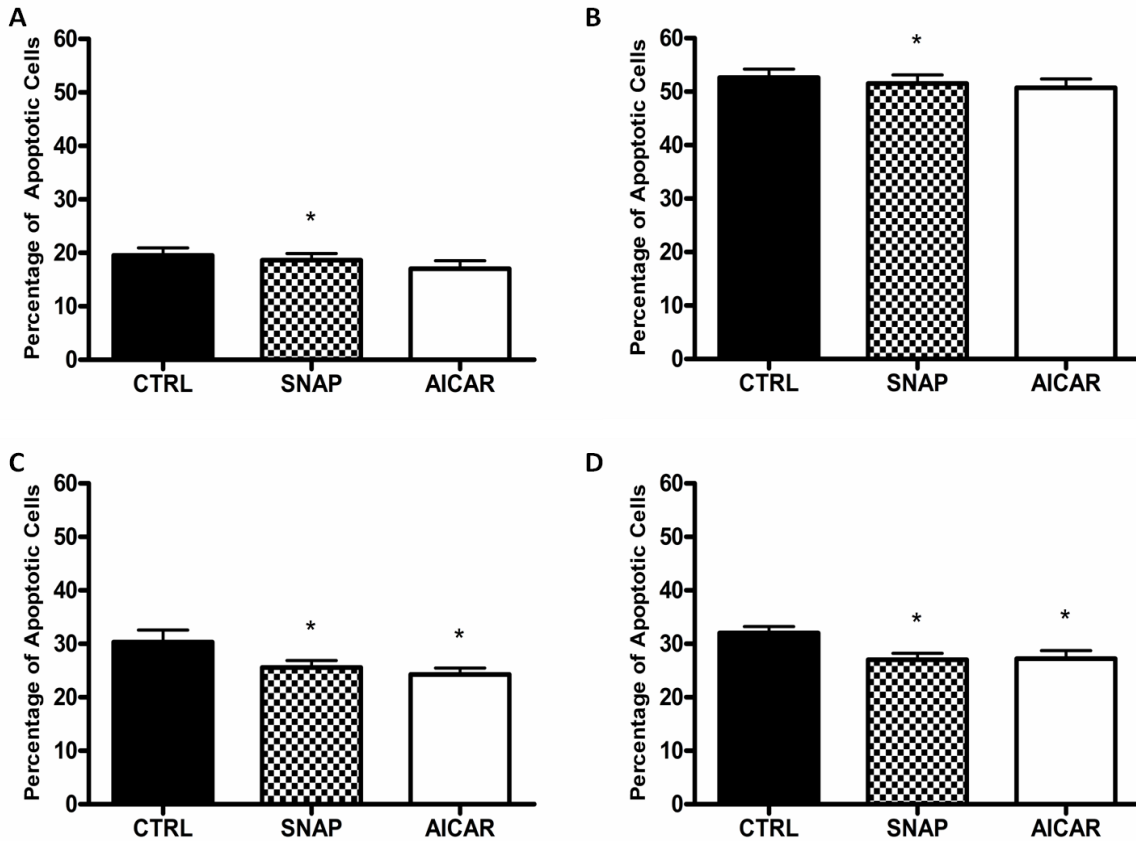
Figure 8. Cell Count / Size Analysis Following Exposure to Apoptotic Stimuli. Proportion of apoptotic cells under basal conditions (A) and following exposure to 2μM staurosporine for 4 hours (B), 2mM H_2O_2 for 24 hours (C), or 50μM C_2-ceramide for 24 hours (D). *$p<0.05$ relative to CTRL within a particular apoptosis-inducing condition. (n=5)

Caspase Activity Assay

To determine the enzyme activity for caspase-3 and caspase-9, a spectrofluorometric assay of cell lysates was performed. Pilot studies using L6 myoblasts showed that increasing concentrations of staurosporine or H_2O_2 leads to a step-wise increase in caspase-3 activity (*see appendix A*). In the current study, under basal conditions, there were no significant differences in caspase-3 or caspase-9 activity in SNAP-, or AICAR- treated cells in comparison to controls (Fig. 9,10). Exposure to staurosporine, H_2O_2, and C_2-ceramide resulted in a significant increase ($p<0.05$) in caspase-3 activity in all cells compared to basal controls (Fig. 9). Similarly, exposure to the apoptosis inducing agents elevated caspase-9 activity, however, this was only

significant ($p<0.05$) in all cells following staurosporine exposure and in control cells following C_2-ceramide exposure (Fig. 10). Additionally, following exposure to staurosporine and C_2-ceramide, caspase-3 activity was significantly reduced ($p<0.05$) by 16% and 19% in the SNAP-treated cells compared to their respective control cells (Fig. 9). Similarly, in cells treated with SNAP, caspase-9 activity was significantly reduced ($p<0.05$) by 18% and 17% compared to control cells following exposure to staurosporine and C_2-ceramide, respectively (Fig. 10). In cells treated with AICAR, caspase-3 activity was significantly decreased ($p<0.05$) by 27% and 17% compared to control cells following exposure to staurosporine and C_2-ceramide, respectively (Fig. 9). Additionally, caspase-9 activity was significantly reduced ($p<0.05$) by 20% in AICAR-treated cells when compared to controls treated with staurosporine (Fig. 10). No significant differences in caspase-3 or caspase-9 activity were found in SNAP- or AICAR-treated cells compared to control cells following exposure to H_2O_2 (Fig. 9,10).
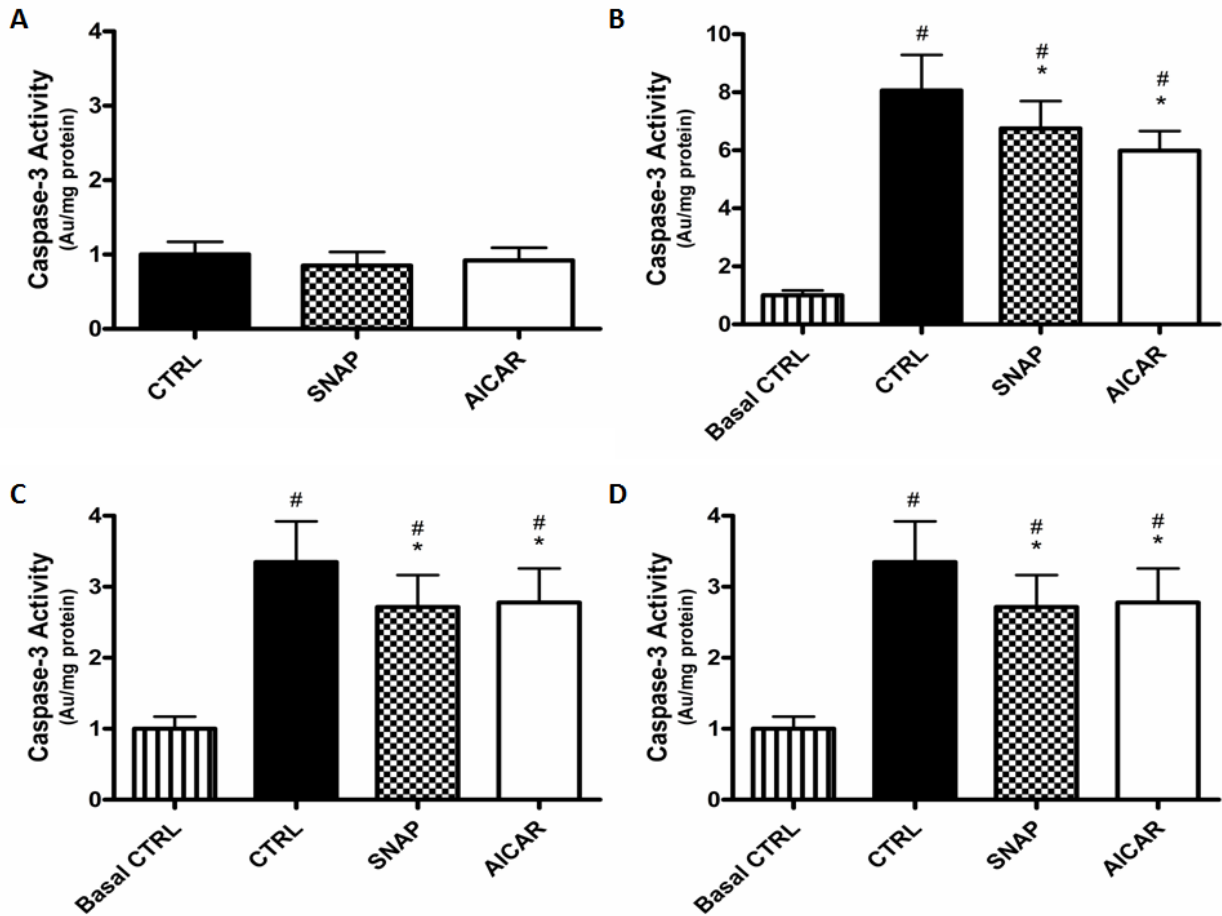
Figure 9. Caspase-3 Activity Following Mitochondrial Biogenesis and Exposure to Apoptotic Stimuli. Caspase-3 activity under basal conditions (A) and following exposure to 2μM staurosporine for 4 hours (B), 2mM H_2O_2 for 24hrs (C), or 50μM C_2-ceramide for 24 hours (D). *p<0.05 relative to CTRL within a particular apoptosis-inducing condition. # p<0.05 relative to Basal CTRL. (n=5)
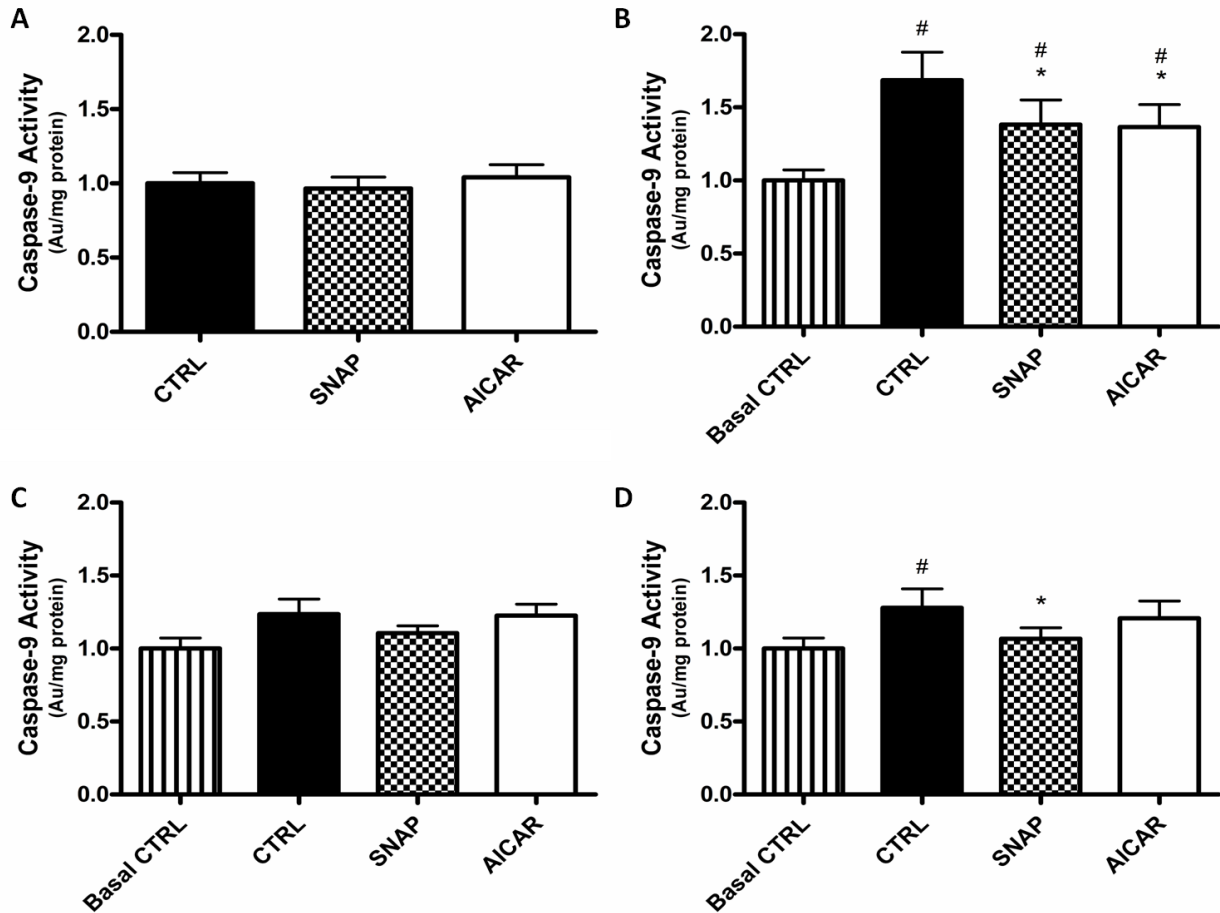
Figure 10. Caspase-9 Activity Following Mitochondrial Biogenesis and Exposure to Apoptotic Stimuli. Caspase-9 activity under basal conditions following mitochondrial biogenesis (A) and following exposure to 2μM staurosporine for 4 hours (B), 2mM H_2O_2 for 24hrs (C), or 50μM C_2-ceramide for 24 hours (D). *p<0.05 relative to CTRL within a particular apoptosis-inducing condition. # p<0.05 relative to Basal CTRL. (n=5)

Western Blot Analysis

Antioxidants

To examine the antioxidant capacity of the cells, the proteins Catalase, MnSOD, and CuZnSOD were examined. In healthy cells, Catalase expression was increased (p<0.05) by 27% and 23% in SNAP- and AICAR-treated cells, respectively, when compared to control cells (Fig.11). Additionally, Catalase expression was significantly elevated (p<0.05) by 17%

and 26% in SNAP- and AICAR-treated cells, respectively, compared to control cells when exposed to staurosporine (Fig.11). Catalase expression was not significantly different between groups following exposure to H_2O_2 or C_2-ceramide (Fig.11). As previously stated, MnSOD content under basal conditions was significantly elevated ($p<0.05$) by 22% and 26% in SNAP- and AICAR-treated cells, respectively, compared to untreated control cells. MnSOD expression was not significantly different between groups following exposure to staurosporine, H_2O_2 or C_2-ceramide (Fig.11). Similarly, CuZnSOD expression was not significantly different between groups under basal conditions or following staurosporine and C_2-ceramide (Fig.11). However, CuZnSOD expression was found to be significantly lower ($p<0.05$) by 17% in the SNAP-treated cells following H_2O_2 exposure, whereas there was no change in AICAR-treated cells (Fig.11).

Figure 11. Relative Antioxidant Protein Content Following Mitochondrial Biogenesis and Exposure to Apoptotic Stimuli. Western blot analysis and representative blots of the antioxidants Catalase, MnSOD, and CuZnSOD under basal conditions (A) and following exposure to 2μM staurosporine for 4 hours (B), 2mM H_2O_2 for 24 hours (C), or 50μM C_2-ceramide for 24 hours (D). *p<0.05 relative to CTRL within a particular apoptosis-inducing condition. # p<0.05 relative to Basal CTRL. (n=5)*

Anti-apoptotic Proteins

ARC expression in the AICAR-treated cells was not significantly different from control cells under basal conditions or following exposure to H_2O_2 and C_2-ceramide (Fig. 12). However, ARC expression was 13% (p=0.07) lower in the AICAR-treated cells in comparison to control cells following staurosporine treatment (Fig. 12). ARC expression in SNAP-treated

cells was not significantly different from controls under basal conditions (Fig. 12). Interestingly, ARC expression was significantly lower (p<0.05) by 14% in SNAP-treated cells compared to control cells following exposure to H_2O_2. Similarly, ARC expression was reduced 20% (p=0.07) and 23% (p=0.06) in SNAP-treated cells compared to control cells following staurosporine and C_2-ceramide exposure, respectively (Fig. 12).

Under basal conditions, XIAP expression was not different in SNAP or AICAR treated cells when compared to controls (Fig. 12). Exposure to the apoptotic stimulants resulted in degradation of XIAP, such that XIAP could not be sufficiently detected via western blot analysis.

Bcl-2 expression was not different in AICAR-treated cells compared to control cells under any condition (Fig. 12). Interestingly, under basal conditions, Bcl-2 expression was 24% higher (p=0.054) in SNAP-treated cells compared to controls (Fig. 12). Similarly, Bcl-2 expression was significantly higher (p<0.05) by 7% in SNAP-treated cells following exposure to H_2O_2 (Fig. 12). There was no difference in Bcl-2 expression between SNAP treated cells and controls following staurosporine and C_2-ceramide exposure (Fig. 12).
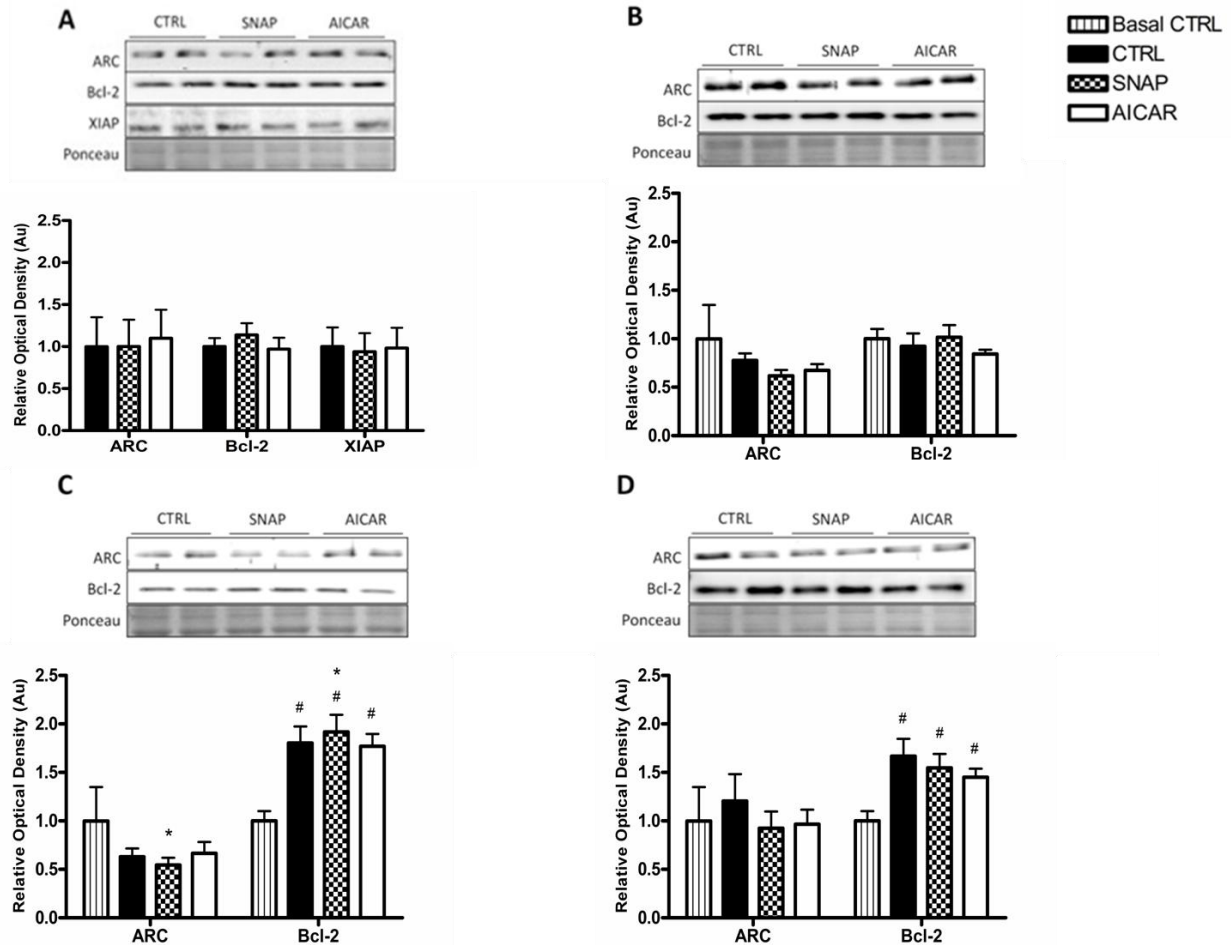
Figure 12. Relative Anti-Apoptoic Protein Content Following Mitochondrial Biogenesis and Apoptotic Stimuli. Western blot analysis of anti-apoptotic proteins ARC, Bcl-2, and XIAP under basal conditions (A) and following exposure to $2\mu M$ staurosporine for 4 hours (B), $2mM$ H_2O_2 for 24 hours (C), or $50\mu M$ C_2-ceramide for 24 hours (D). *p<0.05 relative to CTRL within a particular apoptosis-inducing condition. # p<0.05 relative to Basal CTRL. (n=5)
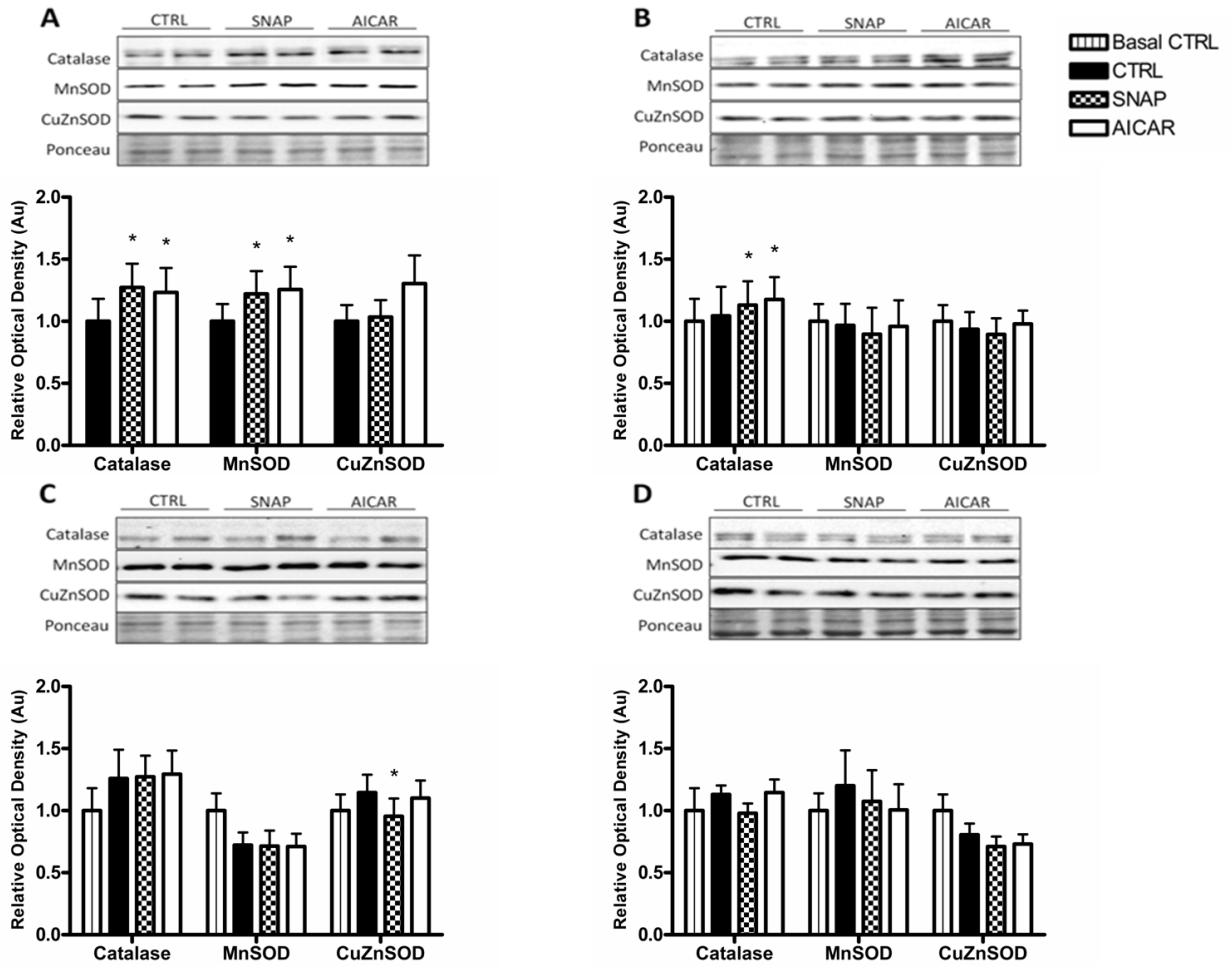
Pro-apoptotic Proteins

Bax content was not significantly different in SNAP- or AICAR-treated cells in comparison to control cells following mitochondrial biogenesis (basal) or exposure to all apoptotic stimuli (Fig. 13). Under basal conditions, the pro-apoptotic protein SMAC was not significantly different from controls in SNAP-treated cells but was 14% higher (p<0.05) in AICAR-treated cells. SMAC content was not significantly different from controls in SNAP- or

AICAR-treated cells following exposure to staurosporine and H_2O_2 (Fig. 13). However, SMAC

content was 19% (p=0.08) and 21% (p=0.1) lower in SNAP and AICAR groups, respectively,

compared to controls following exposure to C_2-ceramide (Fig. 13). As previously stated, under

basal conditions AIF content was significantly elevated (p<0.05) by 19% in both SNAP- and

AICAR-treated cells compared to untreated control cells. However, AIF content was not

significantly different from control cells in SNAP- or AICAR-treated cells following exposure

to staurosporine, H_2O_2, or C_2-ceramide (Fig. 13).
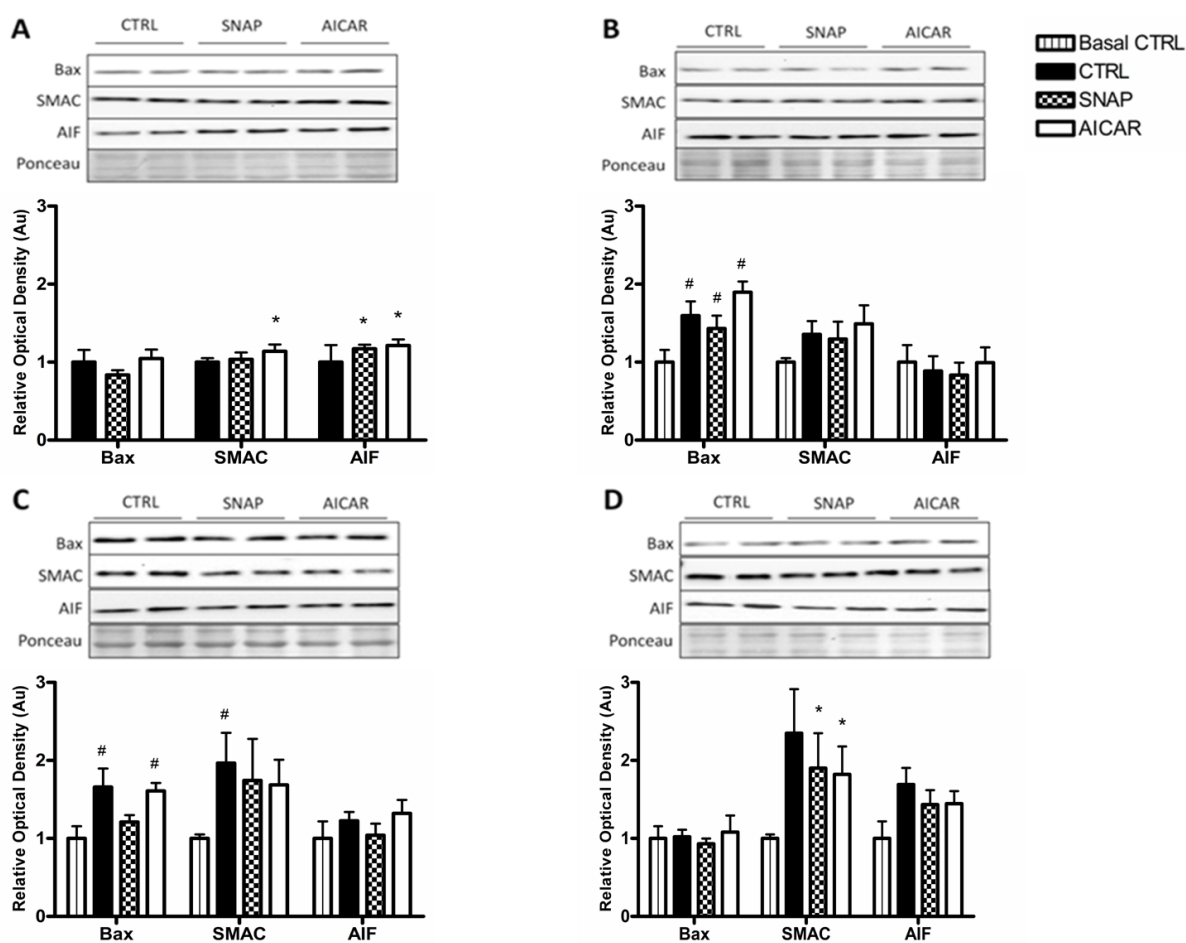
Figure 13. Relative Pro-Apoptotic Protein Content Following Mitochondrial Biogenesis and Exposure to Apoptotic Stimuli. Representative western blots and analysis of the pro-apoptotic proteins Bax, SMAC, and AIF under basal conditions (A) and following exposure to 2μM staurosporine for 4 hours (B), 2mM H_2O_2 for 24 hours (C), and 50μM C_2-ceramide for 24 hours (D). *p<0.05 relative to CTRL within a particular apoptosis-inducing condition. # p<0.05 relative to Basal CTRL. (n=5)

Cell Cycle Analysis of Apoptotic Cells

Cell cycle analysis was performed as another measure to examine the proportion of apoptotic cells. Under basal conditions, the proportion of apoptotic cells was not significantly different in SNAP or AICAR treated cells compared to controls (Fig. 14). Following exposure to staurosporine and H_2O_2, the percentage of apoptotic cells was not significantly different in SNAP- or AICAR-treated cells compared to control cells. The proportion of apoptotic cells was significantly lower ($p<0.05$) in SNAP- and AICAR-treated cells compared to controls following exposure to C_2-ceramide (Fig. 14).
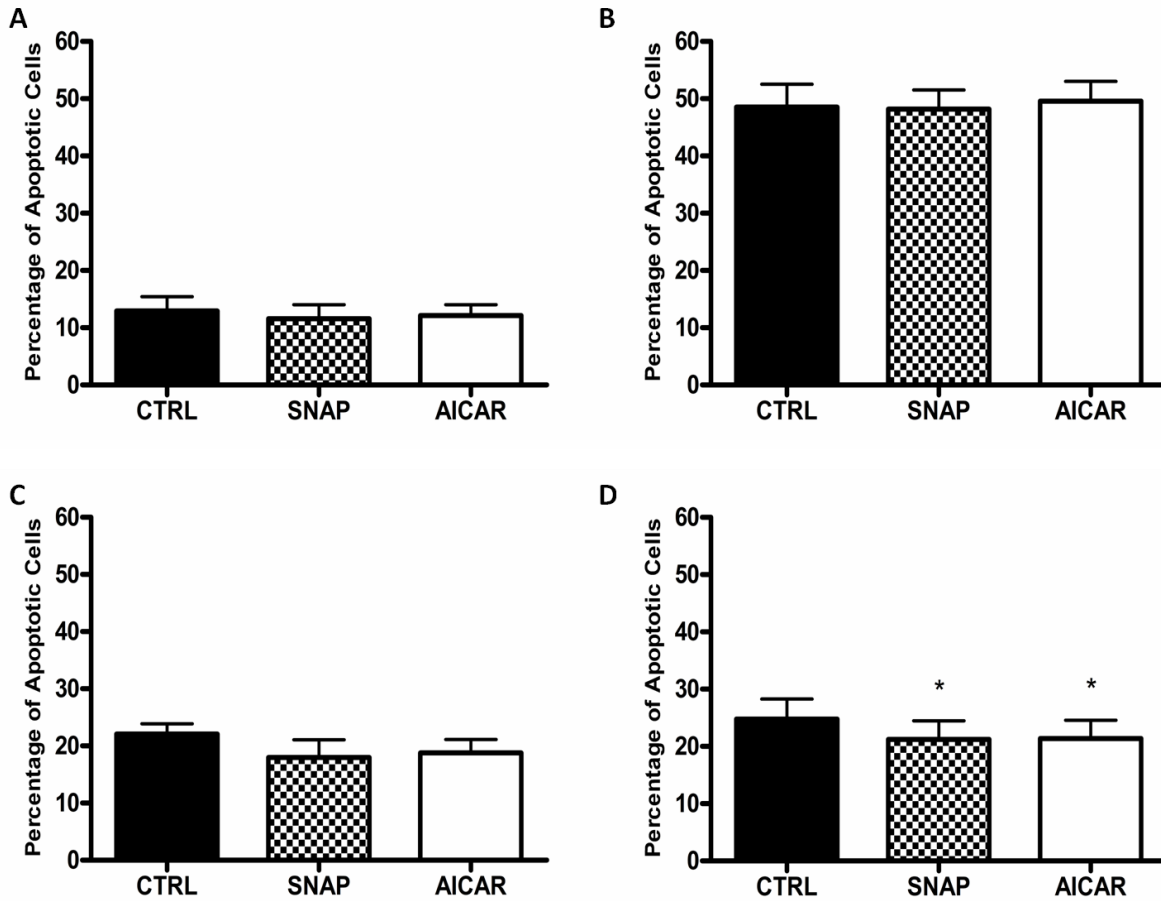
Figure 14. Cell Cycle Analysis of Apoptotic Cells Following Mitochondrial Biogenesis and Exposure to Apoptotic Stimuli. Cell cycle analysis of apoptotic cell population under basal conditions (A) and following exposure to 2μM staurosporine for 4 hours (B), 2mM H_2O_2 for 24 hours (C), or 50μM C_2-ceramide for 24 hours (D). *$p<0.05$ relative to CTRL cells within a particular apoptosis-inducing condition. (n=5)

Discussion

Apoptosis plays a large role in many degenerative skeletal muscle states, including motor nerve denervation, atrophy due to immobilization, and Duchenne muscular dystrophy [1,38]. There is also evidence indicating that dysregulation of apoptotic signaling plays a key role in the pathophysiology of skeletal muscle cell loss with age [33]. Examination of the literature shows that the muscle degeneration observed in some of these states can be attenuated with exercise training and caloric restriction [94,102]. A common adaptation seen in skeletal muscle following both exercise training and caloric restriction is the upregulation of mitochondrial content[49,52,59,78]. Therefore, the purpose of the current study was to examine whether increasing mitochondrial content can provide protection against apoptosis. To isolate this potential mechanism we utilized a cell culture approach in rat L6 myoblasts.

Mitochondrial Biogenesis

In the present study, the NO donor SNAP and the AMPK activator AICAR were used to induce mitochondrial biogenesis. In addition, this study used intermittent exposure to SNAP and AICAR (5 hours/day for 5 days) which we feel is more physiological than others who have used stable overexpression of PGC-1α [55] which results in 2.5-fold increase in mitochondrial content. Exercise and caloric restriction studies have demonstrated NO's role in mitochondrial biogenesis through increased activation of eNOS [48,77,78]. Confirming NO's role in mitochondrial biogenesis is the finding that eNOS knockout animals have reduced mitochondrial content within skeletal muscle [77,80,81]. Furthermore, in cell culture models, it has been demonstrated that NO induces mitochondrial biogenesis through a cGMP mediated pathway that results in elevation of PGC-1α [80]. In the present study, we found that

intermittent exposure of L6 myoblasts to SNAP significantly increased the protein content of

PGC-1 by 1.34 fold, cytochrome c by 1.2 fold, AIF by 1.19 fold, and MnSOD by 1.22 fold,

indicating mitochondrial biogenesis and content was significantly elevated. These findings are

in agreement with other cell culture studies which have used SNAP to induce mitochondrial

biogenesis. For example, McConnel et al. [71] showed that treatment of L6 myotubes with

SNAP significantly increased the protein expression of PGC-1α and the mitochondrial markers

COXI and COXIV by up to 2-fold. Furthermore, cell culture studies in human, rat, and mouse

myoblasts and myotubes have shown that intermittent administration of NO donors (SNAP or

DETA-NO) results in increased PGC-1α and NRF-1 mRNA content and protein expression, as

well as increased expression of other mitochondrial markers such as cytochrome c, COXI,

COXIV, and mtTFA [71,77,80].

AICAR mediates mitochondrial biogenesis through activation of AMPK. Exercise and

cell culture studies suggest that AMPK works by activation and elevation of PGC-1α to induce

mitochondrial biogenesis [72]. In addition, AMPK activation has been shown to result in the

deacetylation of PGC-1α resulting in its activation [104]. The effect of AICAR administration

on AMPK signaling and mitochondrial biogenesis has been well established. For example,

Ojuka et al. [69] demonstrate that exposing L6 myotubes to AICAR results in significantly

elevated AMPK phosphorylation. In addition, this group and others have shown that exposure

of L6 myoblasts to AICAR results in significant increases in the mitochondrial markers ALAS,

cytochrome c, PGC-1α, COXI and COXIV protein expression relative to untreated control

cells [69,70,71]. The current study is in agreement with these findings as we found that acute

treatment with AICAR significantly increased the content of PGC-1, cytochrome c, AIF, and

MnSOD by 1.36, 1.71, 1.19 and 1.26 fold, respectively. Collectively, these results demonstrate

that intermittent exposure to SNAP and AICAR successfully increased mitochondrial biogenesis and content in the L6 myoblasts used in the present study.

Basal Antioxidants, ROS, and Apoptotic Signaling Following Mitochondrial Biogenesis

Although excessive apoptosis has been shown to play a role in a variety of disease states [5,106,6] and muscle atrophy [38,39], regular exercise training as been associated with decreased apoptosis [46,47,90,91] and improved basal levels of a number of apoptosis proteins [46,47,90,91]. As mentioned previously, exercise training has been established to induce mitochondrial biogenesis and it has been suggested that this increase in mitochondrial content could have an anti-apoptotic effect on skeletal muscle; however, this has not been systematically tested. In this study we show that under basal conditions, there is no difference between cells with higher mitochondrial content compared to control cells in the percentage of apoptotic cells measured. Consistent with this data, basal caspase-3 and -9 activities were not significantly different between biogenesis and control cells. However, caspase-3 activity tended to be reduced in SNAP-treated, although this did not reach statistical significance (p=0.09).

We found similar results when examining the apoptotic signaling proteins. Basal levels of the anti-apoptotic proteins ARC, Bcl-2, and XIAP in the biogenesis treated cells were not significantly different from controls cells. Upon examination of the pro-apoptotic proteins, it was found that Bax protein content was not significantly different in biogenesis-treated cells compared to controls. However, AIF content was found to be significantly higher in both SNAP- and AICAR-treated groups while Smac protein content was significantly elevated in the AICAR-treated group but remained unchanged in SNAP-treated group. Although these

results may seem to indicate increased apoptotic signaling in these cells, it is likely that this is not the case. It is important to note that several apoptotic factors have dual roles providing both vital cellular functions and apoptotic signaling. For example, AIF resides in the mitochondria and plays a very important role in the electron transport chain [105,106]. It does not affect apoptotic signaling until receiving appropriate stimuli and is released from the mitochondria [105,106]. Since the cells are in a basal, healthy condition, it is likely that the increase in these proteins reflects the increase in mitochondrial content seen in both the SNAP- and AICAR–treated groups. Therefore, these results indicated that induction of mitochondrial biogenesis by SNAP and AICAR does not alter basal apoptosis levels or the expression of key apoptotic proteins and enzymes.

In contrast, the current study found that SNAP and AICAR treatment resulted in some modifications of the antioxidants under basal conditions. For example, catalase and MnSOD were significantly increased following 5 days of treatment with AICAR and SNAP. However, we did not find any differences in CuZnSOD between groups. In transgenic mice, overexpression of PGC1-α has been shown to increase catalase activity and SOD2 content [107]. Similarly, Irrcher et al. [108] demonstrated that catalase mRNA levels were significantly increased after a single exposure to 1mM AICAR in C2C12 myotubes. Furthermore, in cultured endothelial cells Kukidome et al. [109] revealed that MnSOD mRNA was significantly elevated following exposure to AICAR. *In vivo* and *in vitro* models using thyroid hormone to induce mitochondrial biogenesis have also shown increase in MnSOD content [110,111]. Endurance exercise training has also shown increased MnSOD expression with no change in catalase or CuZnSOD expression in skeletal muscle [112,113,114,115]. Since MnSOD is located only within the mitochondria, it is likely that the increases seen in MnSOD

content are due to a higher mitochondrial volume. Holloszy et al. [113] demonstrated that despite the increase in MnSOD, the ratio of SOD to mitochondria citrate cycle and respiratory chain enzymes were decreased, indicating that increased scavenging of superoxides is unlikely.

Despite the increased MnSOD and catalase levels observed in the present study, total ROS generation (DCF fluorescence) was not decreased in mitochondrial biogenesis cells compared to control cells. In fact, AICAR-treated cells showed a trend towards higher DCF fluorescence compared to controls. Mitochondrial specific ROS production (MitoSOX fluorescence) was not significantly different between mitochondrial biogenesis cells compared to control cells. It is possible that the dosage of SNAP and AICAR used resulted in increased ROS production. For example, Xu et al. [116] have shown that treatment of rat cardiomyocytes with 100μM SNAP results in significantly increased ROS production as determined by DCF fluorescence. Similarly, AICAR administration has also been shown to increase ROS production in neuroblastoma cells [117] and in pancreatic cells [118], AICAR administration has also been shown to increase ROS production. In contrast to these findings, Irrcher et al. [108] reported that 1mM AICAR treatment in C2C12 muscle cells for 24 hours resulted in significantly decreased ROS production compared to control cells. In addition, conditions that lead to increased mitochondrial content *in vivo* (ie. calorie restriction) are associated with reduced ROS production [48,77,78]. It has been suggested that enhanced metabolic efficiency may be responsible for the reduced basal ROS production following mitochondrial biogenesis [79,101]. Interestingly, reduced ROS would be expected to decrease apoptosis in cells. In the present study, we did not find any differences in basal apoptosis or apoptotic signaling which is consistent with the ROS data.

Induction of Apoptosis

In the present study staurosporine, H_2O_2, and C_2-ceramide, all activators of the intrinsic apoptotic pathway, were used to induce apoptosis in L6 myoblasts. Staurosporine is a global intracellular kinase inhibitor which has been shown to significantly increase both intracellular ROS and calcium concentrations [121,122]. Treatment of cells in culture with staurosporine invokes all the responses that are characteristic of apoptotic signaling (ie. cell shrinkage, caspase activation, DNA fragmentation) [120] and it has been well established as an apoptosis inducer in many cell types[120,121,122,123]. In rat cardiomyoctes, staurosporine has been shown to induce apoptosis in a dose dependent manner [120]. Yue et al. demonstrated that incubating cardiomyocytes with 0µM, 0.25µM, 0.5µM, and 1µM for 24hours, resulted in ~8%, 15%, 30%, and 55% of the cells undergoing apoptosis [120]. They also found that caspase-3 activity increased between 5-8 fold with increasing concentrations of staurosporine [120]. A similar dose-response relationship between staurosporine concentration and cell viability has also been shown in neuronal cell culture [124]. These results are consistent with the present findings where we show a dose-response relationship with staurosporine in both the proportion of apoptotic cells and activity of caspase-3. The increased caspase-9 activity following staurosporine administration also indicates that staurosporine is likely acting not only through a caspase-dependant pathway but also through mitochondrial signaling which is consistent with the literature [122,125]. For example, it has been shown that staurosporine administration results in loss of mitochondrial membrane potential[121], release of pro-apoptotic proteins cytochrome c and Smac [123,124], and activation of caspase-3 and -9 [120,122,125]. It has also been demonstrated that caspase-dependent apoptosis can be inhibited using a variety of caspase inhibitors [122,125]. However, in these experiments, despite use of caspase inhibitors,

apoptosis was still shown to occur a short time later, indicating that a caspase-independent mechanism may also be involved [122,125]. Zhang et al. [122] show that staurosporine induces nuclear translocation of AIF to the nucleus despite caspase inhibition, suggesting AIF may be mediating the caspase-independent apoptosis. Although we did not measure AIF release or nuclear translocation, it is possible that staurosporine is acting through both a caspase-dependent and –independent mechanism.

H_2O_2 is commonly used to invoke oxidative stress and induce apoptosis in cell culture. Treatment of cells in culture with exogenous H_2O_2 results in cell death that exhibits all the classical signs of apoptosis such as cell shrinkage, caspase activation, and DNA fragmentation [103,126,127]. Exogenous H_2O_2 administration has been shown to induce apoptosis through the intrinsic apoptotic pathway in a variety of cell types [127,128,129,130]. It is thought that H_2O_2 initiates apoptosis by altering the mitochondrial permeability transition, possibly through Bax activation or free radical generation, allowing for cytochrome c release [128]. Studies have also shown that exogenous H_2O_2 can act on other intracellular structures, such as the endoplasmic reticulum, and invoke a sharp rise in intracellular calcium concentrations, which subsequently activates apoptotic pathways [131,132]. The results from this study show that exposing cells to increasing concentrations of H_2O_2 results in a step-wise increase in the number of apoptotic cells and caspase-3 activation. We also show that exposure to H_2O_2 results in elevation of the pro-apoptotic proteins Bax and Smac, as well as the anti-apoptotic protein Bcl-2. A study by Jiang et al. [133] showed that release of Smac from the mitochondria plays an important role in H_2O_2-induced cell death in C2C12 myoblasts. Exposure of cells to ROS has been shown to increase both pro-and anti-apoptotic proteins in various cell types [134,135]. It has been suggested that this occurs because a number of transcription factors can

be modulated by H_2O_2. For example, nuclear translocation of p53 can be caused by H_2O_2 [136], and the transcription factors, NFκB and AP-1 can be activated by H_2O_2 [135,137]. Once activated, these transcription factors can mediate transcription of both pro-apoptotic and anti-apoptotic genes to either encourage apoptosis or attempt to rescue the cell from death [137,138,139].

Although H_2O_2 has been well established to induce apoptosis in a variety of cells, there is limited data examining the effect of H_2O_2 in skeletal muscle cells. Nevertheless, the results found in the current study correspond with the literature. For example, Stangel et al. [138] examined the potential of skeletal muscle myoblasts to undergo apoptosis when exposed to H_2O_2 and found increased number of TUNEL-positive nuclei and DNA fragmentation in a dose-dependent manner similar to the dose-response in apoptotic cells and caspase activity in the current study. More recently, studies by Caprossi et al. [127] and Siu et al. [103] have revealed the pathway by which H_2O_2 may induce apoptosis in skeletal muscle cells. They demonstrated that elevation of apoptotic DNA fragmentation was accompanied with mitochondrial cytochrome c, Smac, and AIF release, caspase-3 activation, and AIF nuclear translocation [103,127]. Collectively, these findings in C2C12 myotubes, along with findings in brain cells and cardiomyocytes, suggest that H_2O_2 induces apoptosis by both caspase-dependent and -independent mechanisms.

Ceramides have been shown to play a key role in apoptosis and have been implicated as an important second messenger that acts at the level of the mitochondria during TNF-α (extrinsic signaling) induced apoptosis. Treatments of cells and isolated mitochondria with the cell permeable ceramide analog, C_2-ceramide, have been shown to inhibit electron transfer activity and increase ROS production [139]. C_2-ceramide alters intracellular calcium

concentrations [140,141] and has been shown to activate the pro-apoptotic protein Bid [141] another protein that links the extrinsic and intrinsic pathways of apoptosis. C_2-ceramide has also been shown to induce mitochondrial swelling [142], alter mitochondrial membrane potential [140], induce mitochondrial release cytochrome c and AIF [140,142], and activate caspases [141]. The results from the current study are in agreement with the literature as we found that C_2-ceramide induces apoptosis in a dose-dependent manner. We also found that C_2-ceramide increase caspase-3 and caspase-9 activity relative to control cells. In addition, western blot analysis revealed an increase in the pro-apoptotic protein Bax following exposure C_2-ceramide.

Induced Apoptosis Following Mitochondrial Biogenesis

Although mitochondrial biogenesis did not affect basal apoptotic signaling, it was found that upregulating mitochondrial content significantly altered the apoptotic response following exposure to apoptotic stimuli. The most novel and exciting finding in the current study was that increased mitochondrial biogenesis in L6 myoblasts had a protective effect against staurosporine, H_2O_2, and C_2-ceramide induced apoptosis. This is the first study to examine the specific effect of acute induction of mitochondrial biogenesis (through two different chemicals) on both basal apoptotic signaling and stress induced apoptotic signaling using three different apoptosis-inducing agents in a cell culture model. In addition, as mentioned previously we feel this is a more physiological model of mitochondrial biogenesis given the acute exposure and modest increase in mitochondrial biogenesis and content.

The results of this study show that cells treated with SNAP or AICAR had a significantly higher healthy cell population and a significantly lower early and late apoptotic

cell population as determined by AnnexinV/PI staining following staurosporine exposure. Furthermore, caspase-3 and -9 activities were significantly reduced in SNAP- and AICAR-treated cells compared to controls. Similar results were shown in the current study when L6 myoblasts were exposed to C_2-ceramide. The percentage of healthy cells was significantly higher and the percentage of early and late apoptotic cells was significantly lower in the biogenesis cells compared to control cells. Additionally, the percentage of apoptotic cells as determined by cell counting and by the cell cycle assay was significantly lower in both SNAP- and AICAR-treated cells. Furthermore, caspase-3 activity was significantly lower in both SNAP- and AICAR-treated cells while caspase-9 activity was only significantly lower in SNAP-treated cells.

Although to a lesser extent, it was also shown that mitochondrial biogenesis was protective against H_2O_2 induced apoptosis. The results show that there is a significantly higher proportion of healthy cells as well as a significantly lower proportion of early apoptotic cells in the SNAP- and AICAR-treated cells compared to controls cells. In addition, the cell counting/size analysis and cell cycle results show that the proportion of apoptotic cells was significantly lower in the mitochondrial biogenesis groups in comparison to the controls. Interestingly, caspase-3 and -9 activities were not found to be significantly different between groups following H_2O_2 exposure. Bianchi et al. [55] reported similar findings using L6 myotubes. They found that L6 myotubes overexpressing PGC-1α had significantly increased mitochondrial content and were protected against calcium-mediated cell death induced by C_2-ceramide. Interestingly, they also showed that these cells were not protected against staurosporine induced apoptosis. However, Bianchi et al. used a dosage of 1μM staurosporine for 3 hours and their experiments were performed on differentiated L6 myotubes whereas the

current study used a dosage of 2μM staurosporine for 4 hours. In addition, Bianchi et al. used Hoechst staining to determine apoptotic cells, whereas the current study utilized AnnexinV-FITC/PI, cell count/size analysis, cell cycle analysis, and caspase activation were to determine apoptotic cells. The Hoechst staining technique utilized by Bianchi et al. relies on manual counting of apoptotic nuclei whereas the techniques used in the current study are much more sensitive. Furthermore, the dosage of staurosporine used by Bianchi et al. may not have been enough to reveal differences between groups. These differences in methodological approaches may contribute to the differences found in the current study regarding staurosporine-mediated cell death.

With the few exceptions noted before, few differences were observed in the apoptotic protein expression between biogenesis cells and control cells. A notable exception was the change in Smac, Bcl-2, and ARC protein expression. Smac was found to be significantly higher in control cells compared to the mitochondrial biogenesis cells following exposure C_2-ceramide suggesting it may play a role in the increase in apoptotic cells seen in this population. Bcl-2 protein was significantly higher in the SNAP-treated group following exposure to H_2O_2. Bcl-2 seemed to be slightly elevated in the SNAP-treated cells across all apoptosis-inducing groups. NO has been reported to be related to increases in Bcl-2 content in the literature [145,146] and could explain these findings. Interestingly, ARC protein expression was found to be significantly lower in the SNAP-treated group following exposure to H_2O_2. It also showed a trend (p=0.07) towards being decreased in both biogenesis groups following exposure to staurosporine. These interesting results could possibly be explained by how ARC is regulated. It is possible that ARC becomes upregulated in response to cellular stress to prevent further damage and apoptosis. This type of adaptation has been shown to occur in the anti-apoptotic

protein XIAP in response to cellular stress [145]. If ARC regulation behaves in a manner similar to XIAP, then in this situation, it is possible that the biogenesis groups are less stressed following staurosporine administration and therefore ARC does not increase to the same degree as control cells. Further studies will need to be conducted to confirm these hypotheses. Furthermore, many apoptotic inducing agents have been shown to induce differentiation, as well as apoptosis [146,147]. In neuronal cell lines, differentiation has been shown to be associated with protection against apoptosis [148,149]. Moreover, the differentiation state of cells can also affect expression of certain proteins. Unpublished data from our lab has shown that ARC protein content becomes upregulated during differentiation. In the current study, it is possible the control cells, being more sensitive to the apoptotic agents, were also more inclined to begin differentiation. An increase in the number of cells undergoing differentiation could explain the higher ARC protein content seen in the control cells.

Some interesting, yet confounding results were also found when western blot analysis was performed on antioxidant enzymes following exposure to apoptosis inducing agents. For example, CuZnSOD protein content was significantly lower in the SNAP-treated group following exposure to H_2O_2. In contrast, catalase protein content was elevated in SNAP- and AICAR-treated cells following staurosporine exposure. It is likely that the increased catalase level is an artifact of the higher basal catalase levels observed in the SNAP- and AICAR-treated cells. The findings in the current do not follow the trend shown in the literature. For example, Shull et al. [150] have demonstrated that MnSOD mRNA expression is increased 2 hours following oxidant exposure and reach a maximum expression at 24 hours. Furthermore, they showed that MnSOD protein content was elevated at 24 hours and 48 hours following oxidant exposure. Kahl et al. [151] also show significant increases in catalase and MnSOD

mRNA following exposure to H_2O_2. These findings suggest that antioxidant enzymes can be upregulated in response to cellular stress which is in contrast to the present findings. It is possible that the SNAP- and AICAR-treated cells were under less "stress" and therefore, there was no induction of MnSOD and CuZnSOD; however, this remains to be determined.

Some more confounding results were also found upon examining the intracellular ROS generation. Following staurosporine administration, DCF fluorescence was significantly higher in the AICAR-treated group and tended to be higher in the SNAP-treated group ($p=0.075$). These results could be explained by the binding characteristic of the fluorescent dye. Following oxidation by cellular reactive oxygen species, the resulting fluorescent product, DCF, is retained inside living cells due to electric charges and emits light with intensity proportional to the level of cellular oxidative stress [152]. Using hydroethidine (HE), a dye similar to DCF-HA (it must be oxidized to fluoresce), Torres-Roca et al. [153] demonstrated that a population of late stage apoptotic cells was HE negative, indicating they were likely unable to oxidize HE due to lack of metabolism. Similarly, Wang et al. [154] reported that apoptotic cells had decreased production of reactive oxygen intermediates. Collectively, these findings indicate that DCF may not be able to be retained in apoptotic cells due to cellular membrane rupture and loss of electric charge. Our findings show that the percentage of healthy cells was significantly lower and the percentage of apoptotic cells was significantly higher in the control cells. The presence of more apoptotic cells within this population would result in a lower mean DCF fluorescence and could account for the current findings.

Potential Mechanisms for Protection

Some potential mechanisms for protection against apoptosis can be proposed from the results of the current study and by examination of the literature. One potential mechanism often cited in the literature is that increased antioxidant capacity can reduce damage from ROS and protect cells from apoptosis. A study by Pong et al. [155] found that the two synthetic superoxide dismutase/catalase mimetics (EUK-134 and EUK-189) were able to attenuate the apoptotic response in neuronal cells following staurosporine exposure. Similarly, overexpression models of catalase [156,157], MnSOD, and CuZnSOD [158] have shown to decrease cell susceptibility to apoptotic cell death. These studies indicate that the upregulation of MnSOD and catalase observed in the current study in response to SNAP and AICAR treatment could partially explain the decreased susceptibility of myoblasts to apoptosis.

Another potential mechanism of protection could be through increased intracellular calcium buffering capacity. Mitochondria can act as a 'calcium sink' to buffer intracellular concentrations by up-taking free calcium from the cytosol [55]. High intracellular calcium concentrations can lead to increased activation of pro-apoptotic factors such as calpains and Bax [22,23,24]. Interestingly, a direct link has been shown to exist between the endoplasmic reticulum (ER) and the mitochondria, where calcium release from the ER under stress conditions is immediately taken up by the mitochondria [159]. Although buffering calcium from the mitochondria is important, too much calcium intake into the mitochondria can cause swelling, pore formation, or bursting of the mitochondria leading to apoptosis through the release of pro-apoptotic proteins such as cytochrome c, Smac and AIF [160]. Staurosporine and C_2-ceramide have been shown to cause large increases in intracellular calcium concentrations [123,140,141], therefore it is possible that the greatest protection against

apoptosis observed in this study to these agents may have been due to increased calcium buffering as a result of increased mitochondrial volume. In a study performed by Bianchi et al. [55], it was reported following that in cells with increased mitochondrial content, individual mitochondria showed significantly lower mitochondrial calcium concentrations than the cells with lower mitochondrial content. The increased mitochondrial volume in these cells allows intramitochondrial calcium to distribute in a larger volume, resulting in reduced overall mitochondrial calcium concentration. Collectively, these results suggest increasing mitochondrial content allows for the increased removal of calcium from the cytosol while at the same time minimizing mitochondrial swelling thereby decreasing apoptotic activation by calcium.

Summary and Conclusions

In summary, this is the first study to demonstrate that upregulation of mitochondrial content at physiological levels can protect L6 myoblast from apoptosis induced by multiple agents. The results of this study show that apoptotic signaling, measured by AnnexinV-FITC/PI staining, cell cycle assays, caspase-3 and -9 activity, cell size/counting analysis, and antioxidant protein content indicate that upregulating mitochondrial is protective against staurosporine-, H_2O_2-, and C_2-ceramide-induced apoptosis. Mitochondrial ROS production was not significantly altered between groups while total cellular ROS production seemed to be slightly increased in the SNAP- and AICAR-treated groups. These results are in disagreement with the hypothesis but could be explained by the higher retention and oxidation of DCF in living cells. Overall, it was determined that increasing mitochondrial content in L6 myoblasts provides protection against stress induced apoptosis, and it is likely that this protection is

mediated by a combination of increased antioxidant capacity and mitochondrial calcium buffering capacity.

Limitations

The current study was performed on L6 myoblasts. Myoblasts are undifferentiated muscle cells but are characteristically different from differentiated myotubes and terminally differentiated muscle *in vivo*. The goal of the experiment was to isolate the mitochondrial biogenesis effect to determine if increased mitochondrial content provided protection against apoptosis. As AICAR and SNAP have been used for numerous applications and have shown to induce several effects within cells, it is possible that other cellular responses (independent of mitochondrial biogenesis) could also be responsible for the protective effects observed. Nevertheless, we used two different agents to induce mitochondrial biogenesis and found similar results.

Additionally, this study utilized western blotting techniques to determine the mechanisms associated with the protecting the cells from apoptosis. However, the incubation times of the apoptosis inducing agents were either 4 hours (staurosporine) or 24 hours (H_2O_2 and C_2-ceramide) which may not have been long enough to observe changes in some proteins. Analysis of mRNA content could have been useful to obtain a more in depth understanding of the differences between control and biogenesis groups. Furthermore, western blots in the current study were performed on whole cell lysates. A better understanding of the molecular pathways regulating the apoptotic signaling could be gained by performing western blots on subcellular fractions.

For DCF and MitoSOX analysis, the initial flow cytometry set-up was performed on healthy/viable L6 myoblast populations. Upon completion of the experiments and upon analysis of the data, it was determined that the flow cytometer settings used did not allow for discrimination of the viable cell population from the apoptotic cell population using forward or side scatter properties. Furthermore, a dual staining procedure examining propidium iodide and DCF fluorescence simultaneously would allow for easy discrimination of healthy cells from apoptotic cells while at the same time allowing for the examination of differences in DCF fluorescence in these two populations. Adjustments of the instrument settings or use of the dual staining method may provide a more accurate representation of mitochondrial and total cellular ROS generation within the cells.

Future Directions

Future cell culture studies should examine the mechanism by which these cells are protected. Further insight into the antioxidants and calcium buffering is needed to determine how the protective effect observed in these cells is mediated. In addition, future studies should examine whether these protective effects are also seen in terminally differentiated myotubes. More specifically, it should be examined whether differentiated myotubes are more protected against H_2O_2 or C_2-ceramide induced cell death. As differentiated myotubes are multinucleated and contain a much more developed sarcoplasmic reticulum than myoblasts, it is possible that differences in apoptotic susceptibility to different inducing agents may become evident.

Furthermore, future studies should evaluate these findings at the whole tissue level. Many studies have been published examining endurance training and its effect on basal apoptotic signaling in both healthy and disease states. Further research needs to be completed

to determine the individual protective mechanisms by which exercise and mitochondrial

biogenesis are elucidating their effects.

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Appendices
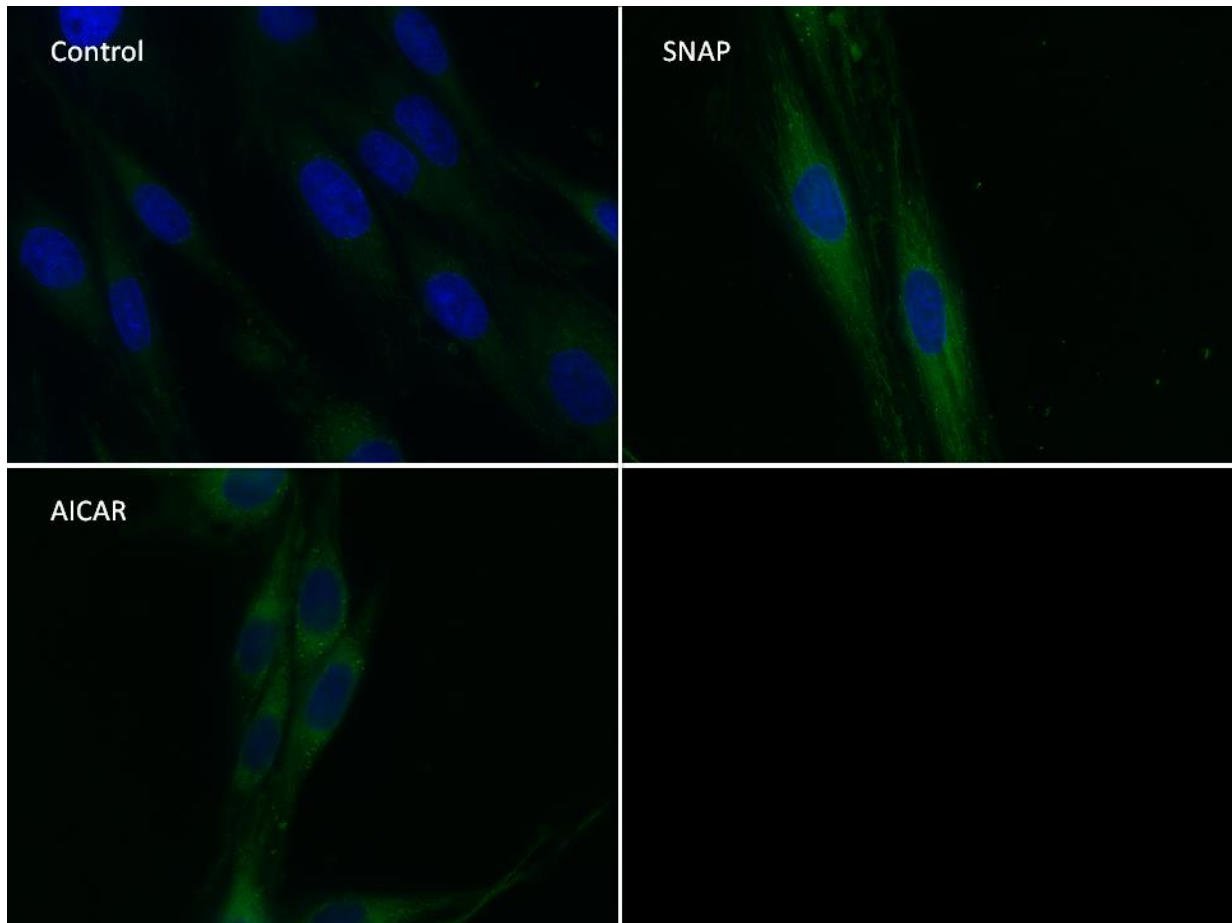
Appendix A – Supplementary Data

Figure A1. MitoTracker Green showing increased mitochondrial content in response to intermittent exposure to SNAP and AICAR. Briefly, cells seeded on glass coverslips and were exposed to nothing (Control), 100µM SNAP or 2mM AICAR for 5hrs/day for 5 days. Cells were then washed with PBS. Fresh PBS was added to the dish and cells were stained with MitoTracker Green (400nM) and DAPI for 30 minutes in the dark at 37°C. Cells were then washed four times with PBS to remove excess dye and were imaged at 63X using a fluorescent microscope.
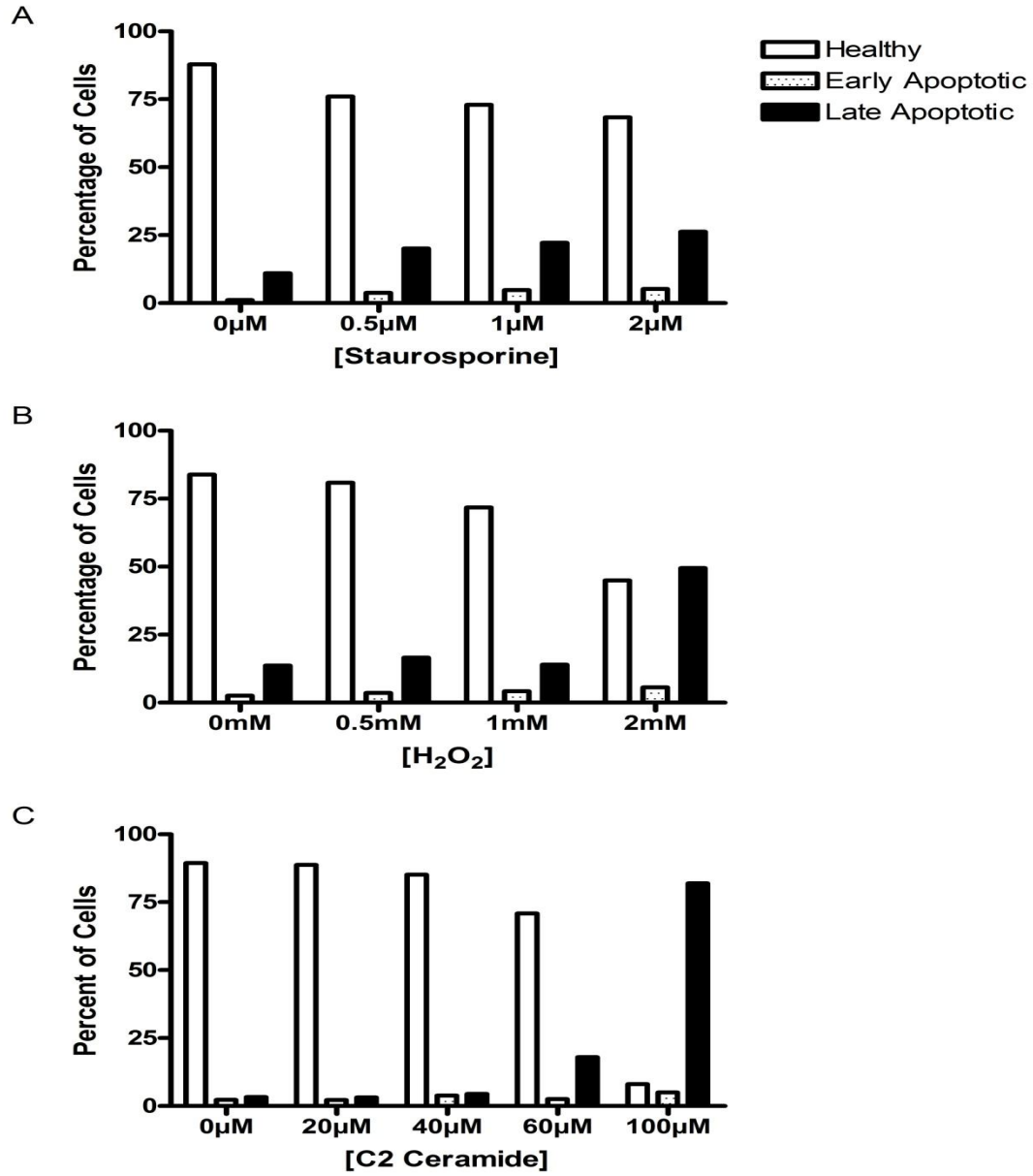
Figure A2. AnnexinV-FITC/PI Staining Dose Response to Apoptotic Stimuli. Analysis of AnnexinV-FITC/PI staining following exposure to increasing concentrations of staurosporine (A), H_2O_2 (B), and C_2-ceramide (C).
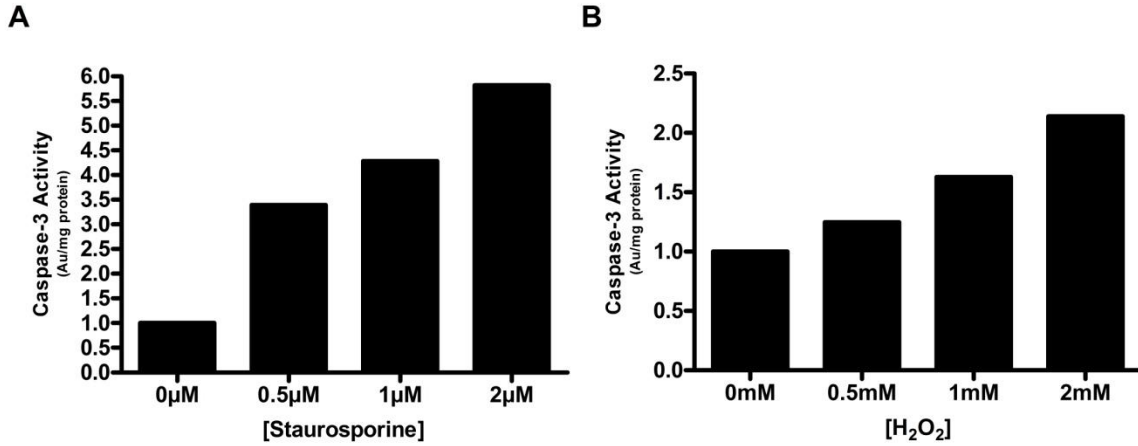
Figure A3. Apoptotic Stimuli Dose-Response Caspase-3 Activity. Pilot results showing that with increasing concentrations of staurosporine (A) and H_2O_2 (B), there is a step-wise increase in caspase-3 activity.
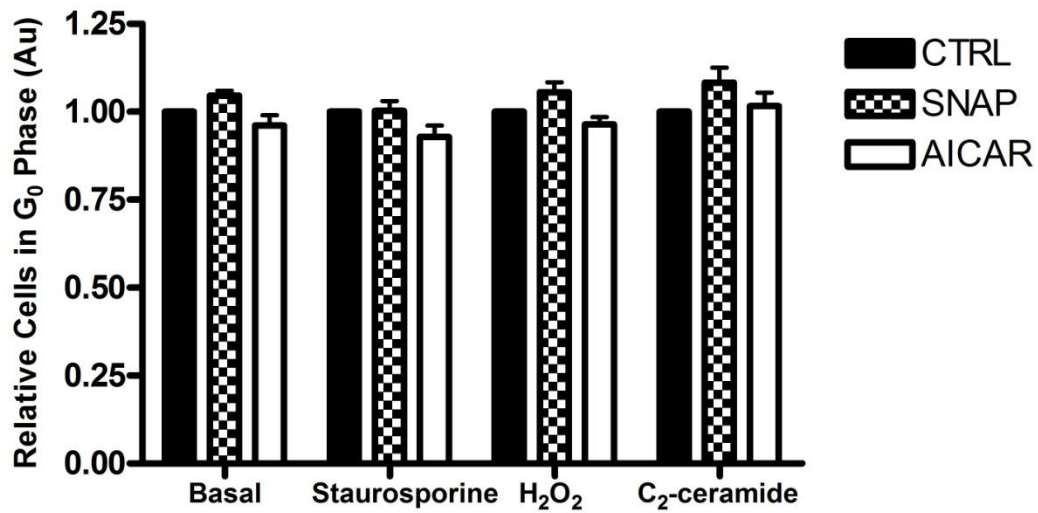

Figure A4. Relative proportion of cells in the G_0 phase. To examine if cells were in different phase of cell cycle, the relative proportion of cells in the G_0 phase was examined under basal conditions, or following treatment with staurosporine, H_2O_2, or C_2-ceramide. There were no statistically significant difference between groups.

Mitochondrial Biogenesis:

To verify that induction of apoptosis is possible by treatment with the AMPK activator

AICAR and the nitric oxide donor SNAP, western blots for AIF, cytochrome c, and PGC-1α

were performed. The results show that intermittent administration of AICAR for 5 hours a day

for results in an increase in AIF, cytochrome c, and PGC-1α in comparison to controls (Fig.

A5). Since AIF and cytochrome c are both markers of mitochondrial content, and PGC-1α is a

marker of mitochondrial biogenesis, the results indicate that mitochondrial biogenesis was
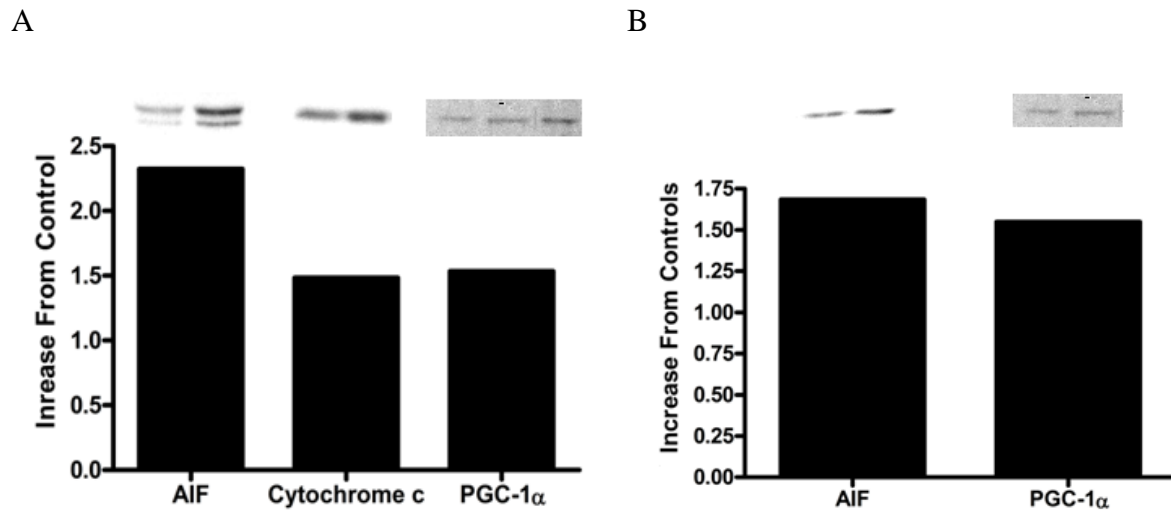
successfully induced.

A   B

Figure A5. Mitochondrial Biogenesis. Treatment of myoblasts with AICAR for 5
hours per day for 5 days results in upregulation of AIF, SNAP, and PGC-1α in comparison to
control cells (A). Treatment of myoblasts with SNAP for 5 hours per day for 5 days results in
upregulation of AIF and PGC-1α in comparison to untreated control cells.

Annexin/PI Flow Cytometry Assay:

To determine an appropriate concentration of staurosporine (STS) and hydrogen

peroxide (H_2O_2) to use to induce apoptosis and verify that apoptotic cells could be identified

using this protocol, varying increasing levels of STS or H_2O_2 were administered to cells. For STS, final concentrations of 0μM, 0.5μM, 1.0μM, and 2.0μM were used while for H_2O_2 final concentration of 0mM, 0.5mM, 1.0mM, and 2mM were used. The attempt to separate apoptotic and necrotic population from healthy cells was successful using this assay technique (Fig. A6). The technique was further verified by the fact that increasing concentrations of STS or H_2O_2 resulted in increasing prevalence of apoptotic cells and a reduction in the healthy cell population.
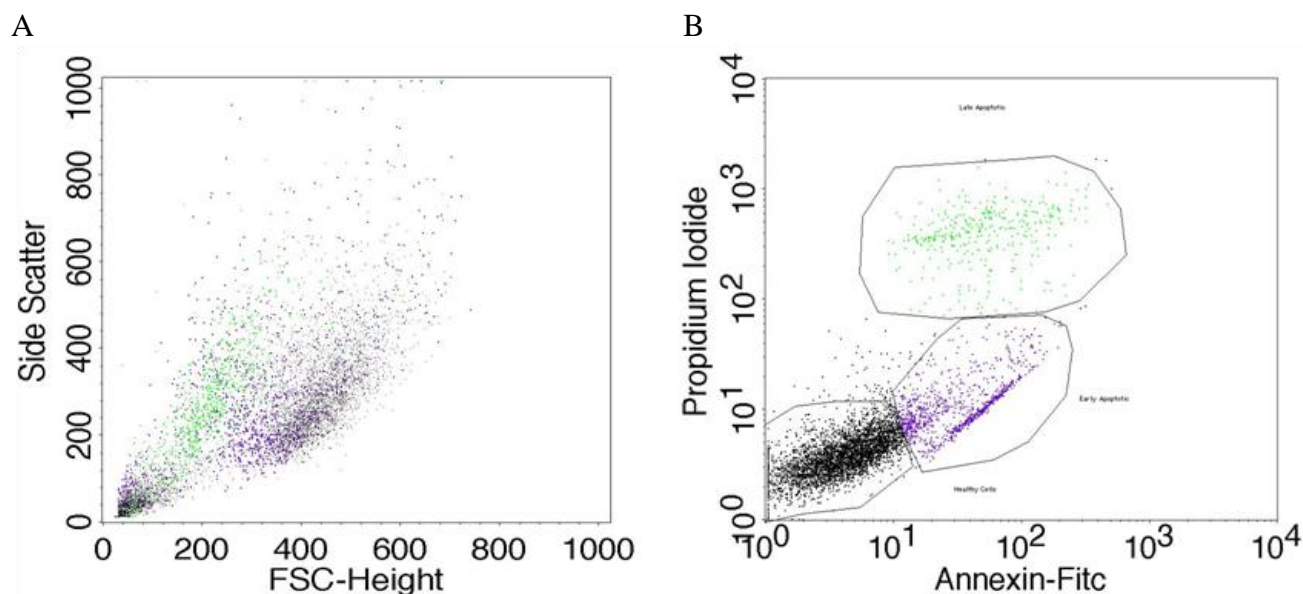
A

B

Figure A6. Flow cytometry analysis of L6 myoblast treated with H_2O_2. (A) shows the original scatter plot of all myoblasts while (B) illustrates the separation of apoptotic and necrotic cells from the healthy population. In both (A) and (B), healthy cells do not express any fluorescence and are represented by the red dots. Purple dots represent cells that express only AnnexinV-FITC fluorescence and are classified as early apoptotic. Late apoptotic cells are those that positively fluoresce both AnnexinV-FITC and PI and are represented by green dots.

Caspase Activity Assay:

In concurrence with the AnnexinV/PI flow cytometry assay, the effect of increasing concentrations of H_2O_2 and STS on caspase-3 activity was analyzed. Increasing dosages of H_2O_2 or STS resulted in an increase in caspase-3 activity in control cells (Fig. A7). It was also shown 2 μM STS administration resulted in a slight decrease of caspase-3 in AICAR treated groups and a slight increase in caspase-3 activity in SNAP treated group. Induction of apoptosis with 2mM H_2O_2 resulted in decreased caspase-3 activity in groups administered AICAR and SNAP (Fig. A7).
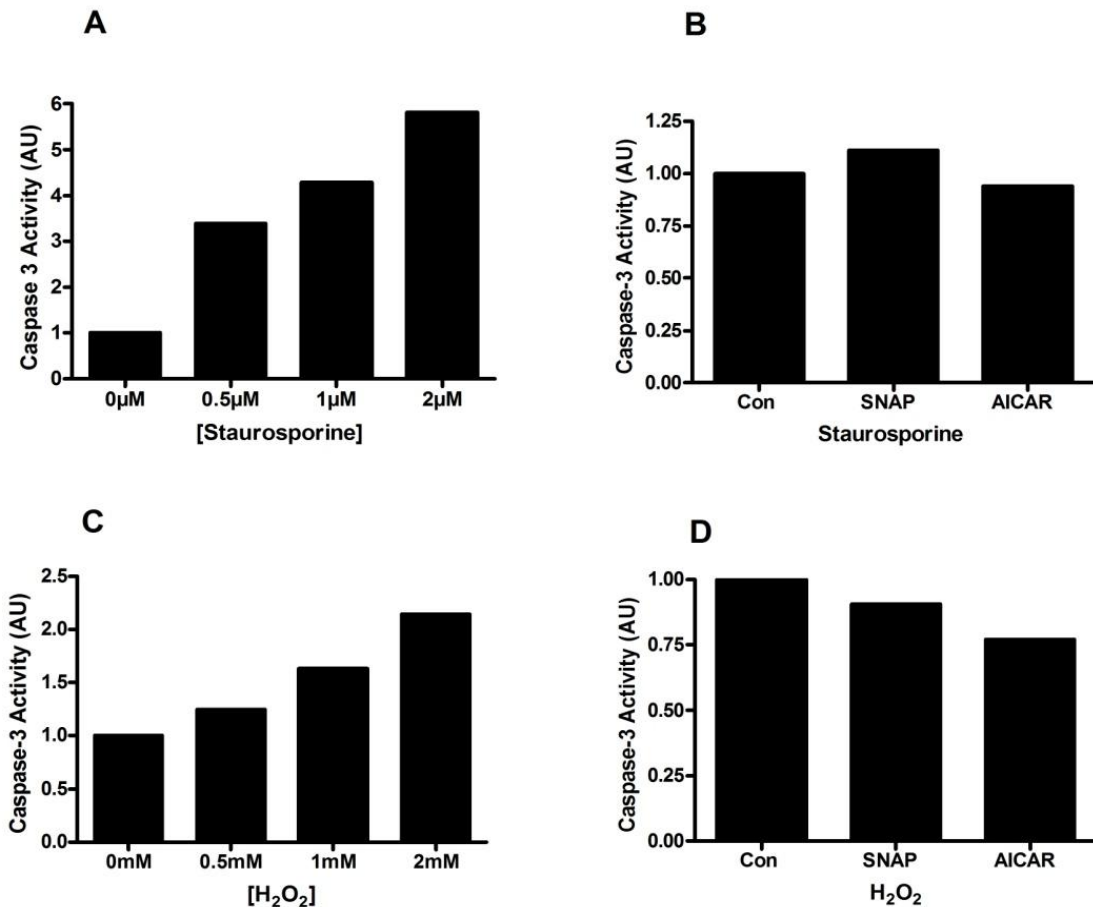

Figure A7. Casaspe Activity. (A) and (C) demonstrate effect of increasing STS and H_2O_2 concentration respectively on caspase-3 activity. (C) demonstrates effect of 5days SNAP and AICAR administration on STS induced activation of caspase-3. (D) demonstrates effect of 5 days of SNAP and AICAR administration on H_2O_2 induced caspase-3 activation.

Cell Cycle Assay

The cell cycle assay allows for cells to be separated according to their phase in the cell cycle. The resultant peaks found in the flow cytometry analysis represent cells in the G_o/G_1, S, and G_2/M phase. The assay also allows us to separate out apoptotic cells. To verify this procedure, control cells were treated with 2mM H_2O_2 to induce apoptosis and the subsequent results are shown in Fig. A8.
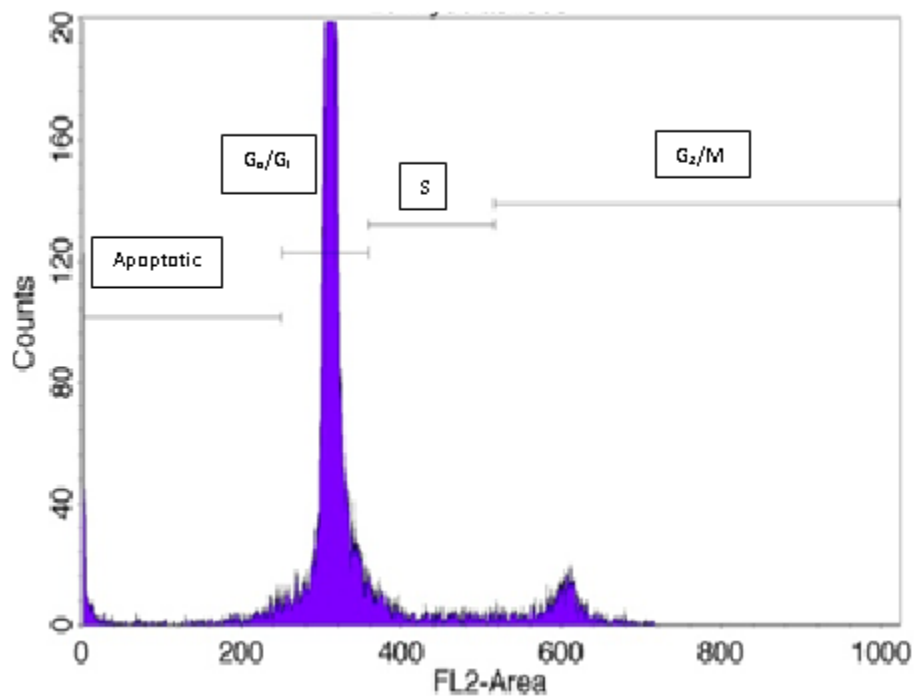

Figure A8. Demonstrtion of propidium iodide (PI) cell cycle flow cytometry assay. Cells can be separated into four groups based on PI fluorescence in the FL2-area channel: apoptotic, G_o/G_1, S, and G_2/M. Apoptotic cells express lower fluorescence in the FL2-Area compared to channel due to breakdown of nuclear material and DNA.