Inactivity-induced skeletal muscle atrophy: a brief review

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ABSTRACT

Skeletal muscle is an adaptable tissue that responds rapidly to both increased contractile activity and inactivity. For example, lengthy periods of skeletal muscle disuse (e.g., bed rest) result in a decline of muscle protein and muscular strength. Our understanding of the processes that contribute to disuse muscle atrophy has expanded markedly during the past two decades and this review will provide an overview of the mechanisms responsible for disuse-mediated muscle atrophy. The first segment of this review will outline the experimental models commonly used to investigate disuse muscle atrophy. The second section will discuss our current understanding of muscle proteases whereas the final sector will identify the role that reactive oxygen species play in inactivity-induced muscle atrophy.

Key-words: *redox, oxidants, antioxidants, proteasome, calpain, caspase-3, reactive oxygen species*

RESUMO

Atrofia do músculo esquelético induzida pela inactividade: uma breve revisão

O músculo esquelético é um tecido adaptável respondendo quer ao aumento da actividade contráctil, quer à inactividade. Por exemplo, períodos prolongados de desuso resultam no declínio das proteínas e da força muscular. O entendimento acerca dos processos que contribuem para a atrofia muscular induzida pelo desuso aumentou notavelmente nas duas últimas décadas e a presente revisão providencia uma síntese dos mecanismos responsáveis pela atrofia muscular esquelética mediada pelo desuso. Numa primeira parte, a presente revisão foca-se nos modelos experimentais comummente usados na investigação da atrofia muscular esquelética induzida pelo desuso. Na segunda parte, discute-se o entendimento actual das próteases musculares enquanto na parte final identifica-se o papel das espécies reactivas de oxigénio na atrofia do músculo esquelético induzida pela inactividade.

Palavras-chave: redox, oxidantes, anti-oxidantes, proteasoma, calpaína, caspase-3, espécies reactivas de oxigénio

INTRODUCTION

Long periods of skeletal muscle inactivity (e.g., due to limb immobilization, bed rest, physical inactivity, or space flight) result in a loss of muscle mass and strength[1]. Understanding the signaling pathways that regulate disuse muscle atrophy is important in developing protective countermeasures against this type of skeletal muscle wasting[2]. In this regard, evolving studies in skeletal muscle biology have markedly elevated our knowledge of the factors leading to muscle atrophy during both disuse and other wasting disorders (e.g., cancer-mediated cachexia). This review is designed to provide a concise summary of our current understanding of those factors that control disuse muscle atrophy. First, we will present an overview of the experimental models used to investigate disuse muscle atrophy. Second, we will provide a summary of the proteolytic pathways that promote disuse muscle atrophy. We will then discuss evidence linking redox disturbances (i.e., oxidative stress) to disuse muscle atrophy. The final section of this report will identify unanswered questions related to the control of muscle protein breakdown and the loss of skeletal muscle nuclei. By recognizing voids in our knowledge about the mechanisms responsible for disuse muscle atrophy, we anticipate that this report will stimulate future research aimed at improving our understanding in this important area of inquiry.

ANIMAL MODELS TO INVESTIGATE DISUSE MUSCLE ATROPHY

It is clear that prolonged periods of inactivity results in skeletal muscle atrophy. Because of ethical considerations, it is often difficult and sometimes impossible to investigate the cellular mechanisms responsible for disuse muscle atrophy in humans. Therefore, animal models have been created to simulate the range of inactivity conditions that produce muscle atrophy in humans. For instance, animal models using hindlimb suspension to unload the hindlimb skeletal muscles are commonly used to mimic prolonged bed rest and space flight in humans (Table 1). Furthermore, animal models of limb immobilization (i.e., casting) are commonly used in research. These animal models are powerful experimental paradigms that provide the researcher with well-controlled conditions to study disuse muscle atrophy. Moreover, animal models provide the scientist with full access to skeletal muscle tissue to perform extensive biochemical analysis of protease activation and the pathways responsible for muscle atrophy. Finally, cultured muscle cells are an emerging model to study skeletal muscle atrophy. The advantages of using cell culture versus whole animal models to study skeletal muscle atrophy are numerous. For example, cells in culture provide a controlled experimental environment whereby gene expression can be amplified (e.g., gene transfection) or knocked down using a variety of gene silencing techniques (e.g., siRNA). Hence, cell culture models provide investigators with the opportunity to study the impact of single proteins or polypeptides on cell signaling pathways linked to muscle protein balance. Although cell culture models provide advantages, this reductionist approach also has disadvantages because muscle cell lines in culture may differ in several ways from intact skeletal muscle fibers in live animals. Therefore, many laboratories use both cell culture and animal models to study the mechanisms responsible for skeletal muscle atrophy.

Table 1. Conditions leading to skeletal muscle atrophy in humans and the corresponding animal and cell culture model.

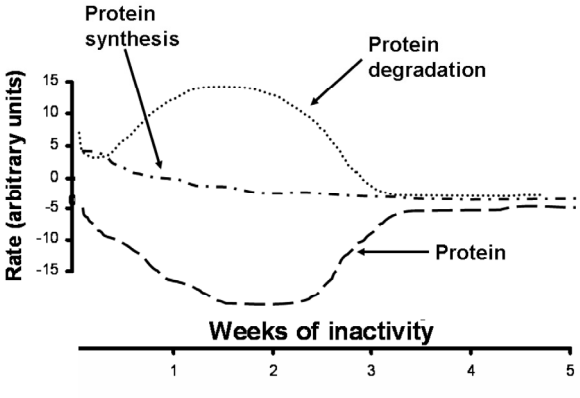
Non-pathological conditions resulting in human skeletal muscle atrophy	Animal model
Limb immobilization	Limb immobilization
Space flight	Hind-limb suspension
Bed rest	Hind-limb suspension
Limb immobilization, space flight, or bed rest	Cell culture using muscle cell lines

DISUSE MUSCLE ATROPHY: ROLE OF PROTEIN SYNTHESIS VS. DEGRADATION

Skeletal muscle atrophy can occur due to a decrease in protein synthesis, an increase in the rate of protein degradation, or a combination of both increased proteolysis and depressed protein synthesis. Using animal models of disuse atrophy it has been shown that inactivity-induced muscle atrophy occurs due to both a decrease in protein synthesis and an increase in the rate of proteolysis[3, 4] (Figure 1). In the hindlimb suspension model of skeletal muscle atro-

phy, the rate of protein synthesis declines rapidly following the onset of muscle unloading[3]. This decrease in muscle protein synthesis reaches a new steady-state level at roughly 48 hours[3]. Further, the reduction in protein synthesis is followed by a large and rapid increase in proteolysis mediated by several key proteases. An overview of the key proteolytic pathways that are active in skeletal muscle follows.

Figure 1. Inactivity-induced muscle atrophy occurs due to both a decrease in protein synthesis and an increase in the rate of proteolysis. The figure illustrates that the rate of protein synthesis declines following the onset of inactivity. Further, the reduction in protein synthesis is followed by a large and rapid increase in proteolysis. Redrawn from data contained in reference 3.



PROTEOLYTIC PATHWAYS IN SKELETAL MUSCLE

Numerous proteolytic systems contribute to the degradation of muscle proteins. The most widely investigated proteases in skeletal muscle are lysosomal proteases, Ca^{2+} activated proteases (i.e. calpain), caspase-3 and the proteasome system. Although lysosomal proteases are activated in skeletal muscle undergoing disuse atrophy, their importance has been questioned[5-7]. Nonetheless, new evidence suggests that lysosomal proteases may contribute to autophagy during muscle atrophy and therefore, may play a more important role than previous envisioned[8]. Additional work is required to clarify the precise role that the autophagic/lysosomal proteolytic pathway plays in disuse skeletal muscle atrophy. Abundant evidence demonstrates that both calpain and the proteasome system play important roles in muscle protein breakdown during muscle atrophy[6, 7, 9, 10]. Moreover, emerging evidence reveals that another protease, caspase-3, may also contribute to select forms of muscle atrophy[11, 12]. The majority of muscle proteins (50-70%) are located within actomyosin complexes[13]. While the proteasome system can degrade monomeric actin and myosin, this protease cannot degrade intact actomyosin complexes[10, 14]. It follows that myofilaments must be released from the sarcomere as monomeric proteins prior to degradation by the proteasome system[13, 15]. This fact indicates that myofilament release is the rate-limiting step in muscle protein degradation. In this regard, it is clear that both calpain and caspase-3 are capable of producing actomyosin disassociation resulting in myofilament release[11, 13, 14]. Therefore, activation of one or both of these proteases is a requirement to achieve proteolytic degradation of myofilaments during muscle disuse.

Calpain-mediated proteolysis: Calpains (calpain I and II) are Ca^{2+}-dependent cysteine proteases that are activated in skeletal muscle during periods of inactivity[14, 16]. Calpain releases sarcomeric proteins by cleaving cytoskeletal proteins (e.g., titin, nebulin) that anchor the contractile elements[6, 17]. Moreover, it appears that calpain can also degrade over 100 skeletal muscle proteins including myosin heavy protein[14].

At present, a detailed understanding of those factors that regulate calpain is not available. Nonetheless, it is established that calpain activity is controlled by several factors including cytosolic calcium levels and the concentration of the endogenous calpain inhibitor, calpastatin[14, 16]. Specifically, it appears that calpain activity is enhanced by any factor that increases cytosolic calcium concentrations and/or decreases calpastatin levels[14]. In this regard, it is established that skeletal muscle inactivity is associated with increased cytosolic calcium levels and calpain activation[18]. The mechanism regulating this inactivity-mediated calcium overload remains unknown and is an important topic for future work.

Caspase-3 and muscle atrophy: Numerous signaling pathways can trigger the activation of a collection of proteases called "caspases"[19]. Collectively, caspases are classified as endoproteases that breakdown proteins and, in some cases, promote programmed cell death (apoptosis). In the cell, caspases are expressed

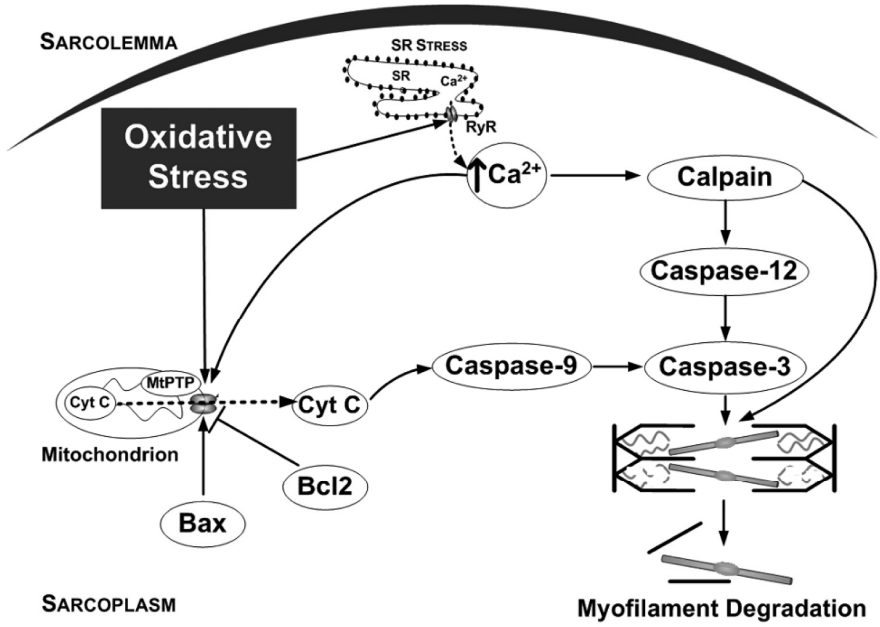


Figure 2. Simplified overview of signaling pathways leading to activation of caspase-3. In one scenario (right), caspase-3 can be activated by oxidative stress leading to SR calcium release, increased calpain activity, and calpain-mediated activation of caspase-12. In another scenario (left), oxidative stress and elevated calcium promote cytochrome C release from the mitochondria leading to caspase-9 activation and subsequent activation of caspase-3. Also, note the multiple lines of cross talk between pathways. SR = sarcoplasmic reticulum; RyR = ryanodine receptor; Cyt C = cytochrome C; MtPTP = mitochondrial permeability transition pore.

as inactive precursors (called procaspases) and activation of these proteases results in events leading to protein breakdown and apoptosis.

Emerging evidence suggests that caspase-3 may play an important role in muscle protein degradation during both diabetes-induced and inactivity-induced muscle atrophy[11, 12]. For example, caspase-3 activation promotes degradation of actomyosin complexes, and, inhibition of caspase-3 activity suppresses the overall rate of proteolysis in diabetes-mediated cachexia[11].

Control of caspase-3 activation is multifaceted and involves numerous interconnected signaling pathways. In the case of diabetes-induced muscle atrophy, it seems feasible that caspase-3 is activated by activation of caspase-12 (via a calcium release pathway); and/or activation of caspase-9 (via a mitochondrial pathway). A key interaction between these caspase-3 activation pathways is that both of these corridors can be activated by reactive oxygen species (ROS)[19, 20] (Figure 2). The calcium release pathway activates caspase-3 activity via a signaling path that ends in a caspase-12-mediated activation of caspase-3[19]. Notice that calpain activation can also contribute to caspase-3 activation via this calcium-mediated pathway[21] (Figure 2). The mitochondrial pathway of caspase-3 activation is convoluted and can be commenced by several interacting signals including reactive oxygen species (ROS) and a high pro- to anti-apoptotic protein ratio in the mitochondria (Figure 2)[22]. ROS can promote mitochondrial release of cytochrome C resulting in caspase-9 activation and the ensuing activation of caspase-3[22]. Finally, the endogenous calpain inhibitor, calpastatin, is a substrate for both caspase-3 and calpain. It follows that elevated caspase-3 or calpain activity will diminish calpastatin levels in cells and encourage calpain activation[11, 14, 23]. Moreover, increased calpain activity can lead to the activation of caspase-3[21]. Hence, cross-talk between the calpain and caspase-3 proteolytic systems could play a regulatory role in myofilament release in skeletal muscle during prolonged periods of inactivity.

Role of the proteasome in proteolysis: In proteasome-mediated proteolysis, proteins can be degraded by either the 26S proteasome or the 20S proteasome[5, 24, 25]. The 26S proteasome is the complete proteasome unit and is composed of three connected units:

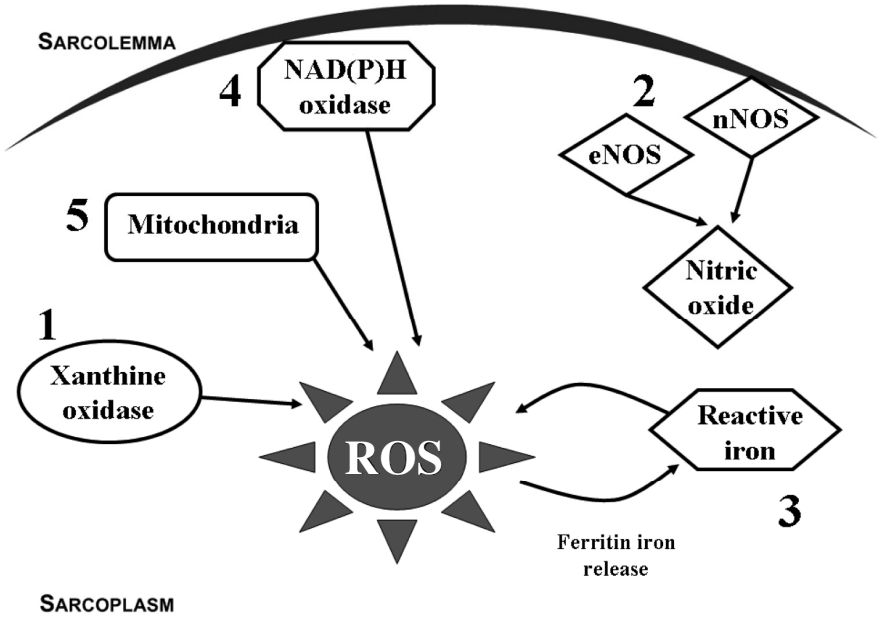


Figure 3. At least five different ROS production pathways exist in skeletal muscle: 1) generation of ROS by the xanthine oxidase pathway; 2) production of NO via nitric oxide synthase (NOS); 3) formation of ROS (hydroxyl radicals) by increased cellular levels of reactive iron; 4) NADPH oxidase; and 5) mitochondrial production of superoxide radicals.

the 20S core proteasome with a regulatory 19S complex connected to each end[26]. The 19S regulatory complex possesses ATPase activity and plays an important role in ATP dependent degradation of ubiquitinated proteins[26]. In the 26S proteasome proteolytic pathway, ubiquitin covalently links with protein substrates and identifies them for degradation. The ubiquitinated protein is then recognized and bound to the 19S regulatory unit of the 26S proteasome. Energy released during ATP hydrolysis removes the polyubiquitin chain and unfolds the substrate protein. The unfolded protein is then fed into the 20S core proteasome where it is degraded in a manner that does not require energy from ATP[25]. Importantly, it is clear that the 20S core proteasome can selectively degrade oxidatively modified proteins without these proteins undergoing ubiquitination[24, 25]. Hence, it is feasible that oxidative stress can accelerate muscle protein breakdown via 20S core proteasome alone.

The process of linking ubiquitin to protein substrates requires the ubiquitin-activating enzyme (E1), specific ubiquitin-conjugating enzymes (E2), and often, a specific ubiquitin protein ligase enzyme (E3) is also required. The ubiquitination of a unique protein is accomplished by one of several different E2s and by a specific E3 ligase. For example, it has been reported that the specific ubiquitin-conjugating enzyme $E2_{14k}$ is a required controller of skeletal muscle ubiquitin-protein conjugation[27]. Moreover, $E2_{14k}$ works with a specific E3 ligase (E3a) to promote muscle protein breakdown in a variety of catabolic conditions. Further, two unique ubiquitin E3 ligases, atrogin-1 (also called muscle atrophy F-box) and muscle ring finger-1 have been discovered in skeletal muscle and it is now clear that these ligases are important in skeletal muscle atrophy[28, 29].

RADICAL PRODUCTION IN MUSCLE FIBERS SERVES AS AN UPSTREAM SIGNAL TO TRIGGER PROTEOLYSIS

It is well established that radicals and other ROS are produced in both inactive and contracting skeletal muscles[30, 31]. When oxidant production in cells exceeds the antioxidant capacity to buffer oxidants, oxidative damage occurs resulting in damage to cellular lipids, proteins, and nucleic acids. High levels of cellular oxidant stress leads to cellular dysfunction and in extreme cases, cell death can occur.

Historically, compared to non-contracting muscles, it was believed that ROS production is greatest in contracting skeletal muscles. Nonetheless, it is now clear that inactive skeletal muscles produce more ROS than contracting skeletal muscles and that oxidative injury occurs during periods of disuse in locomotor skeletal muscles[32-38] and in the unloaded diaphragm during prolonged mechanical ventilation (MV)[39, 40]. Unfortunately, it is unknown which ROS producing pathways are responsible for this inactivity-induced oxidative injury in skeletal muscle fibers. Nonetheless, it seems plausible that oxidative stress in inactive skeletal muscle may be due to the interaction of at least five different oxidant production pathways[30]: 1) generation of ROS by the xanthine oxidase pathway; 2) production of NO via nitric oxide synthase (NOS); 3) formation of ROS (hydroxyl radicals) by increased cellular levels of reactive iron; 4) NADPH oxidase; and 5) mitochondrial production of superoxide radicals (Figure 3).

WHAT ARE THE SIGNALING LINKS BETWEEN OXIDATIVE STRESS AND PROTEOLYSIS?

Several experimental findings connect oxidative stress in inactive skeletal muscle with disuse muscle atrophy. The first suggestion that ROS could be linked to disuse muscle atrophy was provided by Kondo and co-workers[30]. These experiments indicated that prolonged periods of skeletal muscle inactivity was associated with oxidative damage in muscle fibers. Further, this work revealed that disuse muscle atrophy could be partially retarded by the delivery of the antioxidant, vitamin E. The ability of vitamin E to lessen disuse muscle atrophy was later confirmed by Appell, Duarte, and co-workers[41]. Moreover, recent work from our group indicates that prevention of oxidative stress in the diaphragm during MV results in a reduced rate of muscle proteolysis[42, 43]. Collectively, these experiments are consistent with the notion that oxidative stress contributes to disuse muscle atrophy via regulation of proteolysis. Note, however, that not all antioxidant interventions are capable of retarding disuse muscle atrophy[44].

How does prevention of disuse-related oxidative stress in skeletal muscle diminish the rate of muscle proteolysis and atrophy? A definitive answer to this question is not currently available but several possibilities exist. First, it is likely that inactivity-induced oxidative stress in skeletal muscle leads to an increase in free calcium in the cytosol resulting in the activation of calcium-activated proteases (e.g., calpain) in skeletal muscles. This position is supported by evidence that oxidative stress promotes Ca^{+2} overload in cells[18]. A potential mechanism to explain this observation is that ROS can promote the formation of reactive aldehydes (i.e. 4-hydroxy-2,3-trans-nonenal) that can diminish plasma membrane Ca^{+2} ATPase activity and impede Ca^{+2} removal from the cell[45].

Another connection between oxidative stress and skeletal muscle atrophy is related to the regulation of caspase-3 activity. The control of caspase-3 activity is complicated and involves numerous signaling pathways. In the case of inactivity-induced muscle atrophy, it is feasible that caspase-3 is activated by either the activation of caspase-12 (via a calcium release pathway) or by the activation of caspase-9 (via a mitochondrial pathway). An important interaction between these caspase-3 activation pathways is that both of these paths can be activated by ROS[19].

The calcium release pathway can promote caspase-3 activity via a signaling corridor that ends with a caspase-12-derived activation of caspase-3[19]. Note that this pathway can be accelerated by increased calpain activity and other signaling molecules[19].

The mitochondrial pathway of caspase-3 activation is intricate and can be initiated by several interacting signals including ROS and a high pro- to anti-apoptotic protein ratio in the mitochondria. Oxidants can lead to mitochondrial release of cytochrome C resulting in the activation of caspase-9 followed by the activation of caspase-3. Moreover, numerous pro- (e.g. Bax) and anti-apoptotic (e.g. Bcl-2) proteins exist in the cell. A high Bcl-2 to Bax ratio in the cell promotes mitochondria integrity whereas a high Bax to Bcl-2 ratio favors mitochondrial release of cytochrome C leading to the activation of caspase-9 and subsequently activation of caspase-3[19].

Another bond between oxidative stress and muscle disuse atrophy involves the redox regulation of gene expression of proteins involved in the proteasome proteolytic system[10, 46]. For instance, oxidative stress has been shown to up-regulate the expression of $E2_{14k}$, muscle atrophy F-box/atrogin1, and muscle

ring finger-1 in myotubes[27]. Theoretically, increased expression of E3 ubiquitin ligases (i.e., atrogin1, and muscle ring finger-1) in skeletal muscle would lead to accelerated proteasome proteolysis and muscle atrophy[28]. In this regard, Li et al.[27] has argued that oxidant stress accelerates muscle protein breakdown by augmentation of the 26S proteasome system. Further, growing evidence also indicates that the 20S core proteasome can degrade oxidatively modified proteins without ubiquitination[24, 25]. Collectively, these results indicate that oxidant stress can accelerate muscle protein breakdown via both the 26S and 20S core proteasome.

SUMMARY AND CONCLUSIONS

Skeletal muscle is a plastic tissue that responds to increased contractile activity and diminished contractile activation. Indeed, prolonged periods of skeletal muscle inactivity results in a decrease of muscle protein and muscular strength. Disuse skeletal muscle atrophy results from both a decrease in protein synthesis and an increase in proteolysis. Our understanding of the proteases that contribute to muscle wasting has expanded rapidly during the past several years. In this regard, it now clear that several different proteolytic systems work as a unit to degrade muscle proteins. Although the molecular components of these pathways have been characterized, the regulatory networks that control their function remain enigmatic. However, several lines of evidence link ROS to disuse muscle atrophy via redox control of proteolysis. Specifically, a growing number of studies suggest that antioxidants can serve as therapeutic agents in delaying the rate of disuse muscle atrophy. These preliminary findings are encouraging and may ultimately lead to therapeutic countermeasures to retard inactivity-induced muscle atrophy.

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