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FORUM REVIEW ARTICLE

Mechanistic Links Between Oxidative Stress and Disuse Muscle Atrophy

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Abstract

Long periods of skeletal muscle inactivity promote a loss of muscle protein resulting in fiber atrophy. This disuse-induced muscle atrophy results from decreased protein synthesis and increased protein degradation. Recent studies have increased our insight into this complicated process, and evidence indicates that disturbed redox signaling is an important regulator of cell signaling pathways that control both protein synthesis and proteolysis in skeletal muscle. The objective of this review is to outline the role that reactive oxygen species play in the regulation of inactivity-induced skeletal muscle atrophy. Specifically, this report will provide an overview of experimental models used to investigate disuse muscle atrophy and will also highlight the intracellular sources of reactive oxygen species and reactive nitrogen species in inactive skeletal muscle. We then will provide a detailed discussion of the evidence that links oxidants to the cell signaling pathways that control both protein synthesis and degradation. Finally, by presenting unresolved issues related to oxidative stress and muscle atrophy, we hope that this review will serve as a stimulus for new research in this exciting field. *Antioxid. Redox Signal.* 15, 2519–2528.

Introduction

SKELETAL MUSCLE IS THE LARGEST ORGAN in the human body and comprises 40%–50% of total body weight. Prolonged periods of skeletal muscle disuse (*e.g.*, limb immobilization or chronic bed rest) lead to fiber atrophy resulting in muscular weakness and a decreased quality of life. The development of an intervention to prevent disuse muscle atrophy requires a detailed understanding of the cellular signaling pathways that regulate both protein synthesis and protein breakdown in muscle. Ongoing research in muscle biology has improved our understanding of those factors that contribute to inactivity-induced muscle atrophy, and evolving evidence reveals that disturbed redox signaling, due to increased production of reactive oxygen species (ROS), is an important regulator of cell signaling pathways that control both proteolysis and protein synthesis in skeletal muscle. For example, exposure of skeletal muscle myotubes to ROS (*i.e.,* hydrogen peroxide) can activate proteases leading to protein degradation (38, 42). Further, growing evidence suggests that ROS can impede cell signaling pathways that promote protein synthesis (48, 73). Collectively, these results are consistent with the concept that oxidative stress can play an important regulatory role in disuse skeletal muscle atrophy.

The goal of this review is to provide a summary of our current knowledge regarding the signaling links between ROS and the loss of cellular protein during disuse skeletal muscle atrophy. The first section of this report will provide an overview of experimental models used to investigate disuse muscle atrophy followed by a "big-picture" summary of the events leading to muscle atrophy. Next, we will discuss the signaling pathways connecting ROS to decreased protein synthesis and increased proteolysis. Finally, we will close with a discussion of voids in our knowledge about oxidative stress and disuse muscle atrophy in hopes of stimulating future research in this field.

Disuse Muscle Atrophy: Experimental Models

As discussed previously, long periods of bed rest, limb immobilization, space flight, or reduced respiratory muscle activity during mechanical ventilation results in skeletal muscle atrophy in humans and other animals. Due to the complexities involved in studying the mechanisms responsible for disuse muscle atrophy in humans, animal models are often used to study the biological foundation for muscle atrophy. Indeed, several laboratory animal models have been developed to simulate the various types of human disuse muscle atrophy (Fig. 1). For instance, rodent models of limb immobilization (*i.e.,* casting) are widely used to investigate the impact of muscle inactivity on muscle fiber size and cellular signaling pathways. Additionally, a tail suspension technique to unload hindlimb locomotor muscles of rodents is often employed to mimic the human muscle atrophy that occurs during prolonged space flight or bed rest. Further,

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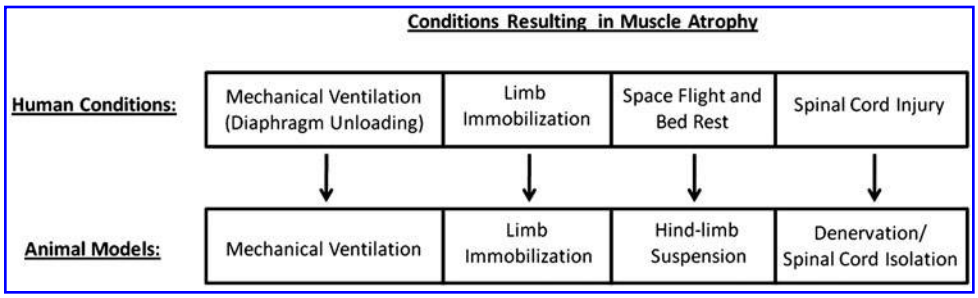

FIG. 1. Human conditions that promote skeletal muscle atrophy and the corresponding animal models that are used to investigate them.

using animal models, experimental manipulations such as denervation or spinal cord isolation have been employed to investigate the impact of neuromuscular diseases on skeletal muscle structure and function. These models differ from the aforementioned models of muscle inactivity (*e.g.,* hindlimb immobilization and tail suspension) and are designed to investigate the impact of both muscle inactivity and the loss of neurotropic factors on skeletal muscle structure/function.

Although studies of disuse muscle atrophy in humans are often difficult to complete due to the invasive nature of muscle biopsies, several human paradigms of disuse muscle atrophy also exist. For instance, human models of limb immobilization, prolonged bed rest, and unilateral lower limb suspension (*i.e.,* immobilizing one leg and using crutches to walk) have been used to investigate inactivity-induced skeletal muscle atrophy in humans.

Controlled mechanical ventilation is a unique and clinically important situation that promotes inactivity-induced atrophy in respiratory muscles. In human medicine, controlled mechanical ventilation is a life-saving intervention for patients suffering from respiratory failure. During this mode of mechanical ventilation the ventilator delivers all of the breaths while the patients' respiratory muscles are inactive. Both human and animal studies have revealed that prolonged mechanical ventilation results in rapid inspiratory muscle (*i.e.,* diaphragm) atrophy. For example, as few as 18 hours of mechanical ventilation can result in significant (*e.g.*, >15% reduction in fiber cross-sectional area) diaphragmatic atrophy in both humans and rodents (36, 59). A unique aspect of this ventilator-induced respiratory muscle atrophy is the rapidity of the atrophic response. Indeed, a comparable level of disuse muscle atrophy in locomotor skeletal muscles would require 96 hours of muscle unloading (43, 68).

Disuse Muscle Atrophy: Relative Roles of Protein Synthesis and Proteolysis

The conservation of skeletal muscle mass is dependent on the balance between the rates of protein synthesis and degradation. Numerous animal studies, using a variety of experimental models, demonstrate that inactivity-induced locomotor and respiratory skeletal muscle atrophy occurs due to both increased proteolysis and decreased muscle protein synthesis (58, 68). Specifically, studies reveal that the rate of protein synthesis declines quickly (*i.e.*, within 6 hours) after the onset of muscle inactivity and reaches a new "lower" steady-state of muscle protein synthesis within 18–48 h (58, 68). Further, studies also show that disuse muscle atrophy is associated with a large increase in muscle proteolysis (59, 68). Therefore, in animals, the net loss of skeletal muscle protein during prolonged muscle inactivity in is due to both decreased protein synthesis and increased protein breakdown.

In contrast to the consensus view that disuse muscle atrophy in animals occurs due to both decreased protein synthesis and increased breakdown, a recent review argues that the primary factor promoting disuse limb muscle atrophy in humans is a decrease in protein synthesis (50). In this regard, the human literature is consistent that locomotor skeletal muscle inactivity results in decreased muscle protein synthesis. In contrast, the literature is inconsistent regarding the influence of prolonged inactivity on human limb muscle proteolysis. For example, some studies indicate that prolonged bed rest or immobilization-induced human limb muscle atrophy is associated with limited increases in protease activity or muscle protein breakdown (19, 20). Conversely, other reports show that prolonged periods of muscle inactivity in humans is associated with elevated rates of muscle protein breakdown and increased protease activation (25, 35, 36, 67). Based upon these divergent findings, a recent report has concluded that additional studies are required to clearly define the role that proteolysis plays in disuse atrophy in human limb muscle (40).

In contrast to the controversy regarding the impact of inactivity on human limb muscle proteolysis, the mechanical ventilation literature is consistent and indicates that mechanical ventilation-induced inactivity in human respiratory muscles results in the activation of major proteolytic systems (*e.g.*, calpain, caspase-3, proteasome, and autophagy) (25, 35, 36). Similar results have been reported in animal studies of mechanical ventilation-induced respiratory muscle atrophy (16, 59, 72). Collectively, these consistent findings support the concept that inactivity rapidly increases proteolysis in respiratory muscles. These results are not surprising, given that mechanical ventilation-induced diaphragmatic atrophy in humans and animals has been reported to occur in as few as 18 hours (36, 43, 59, 72).

Redox Regulation of Disuse Muscle Atrophy

The notion that redox disturbances play a significant role in the control of disuse muscle atrophy was first introduced in 1991 (31). However, this concept did not receive significant experimental attention until recent years. In the following segments, we will provide a historical overview of the evidence linking oxidative stress to disuse muscle atrophy, followed by a discussion of the potential sources of ROS production in inactive skeletal muscle. We will conclude with a detailed discussion of the impact that inactivity-induced oxidative stress has on muscle protein synthesis and degradation.

Oxidative stress and disuse muscle atrophy: Historical overview

The first report linking oxidative stress to disuse muscle atrophy appeared almost 2 decades ago (31). This study, using a rat immobilization model of skeletal muscle disuse, revealed that muscle inactivity is associated with increased muscle lipid peroxidation and that inactivity-induced muscle atrophy can be partially prevented by treatment with the antioxidant vitamin E. Since this early observation, numerous animal studies have confirmed that prolonged skeletal muscle inactivity promotes oxidative stress in the inactive muscle and a growing number of studies reveal that "select" antioxidants can delay disuse muscle atrophy (3, 6, 72). These results have prompted many investigators to conclude that inactivity-induced redox disturbances play an important role in disuse muscle atrophy.

Sources of oxidant production in quiescent skeletal muscles

It is established that ROS are produced in both inactive and contracting skeletal muscles (31, 56). When ROS production in cells exceeds the antioxidant capacity to maintain the normal redox balance, a pro-oxidant state occurs and disturbed redox signaling follows.

Until the early 1990s, it was widely believed that ROS production was limited in noncontracting skeletal muscle and that oxidative damage is not present in inactive muscles. However, many human and animal studies now indicate that oxidative injury occurs in muscle fibers during periods of disuse in locomotor skeletal muscles (10, 31, 32, 34) and in the diaphragm during prolonged mechanical ventilation (25, 36). At present, we do not have a complete understanding of the oxidant producing pathways that are responsible for inactivity-induced oxidative stress in skeletal muscles. Excess production of nitric oxide (NO) has been associated with muscle wasting during some pathological myopathies, such as sepsis and chronic heart failure (1, 8, 46). However, whether NO production increases in skeletal muscle during unloading-induced atrophy is uncertain. Suzuki and colleagues, using a hindlimb suspension model of muscle inactivity, demonstrated that this type of locomotor muscle atrophy was accompanied by a rapid dissociation of nNOS from the dystroglycan complex and increased NO production measured by electron paramagnetic resonance spectrometry (64). This increased NO production was deemed significant since genetic or pharmacological inhibition of nNOS activity attenuated muscle atrophy during 14 days of unloading. In contrast, no evidence of increased NO production or nitrositive stress was detected in the rat diaphragm during 18 h of mechanical ventilation (70). Hence, whether excess NO production contributes to redox disturbance during muscle unloading is controversial, and additional research is required to determine if increased NO production contributes to disuse-muscle atrophy. Therefore, because of the paucity of data regarding NO and muscle atrophy, the remainder of this review will focus on the well-established role that ROS plays in inactivity-induced muscle atrophy.

With regard to the sites of oxidant production in inactive skeletal muscle, new evidence suggests that mitochondria may be an important source of ROS production in inactive diaphragm muscle (28). Indeed, mitochondria isolated from diaphragm muscle of mechanically ventilated animals release $\sim$ 40% more ROS in state 4 respiration compared to mitochondria removed from control animals (28). The mechanisms responsible for this inactivity-induced increase in mitochondria ROS production remain unknown. It is also possible that both xanthine oxidase and NADPH oxidase make small contributions to inactivity-induced ROS production in muscle (44, 71) (Fig. 2).Hence, it appears that inactivity-induced ROS production in skeletal muscle is derived from several different sites and additional work is required to complete our understanding of the control of ROS producing pathways in skeletal muscle during prolonged periods of inactivity.

Oxidative stress can inhibit protein synthesis

Protein synthesis in cells is accomplished by a complex network of signaling pathways that culminate in the translation of mRNA into a specific protein. The rate of protein synthesis is largely controlled by the efficiency of translation that is regulated primarily at the level of initiation (29). In eukaryotes, a key step in translation is the binding of the initiation factor eIF4F to mRNA molecules with a 5′-terminal 7-methylGTP cap (49). In animal cells, the eIF4F is a complex composed of three subunits (48): a) eIF4E, the cap binding subunit; b) eIF4A, an ATP-dependent RNA helicase; and c)

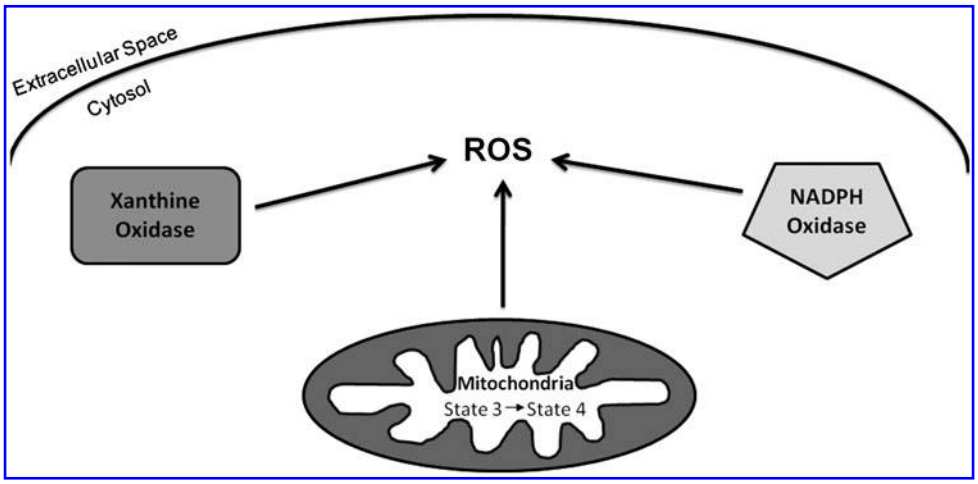

FIG. 2. Simplified diagram illustrating pathways capable of producing superoxide (O_2^-) in skeletal muscle during periods of disuse. Candidates for the production of reactive oxygen include NADPH oxidase, xanthine oxidase, and muscle mitochondria.

eIF4G that serves as a scaffold protein for assembly of eIF4E and eIF4A to form the eIF4F complex.

The eIF4E subunit is one of the main regulators of the assembly of the eIF4F complex because it is present in limited molar amounts (18). The availability of eIF4E to form the eIF4F complex is controlled by its reversible association with the 4E-binding proteins (*e.g.*, 4E-BP1) (29). Hypophosphorylated 4E-BP1 can block eIF4F assembly because this molecule competes with eIF4G for binding to eIF4F (29). However, phosphorylation of 4E-BP1 via the mammalian target of rapamycin (mTOR) causes 4E-BP1 to dissociate from eIF4E, allowing the initiation complex to form and translation to proceed (29, 49) (Fig. 3). Conversely, dephosphorylation of 4E-BP1 by a protein phosphatase (*e.g.*, PP1/PP2A) results in increased association of 4E-BP1 with eIF4E and inhibition of the formation of the eIF4F complex (49).

Growing evidence suggests that oxidants depress protein synthesis by decreasing phosphorylation of 4E-BP1, thereby hindering mRNA translation at the level of initiation (2, 48, 49, 60, 73). For example, exposure of cardiac myocytes to oxidative stress generated by H_2O_2 can inhibit global protein synthesis by $\sim$ 90% (49). Further, treatment of myocytes with H_2O_2 promotes increased protein phosphatase activity, resulting in the dephosphorylation of 4E-BP1 and increased association of 4E-BP1 with eIF4E (49). This binding of 4E-BP1 with eIF4E is consistent with the observed H_2O_2-mediated decrease in both translation and protein synthesis. Similarly, oxidant stress has been shown to decrease the level of 4E-BP1 phosphorylation in other cell types (*e.g.*, PC12 cells) (48). Finally, recent work suggests that oxidants may also impede protein synthesis by impairing mTOR assembly and therefore preventing mTOR-mediated phosphorylation of 4E-BP1 (73).

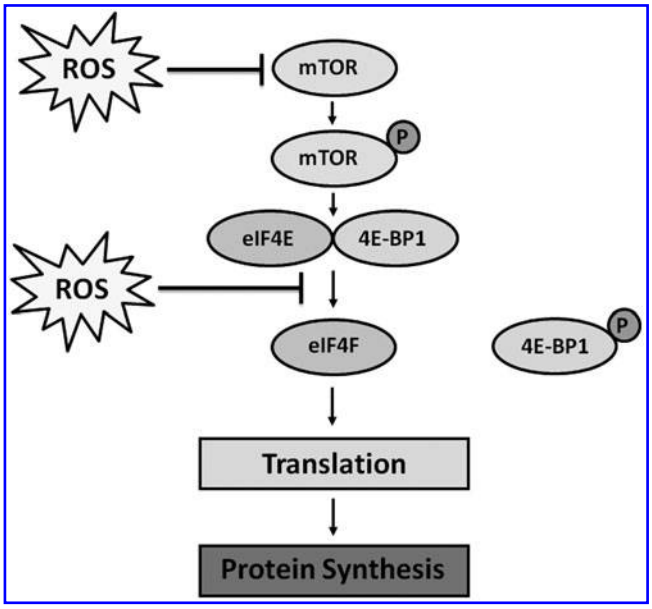

FIG. 3. Reactive oxygen species depress global protein synthesis in cells. A potential mechanism to explain the impact of ROS on protein synthesis is that high levels of ROS can inhibit translation at the level of initiation, in part, by reducing phosphorylation of the eIF4E repressor protein, 4E-BP1. See text for more details.

To summarize, accumulating evidence indicates that oxidative stress can suppress protein synthesis. Further, these reports document the capacity for high levels of H_2O_2 to inhibit mRNA translation at the level of initiation, in part, by reducing phosphorylation of the eIF4E repressor protein, 4E-BP1. Therefore, in theory, the decrease in muscle protein synthesis that occurs during prolonged disuse could be linked to the increased production of ROS that occurs in inactive skeletal muscle. Note, however, that all of the aforementioned studies were conducted on cells in culture and it is currently unknown if oxidative stress plays an important role in the disuse-mediated depression of muscle protein synthesis that occurs in skeletal muscle *in vivo*.

Oxidative stress promotes proteolysis

Growing evidence indicates that oxidative stress can promote muscle protein breakdown in three major ways. First, oxidative stress can increase the gene expression of key components of autophagy, calpain, and the proteasome system of proteolysis. Second, inactivity-induced oxidative stress in skeletal muscle has been shown to activate both calpain and caspase-3. Finally, ROS can also promote proteolysis in muscle fibers by the oxidative modification of myofibrillar proteins which enhances their susceptibility to proteolytic processing. A discussion of each of these links between oxidative stress and proteolysis follows (Fig. 4).

Oxidants increase the expression of proteolytic proteins

The primary proteases in skeletal muscle can be classified into four major categories: a) autophagy (*i.e.*, lysosomal proteases); b) the proteasome system; c) calpains; and d) caspase-3. Growing evidence reveals that cellular oxidative stress can increase the expression of key autophagy proteins, important proteins within the proteasome system, and calpains.

ROS increases expression of key autophagy proteins. Macroautophagy (hereafter referred to as autophagy) is a highly regulated lysosomal pathway for the degradation of non-myofibril cytosolic proteins and organelles (74). During autophagy, cytosolic components are sequestered into double membrane vesicles called autophagosomes, which fuse to lysosomes to form autolysosomes. After autophagosome formation, the cytosolic constituents are degraded by lysosomal proteases (*i.e.*, cathepsins) that are the cellular proteases charged with the removal of both organelles and nonmyofibril cytosolic protein aggregates (45).

Our understanding of the molecular mechanisms that regulate autophagy has advanced in recent years. Autophagy-related genes and the proteins that they express are all described by the common acronym of "Atg" followed by a number to identify the specific gene or protein (*e.g.*, Atg1, Atg2, etc.). The role that each of these Atg proteins play in autophagy continues to be investigated but it appears that at least 16 Atg proteins are involved in autophagy in mammals (45). The steps leading to autophagy and some of the key proteins that contribute to these steps are illustrated in Figure 5.First, the induction of autophagy (*i.e.*, formation of the preautophagasome structure) occurs by activation of the Atg1 complex. Second, the assembly of a partial autophagosome membrane (*i.e.*, nucleation) is achieved by the recruitment of

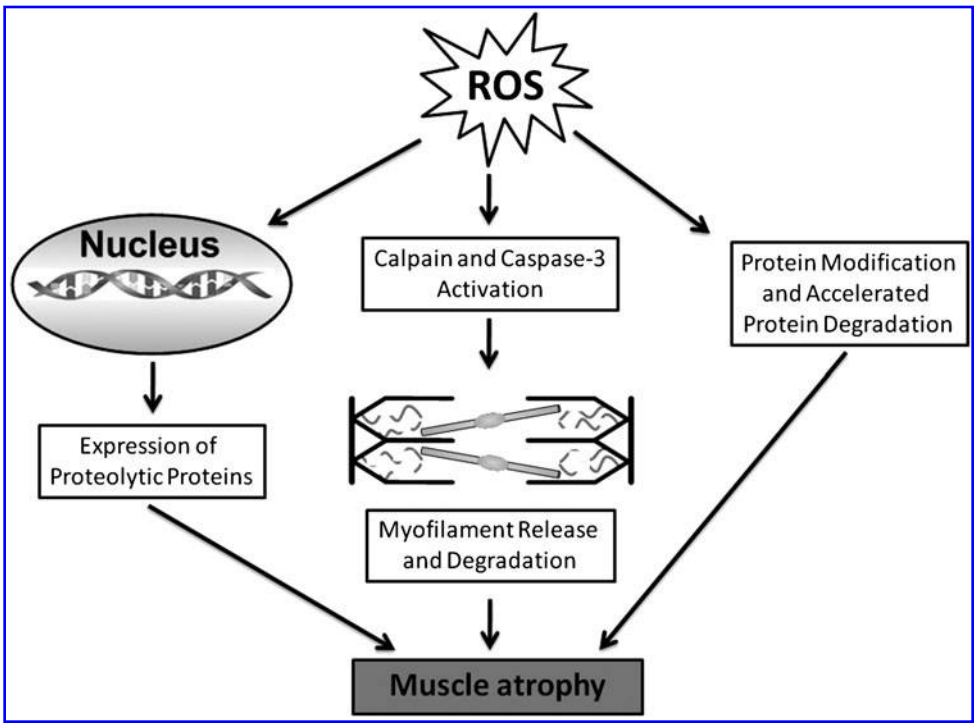

FIG. 4. Oxidative stress can promote muscle protein breakdown in three major ways: 1) Oxidative stress increases the gene expression of key proteins involved in autophagy, calpain, and the proteasome system of proteolysis; 2) Cellular oxidative stress can activate both calpain and caspase-3; 3) Oxidants modify the structure of myofibrillar proteins and enhance their susceptibility to proteolytic processing.

several Atg proteins including the essential Atg6 (called beclin-1 in mammals) (9). The next step in autophagy involves the expansion and completion of the autophagosome and requires numerous autophagy-related proteins, including Atg7 and Atg8 (LC3 in mammals). The subsequent step in autophagy involves the fusion of the autophagosome with the lysosome (5). In this regard, emerging evidence indicates that a lysosomal membrane protein Lamp-2 is essential for autophagosome–lysosome fusion to occur (26). Finally, fusion of the autophagosome and lysosome results in the exposure of autophagosome contents (*i.e.,* cytosolic proteins) to lysosomal proteases, resulting in proteolytic degradation. Important lysosomal proteases expressed in skeletal muscle include cathepsins B, D, and L (5).

Emerging evidence suggests that a baseline level of autophagy is required for maintenance of normal muscle function and mass. Indeed, muscle-specific knockout of Atg7 in mice prevents autophagosome formation and results in dramatic skeletal muscle atrophy and weakness (41). These new findings highlight the importance of a baseline level of autophagy for the clearance of damaged proteins/organelles and maintenance of normal muscle function.

Although it is established that several lysosomal proteases (*i.e.,* cathepsin B, D, and L) are activated in skeletal muscle undergoing disuse atrophy (5), the role that the autophagic proteolytic system plays in muscle atrophy has received limited attention. Nonetheless, recent studies reveal that increases in autophagy (above baseline) contribute to skeletal muscle atrophy due to fasting or denervation (39, 47). Moreover, studies reveal that autophagosomes are formed in diaphragm muscle during prolonged MV, indicating that autophagy contributes to MV-induced diaphragmatic proteolysis (25).

In reference to oxidative stress and autophagy, evidence suggests that increased cellular ROS production promotes the expression of autophagy-related genes (*e.g.,* Beclin-1 and cathepsin L) in nonmuscle cell lines (4, 57, 69) (Fig. 5). Further, a recent report speculates that inactivity-induced oxidative stress can promote expression of autophagy-related proteins in human skeletal muscle (25). Nonetheless, definitive evidence that cellular ROS production increases the expression of Beclin-1 and cathepsin L in skeletal muscle does not currently exist.

ROS increases expression of key proteins within the proteasome system of proteolysis. The total proteasome complex (26S) is comprised of a core proteasome subunit (20S) coupled with a regulatory complex (19S) connected to each end of the 20S core (23). The 26S proteasome degrades ubiquitinated proteins. Therefore the 26S proteasome degradation pathway is active only after ubiquitin covalently binds to protein substrates and marks them for degradation. The binding of ubiquitin to protein substrates is a three-step process that initially requires the ubiquitin-activating enzyme (E1). Following activation, the ubiquitination of specific proteins is provided by one of a variety of ubiquitin-conjugating enzymes (E2s) and by specialized protein ligases (E3s) that recognize specific protein substrates (Fig. 6).For example, the ubiquitin-conjugating enzyme $E2_{14k}$ is an important regulator of skeletal muscle ubiquitin–protein conjugation (38). Further, $E2_{14k}$ interacts with a specific E3 ligase (*i.e.,* E3α) to promote muscle protein degradation in catabolic states. In addition, other unique skeletal muscle ubiquitin E3 ligases (*e.g.,* atrogin1 and muscle ring finger-1) exist and these ligases play essential roles in skeletal muscle atrophy (7, 22, 55, 65).

Reports indicate that oxidative stress promotes increased gene expression of key proteins involved in the proteasome system of proteolysis. For example, *in vitro* experiments have demonstrated that exposure of C2C12 myotubes to H_2O_2 upregulates the expression of specific E2 and E3 proteins that

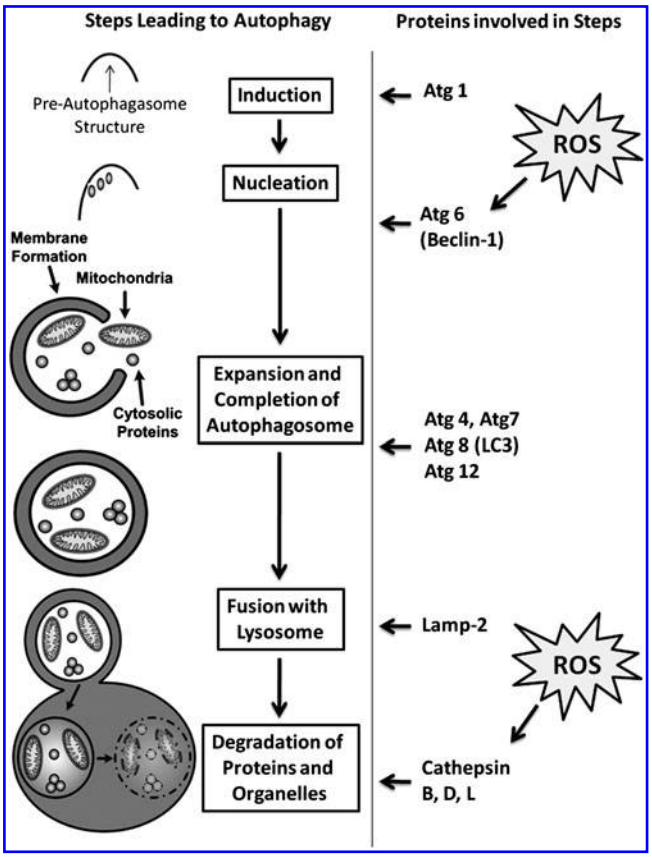

FIG. 5. Steps leading to autophagy and the key autophagy proteins involved in each step. Note that ROS has been shown to increase the expression of Atg6 (Beclin-1) and cathepsins B,D, and L in cell culture.

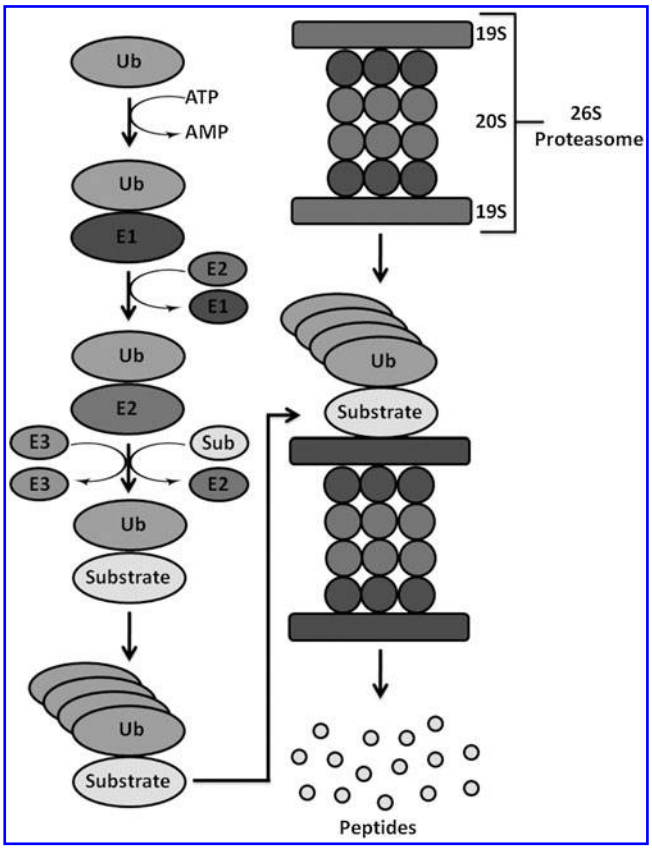

FIG. 6. Some of the key components of the 26S proteasome system of proteolysis. The total proteasome complex (26S) is comprised of a core proteasome subunit (20S) coupled with a regulatory complex (19S) connected to each end of the 20S core. The binding of ubiquitin to protein substrates is a three-step process that initially requires the ubiquitin-activating enzyme (E1), a variety of ubiquitin-conjugating enzymes (E2s), and specialized protein ligases (E3s) that recognize specific protein substrates. See text for more details.

contribute to muscle protein breakdown, including $E2_{14k}$, atrogin1, and muscle ring finger-1 (38, 42). Similarly, TNF-α induced increases in ROS production within myotubes is also associated with increased expression of atrogin1 (37). Collectively, these findings indicate that ROS-induced oxidative stress is capable of promoting myotube expression of key components of the proteasome system of proteolysis.

ROS increases calpain expression. Calpains are Ca^{2+}-dependent cysteine proteases that are located in all vertebrate cells (21). Although numerous members of the calpain family of proteases exist, the two best characterized calpains found in skeletal muscle are calpain 1 and calpain 2 (21). Active calpains have been shown to release sarcomeric proteins by cleaving cytoskeletal proteins (*e.g.*, titin, nebulin) that anchor contractile elements (30, 54). Moreover, calpain is known to degrade several cellular kinases and phosphatases and can also degrade oxidized contractile proteins such as actin and myosin (21, 24, 66).

Recent studies reveal that oxidative stress can increase the expression of calpains in both C2C12 myotubes and human myoblasts. Specifically, exposure of C2C12 myotubes to H_2O_2 has been shown to increase calpain 1 mRNA levels (42). Similarly, exposure of human myoblasts to H_2O_2 increases the expression of both calpain 1 and calpain 2 (11). Together, these reports indicate that ROS-induced oxidative stress is capable of promoting calpain expression in muscle cells in culture. However, it is currently unclear if oxidative stress increases calpain expression in muscle fibers *in vivo*.

Oxidative stress increases protease activation in muscle fibers

As discussed previously, it is established that oxidants can increase the expression of several key proteases. In addition, growing evidence indicates that oxidants can also activate cellular proteases that normally exist in an inactive state. A brief summary of evidence that increased ROS production in muscle cells can activate both calpain and caspase-3 follows.

ROS promotes calpain activation in skeletal muscle. Recent studies reveal that oxidative stress increases calpain activity in muscle cells in culture. For example, exposing C2C12 myotubes to H_2O_2 increases calpain 1 activity and promotes myotube atrophy (42). Also, exposure of human myoblasts to H_2O_2 elevates both calpain 1 and calpain 2 activity (11). Importantly, a recent study has also demonstrated that prevention of oxidative stress via antioxidants can prevent calpain 1

activation in inactive diaphragm muscle *in vivo* (72). Together, these investigations show that increased production of ROS in skeletal muscle is capable of activating calpain both *in vitro* and *in vivo*.

The mechanism(s) responsible for ROS-mediated calpain activation remains unclear. Calpain activity in cells is regulated by several factors, including cytosolic calcium levels and the concentration of the endogenous calpain inhibitor, calpastatin (21). Specifically, calpain activity is increased by a sustained elevation in cytosolic free calcium and/or a decrease in cytosolic levels of the calpain inhibitor, calpastatin (21). In regard to calpain's contribution to disuse muscle atrophy, it is clear that skeletal muscle inactivity is associated with an increase in both cytosolic calcium levels and calpain activity (33). Although the mechanism responsible for this inactivity-mediated calcium overload is unknown, it is feasible that intracellular ROS production could play an important role in disturbances in calcium homeostasis (27). A potential mechanism to link oxidative stress with calcium overload is that ROS-mediated formation of reactive aldehydes (*i.e.*, 4-hydroxy-2,3-trans-nonenal) can inhibit plasma membrane Ca^{+2} ATPase activity (61). It follows that an oxidative stress-induced decrease in membrane Ca^{+2} ATPase activity would impede Ca^{+2} removal from the cell and promote intracellular Ca^{+2} accumulation. Nonetheless, it is currently unknown as to whether this mechanism is the single explanation for inactivity-mediated calcium overload in muscle.

ROS activation of caspase-3 in skeletal muscle. Evidence indicates that active caspase-3 contributes to muscle protein degradation and fiber atrophy (17, 43). In this regard, caspase-3 activation promotes degradation of actomyosin complexes, and inhibition of caspase-3 activity suppresses the overall rate of proteolysis in diabetes-mediated cachexia and myofiber atrophy in mechanical ventilation-induced inactivity of the diaphragm (17, 43).

Recent reports indicate that oxidative stress can activate caspase-3 in muscle fibers *in vitro* and *in vivo*. For example, exposing C2C12 myotubes to H_2O_2 has been shown to activate capase-3 and promote apoptosis (62). Notably, new evidence reveals that antioxidant-mediated protection against inactivity-induced oxidative stress prevents caspase-3 activation in diaphragm muscle *in vivo* (72). Together, these reports are consistent with the concept that inactivity-induced ROS production in skeletal muscle can activate caspase-3.

Control of caspase-3 activity in the cell is complex and involves numerous interconnected signaling pathways. In the case of inactivity-induced muscle atrophy, it has been postulated that caspase-3 is activated by caspase-12 (via a calcium release pathway); and/or activation of caspase-9 (via a mitochondrial pathway) (51). A potential interaction between these caspase-3 activation pathways is that both of these can be activated by ROS (52, 53). Nonetheless, at present, it is unclear which of these pathways is responsible for the ROS-mediated activation of caspase-3 in skeletal muscle during prolonged periods of inactivity.

Oxidation enhances muscle protein degradation via numerous proteases

The third and final potential link between oxidative stress and increased proteolysis in skeletal muscle is that oxidative modification of muscle proteins increases their susceptibility to proteolytic degradation. Using several purified proteases, Davies and colleagues first demonstrated that ROS accelerates the protease-mediated breakdown of proteins (12). This observation has been expanded by others, and it is now established that oxidized proteins are readily degraded by many proteases, including the 20S proteasome, calpains, and caspase-3 (23, 63). For example, recent evidence indicates that oxidation increases the susceptibility of skeletal muscle myofibrillar proteins to degradation by both calpains and caspase-3. In particular, oxidation increases myofibrillar protein breakdown in a dose-dependent manner and following oxidative modification, myosin heavy chain, α-actinin, actin, and troponin I are all rapidly degraded by calpains (I and II) and caspase-3 (63).

The biochemical mechanism to explain why oxidation accelerates protein degradation is as follows. In brief, oxidative modification of muscle proteins can increase their susceptibility to proteolysis, in part due to unfolding of the molecule (12–14). Specifically, oxidative modification of a protein results in a change of the secondary or tertiary structure so that previously shielded peptide bonds are exposed to enzymatic hydrolysis. Based upon this concept, Davies *et al*. has predicted that numerous proteases will degrade oxidized proteins more efficiently than normal proteins (15). Several reports have provided experimental data that are consistent with this concept (12, 13, 23, 63).

Conclusions and Unanswered Questions

In conclusion, inactivity-induced skeletal muscle atrophy occurs in a variety of conditions including prolonged bed rest, limb immobilization, mechanical ventilation, and space flight. Several lines of evidence directly connect ROS to disuse muscle atrophy via ROS-mediated regulation of proteolysis. Specifically, oxidative stress can accelerate proteolysis by: a) increasing gene expression of important proteins involved in several proteolytic systems (*i.e.,* autophagy, calpain, and proteasome); b) activation of calpains and caspase-3; and c) enhancing protein susceptibility to proteolytic processing. Further, it is also feasible that oxidative stress can depress global muscle protein synthesis which will also contribute to a net loss of protein and muscle atrophy.

Although it is clear that ROS-mediated signaling contributes to disuse muscle atrophy, numerous unanswered questions remain. For example, which ROS pathways are active in unloaded skeletal muscle? Moreover, if multiple oxidant production pathways are active, what is the relative contribution of each pathway to the regulation of cell signaling pathways involved in disuse muscle atrophy? Resolution of these issues will provide the information needed to develop therapeutic strategies to prevent oxidant production or scavenge ROS to prevent disturbed redox signaling in the muscle fiber during prolonged periods of inactivity.

Although growing evidence indicates that oxidative stress can suppress protein synthesis of cells in culture, it is unknown if oxidative stress plays an important role in the disuse-mediated depression of muscle protein synthesis that occurs in skeletal muscle *in vivo.* Hence, this topic remains an important area for future work.

Accumulating results suggests that oxidative stress can promote the expression of key proteins involved in autophagy

along with increased expression of both calpains and caspase-3 in cells in culture. Nonetheless, definitive evidence that cellular ROS production increases the expression of Beclin-1 and cathepsin L in skeletal muscle *in vivo* does not currently exist. Similarly, it is currently unclear if oxidative stress increases the expression of both calpain and caspase-3 in muscle fibers *in vivo*.

A final key question is whether production of ROS is a requirement for disuse muscle atrophy or does oxidative stress merely regulate the rate of muscle atrophy? A related question is: do ROS simply act as second messengers to control muscle atrophy or is ROS-mediated protein oxidation a requirement for the rapid onset of disuse muscle atrophy? Both of these questions are important and remain unresolved.

Hopefully, questions outlined in this review stimulate muscle biologists to pursue research in the area of ROS and skeletal muscle atrophy. Future scientific advances in cell signaling will provide the tools required to answer these important questions that will ultimately result in therapeutic approaches to prevent or diminish disuse muscle atrophy.

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Abbreviations Used

Atg = autophagy gene
mTOR = mammalian target of rapamycin
NO = nitric oxide
ROS = reactive oxygen species

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