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Invited Review

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The molecular basis of skeletal muscle atrophy

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Jackman, Robert W., and Susan C. Kandarian. The molecular basis of skeletal muscle atrophy. *Am J Physiol Cell Physiol* 287: C834–C843, 2004; 10.1152/ajpcell.00579.2003.—Skeletal muscle atrophy attributable to muscular inactivity has significant adverse functional consequences. While the initiating physiological event leading to atrophy seems to be the loss of muscle tension and a good deal of the physiology of muscle atrophy has been characterized, little is known about the triggers or the molecular signaling events underlying this process. Decreases in protein synthesis and increases in protein degradation both have been shown to contribute to muscle protein loss due to disuse, and recent work has delineated elements of both synthetic and proteolytic processes underlying muscle atrophy. It is also becoming evident that interactions among known proteolytic pathways (ubiquitin-proteasome, lysosomal, and calpain) are involved in muscle proteolysis during atrophy. Factors such as TNF-α, glucocorticoids, myostatin, and reactive oxygen species can induce muscle protein loss under specified conditions. Also, it is now apparent that the transcription factor NF-κB is a key intracellular signal transducer in disuse atrophy. Transcriptional profiles of atrophying muscle show both up- and downregulation of various genes over time, thus providing further evidence that there are multiple concurrent processes involved in muscle atrophy. The purpose of this review is to synthesize our current understanding of the molecular regulation of muscle atrophy. We also discuss how ongoing work should uncover more about the molecular underpinnings of muscle wasting, particularly that due to disuse.

protein synthesis; protein degradation; nuclear factor-κB; disuse; unloading; cachexia

OVERVIEW

Skeletal muscle atrophy is a change that occurs in muscles of adult animals as a result of the conditions of disuse (e.g., immobilization, denervation, muscle unloading), aging, starvation, and a number of disease states (i.e., cachexia). Regardless of the inciting event, skeletal muscle atrophy is characterized by a decrease in protein content, fiber diameter, force production, and fatigue resistance. The different types of conditions producing atrophy imply different types of molecular triggers and signaling pathways for muscle wasting. This review focuses on our current knowledge of the molecules involved in disuse atrophy.

Although the molecular aspects of atrophy have received increased attention in the literature, the detailed reviews on disuse atrophy are concerned with the characterization of morphological and physiological changes (12, 20, 90). With cachexia, there have been multiple reviews, each having a slightly different focus, that summarize work on putative triggers and signaling molecules and on details of the proteolytic processes governing this type of muscle wasting (37, 38, 57, 58). There are no comprehensive reviews, however, on the molecular basis of disuse atrophy with an organized discussion on the whole process, from the potential triggers and signaling molecules to the final effects on the myofibrillar apparatus.

After the initial physiological stimulus, in which the skeletal muscle no longer bears weight or contracts with tension, molecules involved with disuse atrophy, such as initiating triggers, signaling proteins, and affected targets, carry out the process of muscle protein loss. While it has been well established that muscle disuse due to the removal of weight bearing leads to an early decrease in protein synthesis and an increase in protein degradation rate (28, 64, 91), the upstream molecules regulating these changes are poorly defined. Figure 1 illustrates the organizational hierarchy of molecular components that may be involved in the atrophy pathway. This hierarchy serves as a framework for the discussion in the text. A further purpose of this review is to indicate the areas needed in molecular research on disuse atrophy, and we do, in several cases, compare and contrast disuse data with those data on cachexia, a family of atrophies triggered by humoral changes. Muscle atrophy due to cachexia affects many of the same molecular targets in muscle as that caused by disuse. However, in disuse, the initiating triggers are much less clearly understood, and experimental models are created by physical means (e.g., hindlimb suspension, bed rest, or spaceflight) rather than by the addition of defined triggering chemicals such as TNF-α.

We present this review in three parts, focusing first on the molecular triggers and signals hypothesized to play a role in disuse. These include a discussion of the nuclear factor (NF)-κB pathway, which is just presently being described in

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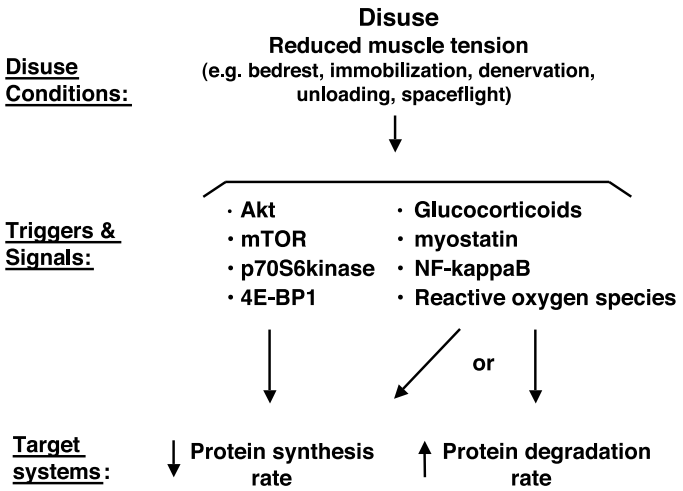

Fig. 1. Flow diagram summarizing the theoretical framework used as the basis for this review. Listed are many of the triggers and signals and the target systems that have been implicated in various disuse conditions that produce skeletal muscle atrophy.

detail for disuse but also has been described using different approaches for cachexia. Next, we present a review of the work on the target systems in atrophy, defined here as protein synthesis and protein degradation (i.e., proteolysis), although ultimate targets could be thought of as the proteins being downregulated, such as myofibril and metabolic proteins. We conclude by indicating how microarray data already are providing candidates for further study of the molecular underpinnings of disuse atrophy. We present the approaches that seem to be the most promising to test the involvement of these new candidates in understanding the molecular basis of disuse atrophy.

MOLECULAR TRIGGERS AND SIGNALING MOLECULES INVOLVED IN MUSCLE ATROPHY

Various triggers and signaling proteins have been studied for roles in regulating disuse atrophy. With the exception of the known signaling proteins directly upstream of translation, it is often not clear whether a trigger or signal affects protein synthesis or degradation in muscle atrophy. The cascade of events that lead to disuse atrophy, beginning with reduced muscle tension and extending to the effects on protein synthesis and degradation, is not known, but several potential triggers and signaling molecules have been identified.

Myostatin

A protein belonging to the TGF-β family, known as myostatin, has been shown to be a strong negative regulator of muscle growth (reviewed in Ref. 78). Knockout or mutation of this protein produces animals with markedly enlarged muscles as a result of hypertrophy and hyperplasia. When postnatal inactivation of myostatin is achieved using the Cre-Lox system, hypertrophy is also seen to the same magnitude as that in a constitutive myostatin knockout mouse (32). Conversely, myostatin can induce atrophy via an inhibitory effect on translation. Systemic administration of this negative growth regulator leads to muscle wasting in mice (107), and treatment of cultured muscle cells with recombinant myostatin has resulted in the loss of protein and reduced protein synthesis rates (89). Moreover, myostatin expression is increased in some types of muscle atrophy (13, 55, 65). Human immunodefiency virus (HIV)-infected men have shown higher levels of serum myostatin (31), indicating that myostatin may contribute to cachexia-type atrophy. It appears that *mdx* mice injected with myostatin antibodies for 3 mo showed an increase in muscle mass (11). Importantly, however, mice with muscle hypertrophy due to knockout of the myostatin gene show muscle atrophy that is equal to or greater than that on wild-type mice in response to unloading (68). This finding indicates that unloading-induced atrophy does not require myostatin. Thus, whereas myostatin may contribute to atrophy in cachexia or disuse, it is not required for disuse atrophy. Testing whether blocking myostatin, with peptides or other drugs, can inhibit disuse will be of further help in understanding its contribution to atrophy in the whole animal.

Glucocorticoids

The synthetic glucocorticoid dexamethasone is widely used to induce muscle proteolysis either in vivo (reviewed in Ref. 36) or in cell culture (se e.g., Ref. 93). In skeletal muscle, glucocorticoids decrease the rate of protein synthesis and increase the rate of protein degradation (27, 66, 97). Both disuse atrophy (see e.g., Ref. 51) and cachexia (reviewed in Ref. 58) are associated with increases in circulating glucocorticoid levels. Moreover, the binding capacity of corticosteriods also was increased markedly with disuse atrophy (84), and so it seemed plausible that glucocorticoids could be an important trigger. However, when adrenalectomized animals underwent unloading, with or without cortisol treatment, atrophy still occurred (51). Importantly, treatment of unloaded rats with an inhibitor of glucocorticoids, RU-38486, also did not inhibit disuse atrophy (95). Thus glucocorticoids do not appear to be required for disuse atrophy. In the case of cachexia, glucocorticoids seem to be a contributing factor to muscle wasting (reviewed in Ref. 58), in part because rats treated with RU-38486 plus TNF-α showed reduced proteolysis, but protein loss was not completely attenuated (35, 106).

TNF-α and Other Cytokines

There is no evidence that TNF-α or other cytokines are involved in disuse atrophy, and our laboratory (47) found no difference in TNF-α protein levels in unloaded muscle. However, there is significant literature on the role of cytokines in cachexia showing that TNF-α and other cytokines such as IL-1 and IL-6 are increased in these conditions (reviewed in Ref. 69). Administration of TNF-α can induce cachexia (69), and blockade of TNF-α by torbafylline in rats with either cancer or sepsis prevents muscle wasting (15). TNF-α treatment alone also leads to increased protein degradation in cultured muscle cells (62). Thus, with cachexia, but not with disuse, cytokines are key triggers of muscle wasting. One likely reason that cytokines are involved with cachexia but do not appear to be involved in disuse atrophy is that the latter is a local phenomenon whereas the former involves systemic triggers.

In addition to TNF-α, a protein named PIF (proteolysis-inducing factor) also has been shown to have the potential for acting as a trigger of atrophy in cancer cachexia (79). Although isolated from more than one tumor type, this molecule is only

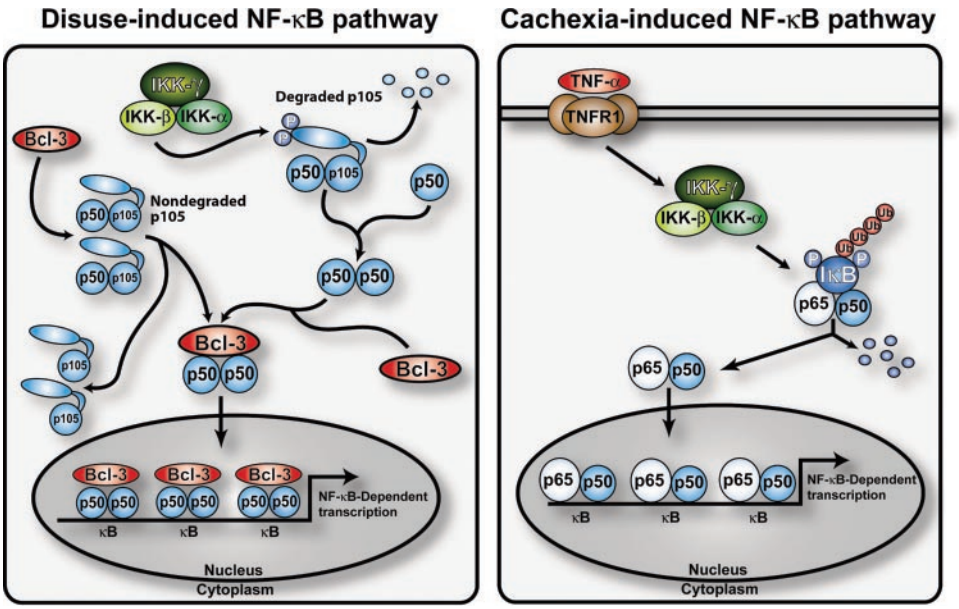

Fig. 2. Comparison of NF-κB pathways involved in disuse vs. cachexia muscle atrophy. Disuse atrophy requires p50 (derived from the processing of p105) and Bcl-3 but not p65. Bcl-3 is known to activate p50 homodimer-mediated gene expression (21) via several possible mechanisms (39, 101). Cachexia involves the canonical pathway requiring the p65 (RelA) family member. The target genes include members of the ubiquitin-proteasome pathway, and there are likely many others. TNFR1, TNF receptor type 1.

in the very early stages of study, but its activity has been linked to NF-κB activation (102). To date, there have been no published studies on the potential role of PIF in disuse atrophy.

NF-κB Signaling

NF-kB represents a family of five transcription factors [p65 (Rel A), Rel B, c-Rel, p52, and p50] that mediate a variety of processes depending on the cell type and upstream trigger. Examples include apoptosis, immunity and inflammation, and development and differentiation (for review, see Ref. 5). All family members are expressed in skeletal muscle (47). Activation of NF-κB is achieved by nuclear transport of heterodimers of NF-κB family members and often occurs by the ubiquitination and degradation of the inhibitory protein IκB, which otherwise binds NF-κB heterodimers and retains their cytosolic residence. p50 and p52 can form homodimers and undergo nuclear translocation. The production of these homodimers comes from the partial processing of their cytoplasmic precursor molecules, p105 (p50) and p100 (p52). The NF-κB transcription factor complex has been implicated in muscle atrophy attributable to both disuse and cachexia, but the specific family members involved in the two types of atrophy are distinct. This is important, because it indicates that there are differences in the molecular signaling for these two types of atrophy and, therefore, that there may be more specific molecules to target in the development of therapies. While our recent work shows that a specific NF-κB pathway is required for disuse atrophy, the upstream triggers or downstream target genes have not been elucidated. In cachexia, the absolute requirement of NF-κB in vivo is not yet firm, but there is more known about the upstream triggers and downstream target genes. It seems that at least one way that NF-κB may induce atrophy is by the transcriptional activation of ubiquitination proteins (60).

Our laboratory (47) first showed several years ago that muscle disuse leads to increased transcriptional activity of NF-κB. The luciferase activity from an NF-κB-dependent reporter plasmid injected into soleus muscles of (7 day) unloaded rats was increased eightfold compared with that in weight-bearing control rats, whereas AP-1- or NFAT (nuclear factor of activated T cells)-dependent reporter plasmids did not increase with unloading. Moreover, while the prototypical NF-κB family member p65 did not show increased nuclear levels, p50 and Bcl-3 (a nuclear IκB family member) were markedly increased. These observations were further supported by supershift assays in nuclear extracts from these muscles. More recent work has shown that, compared with wild-type mice, p50 (*nf-kb1*) knockout mice inhibit the normal decrease in muscle fiber size as the result of 10 days of unloading (Hunter RB and Kandarian SC, unpublished observations). Moreover, injection of an NF-κB reporter plasmid led to a sevenfold increase in luciferase activity in wild-type soleus muscles as the result of unloading but was unaffected by unloading in the p50 knockout mice. The same results were found using *bcl-3* knockout mice, suggesting that p50 and Bcl-3 are required for unloading-induced muscle atrophy. Because p65 does not undergo nuclear translocation during unloading atrophy (47), the NF-κB pathway activated during unloading is different from the prototypical pathway. Models for NF-κB transactivation involving a p50 homodimer bound to Bcl-3 have been described previously (21, 39, 101). The proposed pathway that may be operative during disuse atrophy on the basis of these results and other literature is shown in Fig. 2. The target genes of NF-κB in disuse are currently under investigation.

Experiments showing the involvement of NF-κB in cachexia have been performed in both cell culture and animal models. Cytokines such as TNF-α activate NF-κB in muscle cells, as they do in a variety of cell types (reviewed in Ref. 24). Cytokine activation of NF-κB induces the prototypical p65-p50 heterodimer in muscle. In cell culture, TNF-α upregulates NF-κB, increases myofibrillar proteolysis (62, 63), and suppresses myosin synthesis (33). During TNF-α treatment, genetic inhibition of NF-κB via the expression of a dominant negative form of IκB blocks the increased protein degradation (62).

The list of target genes for NF-κB during cachexia is incomplete but already appears to be complex. Using dominant negative inhibition of NF-kB, investigators have shown that TNF-induced NF-κB activation is responsible for an increase in ubiquitin-conjugating activity and upregulation of the ubiquitin-conjugating E2 enzyme, called UbcH2 (61). Thus at least one transcriptional target of NF-κB, induced by TNF-α, is involved in ubiquitin-proteasome degradation. Another group has shown that TNF-α plus IFN-γ-induced NF-κB activation leads to downregulation of MyoD protein at a posttranslational level, which in turn may lead to decreased myofibrillar synthesis (33). In that study, the NF-κB family member p65 alone could lead to downregulation of MyoD in cultured cells, and expression of a dominant negative IκB molecule rescued the high expression level of MyoD. Thus MyoD may be a second "indirect" target gene of NF-κB. A third target gene of NF-κB in muscle cells is the C3 proteasome subunit. In L6 cells, dexamethasone increased transcription of the C3 proteasome subunit gene by opposing basal NF-κB binding to the promoter region (17). Thus, in L6 cells, NF-κB apparently suppresses transcription of the C3 gene whereas dexamethasone opposes this suppression.

Animal experiments on the role of NF-κB in cachexia are less complete, but existing data are consistent with the cell culture work. One group has shown that muscles from septic rats have transient increases in NF-κB binding activity (72). Another group has shown that injection of NF-κB decoy oligonucleotides into cachexic mice with adenocarcinoma tumors attenuates the muscle wasting but not the tumor growth (52).

Oxidative Stress

Another area that has received some degree of attention is the generation of reactive oxygen species (ROS) in muscle atrophy. Unloading atrophy results in upregulation of Cu,Zn superoxide dismutase and is more damaging because of a concomitant decrease of catalase, glutathione peroxidase, and, possibly, Mn superoxide dismutase (56), the systems that would normally act to metabolize increases in ROS. Treatment of muscle cells with H_2O_2 leads to increased protein breakdown, decreased myosin expression, and increased expression of components of the ubiquitin-proteasome proteolytic pathway (30). That study (30) and other work (60) suggest that components of the ubiquitin-proteosome system are transcriptional targets of ROS signaling in cultured myotubes. Furthermore, ROS (epitomized by H_2O_2) induced activation of both the NF-κB (63) and the FOXO forkhead signaling pathways (22) in muscle cells. In the case of NF-κB, the signaling was associated with protein loss, whereas others have linked FOXO expression to decreased fiber size, via atrogin-1, in muscle (76a). Therefore, it appears possible that the increased ROS in unloading may trigger either or both of the NF-κB and FOXO signaling pathways, perhaps leading to increased proteolysis through the ubiquitin-proteosome pathway, but this has yet to be directly tested.

Myotubes treated with TNF-α have shown increased ROS, evidently released from mitochondria, that activated NF-κB and led to protein loss (59, 62, 63). Catalase treatment has been shown to inhibit the NF-κB activation and protein loss due to TNF-α, whereas addition of H_2O_2 activated NF-κB independently of TNF-α (63). These experiments suggest the possibile role of ROS as a contributing trigger to cytokine-induced muscle wasting.

Upstream Signals Specifically Affecting Protein Synthesis in Atrophy

There have been recent important advances in our understanding of translational mechanisms in skeletal muscle (reviewed in Ref. 71). There are several signaling proteins that can act as upstream regulators of translation initiation that have been associated with the decreased protein synthesis rate caused by disuse atrophy. The protein kinase Akt has been shown to have growth-promoting effects in muscle, and it is a known upstream activator of protein synthesis (75, 88). Proteins downstream of Akt that are involved in regulating protein synthesis are mTOR and p70S6 kinase (reviewed in Ref. 71). One report (10) has shown that overexpression of Akt attenuates denervation-induced atrophy in rodents by 70%. There is also a decrease in total and phosphorylated Akt after 14 days of unloading in rats. While this work suggests that Akt may have an important role, further experiments are needed to determine whether decreased phosphorylation of Akt is a necessary component of disuse atrophy. Muscle unloading also is associated with a decrease in both mTOR (74) and p70S6 kinase phosphorylation (10, 43), which is consistent with the decreased translation rate.

Another molecule whose mRNA is markedly upregulated with muscle unloading and that could affect translation is IGFBP-5 (86). If reflected at the protein level, this would have an inhibitory effect on translation because IGFBP-5 is known to sequester IGF, leading to decreases in protein synthesis (77). IGFBP-5 mRNA also has been shown to be increased with unloading or spinal transection in other laboratories (4, 34, 104). Furthermore, ectopic expression of IGF-I and/or sonic hedgehog attenuated fiber atrophy resulting from gastrocnemius/soleus muscle unloading in mice (2), and this may have been due to an effect on protein synthesis. However, decreases in the expression or activity of neither IGF-I nor sonic hedgehog have been correlated with atrophy in vivo.

TARGET SYSTEMS IN DISUSE ATROPHY

Role of Decreased Protein Synthesis in Disuse Atrophy

It is well established that muscle disuse due to the removal of weight bearing leads to an early decrease in protein synthesis rate (28, 64, 91). While translation of an mRNA to protein occurs in phases called initiation, elongation, and termination, the first two are thought to be more highly regulated (81). With disuse, what we know so far about translational regulation seems to be in the regulation of initiation. Known upstream regulators of translation initiation were discussed above. Downstream of these, 4E-BP-1 is a translation initiation factor that, when unphosphorylated, acts as a strong translational inhibitor by binding eukaryotic initiation factor (eIF)-4E. The amount of 4E-BP1 bound to eIF-4E was increased at 14 days of unloading in rat gastrocnemius muscles (10), suggesting its involvement in the decreased translation rate observed during disuse atrophy. Consistent with this finding, work from our laboratory (86) has shown that unloading atrophy leads to marked increases in inhibitory 4E-BP-1 mRNA. Starvation of

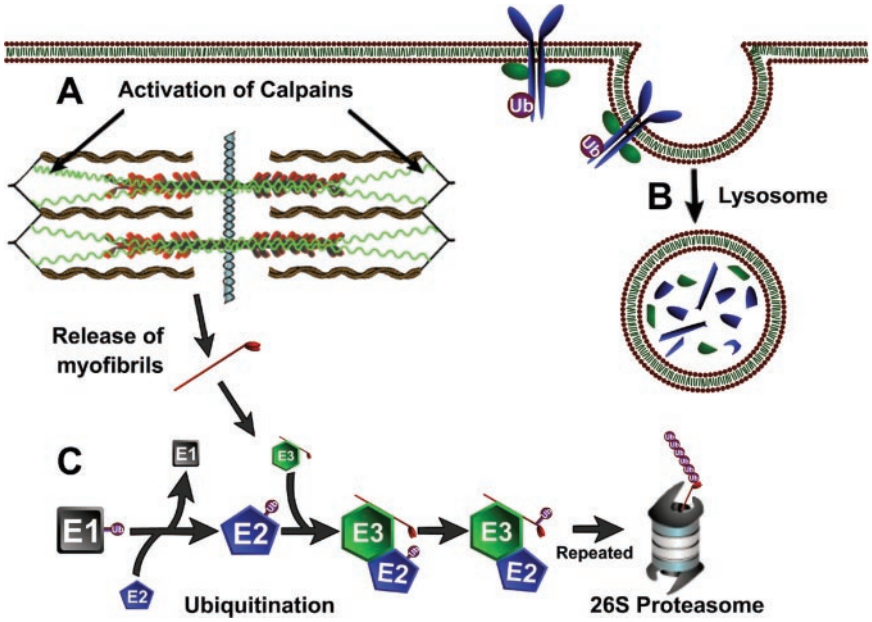

Fig. 3. Three known proteolytic systems implicated in muscle atrophy resulting from disuse or disease: the calcium-dependent calpain system (*A*), the lysosomal protease system (cathepsins; *B*), and the ubiquitin (Ub; *C*)-proteasome system. Recent evidence points toward interactive involvement of these 3 systems in proteolysis. See text for details.

rodents is another model of muscle atrophy associated with increases in 4E-BP-1 mRNA expression (50). In another case, the mRNA for eukaryotic elongation factor 2 (eEF2) kinase (eEF2K), another inhibitor of protein synthesis, was upregulated at 4 days and had a peak increase of fivefold by 7 days (86). Phosphorylation of eEF2 by eEF2K results in a global reduction in ribosomal capacity (100). Therefore, an increase in eEF2K expression, if reflected at the activity level, could lead to a decrease in the protein synthetic capacity in unloaded muscle.

Role of Increased Proteolysis in Muscle Atrophy

There is more known about the role of increased proteolysis than there is of decreased synthesis in disuse atrophy. At least half of total muscle protein is myofibrillar protein, and this fraction is lost at a faster rate than other muscle proteins during atrophy (6, 70). Ongoing work from several laboratories is aimed at determining the relative contributions of three major proteolytic systems to skeletal muscle protein loss: the cytosolic calcium-dependent calpain system, the lysosomal proteases (i.e., cathepsins), and the ATP-dependent ubiquitin-proteasome system. The involvement of the three proteolytic systems discussed in this review during muscle atrophy is shown in Fig. 3. What we are beginning to understand is that these systems work as partners during muscle proteolysis rather than one system being used exclusively during atrophy.

Role of calpains in disuse atrophy. It appears that the bulk of myofibrillar proteolysis resulting from muscle disuse involves the ubiquitin-proteasome pathway (3, 80, 87), yet it is also known that the ubiquitin-proteasome system cannot degrade intact myofibrils (80). In addition, it has been known for some time that the calpains are unable to degrade actin and myosin, although they have activity at a few specific sites (reviewed in Ref. 92). Proteins that are involved in the assembly and scaffolding of myofibrillar proteins such as titin (82), vinculin, C-protein, nebulin, and others (reviewed in Ref. 45) are known calpain substrates.

One of the first experiments to test directly for a role of calcium-mediated protein degradation during skeletal muscle atrophy was published by Marc Tischler's laboratory 14 years ago (96). His group studied protein degradation rates in whole soleus muscle caused by 3 days of unloading, using inhibitors of either calcium-dependent or lysosomal proteolysis. With respect to calcium dependency, unloading-induced protein loss was significantly attenuated when the muscles were directly injected with mersalyl during the unloading period, and protein degradation rate was attenuated when 3-day-unloaded muscles were removed and incubated in a bath with inhibitors of calcium-activated proteases. These changes were not seen when lysosomal inhibitors were tested. Strengths of this study were that an early time point was used, both in vivo and in vitro approaches were included, and the in vivo experiment was administered throughout the period of unloading. These points are likely to be crucial for the action of the calpains because they seem to be involved in myofibrillar disassembly, perhaps the earliest event in muscle-specific protein degradation. A drawback of some studies has been that the atrophy stimulus is elicited for a given number of days, and then the muscle is removed and incubated in a bath with inhibitors (87, 99). A possible reason for the lack of effect of inhibitors may be that the proteolytic system under study has already had a biological effect (partial degradation or disassembly).

A more recent study with a completely different approach also supports the role of calpains in muscle disuse atrophy. An endogenous inhibitor of the calpains, calpastatin, was overexpressed in murine skeletal muscle with the use of a transgenic approach. There was a 30% attenuation of the reduction in muscle fiber cross-sectional area in 10-day-unloaded soleus muscles compared with wild-type littermates (94). There is some inconsistency as to whether there is a change in mRNA or activity of the calpains as the result of disuse. Some studies show increases (34, 87) and some show no change (48, 83, 86). Some of the discrepancies may be related to the natural complexity of calpain regulation (endogenous activators and

inhibitors) and problems with the approaches used to measure it (discussed in Ref. 94), so it is not surprising that there are different outcomes. On the whole though, the calpains remain a likely component of the targets of muscle atrophy, especially as they relate to myofibrillar disassembly.

Other recent work has shown a role for calpains in muscle atrophy resulting from sepsis. Sepsis induced by cecal puncture leads to Z-band disintegration, increased release of myofilaments, and increased mRNA of calpain 1, calpain 2, and calpain 3 in extensor digitorum longus muscle (103). However, treatment of rats with dantrolene attenuates these changes by inhibiting the release of calcium from the sarcoplasmic reticulum. In another study (19), this same group of investigators found that dantrolene attenuates total and myofibrillar protein degradation rates in rats with sepsis. A different group of investigators (44) found similar beneficial results when treating septic rats with dantrolene or when measuring survival rates in a mouse model of endotoxemia. Voisin et al. (99) also suggested a role for the calpains in septic rats injected with *Escherichia coli*. Using inhibitors of either lysosomal or calpain-mediated proteolysis, they showed that these two mechanisms account for 20% of protein degradation during sepsis, and they suggested that this may be a crucial component of total proteolysis. However, taking caution as described above, they incubated the epitrochlearis in a bath with the inhibitors several days after the initiation of sepsis so that none of the proteolytic systems were inhibited before this point.

The caspases, proteins involved in apoptosis that have specific proteolytic activity, were recently shown to have a role in disease-related muscle atrophy (7, 18). The caspases have been proposed to have a role in the initial step in myofibrillar proteolysis by cleavage of actomyosin. In this way, the caspases may be similar to the calpains in making myofibrillar proteins available for ubiquitination. When soluble actomyosin complexes are treated with recombinant caspase-3, an actin proteolytic fragment is produced, as are other proteins that can then be degraded by the ubiquitin-proteasome system (18). These proteolytic fragments also are produced when L6 muscle cells are treated with caspase-3. It was also shown that diabetic rats and rats with cancer have increased muscle caspase activity (7, 18), and muscles of diabetic rats showed reduced proteolysis after treatment with a caspase-3 inhibitor (18). However, there are not yet any studies showing a role of the caspases in disuse atrophy.

Taken together, the studies discussed above all support the notion that calpain- and perhaps caspase-mediated proteolysis may be early rate-limiting steps in myofibrillar protein degradation during muscle atrophy.

Role of lysosomal proteolysis in disuse atrophy. A survey of the literature shows marked increases in various isoforms of cathepsin mRNAs in disuse atrophy (48, 86, 87). Some studies also show increased cathepsin protein or activity levels with unloading (87). However, when atrophying muscle resulting from disuse is treated with agents that block lysosomal acidification (96) or with agents that directly inhibit cathepsins (23, 48, 87), myofibrillar protein degradation rates are not significantly affected and total protein degradation rates are only slightly reduced. A comparison with the cachexia literature shows that the same trends hold there, too (16, 99). These observations are consistent with the current notion that cathepsins do not degrade cytosolic proteins like myofibrils but rather, their major role is to degrade membrane proteins, including receptors, ligands, channels, and transporters (reviewed in Ref. 67). Therefore, one would not expect the inhibition to have much effect on total or myofibrillar protein degradation during muscle atrophy. On the other hand, the membrane-associated proteins that are likely degraded by lysosomal pathways during atrophy, while not contributing significantly to the bulk of total muscle protein, may be key proteins contributing to the atrophied muscle phenotype. The use of muscle-specific cathepsin knockout animals and the measurement of carefully selected dependent variables should prove useful in understanding the exact role of the cathepsins during muscle atrophy.

A related area of research on proteolysis during muscle atrophy is the interaction of lysosomal and ubiquitin-proteasomal mechanisms. Just as the calpain system seems to operate in conjunction with the ubiquitin-proteasome system in degrading myofibrillar protein, it seems that the lysosomal and ubiquitin-proteasomal systems also work together to degrade specific protein substrates (reviewed in Refs. 41, 42). A number of mammalian receptors and ion channels are ubiquitinated and then are degraded by either lysosomal or proteasomal systems. The signal that determines which of these pathways is used is the type of ubiquitin modification that occurs. If a protein is ubiquitinated by a polyubiquitin chain on sequential ubiquitin lysine residues, then it will be recognized and degraded by the proteasome. However, if the protein substrate is mono- or diubiquitinated, then it is degraded by internalization and transport to the lysosome; it is not recognized by the proteasome because of the lack of the polyubiquitin chain. Figure 3 illustrates these two ubiquitination schemes, that is, monoubiquitination for the lysosomal pathway and polyubiquitination for proteasomal degradation. Research on the turnover of the epithelial sodium channel has shown that for the mature, membrane-bound form of the protein, degradation occurs via ubiquitination by the protein ligase Nedd4, leading to endocytosis and lysosomal degradation (reviewed in Ref. 76).

Role of the ubiquitin-proteasome system in disuse atrophy. As mentioned above, there is overwhelming evidence that the bulk of myofibrillar protein degradation during muscle atrophy occurs via the ATP-dependent ubiquitin-proteasome pathway (reviewed in Refs. 49, 58). The process of substrate ubiquitination involves the cooperative interaction of at least three classes of proteins termed E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligating) enzymes (Fig. 3). Details about the complex events involved in the ubiquitination of protein substrates and their subsequent degradation by the proteasome are outlined in excellent reviews such as that by Glickman and Ciechanover (26). Inhibition of the proteasome with agents available since 1994 has shown significant interference of muscle proteolysis in disuse muscle atrophy (reviewed in Ref. 49). In addition, there are significant increases in the expression of components of both the process of ubiquitination and of the many proteasome subunits with disuse (86, 87).

Recently, significant attention has been paid to the increased expression of ubiquitin protein ligases in disuse because, of all the proteins involved in ubiquitinating protein substrates, these seem to have the greatest tissue and substrate specificity (40). For instance, two groups simultaneously identified the muscle-specific F-box containing ubiquitin protein ligase atrogin-1/

MAFbx and found it to be markedly upregulated with atrophy resulting from disuse (9) and also in cachexia (29). When MAFbx knockout mice were subjected to muscle denervation, gastrocnemius atrophy was attenuated by 56% at 14 days (9). Another E3 (i.e., ubiquitin protein ligase) found to be increased by disuse atrophy (unloading, immobilization, or denervation) was also characterized in disuse (9). This was a ring finger-containing ligase that they named MuRF1 (muscle RING finger 1). MuRF knockout mice showed a 36% attenuation of muscle atrophy after 14 days of gastrocnemius denervation (9). Although this work also showed that overexpression of the MAFbx gene could alone induce atrophy in C_2C_{12} myotubes, this result has not been repeatable (Glass D, personal communication). Nevertheless, this work has led to much interest in the role of ubiquitination as a requirement for muscle atrophy.

In our own laboratory (86), we have reconfirmed the upregulation of both atrogin-1 and MuRF1 by muscle unloading using microarrays, and we have found that Nedd4, another E3, is also upregulated. Nedd4 differs from MuRF1 and atrogin-1 in that it is a HECT domain-containing ubiquitin protein ligase. We reconfirmed the threefold increase in mRNA expression during unloading using RT-PCR and showed that protein levels are doubled by 7 days of unloading (53). Nedd4 was localized to the periphery of the myofiber, consistent with the localization of its substrates such as the sodium channel (76) or the IGF-I receptor (98). Work is ongoing to determine whether Nedd4 is required for muscle atrophy. Finally, our data also suggest an upregulation of still another E3, Mdm2. The kinetics of its expression are very close to that of MuRF1 over the time course of muscle unloading. Mdm2 recently has been shown to be involved in the regulation of the IGF-I receptor (25). Thus both Nedd4 and Mdm2 have specificity for a receptor likely involved with myotube growth.

One well-characterized line of investigation has determined that a majority of the proteolysis associated with the ubiquitin-proteosome pathway, at least when studied on soluble muscle proteins, is mediated by the components of the subdivision of the ubiquitin-proteosome pathway referred to as the N-end rule pathway. In this case, it is required that the peptides available for proteolysis have been modified to contain basic or bulky amino acid termini before ubiquitination and degradation and, furthermore, that the component parts of the activation and ligation are by means of E214k and E3α or their redundant homologs, but not by non-N-end rule E2s and E3s (54). However, single knockouts of the E3α or the E214k genes did not inhibit starvation-induced muscle atrophy (57), and, as mentioned above, knockout of non-N-end rule E3s such as MuRF or atrogin-1 resulted in attenuated muscle atrophy due to denervation. These studies suggest that the N-end rule is not essential for atrophy. On the other hand, a role for the N-end rule cannot be ruled out because there are potential redundant genes for E214k and E3α that could be operative in the N-end rule pathway for these knockouts (57).

In sepsis, where components of the N-end rule pathway were previously shown to be upregulated, the non-N-end rule E3s atrogin-1 and MuRF1 also have been found to be upregulated, similar to the case in unloading and starvation (50, 86). As pointed out by Wray et al. (105), the participation of these non-N-end rule E3s and the contribution of the N-end rule pathway, with its requirement for amino-terminal modifications of muscle proteins, remains to be sorted out. However, two points seem worth making. First, there is a consensus in the literature that intact myofilament proteins have to be released before they are available for degradation by the proteasome, and though there is evidence that this may be accomplished at least in part by calpains, it is also possible that non-N-end E3s [for example, MuRF1, which already has been shown to bind to titin, a Z-disk protein (14)] may participate in this. Second, regardless of the relative contribution of the N-end rule pathway, the sensitivity of atrophy to inhibitors of the ubiquitin-proteosome pathway indicates that it has a very important role, both mechanistically and in magnitude, in muscle protein degradation.

FUTURE DIRECTIONS OF STUDY IN ATROPHY REGULATION

New Candidates for Study of Disuse Atrophy

Additional knowledge about the inventory of triggers, signaling pathways, or target genes that could be tested for a role in the process of disuse atrophy comes from a time-course microarray analysis of unloaded soleus muscles (86). For instance, additional upregulated genes involved in ubiquitination, such as Mdm2 and Nedd4, could be studied in further detail (discussed above). Several of the cathepsins that are differentially regulated also need detailed study because they may have an important role in the degradation of membrane-associated proteins with disuse atrophy. As mentioned, oxidative stress likely has an important role in disuse atrophy, and differentially regulated genes (e.g., metallothionein, selenoproteins, glutathione peroxidase, glutathione-*S*-transferase, superoxide dismutase) are good starting points for more in-depth study. While the role that myostatin plays during atrophy is somewhat ambiguous, we have shown that activin IIB (ActIIB) receptor, a myostatin receptor, is upregulated early and is sustained during the course of unloading-induced atrophy. Activation of the ActIIB receptor could possibly increase the sensitivity of muscle cells to myostatin and lead to decreased growth. Several other regulatory genes, differentially expressed with atrophy (86) and deserving further study, have been shown to regulate hypertrophic responses and may have an opposite role in atrophy. These are the tissue inhibitor of metalloprotease (TIMP) (46), cardiac ankyrin-repeated protein (Carp) (1), and interleukin-15 (73). Additional pathways that contain differentially expressed genes with unloading include those involved with myogenic signaling (myoD, Cited2), Notch signaling (transducin-like enhancer of split 4, hairy-related and enhancer of split), IGF signaling (e.g., IGFBP-5), JAK/STAT signaling (e.g., JAK2, STAT5b), amino acid metabolism (e.g., glutamine synthetase), and serine proteases and protease inhibitors, as well as genes involved in synaptic vesicle remodeling, cell proliferation, and cytoskeletal function (see Ref. 86 for discussion of the full data set). Some of the results in this microarray study also have been reported in other studies in which various approaches were used to measure parallel gene expression to examine one or two time points of muscle unloading (8, 85, 104). Overall, these data on global mRNA expression are providing multiple avenues for further study of the regulation of disuse atrophy.

Evolving Molecular Approaches to Study Disuse Atrophy

The progress made during the last several years on the molecular mechanisms of muscle atrophy has been possible

because of advances in understanding cell biology, the continued development of molecular tools, and early rounds of microarray analyses of expressed mRNAs during atrophy. Another avenue that will continue to prove useful is the development of additional knockout mice for putative atrophy-specific genes. These models will help test whether a gene is required for disuse atrophy. Transgenic mice overexpressing candidate gene constructs will be helpful for testing whether a gene is sufficient to induce atrophy. Cell culture models in which genes are overexpressed or knocked down should also prove helpful to work out the biochemical details of the effects of overexpression or inhibition of genes. For both cell culture and whole animal models, future progress will be most effective if efforts focus on determining what molecules are unique to atrophy, and this will require that experiments not be confounded by the involvement of muscle differentiation during genetic or biochemical manipulations. Although much more challenging to develop for animal models, this will involve the creation of conditional (e.g., Cre-Lox, Tet-inducible) and muscle-specific knockout or transgenic mice. The use of adeno-associated viral systems has shown some promise for the introduction of gene products into whole muscle postdifferentiation, and it is an alternative to genetic manipulation at the level of the germ line. For cell culture, averting differentiation will also involve the application of conditional expression systems or viral expression vectors that can be introduced into mature myotubes. Also, the use of small interference RNA to test the knockdown of a specific gene on myotube biochemistry can be achieved by incorporation of the appropriate sequence into an adenoviral expression vector. It will be important that each of these approaches to using genetic tools to assess a gene's role in atrophy should sidestep the confounding problem of expression or knockout during muscle development. Finally, the incorporation of genomics and subsequent proteomics analyses of disuse muscle atrophy will make possible, for the first time, the simultaneous visualization of all the signaling pathways, coordinately acting to produce the physiology underlying atrophy. With this in mind, there are already several new directions unfolding in the search for better understanding of atrophy regulation.

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