Chronic contractile activity upregulates the proteasome system in rabbit skeletal muscle

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Ordway, George A., P. Darrell Neufer, Eva R. Chin, and George N. DeMartino. Chronic contractile activity upregulates the proteasome system in rabbit skeletal muscle. J. Appl. Physiol. 88: 1134–1141, 2000.—Remodeling of skeletal muscle in response to altered patterns of contractile activity is achieved, in part, by the regulated degradation of cellular proteins. The ubiquitin-proteasome system is a dominant pathway for protein degradation in eukaryotic cells. To test the role of this pathway in contraction-induced remodeling of skeletal muscle, we used a well-established model of continuous motor nerve stimulation to activate tibialis anterior (TA) muscles of New Zealand White rabbits for periods up to 28 days. Western blot analysis revealed marked and coordinate increases in protein levels of the 20S proteasome and two of its regulatory proteins, PA700 and PA28. mRNA of a representative proteasome subunit also increased coordinate in contracting muscles. Chronic contractile activity of TA also increased total proteasome activity in extracts, as measured by the hydrolysis of a proteasome-specific peptide substrate, and the total capacity of the ubiquitin-proteasome pathway, as measured by the ATP-dependent hydrolysis of an exogenous protein substrate. These results support the potential role of the ubiquitin-proteasome pathway of protein degradation in the contraction-induced remodeling of skeletal muscle.

protein degradation; muscle plasticity; ubiquitin

Regulated intracellular protein degradation allows organisms to adapt to changing physiological conditions or environmental stresses by rapidly and selectively altering the cellular content of structural, catalytic, and regulatory proteins (17, 41). The ubiquitin-proteasome pathway is a dominant mechanism for protein degradation in eukaryotic cells (23, 38). This two-stage pathway utilizes ubiquitin, a highly conserved 8,500-Da protein, as a molecular marker for the degradation of constituent proteins (23). In the first stage, ubiquitin is covalently attached via its carboxy-terminal glycine residue to free amino groups of target proteins by the sequential action of three conjugating enzymes. This energy-dependent process then progressively attaches additional ubiquitin moieties to lysine 48 of the preceding ubiquitin, resulting in formation of a polyubiquitin chain. In the second stage, the ubiquitinated protein substrate is recognized and degraded by the 26S proteasome, a large protease complex composed of two multisubunit subcomplexes, the 20S proteasome and PA700 (7). The 20S proteasome is a 700,000-Da protease (2, 4, 12). Its 28 subunits are arranged in four stacked heptameric rings that form a hollow cylinder (21, 30). Six subunits of the two inner rings contain catalytic sites facing the lumen of the cylinder and therefore are inaccessible to potential substrates (21).

Proteasome function is activated by each of two regulatory proteins: PA700 (also known as 19S regulatory cap) and PA28 (also known as 11S regulator) (7, 12). PA700 is a 700,000-Da complex consisting of ~20 distinct subunits (25, 32). PA28 is a 180,000-Da ring-shaped heptamer or hexamer consisting of two distinct but homologous subunits (15, 31). Each of these proteins binds to one or both ends of the proteasome cylinder (1, 19). A common functional consequence of PA700 or PA28 binding is a large activation in the proteasome's hydrolysis of short peptides (15, 31). The structural basis for this effect is probably due to the regulator-induced creation of pores in the centers of the outer proteasome rings. These openings allow substrates to gain access to the active sites in the lumen of the proteasome. However, the small diameter of the pores (~13 A) restricts the passage of large folded proteins (21, 30, 45). Nevertheless, the unique properties of PA700 promote the selective degradation of proteins modified by a polyubiquitin chain. For example, at least one PA700 subunit binds polyubiquitin chains with high affinity (13, 44). This property provides the basis for substrate recognition by the 26S proteasome. Furthermore, PA700 probably overcomes
the constraints of narrow pore size by unfolding the substrate and translocating it in a processive manner to the catalytic sites of the proteasome (27). Either or both of these latter actions may be coupled mechanistically to ATP hydrolysis, a known requirement for degradation of ubiquitinated proteins by the 26S proteasome and an inherent property of PA700 (10, 23, 26). Thus PA700 plays multiple mechanistic roles in activation of proteasome function and is obligatory for proteasome-mediated hydrolysis of ubiquitinated proteins.

In contrast to PA700, PA28 cannot activate the degradation of proteins, regardless of their state of ubiquitination (15, 31). Thus, unlike PA700 whose ubiquitin-dependent proteolysis is firmly established, the exact physiological role of PA28 is uncertain. PA28 appears to regulate part of the proteasome's role in the production of antigenic peptides presented by major histocompatibility class I molecules (20). This role would be consistent with its effects on both the rate and specificity of peptide hydrolysis by the proteasome (14, 15, 31). Additional roles in overall cellular protein degradation are likely but undocumented. For example, recent work has shown that a single proteasome molecule may be bound to both PA700 and PA28, suggesting that these regulators can coordinateably modify proteasome function (22)

The general outline of the ubiquitin-proteasome pathway and its role in many physiological processes have been firmly established. In contrast, relatively less is known about how components and/or functions of the pathway are regulated under various conditions. Recently, however, a number of studies, particularly in skeletal muscle, have demonstrated that the expression of components of the pathway, including the 20S proteasome, PA700, ubiquitin, and ubiquitin-conjugating enzymes are increased in several physiological and pathological conditions characterized by increased protein degradation and muscle wasting (11, 28, 35).

Skeletal muscle is a highly plastic tissue that adapts to changing functional demands by altering its constitutent proteins. For example, chronic contractile activity in a muscle not otherwise subjected to such a stimulus promotes changes in the complement of contractile and metabolic proteins that optimize muscle function for this new type of activity (39, 40). Such remodeling involves altered patterns of both protein synthesis and proteolysis (9, 18). We hypothesized that the ubiquitin-proteasome pathway for protein degradation could be an important mechanism for this type of adaptation. To test this hypothesis, we used a well-established model of chronic low-frequency stimulation of the peroneal nerve to determine the effect of chronic contractile activity of the tibialis anterior (TA) muscle on components of the proteasome system. We report that sustained contractile activity promotes rapid and large increases in the expression and function of these components.

**MATERIALS AND METHODS**

Animal surgery and tissue preparation. Adult New Zealand White rabbits weighing ~3 kg were anesthetized by isoflurane inhalation. Under sterile conditions, pulse generators were implanted and their electrodes were placed adjacent to the common peroneal nerve of one hindlimb according to the procedure described by Salmons and Vrbova (40). After a 72-h recovery period, nerves were stimulated continuously at 6–10 Hz for various periods of time as indicated in the text. At completion of stimulation, rabbits were anesthetized with pentobarbital sodium (50 mg/kg iv) for removal of TA muscles. TA muscle obtained from the contralateral, unstimulated hindlimb served as control. All protocols were reviewed and approved by the Institutional Animal Care and Research Advisory Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

For protein and RNA analyses, several pieces of each muscle were quickly frozen in liquid nitrogen and stored at −70°C. Muscle samples were powdered using a precooled mortar and pestle. For the analysis of activity, fresh muscle samples were homogenized in 5 vol of ice-cold buffer (20 mM Tris·HCl, pH 7.6, 20 mM NaCl, 1 mM EDTA, and 1 mM β-mercaptoethanol) using a Polytron homogenizer and were centrifuged at 40,000 g for 30 min. The resulting supernatant (5–10 ml) was dialyzed at 4°C for 12–16 h against two changes (12,000 ml each) of buffer containing 20 mM Tris·HCl, pH 7.6, 20 mM NaCl, 1 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 20% glycerol. Protein concentration was determined by the method of Bradford (5) using BSA as a standard.

Immunohistochemical analysis of muscle extracts. Western blot analysis of muscle extracts was performed by previously described methods using polyconal antibodies against the 20S proteasome (34), PA28 (33), or the p31 subunit of PA700 (43). In brief, after separation by electrophoresis on 12.5% SDS-polyacrylamide Laemmli gels, proteins were electrotransferred to nitrocellulose membranes and incubated with primary and secondary (goat anti-rabbit IgG peroxidase-labeled, American Qualex) antibodies. Blots were visualized by the enhanced chemiluminescence detection method (Amersham) and quantitated by densitometry using known amounts of the respective purified protein.

Northern blot analysis. Total RNA was isolated from ~200 mg of powdered TA muscle by the guanidinium thiocyanate-phenol-chloroform extraction method (6) with the addition of a lithium chloride solubilization step (37). Northern blot analysis was performed as previously described (36). A partial cDNA probe corresponding to 320 bp of mouse proteasome subunit C3 (nucleotides 54–373) was generated by PCR and labeled with [32P]dCTP by random priming (42). After hybridization and washing, the membranes were placed on film (Hyperfilm-MP, Amersham) and exposed. Signal intensity of the developed film was quantified by densitometric scanning (Arcus II scanner, AGFA) and image analysis software (Molecular Analyst, Bio-Rad) and normalized to 28S RNA.

Measurement of proteasome activity. Proteasome activity was determined by measuring the hydrolysis of the synthetic peptide substrate Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (AMC), as described previously (31), using extracts of freshly homogenized and dialyzed muscles. The rate of production of AMC was monitored continuously in a fluorometer. Initial steady-state rates of hydrolysis were used to determine activity as described previously (31). One unit of proteasome activity is defined as the change in product concentration of 1.0 nM/min at 37°C. Assays for different muscle extracts were conducted with equal amounts of protein, and the average values for duplicate assays (which varied by <10%) were calculated in terms of units per milligram of extract protein.
Measurement of proteolysis by the ubiquitin-proteasome pathway. Activity of the ubiquitin-proteasome pathway was assessed by the ATP-ubiquitin-dependent degradation of exogenous [methyl-14C]casein by muscle extracts, as described previously (34). In brief, muscle extracts, prepared as described in Animal surgery and tissue preparation, were incubated for 45 min at 37°C in buffer containing 50 mM Tris·HCl, pH 7.8, 20 mM NaCl, 2 mM DTT, 9 mM MgCl₂, and 7.5 µg [methyl-14C]casein (42,000 dpm) in a final volume of 70 µl. The incubations were conducted in the presence or absence of 5 mM ATP and in the presence or absence of 2 µg/ml exogenous ubiquitin. The extracts contain endogenous ubiquitin, but preliminary experiments demonstrated that the amount was not sufficient to achieve maximal stimulation of ATP-dependent proteolysis. The reactions were terminated by addition of 350 µl of TCA. After at least 60 min on ice, the samples were centrifuged and the acid-soluble peptides produced by proteolysis were measured by scintillation counting of the supernatant. After correction for the small amount of radioactivity made soluble in parallel incubations in the absence of extract, proteolysis was calculated as a percentage of the substrate converted to soluble peptides per hour. Identical amounts of protein were incubated for extracts of different muscles. The assays were conducted in triplicate and had <5% variation among replicates. Preliminary experiments (not shown) demonstrated that the generation of soluble peptides was linear for up to 90 min of incubation.

RESULTS

Effect of chronic contractile activity on components of the proteasome system. Chronic low-frequency electrical stimulation of the rabbit TA muscle has been used commonly as a model for skeletal muscle adaptation. In this model, the normal fast glycolytic TA becomes progressively slow and oxidative as a result of altered protein composition. To assess the possible role of the proteasome system in these alterations, we determined the content of the 20S proteasome during the course of the adaptation. Soluble extracts were prepared from TA muscles that had been stimulated from 1 to 28 days and from their respective contralateral unstimulated muscles. The major soluble proteins visualized by SDS-PAGE were not significantly different among muscles stimulated for up to 10 days (Fig. 1). After longer periods of stimulation, the protein content of some major proteins decreased significantly, whereas the content of others increased. In contrast, the protein composition of the contralateral unstimulated muscles was unchanged during the entire course of muscle stimulation (data not shown).

Western blotting of the muscle extracts with antibodies prepared against the 20S proteasome demonstrated that the content of the proteasome increased greatly during the course of stimulation. A small but consistent increase was observed after 1 day of stimulation, and a maximal increase of about three- to fourfold was reached after 21 days of stimulation (Figs. 2 and 3). Thus the increase in proteasome content occurred before detectable changes in the composition of the major soluble proteins in these extracts. In some but not in all experiments, the proteasome level declined after 21 days of stimulation. These results demonstrate that the concentration of the 20S proteasome increases in
muscles undergoing chronic electrical stimulation and corresponding contraction.

Because the catalytic function of the 20S proteasome in cells is mediated by specific regulatory proteins, such as PA700 and PA28, we also examined the muscle extracts for alterations in the content of these proteins. As with 20S proteasome, the level of each of these proteins increased during the period of electrical stimulation (Fig. 2). In each case, the magnitude of the change was considerably greater than that for the proteasome. For example, PA700 increased by ~12-fold after 21 days of stimulation, and PA28 increased by ~70-fold at this time. As with the proteasome, significant increases were detectable after one day of stimulation and before quantitative and qualitative changes of the major soluble proteins. Furthermore, as with the proteasome, in some experiments such as that shown in Fig. 2, the content of PA700 and PA28 decreased significantly between 21 and 28 days. Together, these results demonstrate that the content of proteins that regulate proteasome activity was greatly increased in TA muscle in response to electrical stimulation and chronic contraction. Similar results on content of 20S proteasome, PA700, and PA28 were obtained with extensor digitorum longus muscles, which also undergo contraction when the peroneal nerve is stimulated (data not shown).

Effect of chronic contractile activity on proteasome mRNA. To determine whether the alteration in proteasome protein was accounted for by increased rates of proteasome synthesis, we analyzed stimulated TA muscles for levels of 20S proteasome mRNA using a representative subunit, C3 (42). Chronic contractile activity evoked a biphasic response (Figs. 4 and 5). The initial response included a rapid increase in message level that was detectable within 4 h of stimulation and reached a maximum after 3 days. After 3 days, the mRNA content decreased and then increased slightly after 21 days. These results indicate that the increased levels of proteasome protein are accounted for by increased levels of proteasome mRNA.
Effect of chronic contractile activity on proteasome activity. To determine whether the observed increases in components of the proteasome system were accompanied by changes in proteasome activity, we assayed extracts of TA muscle that had been contracting for specified times for the ability to hydrolyze the synthetic peptide substrate, Suc-Leu-Leu-Val-Tyr-AMC. This peptide is hydrolyzed specifically by the proteasome under the conditions employed for these assays. Chronic contractile activity of TA muscle caused an increase in proteasome activity that closely approximated the time course and magnitude of the increase in proteasome protein (Fig. 6). Thus proteasome activity increased approximately twofold after 3 days of contraction and threefold after 14 days of contraction. These results indicate that the increased proteasome protein resulted in increased proteasome activity in these extracts.

Effect of chronic contractile activity on the activity of the ubiquitin-proteasome pathway. Skeletal muscle extracts, like extracts of other mammalian cells, can catalyze the degradation of certain exogenous model proteins via the ubiquitin-proteasome pathway. Such substrates are first polyubiquitinated by endogenous conjugating enzymes and then degraded by the 26S proteasome (i.e., a complex of the 20S proteasome and PA700) in an ATP-dependent fashion. This in vitro activity, therefore, represents a multistep enzyme assay that measures the proteolytic capacity of the entire pathway. We determined the effect of chronic contraction on the activity of this pathway by assessing the degradation of [methyl-14C]casein in extracts of 14-day stimulated and contralateral unstimulated TA muscles (Table 1). Extracts of unstimulated TA muscles degraded the substrate at low rates in the absence of ATP and exogenous ubiquitin. When the same extracts were assayed in the presence of ATP and supplemented with exogenous ubiquitin, the degradation of casein was increased by over twofold. The ATP-ubiquitin-stimulated proteolysis was completely inhibited by lactacystin (50 µM), a specific inhibitor of the proteasome, thereby demonstrating the specificity of ATP-ubiquitin-dependent proteolysis (8, 16). Extracts from stimulated TA muscles had about twofold greater protease activity than control extracts in the absence of ATP and ubiquitin but were also stimulated about twofold by ATP and ubiquitin. Lactacystin reduced the ATP-ubiquitin-enhanced rates of degradation to control levels. These results indicate that the activity of the ubiquitin-proteasome pathway was significantly increased in stimulated muscles. They also indicate that another proteolytic pathway may also be affected by chronic contractile activity.

**DISCUSSION**

Chronic contractile activity of the TA, a fast-twitch glycolytic muscle, by electrical stimulation promotes profound alterations in its protein composition. For example, during the course of stimulation, many proteins characteristic of this fiber type are selectively eliminated, whereas proteins characteristic of slow-twitch oxidative fibers are preferentially expressed (39, 40). In addition, the muscle undergoes significant atrophy as a result of increased rates of global protein degradation (9). The present results demonstrate that the chronic contractile activity of this model markedly increases cellular levels of the proteasome and its two protein activators, PA700 and PA28. These increases were associated with corresponding increases in proteasome activity and were detected within 1 day of stimulation. This rapid upregulation of the proteasome system suggests that proteasome-mediated proteolysis may be responsible for some aspects of the remodeling process and for the overall increase in protein degradation that leads to atrophy. The muscle atrophy that occurs in this model cannot be a trivial explanation for the observed changes in levels of components of the proteasome system because there were no significant differences between the ratios of total muscle protein to muscle wet weight for stimulated and unstimulated muscles during the course of stimulation. For example, after 1 day of stimulation when no atrophy was evident, the ratios for unstimulated and stimulated muscles were 65 and 70 mg protein/g muscle, respectively. Similar ratios were observed after 7 days of stimulation (53 vs. 61 mg protein/g muscle), when up to 20% atrophy was detected, and after 21 days of stimulation.
(61 vs. 53 mg protein/g muscle), when up to 40% atrophy was detected. These results exclude the possibility that the observed changes in levels of proteasome system components are passive and increase only relatively to the decline of other cellular constituents. In addition, significant increases in components of the proteasome system occurred well before atrophy was detected. Finally, the magnitude of the increases in proteasome system components (up to 70-fold for PA28) cannot be accounted for by the degree of atrophy produced in this model. Thus the observed changes in proteasome protein are probably due to increased expression of components of the system. Previous work with this experimental model demonstrated that electrical stimulation could promote variable extents of muscle fiber damage and infiltration of the tissue with macrophages (29). In preliminary experiments, we have demonstrated that the stimulated muscles studied here sustained very little fiber damage and macrophage infiltration. Furthermore, the fibers of stimulated muscles show significantly increased immunohistochemical staining for proteasome (Ordway and DeMartino, unpublished observations). Thus it seems unlikely that the increased levels of proteasome reported here are accounted for by muscle necrosis and/or infiltration of the tissue by other cell types.

The patterns of contraction-induced increases in proteasome protein and mRNA shown in the present study are similar to those we observed previously in the same model for expression of the inducible 70-kDa heat stress protein, HSP70, and its mRNA (36). Each pattern is characterized by early and sustained increases in protein content. In contrast, the mRNA levels for each showed an early initial increase, followed by a decline, and then a secondary increase after longer periods of simulation. Recent evidence indicates that molecular chaperones, such as HSP70, may play important roles in mechanisms of protein degradation in general and in the ubiquitin-proteasome pathway in particular (3). Thus the coordinated changes in expression of HSP70 and the proteasome system may be linked functionally.

The increased protein levels of the proteasome and its regulatory proteins documented here were mirrored by increased proteasome activity, as measured by hydrolysis of the proteasome-specific synthetic peptide, Suc-Leu-Leu-Val-Tyr-AMC, one of the three characteristic activities of the proteasome. Unfortunately, the other two hydrolytic activities of the proteasome (the "trypsin-like" and the "post-glutamyl peptide-hydrolyzing" activities) cannot be assayed quantitatively in crude extracts because other proteases also cleave these peptides at high rates. Nevertheless, under the assay conditions used here, hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC (the "chymotrypsin-like" activity) is specific for the proteasome. The magnitude of the activity is determined by the relative concentrations of the proteasome, PA700, and PA28, since these activators greatly enhance the proteasome's hydrolysis of this substrate (9). In this regard, the very large increases in levels of PA700 and PA28 might have been expected to produce greater than the observed three- to fourfold increases in activity. These results may indicate that the extracts contain other proteins that inhibit proteasome activity or that PA700 and PA28 have functions other than proteasome stimulation. In fact, each of these proteins may have chaperone-like activities that could be subjected to regulation in this model (3). In any case, the data clearly demonstrate an increased cellular capacity for proteasome activity in extracts of stimulated muscles.

In addition to using assays that directly measure proteasome activity, we also assessed the activity of the entire ubiquitin-proteasome pathway in muscle extracts by measuring the ATP-ubiquitin-dependent degradation of an exogenous protein. In contrast to the hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC, which requires no modification (e.g., ubiquitination) to be degraded by the proteasome, the ATP-dependent degradation of casein depends on its ubiquitination. Because ubiquitination is accomplished by a cascade of reactions, the assay represents a multistep reaction that could be rate limited by any one of the required components involved in ubiquitination as well as by the 26S proteasome. Thus this assay is a measure of the total functional capacity of the pathway. Additional work will be required to determine whether levels of other pathway components are regulated in this model.

The present results add a new example to a growing list of conditions under which the cellular content and activity of the proteasome system are altered. Interestingly, many of these examples come from skeletal muscles undergoing atrophy in response to various pathological states (24, 28, 35, and references therein). Such results are somewhat surprising because the proteasome is normally present at high levels and has not been considered to be a rate-limiting factor in the ubiquitin pathway of protein degradation. In contrast, the rate at which proteins are ubiquitinated has been considered rate limiting for proteolysis and is known to be regulated under a variety of conditions (23). These results may indicate that the proteasome also participates in ubiquitin-independent pathways, although known examples of this type of activity are rare. Nevertheless, some studies have shown that changes in the proteasome levels and/or activity occur concomitantly with changes in levels of ubiquitin or ubiquitin-conjugating enzymes. Such results suggest that many components of this multicomponent pathway may be regulated coordinately (35, 28). Obviously, considerable work will be required to determine the exact mechanisms and significance of the regulation of multiple protein components of this pathway as part of the response of the cell in achieving new phenotypes.

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