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Research Article

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Inhibitors of the Proteasome Reduce the Accelerated Proteolysis in Atrophying Rat Skeletal Muscles

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Abstract

Several observations have suggested that the enhanced proteolysis and atrophy of skeletal muscle in various pathological states is due primarily to activation of the ubiquitin-proteasome pathway. To test this idea, we investigated whether peptide aldehyde inhibitors of the proteasome, *N*-acetyl-leucyl-leucyl-norleucinal (LLN), or the more potent CBZ-leucyl-leucyl-leucinal (MG132) suppressed proteolysis in incubated rat skeletal muscles. These agents (e.g., MG132 at 10 μM) inhibited nonlysosomal protein breakdown by up to 50% ($P < 0.01$), and this effect was rapidly reversed upon removal of the inhibitor. The peptide aldehydes did not alter protein synthesis or amino acid pools, but improved overall protein balance in the muscle. Upon treatment with MG132, ubiquitin-conjugated proteins accumulated in the muscle. The inhibition of muscle proteolysis correlated with efficacy against the proteasome, although these agents could also inhibit calpain-dependent proteolysis induced with Ca^{2+}.

These inhibitors had much larger effects on proteolysis in atrophying muscles than in controls. In the denervated soleus undergoing atrophy, the increase in ATP-dependent proteolysis was reduced 70% by MG132 ($P < 0.001$). Similarly, the rise in muscle proteolysis induced by administering thyroid hormones was reduced 40–70% by the inhibitors. Finally, in rats made septic by cecal puncture, the increase in muscle proteolysis was completely blocked by MG132. Thus, the enhanced proteolysis in many catabolic states (including denervation, hyperthyroidism, and sepsis) is due to a proteasome-dependent pathway, and inhibition of proteasome function may be a useful approach to reduce muscle wasting. (*J. Clin. Invest.* 1997. 100:197–203.) Key words: sepsis • denervation • hyperthyroidism • protein degradation • ubiquitin

Introduction

The balance between rates of protein synthesis and degradation in muscle determines its protein content, and thus its size and functional capacity (1). If protein degradation exceeds synthesis, muscle atrophy will occur, as seen after nerve injury or in various systemic diseases, including cancer cachexia, sepsis, renal failure, and AIDS (2, 3). Studies with isolated rat skeletal muscles have revealed at least four proteolytic processes in this tissue (4–6): (*a*) a lysosomal pathway mediated by proteases (cathepsins) localized within this organelle; (*b*) a cytosolic Ca^{2+}-dependent process, which involves the Ca^{2+}-activated proteases (calpains); (*c*) an ATP-requiring process, demonstrated after inhibition of lysosomal and Ca^{2+}-dependent proteases; and (*d*) an energy-independent pathway, the biochemical basis of which is uncertain. Most of the protein degradation in cultured mammalian cells requires ATP, and for most proteins this process involves the polypeptide cofactor, ubiquitin (7), and a large (2,000 kD) ATP-dependent protease, the proteasome (8). In this pathway, ubiquitin is covalently ligated to the protein substrate, and this modification marks it for rapid degradation by the 26S proteasome complex, which contains multiple peptidase and ATPase activities, and subunits for recognition of ubiquitin-protein conjugates (8).

Recent studies have explored the relative importance of these pathways in different models of muscle wasting, including cancer (9), fasting (10–12), denervation atrophy (6, 11, 12), acidosis (13), glucocorticoid treatment (10), and sepsis (14, 15). In all of these catabolic states, increased proteolysis was demonstrated in incubated muscles and was not reduced by inhibition of lysosomal function or of the calpains. However, ATP-depletion greatly reduced or eliminated the difference in proteolytic rates. Furthermore, in many of these conditions there is a concomitant increase in the muscle content of mRNA for ubiquitin and subunits of the proteasome, and in levels of ubiquitin-conjugated proteins (9, 11, 13). These observations suggest that activation of the ubiquitin-proteasome pathway is responsible for muscle wasting in these conditions (3). However, these arguments are indirect, and the quantitative importance of this pathway in the increased proteolysis is uncertain.

Recently, several tripeptide aldehydes were shown to be competitive inhibitors of the proteasome's peptidase activities and to inhibit degradation of ubiquitinated proteins by 26S proteasomes (16, 17). Moreover, these inhibitors were shown to readily enter cultured cells and to reduce protein breakdown (17). In the present studies, we examined the effects of two such compounds, *N*-acetyl-leucyl-leucyl-norleucinal (LLN,[1] also called calpain inhibitor I and MG101) and the more potent inhibitor, carbo-benzoxy-leucyl-leucyl-leucinal (MG132) on different proteolytic pathways and protein synthesis in incubated muscles. These agents were then used to determine if a proteasome-dependent process actually increases during muscle atrophy, and whether such inhibitors might be of po-

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1. *Abbreviations used in this paper:* EDL, extensor digitorum longus; LLM, *N*-acetyl-leucyl-leucyl-methioninal; LLN, *N*-acetyl-leucyl-leucyl-norleucinal; MG132, *N*-carbo-benzoxy-leucyl-leucyl-leucinal; T_3, triiodothyronine.

tential therapeutic use in combating the body nitrogen loss in human disease.

We studied three models of muscle wasting closely related to human disease: denervation atrophy induced by section of the sciatic nerve; sepsis resulting from cecal puncture; and thyrotoxicosis induced by triiodothyronine (T_3) treatment. Upon denervation, the rat soleus muscle loses up to 40% of its protein content (6), especially myofibrillar proteins, and ATP-dependent protein breakdown increases markedly (12). Cecal puncture causes a fulminant form of sepsis (14), in which nonlysosomal ATP-dependent proteolysis in muscle rises. Loss of thyroid function leads to a reduction in muscle protein breakdown, while hyperthyroidism induced by T_3 administration enhances proteolysis back to control or even to excessive levels (18). Preliminary studies of ATP-depleted muscles suggested that proteolysis activated by T_3 involves a nonlysosomal process (19, 20). Here we show that the increased proteolysis in each of these models of muscle atrophy is suppressed by inhibitors of the proteasome.

Methods

Materials. LLN was obtained from Calbiochem Corp. (San Diego, CA) and MG132 was generously provided by ProScript, Inc. (formerly Myogenics, Inc., Cambridge, MA).

Animals. Male CD strain rats (Charles River Laboratories, Wilmington, MA) were given free access to a standard laboratory diet before killing by cervical dislocation (60–70 g final body wt). For studies of denervation, the sciatic nerve of one hindlimb was transected, and after 3 d the contralateral normal and denervated soleus muscles were isolated and incubated, as described previously (11). For studies of thyroid hormone action, hypophysectomized rats were treated with subcutaneous injection of T_3 (100 μg/100 g body wt per d) or with equal volumes of the saline vehicle for 4 d (20). For studies of sepsis, rats anesthetized with pentobarbital were subjected to cecal ligation and puncture or to a sham operation, as described by Hasselgren and co-workers (14), and fasted overnight. The rats were killed 16 h after operation, and their muscles were incubated in vitro.

Protein turnover in incubated muscles. Protein breakdown was measured as the release of tyrosine by muscles incubated in vitro in KRB, as described previously (4, 21). For measurements of protein degradation in hindlimb muscles, soleus or extensor digitorum longi were secured to inert supports at resting length. The validity of these approaches has been discussed elsewhere in detail (2). Incubation conditions for individual experiments are described in their respective legends. For the measurement of changes in the sizes of free intracellular tyrosine pools during incubation, all muscles were preincubated for 1 h. One muscle was then placed into trichloroacetic acid to measure pool size at time 0, and the paired hindlimb or quarter-diaphragm muscle was transferred to fresh media for measurement of rates of proteolysis (tyrosine release from proteins). At the end of this 2-h incubation, muscles were placed into acid to extract the final free amino acid pool (21). For the measurement of the ATP-dependent process, muscles were incubated in Ca^{2+}-free media containing 10 mM glucose, 0.5 mM cycloheximide, 0.1 U/ml insulin, branched chain amino acids at three times their plasma concentration, 25 μM E-64, and 10 mM methylamine to suppress lysosomal and Ca^{2+}-dependent proteolysis, as described previously (4). These conditions were used for studies of the ATP-dependent proteolytic process in atrophying muscles (see Tables V, VI, and VII). Unless otherwise indicated, muscles were incubated in the presence of cycloheximide (0.5 mM) to prevent reincorporation of tyrosine into protein. For measurement of protein synthesis, no cycloheximide was added, and tissues were incubated with [^{14}C]phenylalanine (0.5 mM, 0.05 μCi/ml) (21).

Assay of ubiquitin-protein conjugates. The content of ubiquitin-protein conjugates in the muscle was assayed after homogenization of muscles in a buffer containing 0.25 M sucrose, 50 mM Tris, pH 7.4, 2 mM ATP, 10 mM $MgCl_2$, and 1 mM DTT. The crude extract was boiled in SDS, followed by polyacrylamide electrophoresis on 7.5% gels in the presence of SDS (20 μg protein/lane) and Western blotting. The blots were blocked with 2% milk/1% Triton/0.5 M KCl in PBS, exposed to an antiubiquitin antibody (East Acres Biologicals, Southbridge, MA) at 1:1,000 dilution, and then developed with an anti-IgG alkaline phosphatase conjugate (Bio-Rad, Hercules, CA).

Results

Incubation of the diaphragm muscle with either LLN or MG132 for 2 h led to a highly significant inhibition of overall protein degradation. With LLN, this inhibition reached 50% ($P < 0.001$) at high concentrations (140 μM) (Fig. 1). Similar inhibition occurred with MG132, which was consistently more effective in the incubated muscles, as in other cells (see below). Despite this clear reduction in proteolysis, LLN had no significant effect on overall protein synthesis in the muscle even at high concentrations, or on the size of the free intracellular tyrosine pools. Thus, at all concentrations tested, LLN significantly suppressed the negative protein balance in the diaphragm.

MG132 was shown to be at least a 10-fold more potent inhibitor of protein breakdown by isolated proteasomes than LLN (17). We therefore compared the effects of LLN and MG132 on protein degradation and free tyrosine pools in the diaphragm (Table I). However, because these agents can also inhibit calpains and lysosomal thiol proteases (17), incubation

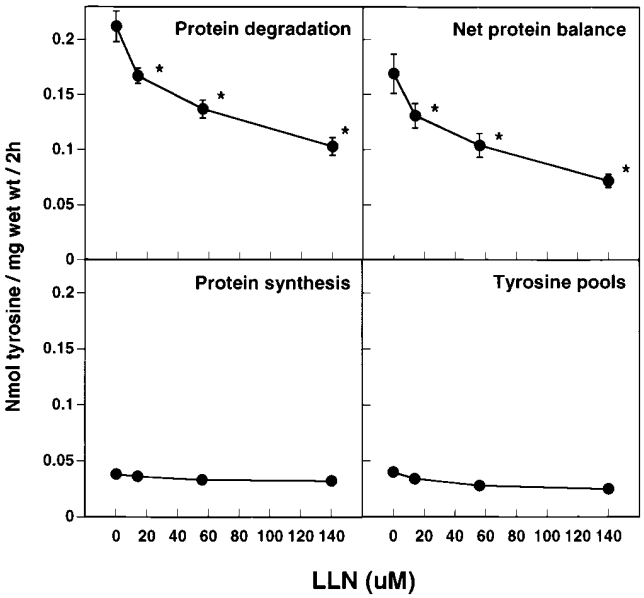

Figure 1. Effects of increasing concentrations of LLN on protein turnover and amino acid pools in diaphragm muscle during in vitro incubation. Shown are the means±SEM for six quarter-diaphragm muscles in each group. Significant effect, $*P < 0.01$. Muscles were incubated in media containing glucose (10 mM), branched chain amino acids at three times normal plasma concentration, insulin (0.1 U/ml), and [^{14}C]phenylalanine (0.5 μCi/ml, 0.5 mM) for the simultaneous measurement of protein synthesis and degradation, as described elsewhere (21). Rates of protein degradation were calculated as the sum of the measured rates of protein synthesis and net protein balance (tyrosine production).

Table I. Inhibition of Nonlysosomal Protein Degradation by LLN and MG132 in Incubated Rat Diaphragm Muscles

Inhibitor	Tyrosine release into medium	Change in tyrosine pool	Total tyrosine production
	nmol tyr/mg wet wt/2 h		
None	0.420±0.024	−0.019±0.011	0.402±0.016
LLN (100 μM)	0.203±0.004	−0.022±0.007	0.181±0.008
Difference	0.217±0.021	NS	0.221±0.017
% Inhibition	52%*		55%*
MG132 (10 μM)	0.211±0.013	−0.013±0.003	0.198±0.016
Difference	0.209±0.028	NS	0.204±0.024
% Inhibition	50%*		51%*

Shown are the means±SEM for quarter-diaphragm muscles from six rats. Significant difference *$P < 0.01$. To suppress lysosomal and Ca^{2+}-dependent proteolytic processes, muscles were incubated in Ca^{2+}-free media containing 10 mM glucose, 0.5 mM cycloheximide, 0.1 U/ml insulin, branched chain amino acids at three times their plasma concentration, 25 μM E-64, and 10 mM methylamine. Intracellular tyrosine pools were determined as described in Methods.

conditions were chosen which block the lysosomal and Ca^{2+}-dependent processes in the muscles (4). LLN at 100 μM and MG132 at 10 μM caused an identical 50% fall ($P < 0.01$) in nonlysosomal proteolysis in the diaphragm, and had no significant effect on intracellular tyrosine pools (Table I). With incubated leg muscles, in either the dark soleus or the pale extensor digitorum longus (EDL), a similar inhibition of proteolysis was found (with no change in intracellular pools). Thus, the relative efficacy of these two agents against muscle proteolysis correlated with their relative potency against the proteasome.

To test if these peptide aldehydes acted by inhibition of the proteasome, we incubated diaphragm muscles with the structural analog of LLN, *N*-acetyl-leucyl-leucyl-methioninal (LLM), which is much less active against the proteasome, but has similar potency against calpains and lysosomal cysteine proteases (17). In marked contrast to LLN or MG132, LLM had no effect on protein degradation regardless of the incubation conditions (Table II). When the diaphragms were incubated in KRB alone (Table II), LLN (50 μM) inhibited overall proteolysis in the muscle by 23% ($P < 0.01$). Under these incubation conditions, the lysosomal and calpain-dependent proteolytic processes in the muscle are activated. Upon incubation with amino acids, insulin, and specific inhibitors to suppress these processes (4), LLN appeared more effective, and inhibited the remaining protein breakdown by 42% ($P < 0.01$). These findings provide strong evidence that these agents are functioning by inhibiting the ATP-dependent process.

Table II. Comparison of Effects of LLN and LLM on Protein Breakdown in Incubated Rat Diaphragm Muscles

Addition	Total proteolysis	Nonlysosomal proteolysis
	nmol tyr/mg wet wt/2 h	
None	0.333±0.015	0.214±0.018
+ LLN (50 μM)	0.256±0.022	0.126±0.008
Difference	0.077±0.020	0.089±0.018
% Inhibition	23%*	42%*
+ LLM (50 μM)	0.306±0.020	0.185±0.009
Difference	NS	NS

Shown are the means±SEM for quarter-diaphragms from six rats. Significant difference *$P < 0.01$. Total proteolysis was measured in media containing glucose (10 mM) and Ca^{2+} (2.5 mM). Nonlysosomal proteolysis was measured in muscles incubated as in Table I.

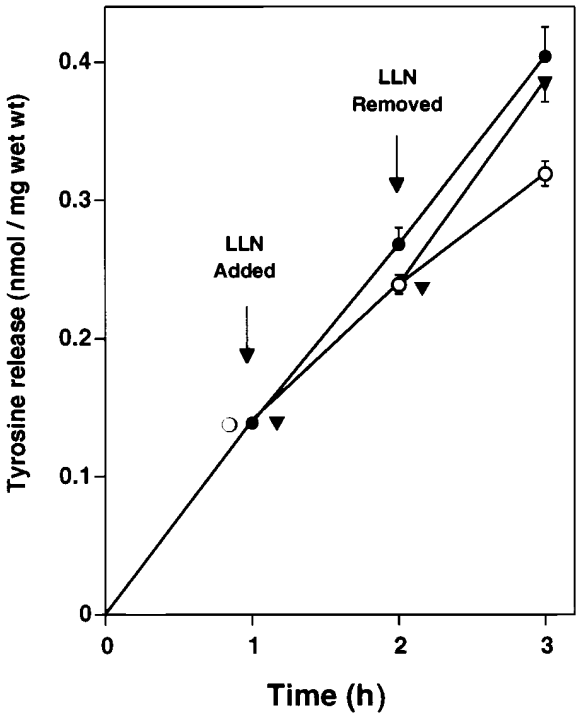

Figure 2. Reversible inhibition of protein breakdown by LLN in incubated diaphragm muscles. Shown are the means±SEM for six quarter-diaphragm muscles in each group. (●) Muscles incubated in the absence of inhibitor; (○) Muscles incubated without inhibitor until addition of LLN (50 μM) at 1 h; (▼) Muscles incubated without inhibitor until LLN was added at 1 h, and subsequently removed at 2 h. Muscles were incubated in media containing cycloheximide (0.5 mM) for measurement of nonlysosomal proteolysis independent of Ca^{2+} as described in Methods.

The inhibition of muscle protein breakdown by the aldehydes was rapid and reversible. As shown in Fig. 2, in the absence of LLN, this degradative process in the diaphragm was linear over a 3-h period of incubation. When LLN (50 μM) was added to the medium, a significant inhibition of proteolysis was observed within 1 h (22%, $P < 0.01$). The extent of this inhibition increased slightly during the second hour of incubation with the inhibitor (32%, $P < 0.01$). When LLN was removed from the medium after 1 h of incubation, protein breakdown measured during the subsequent hour in the absence of inhibitor returned to control values (0.404±0.021 nmol tyr/mg/2 h in untreated control vs. 0.384±0.013 after removal of LLN). Therefore, the inhibition of proteolysis was completely reversible, as was also found in lymphoblasts (17) and other cells (22) with this agent or with other peptide aldehydes (e.g., MG132).

Because these peptide aldehydes can also inhibit sulfhydryl proteases, including lysosomal cathepsin B and the neutral

Table III. Effect of LLN on the ATP-dependent Proteolytic Process in EDL Muscles

Addition	Total proteolysis	Proteolysis in ATP-depleted muscle	ATP-dependent proteolysis
	nmol tyr/mg wet wt/2 h		
None	0.206±0.010	0.134±0.006	0.070±0.006*
+ LLN (75 μM)	0.122±0.004	0.096±0.006	0.026±0.004*
Difference	0.082±0.008	0.038±0.002	0.044±0.006*
% Inhibition	40%*	28%*	65%*

Shown are the means±SEM for EDL muscles from six rats. Significant difference, $*P < 0.001$. The paired EDL was incubated at resting length under conditions which suppress the lysosomal and Ca^{2+}-dependent proteolytic processes, as described in Methods. Glucose was removed from the medium of one muscle and dinitrophenol (0.5 mM) and 2-deoxyglucose (10 mM) were added to deplete nearly all intracellular ATP (4).

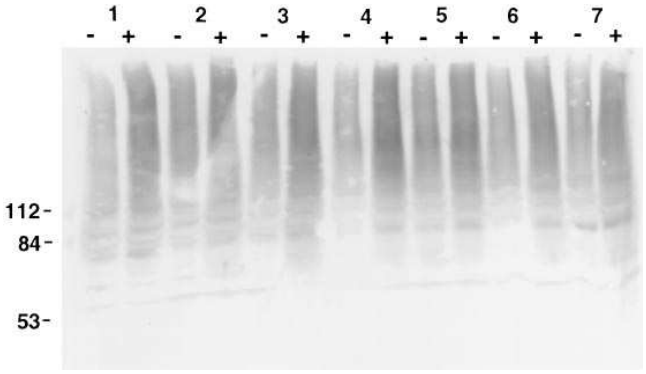

Figure 3. Accumulation of ubiquitin-protein conjugates in diaphragm muscles during incubation with MG132. Each paired lane (−: control; +: incubation with MG132) shows results for the paired hemidiaphragm muscles from one rat. Molecular weight markers are shown in kilodaltons. Muscles were incubated in the presence or absence of MG132 (10 μM) for 2 h under conditions favoring nonlysosomal proteolysis, independent of Ca^{2+}. The content of ubiquitin-conjugates was analyzed by SDS-PAGE and Western blot, using an antiubiquitin antibody as described in Methods.

calpains (17), we determined whether these agents specifically affect the ATP-dependent proteolytic process in muscle (i.e., the component of protein breakdown sensitive to ATP depletion) (Table III). Protein breakdown was compared in contralateral soleus muscles under conditions that suppress the lysosomal and Ca^{2+}-dependent processes (4). When one of the paired muscles was depleted of ATP by the addition to the medium of dinitrophenol and 2-deoxyglucose (4), total protein breakdown was reduced by 34% ($P < 0.001$). However, when muscles were incubated with LLN, ATP depletion had less effect on lowering proteolysis. In other words, the ATP-dependent component of protein breakdown equaled 0.035±0.003 nmol tyr/mg/2 h in the absence of LLN, but only 0.013±0.002 in the presence of the aldehyde. Since LLN reduced the ATP-dependent process by 65% ($P < 0.001$), this process appears to require the proteasome and to be the primary site of these inhibitory effects.

Inhibiting the activity of the proteasome should prevent the rapid degradation of ubiquitin-conjugated proteins in the muscle. In fact, as shown in Fig. 3, incubation of hemidiaphragm muscles with MG132 (10 μM) caused an accumulation of ubiquitin-conjugated proteins. In these experiments, the tissue extracts were analyzed by SDS-PAGE and Western blotting with antiubiquitin antibodies. After incubation for 2 h with inhibitor, a 2–4-fold increase was observed specifically in high-molecular -weight ubiquitin-protein conjugates, which correspond to the preferred substrates for the 26S proteasome (8).

These findings (Table III and Fig. 3) indicate that the proteasome is the major target in muscle for the actions of peptide aldehydes on protein breakdown. As indicated above, these compounds can also inhibit the neutral Ca^{2+}-activated proteases (calpains) found in the soluble fraction of muscle and other cells (17). Under normal physiological conditions, the calpains do not appear to contribute significantly to overall protein degradation in muscle (6). However, after muscle injury and in certain models of muscular dystrophy, the Ca^{2+}-dependent pathway may be activated (2). To test whether the peptide aldehydes might also inhibit the Ca^{2+}-dependent proteolytic process, we incubated muscles with the Ca^{2+} ionophore, A23187 (50 μM) (23). As shown in Table IV, the addition of A23187 to the incubation medium led to a large (+175%, $P < 0.001$) stimulation of protein breakdown in the diaphragm muscle. If LLN (75 μM) was present, the ionophore was much less effective in activating proteolysis, and the Ca^{2+}-dependent proteolytic process was reduced by 55% ($P < 0.001$). Thus, potentially, these inhibitors can also block calpain actions in vivo.

Inhibition of the enhanced proteolysis in denervation atrophy. In a wide variety of catabolic states associated with muscle atrophy and negative nitrogen balance (9–11, 13, 14, 20), an ATP-dependent, nonlysosomal proteolytic pathway appears to increase, as shown by a variety of indirect approaches (3). To test directly for involvement of the proteasome in this process, we examined whether protein breakdown in atrophying muscles showed an increased sensitivity to LLN or MG132. During denervation atrophy, the ATP-dependent proteolytic process increases progressively in the muscle, and ubiquitin-conjugation rises (11). As shown in Table V, 3 d after denervation of the soleus, nonlysosomal proteolysis was 52% ($P < 0.001$) greater than in the innervated muscle of the contralateral hindlimb. MG132 (10 μM) inhibited protein breakdown from 33 to

Table IV. Effect of LLN on Proteolysis Induced by the Ca^{2+} Ionophore, A23187, in Diaphragm Muscles

Addition	Proteolysis in		Ca^{2+}-dependent component
	Control	+A23187	
	nmol tyr/mg wet wt/2 h		
None	0.282±0.014	0.774±0.020	0.494±0.022*
+ LLN (75 μM)	0.146±0.006	0.370±0.012	0.224±0.012*
Difference	0.136±0.012	0.406±0.020	0.270±0.018*
% Inhibition	48%*	52%*	55%*

Shown are the means±SEM for hemidiaphragms from six rats. Significant difference, $*P < 0.001$. The paired hemidiaphragms were incubated with glucose (10 mM) and Ca^{2+} (2.5 mM), and the Ca^{2+} ionophore A23187 (50 μM) was added to activate the Ca^{2+}-dependent proteolytic process (4).

Table V. Comparison of Effects of MG132 on Protein Breakdown in Control or Denervated Soleus Muscles

Addition	Proteolysis in Control	Proteolysis in Denervated	Increase upon denervation
	nmol tyr/mg wet wt/2 h		
None	0.312±0.012	0.476±0.013	0.164±0.013*
+ MG132 (10 μM)	0.206±0.007	0.260±0.006	0.054±0.008*
Difference	0.106±0.013	0.216±0.014	0.110±0.015*
% Inhibition	33%*	45%*	68%*

Shown are the means±SEM for paired muscles from six animals. Significant difference, $*P < 0.001$. Rats (60–70 g) were killed 3 d after section of the sciatic nerve. The contralateral innervated hindlimb was subjected to sham operation, and its soleus muscle used as a paired control. Muscles were incubated at resting length under conditions for measurement of nonlysosomal proteolysis independent of Ca^{2+} as described in Methods.

45% ($P < 0.001$) in all muscles studied, whether denervated or control. However, the absolute reduction in proteolysis was twofold higher ($P < 0.001$) in the denervated muscle than in the control (0.216±0.014 vs. 0.106±0.013 nmol tyr/mg/2 h). Furthermore, the difference in rates of proteolysis between denervated and control muscles in the presence of MG132 was only 1/3 of that in its absence (0.054±0.008 vs. 0.164±0.013 nmol tyr/mg/2 h). Thus, the ATP-dependent component of proteolysis, which is activated by muscle denervation, is particularly sensitive to the inhibitor (68% inhibition at 10 μM), and therefore seems to be mediated by the proteasome.

Effects of thyroid hormone on the proteasome pathway. Other potent stimulators of overall proteolysis (18) and the ATP-dependent degradative process in muscle (19, 20) are the thyroid hormones, and excessive thyroid function can cause significant muscle wasting (2, 18). Conversely, thyroidectomized or hypophysectomized rats show reduced overall protein breakdown and less ATP-dependent proteolysis in their muscles than controls. Interestingly, when muscles from hypophysectomized rats were incubated with LLN or MG132 for 2 h, no significant inhibition of protein breakdown was seen (Table VI). To study the effects of thyroid hormones independently of other pituitary-dependent hormones, we treated hypophysectomized rats with high levels of T_3 for 4 d. Nonlysosomal protein breakdown increased by 41% ($P < 0.01$) in the incubated diaphragm after treatment with T_3 (Table VI). When incubated with either peptide aldehyde, the muscles from T_3-treated animals showed over a 30% ($P < 0.01$) reduction in overall protein breakdown. Importantly, the absolute increase in protein degradation induced by T_3 was reduced markedly by both inhibitors. For example, LLN caused a 67% ($P < 0.05$) reduction in the increase in proteolysis induced by T_3 (0.107±0.023 nmol tyr/mg/2 h vs. 0.035±0.013 in its presence).

These findings indicate for the first time a clear effect of thyroid hormones in activating the proteasome-dependent proteolytic pathway. Although complete food deprivation accelerates ATP-dependent proteolysis in skeletal muscle (12), the increase in proteolysis induced by T_3 cannot be explained by alterations in food intake during hormone treatment. The treatment of rats with T_3 caused a substantial increase in food intake, regardless of the dose of hormone used (data not shown). This increased caloric intake of the T_3-treated rats would be expected to suppress proteolysis rather than to increase this process (1, 2, 12). In addition, thyroidectomy or hypophysectomy actually reduced protein breakdown (18–20) while causing a fall in food intake, exactly opposite to what occurs in fasting. Thus, the increase (or decrease) in muscle proteolysis that is sensitive to the aldehyde inhibitors must be due to the rise (or fall) in thyroid hormone.

Influence of proteasome inhibition in septic rats. Recently, it was shown that the ATP-dependent proteolytic process in muscle increases when rats are made septic by cecal ligation and puncture, particularly in white muscles such as the EDL, where rapid atrophy occurs (14). To determine if this enhancement of proteolysis also involved proteasomes, we studied muscles of rats 16 h after cecal ligation and puncture (Table

Table VI. Effect of Proteasome Inhibitors on Proteolysis in Diaphragm Muscles from Hypophysectomized Rats and Ones Treated with T_3

Addition	Proteolysis in Control	Proteolysis in T_3-treated	Increase with T_3
	nmol tyr/mg wet wt/2 h		
None	0.260±0.020	0.367±0.011	0.107±0.023*
+ LLN (100 μM)	0.208±0.008	0.243±0.010	0.035±0.013‡
Difference	0.052±0.027	0.124±0.020	0.072±0.026‡
% Inhibition	NS	34%*	67%‡
+ MG132 (10 μM)	0.184±0.010	0.246±0.008	0.062±0.013*
Difference	0.076±0.023	0.121±0.008	0.045±0.024‡
% Inhibition	NS	33%*	42%*

Shown are the means±SEM for diaphragm muscles from six animals in each group. Significant difference, $*P < 0.01$, $‡P < 0.05$. To study the effects of T_3 independently of pituitary hormones, hypophysectomized (i.e., hypothyroid) rats (90–120 g) were treated with saline or with T_3 (100 μg/100 g body wt/d) dissolved in saline for 4 d before killing. Muscles were incubated under conditions for measurement of nonlysosomal proteolysis independent of calpains as described in Methods.

Table VII. Effect of MG132 on Proteolysis in EDL Muscles of Septic Rats Induced by Cecal Ligation and Puncture

Addition	Proteolysis in Sham control	Proteolysis in Septic animals	Increase with sepsis
	nmol tyr/mg wet wt/2 h		
None	0.250±0.015	0.344±0.013	0.094±0.019*
+ MG132 (10 μM)	0.162±0.015	0.178±0.004	0.016±0.015 (NS)
Difference	0.087±0.008	0.166±0.011	0.079±0.024*
% Inhibition	35%*	48%*	82%‡

Shown are the means±SEM for EDL muscles from six animals in each group. Significant difference $*P < 0.001$, $‡P < 0.01$. Rats (60–70 g) were subjected to cecal ligation and puncture or sham operation 16 h before killing. All animals were fasted overnight. Muscles were incubated for measurement of nonlysosomal proteolysis independent of calpains as described in Methods.

VII). The nonlysosomal, Ca^{2+}-independent protein breakdown in the EDL muscles from these septic rats increased 38% ($P < 0.001$) above levels in muscles of sham-operated controls. However, if MG132 was present in the incubation medium, this activation of proteolysis by sepsis was completely abolished. In other words, the fraction of protein breakdown sensitive to the inhibitor was almost twofold greater ($P < 0.001$) in the EDL of septic rats than in controls (0.166 ± 0.011 vs. 0.087 ± 0.008 nmol tyr/mg/2 h). Thus, in three distinct experimental models where there is profound muscle wasting, these proteasome inhibitors selectively reduced or eliminated the accelerated proteolysis.

Discussion

A number of findings presented here demonstrate that the peptide aldehyde inhibitors suppress protein breakdown and improve overall protein balance in rat muscles by inhibiting the ubiquitin-proteasome–dependent pathway: (*a*) these agents were found to primarily reduce the ATP-requiring component of protein degradation that is not sensitive to inhibitors of lysosomal function or of calpains; (*b*) they caused an accumulation of ubiquitin-protein conjugates, which are normally degraded by the 26S proteasome complex; and (*c*) these agents were most effective against the accelerated ATP-dependent protein breakdown in three different models of muscle atrophy. Prior studies had suggested that the ubiquitin-proteasome–dependent process was increased in these models (3), based on indirect findings of increased levels of mRNA for ubiquitin and for certain proteasome subunits, and of increased levels of ubiquitin-conjugated proteins. The present findings thus provide further direct support for the conclusion that muscle wasting results primarily from an acceleration of the ubiquitin-proteasome pathway. Furthermore, our studies provide the first evidence that thyroid hormones are an important regulator of the proteasome-dependent proteolytic process.

It seems extremely likely that the suppression of protein breakdown in muscle by LLN and MG132 occurs by inhibition of the active sites of the proteasome. The relative potency of these inhibitors paralleled their effects on the 20S and 26S proteasomes purified from rabbit muscle (16, 17). Also, the analog LLM, which only weakly inhibits the proteasome (17), had no effect on proteolysis in the incubated muscles. The reversibility observed for the inhibition of muscle protein breakdown by LLN is also consistent with a hemiacetal adduct between these inhibitor(s) and the proteasome's active site threonine residue (24), as has been shown to occur upon binding of LLN to archael proteasomes by x-ray diffraction analysis (16). Because MG132 like LLN binds through its aldehyde moiety to the threonine residue in the proteasome active site, its effects should also be readily reversible, as has been seen in other cells (17, 22). Furthermore, our findings in muscle are consistent with previous observations indicating that the peptide aldehydes reversibly inhibit the degradation of long-lived proteins in mammalian cells (17).

It is noteworthy that proteolysis in the isolated skeletal muscles was less sensitive to these inhibitors than protein degradation in cultured lymphocytes, fibroblasts, and HeLa cells (17). The concentrations of LLN and MG132 which inhibited overall degradation in rat skeletal muscles by 40–50% blocked degradation of most proteins in cultured cells by 85–90% (17). Possibly, in intact skeletal muscle, there is slower uptake of the peptide aldehydes or more rapid degradation of these inhibitors (e.g., more rapid oxidation of the aldehyde moiety or hydrolysis of the peptide backbone). The proteasome-dependent proteolytic process in differentiated muscle could be inherently less sensitive to these inhibitors than in cultured cells. In fact, in related studies, we have found that the ubiquitin-proteasome pathway in rat skeletal muscle homogenates (25) and breakdown of ubiquitin-conjugated proteins by purified muscle proteasomes (17) were only partially inhibited by these agents at 50 μM concentrations. The relative resistance of proteolysis in intact muscle may also indicate that a significant fraction of muscle proteolysis is by a nonlysosomal pathway not involving the proteasome. It is noteworthy in this regard that in incubated rat muscles, 30–50% of the protein breakdown appears to be independent of lysosomes, calpains, or ATP depletion (4) and was not affected by the peptide aldehydes in the present studies (Tables I and III). In contrast, in growing fibroblasts (26) and in lymphocytes (17), < 20% of the protein degradation appears independent of these well-characterized pathways. Thus, a novel proteolytic system which remains to be identified may be responsible for degrading certain proteins in incubated muscles. However, degradation of myofibrillar proteins in cell-free systems from muscle (25) and the accelerated proteolysis in various models of muscle atrophy (3) is primarily by the ATP-requiring proteasomal pathway.

The inhibitors studied here, although very effective against the proteasome, can inhibit calpains and certain lysosomal proteases (17). Therefore, for definitive interpretation of results with these aldehydes in intact cells, it is necessary to also evaluate the possible contributions of these enzymes. For example, in our experiments, LLN significantly blunted the activation of proteolysis in muscle incubated with a Ca^{2+} ionophore, which appears to be due to the calpains. These inhibitors are also active against lysosomal sulfhydryl proteases, such as cathepsin B (17). However, in preliminary studies, we found that LLN had only negligible effects on the lysosomal proteolytic process in muscle, i.e., they caused only a small reduction in the component of protein degradation induced by insulin deprivation and sensitive to methylamine (a weak base that inhibits lysosomal function) (data not shown). Furthermore, prior studies established that the Ca^{2+}-dependent or lysosomal degradative processes make only a small contribution to overall proteolysis in muscle under the incubation conditions used here (6). In fact, in contrast to the effects seen with LLN or MG132, inhibitors of calpains or lysosomal proteases do not reduce significantly the increase in proteolysis in muscle upon fasting (10–12), denervation (6), or sepsis (14), which is an ATP-dependent process. Activation of calpain-dependent proteolysis may occur upon anoxia, after mechanical trauma to muscle, and perhaps in certain animal models of muscular dystrophy, when intracellular Ca^{2+} rises (2). Since LLN and MG132 may reduce the activation of Ca^{2+}-dependent proteolysis, such inhibitors may be useful in combating acute muscle damage from activation of calpains in these pathological states, as well as the slower atrophy caused by activation of the ubiquitin-proteasome pathway.

We found that rates of protein synthesis were not altered by LLN or MG132. Since protein synthesis is very sensitive to even a small reduction in ATP levels (27), ATP content must have been well-maintained after exposure to the inhibitors. Accordingly, Bailey et al. (28) found recently that MG132 at

higher concentrations than used here (30 μM) did not reduce rates of protein synthesis or intracellular ATP concentrations in muscles of rats with metabolic acidosis. Related studies have also indicated that cell morphology is not altered for many hours after exposure to LLN or MG132 (17). The apparent lack of acute toxicity of these compounds should make them very useful reagents to diagnose the involvement of the proteasome in a variety of cellular adaptations and disease processes (29).

These findings on muscles atrophying due to denervation, hyperthyroidism, or sepsis raise the obvious possibility that inhibition of the proteasome-dependent pathway may have therapeutic actions in improving muscle protein balance and reducing various types of muscle wasting in animals and humans. The present observations are also consistent with the view that activation of the ubiquitin-proteasome pathway represents a general mechanism responsible for many types of muscle atrophy (3). After completion of this work, Mitch and colleagues demonstrated that MG132 also could reduce the increased proteolysis in muscles of rats with metabolic acidosis (28) or diabetes (30). Because of the many important functions of the ubiquitin-proteasome pathway, complete inhibition of proteasome function eventually must have toxic effects in intact animals, for example due to the inhibition of the cell cycle (by blocking degradation of the mitotic cyclins [31]), accumulation of abnormal proteins (8), and inhibition of class I antigen presentation (17). However, partial inhibition of the ubiquitin-proteasome pathway may not have deleterious consequences, even in prolonged treatments. These findings certainly demonstrate the potential of proteasome inhibitors to improve nitrogen balance and to preserve muscle mass in catabolic states where there is severe cachexia and muscle wasting. Potentially even greater selectivity for pharmacologic intervention and avoidance of toxic consequences will be achieved by alternative strategies such as inhibition of protein ubiquitination (16). More systematic studies will be necessary to evaluate the longer-term effects of partial inhibition of proteasome function on muscle and whole body physiology.

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