Sepsis-induced increase in muscle proteolysis is blocked by specific proteasome inhibitors

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Hoblerr, Scott C., Greg Tiao, Josef E. Fischer, John Monaco, and Per-Olof Hasselgren. Sepsis-induced increase in muscle proteolysis is blocked by specific proteasome inhibitors. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R30–R37, 1998.—Recent studies suggest that sepsis stimulates ubiquitin-dependent protein breakdown in skeletal muscle. The 20S proteasome is the catalytic core of the ubiquitin-dependent proteolytic pathway. We tested the effects in vitro of the proteasome inhibitors N-acetyl-L-leucinyl-L-leucinal-L-norleucinal (LLnL) and lactacystin on protein breakdown in incubated muscles from septic rats. LLnL resulted in a dose- and time-dependent inhibition of protein breakdown in muscles from septic rats. Lactacystin blocked both total and myofibrillar muscle protein breakdown. In addition to inhibiting protein breakdown, LLnL reduced muscle protein synthesis and increased ubiquitin mRNA levels, probably reflecting inhibited proteasome-associated ribonuclease activity. Inhibited muscle protein breakdown caused by LLnL or lactacystin supports the concept that the ubiquitin-proteasome pathway plays a central role in sepsis-induced muscle proteolysis. The results suggest that muscle catabolism during sepsis may be inhibited by targeting specific molecular mechanisms of muscle proteolysis.

ubiquitin; N-acetyl-L-leucinyl-L-leucinal-L-norleucinal; lactacystin; 20S proteasome; catabolic

ONE OF THE MOST PROMINENT metabolic changes during sepsis is the catabolic response in skeletal muscle, characterized by a substantial increase in protein breakdown, in particular, myofibrillar protein breakdown (4, 10). One of the consequences of muscle protein breakdown is the release of amino acids from muscle tissue. A large portion of these amino acids is used by the liver for acute phase protein synthesis and gluconeogenesis (26). Other amino acids, particularly glutamine, are taken up by enterocytes and cells of the immune system and serve as an important source of energy for these cells (20, 32). Thus the catabolic response in skeletal muscle may be beneficial to the organism, at least during the early phase of sepsis. In severe and protracted sepsis, however, continued muscle protein breakdown results in muscle wasting and fatigue, which may impair recovery and lead to an increased risk of thromboembolic and pulmonary complications if ambulation is delayed and respiratory complications if ambulation is delayed and respiratory complications if ambulation is delayed. In recent studies, we found evidence that the catabolic response in skeletal muscle during sepsis is primarily caused by upregulated, energy-dependent, nonlysosomal protein breakdown (30). The most important energy-dependent proteolytic mechanism is the ubiquitin-dependent protein breakdown. In this proteolytic pathway, proteins that are to be degraded are first conjugated to ubiquitin, whereafter they are recognized by the 26S proteolytic complex (6, 11). The catalytic core of the 26S proteasome is the 20S proteasome, which is a barrel-shaped particle composed of four stacked rings (6, 29). Each ring consists of seven subunits, the outermost of which are called α-subunits and the innermost of which are called β-subunits. The functions of the α-subunits include substrate recognition and unfolding, whereas the hydrolytic sites are located on the β-subunits (29).

Although previous findings of upregulated energy-dependent protein breakdown and increased ubiquitin mRNA levels in skeletal muscle during sepsis strongly support a role of the ubiquitin-dependent mechanism in sepsis-induced muscle proteolysis (30), the results do not prove that sepsis-induced muscle catabolism is caused by increased activity in this proteolytic pathway. One way to further test the role of the ubiquitin-proteasome mechanism in sepsis-induced muscle proteolysis would be to use inhibitors of the proteolytic pathway. The recent description of specific 20S proteasome blockers has made such experiments possible (5, 24). The effects of these substances on muscle protein metabolism are not well understood.

The present study was designed to test the hypothesis that sepsis-induced muscle protein breakdown can be blocked by specific 20S proteasome inhibitors. We found that when muscles from septic rats were treated in vitro with the proteasome inhibitor N-acetyl-L-leucinyl-L-leucinal-L-norleucinal (LLnL) (24), protein breakdown was inhibited in a time- and dose-dependent fashion. Similar results were obtained with another 20S proteasome inhibitor, lactacystin (5), further supporting the role of the ubiquitin-proteasome pathway in sepsis-induced muscle proteolysis.

MATERIALS AND METHODS

Animals and experimental model. Sepsis was induced by cecal ligation and puncture (CLP) in male Sprague-Dawley rats weighing 40–60 g, as described previously (10, 22). Control rats underwent sham operation, i.e., laparotomy and manipulation but no ligation or puncture of the cecum. All rats were resuscitated with 10 ml saline/100 g body wt, which was administered subcutaneously on the back at the time of surgery. The rats had free access to water, but food was withheld after surgery to avoid any influence on metabolic
changes of different food intake between the two groups of rats. This experimental model of sepsis is clinically relevant because it resembles the situation in patients with sepsis caused by intra-abdominal abscess and devitalized tissue. The model was characterized with respect to hemodynamic and metabolic changes and survival rates in previous studies from our (22) and other laboratories (2). Rats weighing 40–60 g were used in the present experiments because lower-extremity muscles from rats of this size are thin enough to allow for adequate tissue oxygenation and viability during in vitro incubation (7). All experiments were conducted and animals were cared for in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Cincinnati.

Muscle incubations. Sixteen hours after CLP or sham operation, total and myofibrillar protein breakdown rates were determined by measuring net release of tyrosine and 3-methylhistidine (3-MH), respectively, from incubated extensor digitorum longus muscles, as described in detail previously (9, 10). In other muscles, protein synthesis rates were determined by measuring incorporation of [14C]phenylalanine into protein by incubating muscles with a flooding dose of the precursor amino acid, as described previously (15). For determination of protein breakdown rates, the incubation medium consisted of calcium-free oxygenated (95% O2–5% CO2) Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 10 mM glucose, 0.1 µM/ml insulin, and five times the normal rat plasma concentrations of the branched-chain amino acids leucine, isoleucine, and valine (18). Muscles were preincubated for 30 min and were then transferred to fresh medium and incubated for 2 h unless stated otherwise. All incubations were performed in a shaking water bath at 37°C. Groups of muscles were preincubated and incubated in the absence or presence of different concentrations of LLnL (Sigma, St. Louis, MO) or lactacystin (kindly provided by Dr. E. J. Corey, Harvard University, Cambridge, MA) (5). The incubation medium was calcium free, to avoid the influence of calcium-dependent protein breakdown, and contained insulin and branched-chain amino acids to avoid the influence of lysosomal protein breakdown (30). Tyrosine and 3-MH were determined spectrophotometrically (30).

Western blot analysis. Free and conjugated ubiquitin were determined by Western blot analysis in muscles incubated in the absence or presence of proteasome inhibitor. After the 2-h incubation, the muscles were sonicated for 5–10 s, after which they were homogenized using a Dounce homogenizer (Kontes Glass, Vineland, NJ). Homogenization was carried out in 1 ml of buffer containing 50 mM tris(hydroxymethyl)aminomethane-HCl, pH 7.4, 1% sodium dodecyl sulfate, 5 mM EDTA, 0.25 mM sucrose, 2 µg/ml aprotinin, 1 mM phenylmethane sulphonyl fluoride, and 5 mM N-ethylmaleimide. After centrifugation at 12,000 g for 15 min, the supernatant was used for determination of protein (17), and Western blot analysis was performed using a purified rabbit polyclonal antibody to sodium dodecyl sulfate-denatured ubiquitin (kindly supplied by Dr. Arthur Haas, University of Wisconsin, Madison, WI) (8).

Northern blot analysis. We determined ubiquitin mRNA levels by Northern blot analysis, as described in detail previously (30), using cDNA probes for ubiquitin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) labeled by random priming with [32P]dATP or [32P]dCTP (Stratagene, La Jolla, CA). The ubiquitin cDNA probe used here was obtained as described previously from our laboratory (30). The GAPDH cDNA probe was purchased from American Type Culture Collection (Rockville, MD). Autoradiographs were quantitated on a phosphoimager using the Image Quant program (Molecular Dynamics, Sunnyvale, CA), and the relative mRNA abundance was expressed as the ratio between ubiquitin mRNA and GAPDH mRNA.

Statistics. Results are presented as means ± SE. Student's t-test or analysis of variance followed by Tukey's test was used for statistical comparisons.

RESULTS
In initial experiments, we examined the relationship between different concentrations of LLnL and the incubation medium and inhibition of muscle protein breakdown. Because the focus of the present study was to examine the effect of proteasome blockers on sepsis-induced muscle proteolysis, muscle from septic rats was used in these experiments. LLnL inhibited protein breakdown in a dose-dependent fashion, with a maximal effect noticed at 100 µM (Fig. 1).

When muscles were incubated for various periods of time in the presence of 100 µM LLnL, a slight decrease in tyrosine release was noticed as early as 30 min after the start of incubation, and this inhibition became statistically significant after 60 min of incubation (Fig. 2). This response to LLnL suggests that it is rapidly taken up by the muscle cell and readily reaches the 20S proteasome. Evidence that LLnL (and other peptide aldehydes) readily penetrates cell membranes was found in cultured lymphoblasts as well (24). On the basis of these initial results, muscles were incubated in the presence of 100 µM LLnL for 2 h in most subsequent experiments.

We next examined the effects of LLnL on protein breakdown in muscles from sham-operated and septic rats.
rats. Protein breakdown, measured as tyrosine release, was increased by ~75% in muscles from septic rats (Fig. 3), confirming previous reports from our laboratory (10, 30). Because the incubation medium was calcium free and contained insulin and branched-chain amino acids, the results mainly reflected increased nonlysosomal, calcium-independent protein breakdown. This experimental design was used because a recent study suggested that sepsis selectively stimulates ubiquitin-dependent muscle protein breakdown without affecting lysosomal or calcium-dependent proteolysis (30). Addition of LLnL to the incubation medium (100 µM) inhibited protein breakdown in muscles from both nonseptic and septic rats (Fig. 3). In the presence of LLnL, there was no significant difference in protein breakdown rates between muscles from sham-operated and septic rats, suggesting that the proteasome inhibitor blocked the sepsis-induced increase in muscle proteolysis. Myofibrillar protein breakdown rates could not be determined in the experiments in which LLnL was tested because the substance interfered with the HPLC method used to measure 3-MH (unpublished observation).

The inhibition by LLnL of protein breakdown in muscle from sham-operated rats noticed in Fig. 3 may reflect inhibition of protein breakdown that was increased after sham operation and 16-h fasting. Alternatively, the results could be consistent with a proteasome-dependent component of basal protein breakdown. To dissect these possibilities, we examined the effect of LLnL on protein breakdown in muscles from untreated, fed rats. In these muscles as well, protein breakdown was inhibited in the presence of 100 µM LLnL (from 260 ± 15 to 183 ± 8 nmol tyrosine·g wet weight⁻¹·h⁻¹, P < 0.05, n = 7 in each group), suggesting that basal muscle protein breakdown is at least partly regulated by 20S proteasome activity.

Because the ubiquitin-proteasome proteolytic pathway is energy dependent (6, 11, 30), it is possible that the effect of LLnL on muscle protein breakdown noticed here was caused by reduced tissue energy levels. To test that possibility, we next measured ATP levels in muscles that were incubated with or without LLnL. Results from those experiments demonstrated that muscle ATP levels were maintained during the 2-h incubation and were not influenced by LLnL (Fig. 4). Thus the inhibition of protein breakdown by LLnL did not seem to reflect tissue energy depletion.

Previous studies suggest that proteasomes regulate not only intracellular protein breakdown but protein synthesis as well. For example, proteasomes interfered with protein synthesis in cultured HeLa cells (13) and prevented the formation of 80S initiation complexes in...
a reticulocyte cell-free system (12). To test if proteasomes regulate protein synthesis in muscle tissue as well, we next determined the incorporation rate of phenylalanine into protein in muscles incubated in the absence or presence of LLLnL. When tissue was incubated in the presence of 100 µM LLLnL, protein synthesis was significantly inhibited in muscles from both sham-operated and septic rats (Fig. 5).

One important aspect of a metabolic inhibitor is whether its effect is reversible or not. We tested the reversibility of the effect of LLLnL by measuring protein breakdown in muscles that had first been incubated in the presence of LLLnL and were then washed in medium without LLLnL. When muscles were first incubated for 2 h in the presence of 100 µM LLLnL and then washed two or four times (each wash 15 min) in fresh medium without LLLnL, the inhibitory effect of LLLnL was partially reversed (Fig. 6). The effect of additional washing was not tested because of concern for tissue viability during more-extended periods of incubation. Thus it is not known from the present results whether the remaining inhibition of protein breakdown after washing the tissue four times represented a nonreversible component of the LLLnL-induced inhibition of protein breakdown. Although this question was not resolved by the present experiments, the results are important because the partial reversibility suggests that at least some of the regulatory mechanisms of protein breakdown can be restituted after treatment with LLLnL.

Because in the present study muscles were incubated in calcium-free medium containing insulin and branched-chain amino acids, the influence of calcium-dependent and lysosomal protein breakdown was minimized. Therefore, the effects of LLLnL noticed here were most likely specific for proteasome-dependent proteolysis. To further test the specificity with respect to the 20S proteasome mechanism, we next measured protein breakdown rates in muscles incubated in the absence or presence of 100 µM lactacystin, the most selective proteasome inhibitor presently known (5). In this experiment, lactacystin inhibited both total and myofibrillar protein breakdown, supporting the concept that a substantial part of protein breakdown in septic muscle is proteasome dependent (Fig. 7).

From a theoretical standpoint, inhibition of the 20S proteasome may result in a “proximal” accumulation of ubiquitinated proteins. Indeed, a significant increase in ubiquitin-conjugated proteins was noted in cultured CIR.B27 cells after treatment with LLLnL (14). We tested whether exposure of incubated muscles to the proteasome blocker gave rise to a similar accumulation of ubiquitinated proteins. Western blot analysis demonstrated a substantial increase in high-molecular-weight ubiquitin-conjugated proteins after treatment of incubated muscles from septic rats with 100 µM LLLnL for 2 h (Fig. 8). No significant changes were noted in the small-molecular-weight fractions of ubiquitinated proteins or in free ubiquitin after exposure of the muscles to LLLnL.

We next determined whether LLLnL treatment of incubated muscles from septic rats gave rise to changes in ubiquitin mRNA levels. Northern blot analysis of RNA extracted from septic muscles showed an ~50% increase in ubiquitin mRNA levels after treatment in vitro with LLLnL (Fig. 9). Increased tissue levels of ubiquitin mRNA may reflect stimulated transcription of the ubiquitin gene or increased stability of the messenger. Because of the rapid increase in ubiquitin mRNA levels, noted already after 2 h, and because of previous reports of proteasome-associated ribonuclease (RNase) activity (23), it is likely that the increased ubiquitin mRNA levels noticed here after treatment of muscles with LLLnL reflected increased stability (reduced breakdown) of mRNA.
Determination of actual transcription rates was not done in the present study because initial attempts to perform nuclear run-on assays were unsuccessful, mainly due to difficulties in isolating nuclei from the small muscles used in the present study. Instead, to test the possible contribution of stimulated transcription to the increase in ubiquitin mRNA levels, muscles were incubated with or without 100 µM LLnL in the presence of actinomycin D (5 µg/ml). In other experiments we found that this concentration of actinomycin D effectively blocked RNA synthesis in incubated muscles as evidenced by reduction of [3H]uridine incorporation into RNA to background levels (unpublished observation). Actinomycin D did not prevent the increase in ubiquitin mRNA levels caused by LLnL (Fig. 10), suggesting that the effect of LLnL did not reflect an increased transcription rate of ubiquitin but probably reflected increased stability of ubiquitin mRNA, possibly secondary to inhibited RNase activity (23).

**DISCUSSION**

In the present study, treatment in vitro of incubated muscles from septic or control rats with one of the proteasome blockers LLnL or lactacystin resulted in a significant inhibition of protein breakdown. In the presence of LLnL, the difference in protein breakdown rates in muscles from sham-operated and septic rats
was abolished, suggesting that the sepsis-induced increase in muscle proteolysis is mainly caused by increased proteasome-dependent protein breakdown. The data support the interpretation of the results in a recent report from our laboratory that sepsis stimulates the energy-ubiquitin-dependent proteolytic pathway in skeletal muscle (30). The finding in the current study of inhibited protein breakdown after treatment with LLnL of muscles from nonseptic rats as well suggests that part of the basal protein breakdown in skeletal muscle is also regulated by proteasome activity.

It should be noted that the present results do not rule out a potential involvement of other proteolytic mechanisms during sepsis. It is possible, for example, that the relative importance of different pathways varies during different states of sepsis, with different mechanisms being responsible for the initial and final degradation of muscle proteins.

In a recent study, Rock et al. (24) tested the effects of various proteasome blockers, including LLnL, N-acetyl-L-leucinyl-L-leucinyl-methional, and N-carbobenzoxy-L-leucinyl-L-leucinyl-L-norvalinal on protein breakdown in cultured lymphoblasts and in isolated proteasomes from rabbit muscle homogenates. Results from that study showed that the proteasome blockers inhibited the breakdown of different classes of proteins and that different proteolytic sites of the 20S proteasome were blocked. In addition, evidence was found that blocking the proteasome activity interfered with the presentation of major histocompatibility class I molecules, illustrating the important role of proteasome-dependent proteolysis in antigen presentation.

It should be noted that LLnL is not a completely specific proteasome inhibitor but that the drug may block calpains and lysosomal cysteine proteases as well (25). In a recent study, we found evidence that sepsis selectively stimulates energy-ubiquitin-dependent proteolysis without increasing calcium-dependent or lysosomal protein breakdown in skeletal muscle (30). In the present study, muscles were incubated in calcium-free medium containing insulin and branched-chain amino acids to minimize the influence of calcium-dependent and lysosomal protein breakdown. Therefore, the effects of LLnL noticed here most likely reflected inhibition of proteasome-dependent protein breakdown. One potential concern with this experimental approach is that muscles from septic rats may be partially resistant to insulin and branched-chain amino acids, thereby underestimating the proteasome-dependent component of muscle proteolysis during sepsis. This is why the experiments using lactacystin, a highly selective proteasome blocker (5), were important. The inhibition of protein breakdown observed after treatment of the muscles with lactacystin supports the interpretation that a substantial part of the protein breakdown in the incubated muscles was regulated by the 20S proteasome.

The effects of lactacystin on protein breakdown were tested in a recent study by Fenteany et al. (5) in which 20S proteasomes isolated from bovine brain were used. By using specific fluorogenic peptide substrates, evidence was found that lactacystin inhibited the trypsin-like, chymotrypsin-like, and peptidylglutamyl-peptidyl hydrolyzing activities of the proteasome and that at least some of the effect was caused by covalent binding of lactacystin to and modification of the NH2-terminal threonine of the mammalian proteasome subunit of MB1 (also called MB1). In contrast to LLnL, lactacystin did not inhibit calpain and cathepsin B or any other protease tested, and it was concluded that lactacystin is the most selective proteasome inhibitor presently known (5).

In several previous reports, we found evidence that sepsis mainly stimulates the breakdown of myofibrillar proteins in skeletal muscle and that most of the increase in total protein breakdown reflects increased myofibrillar breakdown (10, 30). In the present experiments, in which LLnL was used, we were not able to determine release of 3-MH, which is a specific measure of myofibrillar protein breakdown (33), due to interference of LLnL with the HPLC method used to measure 3-MH (unpublished observation). Our data, therefore, do not allow us to conclude that LLnL inhibited the breakdown of myofibrillar proteins. It is likely, however, that the proteasome inhibitor blocked myofibrillar protein breakdown because we (30) and others (16) have found evidence that myofibrillar proteins are degraded by an energy-dependent, nonlysosomal mechanism, most likely the ubiquitin-proteasome pathway. The results from the experiments in which lactacystin was used support the concept that myofibrillar protein breakdown in septic muscle is proteasome dependent. Although the present results most likely reflected inhibited myofibrillar protein degradation, it is possible that the breakdown of other proteins as well was inhibited by LLnL and lactacystin. Previous studies in lymphoblasts suggest that proteasome blockers inhibit the breakdown of different classes or proteins, in-
In previous studies, proteasomes interfered with initiation of protein synthesis and blocked the formation of 80S initiation complexes (12). Thus the present result of inhibited, rather than stimulated, protein synthesis in muscles incubated with LLnL was unexpected. The mechanism behind this finding is unclear at present. A general toxic effect of LLnL is an unlikely mechanism, considering the maintained ATP levels in muscles incubated with the proteasome blocker.

The present results support the concept that the proteasomes are multifunctional complexes regulating not only intracellular protein breakdown but also RNA stability and protein synthesis. Thus intervening with blocking agents may have multiple metabolic consequences. It will be an important area for future research to determine whether the proteolytic activity of the 20S proteasome can be blocked selectively and whether such treatment will inhibit muscle proteolysis induced by sepsis and other catabolic conditions.

**Perspectives**

Muscle catabolism is typically seen in patients with sepsis (4), and in a recent study we found evidence of upregulated expression of the ubiquitin-proteasome pathway in human muscle tissue during sepsis (31). The present results are therefore important from a clinical standpoint because they suggest that muscle catabolism during sepsis can be inhibited by targeting specific molecular mechanisms of muscle proteolysis. Before that goal can be achieved, however, a number of additional questions need to be addressed. For example, it is not known at present which is the rate-limiting step in the ubiquitin-proteasome pathway in skeletal muscle during sepsis. The initiating event in myofibrillar protein breakdown and the mechanism(s) by which myofibrillar proteins become susceptible to ubiquitin conjugation also remain to be determined. The finding in the present study that the 20S proteasome may be responsible not only for protein breakdown but also for regulation of protein synthesis and mRNA stability suggests that blocking components of the ubiquitin pathway other than the 20S proteasome may result in a more specific and selective inhibition of the catabolic response to sepsis.

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