Activity and expression of the 20S proteasome are increased in skeletal muscle during sepsis

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Hoblér, Scott C., Arthur Williams, David Fischer, Jing Jing Wang, Xiaoyan Sun, Josef E. Fischer, John J. Monaco, and Per-Olof Hasselgren. Activity and expression of the 20S proteasome are increased in skeletal muscle during sepsis. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R434–R440, 1999.—Recent studies suggest that sepsis stimulates ubiquitin-dependent proteolysis in skeletal muscle. In this proteolytic pathway, ubiquitinated proteins are recognized, unfolded, and degraded by the multicatalytic 26S protease complex. The 20S proteasome is the catalytic core of the 26S protease complex. The role of the 20S proteasome in the regulation of sepsis-induced muscle proteolysis is not known. We tested the hypothesis that sepsis increases 20S proteasome activity and the expression of mRNA for various subunits of this complex. Proteolytic activity of isolated 20S proteasomes, assessed as activity against fluorogenic peptide substrates, was increased in extensor digitorum longus muscles from septic rats. The proteolytic activity was inhibited by specific proteasome blockers. Northern blot analysis revealed an approximately twofold increase in the relative abundance of mRNA for the 20S α-subunits RC3 and RC9 and the β-subunit RC7. However, Western blot analysis did not show any difference in RC9 protein content between sham-operated and septic rats. The increased activity and expression of the 20S proteasome in muscles from septic rats lend further support for a role of the ubiquitin-proteasome pathway in the regulation of sepsis-induced muscle proteolysis.

A prominent metabolic consequence of sepsis is the catabolic response in skeletal muscle, characterized by a substantial increase in protein breakdown, in particular myofibrillar protein breakdown (16). The increased protein breakdown results in release of amino acids from muscle tissue. A large portion of these amino acids are used by the liver for acute phase protein synthesis and gluconeogenesis (30). 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 (25, 36). 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 for thromboembolic and pulmonary complications if ambulation is delayed and respiratory muscles are affected. Increased knowledge of the mechanisms regulating muscle proteolysis during sepsis therefore is of great clinical significance and may be important for the development of future therapeutic modalities to inhibit the catabolic response in patients with sepsis.

Intracellular protein breakdown is regulated by different proteolytic pathways, including lysosomal and nonlysosomal pathways (11). Recent studies in both experimental animals and patients with sepsis provided evidence that the sepsis-induced muscle catabolism is associated with upregulated energy-ubiquitin-dependent protein breakdown (33, 34). In this proteolytic pathway, proteins are conjugated to ubiquitin, whereafter they are degraded by the 26S proteolytic complex (14, 17). The catalytic core of the 26S proteasome is the 20S proteasome, which is a barrel-shaped particle composed of four stacked rings with seven subunits in each ring (2). The two outer rings are comprised of α-subunits and the two inner rings subunits. The functions of the α-subunits include interaction between the 20S proteasome and various regulators, whereas the hydrolytic sites are located on the inner side of some of the subunits. The 20S protease possesses at least five peptidase activities, i.e., the trypsin-like, chymotrypsin-like, peptidylglutamyl peptidase, branched-chain amino acid-preferring, and small neutral amino acid-preferring activities (26). The influence of sepsis on the expression and activity of the 20S proteasome in skeletal muscle is not known.

The present study was designed to test the hypothesis that the activity of the 20S proteasome and the expression of various proteasome subunits are increased in skeletal muscle during sepsis. We found that 20S proteasomes isolated from muscles of septic rats displayed increased proteolytic activity and that the expression of several proteasome subunits was upregulated. The results support a role for proteasome-dependent muscle proteolysis during sepsis.

MATERIALS AND METHODS

Sepsis was induced in male Sprague-Dawley rats (40–60 g body wt) by cecal ligation and puncture (CLP) as described previously (16, 33). Control rats were sham operated, i.e., they underwent laparotomy and manipulation, but no ligation or puncture, of the cecum. All animals were resuscitated with 10 ml/100 g body weight of normal saline administered subcutaneously on the back at the time of surgery. Metabolic studies were performed up to 16 h after CLP or sham...
operation. Rats had free access to drinking water, but food was withheld after the surgical procedures to avoid the influence on metabolic changes of any difference in food intake between the experimental groups. This septic model was used in several previous reports from our laboratory and resulted in a reproducible and pronounced increase in total and myofibrillar protein breakdown rates and upregulated expression of ubiquitin mRNA in skeletal muscle (16, 33). The model is clinically relevant because it resembles the situation in patients with sepsis caused by fecal peritonitis and intra-abdominal devitalized tissue. In previous reports that used this model, rats weighing 40–60 g were used because their extremity muscles are thin enough to allow for measurement of protein breakdown rates during incubation in vitro (12, 15). Rats of the same size were used in the present experiments to make it possible to compare results with previous results of increased protein breakdown rates in incubated muscles 16 h after CLP (16, 33).

Isolation of 20S proteasomes and measurement of proteolytic activity. At different time points up to 16 h after sharn operation or CLP, rats were anesthetized with pentobarbital sodium (35 mg/kg ip) and the extensor digitorum longus (EDL) and soleus muscles were harvested, frozen in liquid nitrogen and stored at −70°C until analysis. The EDL (a fast-twitch muscle) and soleus (a slow-twitch muscle) muscles were studied here because in previous reports total and myofibrillar energy-dependent protein breakdown and expression of the ubiquitin-proteasome proteolytic pathway were substantially increased in EDL muscle 16 h after CLP (16, 33). Isolation of 20S proteasomes and measurement of proteolytic activity. At different time points up to 16 h after sharn operation or CLP, rats were anesthetized with pentobarbital sodium (35 mg/kg ip) and the extensor digitorum longus (EDL) and soleus muscles were harvested, frozen in liquid nitrogen and stored at −70°C until analysis. The EDL (a fast-twitch muscle) and soleus (a slow-twitch muscle) muscles were studied here because in previous reports total and myofibrillar energy-dependent protein breakdown and expression of the ubiquitin-proteasome proteolytic pathway were substantially increased in EDL muscle 16 h after CLP (16, 33).

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Tween, after which it was exposed to the primary antibody (purified rabbit polyclonal antibody to rat C9 produced in the laboratory of J. Monaco) at a dilution of 1:5,000 in TBS, 0.1% Tween, and 5% fat-free dried milk overnight at 4°C. The RC9 signal was visualized with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG antibody (1:3,000) and epichemiluminescence substrate in accordance with the manufacturer’s protocol (Amersham, Arlington Heights, IL). The membrane was exposed to Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY). Bands were quantitated with the BioMAX 1D 1.51 Kodak Scientific Imaging System (Eastman Kodak, Rochester, NY).

Statistics. Results are presented as means ± SE. Student’s t-test or ANOVA followed by Tukey’s test was used for statistical analysis.

RESULTS

In initial experiments, proteasomes were isolated from EDL muscles 16 h after sham operation or CLP. The proteasome activity against LLVY was increased by ~50% and that against LLE was doubled in muscles from septic rats (Fig. 1), suggesting that the chymotrypsin-like and peptidylglutamyl peptidase activities of the proteasome were increased during sepsis.

To examine how soon after induction of sepsis proteasome activity increased, chromotrypsin-like activity was measured in EDL muscles from groups of rats 2, 4, 8, and 16 h after sham operation or CLP. Activity against LLVY was significantly increased 4 h after induction of sepsis and remained elevated throughout the rest of the experimental period (Fig. 2).

To validate the methods used here to isolate 20S proteasomes and to study their proteolytic activity, we next tested the effect of different protease inhibitors. The lysosomal inhibitor leupeptin (100 µM) did not affect proteasome activity against LLVY or LLE in EDL muscles from sham-operated or septic rats (Fig. 3). The proteasome blocker N-acetyl-leu-leu-norleucinal (LLnL; 100 µM) (29) inhibited the activity of proteasomes against both substrates in muscles from sham-operated and septic rats. Lactacystin (100 µM), which is a more specific proteasome blocker than LLnL (10) and mainly blocks chymotrypsin-like proteasome activity (3), inhibited the activity against LLVY of proteasomes from both sham-operated and septic rats, but had no significant effect on the activity against LLE (Fig. 3).

The involvement of the 20S proteasome in the catabolic response to sepsis was further tested by examining the expression of mRNA for the α-subunits RC3 and RC9 and the β-subunit RC7 of the 20S proteasome. These subunits were studied because their expression was increased in muscle tissue in other catabolic conditions, including metabolic acidosis (22), denervation (21), and fasting (37). Sepsis resulted in a ~70% increase in the concentrations of RC3, RC9, and RC7 in EDL muscles (Fig. 4).

In additional experiments, we examined whether sepsis resulted in an increased amount of the 20S proteasome in the EDL muscle. This was done by determining the levels of RC9 protein in muscles from sham-operated and septic rats by means of Western blot analysis. C9 is a ubiquitous 20S proteasome subunit (28), and changes in the amount of 20S proteasome should therefore be reflected by changes in C9 protein. Western blot analysis showed that sepsis did not result in changes in C9 protein levels in EDL muscles (Fig. 5).

In previous studies, evidence was found that the catabolic response to sepsis is particularly pronounced in white, fast-twitch muscles (e.g., EDL muscles) with only minor changes in protein breakdown rates and gene expression of different components of the ubiquitin-proteasome pathway occurring in red, slow-twitch muscle (e.g., soleus muscles) (16, 35). To test whether a similar differential response to sepsis occurs with regard to 20S proteasome activity and expression, we next isolated 20S proteasomes from soleus muscles of sham-operated and septic rats. The proteasome activity against LLVY and LLE was not affected by sepsis in soleus muscles (Fig. 6). Also, mRNA levels for RC3,
RC7, and RC9 were unchanged in soleus muscles during sepsis (Fig. 7).

**DISCUSSION**

In the present study, the activities against the peptide substrates LLVY and LLE were increased in 20S proteasomes isolated from EDL muscles of septic rats concomitant with increased expression of mRNA for several of the 20S proteasome subunits. Because the amount of RC9 protein was not increased in the same muscles, the increased proteasome activity most likely reflected increased specific activity, i.e., an increase in activity per 20S proteasome unit. The mechanism(s) of increased 20S proteasome activity in septic muscle is not known from the present experiments but may be related to increased activity of the regulating protein PA28 (11S) (6, 23) or an altered composition of the 20S proteasome (2). There is evidence that the proteasome fraction generated by the isolation method used in the present report contains PA28 (J. Monaco, unpublished observation). It is not likely that the proteasome activity was influenced by the PA700 (19S) regulatory protein because the assay was performed in the absence of ATP. Regardless of the mechanism of increased 20S proteasome activity, the present results support
the concept that sepsis-induced muscle proteolysis is associated with upregulated expression and activity of the proteasome-dependent proteolytic pathway (33–35). This interpretation was further supported by the finding that 20S proteasome activity and expression were not influenced by sepsis in soleus muscles. Thus the changes in 20S proteasome activity and expression noted here in different types of skeletal muscle, paralleled changes in protein breakdown rates and expression of ubiquitin reported previously (16, 35). In addition, in recent studies from our (9, 18) and other laboratories (32), the catabolic response in skeletal muscle was blocked by specific proteasome inhibitors, lending further support to the role of the ubiquitin-proteasome pathway in sepsis-induced muscle catabolism.

The effects of the proteasome inhibitors LLnL and lactacystin on the 20S proteasome activity were tested here. Although the peptide aldehyde LLnL has been widely used in previous studies as a proteasome blocker (9, 18, 29, 32), this substance is not completely specific but inhibits calpains and lysosomal cysteine protease activity as well (29). In contrast, lactacystin is a specific proteasome blocker that irreversibly inhibits the chymotrypsin-like peptidase activity of the 20S proteasome (10). In recent studies, evidence was found that lactacystin spontaneously hydrolyzes into clastolactacystin β-lactone, which is the active proteasome inhibitor (7). Lactacystin (β-lactone) exerts its 20S proteasome blocking effect by irreversibly binding to β-subunits that have N-terminal threonines, i.e., LMP2, LMP7, MECL-1, X, Y, and Z (10). The differential effects of leupeptin (a predominantly lysosomal protease inhibitor) on one hand and LLnL and lactacystin on the other hand noted here support the interpretation that the activity against the peptide substrates used here reflects proteasome activity.

The influence of sepsis on the activity of isolated 20S proteasomes from skeletal muscle has not been reported previously. However, muscle 20S proteasome activity has been examined in other catabolic conditions. The effect of starvation on muscle 20S proteasomes was reported by Dahlmann et al. (4). They found that neither the total amount of 20S proteasome nor its peptidase activity increased during starvation. Rather, the specific activity was decreased and the 20S proteasome content per muscle remained at the same level as in normal rats. In contrast, recent experiments in our laboratory (unpublished observation) provided evidence that muscle 20S proteasome activity was increased after burn injury, another condition characterized by muscle catabolism. Thus the 20S proteasome may respond differently to different catabolic conditions.

The regulation of the proteolytic activity of isolated 20S and 26S proteasomes from normal rabbit skeletal muscle was examined in a recent study by Craiu et al. (3). Similar to the present report, lactacystin (and its active breakdown product β-lactone) effectively blocked the hydrolysis of LLVY, consistent with inhibition of the chymotrypsin-like proteasome activity. In contrast, the peptidylglutamyl-like peptidase activity was relatively resistant to the effect of β-lactone (3), which probably explains why lactacystin did not significantly block the hydrolysis of LLE in the present study. In addition to examining the hydrolysis of different peptide substrates by the proteasomes, Craiu et al. (3) determined the breakdown of casein by purified muscle proteasomes. Lactacystin and β-lactone inhibited casein breakdown by the 20S and 26S proteasomes as well, although the concentrations necessary to inhibit protein breakdown were higher than to reduce peptide hydrolysis. Those results are important because they suggest that changes in proteasome activity against peptide substrates reflect changes in proteasome proteolytic activity.
RC7, is inactive (24). It is not known from the present study whether the increased mRNA levels for RC7 were related to the increased proteasome proteolytic activity, but it may be speculated that the expression of both inactive and active β-subunits is increased in catabolic conditions, thus allowing for increased interaction between different β-subunits and stimulated proteolytic activity. Because the mRNA levels for the two α-subunits examined here were increased as well, it is possible that the expression of all or at least most proteasome subunits (α and β) is increased in skeletal muscle during different catabolic conditions (21, 22, 34, 35, 37), suggesting that the expression of several of the proteasome subunits is upregulated in parallel in catabolic muscle.

It is not known from the present experiments whether the increased mRNA levels for the proteasome subunits reflected increased gene transcription, increased mRNA stability, or a combination of these mechanisms. In a recent study, we found evidence that increased ubiquitin mRNA levels in septic muscle were not caused by increased mRNA stability, but most likely reflected increased transcription of the ubiquitin gene (35). In other studies, Price et al. (27) found that increased mRNA levels for ubiquitin and the proteasome subunits RC3, RC5, and RC9 in skeletal muscle of insulinopenic rats were caused by stimulated gene transcription. Thus it is likely that the increased mRNA levels for RC3, RC9, and RC7 noted here in muscles of septic rats were caused by increased transcription rates, although further experiments are needed to test that notion.

The unchanged RC9 protein levels in muscles from septic rats were surprising in light of the increased RC9 mRNA levels. The finding may have several explanations, including increased breakdown of RC9 protein in addition to increased RC9 synthesis. The results could also be consistent with reduced translational efficiency of the RC9 mRNA. In addition, the finding may reflect different time courses for RC9 mRNA and protein levels, and it is possible that protein levels were elevated at a later time point than studied here. More experiments are needed to determine the mechanism(s) behind the unchanged RC9 protein levels in light of the increased mRNA levels.

Although the present results suggest that sepsis stimulates proteasome-dependent protein breakdown in skeletal muscle, the data need to be interpreted with caution for several reasons. First, the proteolytic activity was measured in a cell-free system with the use of artificial substrates, and because no ATP was added to the system, activity of the 20S rather than the 26S proteasome accounted for the results. This differs from the situation in vivo, where ubiquitinated proteins are degraded by the 26S proteasome by an energy-dependent mechanism (14, 17). The activity of isolated 20S proteasomes was measured in the present study because the 20S proteasome is the catalytic core of the 26S proteasome (1, 2). The present experimental design, therefore, allowed for the individual assessment of one of the key components of the ubiquitin-proteasome pathway. In addition, there is evidence that the 20S proteasome, rather than the 26S proteasome, is responsible for the breakdown of oxidized proteins (13), which is of particular importance for the present study because sepsis results in increased levels of oxidatively damaged proteins in skeletal muscle (8).

Second, even if the 20S proteasome proteolytic activity is increased in skeletal muscle during sepsis, it is not known if this is a rate-limiting step of sepsis-induced muscle proteolysis. Indeed, although recent studies suggest that sepsis is associated with increased expression of ubiquitin, ubiquitin-conjugating enzymes, and several of the 20S proteasome subunits (19, 33–35), it is not known whether muscle proteolysis during sepsis is activated or regulated by the ubiquitin-proteasome pathway. It is possible, for example, that the proteolytic pathway is upregulated secondary to increased amounts of substrates made available to the system. Third, only two proteolytic activities were tested in the present study, and it remains to be determined if other 20S proteasome activities as well are stimulated by sepsis.

Finally, 20S proteasomes were isolated from muscles of small growing rats with septic peritonitis. Although this experimental model was associated with upregulated energy-dependent muscle protein breakdown and increased expression of various components of the ubiquitin-proteasome pathway (33, 35), further studies are needed to determine if the 20S proteasome activity is increased in muscle from adult animals and patients with sepsis. In light of a recent study from this laboratory in which the expression of ubiquitin and the proteasome subunit HC3 was increased in muscle from septic patients (34), it is possible that sepsis results in increased 20S proteasome activity in human muscle as well.

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