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Tripeptidyl-peptidase II expression and activity are increased in skeletal muscle during sepsis

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Abstract

Ubiquitin–proteasome-dependent protein degradation plays a central role in sepsis-induced muscle wasting. Because the proteasome degrades proteins into small peptides rather than free amino acids, it is likely that additional mechanisms downstream of the proteasome are involved in sepsis-induced muscle proteolysis. Recent studies suggest that the extralysosomal peptidase tripeptidyl-peptidase II (TPP II) degrades peptides generated by the proteasome. We hypothesized that TPP II expression and activity are increased in skeletal muscle during sepsis. Sepsis was induced in rats by cecal ligation and puncture. Control rats were sham-operated. TPP II activity was determined by using the specific substrate Ala-Ala-Phe-7-amido-4-methylcoumarin (AAF-AMC). TPP II protein and gene expression were determined by Western blot and real-time PCR, respectively. Sepsis resulted in increased activity and protein and gene expression of TPP II in extensor digitorum longus muscles. This result was blunted by the glucocorticoid receptor antagonist RU 38486, indicating that glucocorticoids participate in the upregulation of TPP II in skeletal muscle during sepsis. The results suggest that proteolytic mechanisms downstream of the proteasome may be important for the complete degradation of muscle proteins during sepsis.

Keywords: Muscle wasting; Ubiquitin; Proteasome; Glucocorticoids

Sepsis is associated with a catabolic response in skeletal muscle, mainly reflecting increased degradation of proteins, in particular myofibrillar proteins [1,2]. Among factors regulating sepsis-induced muscle proteolysis, glucocorticoids play a prominent role [3,4]. Muscle wasting during sepsis results in muscle weakness and fatigue that may delay or prevent ambulation. When respiratory muscles are affected [5], there is risk for pulmonary complications and prolonged need for ventilatory support. Thus, understanding the molecular regulation of muscle proteolysis during sepsis has important clinical implications.

Previous studies from this and other laboratories provided evidence that sepsis-induced muscle wasting at least in part reflects ubiquitin–proteasome-dependent protein breakdown [6,7]. The gene expression of various components of the ubiquitin–proteasome pathway, including ubiquitin, the ubiquitin-conjugating enzyme $E2_{14k}$, the ubiquitin ligase E3α, and multiple subunits of the 20S proteasome was increased in muscle from septic rats and patients [6–11]. In addition, energy-dependent protein degradation was increased in septic muscle [6] and the sepsis-induced muscle proteolysis was blocked by specific proteasome inhibitors, both in vivo [12] and in vitro [13]. Increased rate of ubiquitination of proteins provided further support for the important role of ubiquitin–proteasome-dependent protein degradation in muscle wasting [14].

Although the ubiquitin–proteasome proteolytic pathway plays a central role in sepsis-induced muscle wasting, other mechanisms may be involved as well. Because the proteasome degrades proteins into small polypeptides, rather than free amino acids, further proteolytic activity, "beyond the proteasome" [15,16], is probably important for the breakdown of muscle proteins during sepsis. There is evidence that multiple

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peptidases are involved in the cytosolic degradation of small polypeptides in various cell types [15–17]. One such peptidase is tripeptidyl-peptidase II (TPP II). TPP II was first described approximately 20 years ago as an extralysosomal tripeptide-releasing aminopeptidase in rat liver cytosol [18] but it is not until recently that the potential relationship between the proteasome and TPP II has been appreciated [15,16,19–22]. TPP II is a "giant" protease, even larger than the 26S proteasome, with a molecular weight $>10^6$ [18,23]. It consists of multiple subunits, each with a molecular weight of 138 kDa. There is evidence that TPP II needs to be assembled into its oligomeric complex for maximal proteolytic activity [24]. Interestingly, there are certain structural similarities between the proteasome and TPP II. Electron microscopic examination of TPP II displayed a rod-shaped particle about 50 nm in length and 17 nm in diameter with a longitudinal segmentation pattern and an internal channel from end-to-end [20,25]. Considering the fact that TPP II can only degrade oligopeptides [23], it is possible that the degradation of peptides takes place in the central channel, similar to the proteasome.

The biological role of TPP II and its involvement in pathophysiological conditions are not well understood and the influence of sepsis on TPP II expression and activity in skeletal muscle has not been reported. The purpose of the present study was to test the hypothesis that sepsis upregulates the expression and activity of TPP II in skeletal muscle and that glucocorticoids at least in part regulate TPP II activity during sepsis.

Materials and methods

Experimental animals. Sepsis was induced in male Sprague–Dawley rats (40–60 g body weight) by cecal ligation and puncture (CLP) as described previously [2,3,6]. Other rats were sham-operated, i.e., they underwent laparotomy and manipulation, but no ligation or puncture, of the cecum. All rats were resuscitated with 10 ml/100 g body weight of normal saline administered subcutaneously on the back at the time of surgery. Animals were allowed free access to water but food was withheld after the surgical procedures to avoid the influence of differences in food intake between sham-operated and septic rats.

In some experiments, rats were treated with 10 mg/kg of the glucocorticoid receptor antagonist RU 38486 [26] or corresponding volume of solvent by gavage 2 h before sham-operation or CLP. In previous studies, this treatment inhibited sepsis-induced protein breakdown and ubiquitin gene expression in skeletal muscle [3,27]. In other experiments, rats were treated with 15 mg/kg of the proteasome inhibitor *N*-benzyloxycarbonyl-Ile-Glu-(*O*-*t*-butyl)-Ala-leucinal (PSI; Calbiochem, San Diego, CA) or corresponding volume of solvent administered intraperitoneally 2 h before sham-operation or CLP. In a recent study in this laboratory, 15 mg/kg of PSI prevented sepsis-induced muscle proteolysis in rats [12].

At various time points after sham-operation or CLP, extensor digitorum longus (EDL) and soleus muscles were removed and frozen at −80 °C until further analysis. In some experiments, liver tissue was also harvested and frozen at −80 °C until further use. The experiments were conducted and the animals cared for in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals*. The Institutional Animal Care and Use Committee at the University of Cincinnati approved the experimental protocol.

TPP II activity. To purify TPP II, tissue samples were homogenized in ice-cold homogenization buffer (pH 6.8) consisting of imidazole–HCl 20 mM, KCl 100 mM, EGTA 20 mM, $MgCl_2$ 2 mM, sucrose 10%, and ATP 1 mM. The homogenates were centrifuged for 15 min at 1500*g*. The supernatant was centrifuged for 15 min at 15,000*g*, followed by 60 min at 100,000*g*, and 180 min at 100,000*g*. The final pellet was resuspended in ice-cold storage buffer (Tris–HCl 50 mM, glycerol 20%, $MgCl_2$ 5 mM, β-mercaptoethanol 0.5 mM, ATP 1 mM; pH 7.4). Protein concentration in the samples was determined according to Lowry et al. [28].

To measure TPP II activity, aliquots of the samples were added to assay buffer (pH 7.5) consisting of Tris–HCl 50 mM, $MgCl_2$ 5 mM, DTT 2 mM, and ATP 2 mM. The TPP II-specific fluorogenic substrate Ala-Ala-Phe-7-amido-4-methylcoumarin (AAF-AMC) was added at concentrations described in Results. The mixture was incubated at 37 °C for 45 min. Fluorescence was measured using a CytoFluor 2350 spectrophotometer (Millipore, Bedford, MA) at 360 nM excitation and 450 nM emission wavelengths. To test the specificity of the assay, one of the TPP II-specific inhibitors, Ala-Ala-Phe-chloromethylketone (AAF-CMK) (Sigma, St. Louis, MO) or butabindide (provided by Dr. R. Ganellin, University College London, London, UK), was added to the assay.

Western blot analysis. Aliquots (25 μg protein) from purified TPP II samples prepared as described above were separated electrophoretically on an 8–16% Tris–Glycine gel (Bio-Rad, San Diego, CA). The proteins were transferred to nitrocellulose membranes and Western blot analysis was performed using a polyclonal antibody to TPP II (OEM Concepts, Toms River, NJ). A polyclonal antibody to chicken IgY (OEM Concepts) was used as a secondary antibody.

In additional experiments, polyubiquitinated proteins in EDL muscles were determined by Western blot analysis as described in detail previously [12].

Quantitative real-time PCR. Tissue RNA was isolated as described by Chomczynski and Sacchi [29]. RNA was resuspended in RNase/DNase-free water (Gibco/Invitrogen, Carlsbad, CA) and quantified with an Agilent 2100 Bioanalyzer using the RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA). First-strand cDNA synthesis was performed using the SuperScript first-strand synthesis system for real-time PCR (Life Technologies, Rockville, MD) with oligo(dT) as the primer according to manufacturer's protocol. As an additional quality control, Arabidopsis thaliana mRNA was added to each RNA sample prior to cDNA synthesis. Real-time PCR was performed in a Smart Cycler (Cepheid, Sunnyvale, CA) using the LightCycler DNA Master SYBR Green I dye intercalation assay (Roche Molecular Biochemicals, Indianapolis, IN). Primers (forward, 5′-TCA GAG AAC TCA GTG GCG TGT GG-3′; and reverse 5′-TGT CGG AGG CAG TAG GAA AGC AG-3′) were generated to mouse TPP II (Accession No. NM_009418) and used to amplify an 89-bp fragment. Measurements were taken at the end of the 72 °C extension step in each cycle, and the second-derivative method was used to calculate the threshold cycle. Melt curve analysis showed a single sharp peak for all samples. Real-time PCR was also performed with primers specific for Arabidopsis thaliana. Fluorescence growth curves and threshold cycle for Arabidopsis thaliana mRNA were equal for all samples ensuring equal cDNA loading.

Statistics. Results are given as means ± SEM. Analysis of variance followed by Tukey's test or Student's *t* test was used to determine statistical significance.

Results

In initial experiments, the optimal conditions for measurement of TPP II activity in rat muscle tissue were

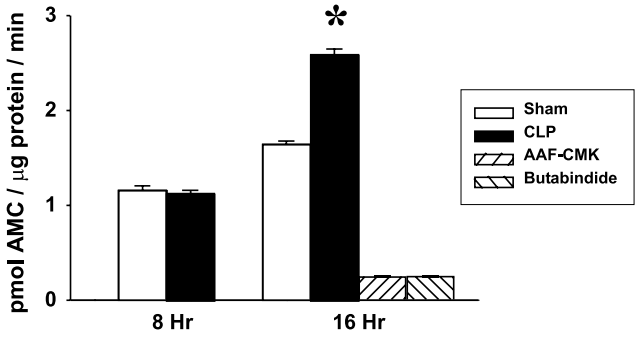

Fig. 1. TPP II activity in EDL muscles of sham-operated and septic (CLP) rats. Muscles were studied 8 and 16 h after sham-operation or CLP. The specificity of the assay was tested by adding AAF-CMK (100 μM) or butabinidide (2 μM) to the assay as indicated in the figure. Results are means ± SEM with $n \geqslant 6$ for each group; $^*p < 0.05$ vs sham.

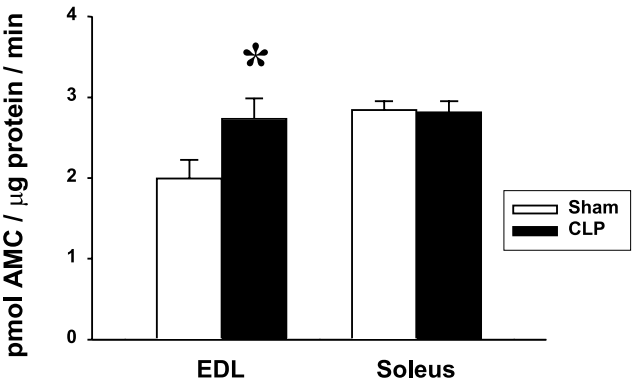

Fig. 2. TPP II activity in EDL and soleus muscles from sham-operated and septic rats. Muscles were studied 16 h after sham-operation or CLP. Results are means ± SEM with $n = 6$ in each group; $^*p < 0.05$ vs sham.

established. When increasing amounts of substrate (AAF-AMC) were added to the assay, the system was saturated at a substrate concentration of 100–200 μM. There was a linear relationship between amount of enzyme added to the assay and proteolytic activity up to an amount of 15 μg protein. The release of AMC from the substrate was linear for at least 60 min during incubation. Based on these results, 10 μg of TPP II sample was incubated with 200 μM AAF-AMC for 45 min in subsequent experiments.

TPP II activity was increased by approximately 40% in EDL muscle 16 h after CLP (Fig. 1). There was no difference in TPP II activity between muscle from sham-operated and septic rats at 8 h. When AAF-CMK or butabinidide was added to the assay, release of AMC was almost completely blocked, confirming that the assay measured TPP II activity.

In previous studies, we found that the catabolic response to sepsis was particularly pronounced in white, fast-twitch skeletal muscle with no or only minor changes noticed in red, slow-twitch muscle [2,30]. To test if TPP II as well is differentially regulated in different types of skeletal muscle during sepsis, TPP II activity was measured in the red, slow-twitch soleus and the white, fast-twitch EDL muscle 16 h after CLP. Whereas sepsis increased TPP II activity in EDL muscle, there was no difference in TPP II activity in soleus muscles from the same sham-operated and septic rats (Fig. 2). The basal TPP II activity was higher in soleus than in EDL muscle.

To examine whether the sepsis-induced increase in TPP II activity in the EDL muscle was associated with increased amount of the peptidase, TPP II levels were determined by Western blot analysis. TPP II protein levels were higher in EDL muscles from septic rats than in muscles from sham-operated rats (Fig. 3A). There was no difference in TPP II levels in soleus muscles from sham-operated and septic rats. Similar to TPP II activity, basal

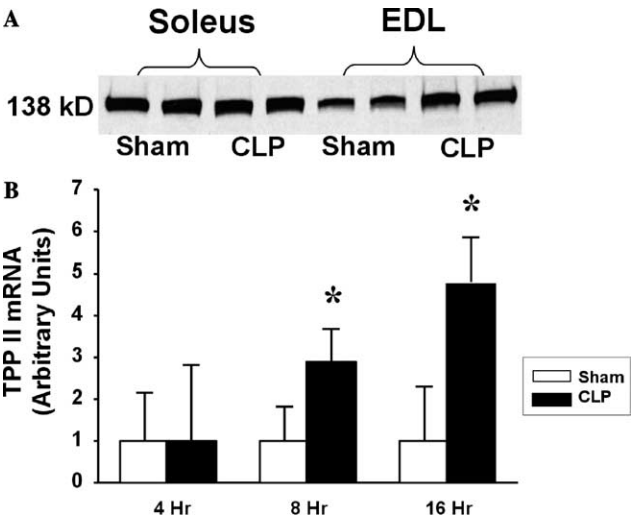

Fig. 3. (A) TPP II protein levels determined by Western blot analysis in rat soleus and EDL muscles 16 h after sham-operation or CLP. (B) TPP II mRNA levels in EDL muscles from sham-operated and septic rats determined by real-time PCR at various time points after sham-operation or CLP. The arbitrary unit for mRNA in muscles from sham-operated rats was set at 1 at each time point. Thus, comparisons cannot be made between the different time points but only between sham and CLP at each time point. Results are means ± SEM with $n = 6$ or 7 for each group; $^*p < 0.05$ vs corresponding sham group.

TPP II protein levels were higher in soleus than in EDL muscle.

To test whether the increased protein expression of TPP II was associated with increased gene expression, TPP II mRNA levels were determined by real-time PCR. TPP II mRNA levels were increased in EDL muscles 8 and 16 h after CLP (Fig. 3B) suggesting (but not proving) that the increase in TPP II protein levels may at least in part reflect transcriptional upregulation of TPP II synthesis.

In previous studies we found evidence that glucocorticoids are an important mediator of sepsis-induced

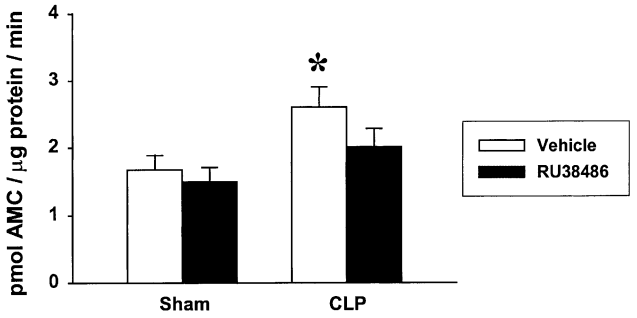

Fig. 4. TPP II activity in EDL muscles 16 h after sham-operation or CLP in rats that had been treated with 10 mg/kg of RU 38486 2 h before sham-operation or CLP or corresponding volume of vehicle by gavage. Results are means ± SEM with $n = 6$ or 7 in each group; *$p < 0.05$ vs all other groups.

muscle proteolysis [3,27]. To examine whether glucocorticoids regulate TPP II activity in skeletal muscle during sepsis, rats were treated with the glucocorticoid receptor antagonist RU 38486. The increase in muscle TPP II activity induced by sepsis was blunted in rats treated with RU 38486 (Fig. 4) suggesting that glucocorticoids participate in the activation of muscle TPP II during sepsis. RU 38486 did not influence TPP II activity in muscles from sham-operated rats.

Inhibited TPP II activity in septic rats treated with RU 38486 can be consistent with at least two different models. First, it is possible that glucocorticoids upregulate TPP II activity secondary to increased supply of substrates (oligopeptides) from activated ubiquitin–proteasome-dependent proteolysis. Second, the result could be consistent with stimulation of TPP II by glucocorticoids independent of proteasome activity. To test whether TPP II activity reflects supply of oligopeptides from the proteasome, rats were treated with 15 mg/kg of the proteasome inhibitor PSI [31]. In recent studies we found that treatment of rats with this dose of PSI reduced sepsis-induced ubiquitin–proteasome-dependent muscle proteolysis [12]. Here we found (as expected) that treatment of septic rats with PSI resulted in increased amounts of polyubiquitinated proteins in EDL muscles, consistent with accumulation of ubiquitinated proteins secondary to inhibited proteasome activity (Fig. 5A). The same treatment did not influence TPP II activity (Fig. 5B) suggesting that TPP II activity may not be regulated by the supply of peptides generated by the proteasome.

In contrast to the catabolic response to sepsis in white, fast-twitch skeletal muscle, sepsis results in an anabolic response in liver characterized by increased protein synthesis, in particular increased synthesis of acute phase proteins [32,33]. Changes in protein degradation in liver during sepsis are not well understood although in one study, liver protein degradation was unchanged in septic rats [33]. The influence of sepsis on TPP II activity in liver has not been reported. We next determined TPP II activity in liver 8 h after sham-operation or CLP. Sepsis

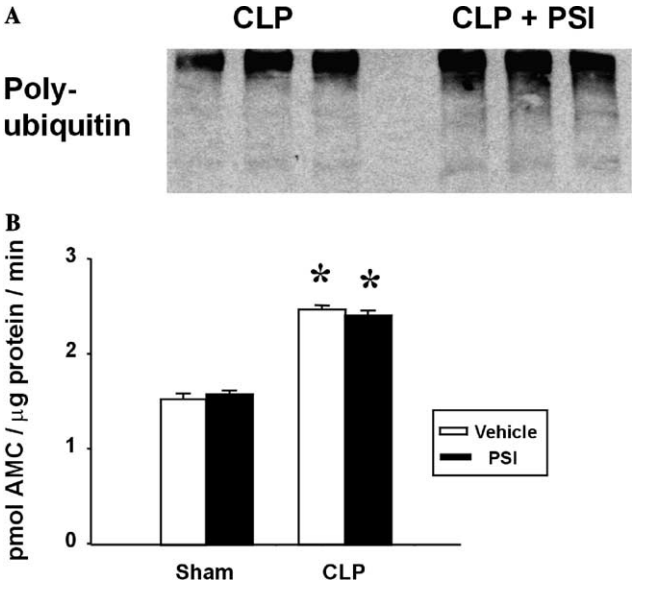

Fig. 5. (A) Polyubiquitinated proteins in EDL muscles in septic rats (CLP) and in septic rats that had been treated with 15 mg/kg PSI 2 h before CLP (CLP + PSI). (B) TPP II activity in EDL muscles from sham-operated and septic rats that had been treated with 15 mg/kg PSI or corresponding volume of vehicle 2 h before sham-operation or CLP. Results are means ± SEM with $n = 6$ or 7 in each group; *$p < 0.05$ vs corresponding sham group.

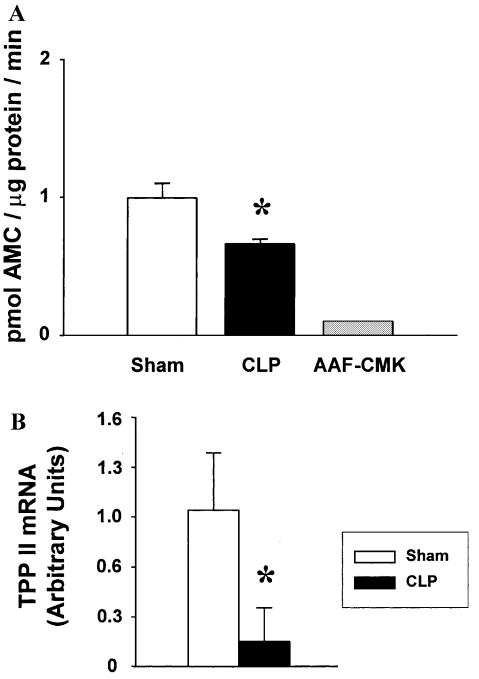

Fig. 6. (A) TPP II activity in liver tissue of sham-operated and septic rats. Liver tissue was studied 8 h after sham-operation or CLP. Specificity of the assay was tested by adding AAF-CMK (100 μM) to the assay. Results are means ± SEM with $n = 6$ or 7 in each group; *$p < 0.05$ vs sham. (B) TPP II mRNA levels determined by real-time PCR in liver tissue from sham-operated and septic rats. Liver tissue was examined 8 h after sham-operation or CLP. Results are means ± SEM with $n = 6$ in each group; *$p < 0.05$ vs sham.

resulted in an approximately 30% reduction of TPP II activity in liver tissue and this was accompanied by reduced TPP II mRNA levels (Fig. 6). These results are important because they demonstrate that changes in TPP II activity and expression during sepsis are tissue specific and because they suggest that there is a correlation between changes in protein metabolism and TPP II activity during sepsis with TPP II activity being increased in catabolic muscle and decreased in anabolic liver.

Discussion

In the present study, sepsis in rats resulted in increased expression and activity of TPP II in skeletal muscle. Similar to changes in protein breakdown rates and gene expression of the ubiquitin–proteasome proteolytic pathway [2,30], the sepsis-induced changes in TPP II occurred in white, fast-twitch skeletal muscle with no changes noticed in red, slow-twitch muscle. These observations support the concept that increased TPP II expression and activity are an integral part of sepsis-induced muscle wasting.

Protein metabolism is differentially regulated in different organs and tissues during sepsis [34]. For example, sepsis is typically associated with a catabolic response in skeletal muscle and an anabolic response in liver. The results in the present study suggest that a correlation exists between changes in TPP II activity and overall changes in protein metabolism in different organs and tissues with increased TPP II activity in catabolic white, fast-twitch muscle, unaltered TPP II activity in red, slow-twitch muscle (in which changes in protein breakdown during sepsis are minor), and reduced TPP II activity in liver (in which changes in protein metabolism are characterized by an anabolic response).

To the best of our knowledge, the influence of sepsis on TPP II activity in skeletal muscle has not been reported previously. However, TPP II activity was examined previously in skeletal muscle in other pathophysiological conditions. For example, the activity of the protease was measured in degenerating and regenerating rat soleus muscle after local injection into the muscle of snake venom [35]. Results in that study showed that there was an initial (12–24 h) decrease followed by an increase in TPP II activity 3–4 days after injection of the snake venom. In other studies, ethanol intake [36,37] or treatment of rats with prednisone [38] reduced muscle TPP II activity with varying response in different muscles. Similar to the finding in the present report, the basal TPP II activity was higher in soleus than in EDL muscle in previous studies [38]. It should be noted that in the previous studies [35–38], TPP II activity was measured with AAF-AMC as substrate but without aminopeptidase inhibitor. Therefore, part of the measured activity may have reflected sequential cleavage of the substrate by aminopeptidases rather than TPP II activity.

The present observation that TPP II activity in EDL muscles was reduced in septic rats treated with RU 38486 suggests that glucocorticoids at least in part regulate TPP II during sepsis. This is important because in previous studies evidence was found that glucocorticoids may be the most significant mediator of sepsis-induced muscle wasting [4]. It should be noted, however, that the regulation of sepsis-induced muscle proteolysis is probably multifactorial and other regulators may be important as well, including the proinflammatory cytokines IL-1 and TNF [39,40]. The influence of these factors on TPP II activity in skeletal muscle remains to be determined.

Although the present results support the concept that glucocorticoids partly regulate TPP II activity in skeletal muscle during sepsis, the mechanism by which this regulation occurs is not known. In previous studies, evidence was found that glucocorticoids regulate the gene expression and activity of the ubiquitin–proteasome pathway. Therefore, it is possible that glucocorticoids regulate TPP II activity by increasing proteasome-dependent protein degradation, providing TPP II with an increased amount of substrates (oligopeptides). However, in the present study, treatment of rats with the proteasome inhibitor PSI did not reduce TPP II activity, suggesting that TPP II may not be regulated by proteasome activity. Thus, it is possible that glucocorticoids regulate the ubiquitin–proteasome system and TPP II in parallel rather than "in tandem." More experiments are needed to define mechanisms by which glucocorticoids influence muscle TPP II during sepsis. Regardless of mechanisms that may be involved in sepsis-induced increase in TPP II activity, the present results of unchanged TPP II activity in sham-operated rats treated with RU 38486 suggest that basal TPP II activity is not regulated by glucocorticoids.

Previous studies suggest that the assembly of the TPP II subunits into a large complex is important for activation of the enzyme [16,24] but factors regulating the association and dissociation of the TPP II subunits are not known. The influence of sepsis (and glucocorticoids) on the process of TPP II complex formation remains to be determined.

Despite the fact that TPP II was described almost 20 years ago [18], the biological and pathophysiological roles of the peptidase are not well understood. Recent studies suggest that TPP II may substitute for the proteasome in cells in which proteasomal activity has been lost [19,20] although this is somewhat controversial [22]. Other reports support the concept that TPP II is an important component of overall intracellular protein degradation, providing for a mechanism of proteolysis downstream of the proteasome [15,16,21,41]. The present result of activated TPP II in muscle from septic

rats is consistent with a model in which degradation of peptides generated by the proteasome is increased in catabolic skeletal muscle.

Both the proteasome and TPP II belong to an emerging class of "giant proteases" regulating intracellular proteolysis [15,16]. Common features of these proteases, in addition to their large size, include the fact that they are composed of multiple subunits and harbor the proteolytic sites in an internal cavity or central channel. This self-compartmentalization is probably one of the mechanisms accounting for the specificity and restrictive nature of the proteolytic activity of giant proteases. The present study provides the first evidence that, in addition to the proteasome, other giant proteases as well may be involved in sepsis-induced muscle wasting. In addition to TPP II, bleomycin hydrolase [42] and dipeptidyl peptidase III [43] are large multicomponent proteases that may be involved in degradation of polypeptides downstream of the proteasome but their involvement in muscle wasting is not known.

It should be noted that TPP II degrades polypeptides into tripeptides (TPP II has been called "the enzyme that can count to three" [16]) and additional mechanisms are probably involved in the complete hydrolysis of peptides into free amino acids. Several aminopeptidases have been described that participate in the complete degradation of tripeptides and dipeptides into free amino acids [15,17,21,41]. Which aminopeptidase(s) that are involved in the complete degradation of small peptides in skeletal muscle during sepsis (or any other catabolic condition) remains to be determined.

The present results are important because they support a model in which multiple sequential steps are involved in sepsis-induced muscle proteolysis. Although the ubiquitin–proteasome pathway plays a central role in muscle protein breakdown during sepsis, other mechanisms, both upstream and downstream of the proteasome, are probably involved in the development of muscle wasting during sepsis. Potential pre-proteasomal, para-proteasomal, and post-proteasomal mechanisms involved in muscle catabolism were reviewed recently elsewhere [44].

Acknowledgments

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