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Sepsis-Induced Muscle Proteolysis Is Prevented by a Proteasome Inhibitor *in Vivo*

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Sepsis-induced muscle proteolysis mainly reflects ubiquitin-proteasome-dependent protein degradation. The effect of *in vivo* administration of a proteasome inhibitor on muscle protein breakdown during sepsis is not known. We treated rats with the proteasome inhibitor *N*-benzyloxycarbonyl-Ile-Glu-(*O*-*t*-butyl)-Ala-leucinal (PSI) or corresponding volume of vehicle i.p. 2 h before sham-operation or induction of sepsis by cecal ligation and puncture. The sepsis-induced increase in total and myofibrillar muscle protein breakdown was inhibited in rats treated *in vivo* with PSI and a maximal effect was seen following 15 mg/kg of the proteasome inhibitor. Results from *in vitro* experiments in which incubated muscles were treated with 100 μM PSI suggest that the drug has a direct effect on muscle and that the effect is specific for the proteasome. The results are important because they suggest that it may be possible to prevent or improve the cachectic response in skeletal muscle during sepsis by treatment with a proteasome inhibitor. © 2000 Academic Press

Key Words: **muscle; cachexia; sepsis; ubiquitin; proteasome; inhibitor.**

Muscle cachexia during sepsis and following severe injury is mainly caused by increased protein breakdown, in particular myofibrillar protein breakdown (1). Skeletal muscle is the major source of nitrogen loss in injured and septic patients (2). Some of the consequences of the catabolic response to sepsis include muscle weakness and fatigue, resulting in delayed ambulation and increased risk for thromboembolic and pulmonary complications (3). A better understanding of the regulation of muscle protein breakdown during sepsis and methods to treat sepsis-induced muscle proteolysis, therefore, have important clinical implications.

Intracellular protein degradation is regulated by multiple proteolytic pathways, including lysosomal, calcium-calpain-mediated and ubiquitin-proteasome-dependent mechanisms (4, 5). Studies in our and other laboratories have provided evidence that sepsis-induced muscle protein breakdown is associated with upregulated expression and activity of the ubiquitin-proteasome pathway (6–10), similar to a number of other catabolic conditions, such as burn injury (11), starvation (12), denervation (13), AIDS (14), and cancer (15). In this proteolytic pathway, protein substrates are first conjugated to multiple molecules of ubiquitin whereafter they are recognized and degraded by the multicatalytic 26S proteasome (16).

Inhibition of proteasome activity may be one way to reduce muscle protein breakdown during sepsis and other catabolic conditions. Indeed, we recently reported that treatment with a proteasome blocker *in vitro* of incubated catabolic muscles from rats with burn injury (17) or sepsis (18) inhibited total and myofibrillar protein breakdown but the effect of *in vivo* administration of a proteasome inhibitor on sepsis-induced muscle cachexia has not been reported previously.

In the present study, we treated rats *in vivo* with the proteasome inhibitor *N*-benzyloxycarbonyl-Ile-Glu-(*O*-*t*-butyl)-Ala-leucinal (PSI) which blocks the chymotrypsin-like activity of the proteasome (19). PSI was used in a recent report to treat deoxycorticosterone acetate-salt hypertensive rats and was found to decrease aortic proteasome activity and reduce blood pressure (20). Here we found that treatment of rats *in vivo* with PSI inhibited chymotrypsin-like proteasome activity in skeletal muscle and prevented sepsis-induced muscle proteolysis.

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 in detail previously (1, 6, 7, 9). Other rats underwent sham-operation, i.e., laparotomy and manipulation, but no ligation or puncture, of the cecum. All rats were resuscitated with 10 ml/100 g body weight of saline administered subcutaneously on the back at the time of surgery to prevent hypovolemia and septic shock. CLP is a clinically relevant septic model because it resembles the situation in patients with septic peritonitis caused by intra-

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abdominal abscess and devitalized tissue. The model was characterized with regards to metabolic and hemodynamic changes and mortality rates in previous studies from our (21) and other laboratories (22). Rats weighing 40–60 g were used in the present study because isolated lower extremity muscles from rats of this size are thin enough to allow measurement of protein breakdown rates during incubation *in vitro* (23).

Groups of rats were treated with different doses of PSI, administered intraperitoneally 2 h before sham-operation or CLP. Control rats received corresponding volume (0.2 ml/100 g) of solvent (DMSO). Metabolic studies were performed in the extensor digitorum longus (EDL) muscle 16 h after sham-operation or CLP. This muscle was studied because in previous experiments we found that the catabolic response to sepsis was particularly pronounced in white, fast-twitch muscle (1). In other studies, rats were in a hyperdynamic, hypermetabolic phase of sepsis approximately 16 h after CLP (21, 22).

All animals were cared for and experiments were performed 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 protein breakdown rates. Total and myofibrillar protein breakdown rates were measured in incubated EDL muscles as net release of tyrosine and 3-methylhistidine (3-MH), respectively, as described in detail previously (1, 6, 7). After pre-incubation for 30 min, muscles were incubated at resting length for 2 h in 3 ml oxygenated Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 10 mM glucose and 0.5 mM cycloheximide. Net release of tyrosine and 3-MH was determined by measuring release of the amino acids into the incubation medium during the incubation and by correcting for changes in intracellular tyrosine and 3-MH. This method to measure protein breakdown rates in incubated muscles was used in several previous studies from our and other laboratories (1, 6, 7, 12, 13). Muscles remain viable during incubation for at least 2 h and release of tyrosine and 3-MH is linear during that period of time (24). Muscles were incubated fixed at resting length rather than flaccid because in previous studies, energy and protein balance were better maintained in muscle at resting length (24, 25).

When the direct effect of PSI on muscle protein breakdown was tested, muscles were both pre-incubated and incubated in the absence or presence of 100 μM PSI. Because PSI was dissolved in DMSO, a corresponding volume of DMSO (7 μl/ml incubation medium) was added to muscles incubated without PSI. In some experiments, muscles were incubated in calcium-free medium containing 10 mM methylamine, 0.1 mU/ml bovine insulin and 5 times normal rat plasma concentrations of the branched chain amino acids leucine, isoleucine and valine as described previously (6). This modification of the incubation medium blocks lysosomal and calcium-dependent proteolysis and, therefore, any effect of PSI seen under these experimental conditions more specifically reflects proteasome-dependent protein degradation.

Proteasome activity. 20S proteasomes were isolated from EDL muscles after homogenization and multiple centrifugations as described in detail recently (26). To increase the yield of proteasomes, muscles from 5 rats were pooled for each assay. The proteolytic activity of the proteasomes was determined by measuring the activity against the fluorogenic substrates succinyl-leu-leu-val-tyr-7-amido-4-methylcoumarin (LLVY) and N-carbenzoxy-leu-leu-glu-7-amido-4-methylcoumarin (LLE) (Sigma, St Louis, MO) as described in detail recently (26). These substrates are preferentially hydrolyzed by the chymotrypsin-like and peptidyl-glutamyl-peptidase activities of the 20S proteasome, respectively (27).

Western blot analysis. EDL muscles were homogenized in 1 ml of ice-cold buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiotreitol, and 1 mM phenyl methyl sulfonylfluoride) and the homogenates were incubated on ice for 10 min. The samples were then centrifuged at 850*g* for 10 min at 4°C. The supernatants were discarded and the pellets were resuspended in 1 μl of buffer A with 0.1% Triton X-100 per μg of tissue, incubated for 10 min on ice and centrifuged as described above. The supernatant was assayed for protein concentration (Bio-Rad Laboratories, Hercules, CA) and stored at −80°C until further analysis.

Aliquots of cytoplasmic fractions containing 25 μg protein were boiled in equal volumes of loading buffer (125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, and 10% 2-mercaptoethanol) for 3 min, separated by electrophoresis on a 4–20% Tris-glycine gradient gel (Novex, San Diego, CA) and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dried milk in Tris-buffer saline (TBS), pH 7.6, containing 0.05% Tween-20 (TTBS) for 30 min and incubated with a monoclonal mouse anti-rat antibody to ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA) for 90 min. After washing twice in TTBS, the blots were incubated with a peroxidase-conjugated goat anti-mouse IgG secondary antibody for 45 min. The blots were washed in TTBS for 5 min × 3, then in TBS for 5 min, incubated in enhanced chemiluminescence reagent (ECL, Amersham Life Sciences, Buckingham, England), and exposed on radiographic film (Eastman-Kodak, Rochester, NY).

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

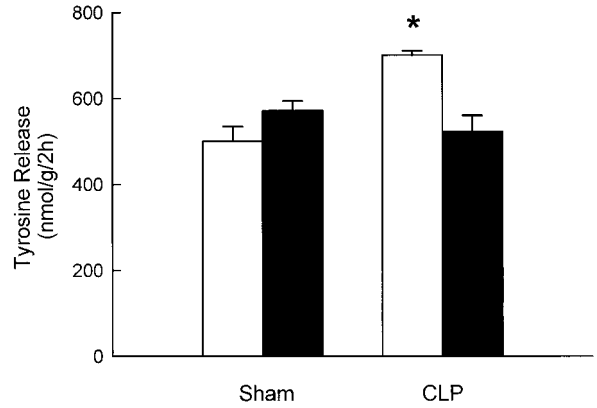

FIG. 1. Total protein breakdown rates in incubated EDL muscles from sham-operated and septic (CLP) rats. Groups of rats were treated with 15 mg/kg of PSI (filled bars) administered i.p. 2 h before sham-operation or CLP or corresponding volume of vehicle (open bars). Results are means ± SEM with n = 7 or 8 in each group. *$P <$ 0.05 vs all other groups by ANOVA. Almost identical results were observed in three repeated experiments.

RESULTS

Sixteen hours after CLP, septic rats showed signs of illness in the form of piloerection, exudate around the eyes and nostrils, and diarrhea. There was no apparent difference in the clinical appearance between septic rats treated with PSI or vehicle. Mortality rate 16 h after CLP was approximately 40% with no difference between PSI- and vehicle-treated rats. There was no mortality among sham-operated rats receiving vehicle or PSI.

Sepsis resulted in an approximately 40% increase in total muscle protein breakdown, measured as release of tyrosine in incubated EDL muscles (Fig. 1). This

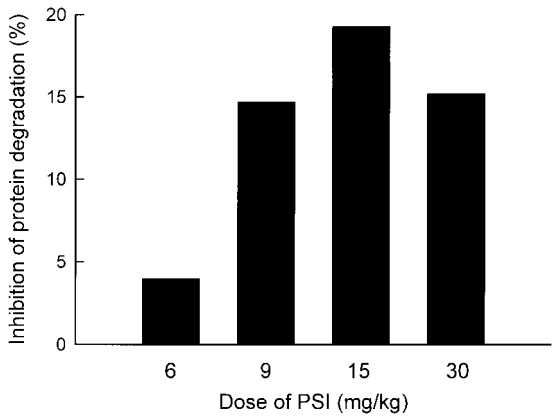

FIG. 2. Inhibition of muscle protein degradation in septic rats treated with different doses of PSI i.p. 2 h before CLP. The inhibition was calculated from the difference in protein degradation rates in septic rats receiving PSI or corresponding volume of vehicle. The calculations were based on n ≥ 7 for both vehicle- and PSI-treated rats at each dose.

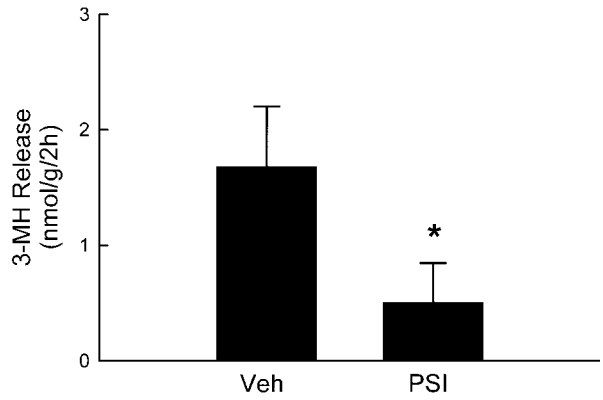

FIG. 3. Myofibrillar protein breakdown in incubated EDL muscles from septic rats treated with vehicle (Veh) or 15 mg/kg PSI i.p. 2 h before CLP. $N = 12$ in each group. $^*P < 0.05$ by Student's t test.

result is similar to previous reports from our laboratory and confirms the catabolic response in the septic model used here (1, 6, 7). The sepsis-induced increase in muscle protein breakdown was prevented in rats that were treated with 15 mg/kg of PSI 2 h before CLP (Fig. 1). Protein breakdown rates in muscles from sham-operated rats were not influenced by treatment with the proteasome inhibitor.

We next determined whether the effect of PSI on sepsis-induced muscle proteolysis was dose-dependent. In a previous report, treatment of rats *in vivo* with 3 mg/kg of PSI reduced blood pressure and proteasome activity in the aortic wall (20). When this dose was used in the present study, muscle protein breakdown was not influenced in sham-operated or septic rats (not shown). We therefore used higher doses of PSI (from 6 to 30 mg/kg) and found that a maximal effect of the treatment was achieved with a dose of 15 mg/kg (Fig. 2). This dose of PSI was used in all subsequent experiments.

Results in previous studies provided evidence that most of the increase in muscle protein breakdown during sepsis reflected myofibrillar protein degradation, measured as release of 3-MH from incubated muscles (1). In order to determine whether treatment with PSI inhibited myofibrillar protein breakdown, we measured 3-MH release in muscles from rats treated with solvent or PSI. Because our initial experiment showed that PSI reduced protein breakdown in septic but not in sham-operated rats (see Fig. 1), 3-MH release was measured in muscles from rats that underwent CLP. Treatment of septic rats with PSI (15 mg/kg) reduced myofibrillar protein breakdown by approximately 75% (Fig. 3).

To examine the effect of PSI treatment on proteasome activity, proteasomes were isolated from EDL muscles 16 h after CLP in groups of rats that were treated with PSI or solvent. Proteasome activity against the fluorogenic substrate LLE was not affected by PSI whereas the activity against LLVY was reduced by approximately 40% (Fig. 4). This result suggests that treatment with PSI reduced the chymotrypsin-like activity of the proteasome, similar to previous

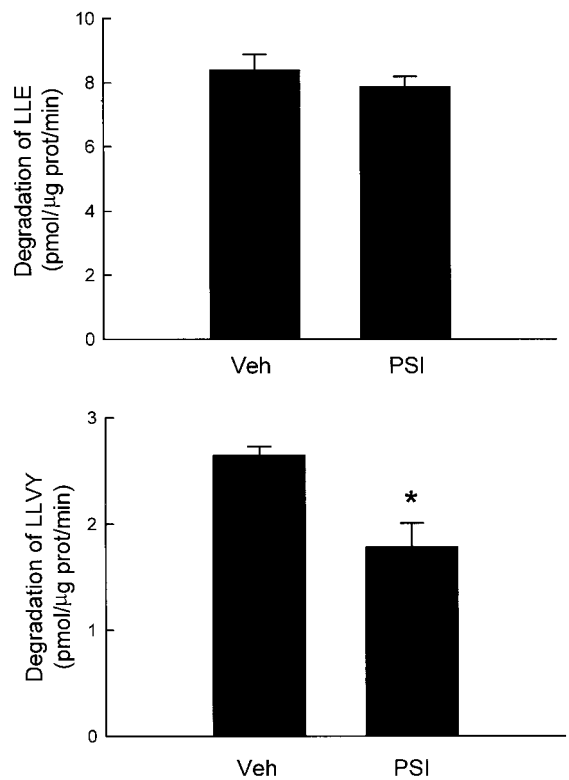

FIG. 4. Activity of isolated muscle proteasomes against the fluorogenic substrates LLE (upper panel) and LLVY (lower panel). Muscles were from septic rats treated with vehicle (Veh) or 15 mg/kg PSI i.p. 2 h before CLP. $N = 4$ in each group with muscles from 5 rats pooled for each assay. $^*P < 0.05$ vs Veh by Student's t test.

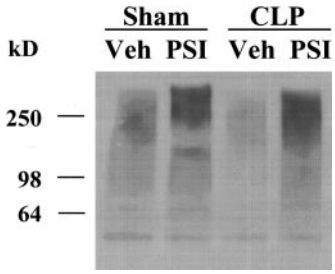

FIG. 5. Ubiquitin-protein conjugates in muscle extracts from rats that were treated with vehicle (Veh) or 15 mg/kg PSI i.p. 2 h before sham-operation or CLP. The figures in the left-hand panel indicate the size of different molecular weight markers. Similar results were observed in three separate experiments.

results *in vitro* in different cell lines (19, 28, 29) and in the aortic wall of hypertensive rats (20).

From a theoretical standpoint, inhibition of the proteasome may give rise to an accumulation of ubiquitinated proteins. Indeed, in previous *in vitro* experiments, treatment of a cultured mouse neuronal cell line with PSI resulted in accumulation of ubiquitin-protein conjugates (19). In the present study, Western blot analysis showed that treatment of rats with PSI resulted in increased muscle levels of ubiquitinated proteins (Fig. 5). The most pronounced effect was seen in proteins with a high molecular weight, suggesting (but not proving) that the result reflected accumulation of ubiquitinated myofibrillar proteins.

Because inhibition of the proteasome *in vivo* may give rise to multiple metabolic and inflammatory changes, it is possible that the inhibition of muscle protein breakdown noticed in the present experiments was secondary to other effects induced by PSI. It was therefore important to test whether PSI has a direct effect on muscle protein breakdown. Although previous reports suggest that different proteasome inhibitors can inhibit protein breakdown by a direct effect in incubated muscles (17, 18, 30), the influence of PSI added to incubated muscle *in vitro* is not known. When incubated EDL muscles from sham-operated and septic rats were treated *in vitro* with 100 μM PSI, protein breakdown was reduced by approximately 30% and 60% in non-septic and septic muscles, respectively, consistent with a direct effect of the proteasome inhibitor in skeletal muscle (Fig. 6). When PSI was added to the incubated muscles, protein breakdown rates were almost identical in muscles from sham-operated and septic rats, supporting the concept that sepsis-induced muscle proteolysis mainly reflects proteasome-dependent protein degradation.

Because peptide aldehydes, including PSI, are inhibitors of both serine and cysteine proteases, their inhibition of protein breakdown may not be completely specific for the proteasome but may also reflect inhibition of other proteases, such as calcium-dependent and lysosomal cysteine proteases (31). It was important,

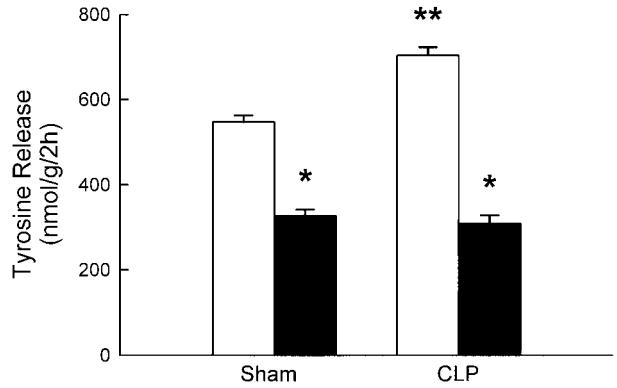

FIG. 6. The effect of PSI *in vitro* on muscle protein breakdown. EDL muscles from sham-operated and septic (CLP) rats were incubated in the absence (open bars) or presence (filled bars) of 100 μM PSI. $N = 7$ in each sham group and $n = 8$ in each CLP group. *$P <$ 0.05 vs muscles incubated without PSI; **$P < 0.05$ vs corresponding sham group by ANOVA.

therefore, to test if the effect of PSI noted here was specific for proteasome-dependent protein breakdown. This was done by incubating muscles under conditions in which lysosomal and calcium-dependent protein breakdown was inhibited, i.e., in calcium-free medium containing insulin, methylamine and branched chain amino acids as described previously (6). Any effect caused by PSI under such conditions should reflect non-lysosomal, calcium-independent proteolysis. When PSI was added to muscles incubated in calcium-free medium containing insulin, methylamine and branched chain amino acids, a similar inhibition of protein breakdown rates was noticed as in muscles incubated in normal medium (Fig. 7). The lower overall protein breakdown rates noticed in this experiment as compared to the experiment in which muscles were

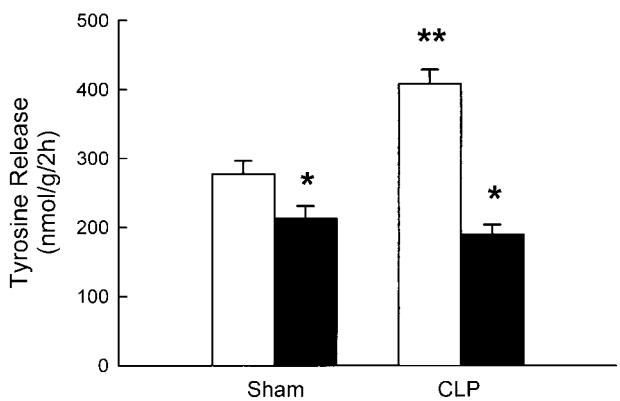

FIG. 7. The effect of PSI on protein breakdown in muscles incubated in calcium-free medium containing methylamine, insulin and five times normal plasma concentrations of branched-chain amino acids. Muscles were incubated in the absence (open bars) or presence (filled bars) of 100 μM PSI. $N = 8$ in each group; *$P < 0.05$ vs muscles incubated without PSI; **$P < 0.05$ vs corresponding sham group by ANOVA.

incubated in normal medium (see Fig. 6) suggest that lysosomal and calcium-dependent protein degradation contribute to basal protein breakdown in skeletal muscle.

DISCUSSION

In the present study, treatment of rats with the proteasome inhibitor PSI prevented the sepsis-induced increase in muscle protein breakdown. To the best of our knowledge, this is the first report of reduced muscle catabolism following administration of a proteasome inhibitor *in vivo*. The results lend further support to the concept that muscle proteolysis during sepsis, at least in part, reflects proteasome-dependent protein degradation (5, 6). Other observations implicating the ubiquitin-proteasome system in sepsis-induced muscle cachexia include increased gene expression of ubiquitin (6, 9), enzymes involved in the ubiquitination of proteins (32, 33), and proteasome subunits (26) as well as inhibition of protein breakdown in septic muscles treated *in vitro* with different proteasome blockers (18, 30).

Because the ubiquitin-proteasome system is involved in a multitude of cellular functions, such as regulation of the activity of transcription factors, most notably NF-κB (29), and regulation of the cell cycle (28), it is possible that the effects of PSI on muscle protein breakdown noticed in the present study were indirect and did not reflect inhibition of muscle proteasome activity. Reduced chymotrypsin-like activity of proteasomes isolated from muscles of septic rats treated with PSI and increased amounts of ubiquitinated proteins in the same muscles suggest, however, that the inhibition of muscle proteolysis was at least associated with inhibited proteasome activity in skeletal muscle. The *in vitro* experiments in which incubated muscles were treated with PSI provided further evidence that the inhibition of muscle protein breakdown was specifically caused by inhibition of the proteasome and that this was a direct effect on muscle.

The proteasome inhibitor used in the present study blocks the chymotrypsin-like activity of the proteasome (19). This is important in light of recent observations suggesting that the chymotrypsin-like activity is rate-limiting in proteasome-dependent protein degradation (34). The finding that treatment of rats with PSI inhibited the proteasome activity against the substrate LLVY but not against LLE is consistent with inhibition of the chymotrypsin-like activity whereas the peptidyl-glutamyl peptidase activity did not seem to be influenced by the treatment.

The effect of PSI on muscle protein breakdown has not been reported previously. In other studies, the drug blocked the cell cycle in cultured HeLa cells (28) and interfered with induction of nitric oxide synthase in macrophages by blocking NF-κB activation (29). When cultured neuronal cells were treated with PSI, ubiquitin protein conjugates accumulated in the cells, consistent with a "back up" of ubiquitinated substrates when the proteasome was blocked (19). In the present study, treatment of rats with PSI resulted in a similar accumulation of ubiquitinated proteins.

Although the present study is the first in which a proteasome inhibitor was used *in vivo* to reduce the catabolic response in skeletal muscle, proteasome inhibitors were administered *in vivo* in other experiments designed to test the role of the proteasome in other disease states. For example, treatment of deoxycorticosterone acetate-salt hypertensive rats with PSI *in vivo* reduced blood pressure secondary to inhibited proteasome activity in the aortic wall (20). Oral administration of the proteasome inhibitor MG-341 attenuated mucosal inflammation in an experimental model of colitis in rats (35). The cytokine release in endotoxemic mice was blocked by treatment *in vivo* with one of the proteasome inhibitors N-acetyl-leucinyl-leucinyl-norleucinal (36) or 5-methoxy-1-indanone-3-acetyl-leu-D-leu-1-indanylamide (37). In recent studies, the effects of different members of a new series of proteasome inhibitors (boronic acid analogues) were tested. In one of those reports, treatment of rats *in vivo* with the drug PS-519 protected the heart from ischemia-reperfusion damage (38). In another study, intravenous treatment of tumor-bearing mice with PS-341 substantially reduced the tumor size and when the drug was injected directly into the tumor, the tumor was eradicated in 40% of the mice (39).

It should be noted that although the observations in the present study suggest that it may be possible to prevent sepsis-induced muscle cachexia with a proteasome inhibitor, the results need to be interpreted with caution for several reasons. First, rats were treated with PSI 2 h before the induction of sepsis and it remains to be determined whether the drug can reduce the catabolic response in skeletal muscle if administered after the onset of sepsis. Second, the influence of PSI on long-term survival in the present experimental model is not known since all animals were sacrificed 16 h after CLP when muscles were harvested for metabolic studies. Third, the anti-catabolic effects of PSI in other conditions characterized by muscle cachexia remain to be tested. Finally, it is not known if the present results are specific for PSI or whether similar effects would be seen after treatment with other proteasome inhibitors.

Despite these limitations, the results reported here are important because they suggest that treatment with a proteasome inhibitor *in vivo* can improve the metabolic situation in skeletal muscle during sepsis. The results also lend strong support to the concept that sepsis-induced muscle proteolysis, at least in part, reflects proteasome-dependent protein degradation. It will be important in future studies to test whether

muscle protein breakdown can be reduced by treatment with a proteasome inhibitor after the onset of sepsis and whether the treatment can prevent muscle cachexia in other catabolic conditions as well.

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