Treatment of rats with calpain inhibitors prevents sepsis-induced muscle proteolysis independent of atrogin-1/MAFbx and MuRF1 expression

Moin U. Fareed,1 Amy R. Evenson,1 Wei Wei,1 Michael Menconi,1 Vitaliy Poylin,1 Victoria Petkova,2 Bernadette Pignol,3 and Per-Olof Hasselgren1

Departments of 1Surgery and 2Hematology/Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts; and 3Department of Neurobiology, Ipsen Research Laboratories, Institut Henri Beaufour, Les Ulis, France

Submitted 14 September 2005; accepted in final form 25 January 2006

MUSCLE WASTING DURING SEPSIS is mainly caused by an increase in protein breakdown, in particular, breakdown of the myofibrillar proteins actin and myosin (17, 36). Previous studies from our and other laboratories suggest that muscle proteolysis in sepsis and a number of other catabolic conditions, such as burn injury, cancer, and uremia, reflects ubiquitin-proteasome-dependent proteolysis (11, 13, 17, 36, 44). Proteins degraded by this mechanism are first conjugated to multiple molecules of ubiquitin followed by degradation by the 26S proteasome (22). Ubiquitination of the protein substrates is regulated by multiple enzymes, including the ubiquitin-activating enzyme E1, ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s). Among these enzymes, the E3s are particularly important because they account for substrate specificity in the system. In recent studies, the expression of two newly discovered muscle-specific ubiquitin ligases, atrogin-1/MAFbx and MuRF1, was substantially increased in skeletal muscle during various muscle-wasting conditions, including sepsis (5, 15, 45), and increased mRNA levels for atrogin-1/MAFbx and MuRF1 have been suggested to be reliable molecular markers for muscle atrophy (28).

Although ubiquitin-proteasome-dependent protein degradation plays an important role in sepsis-induced muscle proteolysis (17, 23, 36), it should be noted that the proteasome does not degrade intact myofibrils (27). Therefore, it is likely that myofibrillar proteins are first released from the sarcomere before they are ubiquitinated and degraded by the proteasome. We and others have proposed a model in which calpain-dependent degradation of Z-band-associated proteins results in the release of myofilaments from the sarcomere followed by ubiquitination and subsequent proteasomal degradation of the myofilaments (14, 17, 33, 34).

Results in a recent study from our laboratory provided further evidence for a role of increased calpain activity in sepsis-induced muscle proteolysis (42). In that study, induction of sepsis in rats by cecal ligation and puncture (CLP) resulted in increased calpain activity in skeletal muscle. Interestingly, the increase in calpain activity was caused by reduced activity of the endogenous calpain inhibitor, calpastatin. This observation supports the results in a recent study by Tidball and Spencer (37), in which muscle-specific overexpression of calpastatin reduced muscle atrophy in a model of muscle wasting, consisting of hindlimb unloading. Other studies as well have supported the concept that the net calpain activity in muscle is determined by the calpain/calpastatin ratio (3, 7).

Because calpain-dependent release of myofilaments from the sarcomere may be an early, and perhaps rate-limiting, component of sepsis-induced muscle wasting, it is possible that inhibition of calpain activity can block sepsis-induced muscle proteolysis. Indeed, in our recent study on sepsis-induced muscle wasting, incubation in vitro of muscles from septic rats in the presence of the calpain inhibitor calpeptin reduced calpain activity and abolished the sepsis-induced increase in protein breakdown (42). Although those results suggest that inhibition of calpain activity may reduce sepsis-

ubiquitin ligases; calpains
induced muscle proteolysis, several important questions remain to be answered. First, it is not known whether treatment in vivo with a calpain inhibitor can block sepsis-induced muscle protein breakdown, a question that has important clinical implications. Second, the influence of calpain inhibition on the ubiquitin-proteasome proteolytic pathway has not been reported. Testing the regulation by calpain inhibitors of the ubiquitin-proteasome system may shed light on the interaction between the proteolytic pathways in atrophying muscle. Finally, because most pharmacological inhibitors of calpain activity, including calpeptin, are not completely specific, it is important to examine the effects of additional calpain inhibitors to ascertain a role of calpain activity in sepsis-induced muscle wasting.

In the present study, we tested the hypothesis that treatment of rats in vivo with a calpain inhibitor prevents sepsis-induced increase in muscle protein degradation. To test that hypothesis, rats were treated with calpeptin (12) or the novel calpain inhibitor BN82270. BN82270 is a hybrid molecule consisting of a calpain-inhibitory component and an antioxidant that was found recently to be a potent inhibitor of cellular calpain activity and lipid peroxidation (2). In addition, we examined whether inhibition of calpain activity influences the ubiquitin-proteasome system in skeletal muscle during sepsis. This was done by measuring mRNA levels for atrogin-1/MAFbx and proteasome-dependent proteolysis in incubated muscles from septic rats.

MATERIALS AND METHODS

Experimental animals. Sepsis was induced in male Sprague-Dawley rats (50–70 g) by CLP, as described previously (23, 36, 42, 43). Other rats underwent a sham operation consisting of laparotomy and manipulation, but no ligation or puncture, of the cecum. Saline (100 ml/kg body wt) was administered subcutaneously on the back of each rat at the time of surgery to prevent hypovolemia and septic shock. Muscles were studied 16 h after CLP or sham operation. Animals had free access to water, but food was withheld after the surgical procedures to avoid the influence of differences in food intake on metabolic changes in sham-operated and septic rats. The septic model used here results in a reproducible increase in muscle protein breakdown and activation of the calpain and ubiquitin-proteasome proteolytic mechanisms, as described in several previous reports from our laboratory (23, 36, 42, 43, 45). The experimental protocol was approved by the Institutional Animal Care and Utilization Committee at the Beth Israel Deaconess Medical Center (Boston, MA). Animals were treated and cared for in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals.

Groups of rats were treated with one of the calpain inhibitors benzylxycarbonyl leucyl-norleucinal (calpeptin) (12) or BN82270 (2). The drugs were administered subcutaneously 2 h before and 8 and 15 h after sham operation or CLP, using doses described in the RESULTS. Control rats received corresponding volumes of vehicle (0.1% DMSO in saline for calpeptin and 10% montanox in saline for BN82270).

Muscle incubations. Sixteen hours after CLP or sham operation, extensor digitorum longus (EDL) muscles were dissected, mounted on stainless steel supports at resting length, and incubated under physiological conditions in a shaking water bath at 37°C, as described in detail previously (18, 19). Protein breakdown rates were determined by measuring net release of free tyrosine (40). In some experiments, paired muscles were incubated in the absence or presence of BN82270 (Ipsen, Les Ulis, France), the oxygen radical scavenger 2,6-di-tert-

butyl-4-methylphenol (BHT) (8) (Sigma, St Louis, MO), H2O2 (Sigma), cathepsin B inhibitor II (Calbiochem, EMD Biosciences, San Diego, CA), the cathepsin D inhibitor pepstatin (Roche Diagnostics Biochemical Products, Indianapolis, IN), cathepsin L inhibitor IV (46) (Calbiochem), and the proteasome inhibitor β-lactone (Boston Biochem, Cambridge, MA) at concentrations described in the RESULTS.

Calpain activity. Muscle calpain activity was determined by measuring the degradation of the fluorogenic calpain-specific substrate 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-proiono acid labeled casein, as described in detail recently (35, 42). This method measures total calpain activity, that is, both µ- and m-calpain activity and possibly the activity of other calpains as well.

Cathepsin L activity. Muscles were homogenized in 1% Triton X-100 in PBS (pH 7.4), and the homogenates were centrifuged at 10,000 g for 20 min at 4°C. Protein concentration in the supernatant was determined by the Bradford method (1), and aliquots (100 µg protein) were used for measurement of cathepsin L activity. Cathepsin L activity was determined by using the fluorogenic peptide substrate Z-Arg-Ala-Arg-AMC (Calbiochem) and the InnoZyme cathepsin L activity kit (Calbiochem), following the manufacturer’s instructions. Purified cathepsin L from human liver (Calbiochem) was used as a positive control.

Real-time PCR. Atrogin-1/MAFbx and MuRF1 mRNA levels in EDL muscles were determined by real-time PCR. Tissue RNA was extracted and real-time PCR was performed, as described in detail recently (42). The forward, reverse, and double-labeled oligonucleotides for atrogin-1/MAFbx were as follows, respectively: 5′-CTT TCA ACA GAC TGG ACT TCT CGA-3′, 5′- CAG CTC CAA CAG CCT TAC TAC GT-3′, and 5′- TGC CAT CCT GGA TTC CAC AAG ATT CAA C-3′. The corresponding sequences for MuRF1 were as follows: 5′- GGA CTC CTG CCG AGT GAC C-3′, 5′- GCC TCA AAC TTG TGG CTC AG-3′, and 5′- AGG AAA ACA GCC ACC AGG TGA AGG AGG-3′. Amplification of 18S rRNA was performed in the same reaction tubes as an internal standard with an alternatively labeled probe (VIC-labeled probe) to distinguish its product from that derived from atrogin-1/MAFbx or MuRF1 RNA. Atrogin-1/MAFbx and MuRF1 mRNA concentrations were normalized to the 18S mRNA levels. Measurements were performed in duplicate for each standard and rat muscle sample.

Cultured L6 muscle cells. L6 rat skeletal muscle cells were obtained from ATCC (Manassas, VA) and were grown in DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 10% CO2. Using the lipofectin method, the cells were transfected for 24 h with a vector containing full-length cDNA for human calpastatin (pCIG-CDP1, kindly provided by Dr. Masatoshi Maki, Nagoya, Japan) or an empty vector (pCAGGS-ires2-nucEGFP, kindly provided by Dr. Chester Chamberlain, Department of Molecular and Cell Biology, Harvard University, Boston, MA). Cellular levels of calpastatin were determined by Western blot analysis as described in detail previously (42). For the measurement of protein degradation rates, cells were labeled with 1.0 µCi/ml [3,5-3H]tyrosine for 48 h followed by determination of release of trichloroacetic acid-soluble [3H]tyrosine, as described in detail previously (41, 42). Protein degradation rates were calculated as described (24, 41, 42) and expressed as %/24 h.

Statistical analysis. Statistical analysis was performed by using Student’s t-test or ANOVA followed by Holm-Sidak’s or Dunn’s method, as appropriate. Results are reported as means ± SE.

RESULTS

In a recent study, we found that treatment in vitro of incubated muscles from septic rats with calpeptin resulted in reduced protein breakdown rates (42). It is not known from those experiments whether treatment of rats in vivo with calpeptin inhibits sepsis-induced protein breakdown in skeletal muscle. To test the in vivo effects of calpeptin, we treated rats
with three repeated doses of calpeptin (each dose 2 mg/kg) injected subcutaneously 2 h before and 8 and 15 h after sham operation or CLP followed by measurement of muscle protein breakdown rates 1 h after the last injection of calpeptin. Treatment with this amount of calpeptin resulted in an \( \approx 15\% \) reduction of protein degradation in muscles from septic rats, whereas muscle protein breakdown in sham-operated rats was not affected (Fig. 1). To examine whether the effect of calpeptin was dose dependent, we next treated septic rats with an increased amount of calpeptin (three doses of 4 mg/kg). This treatment reduced protein breakdown in muscle from septic rats by \( \approx 25\% \) (Fig. 1).

Because calpeptin is not completely specific in its calpain-inhibitory effect (but may inhibit other cysteine proteases as well, including cathepsin B, D, and L), we next wanted to compare the effects of calpeptin with those of another calpain inhibitor. For that purpose, rats were treated with three repeated doses (60 mg/kg each) of the novel calpain inhibitor BN82270 (2) injected subcutaneously in sham-operated and septic rats at the same time points and intervals as used for the calpeptin treatment. Treatment of rats in vivo with BN82270 reduced muscle protein breakdown rates by \( \approx 30\% \) in septic rats but did not influence muscle protein degradation in sham-operated rats (Fig. 2).

Our recent observation that exposing incubated muscles in vitro to calpeptin reduced protein breakdown rates (42) suggests that calpeptin exerts a direct effect in skeletal muscle. We next tested whether BN82270 also may have a direct effect in skeletal muscle. When incubated muscles from sham-operated and septic rats were treated in vitro with 100 \( \mu M \) BN82270, protein breakdown rates were reduced in muscles from both sham-operated and septic rats (Fig. 3A) consistent with the concept that BN82270 can exert a direct effect in skeletal muscle. The reason why BN82270 reduced protein breakdown when added to incubated muscles from sham-operated rats but not when administered in vivo to sham-operated rats (see Fig. 2) is not known, but it is possible that the concentration of 100 \( \mu M \) BN82270 used in the in vitro experiments was higher than the circulating levels achieved when rats were treated with three doses of 60 mg/kg of BN82270. The observation that BN82270 reduced protein breakdown when added to muscles

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** The effect of calpeptin on sepsis-induced muscle proteolysis. Groups of rats were treated with 2 or 4 mg/kg of calpeptin or corresponding volume of vehicle administered subcutaneously 2 h before and 8 and 15 h after sham operation or CLP. Extensor digitorum longus (EDL) muscles were harvested 16 h after sham operation or CLP and incubated for 2 h under physiological conditions. Protein breakdown rates were determined as net release of tyrosine. Results are means \( \pm \) SE, with \( n = 8 \) in each group. \(* P < 0.05\) vs. sham groups; \(+ P < 0.05\) vs. corresponding vehicle group by ANOVA.

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** The effect of BN82270 on muscle protein breakdown in sham-operated and septic rats. Groups of rats were treated with vehicle or 60 mg/kg of BN82270 administered as three repeated subcutaneous injections 2 h before and 8 and 15 h after sham operation or CLP. Protein breakdown rates were determined in incubated EDL muscles 16 h after sham operation or CLP. \(* P < 0.05\) vs all other groups. \(+ P < 0.05\) vs. corresponding sham group by ANOVA; \( n = 8 \) in each group.

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** The effects of BN82270 on protein breakdown rates (A) and calpain activity (B) in incubated muscles from sham-operated and septic rats. EDL muscles were harvested 16 h after sham operation or CLP and incubated in the absence (control) or presence of 100 \( \mu M \) BN82270. \(* P < 0.05\) vs. corresponding sham group; \(+ P < 0.05\) vs. corresponding control group by ANOVA; \( n = 8 \) in each group.
from rats that had undergone CLP suggests that BN82270 can reduce muscle protein breakdown that has already been increased during sepsis. Because similar results were observed when muscles from septic rats were incubated in the presence of calpeptin (42), it is possible that treatment with calpain inhibitors may be efficacious not only in the prevention, but also in the treatment of sepsis-induced muscle wasting. Similar to the effects of calpeptin (42), calpain activity was reduced in muscles incubated in the presence of BN82270 in vitro (Fig. 3B).

Because the proteasome does not degrade intact myofibrils (27), a model of muscle wasting has been proposed in which calpain-dependent release of myofilaments from the sarcomere may be an early, and perhaps rate-limiting, component of increased muscle proteolysis (14, 17, 33, 43). In this model, myofilaments that have been detached from the sarcomere are ubiquitinated and subsequently degraded by the 26S proteasome. It is not known whether activation of the ubiquitin-proteasome pathway depends on increased calpain activity and calpain-dependent release of myofilaments from the sarcomere (providing the ubiquitin-proteasome system with increased amounts of substrates) or whether the two proteolytic mechanisms are activated in “parallel.” Because the expression of the ubiquitin ligases atrogin-1/MAFbx and MuRF1 provides a sensitive marker for ubiquitin-proteasome-dependent proteolysis and muscle wasting (5, 15, 28, 45), we next determined mRNA levels for atrogin-1/MAFbx and MuRF1 in muscles from BN82270-treated rats. Sepsis resulted in a pronounced (20–30 fold) increase in muscle atrogin-1/MAFbx and MuRF1 mRNA levels, and these effects of sepsis were not prevented when rats were treated with three doses of 60 mg/kg of BN82270 (Fig. 4). This observation suggests that activation of the ubiquitin-proteasome pathway does not depend on increased calpain-dependent proteolysis. Thus it is possible that the calpain and ubiquitin-proteasome systems are upregulated in “parallel” during sepsis rather than in sequence.

This interpretation is made with caution, however, because unchanged mRNA levels for atrogin-1/MAFbx and MuRF1 in muscles from BN82270-treated rats do not necessarily mean that BN82270 cannot inhibit proteasome activity. To examine that question, we performed experiments in which paired EDL muscles from septic rats were incubated in the absence or presence of 100 μM β-lactone, a highly specific proteasome inhibitor (10) and calculated the proteasome-dependent proteolysis as β-lactone-inhibited protein breakdown. The effect of β-lactone was tested in muscles incubated in the absence or presence of 100 μM BN82270. Calculated in this way, the proteasome-dependent protein degradation (i.e., the difference in protein degradation between muscles incubated with or without β-lactone) was almost identical (100 and 112 nmol/g wet wt × 2 h) in muscles incubated in the absence or presence of BN82270 (Fig. 5), suggesting that BN82270 did not reduce overall proteasome activity under the present experimental conditions.

In addition to being a calpain inhibitor, BN82270 is an inhibitor of lipid peroxidation as described by Auvin et al. (2). To test whether the BN82270-induced inhibition of muscle protein breakdown noticed in the present experiments reflected oxygen radical scavenging, muscles were incubated in the presence of 5 mM H2O2. H2O2 induces the formation of Reactive Oxygen Species (ROS), which can cause oxidative stress and subsequent muscle wasting. By using a specific proteasome inhibitor, we can isolate the effect of BN82270 on proteasome-dependent protein degradation. In this experiment, we incubated muscles from septic rats in the presence of 100 μM β-lactone and 100 μM BN82270 and calculated the proteasome-dependent protein degradation as before. The results showed that the proteasome-dependent protein degradation was almost identical (100 and 112 nmol/g wet wt × 2 h) in muscles incubated in the absence or presence of BN82270 (Fig. 5), suggesting that BN82270 did not reduce overall proteasome activity under the present experimental conditions.

To test whether the BN82270-induced inhibition of muscle proteolysis reflected oxygen radical scavenging, we performed experiments in which paired EDL muscles from septic rats were incubated in the absence or presence of 100 μM BN82270. In these experiments, we incubated muscles from septic rats in the presence of 100 μM β-lactone and 100 μM BN82270 and calculated the proteasome-dependent protein degradation as before. The results showed that the proteasome-dependent protein degradation was almost identical (100 and 112 nmol/g wet wt × 2 h) in muscles incubated in the absence or presence of BN82270 (Fig. 5), suggesting that BN82270 did not reduce overall proteasome activity under the present experimental conditions.

Fig. 4. The effect of BN82270 on sepsis-induced expression of atrogin-1 (A) and MuRF1 mRNA (B) in skeletal muscle. BN82270 (each dose 60 mg/kg) or corresponding volume of vehicle was administered subcutaneously 2 h before and 8 and 15 h after sham operation or CLP in rats. EDL muscles were harvested 16 h after sham operation or CLP, and mRNA levels were determined by real-time PCR. *P < 0.05 vs corresponding sham group by ANOVA; n = 8 in each group.

Fig. 5. The influence of BN82270 on β-lactone-induced inhibition of protein degradation in incubated EDL muscles from septic rats. Paired EDL muscles from septic rats were incubated in the absence or presence of 100 μM BN82270. One of the muscles was treated with 100 μM β-lactone, and the paired muscle was incubated with the same concentration of solvent. Thus the effects of β-lactone were tested in the absence or presence of BN82270, as indicated. *P < 0.05 vs. control; n = 6 in each group.
hydroxyl radicals, which can initiate radical chain processes, such as lipid peroxidation, protein oxidation, and DNA oxidation. Similar to previous reports (29, 31), exposure of muscles to H$_2$O$_2$ resulted in increased protein breakdown rates (Fig. 6A). This effect of H$_2$O$_2$ was seen also in muscles incubated in the presence of 100 $\mu$M BN82270, suggesting that BN82270 did not effectively scavenge oxygen radicals in incubated rat muscles under the present experimental conditions. Incubation of muscles in the presence of H$_2$O$_2$ resulted in a significant increase in muscle wet weight (Fig. 6B). Because muscle dry weight did not change during incubation with H$_2$O$_2$ (data not shown), the increase in wet weight probably reflected oxygen radical-mediated membrane injury and increased cellular water. The increase in muscle weight during incubation in the presence of H$_2$O$_2$ was not blocked by BN82270, providing further support for the concept that BN82270 did not effectively scavenge oxygen radicals under the present experimental conditions.

To further test the potential role of oxygen radicals in the regulation of muscle protein breakdown, we next incubated muscles from sham-operated and septic rats in the presence of the oxygen radical scavenger BHT (9). The concentration of BHT used in these experiments (0.2 mM) was equal to or greater than concentrations shown in previous studies to effectively scavenge oxygen radicals in various cell types and tissues (8). Protein breakdown rates were not influenced by BHT in muscles from sham-operated or septic rats in the present experiments (data not shown). Taken together with the results in Fig. 6, this observation suggests that BN82270 did not inhibit protein degradation in incubated muscles secondary to oxygen radical scavenging.

Because both calpeptin and BN82270 are cysteine protease inhibitors (2, 12), it was important to examine the potential role of cysteine proteases other than the calpains, such as cathepsins B, D, and L, in sepsis-induced muscle proteolysis. When muscles from sham-operated and septic rats were incubated in the presence of cathepsin B inhibitor II (4 $\mu$M) or the cathepsin D inhibitor pepstatin (10 $\mu$g/ml), protein breakdown rates were not affected (Fig. 7, A and B). These results suggest that the effects on protein breakdown in incubated muscles of BN82270 noticed in the present study and of calpeptin noticed in our recent report (42) did not reflect inhibition of cathepsin B or D activity. In contrast, incubation of muscles in the presence of 80 $\mu$M cathepsin L inhibitor IV (26) resulted in reduced protein degradation in muscles from both sham-operated and septic rats (Fig. 7C).

Because previous studies suggest that the expression of cathepsin L is increased in atrophying muscle (9, 26) and because we found here that a cathepsin L inhibitor reduced protein breakdown rates in incubated muscles (see Fig. 7C), it was important to further test the role of cathepsin L in the inhibition of protein breakdown rates and calpain activity that is noticed in muscles treated with calpain inhibitors. We therefore next tested the effect of BN82270 on cathepsin L activity in incubated muscles from sham-operated and septic rats. Cathepsin L activity was reduced by BN82270 in muscles from septic rats (Fig. 8A). When muscles were incubated in the presence of cathepsin L inhibitor IV, calpain activity was reduced in muscles from sham-operated and septic rats (Fig. 8B). Taken together, the results in Fig. 8 suggest that the inhibition of protein breakdown in septic muscles caused by BN82270 may, at least in part, reflect inhibited cathepsin L activity, in addition to inhibited calpain activity. Furthermore, the effect of cathepsin L inhibitor IV on muscle protein degradation (see Fig. 7C) may reflect inhibition of both cathepsin L and calpain activity. Thus both cathepsin L and calpain activity may contribute to sepsis-induced muscle proteolysis, providing support for the concept that multiple proteolytic mechanisms are involved in muscle wasting during sepsis (9, 17, 20, 39).

Because the present results suggest that the effects of the calpain inhibitors on muscle proteolysis may reflect inhibition of both calpain and cathepsin L activity (but not scavenging of oxygen radicals), we performed additional experiments to further test the hypothesis that inhibition of calpain activity can reduce protein degradation in muscle-wasting conditions. In those experiments, protein degradation rates were measured in dexamethasone-treated myocytes overexpressing the endogenous calpain inhibitor calpastatin. Dexamethasone-treated myocytes and myotubes are frequently used as in vitro models of muscle wasting because muscle protein degradation is mainly regulated by glucocorticoids during sepsis and several other catabolic conditions as well (5, 16, 24, 26). Transfection of myocytes with a plasmid containing calpastatin DNA re-

![Fig. 6. The influence of BN82270 on H$_2$O$_2$-induced changes in protein breakdown rates (A) and wet weight (B) in incubated rat muscles. Paired EDL muscles from normal untreated rats were incubated in the absence or presence of 100 $\mu$M BN82270. One of the muscles was treated with 5 mM H$_2$O$_2$, and the paired muscle was incubated with the same concentration of solvent. Thus the effects of H$_2$O$_2$ were tested in the absence or presence of BN82270, as indicated. *$P < 0.05$ vs. control; $n = 8$ in each group.](http://ajpregu.physiology.org/)

AJP-Regul Integr Comp Physiol • VOL 290 • JUNE 2006 • www.ajpregu.org
sulted in increased levels of calpastatin protein (Fig. 9A). When myocytes transfected with an empty vector were treated with dexamethasone, protein degradation increased by ~25% (Fig. 9B) similar to previous reports (24, 41, 42). This effect of dexamethasone was significantly reduced in myocytes overexpressing calpastatin. In a recent study, we found that calpeptin abolished the dexamethasone-induced increase in protein degradation in cultured myotubes (42). Taken together, those results and the present observations support the concept that inhibition of calpain activity can block the increase in protein breakdown seen in catabolic conditions, at least when regulated by glucocorticoids. Of note in the present study was the finding that basal protein degradation was not reduced in myocytes expressing high levels of calpastatin (see Fig. 9B). This observation suggests that calpain activity may be more important for the regulation of protein breakdown in muscle subjected to catabolic stimuli than in normal muscle.

**DISCUSSION**

In the present study, treatment of rats in vivo with the calpain inhibitors calpeptin and BN82270 prevented sepsis-induced increase in muscle proteolysis. In additional experiments, treatment of muscles in vitro with BN82270 reduced calpain activity and protein breakdown rates, suggesting that calpain inhibitors have a direct effect on skeletal muscle. Although the major effect of calpeptin and BN82270 is inhibition of calpain activity (2, 12), neither drug is completely
intensive care unit. Therefore, treatments that can reduce the need for prolonged ventilatory support and extended stay in the intensive care unit are important clinical implications. Muscle wasting during severe sepsis and other muscle-wasting conditions. It should be noted that although muscle wasting during severe and prolonged sepsis results in muscle weakness and fatigue, it may be argued that increased muscle proteolysis may actually be beneficial to the organism, at least during early sepsis and during the initial phase after injury, by providing essential amino acids to vital organs and tissues. Indeed, when critically ill patients were treated with human growth hormone, the mortality rate was increased, suggesting that prevention of muscle breakdown in critical illness may actually be harmful (34). Results in that study, however, are controversial, and in several other reports (21, 25, 32, 38), treatment of critically ill patients with human growth hormone did not result in adverse effects but improved nitrogen balance and wound healing. As suggested by others (30, 32) and by Takala et al. (34), it is possible that the increased mortality in human growth hormone-treated patients in the study by Takala et al. (34) reflected suboptimal control of blood sugar levels rather than prevention of muscle proteolysis.

Second, because most pharmacological inhibitors of enzyme activities are not completely specific, we tested the effects of two drugs in the present study. Thus, in addition to the well-established calpain inhibitor calpeptin (12), which was used in vitro in our previous study (42), we also examined the effects of the novel calpain inhibitor BN82270 in the present experiments. BN82270 was described recently by Auvin et al. (2). It is a hybrid compound consisting of a calpain-inhibitory component acting on the cysteine residue of the calpain-active site and a phenothiazine antioxidant bound together via a L-leucine linker. BN82270 was reported recently to be a potent inhibitor of cellular calpain activity and lipid peroxidation and provided protection against glial cell death induced by maitotoxin (2). The present study is the first report in which the effects of BN82270 on sepsis-induced muscle proteolysis and calpain activity were tested.

Third, in the present study, we assessed the potential role of nonspecific effects of the calpain inhibitors by comparing their effects with those of various other cysteine protease inhibitors and by determining the role of oxygen radical scavenging and proteasome activity. Results from those experiments suggest that some of the effects of BN82270 on protein breakdown rates in incubated muscles may reflect inhibition of cathepsin L activity (in addition to inhibition of calpain activity) and, conversely, that some of the effects of a commonly used cathepsin L inhibitor may reflect inhibition of calpain activity. Those observations are important because they underscore the potential weakness of experiments in which pharmacological inhibitors are used to block specific proteases and emphasize the importance of always taking possible nonspecific effects into account when such inhibitors are used. From a clinical standpoint, of course, it may be argued that the potential to prevent sepsis-induced muscle proteolysis by treatment with calpeptin or BN82270 is important regardless of whether the drugs exert their effect by inhibiting calpain activity, cathepsin L activity, or both. Similarly, treatment with a cathepsin L inhibitor may be clinically relevant to prevent sepsis-induced muscle proteolysis, regardless of whether the inhibitor blocks cathepsin L or calpain activity or both.

Fourth, the present study provides the first evidence that overexpression of the endogenous calpain inhibitor calpastatin
reduces the catabolic effects of glucocorticoids in skeletal muscle. Although those experiments do not resolve the issue of whether calpeptin and BN82270 prevent sepsis-induced muscle proteolysis mainly by inhibiting calpain activity, they support the concept that inhibition of calpain activity may reduce protein degradation in muscle-wasting conditions, at least when regulated by glucocorticoids.

Finally, and perhaps most important from a mechanistic standpoint with regard to sepsis-induced muscle proteolysis, the present study provides evidence that the gene expression of the ubiquitin-proteasome system is upregulated independent of the calpain system in septic muscle. This interpretation was further supported by the finding that BN82270 did not reduce total proteasome-dependent protein degradation, calculated as β-lactone-inhibited proteolysis, in incubated muscles from septic rats. Because BN82270 is a cysteine protease inhibitor, and some proteasome peptidases may be sensitive to cysteine protease inhibitors, it may seem surprising that proteasome-dependent protein degradation was not influenced by BN82270. It should be noticed, however, that the present results do not rule out the possibility that individual proteasome peptidase activities may be influenced by BN82270 without resulting in inhibition of total proteasome-dependent protein degradation. Although additional experiments would be needed to test that possibility, the present results are important because they support the notion that treatment with BN82270 inhibits calpain activity in skeletal muscle without influencing mRNA levels for atrogin-1/MAFbx and MuRF1 or overall proteasome activity. Furthermore, when the degradation of Suc-Leu-Leu-Val-Tyr-AMC was determined in a cell-free system consisting of purified rabbit 20S proteasomes, BN82270 at a concentration of 10 μM did not influence proteasome activity, whereas the same concentration of the proteasome inhibitor MG 132 inhibited the proteasome activity by more than 90% (Pignol B, unpublished observations) further supporting the concept that BN82270 is not a proteasome inhibitor.

In previous studies, we and others have proposed a model in which muscle catabolism is regulated by an “upstream” activation of calpains, resulting in the release of myofilaments from the sarcomere followed by the ubiquitination and proteosomal degradation of the myofilaments (14, 17, 33, 43). It is not known whether these mechanisms are regulated in tandem or in parallel. Although the present results do not definitively answer that question, they support a model in which the ubiquitin-proteasome system is upregulated independent of calpain-regulated release of myofilaments. Thus sepsis may stimulate the calpain and ubiquitin-proteasome systems in parallel, making the ubiquitin-proteasome system “prepared” to handle an increased flux of substrates even before they are generated. This model is not only of theoretical interest because it suggests that inhibition of an upstream mechanism, such as the calpain-dependent release of myofilaments, may be essential in the prevention and treatment of muscle wasting. The present study suggests that such an approach may indeed be successful in the treatment of sepsis-induced muscle proteolysis. It should be noted that the present results suggest that other treatment strategies, including inhibition of cathepsin L activity, may also be efficacious in the prevention and treatment of sepsis-induced muscle proteolysis. Because multiple mechanisms are probably involved in the development of muscle wasting, it is possible that treatment with a combination of various inhibitors may be the most efficient way to prevent and treat muscle wasting during sepsis and perhaps other catabolic conditions as well.

GRANTS

The study was supported in part by National Institutes of Health (NIH) Grants DK37908 and NR08545. A. R. Evenson was supported in part by an Individual Research Service Award from the NIH.

REFERENCES


