

Regulation of the Ubiquitin Proteasome System in Mechanically Injured Human Skeletal Muscle

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Summary

Metabolic consequences of direct muscle trauma are insufficiently defined. Their effects on the ubiquitin-proteasome pathway (UPP) of protein degradation in human skeletal muscles are as yet unknown. Thus, we investigated whether the UPP is involved in the metabolic response evoked in directly traumatized human skeletal muscles. Biopsies were obtained from contused muscles after fractures and from normal muscles during elective implant removal (control). As estimated by western blot analyses, concentrations of free ubiquitin and ubiquitin protein conjugates were similar in extracts from injured and uninjured muscles. Ubiquitin protein ligation rates were reduced after injury (1.5 ± 0.2 vs. 1.0 ± 0.15 fkat/ μ g; $p=0.04$). Chymotryptic-, tryptic- and caspase-like proteasome peptidase activities (total activity minus activity in the presence of proteasome inhibitors) increased significantly after trauma ($p=0.04 - 0.001$). Significant increases in total chymotryptic- and caspase-like activities were attributable to proteasome activation. Our results extend the possible role of the UPP in muscle wasting to direct muscle trauma. They further suggest that the effects of direct mechanical trauma are not limited to the proteasome and imply that ubiquitin protein ligase systems are also involved. Based on the potential role of the UPP in systemic diseases, it might also be a therapeutic target to influence muscle loss in critically ill blunt trauma patients, in which large proportions of muscle are exposed to direct trauma.

Key words

Catabolism • Muscle cachexia • Ubiquitin-protein ligase complexes • Epoxomicin • Adamantane-acetyl-(6-aminohexanoyl)₃-(leucinyloxy)₃-vinyl-(methyl)-sulfone

Introduction

Muscle protein wasting is a prominent feature of the metabolic response to a variety of catabolic disease states, such as sepsis, severe trauma, burns, cancer or chronic kidney disease, and its important clinical

consequences have been widely recognized (Hasselgren and Fisher 2001, Du and Mitch 2005, Pereira *et al.* 2005, Wing 2005). Besides systemic activation of muscle protein wasting through a variety of circulating mediators in most of the diseases, the pathophysiology of severe blunt trauma is complicated by the fact that large

proportions of skeletal muscle are also exposed to direct mechanical trauma.

The molecular mechanisms leading to increased muscle protein degradation have been studied mostly in systemic disease processes. Studies in rodents and patients suggested that stimulation of the ATP-dependent ubiquitin proteasome pathway (UPP) of protein degradation contributes significantly to skeletal muscle wasting (Mitch and Goldberg 1996, Mansoor *et al.* 1996, Tiao *et al.* 1997, Hasselgren and Fisher 2001, Du and Mitch 2005, Wing 2005). In the UPP, ubiquitin (Ub) protein ligase systems, which traditionally consist of a Ub activating enzyme (E1), a Ub carrier or conjugating enzyme (E2) and a Ub protein ligase (E3), catalyze the covalent ligation of Ub to intracellular proteins in an ATP-dependent process (ubiquitination or ubiquitylation). The ubiquitylated protein is then destined for degradation by the multicatalytic Mg^{2+} /ATP-dependent 26S proteasome, which consists of the 20S proteasome, comprising the proteolytic core, and capped by two 19S regulators, which confer Mg^{2+} /ATP-dependency and ubiquitylated substrate specificity (Hershko and Ciechanover 1998, Wolff and Hilt 2004).

The consequences of direct muscle damage are less well defined and its effects on the UPP in human skeletal muscle are unknown. Therefore, we investigated whether the UPP is affected by direct mechanical trauma in skeletal muscles from patients. For its characterization, we analyzed free Ub and Ub protein conjugates, total cytosolic Ub protein ligation rates (Majetschak *et al.* 2000) and the three main proteasome peptidase activities in skeletal muscle extracts. Since differentiation between systemic and local effects on the UPP is confounded by the systemic inflammatory response to trauma in severely injured patients, we used muscle biopsies from patients with isolated fractures undergoing primary operative repair as a clinical model of direct mechanical trauma.

Methods

The protocol used was approved by the Ethics Committee of the Faculty of Clinical Medicine Mannheim. Informed consent was obtained from each patient. The study population consisted of 29 patients in the Department of Trauma Surgery (Table 1). Patients fulfilled the following criteria: age > 18 years, no malignant or infectious disease, rheumatoid arthritis, immunosuppressive medication and known liver, kidney or pancreas disease requiring treatment. Fifteen muscle

biopsies were obtained from 14 patients who underwent primary operative fracture repair within 8 h after trauma. Biopsies (approximately 100 mg) were obtained during the operation from macroscopically contused muscle adjacent to the fracture. As controls, 16 biopsies from macroscopically normal muscle were obtained from 15 patients who underwent elective implant removal. Biopsies were snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Muscle extracts were prepared using a total protein extraction kit (BioChain, Hayward, CA) according to the manufacturer's instructions and protein concentrations were determined (Lowry *et al.* 1951). Immunoblotting to Ub with rabbit anti-Ub (1:500 v/v, Sigma, St. Louis, MO) and densitometric quantification of the chemiluminescence signal were performed as described previously in detail (Majetschak *et al.* 2003, Ponelies *et al.* 2005).

The total ubiquitylation rate of cytosolic proteins, as a result of the total Ub-protein ligase activity, was measured as incorporation of biotinylated Ub into the sum of cytosolic proteins (Majetschak *et al.* 2000, Ponelies *et al.* 2005). The ubiquitylation rate is given in katal (1 mol biotinylated Ub incorporated into cytosolic proteins per s). Peptidase activities were measured employing fluorogenic peptide substrates *N*-Suc-LLVY-7-amino-4-methylcoumarin (AMC) (chymotryptic-like, 100 μM), *Z*-AAR-AMC (tryptic-like, 200 μM) or *Z*-LLE-7-AMC (caspase-like 100 μM) (all from Merck, Schwalbach, Germany). Reaction mixtures contained 1 mM DTT, 5 mM ATP, 5 mM $MgCl_2$, 50 mM TrisHCl, pH 8.0, peptide substrate and 0.5 mg/ml freshly prepared muscle extract and were incubated for 60 min at 37°C . Free AMC cleaved from the substrates was measured in 15 min intervals on a fluorescence reader (λ excitation/emission = 360/465 nm, Genios, Tecan, Crailsheim, Germany) against standard curves of AMC (Sigma). To differentiate the proteasome from other peptidase activities, the proteasome inhibitors epoxomicin (1 μM ; Meng *et al.* 1999) or adamantane-acetyl-(6-aminohexanoyl)₃-(leucinyloxy)-vinyl-(methyl)-sulfone (15 μM , ADA; Kessler *et al.* 2001) were added to the incubation mixtures for the assessment of chymotryptic-like or tryptic- and caspase-like peptidase activities, respectively. Both proteasome inhibitors were from Biomol (Hamburg, Germany). Proteasome (total peptidase activity minus peptidase activity in the presence of proteasome inhibitor) and proteasome-independent (peptidase activity in the presence of proteasome inhibitor) peptidase activity were determined as mol of AMC cleaved per min. Enzyme time-progression curves showed linearity for all three

Table 1. Patient characteristics and muscle biopsies

Biopsy	Age	Sex	Muscle	Diagnosis
<i>Control</i>				
1	18	F	Gluteus medius	Implant removal
2	41	M	Erector spinae	Implant removal
3	41	M	Extensor carpi radialis	Implant removal
4	40	M	Vastus lateralis	Implant removal
5	56	F	Deltoideus	Implant removal
6	54	M	Gluteus medius	Implant removal
7	64	F	Vastus lateralis	Implant removal
8	28	M	Vastus lateralis	Implant removal
9	35	F	Erector spinae	Implant removal
10	60	F	Tibialis ant.	Implant removal
11	64	F	Erector spinae	Implant removal
12	52	F	Peroneus longus	Implant removal
13	64	F	Vastus lateralis	Implant removal
14	54	M	Peroneus longus	Implant removal
15	24	M	Gluteus medius	Implant removal
16	64	F	Erector spinae	Implant removal
Total:	48±16	9 F/ 7 M		
<i>Injured</i>				
1	48	F	Deltoideus	Proximal humerus fracture
2	51	F	Triceps humeri	Supracondylar humerus fracture
3	79	F	Deltoideus	Proximal humerus fracture
4	50	M	Erector spinae	Vertebral fracture (L4)
5	58	M	Deltoideus	Proximal humerus fracture
6	58	M	Biceps brachii	Proximal humerus fracture
7	29	M	Erector spinae	Vertebral fracture (T9)
8	41	M	Extensor carpi ulnaris	Radius and ulna fracture
9	78	F	Deltoideus	Proximal humerus fracture
10	85	F	Gluteus medius	Proximal femur fracture
11	26	F	Peroneus longus	Proximal tibia fracture
12	32	M	Soleus	Proximal tibia fracture
13	78	F	Deltoideus	Proximal humerus fracture
14	85	F	Gluteus medius	Proximal femur fracture
15	76	F	Extensor digitorum	Distal radius fracture
Total:	58±22	9 F/ 6 M		

Data are mean ± SD. F: Female. M: Male. Biopsies 2 and 3 (control) and biopsies 5 and 6 (injured) were obtained from the same patient. Age and gender distribution were similar in both groups ($p > 0.05$, t-test and χ^2 -test, respectively).

peptidase activities. The data are described as the mean ± SD. Statistics were calculated with the SPSS for Windows Release 13.0 program (t-test, chi-square test (χ^2)). A two-tailed $p < 0.05$ was considered as significant.

Results and Discussion

Representative immunoblotting experiments are shown in Fig. 1A and revealed no visible differences between

control and injured muscles. Densitometric quantification confirmed that the concentrations of total Ub (control (n=10): 13 ± 1 ng/ μ g protein; injured (n=11): 13.3 ± 1 ng/ μ g protein), free Ub (control (n=10): 1.5 ± 0.06 ng/ μ g protein; injured (n=11): 1.6 ± 0.1 ng/ μ g protein) and Ub protein conjugates (control (n=10): 11.5 ± 1 ng/ μ g protein; injured (n=11): 11.7 ± 1 ng/ μ g protein) were similar ($p > 0.05$ for all comparisons).

Figure 1B shows the initial progress curves of Ub protein ligation. We observed a lag-phase of about 2.5 min with a subsequent linear increase in ubiquitylation. Based on the initial progress curves, Ub protein ligation rates were 1.5 ± 0.2 fkat/ μ g in the controls (n=11) and 1.0 ± 0.15 fkat/ μ g in injured muscles (n=12), which was significantly lower (Fig. 1C, $p = 0.04$). We have shown recently that Ub protein ligation is also significantly reduced in human mononuclear cells after endotoxin stimulation *in vitro* and in mononuclear cells from patients with sepsis (Majetschak *et al.* 2000, Ponelies *et al.* 2005). Although the clinical relevance of reduced ubiquitylation rates and the underlying mechanism remain to be determined, we interpret its significant reduction in injured muscle as a common cell response to infectious and non-infectious inflammation in general, that could be aimed to prevent uncontrolled proteolysis.

Total peptidase activities were higher in injured (n=5) than in control (n=5) muscles (tryptic-like: 2.9 ± 0.8 and 1.7 ± 0.5 pmol/min ($p = 0.4$), chymotryptic-like: 1.5 ± 0.2 and 0.6 ± 0.07 pmol/min ($p = 0.006$), caspase-like: 0.4 ± 0.05 and 0.1 ± 0.01 pmol/min ($p < 0.001$) for injured and control, respectively). This finding is consistent with previous data from rodents, which showed a marked decrease in total protein content and an increase in overall proteolytic rates in skeletal muscle after direct mechanical trauma (Fisher *et al.* 1990, Farges *et al.* 2002). Addition of proteasome inhibitors reduced tryptic-like peptidase activities by 17 ± 5 % (control) and 21 ± 4 % (injured), chymotryptic-like activities by 79 ± 3 % (control) and 78 ± 2 % (injured), and caspase-like activities by 94 ± 5 % (control) and 95 ± 1 % (injured) without significant differences between control and injured muscle. The proteasome peptidase activities (Fig. 1D-F, grey bars) were significantly higher for all three substrates in injured muscles. In contrast, we did not observe significant differences between control and injured muscles for the proteasome-independent tryptic- and caspase-like activities (Fig. 1D-F, open bars). Thus, the results suggest that increased total chymotryptic- and caspase-like peptidase activities can be attributed

predominantly to proteasome activation.

Our finding of proteasome activation contradicts a recent study which reported unchanged proteasome activity at 48 h after direct mechanical injury, assessed as MG132-dependent tyrosin release from dissected gastrocnemius strips (Farges *et al.* 2002). The different experimental procedures that were used to assess proteasome contribution could possibly explain these controversial findings. The peptide aldehyde MG132 is a reversible inhibitor of the chymotryptic-like site. Epoxomicin and ADA are irreversible inhibitors. While epoxomicin, which is one of the most selective proteasome inhibitors, predominantly inhibits chymotryptic-like proteasome activity, ADA has comparable affinity for all catalytic subunits (Meng *et al.* 1999, Kessler *et al.* 2001, Kisselev and Goldberg 2001). Both methods, i.e. measuring tyrosin release from dissected muscle strips and measurement of peptidase activities in cell free extracts, have major limitations and provide only indirect assessment of proteasome activity *in vitro* (Kisselev and Goldberg 2001, Rodgers and Dean 2003, Kukan 2004). Furthermore, proteasome activity was assessed at a time point when total proteolytic rates peaked in the experimental model (Farges *et al.* 2002), while muscle biopsies were collected at an earlier time point in patients. Although the time course of proteolytic activation in injured human muscle is unknown, the kinetics of proteasome activation after injury may not necessarily resemble kinetics of total or non-proteasomal proteolytic activation in rodents or humans. This hypothesis is supported by peptidase activity measurements in muscle extracts from a patient (Table 1, #11) who underwent a second-look operation at 72 h after injury and during which we could obtain a second biopsy. Comparison of peptidase activities within 8 h and at 72 h after trauma showed that all three proteasome activities increased 1.5 to 4-fold by 72 h (pmol/min: tryptic-like – 0.2 (<8 h) and 0.76 (72 h); chymotryptic-like – 0.89 (<8 h) and 1.38 (72 h); caspase-like – 0.22 (<8 h) and 0.55 (72 h)). Proteasome independent tryptic-like activity increased 10-fold, chymotryptic-like activity 1.2-fold, whereas proteasome independent caspase-like activity decreased 2-fold (pmol/min: tryptic-like – 0.4 (<8 h) and 4.0 (72 h); chymotryptic-like – 0.28 (<8 h) and 0.33 (72 h); caspase-like – 0.02 (<8 h) and 0.01 (72 h)). Another possible explanation is that the severity of mechanical injury in rats might have been below the biological threshold of proteasome activation. Since we detected a significant increase in total peptidase activities

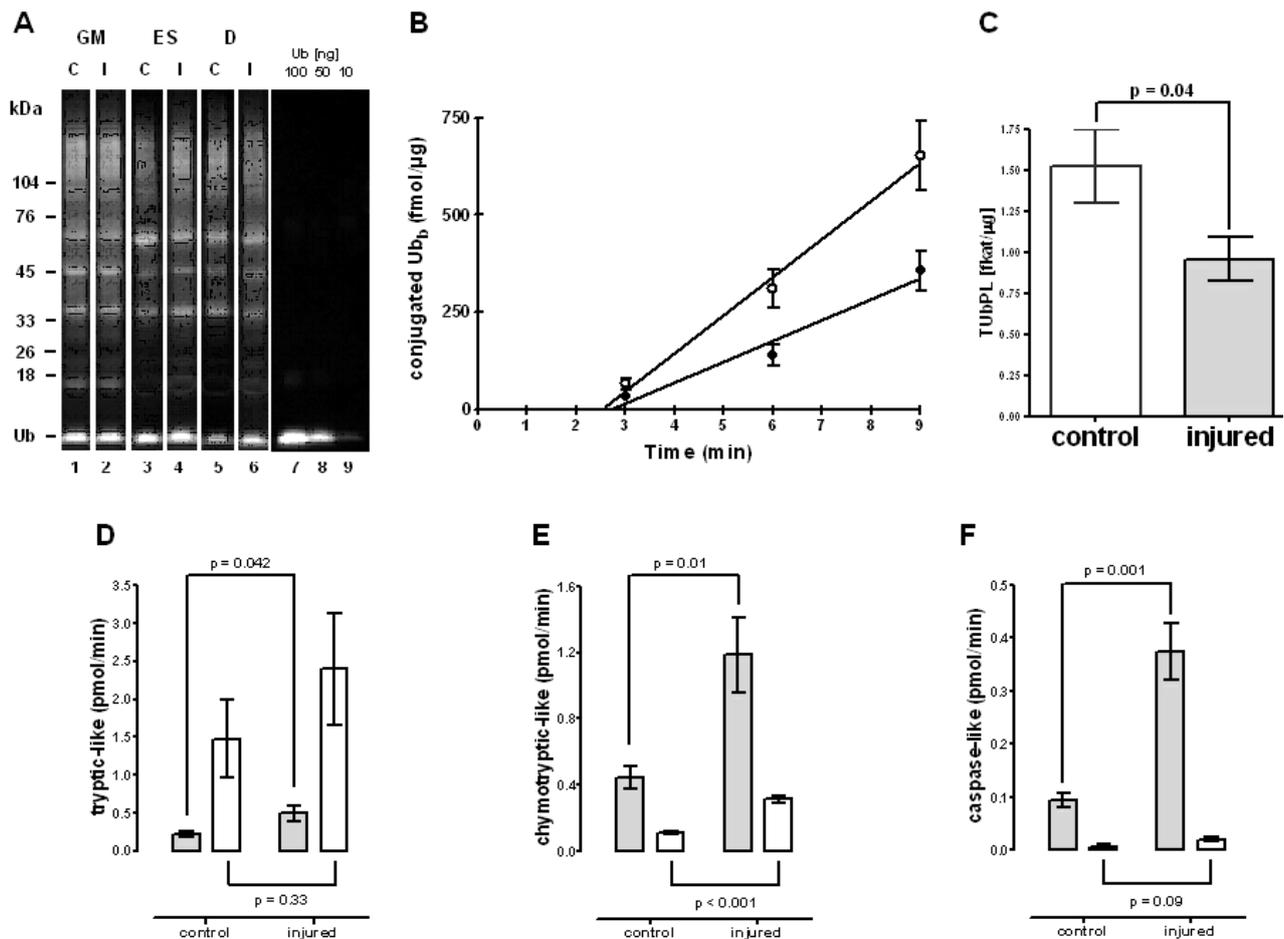


Fig. 1. **A.** Typical immunoblot for ubiquitin in control and injured muscle extracts. Ub: Ubiquitin. Left: Position of the molecular mass standards. C: Control muscle. I: Injured muscle. GM: Gluteus medius muscle. ES: Erector spinae muscle. D: Deltoid muscle. Lanes 1 – 6: Each lane contains 30 μg of muscle extract protein. Lanes 1 and 2: GM, control and injured. Lanes 3 and 4: ES, control and injured. Lanes 5 and 6: D, control and injured. Lanes 7-9: Ub standards, 100, 50 and 10 ng, respectively. **B.** Initial progress curves of ubiquitin protein ligation in muscle extracts. Ub_b: Biotinylated ubiquitin. Data are mean ± SD. Open circles: Control (n=11). Filled circles: Injured (n=12). Line: Linear regression curves ($r^2=0.96-0.98$). **C.** Total ubiquitin protein ligation rates (TUbPL) in muscle extracts. Data are mean ± SD. Open bars: Control (n = 11). Grey bar: Injured (n=12). **D.-F.** Peptidase activities in muscle extracts. Data are mean ± S.D. Open bars: Proteasome independent peptidase activity (n=5). Grey bars: Proteasome peptidase activity (n=5). **D.** Tryptic-like peptidase activity. **E.** Chymotryptic-like peptidase activity. **F.** Caspase-like peptidase activity.

at a time point when total proteolysis was not significantly increased in the animal model (<8 h after trauma), the mechanical impact in our study appears to be higher and could possibly not have been high enough to affect the ubiquitin proteasome system in the experimental model.

It is certainly a limitation of the present study that we were unable to quantify and compare the mechanical impact with experimental data in rats. However, all injured muscle biopsies were obtained from areas adjacent to the fracture indicating a biologically relevant soft tissue trauma. Another limitation is that extracts were prepared without addition of protease or Ub hydrolase inhibitors. Thus, quantification of the cellular Ub pool and Ub protein ligation rates could have been

affected by increased proteolysis or altered Ub hydrolase activities in the extracts. However, we did not detect alterations of the cellular Ub pool after direct mechanical trauma, as would have been expected if altered protein degradation or hydrolysis of Ub-protein conjugates had occurred *in vitro*. In addition, our preliminary data show that activation of proteasome peptidase activity in skeletal muscle is not directly reflected by alterations of the cellular Ub pool in animals after traumatic shock and hemorrhage (Patel and Majetschak, unpublished observation). Therefore, it cannot be ruled out that changes would have been detectable at other time points.

In conclusion, the present study supports previous findings that increased proteolytic rates are a determinant of protein loss in direct muscle trauma

(Farges *et al.* 2002). In line with studies on systemic catabolic disease states, our results further support the hypothesis that the UPP is critical during muscle wasting and extend its role to direct muscle trauma. Furthermore, they suggest that the effects of mechanical trauma are not limited to the proteasome and imply that Ub protein ligase systems are also affected. Therefore, the UPP might be a target to influence muscle loss in critically ill

blunt trauma patients, e.g. by the therapeutic use of proteasome inhibitors.

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References

- DU J, MITCH WE: Identification of pathways controlling muscle protein metabolism in uremia and other catabolic conditions. *Curr Opin Nephrol Hypertens* **14**: 378-382, 2005.
- FARGES MC, BALCERZAK D, FISHER BD, ATTAIX D, BECHET D, FERRARA M, BARACOS VE: Increased muscle proteolysis after local trauma mainly reflects macrophage-associated lysosomal proteolysis. *Am J Physiol* **282**: E326-E335, 2002.
- FISHER BD, BARACOS VE, SHNITKA TK, MENDRYK SW, REID DC: Ultrastructural events following acute muscle trauma. *Med Sci Sports Exerc* **22**: 185-193, 1990.
- HASSELGREN PO, FISCHER JE: Muscle cachexia: current concepts of intracellular mechanisms and molecular regulation. *Ann Surg* **233**: 9-17, 2001.
- HERSHKO A, CIECHANOVER A. The ubiquitin system. *Annu Rev Biochem* **67**:425-479, 1998.
- KESSLER BM, TORTORELLA D, ALTUN M, KISSELEV AF, FIEBIGER E, HEKKING BG, PLOEGH HL, OVERKLEEF T HS: Extended peptide-based inhibitors efficiently target the proteasome and reveal overlapping specificities of the catalytic beta-subunits. *Chem Biol* **8**: 913-929, 2001.
- KISSELEV AF, GOLDBERG AL: Proteasome inhibitors: from research tools to drug candidates. *Chem Biol* **8**: 739-758, 2001.
- KUKAN M: Emerging roles of proteasomes in ischemia-reperfusion injury of organs. *J Physiol Pharmacol* **55**: 3-15, 2004.
- LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265-75, 1951.
- MAJETSCHAK M, SUCIU DM, HASLER K, OBERTACKE U, SCHADE FU, JENNISEN HP: Cytosolic protein ubiquitylation in normal and endotoxin stimulated human peripheral blood mononuclear cells. *J Endotoxin Res* **6**: 483-488, 2000.
- MAJETSCHAK M, KREHMEIER U, BARDENHEUER M, DENZ C, QUINTEL M, VOGGENREITER G, OBERTACKE U: Extracellular ubiquitin inhibits the TNF α response to endotoxin in peripheral blood mononuclear cells and regulates endotoxin hyporesponsiveness in critical illness. *Blood* **101**: 1882-1890, 2003.
- MANSOOR O, BEAUFRERE B, BOIRIE Y, RALLIERE C, TAILLANDIER D, AUROUSSEAU E, SCHOEFLER P, ARNAL M, ATTAIX D: Increased mRNA levels for components of the lysosomal, Ca²⁺-activated, and ATP-ubiquitin-dependent proteolytic pathways in skeletal muscle from head trauma patients. *Proc Natl Acad Sci USA* **93**: 2714-2718, 1996.
- MENG L, MOHAN R, KWOK BH, ELOFSSON M, SIN N, CREWS CM: Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. *Proc Natl Acad Sci USA* **96**: 10403-10408, 1999.
- MITCH WE, GOLDBERG AL: Mechanisms of muscle wasting: the role of the ubiquitin-proteasome pathway. *New Engl J Med* **335**: 1897-1905, 1996.
- PEREIRA C, MURPHY K, JESCHKE M, HERNDON DN: Post burn muscle wasting and the effects of treatments. *Int J Biochem Cell Biol* **37**: 1948-1961, 2005.
- PONELIES N, KREHMEIER U, HIRSCH T, DENZ C, PATEL MB, MAJETSCHAK M: Cytosolic ubiquitin levels and ubiquitylation rates in human peripheral blood mononuclear cells during sepsis. *Shock* **24**: 20-25, 2005.

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- RODGERS KJ, DEAN RT: Assessment of proteasome activity in cell lysates and tissue homogenates using peptide substrates. *Int J Biochem Cell Biol* **35**: 716-727, 2003.
- TIAO G, HOBLER S, WANG JJ, MEYER TA, LUCHETTE FA, FISCHER JE, HASSELGREN PO: Sepsis is associated with increased mRNAs of the ubiquitin-proteasome proteolytic pathway in human skeletal muscle. *J Clin Invest* **99**: 163-168, 1997.
- WING SS: Control of ubiquitination in skeletal muscle wasting. *Int J Biochem Cell Biol* **37**: 2075-2087, 2005.
- WOLF DH, HILT W: The proteasome: a proteolytic nanomachine of cell regulation and waste disposal. *Biochem Biophys Acta* **1695**: 19-31, 2004.
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