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Direct Effects of Proteasome Inhibitor AdaAhx$_3$L$_3$VS on Protein and Amino Acid Metabolism in Rat Skeletal Muscle

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Summary

Proteasome inhibitors are novel potential drugs for therapy of many diseases, and their effects are not fully understood. We investigated direct effects of peptide vinylsulfone inhibitor AdaAhx$_3$L$_3$VS on protein and amino acids metabolism in rat skeletal muscle. Soleus and extensor digitorum longus muscles were incubated in a medium containing 30 μmol/l AdaAhx$_3$L$_3$VS or no inhibitors. Total proteolysis was determined according to the rates of tyrosine release into the medium during incubation. The rates of leucine oxidation and protein synthesis were evaluated during incubation in medium containing L-[1-^{14}C]leucine. Amino acid concentrations in the medium were measured using HPLC. AdaAhx$_3$L$_3$VS decreased tyrosine release into the medium by 21 and 19 %, decreased leucine incorporation into proteins by 22 and 12 %, and increased leucine oxidation by 24 and 19 % in soleus and extensor digitorum longus muscles, respectively. The release of amino acids into the medium was reduced. We conclude that AdaAhx$_3$L$_3$VS significantly decreased proteolysis and protein synthesis and increased leucine oxidation.

Key words

Proteasome inhibitor • Peptide vinylsulfone • Protein metabolism • Amino acids

Introduction

Ubiquitin-proteasome dependent proteolytic system is ATP-dependent intracellular proteolytic machinery, localized in the cytosol and nucleus. Proteins to be degraded are mostly tagged by the polyubiquitin chain in a process precisely regulated by a system of ubiquitinating and deubiquitinating enzymes that assure the specificity and control of proteolysis. The degradation proceeds in a large proteolytic complex referred to as the 26S proteasome, which contains catalytic residues with "peptidyl glutamyl peptide hydrolyzing" activity (recently suggested to be called "caspase-like activity"), "trypsin-like" cleavage sites, and "chymotrypsin-like" active cleavage sites. The ubiquitin-proteasome system participates in protein turnover under physiological conditions and the quantity of degraded proteins through the proteasome increases during various catabolic conditions (Tiao *et al.* 1994, Mitch and Price 2003). The ubiquitin-proteasome system is responsible for the breakdown of a large variety of cell proteins involved in important biological processes including the cell cycle, cell growth, gene expression, DNA repair, stress response and also programmed cell death (Drexler 1998, Hershko *et al*. 2000).

The development of proteasome inhibitors has greatly extended the information about the role of the proteasome in mammalian cells since 1994. These

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inhibitors have recently been introduced as novel therapeutic agents for the treatment of cancer and potentially other diseases, e.g. stroke and inflammatory diseases (Di Napoli and Papa 2003, Mack *et al*. 2003, Davis *et al*. 2004). The first inhibitors developed were peptide aldehydes and they are still most widely used for research purposes (Kisselev and Goldberg 2001), but they also inhibit cysteine and serine proteases. Other proteasome inhibitors containing a peptide portion are peptide boronates with a great potential for clinical use due to their specificity and potency, and irreversible peptide vinylsulfones. Peptide vinylsulfones were first described as cysteine protease inhibitors (Palmer *et al*. 1995), and their ability to act as irreversible inhibitors of the proteasome was discovered two years later by Bogyo *et al*. (1997). Natural inhibitors lactacystin and β-lactone, and epoxyketones epoxomycin and eponemycin are very selective. Research is now focused on the development of new inhibitors as drug candidates and searching new indications for these agents. However, it should be stressed that the inhibition of the proteasome as a therapeutic approach has certainly had a great impact in organism, which is not yet fully appreciated and understood. Particularly, considerable alteration of the protein metabolism should be expected. Several studies have shown that proteasome inhibition decreases proteolysis in skeletal muscles, but the effect on protein synthesis and amino acid metabolism is not clear.

The aim of our work was to evaluate the effect of $AdaAhx_3L_3VS$ on basic parameters of protein metabolism in incubated rat skeletal muscle – proteolysis, protein synthesis, and also leucine oxidation. Leucine is one of three essential branched-chain amino acids (BCAA) that are known to be regulators of protein synthesis and protein breakdown (Harris *et al.* 2004). Leucine oxidation has been shown to be increased in tumour-bearing rats (Argiles and Lopez-Soriano 1990) and we therefore examined how it is influenced by a proteasome inhibitor as novel therapeutic agent for cancer. In addition, we also measured changes in the total amino acid pool released into medium during incubation. $AdaAhx_3L_3VS$ is a peptide vinylsulfone that cannot be used for therapeutic purposes because of its irreversibility, however, it is a useful research tool. The peptide part of the compound is extended, which was shown to increase its potency (to a level comparable to epoxomycin) and selectivity for the proteasome. Moreover, $AdaAhx_3L_3VS$ reveals a remarkably equal inhibition profile towards all the individual catalytic activities of the proteasome (Kessler *et al*. 2001). We therefore suppose that $AdaAhx_3L_3VS$ may well elucidate the effect of proteasome inhibition.

Methods

Materials

Cycloheximide, amino acid standards, Folin-Ciocalteu phenol reagent, acetonitrile, methyl alcohol and albumin were purchased from Sigma Chemical (St. Louis, MO), HPLC eluent and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate from Waters (Milford MA). L-[1-^{14}C]leucine was purchased from Amersham (Buckinghamshire, UK), Aminoplasmal 15 from B. Braun Medicals (Melsungen, Germany), and hydroxide of hyamine from Packard Instruments (Meriden, CT). $AdaAhx_3L_3VS$ was kindly donated by Benedikt M. Kessler (Harvard Medical School). The remaining chemicals were obtained from Lachema (Brno, CR).

Animals

Male Wistar rats (BioTest, Konárovice, CR) weighing 40-60 g, housed under controlled conditions (12-h light-dark cycle, 22 °C, 55-65 % humidity) were used for the study. All experiments were performed according to guidelines set by the Institutional Animal Use and Care Committee of Charles University.

Muscle incubation

Rats were anesthetized with pentobarbital (6 mg/100 g body weight, intraperitoneally). Soleus (SOL) and extensor digitorum longus (EDL) muscles were then quickly dissected and excised with intact tendons. The muscles were mounted on stainless steel clips at approximate resting length and immediately transferred to 2.5 ml of oxygenated Krebs-Henseleit bicarbonate buffer with 6 mM glucose and 2 mU/ml insulin (pH 7.4, 37 °C). Muscles were preincubated for 30 min in a thermostatically controlled bath (37 °C) with a shaking device (70 cycles/min). After preincubation, muscles were quickly rinsed in 0.9 % NaCl and transferred to a second set of vials containing fresh media.

Bilateral muscles were individually preincubated and incubated in a medium with addition of 30 μM $AdaAhx_3L_3VS$, or in a medium with solvent (DMSO), respectively, so that control and experimental incubations from the same rat could be compared.

Protein and amino acid metabolism measurement

Total protein breakdown rates, protein synthesis and leucine oxidation were measured in SOL and EDL as described in detail previously (Šafránek *et al*. 2003). Total proteolysis was determined according to the rates of tyrosine release into the medium during 2 h incubation. Besides tyrosine, we measured concentrations of other amino acids released into the medium. Cycloheximide (0.5 mM) was added into the medium to prevent reincorporation of amino acids in the muscle proteins. Protein synthesis and leucine oxidation were measured during 1 h incubation in the medium containing L-[1-^{14}C]leucine (0.2 μCi/ml). Medium for both preincubation and incubation was enriched with a solution of amino acids – Aminoplasmal 15 (2.2 mM amino acids). At the end of incubation, 0.4 ml of hydroxide of hyamine was added in a hanging well to capture $^{14}CO_2$ generated from oxidized leucine, and the metabolism in the muscle was stopped by the addition of 0.2 ml of 35 % (v/v) perchloric acid solution. The muscles were then frozen in liquid nitrogen, homogenized, proteins precipitated in 6 % (v/v) $HClO_4$, and washed three times in the same solution. The amount of L-[1-^{14}C]leucine incorporated into proteins was determined after their dissolution in 1 M NaOH. Protein synthesis was then expressed as nmol of incorporated leucine/g of muscle protein/hour. Leucine oxidation was calculated as the amount of $^{14}CO_2$ trapped in the hanging well containing hydroxide of hyamine. We expressed protein synthesis per *gram of muscle protein* preferably to *gram of wet muscle weight*, as we did with proteolysis and leucine oxidation, because of the non-constant loss of muscle tissue during homogenization.

Other techniques

Tyrosine and concentrations of other amino acids were measured using HPLC techniques (Waters 2695, Fluorescence detector 2475) after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. Protein content was measured according to Lowry *et al*. (1951). The radioactivity was determined with the liquid scintillation radioactivity counter LS 6000 (Beckman Instruments, Fullerton, CA).

Statistical analysis

The results are expressed as the mean ± S.E.M. Statistical analysis was performed using the paired t-test and two-sample t-test. Statistical software NCSS 2001 was used for the analysis.

Results

The addition of $AdaAhx_3L_3VS$ into the incubation medium decreased proteolysis in SOL muscle by 21 % and in EDL muscle by 19 % (Fig. 1). Concentrations of most measured amino acids in the medium were also significantly lowered: branched-chain amino acids (valine, leucine, and isoleucine), histidine, alanine, methionine, lysine, phenylalanine and threonine, as well as total amino acid concentration. Changes in the concentration of asparagine and glutamine were significant only in EDL muscle, serine concentration was lowered only in SOL muscle, and we found no change in the concentrations of glycine and glutamate (Table 1).

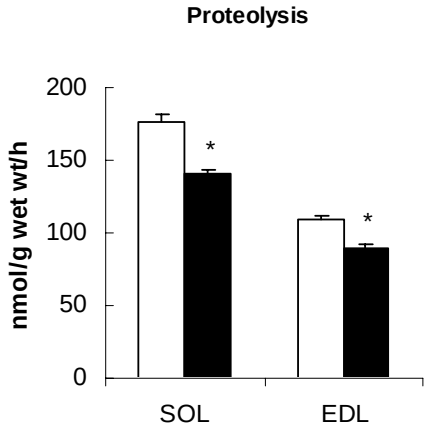

Fig. 1. Proteolysis in incubated skeletal muscles of the rat. Muscles were incubated in the absence (open bars) or presence (filled bars) of 30 μM $AdaAhx_3L_3VS$. Proteolysis was calculated as the amount of tyrosine released into the medium during incubation. N=8; paired t-test *p<0.001 vs. control (vehicle only).

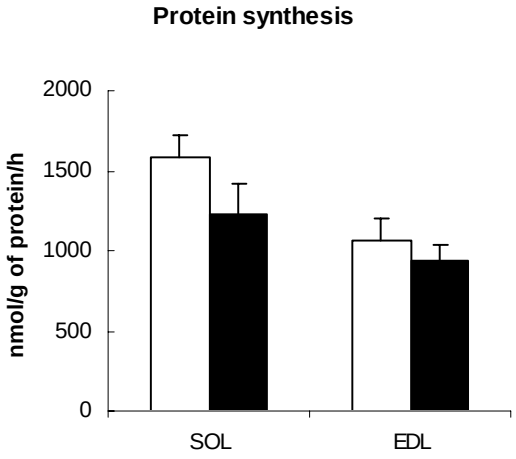

Fig. 2. Protein synthesis in incubated rat skeletal muscles. Muscles were incubated in the absence (open bars) or presence (filled bars) of 30 μM $AdaAhx_3L_3VS$. Protein synthesis was determined according to the amount of L-[1-^{14}C]leucine incorporated into proteins during incubation. N=8; paired t-test *p<0.05 vs. control (vehicle only).

Table 1. Concentrations of amino acids released into the medium during muscle incubation.

Amino acid	**Soleus muscle**		**EDL muscle**	
	Control (n=8)	**AdaAhx$_3$L$_3$VS (n=8)**	**Control (n=8)**	**AdaAhx$_3$L$_3$VS (n=8)**
Glycine	819 ± 31	790 ± 34	568 ± 23	582 ± 28
Serine	335 ± 20	274 ± 19***	172 ± 13	162 ± 16
Asparagine	171 ± 10	150 ± 17	78 ± 7	51 ± 5***
Glutamate	162 ± 9	184 ± 15	98 ± 8	113 ± 15
Glutamine	1977 ± 62	1975 ± 78	1181 ± 52	1100 ± 48*
Histidine	323 ± 11	299 ± 17*	178 ± 10	145 ± 5**
Alanine	842 ± 40	744 ± 37*	584 ± 26	510 ± 30*
Valine	330 ± 12	226 ± 6***	196 ± 6	132 ± 4***
Methionine	125 ± 5	91 ± 2***	72 ± 2	50 ± 2***
Isoleucine	176 ± 8	109 ± 3***	96 ± 4	56 ± 2***
Leucine	451 ± 17	302 ± 7***	264 ± 7	171 ± 5***
Lysine	438 ± 20	402 ± 7*	343 ± 14	318 ± 4*
Phenylalanine	212 ± 8	163 ± 3***	128 ± 2	98 ± 2***
Threonine	405 ± 21	331 ± 16**	332 ± 9	259 ± 15**
Derived values				
Total AA	6960 ± 225	6292 ± 242*	4381 ± 129	3810 ± 100**
BCAA	958 ± 38	637 ± 15***	556 ± 17	359 ± 10***

Values are expressed as nmol/g wet wt/h. BCAA – branched-chain amino acids (LEU, ILE, VAL). Paired t-test *p<0.05, ** p<0.01, *** p<0.001 vs. control (vehicle only).

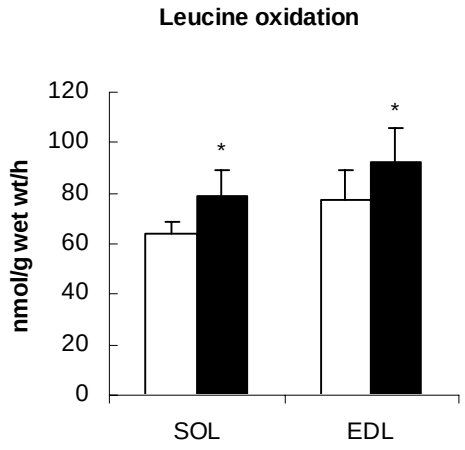

Fig. 3. Leucine oxidation in incubated rat skeletal muscle. Muscles were incubated in the absence (open bars) or presence (filled bars) of 30 μM AdaAhx$_3$L$_3$VS. Leucine oxidation was evaluated according to the amount of $^{14}CO_2$ generated from oxidized leucine. N=8; paired t-test *p<0.05 vs. control (vehicle only).

The protein synthesis was decreased similarly to proteolysis – by 22 % in the soleus muscle and by 12 % in EDL (Fig. 2). Leucine oxidation increased by 24 % in SOL and by 19 % in EDL muscle (Fig. 3).

It should be noted that the parameters of protein metabolism were studied in two types of muscle: in fast twitch (EDL) and slow twitch (SOL) muscles. When comparing the data of control muscles, the proteolysis ($p<0.0001$) and protein synthesis ($p<0.05$) was higher in SOL muscle, while there was no significant difference between leucine oxidation in SOL and EDL muscles. Total amino acid levels released from the SOL muscle were higher then in EDL ($p<0.0001$). These differences reflect the different metabolic activities in the two muscle types. Moreover, the higher proteolysis and amino acid levels in medium can be partly explained by a higher proteasomal activity in SOL than in EDL muscle (Tsujinaka *et al.* 1995).

Discussion

Addition of AdaAhx$_3$L$_3$VS at a concentration of 30 μM into the incubation medium decreased proteolysis in both SOL and EDL incubated muscles. This is in agreement with other studies investigating the effects of

proteasome inhibitors on proteolysis in incubated muscles (Bailey *et al*. 1996, Tawa *et al*. 1997, Hobler *et al*. 1998, Fang *et al*. 1998). Compared to the study of Tawa *et al*. (1997), who observed decreased proteolysis by 50 % in muscles of intact rats incubated with 10 μM peptide aldehyde MG132, $AdaAhx_3L_3VS$ is less potent inhibitor of proteolysis. However, the effect of $AdaAhx_3L_3VS$ seems to be more pronounced than the effect of lactacystin, which reduced proteolysis in rat muscles by 20 % when used at 100 μM concentration (Fang *et al*. 1998).

Significant decrease in protein synthesis observed in both muscle types in the presence of $AdaAhx_3L_3VS$ can be partly explained by a lower supply of amino acids due to the decreased proteolysis. The direct effect on the proteasome, decreased degradation of protein synthesis regulators, may also be involved. These results are in agreement with our previous study using proteasome inhibitor MG 132 (Kadlčíková *et al.* 2004) as well as with those of Hobler *et al*. (1998), who observed decreased protein synthesis in EDL muscles incubated with peptide aldehyde N-acetyl-leucyl-leucyl-norleucinal (LLnL). Moreover, Fang *et al*. (1998) described the same effect of LLnL in EDL, soleus, and diaphragm. However, LLnL had no effect on protein synthesis in the diaphragm even at high concentrations used by Tawa *et al*. (1997), and protein synthesis was influenced neither by MG132 (Bailey *et al*. 1996) nor by β-lactone (Fang *et al*. 1998). The distinct effects of proteasome inhibitors on protein synthesis do not seem to be dependent on muscle type or specificity for the proteasome. Further studies are needed to elucidate the reason of these inconsistent results and the mechanism of action of proteasome inhibitors on protein synthesis.

To provide an overall idea about changes in protein mass by proteasome inhibitors, if there is 203 μmol of tyrosine and 663 μmol of leucine per g of protein (Welle 1999), we can make following calculations:

$$\% \text{ of degraded protein/day} = (X_1 - X_2) \times 24/203 \times 100/300$$

where X_1 is proteolysis in control muscle [nmol of tyrosine/g wet wt/h] and X_2 is proteolysis in experimental muscle [nmol of tyrosine/g wet wt/h].

$$\% \text{ of synthesized protein/day} = (X_1 - X_2) \times 24/663 \times 100/1000$$

where X_1 is protein synthesis in control muscle [nmol of leucine/g of protein/h] and X_2 is protein synthesis in experimental muscle [nmol of leucine/g of protein/h].

According to these formulas, $AdaAhx_3L_3VS$ blocks the degradation of 0.8 % of EDL muscle protein/day and 1.5 % of SOL muscle protein/day. Protein synthesis is decreased during incubation with $AdaAhx_3L_3VS$ by 0.5 % of EDL muscle protein/day and 1.1 % of SOL muscle protein. However, the two methods for determining protein synthesis and protein breakdown are based on different amino acids. When measuring protein synthesis the incorporated ^{14}C-leucine can be released during incubation back into the medium, while the tyrosine released into the medium as a marker of protein breakdown cannot be reincorporated into proteins due to the addition of cycloheximide. Therefore, the values calculated from these formulas are only approximate and it would not be correct to compare these data and to calculate resulting protein balance.

Leucine oxidation was increased in both muscle types in the presence of $AdaAhx_3L_3VS$ despite the fact that the supply of leucine was reduced due to the decreased proteolysis. We therefore suppose that $AdaAhx_3L_3VS$ activates branched-chain alpha-keto acid dehydrogenase (BCKAD), the rate-limiting enzyme in the catabolism of BCAA in skeletal muscle. BCAA are not just simple material for building up proteins of the body, but they are also known to have an important regulatory function in protein metabolism. Increased oxidation of leucine and activated BCKAD indicate wasting of essential BCAA, and are characteristic features of some serious disorders including cancer (Costelli *et al*. 1995), TNF-α and/or endotoxin administration (Holeček *et al*. 1997). To the best of our knowledge, the effect of proteasome inhibitors on leucine oxidation has not been studied yet. When considering the proteasome inhibitors as therapeutic agents, leucine oxidation may be an important parameter to be monitored.

The changes in amino acid concentrations in the medium should be interpreted carefully with respect to the fact that degradation of both structural proteins and enzymes, changes in transport mechanisms, binding to cell structures such as membranes can contribute to the amino acid release from muscle to the medium. The concentrations of many amino acids in the medium containing $AdaAhx_3L_3VS$ were lower than in medium with the solvent only, which can be partly explained by lower proteolysis. The most important were changes in concentrations of BCAA, which were decreased by more than 30 %. It was demonstrated that BCAA are the main donor for synthesis of alanine and glutamine in skeletal muscles (Odessey *et al*. 1974, Haymond and Miles 1982). Alanine and glutamine levels were influenced only slightly and we therefore suppose that the lower concentrations of BCAA in medium containing

$AdaAhx_3L_3VS$ were caused partly by increased oxidation of BCAA, associated with increased synthesis of alanine and glutamine. The decreased lysine concentration may be considered as another marker indicating decreased proteolysis, because it cannot be synthesized from its corresponding α-ketoacid, and its catabolism in muscle is also negligible (the major site for lysine catabolism is liver) (Bender 1985, Hutzler and Dancis 1975). Despite the lower proteolysis we did not observe any change in glycine and glutamate concentrations, probably due to the synthesis of these two amino acids in the muscles. Glycine can be readily synthesized from common metabolic intermediates, and can also be easily converted from serine. Glutamate can be synthesized from α-ketoglutarate in a transaminating reaction that is a key step in the amino acid catabolism.

The method we used in our study – incubation of isolated muscles – enables us to determine direct effects of $AdaAhx_3L_3VS$ that are not modified by regulations and homeostatic mechanisms of the organism. It should be stressed that we used muscles of rats weighing 40-60 g that are permeable enough to assure proper oxygenation of muscle cells and saturation with glucose, amino acids, insulin, and other important components of the incubation medium.

We conclude that $AdaAhx_3L_3VS$ in our study significantly reduced the overall protein turnover in the skeletal muscle. Considering proteasome inhibitors as promising agents for treatment of cancer and other disorders, which are often accompanied by muscle wasting and whole body cachexia, their effect on protein metabolism should also be investigated in more detailed studies. Particularly their effect on the degradation and synthesis of specific molecules such as structural proteins actin and myosin should be elucidated. We suppose that a drug influencing the protein metabolism similarly to $AdaAhx_3L_3VS$ would have no significant effect on protein balance, at least in skeletal muscle, as it decreases both proteolysis and protein synthesis to the same extent.

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References

ARGILES JM, LOPEZ-SORIANO FJ: The oxidation of leucine in tumour-bearing rats. *Biochem J* **268**: 241-244, 1990.

BAILEY JL, WANG X, ENGLAND BK, PRICE SR, DING X, MITCH WE: The acidosis of chronic renal failure activates muscle proteolysis in rats by augmenting transcription of genes encoding proteins of the ATP-dependent ubiquitin-proteasome pathway. *J Clin Invest* **97**: 1447-1453, 1996.

BENDER DA: Amino acids synthesized from aspartate: lysine, methionine (cysteine) and threonine. In: *Amino Acid Metabolism.* John Wiley and Sons Ltd. (ed), Chichester, UK, 1985, pp 145-174.

BOGYO M, MCMASTER JS, GAZCYNSKA M, TORTORELLA D, GOLDBERG AL, PLOEGH H: Covalent modification of the active site Thr of proteasome β-subunits and the *E. coli* homologue HsIV by a new class of inhibitors. *Proc Natl Acad Sci USA* **94**: 6629-6634, 1997.

COSTELLI P, LLOVERA M, GARCIA-MARTINEZ C, CARBO N, LOPEZ-SORIANO FJ, ARGILES JM: Enhanced leucine oxidation in rats bearing an ascites hepatoma (Yoshida AH-130) and its reversal by clenbuterol. *Cancer Lett*. **91**: 73-78, 1995.

DAVIS NB, TABER DA, ANSARI RH, RYAN CW, GEORGE C, VOKES EE, VOGELZANG NJ, STADLER WM: Phase II trial of PS-341 in patients with renal cell cancer: a University of Chicago phase II consortium study. *J Clin Oncol* **22**: 115-119, 2004.

DI NAPOLI M, PAPA F: MLN-519. Millennium/PAION. *Curr Opin Investig Drugs* **4**: 333-341, 2003.

DREXLER HC: Programmed cell death and the proteasome. *Apoptosis* **3**: 1-7, 1998.

FANG CH, WANG JJ, HOBLER S, LI BG, FISCHER JE, HASSELGREN PO: Proteasome blockers inhibit protein breakdown in skeletal muscle after burn injury in rats. *Clin Sci* **95**: 225-33, 1998.

HARRIS RA, JOSHI M, JEOUNG NH: Mechanisms responsible for regulation of branched-chain amino acid catabolism. *Biochem Biophys Res Commun* **313**: 391-396, 2004.

HAYMOND MW, MILES JM: Branched chain amino acids as a major source of alanine nitrogen in man. *Diabetes* **31**: 86-89, 1982.

HERSHKO A, CIECHANOVER A, VARSHAVSKY A: The ubiquitin system. *Nat Med* **6**: 1073-1081, 2000.

HOBLER SC, TIAO G, FISCHER JE, MONACO J, HASSELGREN PO: Sepsis-induced increase in muscle proteolysis is blocked by specific proteasome inhibitors. *Am J Physiol* **274**: R30-R37, 1998.

HOLEČEK M, SKOPEC F, ŠPRONGL L, MRÁZ J, SKALSKÁ H, PECKA M. Effect of alanyl-glutamine on leucine and protein metabolism in irradiated rats. *Amino Acids* **22**: 95-108, 2002.

HOLEČEK M, ŠPRONGL L, SKOPEC F, ANDRYS C, PECKA M: Leucine metabolism in TNF-alpha- and endotoxin-treated rats: contribution of hepatic tissue. *Am J Physiol* **273**: E1052-E1058, 1997.

HUTZLER J, DANCIS J: Lysine-ketoglutarate reductase in human tissues. *Biochim Biophys Acta* **377**: 42-51, 1975.

KADLČÍKOVÁ J, HOLEČEK M, ŠAFRÁNEK R, TILŠER I: Poteasome inhibitor MG 132 has different effect on protein synthesis in healthy and septic rats. *Physiol Res* **53**: 15P, 2004.

KESSLER BM, TORTORELLA D, ALTUN M, KISSELEV AF, FIEBIGER E, HEKKING BG, PLOEGH HL, OVERKLEEFT 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.

LOWRY OH, ROSEBROUGH AL, FARR AL, RANDAL R: Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265-275, 1951.

MACK PC, DAVIES AM, LARA PN, GUMERLOCK PH, GANDARA DR: Integration of the proteasome inhibitor PS-341 (Velcade) into the therapeutic approach to lung cancer. *Lung Cancer* **41**: S89-S96, 2003.

MITCH WE, PRICE SR: Mechanisms activating proteolysis to cause muscle atrophy in catabolic conditions. *J Ren Nutr* **13**: 149-152, 2003.

ODESSEY R, KHAIRALLAH EA, GOLDBERG AL: Origin and possible significance of alanine production by skeletal muscle. *J Biol Chem* **249**: 7623-7629, 1974.

PALMER JT, RASNICK D, KLAUS JL, BROMME D: Vinylsulfones as mechanism-based cysteine protease inhibitors. *J Med Chem* **38**: 3193-3196, 1995.

ŠAFRÁNEK R, HOLEČEK M, KADLČÍKOVÁ J, ŠPRONGL L, MISLANOVÁ C, KUKAN M, CHLÁDEK J: Effect of acute acidosis on protein and amino acid metabolism in rats. *Clin Nutr* **22**: 437-443, 2003.

TAWA NE JR, ODESSEY R, GOLDBERG AL: Inhibitors of the proteasome reduce the accelerated proteolysis in atrophying rat skeletal muscles. *J Clin Invest* **100**: 197-203, 1997.

TIAO G, FAGAN JM, SAMUELS N, JAMES JH, HUDSON K, LIEBERMAN M, FISCHER JE, HASSELGREN PO: Sepsis stimulates nonlysosomal, energy-dependent proteolysis and increases ubiquitin mRNA levels in rat skeletal muscle. *J Clin Invest* **94**: 2255-2264, 1994.

TSUJINAKA T, HOMMA T, EBISUI C, FUJITA J, KIDO Y, YANO M, SHIBATA H, TANAKA T, MORI T: Modulation of muscle protein metabolism in disseminated intravascular coagulation. *Eur Surg Res* **27**: 227-230, 1995.

WELLE S: Whole-body protein turnover. In*: Human Protein Metabolism*. WELLE S (ed), Springer, New York, 1999, pp 30-48.

Reprint requests

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