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Effects of proteasome inhibitors MG132, ZL_3VS and $AdaAhx_3L_3VS$ on protein metabolism in septic rats

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Summary

Proteasome inhibitors are novel therapeutic agents for the treatment of cancer and other severe disorders. One of the possible side effects is influencing the metabolism of proteins. The aim of our study was to evaluate the influence of three proteasome inhibitors MG132, ZL_3VS and $AdaAhx_3L_3VS$ on protein metabolism and leucine oxidation in incubated skeletal muscle of control and septic rats. Total proteolysis was determined according to the rates of tyrosine release into the medium during incubation. The rates of protein synthesis and leucine oxidation were measured in a medium containing L-[1-^{14}C]leucine. Protein synthesis was determined as the amount of L-[1-^{14}C]leucine incorporated into proteins, and leucine oxidation was evaluated according to the release of $^{14}CO_2$ during incubation. Sepsis was induced in rats by means of caecal ligation and puncture. MG132 reduced proteolysis by more than 50% and protein synthesis by 10–20% in the muscles of healthy rats. In septic rats, proteasome inhibitors, except ZL_3VS, decreased proteolysis in both soleus and extensor digitorum longus (EDL) muscles, although none of the inhibitors had any effect on protein synthesis. Leucine oxidation was increased by $AdaAhx_3L_3VS$ in the septic EDL muscle and decreased by MG132 in intact EDL muscle. We conclude that MG132 and $AdaAhx_3L_3VS$ reversed protein catabolism in septic rat muscles.

Keywords
proteasome inhibitors, protein metabolism, sepsis

The ubiquitin–proteasome system (UPS) is a proteolytic system that is localized in cytosol and nucleus and requires ATP and mostly also the cofactor ubiquitin for proteolysis. Proteins to be degraded by UPS are tagged by polyubiquitin chain in a process precisely regulated by a system of ubiquitinating and deubiquitinating enzymes that assure the specificity and control of proteolysis. The proteasome contains protease active sites that account for the cleavage preferences: chymotryptic, tryptic and peptidylglutamyl (Glickman & Ciechanover 2002). The great importance of the UPS is now evident. It is responsible for the breakdown of a large variety of cell proteins, involved in important biological processes, including cell cycle, cell growth, gene expression, DNA repair, stress response and also programmed cell death (Drexler 1998; Hershko *et al*. 2000). The activated breakdown of myofibrillar proteins, i.e. actin and myosin, in many serious disorders,

including cancer, renal failure, acidosis, infections, burns and sepsis, correlates with enhanced activity of the UPS (Tiao *et al.* 1994; Mitch & Price 2003).

Many proteasome inhibitors have been developed (Kisselev & Goldberg 2001). They are divided into several groups according to the chemical structure and proteasome specificity. Most of them (except the fungal metabolite, lactacystine) are based on short oligopeptidic sequences with an electrophilic structure at the C-terminus. This electrophilic structure can be an aldehyde, a boronate (reversible inhibitors), an epoxyketone or a vinylsulfone (irreversible inhibitors) (Lee & Goldberg 1998). Proteasome inhibitors have been introduced as novel therapeutic agents. The first drug of this class is Bortezomib (Velcade®, Millennium Pharmaceuticals, Cambridge, MA, USA), used for the treatment of multiple myeloma. This and other novel proteasome inhibitors are currently undergoing clinical trials as potential treatment for a growing number of other cancer types (Mack *et al.* 2003; Davis *et al.* 2004) and other diseases, such as stroke and inflammatory diseases (Di Napoli & Papa 2003).

The mechanism of action of proteasome inhibitors predicts large-scale effects in the organism. In general, proteasome inhibitors seem to have manageable adverse effects, e.g. thrombocytopenia, fatigue and peripheral neuropathy (Richardson *et al.* 2003). As it is presently believed, the proteasome is responsible for a substantial part of proteolysis in physiological state. Several authors studied the ability of proteasome inhibitors to reduce enhanced protein degradation in various catabolic diseases, including denervation, hyperthyroidism, burn and sepsis (Tawa *et al.* 1997; Fang *et al.* 1998; Hobler *et al.* 1998; Fischer *et al.* 2000), and their findings support the hypothesis that the ubiquitin–proteasome pathway plays a central role in muscle wasting in these catabolic states.

The aim of the present study is to evaluate the direct effects of MG132 and new proteasome inhibitors – ZL_3VS and $AdaAhx_3L_3VS$ – on protein and leucine metabolism in skeletal muscle. In addition to evaluating changes in proteolysis, we studied also changes in protein synthesis and leucine oxidation, as the effects of proteasome inhibitors on these parameters are not clear. The experiments were performed by means of isolated muscles of both intact and septic rats. MG132 belongs to the group of peptide aldehydes. It is a strong inhibitor of the proteasome, but inhibits partly also cathepsins and calpains. ZL_3VS is a vinylsulphone inhibitor and $AdaAhx_3L_3VS$ is a vinylsulphone inhibitor with extended peptide portion, which reveals equal potency to all individual catalytic activities of the proteasome (Kessler *et al.* 2001).

Methods

Materials

MG132, cycloheximide, amino acids, Folin-Ciocalteu phenol reagent and albumin were purchased from Sigma Chemical (St Louis, MO, USA); L-[1-^{14}C]leucine was purchased from Amersham (Buckinghamshire, UK); Aminoplasmal-15 from B. Braun Medicals (Melsungen, Germany); hydroxide of hyamine from Packard Instruments (Meriden, CT, USA). ZL_3VS and $AdaAhx_3L_3VS$ were synthesized by Benedikt M. Kessler and colleagues (Harvard Medical School, Boston, MA, USA). The remaining chemicals were obtained from Lachema (Brno, Czech Republic).

Animals

Male Wistar rats (BioTest, Konarovice, Czech Republic) 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 the guidelines set by the Institutional Animal Use and Care Committee of Charles University, Praha, Czech Republic.

Induction of sepsis

Sepsis was induced in rats by means of caecal ligation and puncture (CLP). Under ether anaesthesia, rats underwent laparotomy and the caecum was ligated below the ileocaecal valve and was punctured twice with a No.18 gauge needle. The abdomen was closed with a running 2–0 silk suture and 10 ml of sterile saline solution per 100 g of body weight was administered subcutaneously on the back for hydration (Pedersen *et al.* 1989). Mortality 18 h after CLP was 27%. Sham-operated control rats underwent laparotomy and manipulation, but no ligation and puncture, of the caecum.

Muscle incubation

Rats were anaesthetized with pentobarbital (6 mg/100 g body weight, intraperitoneally) 18 h after CLP or sham operation, and blood was taken for analysis. 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 they were transferred to 2.5 ml of oxygenated Krebs-Henseleit bicarbonate buffer with 6 mM glucose and 2 mU/ml of insulin (pH 7.4, 37 °C). Muscles were pre-incubated

for 30 min in a thermostatically controlled bath (37 °C) with a shaking device (70 cycles/min). After pre-incubation, muscles were quickly rinsed in 0.9% NaCl and were transferred to a second set of vials containing fresh media.

Bilateral muscles were individually pre-incubated and were incubated in a medium with the addition of 30 μM MG132, ZL_3VS or $AdaAhx_3L_3VS$, or in a medium without proteasome inhibitors, respectively, so that incubations with and without proteasome inhibitors from the same rat could be compared.

Protein metabolism measurement

Total proteolysis was determined according to the rates of tyrosine release into the medium during 2-h incubation, and was expressed as nmol/g wet weight/h. Cycloheximide (0.5 mM) was added into the medium in order 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 pre-incubation 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 into the hanging well in order to capture $^{14}CO_2$ generated from oxidized leucine and the reaction was stopped by means of the addition of 0.2 ml of 35% (v/v) perchloric acid solution. After 1 h of shaking at room temperature, the muscles were frozen in liquid nitrogen, homogenized, protein 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 dissolution of homogenized muscles in 1 M NaOH. Protein synthesis was then expressed as nmol of incorporated leucine/g of muscle protein/h. Leucine oxidation was calculated as nmol of $^{14}CO_2$ trapped in the hanging well/g wet weight/h. We expressed protein synthesis as nmol of incorporated leucine 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.

Experimental design

We have performed three separate studies. In the first, we studied changes in protein metabolism caused by the proteasome inhibitor MG132 in the muscles of healthy rats. The second study was performed in order to determine changes in protein metabolism induced by CLP. In the third experiment, we evaluated the influence of proteasome inhibitors on protein metabolism in the muscles of septic rats.

Other techniques

Tyrosine concentration was measured fluorimetrically as described by Waalkes and Udenfriend (1957). 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, USA).

Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and L-lactate dehydrogenase (LDH) were measured by using commercial tests (Boehringer, Mannheim, Germany). Blood count was evaluated by using a blood particle analyser, Coulter Counter JT3 (Coulter Electronics, Luton, UK). Na^+ and K^+ were determined with the help of ion-selective electrodes on AVL 983-S (Block Scientific, Englewood, NJ, USA).

Statistical analysis

The results have been expressed as the mean ± SEM. Statistical analysis was performed by using paired *t*-test, two-sample *t*-test and ANOVA followed by Bonferroni test. Statistical software NCSS 2001 was used for the analysis.

Results

The first experiment was performed in order to evaluate changes in protein metabolism by MG132 in the muscles of intact rats and it revealed that MG132 inhibited proteolysis in both SOL and EDL by more than 50%, and protein synthesis by 21 and 10%, respectively. Leucine oxidation decreased significantly (by 16%) only in EDL muscle (Table 1).

In the second study, we observed a significant decrease in haematocrit, haemoglobin, thrombocytes, Na^+ and Cl^- levels and dry weight of the spleen, the liver and the kidneys in septic rats. LDH, AST, ALT and K^+ levels and the spleen wet weight were increased (Table 2). Haematologic changes indicate that our model of sepsis is most likely accompanied with disseminated intravascular coagulation; reduction of dry-to-wet weight ratio of the spleen, the liver and the kidneys represents their oedema; decrease in Na^+ and Cl^- levels is caused most probably by haemodilution in generalized oedematous state, and increase in K^+ level by cell integrity impairment. Increased hepatic enzyme levels indicate that CLP alters hepatic function and splenomegaly is the marker of a serious infection.

Table 3 summarizes that CLP resulted in negative protein balance in both SOL and EDL muscles – we observed an increase in proteolysis and a decrease in protein synthesis. In addition, an increase in leucine oxidation was observed.

The third experiment was designed in order to evaluate the influence of MG132, ZL_3VS and $AdaAhx_3L_3VS$ on protein

Table 1 Effect of MG132 on protein and amino acid metabolism

	Muscle	Control	MG132
Proteolysis (nmol tyrosine/g wet weight/h)	SOL ($n = 8$)	82 ± 4	39 ± 2*
	EDL ($n = 8$)	75 ± 2	31 ± 1*
Protein synthesis (nmol leucine/g of protein/h)	SOL ($n = 9$)	1393 ± 67	1095 ± 124**
	EDL ($n = 10$)	731 ± 49	658 ± 55**
Leucine oxidation (nmol leucine/g wet weight/h)	SOL ($n = 10$)	74.3 ± 6.8	67.7 ± 6.4
	EDL ($n = 10$)	68.3 ± 4.5	57.4 ± 6.2***

Mean ± SEM. Paired *t*-test *$P < 0.001$, **$P < 0.01$, ***$P < 0.05$. EDL, extensor digitorum longus; SOL, soleus.

Table 2 Parameters of control (sham-operated) and septic rats

	Sham ($n \geq 8$)	CLP ($n \geq 11$)		Sham ($n \geq 8$)	CLP ($n \geq 11$)
Thrombocytes (*10^9/l)	1052.5 ± 54.7	467 ± 53.7*	Alanine aminotransferase (μkat/l)	0.4 ± 0.01	1.3 ± 0.1*
Haematocrit	0.34 ± 0.01	0.29 ± 0.01**	Aspartate aminotransferase (μkat/l)	1.9 ± 0.1	3.9 ± 0.4*
Haemoglobin (g/l)	106.8 ± 2.5	89.1 ± 5.2**	Spleen weight (mg/g of body weight)	3.3 ± 0.2	4.1 ± 0.3**
Na^+ (mmol/l)	134.5 ± 1.4	128.1 ± 0.9*	Dry-to-wet weight ratio of the spleen	0.228 ± 0.003	0.206 ± 0.006**
K^+ (mmol/l)	3.4 ± 0.1	3.8 ± 0.1**	Dry-to-wet weight ratio of the liver	0.268 ± 0.005	0.246 ± 0.002**
Cl^- (mmol/l)	98.1 ± 1.4	95 ± 1.0**	Dry-to-wet weight ratio of the left kidney	0.224 ± 0.002	0.213 ± 0.003**
Lactate dehydrogenase (μkat/l)	2.6 ± 0.5	8.7 ± 1.6**	Dry-to-wet weight ratio of the right kidney	0.224 ± 0.002	0.212 ± 0.004**

Mean ± SEM. Two-sample *t*-test *$P < 0.001$ *vs.* control (sham-operated), **$P < 0.05$ *vs.* control (sham-operated). CLP, caecal ligation and puncture.

Table 3 Protein and amino acid metabolism – changes in muscles of rats made septic by means of caecal ligation and puncture (CLP)

	Muscle	Sham	CLP
Proteolysis (nmol tyrosine/g wet weight/h)	SOL ($n \geq 9$)	78.8 ± 2.3	98.2 ± 5.8*
	EDL ($n \geq 10$)	73.8 ± 1.7	93.7 ± 5.7*
Protein synthesis (nmol leucine/g of protein/h)	SOL ($n = 10$)	1350 ± 74	1142 ± 72*
	EDL ($n = 11$)	699 ± 55	529 ± 53*
Leucine oxidation (nmol leucine/g wet weight/h)	SOL ($n = 11$)	71 ± 7	127 ± 10**
	EDL ($n = 11$)	68 ± 4	132 ± 9**

Mean ± SEM. Two-sample *t*-test *$P < 0.05$ *vs.* control (sham-operated), **$P < 0.001$ *vs.* control (sham-operated). EDL, extensor digitorum longus; SOL, soleus.

Table 4 Effects of proteasome inhibitors on the rates of proteolysis in the muscles of rats made septic by means of caecal ligation and puncture (CLP)

Muscle	CLP ($n = 8$)	CLP + MG132 ($n = 8$)	CLP ($n = 9$)	CLP + ZL_3VS ($n = 9$)	CLP ($n = 8$)	CLP + $AdaAhx_3L_3VS$ ($n = 8$)
SOL	83 ± 13	36 ± 2*	81 ± 4	75 ± 4	68 ± 5	54 ± 3*
EDL	76 ± 8	30 ± 2*	80 ± 5	66 ± 4*	70 ± 3	47 ± 2*

Mean ± SEM in nmol tyrosine/g wet weight/h. Paired *t*-test *$P < 0.05$ *vs.* septic control. We used ANOVA followed by Bonferroni test to see whether the means of MG132, ZL_3VS and $AdaAhx_3L_3VS$ are equal. Because the *P*-value is less than 0.05, we reject that hypothesis.

Table 5 Effects of proteasome inhibitors on the rates of protein synthesis in the muscles of rats made septic by means of caecal ligation and puncture (CLP)

Muscle	CLP ($n = 8$)	CLP + MG132 ($n = 8$)	CLP ($n = 7$)	CLP + ZL_3VS ($n = 7$)	CLP ($n = 8$)	CLP + $AdaAhx_3L_3VS$ ($n = 8$)
SOL	1145 ± 105	1362 ± 150	927 ± 119	927 ± 38	1327 ± 121	1414 ± 87
EDL	525 ± 51	614 ± 75	517 ± 116	360 ± 51	438 ± 31	482 ± 50

Mean ± SEM in nmol leucine/g of muscle protein/h. Paired *t*-test. EDL, extensor digitorum longus; SOL, soleus.

Table 6 Effects of proteasome inhibitors on leucine oxidation in the muscles of rats made septic by means of caecal ligation and puncture (CLP)

Muscle	CLP ($n = 8$)	CLP + MG132 ($n = 8$)	CLP ($n = 8$)	CLP + ZL_3VS ($n = 8$)	CLP ($n = 8$)	CLP + $AdaAhx_3L_3VS$ ($n = 8$)
SOL	136 ± 22	118 ± 19	101 ± 11	106 ± 13	143 ± 12	156 ± 12
EDL	134 ± 17	114 ± 19	129 ± 20	113 ± 15	134 ± 12	157 ± 17*

Mean ± SEM in nmol leucine/g wet weight/h. Paired *t*-test $^*P < 0.05$ *vs.* septic control. EDL, extensor digitorum longus; SOL, soleus.

metabolism in the muscles of septic rats. MG132 and $AdaAhx_3L_3VS$ decreased proteolysis (Table 4) and had no effect on protein synthesis (Table 5) in both SOL and EDL muscles. Moreover, ZL_3VS had no effect on protein synthesis, and had a significant effect on proteolysis only in EDL muscle. $AdaAhx_3L_3VS$ increased leucine oxidation in EDL muscle by 14%, whereas other proteasome inhibitors had no significant effect on leucine oxidation in the septic muscle (Table 6).

Discussion

When MG132 was added into the incubation medium and its effect on muscles of intact rats was investigated, proteolysis and protein synthesis decreased in both SOL and EDL muscles and leucine oxidation decreased significantly only in EDL muscle. The decrease of proteolysis is in agreement with the study of Tawa *et al.* (1997), and supports the concept that a substantial part of proteolysis in skeletal muscles of healthy rats is mediated by the UPS. The decrease in protein synthesis and leucine oxidation may be caused partially by lower supply of amino acids resulting from decreased proteolysis, and/or by other possible effects of MG132 – decreased degradation of protein synthesis regulators through the proteasome and lower consumption of ATP by the proteasome, inactivating branched-chain alpha-keto acid dehydrogenase (BCKAD – the rate-limiting enzyme in the catabolism of branched-chain amino acids valine, leucine and isoleucine) (Lombardo *et al.* 1998). Decreased protein synthesis was also observed (Fang *et al.* 1998; Hobler *et al.* 1998) in EDL muscles incubated in the presence of N-acetyl-L-leucinyl-L-leucinal-L-norleucinal (LLnL). However, neither MG132 nor LLnL induced any changes in protein synthesis in incubated rat diaphragm muscle (Bailey *et al.* 1996) and in epitrochlearis muscle (Tawa *et al.* 1997), respectively. Therefore, it may be suggested that the effect of proteasome inhibitors may differ in different muscle types.

Sepsis induced by CLP caused negative protein balance and increased leucine oxidation in both muscle types. These findings are in agreement with studies describing an increase in proteolysis (Hasselgren *et al.* 1989; Chai *et al.* 2003), a decrease in protein synthesis (Zamir *et al.* 1992) and increased leucine oxidation (Holeček 1996) in the muscles of septic rats. The increased leucine oxidation is caused probably by the BCKAD activation. Its activation was observed in skeletal muscle after the administration of endotoxin, tumour necrosis factor or interleukin-1 to rats in several studies (Nawabi *et al.* 1990; Holeček *et al.* 1997).

The addition of proteasome inhibitors to incubated muscles of septic rats showed their considerable effect on protein balance. MG132 decreased proteolysis by more than 50%, $AdaAhx_3L_3VS$ by around 20%, and the effect of ZL_3VS was significant only in EDL muscle. In the conditions of the present experiment, MG132 was a much stronger inhibitor of proteolysis than $AdaAhx_3L_3VS$. Regarding the two vinylsulphone inhibitors tested, $AdaAhx_3L_3VS$ had a stronger effect on proteolysis. The structure of $AdaAhx_3L_3VS$ is extended with aminohexanoic spacers, what was designed to resemble the natural substrates of the proteasome (unfolded polypeptides), but modified, in order not to be cleaved by the proteasome. This structure has also been shown to be more potent than its shorter counterpart ZL_3VS in cell culture (Kessler *et al.* 2001).

We found no change in protein synthesis in septic muscle incubated with any of the three proteasome inhibitors tested, whereas in intact muscle, MG132 decreased the protein synthe-

sis. Such discrepancies suggest that the changes induced by sepsis could modulate the effect of MG132 on protein synthesis observed in healthy rats so that it does not provoke further decrease in protein synthesis observed in sepsis. Considering the study of Hobler *et al.* (1998), who showed that LLnL decreased protein synthesis in incubated EDL muscles of septic rats, we suggest that the effect on protein synthesis may differ by using various inhibitors of the proteasome.

Leucine oxidation that was increased in the septic muscle was further increased after the addition of $AdaAhx_3L_3VS$ into the medium. Considering the reduced supply of leucine because of the decreased proteolysis, the increase in leucine oxidation should be caused by increased activity of BCKAD.

It should be noted that the parameters of protein metabolism were studied in two types of muscle – fast twitch (EDL) and slow twitch (SOL) muscles – and we observed some distinct effects (Tables 4 and 6). The possible cause can be a higher proteasomal activity in SOL than in EDL muscle that was observed both in control rats and in rats with disseminated intravascular coagulation (Tsujinaka *et al.* 1995). A similar difference in the regulation of proteolysis and protein synthesis in fast and slow muscle fibres was also observed (Tiao *et al.* 1997; Šafránek *et al.* 2003).

It should also be stated that MG132 has also been shown to target cysteine and serine proteases and may, therefore, influence other proteolytic systems in the cells (Lee & Goldberg 1998). The inhibition of calpains and cathepsins, however, requires 10-fold higher concentrations of MG132 than the inhibition of the proteasome (Kisselev & Goldberg 2001). Tawa *et al.* (1997) tested the effect of MG132 in incubated muscle of intact rats with results similar to that in the first part of our work. In their study, conditions were chosen to block the lysosomal and Ca^{2+}-dependent processes, and evidence that MG132 was functioning by inhibiting the UPS was given. We, therefore, suppose that in our current model, the effect of MG132 is mediated mainly by influencing the proteasome.

In conclusion, proteasome inhibitors used in our study showed beneficial effect on proteocatabolism induced by sepsis, as they decreased the protein breakdown and did not change the protein synthesis. This could prove to be useful in potential clinical use of proteasome inhibitors – namely in cancer diseases – that are very often accompanied with muscle wasting and cachexia. Unfortunately, the compounds used in our study do not have any potential to be used in humans, as MG132 is not specific for the proteasome and ZL_3VS and $AdaAhx_3L_3VS$ act irreversibly. Further studies are needed in order to establish whether the proteasome inhibitors with a clinical potential do have similar beneficial effect on negative protein balance. We also suggest that as $AdaAhx_3L_3VS$ increased leucine oxidation in septic muscle the effects of these agents on leucine oxidation should be monitored.

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