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Proteasome inhibitor MG-132 enhances whole-body protein turnover in rat

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Abstract

Proteasome inhibitors are novel therapeutic agents which may be used in treatment of cancer and other severe disorders. We studied the effect of proteasome inhibitor MG-132 on protein and amino acid metabolism. In MG-132-treated rats we observed a significant decrease in proteasome-dependent proteolysis in skeletal muscle and an increase in whole-body protein turnover (i.e., increase in whole-body proteolysis and protein synthesis). Proteasome-dependent proteolysis was activated in the liver and kidney, protein synthesis increased in skeletal muscle, liver, and kidney. Insignificant changes were found in jejunum and colon. MG-132 administration induced a significant increase in concentration of several amino acids in blood plasma and their decrease in jejunum and colon. We conclude that administration of MG-132 affects both protein anabolic and protein catabolic pathways via the direct effect on proteasome-dependent proteolysis and indirect effect on proteolysis and protein synthesis via unidentified mediators.

Keywords: Proteasome; Inhibitor; Protein metabolism; Ubiquitin; Nutrition; Amino acids

Ubiquitin-proteasome pathway is a well-recognized system localized in cytosol and nucleus responsible for degradation of short-lived and abnormal proteins, e.g., proteins related to signal transduction, regulation of the mitotic cycle, antigen presentation, and apoptosis [1]. The involvement of ubiquitin-proteasome system in these events was a powerful stimulus for development of a large spectrum of proteasome inhibitors with different structure and proteasome specificity and their introduction as novel therapeutic agents, particularly for treatment of certain types of cancer, and inflammatory disorders [2]. The first drug of this class is bortezomib, which has been approved for treatment of multiple myeloma [3].

It was demonstrated that ubiquitin-proteasome system also plays an important role in mediating the degradation of long-lived myofibrillar proteins in skeletal muscle and that activation of the proteasome system is the main cause of the accelerated breakdown of skeletal protein in sepsis [4–7]. Consequently, it was hypothesized that targeting specific molecular mechanisms of muscle proteolysis by proteasome inhibitors may inhibit development of muscle wasting in proteocatabolic illness. The hypothesis was confirmed by observation of inhibitory effect of proteasome inhibitors on proteolysis in experiments using skeletal muscles isolated from rats in catabolic states including sepsis, burn injury, cancer, uremia, denervation, and hyperthyroidism [8–12]. In all these in vitro studies, the incubated muscles were treated by proteasome inhibitor. We found only one study in which the inhibitory effect of proteasome inhibitor on muscle proteolysis was demonstrated after in vivo administration [13].

Studies evaluating the effect of in vivo administration of proteasome inhibitors are necessary because of their potential use in clinical practice, where their effect on protein metabolism should be considered as an important side effect which may be utilized in treatment of muscle wasting

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disorders. However, there is no information about the effect of proteasome inhibitors on whole-body protein turnover, particularly on changes in protein breakdown and protein synthesis in specific tissues which could significantly affect protein balance and the outcome of the illness.

The aim of the present study was to evaluate the effect of proteasome inhibitor MG-132 on parameters of protein metabolism of healthy laboratory rat under in vivo conditions. MG-132 belongs to the group of peptide aldehydes, it is a strong inhibitor of proteasome and also partly inhibits calpains, caspases and cathepsins [2]. Calpains and caspases are believed to play a role in disassembly of sarcomeric proteins, an initial step in degradation of myofibrillar proteins of skeletal muscle [14,15]. Cathepsins do not contribute significantly to overall protein breakdown in skeletal muscle [4].

Materials and methods

Animals and materials. Male Wistar rats (BioTest, Konarovice, CR) weighing about 200 g were housed in standardized cages in quarters with controlled temperature and a 12-h light–dark cycle and received Velaz-Altromin 1320 laboratory chow and drinking water ad libitum. All procedures involving animals were performed according to guidelines set by the Institutional Animal Use and Care Committee of Charles University.

L-[1-^{14}C]leucine was purchased from Amersham (Buckinghamshire, UK), [^{14}C]bicarbonate was from Du Pont-NEN (Bad Homburg, Germany), carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132) from Biomol (Plymouth Meeting, PA) and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate from Waters (Milford, MA). Leucine, Folin-Ciocalteu phenol reagent, dimethyl sulfoxide, and albumin were purchased from Sigma Chemical (St. Louis, MO). The remaining chemicals were obtained from Lachema (Brno, CZ).

Experimental design. Two separate studies were performed. In the first study, rats starved 24 h and then were injected intraperitoneally with MG-132 dissolved in dimethylsulfoxide at a dose of 10 mg/kg b.w. The dose of MG-132 was determined on the basis of our in vitro studies [11] and experiments evaluating the effect of different doses of various proteasome inhibitors on aminoacidemia (unpublished). Control animals received a corresponding volume of the solvent. Five hours later, the animals were sacrificed in ether narcosis by exsanguination via the abdominal aorta. Afterwards the gastrocnemius muscle, liver, kidney, and samples of jejunum and colon were quickly removed and frozen in liquid nitrogen for measurement of chymotrypsin-like activity. Concentrations of glucose, lipids, ALT, AST, urea, and zinc were estimated in blood plasma.

The experiments of the second study were performed separately. A polyethylene cannula was inserted into the jugular vein and the rat fasted for 24 h. The next day, the animal was injected intraperitoneally with MG-132 or solvent and placed in a glass metabolic cage. Two hours later, unrestrained and conscious rat was infused with L-[1-^{14}C]leucine (1.9 μCi/ml). A priming dose of 0.7 ml infused within 4 min 35 s was followed by a constant infusion at a rate of 0.40 ml/h for 210 min. The dose of labelled leucine and the rate of infusion were estimated on the basis of our previous studies [16]. The rat was killed by exsanguination via the abdominal aorta during 215th min from the beginning of the infusion. Afterwards the liver, gastrocnemius muscle, kidney, and samples of jejunum and colon were quickly removed and immediately frozen in liquid nitrogen for measurement of protein synthesis.

The parameters of whole body leucine metabolism were evaluated at steady-state conditions by the procedure described in detail previously [16]. The expired CO_2 was trapped at 10-min intervals between the 125th and 185th min of infusion by monoethanolamine. The average value of six measurements of $^{14}CO_2$ radioactivity in expired air at steady-state condition was used for calculations of the leucine oxidation rate. The $^{14}CO_2$ recovery factor (FR) estimated by infusion of [^{14}C]bicarbonate was about 90% both for MG-132 and solvent treated animals. Leucine specific activity (SA_{Leu}), turnover (Q_{Leu}), clearance (C_{Leu}), and decarboxylation (D_{Leu}) rates were calculated using the following formulae:

$$SA_{Leu}\ (\text{dpm}/\mu\text{mol}) = \frac{\text{Leu radioactivity (dpm/ml)}}{\text{Leu concentration (}\mu\text{mol/ml)}}$$

$$Q_{Leu}\ (\mu\text{mol/h}) = \frac{\text{infusion rate (dpm/h)}}{SA_{Leu}\text{ in plasma (dpm/}\mu\text{mol)}}$$

$$C_{Leu}\ (\text{ml/h}) = \frac{Q_{Leu}\ (\mu\text{mol/h})}{\text{plasma Leu (}\mu\text{mol/ml)}}$$

$$D_{Leu}\ (\mu\text{mol/h}) = \frac{^{14}CO_2\text{ production rate (dpm/h)}}{SA_{Leu}\text{ in plasma (dpm/}\mu\text{mol)}\cdot FR}$$

Whole body leucine metabolism was considered to take place within a common metabolic pool represented by free plasma leucine. Due to the fact that exogenous leucine intake (E) was zero in our protocol, Q_{Leu} estimates the leucine released from protein, i.e., the protein breakdown (B) as described by the equation: $Q = In + D = B + E$. By using this formula, rates of protein incorporation into protein (In), the oxidized fraction of leucine ($OF = D \times 100/Q$) and protein balance ($PB = In - B = E - D$) were calculated. Therefore, the absolute values of leucine oxidation and protein balance are of the same value in our experimental conditions.

Measurement of protein synthesis. For the assessment of leucine incorporation into protein, small pieces of tissue (about 0.5 g) were rinsed and homogenized in 2% (v/v) perchloric acid. The precipitated proteins were collected by centrifugation. The supernatant was used for measurement of L-[1-^{14}C]leucine radioactivity and leucine concentration. To avoid contamination by ketoisocaproate, samples were treated with 30% hydrogen peroxide, which causes the carboxyl carbon of ketoisocaproate to be released as CO_2. The pellet was washed three times and then hydrolyzed in 2 N NaOH. Aliquots were taken for protein content [17] and radioactivity measurement. The fractional protein synthesis rates were calculated using the equation derived by Garlick et al. [18]:

$$\frac{Sb}{Si} = \frac{\lambda_i}{(\lambda_i - K_s)} \times \frac{(1 - e^{-K_s t})}{1 - e^{-\lambda i t}} - \frac{K_s}{(\lambda_i - K_s)}$$

where Sb and Si are the specific activities of the protein-bound and free acid-soluble tissue leucine pools, respectively, in disintegrations per minute per micromole; λ_i is the rate constant for the rate of rise of specific activity of leucine in the acid-soluble amino acid pool per day; t is the duration of L-[1-^{14}C]leucine infusion in days, and K_s is the fraction of protein mass renewed each day, in percent per day.

Measurement of chymotrypsin-like activity. The chymotrypsin-like activity of the proteasome was determined using the fluorogenic substrate Suc LLVY-MCA (0.1 mM) according to the method of Gomes-Marcondes et al. [19]. Muscles were homogenized in medium containing 20 mM Tris–HCl (pH 7.5), 2 mM ATP, 5 mM $MgCl_2$, and 1 mM dithiothreitol. The homogenate was then centrifuged for 10 min at 18,000*g* at 4 °C. Cellular supernatant (0.1 ml) was incubated with substrate (0.05 mM) in a total volume of 0.2 ml of 100 mM Tris–HCl (pH 8.0), with or without inhibitor, MG-132 (0.02 mM), for 1 h on ice. The reaction was terminated by addition of 80 mM sodium acetate, pH 4.3 (1 ml). The fluorescence was determined using an excitation wavelength of 340 nm and an emission wavelength of 440 nm (Perkin Elmer luminescence spectrometer LS 50 B). Standard curve was established for 7-amino-4-methylcoumarin (AMC), which permitted the expression of CHTLA activity as nmol AMC/g protein/h. The activity was adjusted for protein concentration of the sample. Only the MG-132-inhibited activity was used for the calculations.

Other techniques. Amino acid concentrations in deproteinized samples of blood plasma or tissues were determined with high-performance liquid chromatography (Waters, Milford, MA) after precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate. Plasma levels of ALT, AST, glucose, triglycerides, and cholesterol were measured using commercial tests (Boehringer, Mannheim, Germany; Elitech, Sées, France, and Lachema, Brno, CZ). Zn^{2+} was determined using ion-selective

electrode on AVL 983-S (Block Scientific, Englewood, NJ). The radioactivity of the samples was measured with the liquid scintillation radioactivity counter LS 6000 (Beckman Instruments, Fullerton, CA).

Statistical analysis. Results are expressed as means ± SE. Statistical analysis was performed using F test and unpaired Student's t test. A difference was considered significant at $P < 0.05$. Statistical software NCSS 2001 (NCSS, Kaysville, Utah) was used for the analysis.

Results

MG-132 treatment induced a significant increase in glycemia and a marked decrease in zinc concentration in blood plasma. There were no differences in plasma concentration of lipids, ALT, AST, and urea (Table 1). A significant decrease in chymotrypsin-like activity (indicating the rate of proteasome-dependent proteolysis in our study) was found in skeletal muscle while an increase was observed in the liver and kidney. The changes in jejunum and colon were insignificant (Fig. 1).

There was a significant increase in whole-body protein synthesis and proteolysis in MG-132-treated animals. However, the effect on protein balance, leucine oxidation, and leucine oxidized fraction was insignificant (Table 2). A significant increase in protein synthesis was observed in skeletal muscle, liver, and kidney (Fig. 2).

Table 3 demonstrates that MG-132 treatment induced a marked increase in amino acid concentrations in blood plasma while changes in skeletal muscle were insignificant. Moderate effect of MG-132 was found in liver (an increase in citrulline) and kidney (a decrease in proline). Significant changes resulting in decrease in total amino acid concentration after MG-132 treatment were found in jejunum and colon. In both tissues decreased asparagine, glycine, citrulline, alanine, tyrosine, valine, methionine, and isoleucine.

Discussion

MG-132 is a strong inhibitor of proteasome and its effect should be expected particularly in tissues where there are few lysosomes and proteasome-dependent proteolysis is the major proteolytic pathway. Such a tissue is skeletal muscle [20] which is the most abundant tissue in the body, accounting approximately 50% of the total body mass. For those reasons, and because the decrease in protein breakdown in skeletal muscle after treatment by proteasome inhibitors has been demonstrated in a number of studies (see introduction), a significant decrease in whole-body proteolysis has been expected after MG-132 treatment.

Table 1
Parameters of control and experimental animals (Study 1)

	Control ($n = 9$)	MG-132 ($n = 9$)
Body weight (g)	212 ± 2	212 ± 2
Blood plasma		
Glucose (mmol/l)	4.28 ± 0.22	6.40 ± 0.45*
Triglycerides (mmol/l)	0.45 ± 0.06	0.31 ± 0.07
Cholesterol (mmol/l)	1.79 ± 0.07	1.68 ± 0.09
HDL cholesterol (mmol/l)	1.28 ± 0.05	1.21 ± 0.06
LDL cholesterol (mmol/l)	0.30 ± 0.03	0.33 ± 1.52
Urea (mmol/l)	4.07 ± 0.27	4.12 ± 0.21
ALT (μmol/l)	0.87 ± 0.08	0.83 ± 0.06
AST (μmol/l)	2.30 ± 0.14	2.11 ± 0.14
Zinc (μmol/l)	31.99 ± 1.97	20.86 ± 0.72*

Values are means ± SE. F test and unpaired Student's t test.
ALT, alanine aminotransferase; AST, aspartate aminotransferase.
* $P < 0.05$.

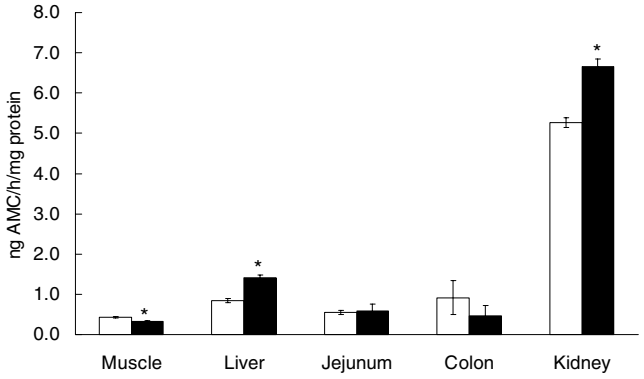

Fig. 1. Chymotrypsin-like activity in proteasome in specific tissues in control (open bars) and MG-132-treated rats (filled bars). Values are means ± SE with $n = 9$ in each group. F test and unpaired Student's t test, *$P < 0.05$ (Study 1).

Table 2
Effect of MG-132 on whole-body protein and leucine metabolism (Study 2)

	Control ($n = 8$)	MG-132 ($n = 8$)
Body weight (g)	204 ± 3	212 ± 3
Proteolysis (μmol Leu kg^{-1} h^{-1})	171 ± 12	232 ± 24*
Protein synthesis (μmol Leu kg^{-1} h^{-1})	147 ± 11	199 ± 20*
Protein balance (μmol Leu kg^{-1} h^{-1})	−24.1 ± 2.8	−32.1 ± 3.9
Leucine oxidation (μmol Leu kg^{-1} h^{-1})	24.1 ± 2.8	32.1 ± 3.9
Leucine oxidized fraction (%)	14.1 ± 1.3	13.9 ± 0.8
Leucine clearance (ml kg^{-1} h^{-1})	1198 ± 65	1338 ± 89

Values are means ± SE. F test and unpaired Student's t test.
* $P < 0.05$.

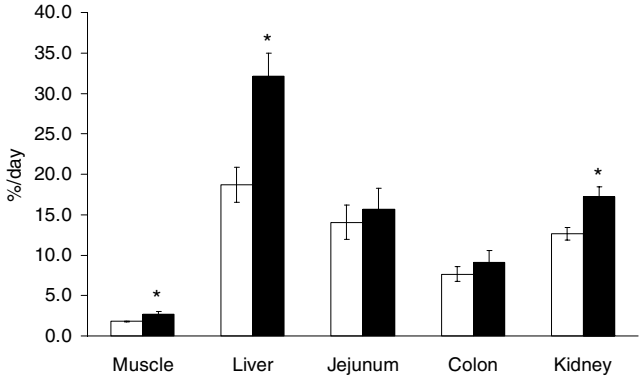

Fig. 2. Fractional rate of protein synthesis in specific tissues in control (open bars) and MG-132-treated rats (filled bars). Values are means ± SE with $n = 8$ in each group. F test and unpaired Student's t test, *$P < 0.05$ (Study 2).

Table 3
Effect of MG-132 on amino acid concentrations in blood plasma and muscle (Study 2)

	Blood plasma (μmol/l)		Skeletal muscle (nmol/g wet tissue)	
	Control ($n = 8$)	MG-132 ($n = 8$)	Control ($n = 8$)	MG-132 ($n = 8$)
Aspartate	5 ± 1	8 ± 1*	1132 ± 144	1297 ± 194
Glutamate	217 ± 20	255 ± 17	4485 ± 611	3755 ± 568
Serine	166 ± 11	218 ± 18*	1124 ± 109	1106 ± 145
Asparagine	41 ± 4	55 ± 3*	366 ± 40	326 ± 39
Glycine	237 ± 27	290 ± 21	1,595 ± 178	1,342 ± 179
Glutamine	336 ± 33	449 ± 28*	4,577 ± 451	4,389 ± 586
Histidine	45 ± 5	61 ± 4*	307 ± 54	300 ± 49
Arginine	51 ± 6	75 ± 5*	—[a]	—[a]
Citrulline	24 ± 3	36 ± 3*	184 ± 23	200 ± 28
Threonine	79 ± 9	105 ± 6*	306 ± 35	287 ± 39
Alanine	203 ± 18	300 ± 24*	958 ± 83	1134 ± 157
Proline	86 ± 7	114 ± 10*	697 ± 82	679 ± 85
Tyrosine	41 ± 5	45 ± 4	77 ± 7	64 ± 7
Valine	168 ± 12	202 ± 18	181 ± 16	176 ± 20
Methionine	33 ± 3	45 ± 3*	27 ± 2	22 ± 4
Isoleucine	84 ± 6	106 ± 9	93 ± 8	84 ± 8
Leucine	144 ± 10	175 ± 16	149 ± 14	143 ± 17
Lysine	285 ± 23	368 ± 26*	527 ± 59	476 ± 45
Phenylalanine	45 ± 4	65 ± 5*	72 ± 8	75 ± 10
Total AA	2291 ± 184	2972 ± 163*	16,856 ± 1,570	15,855 ± 1,966

Means ± SE. F test and unpaired Student's t test.
[a] Arginine was not estimated because of interference with high levels of carnosine.
* $P < 0.05$.

However, despite proteasome-dependent proteolysis in skeletal muscle decreased, an increase in whole-body protein turnover was observed in MG-132-injected animals. Proteolysis increased in liver and kidney, protein synthesis increased in liver, kidney, and muscle.

The discrepancy between our expectations and findings could be explained by effect of proteasome inhibitors on expression, activation or inhibition of various humoral factors, mediators, transcription factors, etc. which could affect protein synthesis and proteolysis. Here are examples:

- proteasome inhibition by MG-132 effectively blocks glucocorticoid receptor down-regulation [21];
- cultured endothelial cells treated with MG-132 increased expression of interleukine Il-6, a cytokine that plays an important role in inflammatory injury [22];
- proteasome inhibition in hepatocytes induces production of massive quantities of the proinflammatory interleukine Il-8, possibly resulting in neutrophil infiltration and liver injury [23];
- proteasome inhibition leads to activation of heat shock factors [24];
- in certain cells, proteasome inhibitors may induce activation of NF-κB through increase in inhibitory κB kinase activation [25].

Some of these changes (i.e., production of proinflammatory cytokines, activation of NF-κB, etc.) are considered as the typical features of systemic inflammatory response in proteocatabolic illness, e.g., sepsis and burn injury. The argument supporting the speculation may be an observation of decreased concentration of zinc, which is a typical finding associated with increased production of proinflammatory cytokines [26].

It should be noted that the response of the body to administration of proteasome inhibitor may differ in healthy state and in stress illness. In a recent study, Cuschieri et al. [27] demonstrated that proteasome inhibition by MG-132 affects lipopolysaccharide-induced signaling in macrophages and results in a conversion to an anti-inflammatory phenotype. The authors observed decreased degradation of IκB resulting in abolished NF-κB activation, and increased Il-10 and decreased Il-8 and TNF-α production.

Although moderate or insignificant changes in amino acid concentrations were observed in skeletal muscle, liver, and kidney, a marked effect of MG-132 was found in blood plasma, jejunum, and colon. The increase in plasma amino acid concentrations may be related to higher increase in whole-body proteolysis in comparison with increase in whole-body protein synthesis or impaired transport of amino acids across the cell membrane. These suggestions are in agreement with higher negativity of protein balance ($P = 0.1185$) in MG-132-treated animals and with decreased concentration of several amino acids in jejunum and colon.

It seems practically important that after MG 132 administration, proteolysis is inhibited and protein synthesis is activated in skeletal muscle, which may result in a marked increase in muscle protein content. As in incubated skeletal muscle an inhibition of protein synthesis was observed when MG-132 was added into incubation medium [11], we assume that the activation of protein synthesis is caused rather by action of some humoral factors than by

the direct influence of MG-132 on protein synthesis. The observation of inhibitory effect of MG-132 on protein synthesis under in vitro conditions in our recent study and increased protein synthesis in skeletal muscle under in vivo conditions in the present one indicate that the observations in vitro may not necessarily reflect the in vivo situation and caution must be exercised in interpreting data obtained from in vitro experiments.

In conclusion, the main effect of MG-132 administration on protein metabolism seems to be the inhibition of proteasome-dependent proteolysis in skeletal muscle and the increase in protein turnover associated with a significant increase in proteasome-dependent proteolysis in the liver and kidney, and protein synthesis in the liver, kidney, and skeletal muscle. These changes are caused by the direct influence of proteasome inhibitor on proteasome-dependent proteolysis and by influence of various factors, activity of which was modified by MG-132 treatment.

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