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The effect of new proteasome inhibitors, belactosin A and C, on protein metabolism in isolated rat skeletal muscle

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The proteasome inhibitors are used as research tools to study of the ATP-dependent ubiquitin-proteasome system. Some of them are at present undergoing clinical trials to be used as therapeutic agents for cancer or inflammation. These diseases are often accompanied by muscle wasting. We herein demonstrate findings about new proteasome inhibitors, belactosin A and C, and their direct effect on protein metabolism in rat skeletal muscle. M. soleus (SOL) and m. extensor digitorum longus (EDL) were dissected from both legs of male rats (40-60g) and incubated in a buffer containing belactosin A or C (30 μM) or no inhibitor. The release of amino acids into the medium was estimated using high performance liquid chromatography to calculate total and myofibrillar proteolysis. Chymotrypsin-like activity (CTLA) of proteasome and cathepsin B, L activity were determined by fluorometric assay. Protein synthesis and leucine oxidation were detected using specific activity of L-[1-^{14}C] leucine added to medium. Inhibited and control muscles from the same rat were compared using paired t-test. The results indicate that after incubation with both belactosin A and C total proteolysis and CTLA of proteasome decreased while cathepsin B, L activity did not change in both SOL and EDL. Leucine oxidation was significantly enhanced in SOL, protein synthesis decreased in EDL. Myofibrillar proteolysis was reduced in both muscles in the presence of belactosin A only. In summary, belactosin A and C affected basic parameters of protein metabolism in rat skeletal muscle. The response was both muscle- and belactosin-type-dependent.

Key words: Belactosin, Proteases, Proteasome, Skeletal muscle.

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The ATP-dependent ubiquitin-proteasome system (UPS) is the intracellular proteolytic machinery localized in the cytosol and nucleus. Proteins to be degraded are mostly tagged by the polyubiquitin chain in a process regulated by ubiquitinating and deubiquitinating enzymes (1). Subsequent proteolysis is performed in a 26S proteasome consisting of a 20S central cylindrical component and one or two 19S regulatory units with ATPase activity and ubiquitin-recognizing sites. The 20S core is organized as a stack of four rings formed by seven subunits (28). The two inner rings are composed of β-subunits, which form a hydrolytic chamber, whereas the outer subunit rings are essential for the assembly and substrate gating (15). On β-subunits, there are at least three well-characterized active sites with cleavage activities referred to as chymotrypsin-like (CTLA), trypsin-like, and post-glutamyl peptide hydrolyzing activity (12).

The UPS is an important proteolytic pathway involved in many intracellular processes. Levels of cyclins during the cell cycle are regulated due to the degradation mediated by the UPS (13, 26). Similarly, the generation of antigenic peptides, and consequently their presentation, is affected by the UPS activity (11, 29). Although there is evidence for the involvement of several regulatory mechanisms in the breakdown of myofibrillar proteins, it is thought that protein degradation in skeletal muscle occurs primarily through the UPS (21). Atrophy of skeletal muscle via UPS activation is common complication to a number of serious disorders. Thus, the UPS represents an appropriate therapeutic target to prevent muscle wasting (35). Considering these facts, the research has recently been focused on the development of new proteasome inhibitors (PIs) as therapeutic agents.

In the course of a microbial screening programme, the new structurally unique Streptomyces metabolites, belactosins, were identified. These substances were referred to as potential new anti-tumour agents with inhibitory effect on proteasome (3). Consequently, methods for the total syntheses of belactosin A, C and a homoanalogue of belactosin C have been developed (5, 20). As belactosin A and C as well as their benzyl-diprotected forms are promising agents for the treatment of cancer, which is often attended by muscle wasting, the aim of our study was to provide basic information about their effect on protein and amino acid metabolism in isolated skeletal muscle of intact rat.

Material and Methods

Animals.– Male Wistar rats (body weight 40-60 g) obtained from BioTest, Konarovice, CZ, were used in this study. The rats were housed under controlled conditions (12-h light-dark cycle, 22 °C, 55-65% relative humidity) with free access to standard laboratory chow and water. All procedures involving animal manipulation were performed in accordance with guidelines set by the Institutional Animal Use and Care Committee of Charles University, Prague, CZ.

Materials.– Armin de Meijere and Oleg Larionov synthesized belactosin A and C in their benzyl-diprotected forms (Fig. 1). L-[1-^{14}C]leucine was purchased from GE Healthcare Life Sciences (Buckinghamshire, GB); amino acids, cycloheximide, Folin-Ciocalteu's phenol reagent, N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-MCA), N-Cbz-Phe-Arg-7-amido-4-methyl-

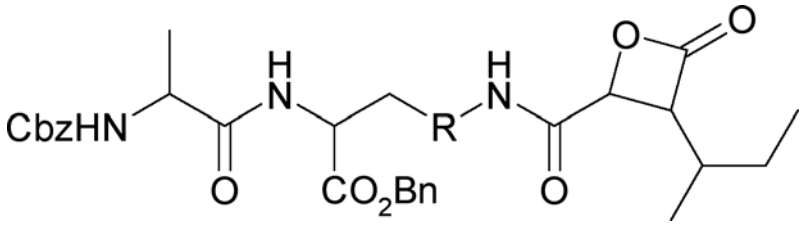

Fig. 1 *Chemical structure of belactosins.*
Diprotected belactosin A (R=C3H4); Diprotected belactosin C (R=C2H4); Cbz, benzyloxycarbonyl; Bn, benzyl.

coumarin hydrochloride (Z-FA-MCA), N-Cbz-Phe-Phe-fluoromethyl ketone (Z-FF-FMK) and albumin were purchased from Sigma Chemical (St Louis, MO, US); hydroxide of hyamine from Packard Instruments (Meriden, CT, US); Aminoplasmal 15 from B. Braun Medicals (Melsungen, DE); MG132 from Biomol (Hamburg, DE). The remaining chemicals were obtained from Sigma Chemicals (St Louis, MO, US), Waters (Milford, MA, US), and Lachema (Brno, CZ).

Muscle incubation.– Rats were anaesthetized with pentobarbital (6 mg/100 g body weight, i.p.) and blood was withdrawn from the bifurcation of aorta. Soleus (SOL) and extensor digitorum longus (EDL) muscles of both legs were dissected as described (25). Isolated muscles were fixed to stainless steel clips to provide slight tension and immediately transferred to 2.5 ml of modified Krebs-Henseleit bicarbonate buffer (in mM: 144 Na^+; 4.2 K^+; 0.8 Mg^{2+}; 1.3 Ca^{2+}; 122.8 Cl^-; 26 HCO_3^-; 0.8 SO_4^{2-}; 2 $H_2PO_4^-$) with 6 mM glucose and 2 mU/ml insulin (pH 7.4, 37 °C). Other components present in the medium were dependent on the measured parameter. The medium was saturated with O_2/CO_2 (95% / 5%). The muscles were preincubated for 30 min in a thermostatically controlled bath (37 °C) with a shaking device (70 cycles/min) to ensure stable intramuscular concentrations of components present in the medium. After the preincubation, the muscles were quickly rinsed in 0.9% NaCl, blotted and transferred to a second set of vials containing fresh incubation media identical in composition and volume with the preincubation one. Up to date there are no data about bioavailability of belactosin A and C. Nevertheless, proteasome inhibition activity of belactosin A and C is similar to lactacystin (3), which is usually used in 10-100 μM concentrations. Thus bilateral muscles were individually preincubated and incubated with the addition of 30 μM belactosin A or C, or in a medium with a solvent (dimethylsulfoxide-final concentration 0.1%) respectively. Control and experimental incubations of the same rat were compared. The viability of incubated muscles was previously confirmed in our laboratory (30) as well as by other authors (8).

CTLA of proteasome.– The CTLA of proteasome was determined using the fluorogenic substrate Suc-LLVY-MCA (14) as follows. Following two-hour incubation in the medium for proteolysis (modified Krebs-Henseleit buffer with 0.5 mmol/l cycloheximide), the muscles were homogenized in 0.4 ml of ice-cold 20 mM Tris buffer, pH 7.5, containing 2 mM ATP, 5 mM $MgCl_2$ and 1 mM dithiothreitol. The homogenates were centrifuged for 10 min at 18 000 g at 4 °C. The cellu-

lar supernatant (0.1 ml) was incubated with 0.1 ml of substrate Suc-LLVY-MCA (0.1 mM), with and without inhibitor MG132 (0.02 mM), for 1 h on ice. Adding 0.4 ml of 100 mM sodium acetate buffer, pH 4.3, stopped the reaction. The fluorescence of the samples was immediately determined at the excitation wavelength of 340 nm and the emission wavelength of 440 nm (Perkin Elmer luminescence spectrometer LS 50 B). The standard curve was established for 7-amino-4-methylcoumarin, which permitted the expression of CTLA as nmol of 7-amino-4-methylcoumarin/g protein/hour. The activity was adjusted for the protein concentration of the supernatant estimating according to LOWRY (24). The differences after the subtraction of inhibited from non-inhibited activities were used for the calculations.

Cathepsin B, L activity.– The activity of cathepsin B, L was determined using the fluorogenic substrate Z-FA-MCA [19, 32] as follows. The muscles were incubated for two hours in the medium for proteolysis and homogenized in 0.6 ml ice-cold 300 mM sodium acetate buffer, pH 5.0, containing 4 mM EDTA, 8 mM dithiothreitol and 0.2 % Triton X-100 (v/v). The homogenates were allowed to stand for 30 min on ice and then centrifuged for 30 min at 18 000 g at 4 °C. The cellular supernatant (0.01 ml) was incubated with 0.2 ml of substrate Z-FA-MCA (0.1 mM), with and without inhibitor Z-FF-FMK (0.04mM). After a 30min incubation at 37°C, the reaction was stopped by adding 1ml of 100mM sodium acetate buffer, pH 4.3, and the activity of cathepsin B, L was determined as described above for CTLA.

Total and myofibrillar proteolysis.– The total and myofibrillar protein breakdown was estimated after a two-hour incubation of the muscle in medium for proteolysis, which prevented the reincorporation of the released amino acids into the proteins. Since tyrosine is neither synthesized nor degraded in the skeletal muscle and since the intracellular pool of tyrosine remains constant during incubation (10), its amount released into the medium reflects total proteolysis in the muscle. The amount of 3-methylhistidine, a characteristic product of the myofibrillar breakdown, released into the medium, served for the myofibrillar proteolysis calculation. The rates of amino acid release were estimated based on their respective concentrations in the medium and the weight of the muscle. 3-methylhistidine was quantified using a high performance liquid chromatography method (Waters, MA, USA) based on the reaction with fluorescamine (23, 37) amino acid concentrations in the medium were determined by a high performance liquid chromatography method after a precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (6, 27).

Leucine oxidation and protein synthesis.– Leucine oxidation and protein synthesis were measured after an one-hour incubation of the muscle in 2.5 ml of modified Krebs-Henseleit bicarbonate medium enriched with amino acids in approximately physiological concentrations (Aminoplasmal 15 with added glutamine, tyrosine, threonine, serine, lysine, cysteine and asparagine in total concentration 2.8 mM) and [1-^{14}C]leucine (0.6 μCi/ml). The final concentration of labelled and unlabelled leucine was 150 μM. The one-hour (shortened) incubation was performed to minimize the bias of results via degradation of proteins. At the end of the incubation period, 0.4 ml of hydroxide of hyamine was added to the container hanging above the incubation medium. The

reaction was stopped by the addition of a 35% (v/v) perchloric acid solution (0.2 ml) into the incubation medium, and the flasks were shaken for one hour to ensure complete absorption of $^{14}CO_2$ into the hyamine hydroxide. The muscles were removed from the incubation flasks, quickly rinsed in cold 6% (v/v) $HClO_4$, blotted, and homogenized in 0.6 ml of 6% (v/v) perchloric acid. The homogenate was centrifuged for 5 min at 12 000 g, and the pellet was used for other measurements. The L-[1-^{14}C]leucine incorporation into the precipitated proteins was estimated after their hydrolysis in 1 M NaOH. Leucine oxidation and protein synthesis were calculated using leucine specific activity in the incubation medium and expressed as nmol of oxidized Leu/g wet weight/hour and nmol of incorporated leucine/g protein/hour, respectively. The radioactivity of the samples was measured with the liquid scintillation radioactivity counter LS 6000 (Beckman Instruments, CA, USA). The protein content of the samples was estimated according to LOWRY (24).

Statistical analysis.– The results are expressed as the mean ± SEM. Paired t-test (statistical software NCSS 2001) has been used for the analysis of the data. Differences were considered significant at $P<0.05$.

Results

CTLA of proteasome and cathepsin B, L activity.– As described in Table I and II, both inhibitors caused a significant reduction of CTLA of proteasome while cathepsin B, L activity was not affected both in SOL and EDL.

Total and myofibrillar proteolysis.– The addition of both belactosin A and C into the incubation medium significantly decreased the total proteolysis in both SOL and EDL (Table I and II). Myofibrillar proteolysis was significantly lowered in both types of muscle only after incubation with belactosin A (Table I).

Leucine oxidation and protein synthesis.– Both belactosin A and C induced a

Table I. *Effect of belactosin A on basic parameters of protein and amino acid metabolism.*

	SOL_{A-}	SOL_{A+}	EDL_{A-}	EDL_{A+}
CTLA of proteasome (n=8) (nmol AMC/g of protein/h)	1150±61	679±44*	1206±119	1022±64*
Cathepsin B,L activity (n=8) (µmol AMC/g of protein/h)	328±35	341±16	278±26	240±21
Total proteolysis (n=10) (nmol TYR/g wet wt/h)	273±14	198±8*	140±4	105±3*
Myofibrillar proteolysis (n=10) (nmol 3MH/g wet wt/h)	1.260±0.043	1.098±0.049*	0.756±0.024	0.613±0.024*
Leucine oxidation (n=8) (nmol LEU/g wet wt/h)	72±7	88±8*	80±13	89±6
Protein synthesis (n=8) (nmol LEU/g of protein/h)	1905±92	1757±86	1573±86	1425±63*

Mean ± SEM; Paired *t*-test; *P<0.05 *vs*. control. Control groups (A-) are compared to the groups consisting of contralateral muscles incubated with belactosin A (A+). SOL-m. soleus, EDL-m. extensor digitorum longus.

Table II. *Effect of belactosin C on basic parameters of protein and amino acid metabolism.*

	SOL_{C-}	SOL_{C+}	EDL_{C-}	EDL_{C+}
CTLA of proteasome (n=8) (nmol AMC/g of protein/h)	1236±86	789±62*	1475±94	1015±66*
Cathepsin B,L activity (n=8) (μmol AMC/g of protein/h)	298±26	259±27	285±31	197±21
Total proteolysis (n=10) (nmol TYR/g wet wt/h)	258±7	230±8*	135±5	112±6*
Myofibrillar proteolysis (n=10) (nmol 3MH/g wet wt/h)	1.122±0.101	1.157±0.096	0.588±0.037	0.528±0.035
Leucine oxidation (n=8) (nmol LEU/g wet wt/h)	64±5	79±7*	75±12	91±5
Protein synthesis (n=8) (nmol LEU/g of protein/h)	1924±90	1769±83	1580±87	1447±66*

Mean ± SEM; Paired t-test; *P<0.05 *vs.* control. Control groups (C-) are compared to the groups consisting of contralateral muscles incubated with belactosin C (C+). SOL-m. soleus, EDL-m. extensor digitorum longus.

Table III. *Effect of belactosin A on the release of amino acids into the medium (nmol/g w. w./hour).*

	SOL_{A-}	SOL_{A+}	EDL_{A-}	EDL_{A+}
Ala	1121 ± 67	1034 ± 42	679 ± 37	594 ± 40*
Asn	295 ± 26	244 ± 17*	139 ± 6	117 ± 5*
Gln	2037 ± 124	1944 ± 97	1307 ± 42	1166 ± 19*
Glu	424 ± 53	385 ± 45	174 ± 21	149 ± 13
Gly	1023 ± 59	876 ± 53	582 ± 30	498 ± 29*
His	333 ± 22	293 ± 13	165 ± 6	141 ± 6*
Ile	324 ± 14	241 ± 8*	197 ± 10	158 ± 8*
Leu	590 ± 27	429 ± 15*	289 ± 11	219 ± 10*
Lys	690 ± 52	560 ± 26*	430 ± 23	375 ± 18*
Met	168 ± 9	125 ± 6*	84 ± 3	65 ± 4*
Phe	308 ± 17	227 ± 11*	149 ± 5	120 ± 6*
Pro	597 ± 34	477 ± 38*	346 ± 13	282 ± 9*
Ser	992 ± 102	868 ± 80	530 ± 29	430 ± 24*
Tau	570 ± 148	613 ± 139	286 ± 66	255 ± 41
Val	473 ± 25	359 ± 14*	227 ± 8	178 ± 8*
Total AA	10158 ± 669	8757± 422	5724 ±187	4852 ± 148*

Mean ± SEM, n=10 in each group. Paired *t*-test; *P < 0.05 *vs.* control. Media of control muscles (A-) are compared to media of contralateral muscles incubated with belactosin A (A+). SOL-m. soleus, EDL-m. extensor digitorum longus, Total AA, sum of all measured amino acids.

significant increase in leucine oxidation in SOL, whereas in EDL this parameter remained unchanged. In contrast, both inhibitors decreased protein synthesis in EDL without a significant effect on the SOL muscle (Tables I and II).

Amino acid release into the incubation medium.– Table III represents the rates of amino acid release from SOL and EDL into the medium after the incubation with belactosin A. The release of most of the amino acids (except glutamic acid and taurine) decreased in EDL, resulting in total amino acid release reduction. In SOL, a decrease in Asn, Pro, Val, Met, Ileu, Leu, Lys and Phe release was detected. After the belactosin C treatment (Table IV), the release of Val, Met, Ileu, Leu and Phe was reduced in both muscles. Additionally, a decrease in Asn, Ala, Pro, Lys and total amino acids was observed in EDL only.

Discussion

The addition of both belactosin A and C inhibited CTLA of proteasome in both SOL and EDL. We did not observe a significant change in cathepsin B, L activity following the belactosin treatment. This indicates that belactosin A and C affect CTLA of proteasome similar to most of the known PIs. Additionally, we provide evidence that belactosin A and C does not inhibit cathepsin B, L activity in skeletal muscle, partially indicating their proteasome specificity. This corresponds with the findings demonstrating that the analogue of belactosin A, KF33955, has a weak effect on other proteases such as elastase, IL-1β converting enzyme and cathepsin B (3).

In agreement with the concept of the substantial role of UPS in skeletal muscle protein degradation, total proteolysis was decreased, as was CTLA of proteasome. The incubation with both belactosin A and C significantly reduced the total proteolysis both in SOL and EDL. Similar results were published after the incubation with $AdaAhx_3L_3VS$ (18) and lacta-

Table IV. *Effect of belactosin C on the release of amino acids into the medium (nmol/g w. w./hour).*

	SOL_{C-}	SOL_{C+}	EDL_{C-}	EDL_{C+}
Ala	1157 ± 53	1098 ± 56	616 ± 19	451 ± 20*
Asn	307 ± 11	302 ± 15	118 ± 2	102 ± 5*
Gln	2013 ± 58	1997 ± 56	1220 ± 41	1106 ± 50
Glu	488 ± 38	568 ± 27	154 ± 17	153 ± 18
Gly	897 ± 29	875 ± 32	489 ± 26	455 ± 30
His	313 ± 12	308 ± 11	166 ± 13	140 ± 13
Ile	307 ± 8	261 ± 9*	155 ± 6	117 ± 7*
Leu	586 ± 17	494 ± 16*	299 ± 12	225 ± 11*
Lys	817 ± 27	763 ± 43	498 ± 14	457 ± 21*
Met	169 ± 5	147 ± 6*	79 ± 3	61 ± 3*
Phe	303 ± 9	267 ± 9*	149 ± 5	123 ± 6*
Pro	649 ± 19	593 ± 22	356 ± 12	297 ± 11*
Ser	892 ± 63	892 ± 76	404 ± 30	379 ± 34
Tau	599 ± 89	699 ± 72	146 ± 25	213 ± 59
Val	463 ± 13	404 ± 14*	234 ± 13	181 ± 10*
Total AA	10218 ± 320	9899 ± 378	5217 ± 158	4571 ± 222*

Mean ± SEM, n=10 in each group. Paired *t*-test; *P < 0.05 *vs*. control. Media of control muscles (C-) are compared to media of contralateral muscles incubated with belactosin C (C+). SOL-m. soleus, EDL-m. extensor digitorum longus, Total AA, sum of all measured amino acids.

cystin (9). In other studies evaluating the effect of MG132 on skeletal muscle of intact rats, as high as a 50% reduction of the total proteolysis was registered (4, 33).

Regarding the myofibrillar proteolysis, we found a decrease in both SOL and EDL incubated with belactosin A, while exposure to belactosin C failed to affect this parameter. These findings are in agreement with decreased 3-methylhistidine release following the treatment of C_2C_{12} myotubes with MG132 (34). However, no impact of lactacystine on this parameter was observed (9). It has been demonstrated that myofibrils cannot be cleaved by proteasome itself (31). However, other proteases (e.g. caspase-3) disintegrate myofibrils and make actin and myosin accessible for proteasomal cleaving (7). We therefore hypothesize that the different effects of belactosin A and C on the myofibrillar proteolysis may be caused by different protease specificity.

As the removal of leucine reduces protein synthesis through changes in translation initiation factors eIF2B and eIF4E (36), and leucine stimulates protein synthesis in skeletal muscle (2), we estimated the effect of belactosins on leucine oxidation. This parameter was enhanced in the presence of both belactosin A and C in SOL. In EDL, leucine oxidation tended to increase, however no significant changes were verified. This is partly in conflict with the findings of increase in leucine oxidation after the $AdaAhx_3L_3VS$ treatment in both muscle types (18) and decrease in this parameter in EDL following an MG132 exposure (17). As the branched-chain alpha-keto acid dehydrogenase is the rate-limiting enzyme of leucine catabolism (22), one of possible explanations of increased leucine oxidation is, that the enzyme might be induced by belactosins.

In EDL, protein synthesis decreased significantly both in the presence of belactosin A and C without a significant effect in SOL. These data correspond with those reporting a lowering of protein synthesis in EDL following an LLnL treatment (16). Similar changes both in EDL and SOL were observed using MG132 and $AdaAhx_3L_3VS$, respectively (17, 18). In contrast, LLnL has no effect on protein synthesis in incubated diaphragm (33). The reduction of protein synthesis in EDL can be related to neither enhance leucine oxidation nor to decreased supply of amino acids due to the lowered proteolysis (see above). Therefore, we hypothesise that belactosin A and C reduced the skeletal muscle protein synthesis via an unknown mechanism.

The total amino acid release was decreased in the presence of both belactosins in EDL and did not change in SOL. A similar effect of $AdaAhx_3L_3VS$ was demonstrated in both muscle types (17). We assume that this effect reflects a muscle-type-dependent response to belactosins rather than a decrease in total proteolysis. However, the changes in amino acid concentrations in the incubation medium should be interpreted carefully with respect to the fact that the amino acid release from the muscle to the medium can be contributed by degradation of both structural proteins and enzymes, changes in transport mechanisms, re-synthesis and catabolism of some amino acids, and binding to cell structures.

We conclude that the effect of belactosins on protein and amino acid metabolism in isolated skeletal muscles of intact rats exhibits some differences compared to the other known PIs. Belactosin A and C differ in action on myofibrillar proteolysis and their impact on protein synthesis and leucine oxidation is muscle-type

dependent. These promising findings were obtained on isolated muscles of healthy rats and provide no data about effect of belactosins *in vivo*. As belactosins are prospective agents for treatment of cancer, their putative effect on cancer-related skeletal muscle wasting should be investigated on appropriate animal models.

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