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Comparative study of the biochemical properties of proteasomes in domestic animals

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immunoproteasomes, sensitivity to proteasome inhibitors, subunit composition, and overall structure of both 20S and 19S particles that has been acquired during the last two decades(mainly because these animals were chosen as experimental models by several groups performing basic studies on the cell biology and biochemistry of proteolysis) (Eleuteriet al., 1997; Cascio et al., 2001). However, for other species there is only scanty information available. Furthermore, direct comparison between species is further rendered difficult due to the extreme heterogeneity of the experimental settings used, including differences in protocols for proteasome extraction and purification, assay conditions for peptidase activity, and chemical structure and concentration of the fluorogenic substrates and inhibitors tested.

2. Materials and methods

2.1. Proteasome purification

Constitutive and immunoproteasomes were purified as described (Cerruti et al., 2007) with minor modifications. Briefly, samples of muscle and spleen from dog and cat were derived from surgical resections, rabbit muscle and spleen were purchased from Pel Freez Biologicals; mouse muscle and spleen were obtained from animals maintained in the animal facility unit of the SPAE (Universityof Torino), in conformity with European laws and policies and with the approval of the Ethical Committee of the University of Torino and the Italian Ministry of Health; horse, swine, goat, sheep and cattle samples were collected at the local slaughterhouse. Tissues were homogenized in ice-cold extraction buffer (50 mM Tris-HCl pH 7.5, 1 mM dithiothreitol, 250 mM sucrose, 5 mM MgCl2, 0.5 mM EDTA, and 2 mM ATP) using an Ultraturax DIAX900 homogenizer (Heidolph Instruments, Kelheim, Germany), centrifuged twice at 21,000 × g for 45 min at 4◦C to remove cell debris, and then ultracentrifuged at 300,000 × g for 3 hours. Pellets obtained were dissolved in ice cold extraction buffer and further centrifuged at 21,000 ×g for 15 min at 4◦C. The protein concentration in supernatants was determined with the Bradford reagent (Sigma-Aldrich) using a standard curve constructed with BSA. Samples were stored at −80◦C until use. Human proteasomes were recovered from the human cell lines HeLa and RPMI8226 that express at high level only constitutive (Nathan et al., 2013) or immunoproteasomes (Cenci et al., 2012) respectively. Cellular extracts and proteasome purification were performed as described (Kisselev and Goldberg, 2005).

2.2. Immunoblot analyses

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Immunoblot analyses of proteasomal non-catalytic sub-units b3, b4, b5, and a6, were performed as previously described (Favole et al., 2012). Briefly, 75 ug of proteins recovered after ultracentrifugation of muscle extracts were separated on a 12% SDS-PAGE gel, and transferred on aImmobilon®-P transfer membrane (Millipore). The membrane was then incubated in blocking buffer (5% BSA, 0.1%Tween-20 in 1 × PBS), followed by incubation with primary monoclonal antibodies (MCP196, MCP106, MCP79,MCP257, Enzo Lifesciences). Bound antibodies were visualized using the ECL technique.

2.3. Proteasome activity assays and susceptibility to inhibitors

Peptidase activities of partially purified proteasomes and immunoproteasomes were measured using 100 uM Suc-LLVY-amc (for chymotrypsin-like activity), 100 uM Z-YVAD-amc (for caspase-like activity), and 100 uM Bz-VGR-amc (for trypsin-like activity) (Bachem) in 20 mM Tris–HCl pH 7.5, 1 mM ATP, 2 mM MgCl2, and 0.2% BSA. The fluorescence of released amc (excitation, 380 nm; emission, 460 nm) was monitored continuously at 37◦C with a Carry Eclipse spectrofluorometer (Varian). Assays were calibrated using standard solutions of the free fluorophore, and the reaction velocities were calculated from the slopes of the initial linear portions of the curves. Substrate consumption at the end of incubation never exceeded 1%. To evaluate the effects of proteasome inhibitors, aliquots of constitutive proteasomes from different species were pre-incubated at room temperature for 20 minutes in the presence or absence (vehicle alone) of 10 uM MG132 (Calbiochem), 5 uM clasto-lactacystin-b-lactone (Biomol), 2 uM or 20 uM epoxomicin (Sigma-Aldrich), 100 uM leupeptin (Sigma-Aldrich) and immediately used for proteasome activity assays as described above.

2.4. Statistical analysis

To compare average measurements of peptidase proteasomal activities among domestic animals, we adopted a Mann-Whitney test. Data were graphically visualized using box plots.

3. Results and Discussion

Peptidase activities of proteasomes obtained from several species of veterinary interest were assessed according to a protocol widely used for human and experimental animal cells and tissues (Kisselev and Goldberg, 2005). Herein, the enzyme was partially purified by ultracentrifugation from homogenates of skeletal muscle that exclusively contain

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constitutive proteasomes (Van Kaer et al., 1994;Kisselev et al., 1999; Cascio et al., 2001), which were characterized with various fluorogenic peptides. Fig. 1A shows the comparative quantification of the chymotrypsin-like, caspase-like and trypsin-like activities of constitutive proteasomes in different species. In agreement with data for humans and mouse reported in literature (Chu-Ping et al.,1994; Akaishi et al., 1996; Coux et al., 1996) that we confirmed in our study (Fig. S1A), chymotryptic activity was higher in all species analyzed, followed by the tryptic and caspase-like activities (Fig. 1A). Consequently, the ratios among the three main proteasomal peptidase specificities were similar in all species assessed, including human and mouse (Table 1). On the other hand, the specific activities of constitutive proteasomes varied among the domestic animals analyzed. Specifically, swine, sheep, horse, and rabbit had significantly lower values of the three activities than those in cat, goat, cattle, and dog (Fig. 1A), that appear more similar to those measured in parallel experiments for human and mouse (Fig. S1A).To further define the biochemical properties of the proteasomal chymotryptic site in domestic animals, we assessed its sensitivity to MG132, clasto-lactacystin b-lactone, and epoxomicin, three of the most potent and specific proteasome inhibitors available. As shown in Fig. 2A, proteasomes from all species analyzed were highly sensitive (inhibition > 90%) to the inhibitory effects of these three compounds, thus demonstrating substantial conservation of the chymotryptic b5 site in mammals. More importantly, these conclusions were further corroborated by the results of an accurate titration of the three compounds that didn't highlight any difference in their inhibitory effects at any of the concentrations assessed (Fig. 2B). This further provides the rationale for the use of these chemicals in research involving domestic animals, as well as their potential therapeutic application. Sensitivity to inhibitors of the other two catalytic b-subunits was also assessed by means of leupeptin (a potent and specific inhibitor of the b2 tryptic subunit) (Kisselev et al., 2006) and epoxomicin (that at the concentration used efficiently suppresses the activity of the b1 caspase subunit) (Kisselevet al., 2006). As shown in Fig. 2A, both inhibitors proved highly effective in suppressing the activity of the target subunit in all species analyzed, thus indicating a substantial conservation in mammals also of b1 and b2 proteasome subunits. A similar analysis of peptidase activities was subsequently performed for immunoproteasomes. To this purpose, the enzyme was purified by ultracentrifugation from the spleen, which constitutively and exclusively expresses this form of the proteasome (Eleuteri et al., 1997;Cascio et al., 2001). As shown in Fig. 1B, this analysis revealed that,

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which, by itself, has values that are higher than those of other herbivorous animals. At the moment, it is unclear if the differences in the levels of proteasomal activities actually reflect some species-specific requirements for an enhanced proteolytic intracellular pathway or merely indicate substantial redundancy of the proteasomal apparatus in some animals. Further studies will be required to clarify this intriguing issue. To further define the biochemical properties of proteasomes from domestic animals, we tested the sensitivity of the chymotryptic site of the b5 subunit to the effects of three highly specific and widely used proteasome inhibitors. Chymotrypsin-like is the main proteasomal activity, and is rate limiting for the turnover of the vast majority of proteins in vivo (Chen and Hochstrasser,1996; Arendt and Hochstrasser, 1997; Heinemeyer et al.,1997). For this reason, most compounds used for scientific and therapeutic purposes were developed to exclusively or preferentially target the active site of b5 (Kisselevand Goldberg, 2001). In our assays, when employed at concentrations that quench the human enzyme, MG132,clasto-lactacystin b-lactone, and epoxomicin efficiently inhibited proteasomes from all animals tested, with a level of inhibition that was always higher than 90%. There were, moreover, no clear differences among species. Importantly, these inhibitors are known to inactivate chymotryptic activity by accommodating into the substrate-binding groove of _5 subunits and selectively blocking the N-terminal catalytic threonine of the enzymatic site either reversibly (MG132) or irreversibly (clasto-lactacystin b-lactone and epoxomicin). Consequently, the uniform and strong effect of these three inhibitors clearly demonstrates substantial conservation of the catalytic mechanism and active site structure of the b5 subunit in these mammals. Moreover, our results demonstrate for the first time the high sensitivity of proteasomes of several domestic animals to three of the most potent and widely available proteasome inhibitors, thus validating their use in the veterinary field as important research tools and as potential therapeutic agents. Finally, we also performed a preliminary investigation of the non-catalytic a subunits that form the two outer rings connecting the internal proteolytic chamber with different proteasome activators (e.g. 19S, PA28, PA200). For this purpose, the cross-reactivity of the four different proteasomal a subunits was tested in western blot analysis using commercial antibodies specific for the corresponding human proteins. Remarkably, two antibodies recognized their specific target with equal, high affinity in all species; one detected its subunit in all animals, although with slight differences in affinity and one reacted the protein equally well in six species, but unexpectedly did not give any signal in dog or cat. Altogether, our study

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demonstrates that there is substantial overall conservation of the a and catalytic b subunits of proteasomes in all the domestic animals investigated, although there are some minor species-specific differences that warrant further investigation. Moreover, our study demonstrates that the inhibitors and antibodies widely utilized in research in humans are also applicable in veterinary research as valuable scientific tools and potential therapeutic agents.

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Appendix A. Supplementary dataSupplementary data associated with this article can befound, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2015.04.005.

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Table 1 Relative ratios of activities of constitutive and immunoproteasomes in domestic animals, mouse and human.

	Constitutive proteasome		Immuno-proteasome	
	CL/CTL (%)	TL/CTL (%)	CL/CTL (%)	TL/CTL (%)
DOG	4.9	25.2	7.8	174.4
CAT	5.5	ND	5.2	ND
RABBIT	7.2	40.7	3.0	94.4
HORSE	7.8	51.9	2.4	82.0
SWINE	12.8	31.1	3.3	66.4
GOAT	4.9	20.8	3.7	133.4
SHEEP	10.8	33.2	3.3	82.5
CATTLE	5.1	28.8	2.9	199.5
MOUSE	10.5	46.0	9.4	145.8
HUMAN	4.21	51.34	2.5	92.9

Caspase-like (CL) and trypsin-like (TL) activities of constitutive and immunoproteasomes were assessed as described in Section 2 and indicated as percentage of chymotryptic-like (CTL) activity. ND, not determined.

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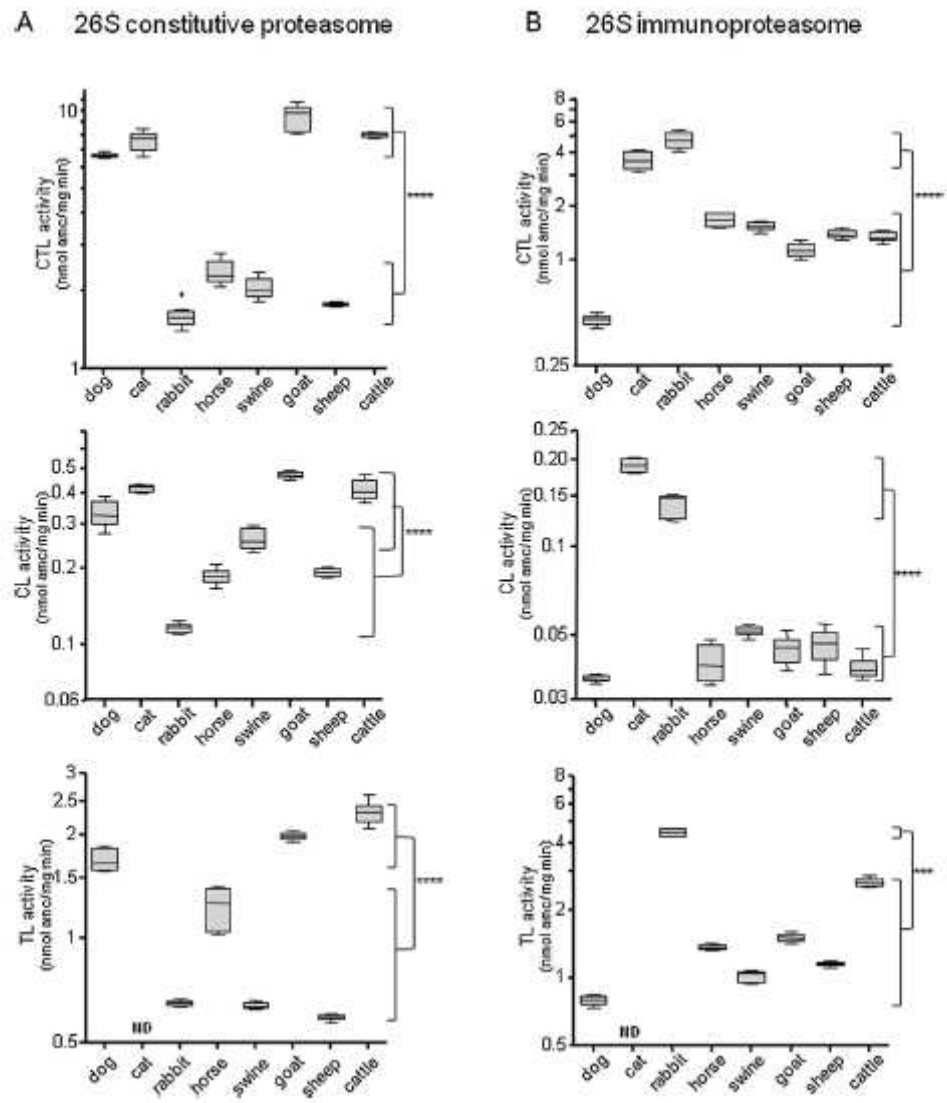

Fig. 1. Peptidase activities of constitutive and immune- proteasomes in domestic animals. Chymotrypsin-like, caspase-like, and trypsin-like activities of constitutive (A) and immune-proteasomes (B) were assessed as described in Section 2. Data are the mean of nine independent measurements ± SEM. ****p < 0.0001. CTL, chymotrypsin-like; CL, caspase-like; TL, trypsin-like; ND, not determined. Reference values for human and mouse enzymes are shown in figure S1.

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A

	CHYMOTRYPSIN-LIKE ACTIVITY (% inhibition)			TRYPSIN-LIKE ACTIVITY (% inhibition)	CASPASE-LIKE ACTIVITY (% inhibition)
	Clasto-lactacystin β-lactone	MG-132	Epoxomicin	Leupeptin	Epoxomicin
DOG	96.3 ± 0.4	98.1 ± 0.3	98.2 ± 0.4	97.8 ± 0.7	90.2 ± 0.8
CAT	93.4 ± 0.7	97.6 ± 0.4	98.0 ± 0.7	ND	89.3 ± 1.2
RABBIT	93.8 ± 2.0	96.6 ± 0.4	93.3 ± 2.7	95.2 ± 1.6	88.8 ± 2.2
HORSE	90.4 ± 1.1	95.8 ± 1.8	94.6 ± 1.0	95.0 ± 1.2	89.0 ± 1.6
SWINE	95.1 ± 0.1	97.5 ± 0.4	96.9 ± 0.2	95.7 ± 2.1	93.1 ± 3.6
GOAT	97.1 ± 0.2	98.9 ± 0.2	98.5 ± 0.2	95.5 ± 1.1	95.8 ± 1.0
SHEEP	92.5± 0.4	95.1 ± 0.3	94.3 ± 0.2	97.2 ± 1.7	93.2 ± 3.4
CATTLE	96.2 ± 0.5	98.5 ± 0.2	98.6 ± 0.2	97.8 ± 1.2	92.7 ± 2.2
MOUSE	95.0 ± 1.2	98.3 ± 0.3	98.9 ± 0.1	98.0 ± 0.1	93.8 ± 2.1

B

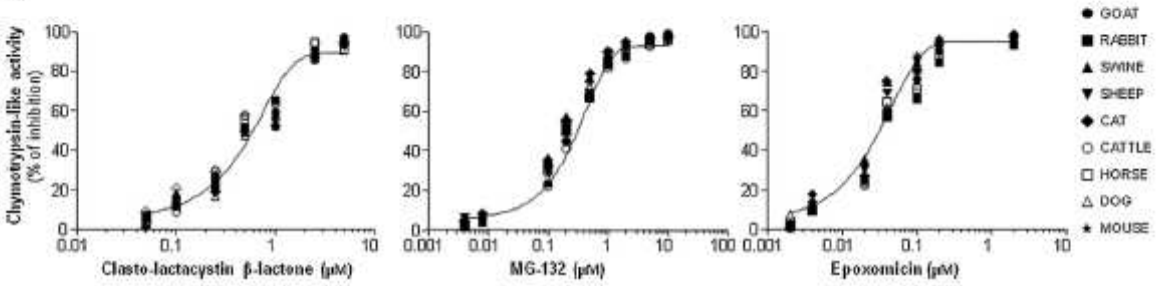

Fig. 2. Effect of inhibitors on peptidase activities of constitutive proteasomes in different animals. (A) The inhibitory effects of clasto-lactacystin b-lactone(5 uM), MG-132 (10 uM), and epoxomicin (2 uM) on the chymotrypsin-like activity, of leupeptin (100 uM) on the trypsin-like activity and of epoxomicin (20 uM) on the caspase-like activity of constitutive proteasomes were assessed as described in Section 2 and indicated as percentage of inhibition relative to vehicle-treated controls. Data are the means of three independent measurements ± S.D. (B) Titration of the three inhibitors of the chymotrypsin-like activity.

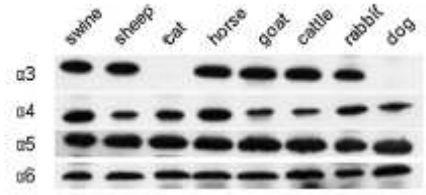

Fig. 3. Western blot analysis of proteasome a-subunits in domestic animals. Western blots of the a3, a4 a5, and a6 proteasomal subunits were performed with specific monoclonal antibodies as described in Section 2.

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