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Short communication

Comparative study of the biochemical properties of proteasomes in domestic animals

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ABSTRACT

Information on the biochemical properties of proteasomes is lacking or, at best, only fragmentary for most species of veterinary interest. Moreover, direct comparison of the limited data available on the enzymatic features of proteasomes in domestic animals is rendered difficult due to the heterogeneity of the experimental settings used. This represents a clear drawback in veterinary research, given the crucial involvement of proteasomes in control of several physiological and pathological processes. We performed the first comparative analysis of key biochemical properties of proteasomes obtained from 8 different domestic mammals. Specifically, we investigated the three main peptidase activities of constitutive and immunoproteasomes in parallel and systematically checked the sensitivity of the chymotryptic site to three of the most potent and selective inhibitors available. Overall, there was substantial similarity in the enzymatic features of proteasomes among the species examined, although some interesting species-specific features were observed.

1. Introduction

The 26S proteasome is an ATP-dependent protease present in both the cytoplasm and the nucleus of eukaryotic cells that is responsible for degradation of the majority of cellular proteins (Voges et al., 1999). This large (2.4 MDa) and abundant multisubunit proteolytic complex consists of the 20S proteasome, in which proteins are degraded, capped at one or both ends by the 19S regulatory particle, which is responsible for recognizing, unfolding, and translocating polyubiquitinated (and some non-ubiquitinated) substrates into the internal proteolytic cavity of the 20S particle (Glickman and Ciechanover, 2002). PA28 is an alternative proteasome activator that enhances hydrolysis of short peptides, but not entire proteins (Raule et al., 2014a). The 20S proteasome is a ~700 kDa barrel-shaped structure composed of four stacked heptameric rings. The two outer rings consist of α-subunits, while the two central rings are made up of β-subunits (Coux et al., 1996). Three of the subunits in the β rings (β1, β2, and β5) contain the proteolytic active sites that are positioned on the interior face of the cylinder. When measured with short fluorogenic substrates, the proteolytic activities of proteasomes have three distinct cleavage preferences: β1 has caspase activity (i.e. cleavage after acidic residues); β2 possesses tryptic activity (i.e. cleavage after basic residues); and β5 displays chymotryptic activity (i.e. cleavage after hydrophobic residues). However, lymphoid cells and cells exposed to cytokines such as IFN-γ or TNF-α express three homologous subunits (β1i/LMP2, β2i/MECL-1, and β5i/LMP7) that replace the constitutive ones in newly assembled, so-called immunoproteasome particles (Cascio, 2014). A variety of

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studies have demonstrated that incorporation of these IFN-gamma induced subunits quantitatively modifies the preferences of proteasomal cleavage in a way that enhances production of a significant number of antigenic peptides (Sijts and Kloetzel, 2011). Additionally, immunoproteasomes have been reported to be important for efficient cytokine production (Groettrup et al., 2010) and have recently been shown to hydrolyze basic proteins, such as histones, at greatly increased rates compared with constitutive proteasomes. This suggests that they have a potential role in regulation of transcription (Raule et al., 2014b).

The crucial role of the ubiquitin proteasomes system in controlling several physiological (e.g. heat shock and unfolded protein responses, apoptosis, transcription, aging, B-cell differentiation, cell cycle regulation, MHC-I antigen presentation) and pathological (e.g. neoplastic transformation, inflammation, neurodegenerative and autoimmune diseases) processes was unambiguously established during the last decade (Lecker et al., 2006). Moreover, proteasome inhibitors have emerged as a promising new class of anticancer agents due to their ability to selectively induce apoptosis in tumor cells, especially in those of hematological origin (Adams, 2004; Cenci et al., 2012). It is, therefore, somehow surprising that there is a distinct lack of data regarding the biochemical properties of proteasomes in domestic animals, and especially the absence of studies that systematically compare the functional and structural characteristics of these proteases in different species. For two mammalian species (i.e. cattle and rabbit) there is a significant amount of data regarding the enzymatic activities of both constitutive and 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) (Eleuteri et 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 (University of 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 $MgCl_2$, 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

Immunoblot analyses of proteasomal non-catalytic subunits α3, α4, α5, and α6, were performed as previously described (Favole et al., 2012). Briefly, 75 μg of proteins recovered after ultracentrifugation of muscle extracts were separated on a 12% SDS-PAGE gel, and transferred on a Immobilon®-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 μM Suc-LLVY-amc (for chymotrypsin-like activity), 100 μM Z-YVAD-amc (for caspase-like activity), and 100 μM Bz-VGR-amc (for trypsin-like activity) (Bachem) in 20 mM Tris–HCl pH 7.5, 1 mM ATP, 2 mM $MgCl_2$, 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 μM MG132 (Calbiochem), 5 μM clasto-lactacystin β-lactone (Biomol), 2 μM or 20 μM epoxomicin (Sigma-Aldrich), 100 μM leupeptin (Sigma-Aldrich) and immediately used for proteasome activity assays as described above.