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Proteasomal activity in skeletal muscle: A matter of assay design, muscle type, and age

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ABSTRACT

The ubiquitin–proteasome system (UPS) is a major degradation system for regulatory and misfolded proteins. UPS function has been implicated to exert a central role in the pathogenesis of various human diseases. Because biochemical analyses are often hampered by the amount of available diseased tissue, we report on the establishment and validation of a luminescence-based proteasomal activity assay applicable to 5-mg quantities of skeletal muscle. We demonstrate that the specific proteasomal activity differs in individual muscle groups and decreases with aging. These findings warrant the use of appropriate controls and a careful interpretation of results in mammalian skeletal muscle pathologies.

The ubiquitin–proteasome system (UPS)[1] is a major nonlysosomal protein degradation machinery of the cell that cleaves regulatory, misfolded, and damaged proteins into small peptides. The ATP-dependent UPS represents a cascade of enzymatic factors, ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin–protein ligases (E3) that are associated with the 26S proteasome and give rise to a large macromolecular complex. The barrel-shaped conventional 26S proteasomal structure is composed of two regulatory cap domains (19S-subunits) and a proteolytically active central core domain (20S-subunit) that harbors the three main catalytic activities: the chymotrypsin-like, trypsin-like, and peptidylglytamyl-like (caspase-like) activities [1–3]. The UPS has been implicated to exert a functional role in the pathogenesis of cancer, diabetes, and aging as well as in a broad variety of protein aggregate diseases [4–7]. The latter include various neurodegenerative disorders (e.g., Parkinson and Huntington diseases), cardiomyopathies (e.g., desminopathy), and progressive myopathies (e.g., myofibrillar myopathies) [8,9]. However, further progress in our understanding of proteasome-associated disease mechanisms is hampered by the fact that tissue samples from biopsy specimens are available only in small amounts.

In the current study, we established and validated a reliable luminescence-based proteasomal activity assay applicable to milligram quantities (typically 5 mg) of murine skeletal muscle tissue. Special emphasis of our work is put on the influence of tissue preparation and storage, the amount of total protein extract yielding a stable luminescent signal with an optimal signal-to-noise ratio, the use of different substrates in conjunction with a specific and irreversible proteasomal inhibitor, differences in the use of individual muscle groups, and the effect of aging.

Materials and methods

Preparation of skeletal muscle tissue

C57BL/6J male mice were sacrificed at the following time points of life: 1.5, 3, 8, and 20 months. Quadriceps femoris and soleus muscles were prepared for further analysis. Tissue samples of approximately 5 mg were dissected from each muscle on ice, rinsed in ice-cold phosphate-buffered saline (PBS) to remove blood, subsequently snap-frozen in liquid nitrogen, and stored at −80 °C.

Protein extraction and quantitation

Ice-cold PBS (100 μl) containing 5 mM EDTA (PBSE) was added to 1 mg of skeletal muscle tissue and sonicated on ice for 20 s with a pulse length of 1 s two times using a pulsed homogenizer (UP

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[1] *Abbreviations used:* UPS, ubiquitin–proteasome system; PBS, phosphate-buffered saline; PBSE, PBS containing EDTA; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS-T buffer, Tris–HCl, NaCl, and Tween 20; cps, counts per second, RLU, relative light units; AdaAhx$_3$L$_3$VS, adamantane-acetyl-(6-aminohexanoyl)$_3$-(leucinyl)$_3$-vinyl-(methyl)-sulfone.

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200S, Hielscher, Teltow, Germany). Tissue lysates obtained in this way were centrifuged at 13,000*g* for 5 min at 4 °C, and the supernatants were subjected to protein quantitation employing a fluorescence-based quantitation assay kit (ProStain Protein Quantification Kit, Active Motif, cat. no. 15001) with bovine serum albumin (100, 50, 25, 12.5, 6.25, 3.125, and 1.56 μg) as standard. Protein extraction buffer PBSE was used as blank. The supernatants were diluted to 1:5, 1:10, and 1:20 and then mixed with a fluorescent dye to a final volume of 200 μl in a nontransparent black 96-well plate (Nunc). The reactions were incubated at room temperature for 60 min, and the fluorescence intensity was measured three times at 485 nm excitation/590 nm emission in a Fluoroskan Ascent FL plate reader (Thermo Fisher Scientific, Germany). The coefficients of variation (r^2) for the standard curves were between 0.97 and 0.99.

SDS–PAGE and Western blot

Next, 3 μg of the protein extracts (supernatants) were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and transferred onto nitrocellulose membranes by the semidry method [10,11]. The membranes were blocked with TBS-T buffer (10 mM Tris–HCl [pH 8.0], 150 mM NaCl, and 0.2% Tween 20) containing 5% milk powder and were probed with an antibody directed against the S4-subunit of the 26S proteasome (anti-26S proteasome S4-subunit, Calbiochem, cat. no. 539167, 1:1000 dilution) followed by anti-rabbit secondary antibody conjugated with peroxidase (Sigma) and chemiluminescence detection. Images were recorded and analyzed using the Fluorchem SP imaging system (Alpha Innotec). The amount of 26S proteasome S4-subunit was determined densiometrically using the Spot Denso tool of the AlphaEaseFC software (Alpha Innotec). The 26S proteasome S4-subunit is part of the 19S regulatory complex [12], and its expression level has been shown to remain unaffected by aging in rat muscle [13].

Proteasome activity assay

For the setup of our proteasome activity assay applicable to small quantities of skeletal muscle tissue, a commercially available indirect enzyme-based luminescent assay was modified (Promega, cat. no. G8621 with substrate for chymotrypsin-like activity [Suc-LLVY-aminoluciferin] and cat. no. G8631 with substrate for trypsin-like activity [Suc-Z-LRR-aminoluciferin]). This assay kit is designed and established to measure the proteasomal activity in purified proteasome using a luminogenic substrate. Thus, the original purpose of this commercially available assay is to detect the effect of test compounds on purified proteasome. In contrast, we modified the assay setup to enable the measurement of the proteasomal activity using soluble total protein extracts from skeletal muscle tissue as described in the following two paragraphs.

The supernatants (soluble protein extracts) prepared from skeletal muscle tissue as described above were diluted to a concentration of 0.2 mg/ml total protein with ice-cold PBSE. If not stated otherwise, 50 μl corresponding to 10 μg of total protein was added to 50 μl of the luminescent reagent containing the Ultra-Glo Luciferase and the signal peptide (specific for either chymotrypsin- or trypsin-like activity) coupled to luciferin. After mixing of the components and preincubation for 60 min in a black 96-well plate at room temperature, the resulting luminescence was measured three times with an integration time of 1 s using a Fluoroskan Ascent FL plate reader in luminometry mode. If available, the luminescent signal should be measured with a plate reader that is equipped with a count photomultiplier (result: counts per second [cps]) instead of a current photomultiplier (result: relative light units [RLU]).

In this setup, the luminescence signal intensity in each supernatant analyzed is proportional to the total peptidase activity (i.e., the summation of the proteasomal activity and unspecific peptidase activities resulting from other enzymes within the protein extract). To calculate the proteasomal activity, dual measurements with or without the addition of 30 μM of the irreversible and highly specific proteasomal inhibitor adamantane-acetyl-(6-aminohexanoyl)$_3$-(leucinyl)$_3$-vinyl-(methyl)-sulfone (AdaAhx$_3$L$_3$VS, Calbiochem, cat. no. 114802) were carried out [14]. The proteasomal activity in supernatants was calculated by subtracting the unspecific background activity (value with proteasomal inhibitor added) from the total peptidase activity (value without inhibitor added). For the final determination of the specific proteasomal activity that is correlated to the amount of proteasome, the calculated value of the proteasomal activity was normalized by using the densiometry value obtained from the 26S proteasome S4-subunit immunoblot analysis.

Results

Assay setup: Determination of optimal probe handling and elimination of unspecific background activities

In general, reliable results of proteasomal activity measurements are related to the stability and quality of the luminescence signal. In a first step, we studied the influence of various amounts of protein (1, 5, 10, 25, and 50 μg) on signal intensity, signal stability, and signal-to-noise ratio (i.e., total peptidase activity divided by unspecific background activity). Using a protein extract from skeletal muscle with a total amount of 5 or 10 μg of protein resulted in a fairly stable luminescence signal between 50 and 70 min (Fig. 1A). In contrast, 25 or 50 μg of protein yielded a marked and distinct loss of signal intensity, whereas 1 μg of protein resulted in only a very weak signal at the baseline.

Further experiments clearly demonstrated that a concentration of 30 μM of the highly specific and irreversible proteasomal inhibitor AdaAhx$_3$L$_3$VS [14] led to maximal inhibition of the proteasomal activity after 50 min of incubation (Fig. 1B), allowing indirect determination (calculation) of the unspecific background activity. A determination of unspecific background activities for 1, 5, 10, 25, and 50 μg of skeletal muscle extract in the presence of 30 μM inhibitor indicated that 1, 5, and 10 μg of protein extract showed minimal signal intensities in conjunction with linear curve progressions after 50 min of incubation (Fig. 1C). Finally, the use of 10 μg of protein in combination with 30 μM inhibitor resulted in an optimal signal-to-noise ratio after 60 min of preincubation when compared with higher and lower protein amounts as well as longer and shorter incubation times (Fig. 1D and data not shown).

To study the influence of protein storage at −80 °C, the proteasomal activity was determined in both conditions directly after the protein extraction and after one cycle of snap-freezing in liquid nitrogen and thawing on ice (Fig. 1E). It is noteworthy that in all supernatants analyzed, freezing of probes resulted in a marked decrease (27–51%) of the proteasomal activity (i.e., the total peptidase activity subtracted by unspecific background activity). As a consequence, only freshly prepared skeletal muscle extracts were used for our studies.

Specific proteasomal activity in relation to proteasomal protein content

The specific proteasomal activity in supernatants extracted from skeletal muscle can be determined only in relation to the proteasomal protein content in each individual sample. Western blot