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Catalytic site-specific inhibition of the 20S proteasome by 4-hydroxynonenal

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Abstract The proteasome is responsible for most intracellular protein degradation and is essential for cell survival. Previous research has shown that the proteasome can be inhibited by a number of oxidants, including 4-hydroxynonenal (HNE). The present study demonstrates that HNE rapidly inhibits the chymotrypsin-like activity of the 20S proteasome purified from liver. Subunits containing HNE-adducts were identified following 2D gel electrophoresis, Western immunoblotting, and analysis by MALDI-TOF MS. At a time when only the chymotrypsin-like activity was inhibited, the α6/C2 subunit was uniquely modified. These results provide important molecular details regarding the catalytic site-specific inhibition of proteasome by HNE.

Keywords: 20S proteasome; 4-Hydroxynonenal; Chymotrypsin-like activity; Proteasome inhibition; MALDI-TOF mass spectrometry

1. Introduction

The proteasome is a multi-subunit proteolytic complex that is responsible for the regulated degradation of most intracellular proteins and is essential for cell survival [1]. The proteolytic core of the proteasome, known as the 20S proteasome, appears as a hollow cylinder of four stacked rings [2]. The two outer rings, each composed of seven distinct α-subunits, serve to stabilize the catalytic core and have been proposed as the site of control for entry of substrates into the catalytic chamber [3]. The two inner rings are composed of seven distinct β-subunits. In mammalian proteasome, three of the seven β-subunits contain the active sites, which perform different proteolytic cleavage. Based on mutational and crystal structural analysis, the three catalytic activities, the peptidylglutamyl hydrolyzing, trypsin-like, and chymotrypsin-like, have been assigned to the β1, β2, and β5 subunits, respectively [4]. As a consequence of an inflammatory stimulus, the constitutive catalytic subunits β1, β2, and β5 can be replaced in nascent proteasomes by the inducible subunits LMP2, MECL, and LMP7, respectively [5].

Because the proteasome plays a central role in key cellular processes, it is essential to understand the mechanistic basis for proteasome inhibition. Previous research has shown that the proteasome can be inhibited by a number of oxidants, including 4-hydroxynonenal (HNE) [6]. HNE is an aldehyde that originates from peroxidation of polyunsaturated fatty acids and forms a mixture of adduct types on the side-chains of cysteine, lysine, and histidine through a Michael-type nucleophilic addition [7]. The addition of HNE adducts on both membrane and soluble proteins has been shown to inhibit protein function [8–11]. Further, direct attachment of HNE to the proteasome has been ascribed to the decline in proteasome activity in studies of cardiac muscle following ischemia reperfusion [12], with cerebral ischemia [13], in kidney homogenates following incubation with HNE [14], and in lymphocytes and spinal cord from aged organisms [15,16]. However, the molecular details responsible for the loss in activity have not been elucidated.

2. Materials and methods

2.1. Materials

Proteasome substrates were purchased from Sigma and Calbiochem. HNE was from Cayman Chemical. Antibodies and their respective companies are as follows: Proteasomal subunits (Affinity Bioreagents), HNE (Alpha Diagnostics and Calbiochem), heat shock protein 90 (HSP90) (StressGen), alkaline phosphatase-conjugated goat anti-rabbit or -mouse secondary antibody (BioRad). All reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Western immunoblotting were supplied by BioRad. Immobline dry strips (pH 3–11, non-linear, 11 cm) were from Amersham Pharmacia Biotech. Immobilon-P polyvinylidene difluoride membrane (PVDF) and C18 ZipTips were from Millipore. The bicinchoninic acid (BCA) protein assay kit was obtained from Pierce. HPLC-grade dimethyl sulfoxide and all reagents for the preparation of samples for mass spectrometry were from Aldrich.

2.2. Preparation and characterization of liver 20S proteasome

The 20S proteasome was purified from frozen rat liver as described [17]. Proteasome was suspended in 50 mM potassium phosphate (pH 7.0) and 0.1 M KCl and aliquots were stored at −80 °C. Protein concentrations were determined using the BCA protein assay and bovine serum albumin as the standard protein. The relative content of LMP7 and β5 subunits was determined by Western immunoblotting (see Section 2.6) comparing the immune reaction of subunit-specific antibodies in the liver 20S with the reaction in protein from retinal pigment epithelial cells containing a known percent of each subunit. In these cells, no LMP7 is present in the absence of cytokines. Induction of the immunoproteasome was accomplished by incubating the cells with 100 U/ml interferon-γ for 72 h. Mixtures of cytokine-free and cytokine-exposed cells were used to generate a standard curve of known percentages of the inducible and constitutive subunits.

2.3. Incubation of proteasome with HNE

20S proteasome was incubated with 20 μM HNE at various times from 0 to 240 min. The molar ratio of the 20S proteasome (mass ~700 kDa) to HNE (mass 156 Da) was approximately 1:10000. Preliminary experiments showed that 20 μM was the optimal concentration to achieve maximal inhibition without the formation of HNE-protein aggregates,

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which would have complicated the 2D gel analysis. The HNE reaction was quenched by either (1) addition of 10-fold excess buffer to measure activity, (2) addition of reducing buffer and immediate 1D electrophoresis of protein, or (3) dialysis of the proteasome in 20 mM Tris (pH 7.4) overnight at 4 °C to prepare prior to 2D electrophoresis.

2.4. Measurement of proteasome activity

The fluorogenic peptides LLVY-AMC (150 μM), LSTR-AMC (200 μM), and LLE-AMC (200 M) were used to measure the chymotrypsin-like, trypsin-like and peptidylglutamyl hydrolyzing activities of the proteasome, respectively. Proteasome (0.3 μg) was pre-incubated with either buffer (control) or HNE for a specific length of time at room temperature. Proteasome activity was measured as described previously [18]. Measurement of proteasome inhibition by HNE was measured on three separate preparations of 20S proteasome. The kinetics of HNE-inhibition was determined from fitting a curve assuming first-order exponential decay to the experimental data, plotted as relative activity versus time of HNE incubation (Origin version 6.0).

2.5. Gel electrophoresis

1D SDS–PAGE separation of proteasomal subunits (1 μg) was accomplished using a 12% resolving gel with a 3% stacking gel [19]. For 2D electrophoresis, proteasomal subunits were first focused across a non-linear pH gradient of 3–10 (11 cm) using a Protean IEF Cell (BioRad). Purified 20S (15 g) was diluted in sample buffer (8 M urea and 4% CHAPS) and focused for 50 000 V h over 23 h. Second dimension was performed using 12% SDS–PAGE [19]. Two gels were run in parallel. One gel was silver stained using the Silver Stain Plus Kit. The alternate gels were used for Western immunoblotting. Images were captured using a Fluor-S MultiImaging system (BioRad).

2.6. Western immunoblotting of 1D and 2D gels

Proteins were electrophoretically transferred to PVDF membrane and probed with either the monoclonal antibody to HSP90, or polyclonal antibodies that recognize proteasomal subunits (β1, β2, β5, LMP2, LMP7, α6/C2, α-subunits, and β-subunits), amino acid-HNE Michael adducts or the reduced forms of HNE (1:1 amino acid–HNE Michael adducts). The specificity of the HNE antibody recognizing 1:1 amino acid:HNE Michael adducts has been thoroughly characterized [20]. Prior to application of the antibody that recognizes the reduced forms of HNE, blotted proteins were reduced by incubating the PVDF membranes in a solution containing 25 mM sodium borohydride in 100 mM MOPS (pH 8.0) for 10 min at room temperature. Sodium borohydride reduces the carbonyl present on Michael adducts [21]. For the HNE antibodies, Immun-blot Amplified Alkaline Phosphatase Kit (1:3000 each of biotinylated goat anti-rabbit IgG, streptavidin, and biotinylated alkaline phosphatase) was used in conjunction with the substrate BCIP-NBT to visualize the immunoreaction. For all other antibodies, the secondary antibody was either goat anti-mouse or -rabbit alkaline phosphatase without the amplification system. Images were captured using a Fluor-S MultiImaging System (BioRad).

2.7. Selection and preparation of proteins for mass spectrometry

Western immunoblotting of 2D gels was used to identify specific protein spots containing HNE adducts. Immune reactive spots were aligned with spots on silver stained gels. The selected spots were excised and proteins digested in-gel overnight at 37 °C with trypsin. Peptides were extracted as described [22].

2.8. Matrix-assisted laser desorption ionization-time of flight mass spectrometry

The peptide mixture was desalted using Millipore C18 ZipTips following the manufacturer's protocol. Data were acquired on a QSTAR XL quadrupole-TOF mass spectrometer (ABI) as described [22]. Measured peptide masses were used to search the NCBI and Swiss-Prot sequence databases for protein identifications and database accession numbers using Mascot (www.matrixscience.com) or BioAnalyst (ABI) software. All searches were performed with a mass tolerance at 50 ppm. Positive identification required a minimum of three peptide matches and a probability score that indicates high concordance between the masses of experimentally derived peptides with theoretical masses of peptides from the matched protein, and a positive identification from at least one product ion spectrum.

2.9. MS/MS sequencing of peptides

Product ion spectra were collected in an information dependent acquisition mode using the enhanced feature of the scan mode with a three second scan time. Experimental fragment masses were used to search the NCBI and Swiss-Prot sequence databases for protein identifications and database accession numbers using Mascot (www.matrixscience.com) or BioAnalyst (ABI) software. Peptides with a minimum of four consecutive b and y ions were considered as an acceptable match.

3. Results

3.1. Selective Inhibition of the 20S proteasome by HNE

To directly test the effect of HNE modification on proteasome function, the 20S proteasome purified from liver was incubated with HNE from 0 to 240 min. Fig. 1 shows a silver-stained polyacrylamide gel and the corresponding Western immunoblot probed for HNE-adducts. Prior to incubation with HNE, there is a prominent reaction in a single protein band at ~26 kDa before exposure to exogenous HNE (Fig. 1B). Following incubation, there is a time-dependent increase in the reaction in a protein band at ~33 kDa and in proteins with migrations at higher molecular masses. By four hours of incubation, HNE-adducts are present in the proteins at the interface between the resolving and stacking gels and near the top of the resolving gel, suggesting the formation of protein aggregates cross-linked by HNE.

In a parallel experiment, the activity of each catalytic site was measured using the fluorogenic peptides LLE-AMC, LSTR-AMC, and LLVY-AMC, to assay the peptidylglutamyl hydrolyzing, trypsin-like, and chymotrypsin-like activities, respectively. We observed a time-dependent inhibition of each catalytic site that differed in both the extent and the rate of inhibition (Fig. 2). The peptidylglutamyl hydrolyzing activity was the least sensitive to HNE; only 25% inhibition was observed during 4 h of incubation. The trypsin- and chymotrypsin-like activities were both inhibited by ~45%. However, rapid inactivation of the chymotrypsin-like activity occurred within 2 min of incubation with HNE, whereas maximal inhibition of the trypsin-like activity occurred in ~158 min. These

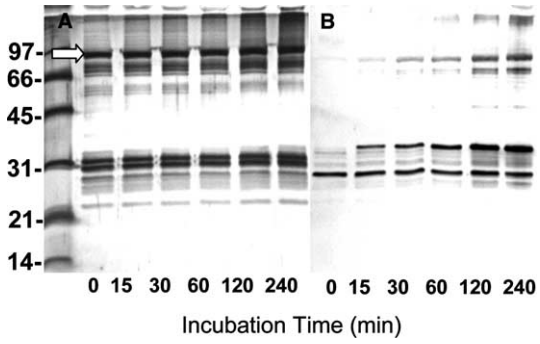

Fig. 1. Time-dependent modification of the 20S proteasome by HNE. (A) Silver-stained gel of proteasomal subunits resolved by 13% SDS–PAGE following incubation with HNE for varying times. Arrow indicates the position of HSP90, determined by a positive immune reaction with anti-HSP90. (B) Western immunoblot probing for HNE adducts on proteins from the 20S proteasome following incubation with HNE for varying times. Protein loads were 1 μg per lane. Molecular mass standards are (in kDa): 97.4, phosphorylase b; 66.2, bovine serum albumin; 45, ovalbumin; 31, carbonic anhydrase; 21.5, soybean trypsin inhibitor; and 14.4, lysozyme.

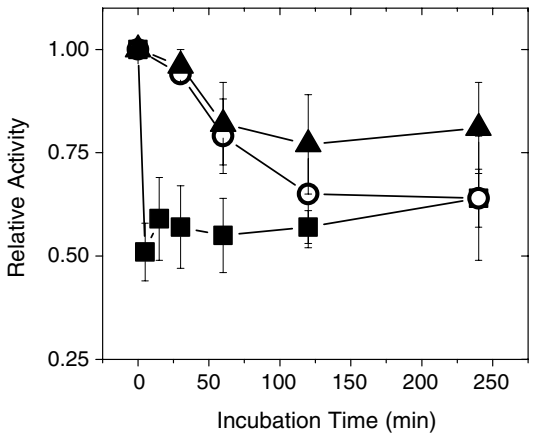

Fig. 2. Time-dependent inactivation of the proteasome by HNE. The peptidylglutamyl hydrolyzing (triangle), chymotrypsin-like (square) and trypsin-like (circle) activities were determined by measuring the proteolysis of the fluorogenic peptides LLE-AMC, LLVY-AMC and LSTR-AMC, respectively. Data are reported as means (±SEM) for duplicate measurements from three separate preparations of purified 20S proteasome. Initial rates of peptide proteolysis in the absence of HNE were 17.2 ± 6.8 (LLVY), 1.1 ± 0.2 (LSTR), and 3.2 ± 1.2 (LLE) nmol/mg/min.

results suggest that the chymotrypsin-like activity is exquisitely sensitive to HNE modification, although prolonged exposure to HNE also affects the activity of the other catalytic sites.

3.2. Identification of subunits containing HNE-adducts

To investigate the mechanistic basis for the rapid inhibition of the chymotrypsin-like activity by HNE, we identified subunits that were modified by HNE using a combination of 2D-PAGE, Western immunoblotting, and mass spectrometry. After localizing the HNE-immune reaction to specific protein spots, the corresponding subunits were identified by performing in-gel trypsin digests and peptide mapping using full scan matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Tandem MS data were acquired to provide unambiguous confirmation of a subunit's identity. To identify the HNE-modifications that were restricted solely to a loss in chymotrypsin-like activity, we chose an incubation time of 15 min. During this short incubation, a strong immune reaction of a single protein band (Fig. 1) correlates with maximal inhibition of chymotrypsin-like activity and no effect on the other activities (Fig. 2).

Following 2D gel resolution of subunits from the 20S catalytic core, we observed a larger number of protein spots than predicted for the 17 catalytic core subunits known from the mammalian proteasome (Fig. 3). Our analysis of individual protein spots by either MS or Western immunoblotting showed that many of the individual subunits were found in multiple spots or smears migrating at the same molecular mass but at different isoelectric points (p*I*). These isoelectric variants from individual proteins are known as "charge trains" and result from post-translational modifications that alter the intrinsic charge of the protein. Post-translational modifications described for individual subunits that alter the protein's p*I* include phosphorylation, acetylation, and glycosylation [23–25]. HNE-modification of the ε-amino group of lysine would also cause the modified protein to migrate to a more acidic position than the unmodified parent protein. Several protein spots on the silver stained gel demonstrated very light negative staining.

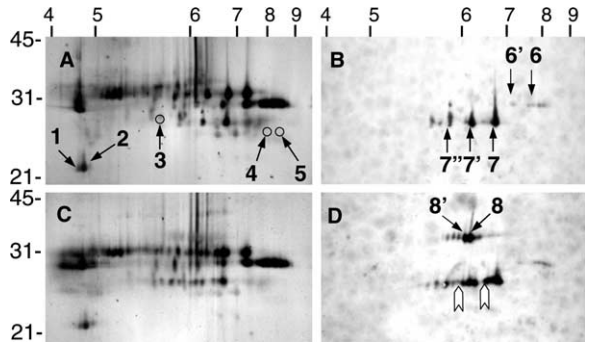

Fig. 3. Two-dimensional gel resolution of proteasomal subunits before and after incubation with HNE. The 20S proteasome purified from liver was incubated in the absence of HNE (control) (A,B) or presence of 20 M HNE for 15 min (C,D). Proteasome (15 μg) was then resolved by 2D gel electrophoresis. One gel was stained with silver and used for mass spectrometric identification of proteins (A,C). Gel A shows the proteins corresponding to the catalytic β-subunits (1, β1i/LMP2; 2, β1/Y; 3, β2/Z; 4, β5/X; and 5, β5i/LMP7), as determined by Western immunoblotting using protein-specific antibodies. The second gel of each pair was used for Western immunoblotting to locate subunits containing HNE-adducts (B,D). Arrows on Western blots indicate HNE reactions unique to that incubation time. Open arrows are modified subunits with more acidic migration than parent protein. All numbered spots were identified by MALDI-TOF MS and data base search (Table 1). The indicated p*I* values (top of A,B) were determined from an experimental calibration curve supplied by Amersham Biosciences. Molecular mass standards are (in kDa): 45, ovalbumin; 31, carbonic anhydrase; and 21.5, soybean trypsin inhibitor.

This type of "reversed" staining with silver is normally associated with proteins containing sugar moieties [26].

As a reference point, we localized the position of the β-subunits containing the catalytic sites by Western immunoblot using subunit-specific antibodies. The protein spots corresponding to each catalytic subunit are indicated on the silver-stained gel from the control (no HNE) sample (Fig. 3A). A positive immune reaction for both the constitutive (β1/Y, β2/Z, and β5/X) catalytic subunits and their inducible counterparts (β1i/LMP2 and β5i/LMP7) was evident in the 20S from liver. (We were unable to obtain an unambiguous immune reaction for β2i/MECL.) The apparent molecular mass and p*I* values were consistent with the theoretical values for subunits without their corresponding propeptide (Table 1). To estimate the percent composition of the inducible (LMP7) and constitutive (β5) subunits responsible for the chymotrypsin-like activity, we compared the immune reaction in the liver 20S with the reaction in cells containing a known percentage of each subunit (data not shown). We estimated that our 20S preparation contained 70% LMP7 and 30% β5.

In the control sample, Western immunoblot analysis with anti-HNE showed a strong immune reaction in five distinct proteins spots migrating between p*I* 5.8 and 7.0 and at a mass of ~26 kDa (Fig. 3B). MS analysis determined that these protein spots were the isoelectric variants of the α2/C3 subunit. A light immune reaction was also observed in multiple spots migrating more acidic to the α2/C3 subunit at a mass of ~28 kDa. These proteins were identified as the α4/C6 subunit. The pattern of migration for these subunits and the HNE-modified subunits identified following 15 min of incubation (see below) was consistent with the theoretical values for their respective molecular mass and p*I* values for the unmodified protein plus isoelectric variants containing post-translational modifications that alter the intrinsic charge (Table 1).

Table 1
Subunit identification of 20S proteasome

Spot[a] no.	20S subunit[b]	Accession No.	Molecular mass		pI		Identification		
			Exp.[c] kDa	Theo.[d] Da	Exp.[e]	Theo.	WB[f]	MALDI-TOF MS	
								% Coverage	# Peptides
1	β1i/LMP2	P28077	23	21 354	4.7	4.77	X		
2	β1/Y	P28073	23	21 964	4.8	4.98	X		
3	β2/Z	Q9JHW0	27	25 314	5.7	5.84	X		
4	β5/X	P28075	26	22 482	8.0	8.33	X		
5	β5i/LMP7	P28064	26	22 733	8.8	8.57	X		
6	α4/C6	XP_342599.1	30	28 439	7.9	8.60		20	3
6′	α4/C6		30	28 439	7.3	8.60		27	7
7	α2/C3	P17220	27	25 795	6.8	7.12		38	7
7′	α2/C3		27	25 795	6.3	7.12		46	7
7″	α2/C3		27	25 795	5.9	7.12		35	6
8	α6/C2	P18420	33	29 517	6.2	6.14		44	12
8′	α6/C2		33	29 517	6.0	6.14		30	6

[a] Protein spots were excised from 2D gels shown in Fig. 3 and analyzed on a QSTAR XL quadrupole MALDI-TOF MS instrument.
[b] First subunit name follows nomenclature by Groll [2].
[c] Exp., experimental molecular mass values were estimated using the experimental calibration curve generated from the R_f of low molecular weight silver stained markers.
[d] Theo., theoretical molecular mass and p*I* values were calculated from the Swiss-Prot sequences without the propeptide sequences.
[e] Exp., experimental p*I* values were estimated using the calibration curve supplied for 11 cm, pH 3–11, non-linear IPG strips (Amersham Biosciences).
[f] WB, Western immunoblotting.

Following 15 min of incubation with HNE, Western immunoblot analysis probing for HNE-adducts showed a prominent, new reaction of multiple spots between p*I* 5.8 and 6.2 and at a mass of ~33 kDa (Fig. 3D). These proteins were identified by MS analysis as the α6/C2 subunit (Fig. 4). Fig. 4A shows a MALDI-TOF mass spectrum of peptides generated by in-gel trypsin digestion from spot 8′. Six peptides covering 30% of the primary sequence for the α6/C2 subunit were identified from the peptide mass fingerprint. The fragmentation of peptide 1431 generated by tandem MS produced a spectrum containing 9 of 12 b ions and 8 of 12 y ions matching residues 19-30 of the α6/C2 subunit (Fig. 4B). Six b- and y-series ions of low intensity (*m*/*z* ranging between 129 and 605, not indicated on spectrum) with the loss of a water or ammonia also matched. Fragmentation of one additional peptide of a singly charged ion of 1779 matched the sequence for residues 4–18 of the α6/C2 subunit (data not shown). These data provide unambiguous identification of the α6/C2 subunit.

One additional change in immune reaction and protein staining occurred with the α2/C3 subunit. After the 15 minute incubation, two new immunoreactive protein spots appeared to the acidic side of spots 7 and 7′, which had been identified as α2/C3. On silver stained gels, these new protein spots were approximately equivalent to the parent spots 7 and 7′. The acidic migration of new protein spots suggests the addition of HNE-adducts on one or more lysine residues.

3.3. Confirmation of antibody specificity

A second antibody that recognizes the reduced form of amino acid:HNE Michael adducts was used to verify the specificity of the antibody used in the previous experiments. As shown in Fig. 5, identical immune reactive bands were observed for 20S incubated with HNE for 15 min (Lanes 2 and 3). The absence of reaction for HNE modified BSA that was not reduced with sodium borohydride prior to exposure to the antibody that recognizes the reduced HNE Michael adducts (Lane 5) also confirms the specificity of this second antibody. Taken together, the immune reaction of two antibodies that recognize different chemical forms of HNE provides convincing evidence for the presence of HNE Michael adducts on proteasomal subunits.

4. Discussion

The goal of this study was to test the effect of HNE on individual catalytic activities and elucidate the molecular details responsible for HNE-induced inhibition by identifying the subunits containing HNE-adducts. Activity measurements performed during incubation with HNE showed rapid inhibition of the chymotrypsin-like activity, followed by slower inhibition of the other catalytic sites. Resolution of modified subunits by 2D gel electrophoresis and Western immunoblotting revealed that the α6/C2 subunit is uniquely modified at a time when only the chymotrypsin-like activity is inhibited. These results provide important molecular details regarding the catalytic site-specific inhibition of the proteasome by HNE. Understanding how the 20S proteasome reacts in the presence of elevated levels of HNE is particularly relevant, since the 20S proteasome has been suggested as the primary mechanism for degrading damaged proteins following an oxidative insult [27,28]. As a caveat, the identity of the proteasome species that is functionally active under different physiological conditions remains controversial.

Inhibition of the chymotrypsin-like activity induced by HNE would have a direct negative impact on many key cell functions regulated by the proteasome, since it is primarily the chymotrypsin-like activity that determines the rate of protein breakdown [4,29,30]. The exquisite sensitivity of the chymotrypsin-like site to modification by HNE was also demonstrated in two different neuronal cell lines following incubation with HNE [31]. In these studies, the loss in activity occurred along with the appearance of proteasome HNE-adducts, increased levels of protein carbonyls and ubiquitinated

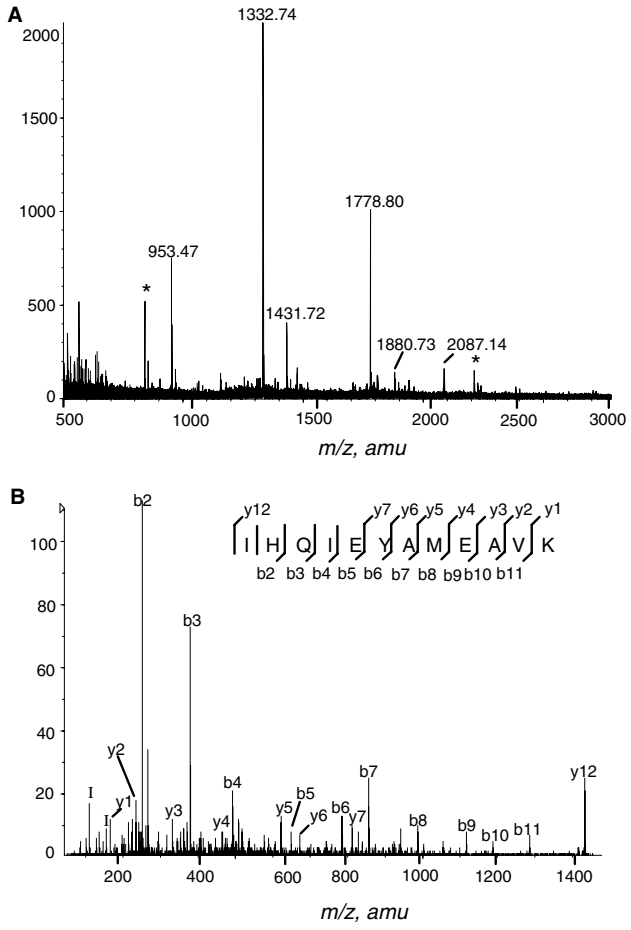

Fig. 4. Mass spectrometric analysis of tryptic peptides from the 20S proteasome subunit α6/C2. (A) Full scan of a MALDI-TOF MS peptide mass fingerprint from spot 8′ (Fig. 3D). The spectrum shows six peptides that matched the theoretical *m/z* values for residues in the sequence from rat 20S proteasome subunit α6/C2. The six matching peptides covered 30% of the sequence (Table 1). * denotes products of trypsin autolysis. (B) Product ion spectrum for fragmentation of the singly charged ion 1431 of the IHQIEYAMEAVK peptide formed by MALDI ionization. The amino acid sequence is displayed above the spectrum and corresponds to residues 19–30 of proteasome subunit α6/C2. The y- and b-ions found experimentally are written above and below the sequence, respectively, and are indicated above the corresponding peak in the spectrum. Immonium ions are denoted as (I) on spectrum. Fragment ion nomenclature was established by Biemann [50]. The data were plotted as a function of *m/z* values after raw mass spectral data were smoothed, centroided, and labeled in BioAnalyst (ABI).

proteins, and cellular changes indicative of apoptosis. Studies showing the partial reversal of peroxide-induced inhibition of chymotrypsin-like activity by DTT and the inhibition of chymotrypsin-like activity by sulfhydryl compounds, such as *N*-ethyl-maleimide [32,33], suggest that at least one of the sites critical for maintenance of chymotrypsin-like activity is a cysteine residue. In support of this idea, other studies have suggested that the reversible *S*-glutathionylation of cysteine may be an important physiological regulator of proteasome chymotrypsin-like activity [33,34]. Taken together, these results indicate that the intracellular redox state may be closely linked to proteasome-mediated proteolysis. Further, HNE modification of a key cysteine is a potential means for rapid inactivation of the chymotrypsin-like activity.

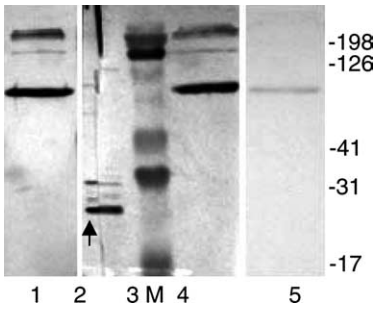

Fig. 5. Comparison of immune reactions between antibodies that recognize different chemical forms of HNE-Michael adducts. Proteins were resolved by 13% SDS–PAGE, then blotted on a PVDF membrane. The lane containing the 20S proteasome was cut in half so that the reaction with the two antibodies could be aligned. Reaction of the antibody that recognizes amino acid:HNE Michael adducts with HNE-modified bovine serum albumin (Lane 1) and 20S proteasome incubated with HNE for 15 min (Lane 2, arrow). Reaction of the antibody that recognizes the reduced form of amino acid:HNE Michael adducts with 20S proteasome incubated with HNE for 15 min (Lane 3, right half of blot) and HNE-modified bovine serum albumin (Lane 4). Both samples were reduced with sodium borohydride prior to exposure to the antibody. Reaction of the antibody recognizing the reduced form of HNE with HNE-modified bovine serum albumin without prior reduction with sodium borohydride (Lane 5). Protein loads were 1 μg. Lane M is the molecular mass marker; corresponding masses are shown on the right.

In the present study, the trypsin-like activity was inhibited to the same extent as the chymotrypsin-like activity, but at a much slower rate. This observation suggests several possibilities, including differential susceptibility to HNE-modification or partial protection of sites critical for the trypsin-like activity. The contaminant, HSP90, which co-purified with the 20S (Fig. 1, arrow), could be the putative protector against HNE-inhibition. In previous studies, exogenous addition of HSP90 to purified 20S proteasome and overexpression of HSP90 in cells protected the trypsin-like activity from inhibition by both HNE and metal-catalyzed oxidation [21,35,36]. In our study, HSP90 is not modified by HNE during the incubation (Fig. 1). Therefore, the mechanism of protection does not include providing a non-proteasome target for HNE molecules, i.e., HSP90 is acting as a ''molecular sponge''. Rather, it is likely that the binding site of HSP90 shields residues that are sensitive to modification by HNE.

The plot of the time-dependent inhibition of the three catalytic activities (Fig. 2) shows that the maximal extent of inhibition is less than 50%. These results suggest that only half of the proteasome population were inhibited even in the presence of HNE in molar excess. One explanation is that at least a portion of the population was in a conformation that makes it less susceptible to HNE modification and subsequent inactivation. Additionally, both the composition of the β-subunits (constitutive or inducible) and ''activation state'' (active or latent) of the purified 20S proteasome have been shown to alter the catalytic activity in response to oxidants or following binding of proteins, such as HSP90 [35–38]. For example, Amici et al. [38] showed that proteasomes isolated from thymus, composed mainly of the inducible subunits, were more sensitive to peroxynitrite inactivation. In contrast, proteasomes isolated from brain, composed mainly of the constitutive subunits, were resistant to peroxynitrite inactivation. Liver 20S proteasome contains both constitutive and inducible β-subunits, as

evidenced in our study by a positive immune reaction with protein-specific antibodies (Fig. 3A) and also reported by others [39–41]. In our preparations, approximately 70% of the chymotryptic subunit is LMP7. In view of the evidence demonstrating increased susceptibility of proteasome containing the inducible subunits to oxidative damage, the 50% maximal inhibition achieved in the current study could reflect the differential susceptibility of the immunoproteasome versus the constitutive proteasome.

A logical prediction of the mechanism of inactivation by HNE would be the direct modification of the β-subunits at either key residues required for activity or at sites that would disrupt specific interactions between adjacent β-subunits. While we did not detect HNE-adducts on β-subunits, it is possible that our method of analysis did not have the required sensitivity to reveal modification of a limited number of HNE-adducts. Additionally, the HNE could have assumed a form, i.e., fluorescent cross links, that is not recognized by the antibodies used in this study. However, we did observe a robust immune response for modification of α-subunits during the HNE-induced inhibition of the proteasome. These results are consistent with a report of HNE modification of only α-subunits for 20S proteasome isolated from heart muscle following an oxidative stress induced by ischemia/reperfusion [12]. Examination of the crystal structure of mammalian 20S proteasome provides potential mechanistic explanations for our results based on the physical arrangement of the α-subunits and the catalytic subunits [42,43]. The α6/C2 subunit is an adjacent neighbor to the β5 subunit that contains the chymotrypsin-like active site. Although the details of how cross-talk occurs between these two subunits are still unclear, the interaction of the α6/C2 subunit with either micro- or macromolecular ligands has a profound effect on the chymotrypsin-like activity. For example, direct binding of lipopolysaccharide to the α6/C2 subunits in proteasome isolated from macrophages or rabbit muscle selectively activated the chymotrypsin-like activity [44]. The α6/C2 subunit is also the putative binding site for the regulatory protein, PA28 [45]. Binding of this macromolecular ligand of the 20S significantly increases the hydrolysis of fluorogenic peptides, especially the peptides hydrolyzed by the chymotrypsin-like site [46].

Prior to incubation with exogenously added HNE, the α2/C3 and α4/C6 contained HNE-adducts. While we cannot rule out that the modification occurred during the purification process, the consistent presence of HNE-adducts on these subunits in three separate liver preparations and in 20S purified from skeletal muscle (unpublished observation) provides strong evidence for their modification in vivo. Further, the positive immune reaction with a second antibody that recognizes the reduced form of amino acid:HNE Michael adducts verifies the selective recognition of two different chemical forms of HNE. These results also imply that HNE-modification is not a random process and that even in a multi-subunit complex such as the proteasome, specific proteins are molecular targets for HNE.

Proteasome function is intimately linked to its subunit composition and structural properties, such as post-translational modifications. An important observation that has recently emerged is the plasticity of the proteasome subunit composition in response to changing cellular environments. In cultured neuronal cells, both low (sublethal) levels of oxidative stress and cellular expression of aggregate-prone proteins resulted in increased expression of the inducible subunit, LMP2 [47,48]. Increased content of the inducible subunits, LMP2 and LMP7, was also reported in aged muscle [18]. While the consequences of altering the subunit composition are not fully understood, it may be a compensatory change that primes the proteolytic machinery to accommodate an increased demand for degradation of specific substrates, i.e., oxidized proteins. These changes could confer protection against subsequent stresses applied to the cell.

Results from this study provide insight into how modification of a specific proteasomal subunit by HNE can affect the activity of a select catalytic site. These results also provide a potential explanation for the selective loss of chymotrypsin-like activity we have previously reported in retinal proteasome from aged rats [49]. However, we also acknowledge that in the cellular context, proteasomal inhibition by HNE-conjugated proteins may also play a significant role in inhibiting proteasome function. Knowledge of the structural modifications that affect proteasome activity, such as altering the subunit composition or oxidative modification, is critical to understanding its function under both normal and pathological conditions.

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