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Inactivation of the proteasome by 4-hydroxy-2-nonenal is site specific and dependant on 20S proteasome subtypes

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Abstract

The proteasome represents a major intracellular proteolytic system responsible for the degradation of oxidized and ubiquitinated proteins in both the nucleus and cytoplasm. We have previously reported that proteasome undergoes modification by the lipid peroxidation product 4-hydroxy-2-nonenal (HNE) and exhibits declines in peptidase activities during cardiac ischemia/reperfusion. This study was undertaken to characterize the effects of HNE on the structure and function of the 20S proteasome. To assess potential tissue-specific differences in the response to HNE, we utilized purified 20S proteasome from heart and liver, tissues that express different proteasome subtypes. Following incubation of heart and liver 20S proteasome with HNE, changes in the 2D gel electrophoresis patterns and peptidase activities of the proteasome were evaluated. Proteasome subunits were identified by mass spectrometry prior to and following treatment with HNE. Our results demonstrate that specific subunits of the 20S proteasome are targeted for modification by HNE and that modified proteasome exhibits selective alterations in peptidase activities. The results provide evidence for a likely mechanism of proteasome inactivation in response to oxidative stress particularly during cardiac ischemia/reperfusion.

Keywords: Proteasome; Oxidative stress; Oxidatively modified proteins; 4-Hydroxy-2-nonenal; Heart; Liver; Mass spectrometry

Cardiac ischemia/reperfusion results in enhanced production of reactive oxygen/nitrogen species and lipid peroxidation products, all of which readily interact with and modify intracellular components such as protein [1–7]. Free radical derived modifications can alter protein structure and, in many cases, inhibit protein function. The proteasome represents a major intracellular proteolytic system responsible for the degradation of oxidized and ubiquitinated proteins in both the nucleus and cytoplasm [8–12]. It has previously been reported that the proteasome exhibits declines in function during cardiac ischemia/reperfusion and aging. Declines in proteolytic activity were accompanied by modification of the proteasome by the lipid peroxidation product 4-hydroxy-2-nonenal (HNE)[2] [6,13–16]. Oxidative stress may therefore decrease the cellular proteolytic capacity and promote accumulation of oxidized and aggregated protein [17]. HNE is a major α-β-unsaturated

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[2] *Abbreviations used:* HNE, 4-hydroxy-2-nonenal; LLVY-AMC, Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin; LSTR-AMC, N-t-Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin; LLE-NA, N-Cbz-Leu-Leu-Glu-β-naphthylamide; MG132, N-Cbz-Leu-Leu-Leucinal; ESI, electrospray ionisation; MS, mass spectrometry; MALDI-TOF–TOF, matrix assisted laser desorption ionisation-time of flight-time of flight.

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aldehyde formed from peroxidation of both ω-3 and ω-6 polyunsaturated fatty acids. HNE is a strong electrophile that can react with nucleophilic functional groups of histidine, lysine, and cysteine to form Michael and Schiff base adducts. These reactions can lead to intra- and inter-protein cross-linking and protein aggregation [18–21]. Furthermore, in light of recent reports, HNE appears to be responsible for many pathological effects associated with oxidative stress. Specifically, HNE has been implicated in inactivation of several key enzymes and proteins including glucose-6-phosphate dehydrogenase [22], glyceraldehyde-3-phosphate dehydrogenase [23], glutathione reductase [24], interleukin 1B converting enzyme [25], glutathione *S*-transferase [26], aldose reductase [27], histone [28], and proteasome [6,13–16]. Moreover, catalytic site-specific inhibition of rat liver 20S proteasome by HNE has been recently reported [29].

The proteasome is responsible for intracellular protein degradation and is involved in the regulation of a variety of cellular processes including cell cycle progression, transcriptional regulation, generation of peptides presented on MCH class-I molecules, stress response, and regulation of apoptosis [8,30,31]. In mammalian cells, the 20S proteasome is a 700 kDa cylindrical structure composed of four heptameric rings that are made of seven different α-type subunits in each of the outer rings and seven different β-type subunits in each of the inner rings $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$. Three of the seven β subunits have the N-terminal threonine residue which acts as the catalytic nucleophile for peptide bond cleavage [32–34]. As observed from structural studies of the proteasome, the three active sites are located inside the central cavity and confer no less than three different cleavage capabilities, chymotrypsin-like (ChT-L), trypsin-like (T-L), and peptidylglutamyl-peptide hydrolase (PGPH) activities carried out by the β5, β2, and β1 subunits, respectively [33]. Protein translocation into the central catalytic chamber may be facilitated by hydrolysis of ATP by 19S (PA700) regulatory complexes. These complexes bind to the ends of the 20S proteasome to form the 26S proteasome that is involved in ATP-dependent proteolysis of ubiquitinated proteins. The 19S complex contains about 20 subunits, of which six are ATPases [9]. Alternatively, the 20S proteasome can bind PA28 (11S) complexes that are composed of two different α and β subunits, both of which are γ-interferon inducible [35]. In addition, the core 20S proteasome exhibit variable subunit compositions. There are 10 β subunit genes in animal cells, whereas only seven β subunits are incorporated into the structure. Three non-essential γ-interferon inducible catalytic β subunits ($i\beta_1$, $i\beta_2$, and $i\beta_5$) can replace their three closely related constitutive counterparts (respectively, β1, β2, and β5), to form what has been termed the immunoproteasome [36]. Thus, competition of the 10 different types of β subunits for integration into the seven-membered β-ring leads to the coexistence of multiple 20S proteasome subtypes within the cell. This heterogeneity in proteasome subtypes not only exists within a single cell, but also between different tissues, and alterations in the distribution of proteasome subtypes have been reported in various pathological states [37–40].

The current study was undertaken to characterize the effects of the lipid peroxidation product HNE on 20S proteasome structure and function. According to the potential variation of the core particle subunit composition, purified 20S proteasomes from both heart and liver were used to assess tissue-dependent differences. Following incubation of purified 20S proteasomes with HNE, changes in the 2D gel electrophoresis patterns and three peptidase activities of the proteasome were evaluated. Proteasome subunits were identified by mass spectrometry prior to and following treatment with HNE. We report here that specific subunits of the 20S proteasome are targeted for modification by 4-hydroxy-2-nonenal and that modified proteasome exhibits selective alterations in peptidase activities. Furthermore, we provide evidence that modification by HNE depends on the 20S core structure, as indicated by comparison of the 2D gel patterns obtained from heart versus liver, tissues that express different predominant proteasome subtypes. Finally, our in vitro results show that HNE targets subunits previously reported to be modified upon cardiac ischemia/reperfusion [6] suggesting that HNE modification constitutes an in vivo mechanism of proteasome inhibition.

Materials and methods

Preparation of cytosolic fractions

Wistar rats (3-month-old, ~300 g) were purchased from Charles Rivers. Following decapitation, the liver and heart were harvested, frozen in liquid nitrogen, and stored at −80 °C. Cytosolic fractions were then prepared from the frozen liver and heart. Tissues were minced, rinsed several times in ice-cold homogenization buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, and 0.5 mM DTT), and homogenized using a Polytron homogenizer (3 pulses of 20 s each, low setting). Homogenates were then centrifuged at 15,000*g* for 2 h at 4 °C. Supernatants were used for further studies.

Proteasome purification

The 20S proteasomes were purified from the cytosolic fractions obtained either from the liver or heart as previously described [41,42] with minor modifications. Briefly, 1 mM of 2-mercaptoethanol was added to pooled cytosolic fractions from liver or heart and then subjected to an ammonium sulphate cut (40 and 60% saturation). The 60% ammonium sulphate pellet was suspended in buffer containing 20 mM Tris–HCl, pH 7.2, 0.1 mM EDTA, and 1 mM 2-mercaptoethanol and dialyzed overnight at 4 °C against the same buffer. Purification was then achieved by a succession of three chromatographic steps utilizing a Beckman Gold HPLC system. The first two steps consisted of ion exchange chromatography using a DEAE column (TosoHaas) and a monoQ HR5/5 column (Amersham

Biosciences) and the third step utilized a gel filtration Superose 6 column (Amersham Biosciences).

Preparation of HNE modified 20S proteasomes

HNE was generated as described [21] and concentrations were determined by measurement of UV absorbance at 224 nm with a molar absorptivity of 13,750 M^{-1}. Purified 20S proteasome (30 μg) from either rat liver or heart was incubated in a final volume of 200 μl, in the presence of various concentrations of HNE (0–2.0 mM) for 15 min at 37 °C, in 25 mM KH_2PO_4, pH 7.4, buffer containing 100 mM KCl. Reactions were quenched by addition of 1.8 ml of 50 mM Tris–HCl, pH 7.4. Proteasomes were then recovered after an ultra-dialysis step (Centricon YM30, Millipore).

Indirect antibody sandwich ELISA for quantification of HNE-modifications

Polyclonal rabbit antibody (600 ng) specific to HNE-modified proteins [43] was adsorbed overnight at 4 °C, in each well of a 96-well microtiter plate (Nunc Maxisorb plate, Nunc). All the washes were done with PBS buffer (Sigma) containing 0.05% (v/v) Tween 20. Nonspecific fixation sites were saturated using Superblock buffer (Pierce). Purified HNE-treated 20S proteasomes (0.5–2 μg) were then incubated in the wells for 3 h at 25 °C. Immobilized HNE-modified proteasomes were incubated (1.5 h, 25 °C) in the presence of mouse monoclonal antibody (10 μg) raised against HNE modifications (Jaica, Japan). Following a final incubation with monoclonal goat anti-mouse antibody conjugated to horseradish peroxidase (1 μg, 1.5 h, 25 °C), Ultra TMB substrate (Pierce) was added and allowed to incubate for ~1 h, reactions were stopped by addition of H_2SO_4, and absorbance at 450 nm measured on an ELISA plate reader (SLT Rainbow Lab instruments).

Peptidase activities of proteasomes

Proteasome fluorogenic peptidase substrates: Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-AMC), N-t-Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (LSTR-AMC), N-Cbz-Leu-Leu-Glu-β-naphtylamide (LLE-NA) and a specific proteasome inhibitor N-Cbz-Leu-Leu-Leucinal (MG132) were obtained from Sigma. Peptidase activities were assayed in triplicate, using a mixture of 1–3 μg of purified native or HNE-modified proteasomes in 25 mM Tris–HCl, pH 7.5, containing the appropriate peptidase substrate (12.5 μM LLVY-AMC, or 40 μM LSTR-AMC, or 200 μM LLE-NA) in a final volume of 200 μl. Kinetic analyses were conducted (30 min) in a temperature-controlled fluorometric microplate reader (Fluostar Galaxy, bMG). Excitation/Emission wavelengths were 350/440 and 340/410 nm for AMC or NA products, respectively. Controls were performed in the presence of 20 μM MG132.

Two-dimensional gel electrophoresis and Western blot analysis

The first dimension was resolved utilizing Immobiline DryStrips (13 cm linear gradient, pH 3–10, Amersham Biosciences) rehydrated overnight with purified native or HNE-modified 20S proteasomes (25–100 μg) suspended in 200 μl of sample buffer (7 M urea, 2 M thiourea, 2% CHAPS, 2% pharmalytes, pH 3–10, 60 mM dithiothreitol, and bromophenol blue). Proteins were focused for 50,000 V h over 23 h at 18 °C. To prepare the Drystrips for the second dimension, the Drystrips were first equilibrated in a buffer containing 50 mM Tris–HCl, pH 8.8, 30% (v/v) glycerol, 2% (w/v) SDS, 6 M urea with 1% (w/v) DTT for 10 min at room temperature, followed by a second equilibration for 10 min using the same buffer except that DTT was replaced by 4% (w/v) iodoacetamide. Subsequently, the Drystrip was applied horizontally over a 15% SDS–polyacrylamide gel and embedded in 0.5% (w/v) agarose gel dissolved in electrophoresis buffer. Proteins were then separated vertically according to Laemmli [44] at a constant voltage for 1700 V h using a Protean II electrophoresis system (Bio-Rad). The resulting two-dimensional gel was either stained with colloidal blue (Sigma), or electroblotted onto a PVDF membrane (Amersham Biosciences). PVDF membranes were probed with polyclonal anti-HNE adducts antibody. Stained gels were digitized with a JX-330 scanner (Sharp), and spot detection was done performed using Imagemaster 2 D elite software (Amersham Biosciences). Spots were then excised and used for further mass spectrometric analysis.

In-gel trypsin digestion

Protein spots were in-gel digested either manually or using a Genesis Protean 150 instrument (Tecan Group). Proteins were in-gel reduced in 10 mM DTT and alkylated with 55 mM iodoacetamide in 50 mM ammonium bicarbonate. The gel spots were washed by alternatively dehydrating gel pieces with acetonitrile and rehydrating them with ammonium bicarbonate buffer 50 mM. They were finally dehydrated in acetonitrile and dried (in a SpeedVac manually or in an incubator at 56 °C) for 15 min. Gel pieces were further rehydrated for 15 min at room temperature in a 0.5 mM solution of modified bovine trypsin (Roche, sequencing grade, EC 3.4.21.4) and samples were incubated for 2 h at 37 °C. Adding 1% aqueous formic acid stopped the reaction and digests were desalted using Zip-Tips™ C18 (Millipore) and stepwise eluted in 50 and 100% acetonitrile. The desalted peptide mixture was concentrated to 2 μl in 1.0% aqueous formic acid. Peptides were then analysed by either LC-MS/MS or MALDI-TOF–TOF.

LC-MS/MS analyses

Liquid chromatography-tandem mass spectrometric (LC-MS/MS) analyses were performed with an electro-

spray ion-trap mass spectrometer (LCQ Advantage; ThermoElectron) coupled on-line with a Surveyor HPLC system (ThermoElectron). A 150 × 0.18 mm ThermoElectron HyPURITY C18 column (5 μm particle diameter; 190 Å pore size) with a mobile phase of solvent A [0.1% (v/v) formic acid in water] and solvent B [0.1% (v/v) formic acid in 80% (v/v) acetonitrile] was used with a linear gradient of 2–60% of mobile phase B over 60 min at a flow rate of 250 μl/min. The flow was split and 2 μl/min was directed to the column. The electrospray needle was operated with a voltage of 2.5 kV, and the heated desolvation capillary was held at 180 °C. Nitrogen was used as the sheath gas. All scans were acquired in positive ion mode. The mass spectrometer was operated in a data-dependent MS/MS mode. The top three most intense ions were selected from the full MS scan (*m*/*z* range 300–2000) for MS/MS analysis. The isolation width of the parent ions was set to 2 *m*/*z* units with 35% normalized collision energy. An *m*/*z* ratio for an ion that had been selected for fragmentation was placed in a list and dynamically excluded from further fragmentation for 1 min. Proteins were identified automatically by the computer program TurboSEQUEST (ThermoElectron).

MALDI-TOF–TOF MS

Sample spots were prepared following the dried droplet procedure with cyano-hydroxycinnamic acid (CHCA) at 4.0 mg/ml in acetonitrile/H_2O/TFA (60:40:0.1, v/v/v) with 10 mM diammonium citrate. Sample (0.3 μL) and matrix solution (0.6 μL) were mixed on stainless steel target and let dry at room temperature. Spectra were acquired on a tandem MALDI-TOF–TOF mass spectrometer TM 4800 (Applera Applied Biosystems Inc.). MS and MS/MS analyses were performed in positive reflectron mode with an accelerating voltage at 20 kV in first source 1, and 2 kV collision energy for fragmentation. Scans (2000) were averaged on the mass range 500–3600 Da. Data were processed with the GPS Explorer™ (Applied Biosystems) software and search against mammalian MSDB database using embedded Mascot software (MatrixScience). External calibration was performed with $[M+H]^+$ species of the calibration mixture Proteomix Peptide mix 4 (LaserBioLabs) namely containing clip 1–5 of Bradykinin (573.315 Da), human angiotensin II (1046.542 Da), neurotensin (1672.918 Da), clip 18–39 of ACTH (2465.199 Da), and oxidized B chain of insulin (3494.651 Da).

Results

Modification of proteasome by 4-hydroxy-2-nonenal

The 20S proteasome was purified from rat liver and incubated for 15 min at 37 °C with varying concentrations of HNE. As shown in Fig. 1, HNE adduction to the 20S proteasome was proportional to HNE concentrations up to

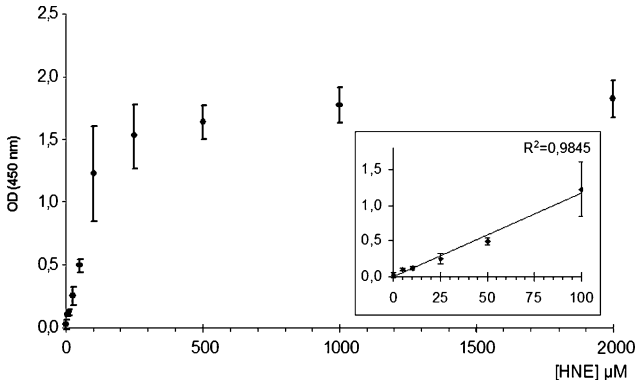

Fig. 1. Concentration-dependent modification of the proteasome by HNE. The 20S proteasome purified from rat liver was incubated for 15 min with various concentrations of HNE (0–2.0 mM). The formation of HNE adducts was monitored by double sandwich ELISA using a polyclonal and a monoclonal antibody recognizing HNE-protein adducts as described in Material and methods. Three independent experiments were conducted and assayed in triplicate.

100 μM. At HNE concentrations greater than 100 μM, the level of modification did not increase indicating that all HNE reactive sites had undergone modification. The same profile was observed with proteasome purified from rat heart (data not shown). To determine whether all proteasome subunits are susceptible to modification or, as previously observed after cardiac ischemia/reperfusion [6], certain subunits are more prone to HNE modification, the 2D gel electrophoresis patterns of proteasome subunits were assessed before and after treatment with 500 μM HNE for 15 min at 37 °C. As shown in Fig. 2, incubation of the proteasome with HNE resulted in the appearance of six new protein spots immediately located on the acidic side of

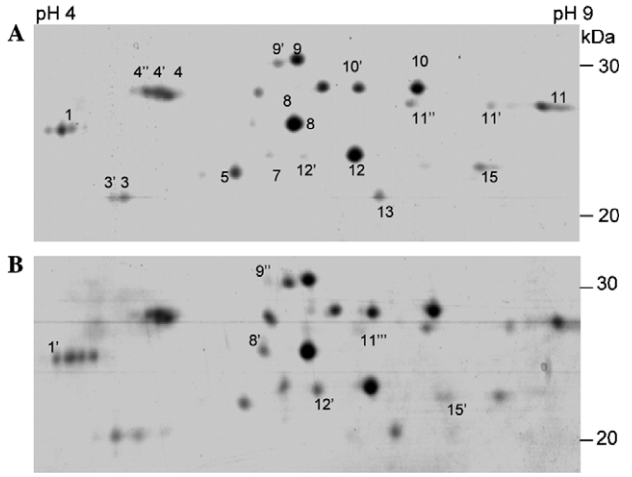

Fig. 2. Effect of HNE modification of heart proteasome on 2D gel electrophoresis subunit distribution. The 20S proteasome purified from rat heart was incubated in the presence or absence of 500 μM HNE for 15 min at 37 °C. The proteasome was then subjected to 2D gel electrophoresis as described in Material and methods and the resulting 2D gel was stained with colloidal blue. (A) Untreated proteasome and (B) proteasome treated with HNE. The protein spots (or group of spots as indicated by an apostrophe following the subunit number) are numbered from 1 to 16. Each sample was evaluated in three separate analyses.

specific proteasome subunits, suggesting that these subunits were susceptible to HNE modification. Separation of proteasome subunits by 2D gel electrophoresis followed by Western blot analysis using anti-HNE antisera indicated that atleast three of the six new protein spots were modified by HNE (data not shown).

Effects of HNE modification on proteasome peptidase activities

Since results of previous studies indicated that HNE is a potent inhibitor of proteasome [11,29,45], we next addressed whether the observed modifications of 20S proteasome subunits were accompanied by alterations in proteasome peptidase activities. Incubation of the proteasome purified from rat liver with HNE for 15 min at 37 °C resulted in inactivation of all three peptidase activities (Fig. 3A). Chymotrypsin-like activity was significantly reduced at HNE concentrations as low as 10 μM while 100 and 500 μM of HNE was required to affect trypsin-like and the peptidylglutamyl peptide hydrolase activities, respectively. The proteasome purified from rat heart exhibited a different peptidase HNE inactivation profile. As shown in Fig. 3B, the trypsin-like activity was more sensitive to HNE than the chymotrypsin-like and the peptidylglutamyl peptide hydrolase activities. Trypsin-like activity was significantly decreased at concentrations of HNE as low as 50 μM, whereas HNE concentrations greater than 100 and 500 μM were required to inhibit chymotrypsin-like and the peptidylglutamyl peptide hydrolase activities, respectively. The 2D gel electrophoresis pattern obtained for rat liver proteasome subunits (Fig. 4A) was different than that obtained for proteasome from rat heart (Fig. 2A). In addition, treatment of liver proteasome with HNE (Figs. 4B and C) resulted in the appearance of seven new protein spots immediately located on the acidic side of specific proteasome subunits, three of which were identical to those observed after HNE treatment of heart proteasome (Fig. 2B). Taken together, these results indicate that the HNE inactivation profile of proteasome peptidase activities

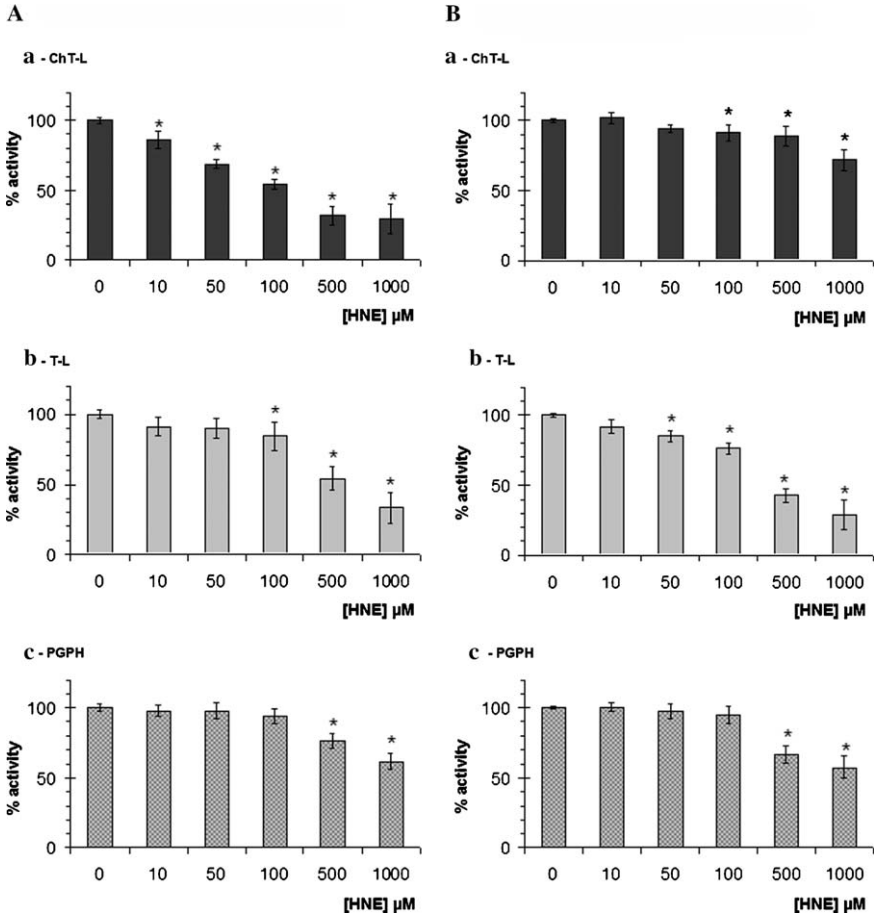

Fig. 3. Differential effects of HNE on proteasome peptidase activities. Proteasome peptidase activities chymotrypsin-like (ChT-L), trypsin-like (T-L), and peptidylglutamyl peptide hydrolase (PGPH) were assayed in triplicate using a mixture of 1.0–3.0 μg of purified native or HNE-modified proteasome in 25 mM Tris–HCl, pH 7.5 containing the appropriate peptidase substrate (12.5 μM LLVY-AMC, or 40 μM LSTR-AMC, or 200 μM LLE-NA) in a final volume of 200 μl. Peptidase activities were measured for 30 min in a temperature controlled fluorometric microplate reader (Fluostar Galaxy, bMG) as described in Material and methods. Activities are presented as percentage of values obtained for native proteasome. (A) Liver proteasome activities and (B) heart proteasome activities with a: chymotrypsin-like; b: trypsin-like; and c: peptidylglutamyl peptide hydrolase peptidase activities. For all conditions tested values represent the mean ± SEM ($N = 5$). p values (Student t test) for the different concentrations of HNE versus no HNE: * $\leqslant 0.05$.

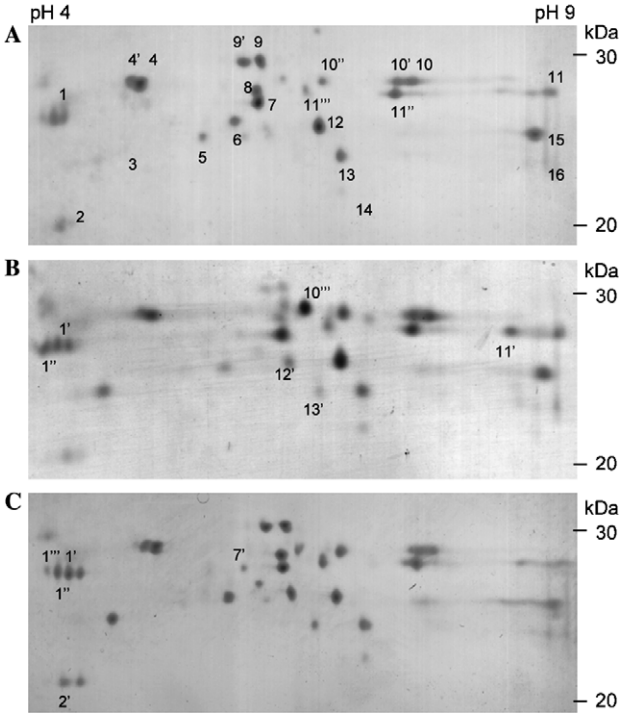

Fig. 4. Effect of HNE modification of liver proteasome on 2D gel electrophoresis subunit distribution. The 20S proteasome purified from rat liver was incubated in the presence or absence of HNE for 15 min at 37 °C. The proteasome was then subjected to 2D gel electrophoresis as described in Material and methods and the resulting 2D gel was stained with colloidal blue. (A) Untreated proteasome, (B) proteasome treated with 50 μM HNE and (C) proteasome treated with 500 μM HNE. The protein spots (or group of spots as indicated by an apostrophe following the subunit number) are numbered from 1 to 16. Each sample was evaluated in three separate analyses.

is dependent on the proteasome source and the subunit composition.

Identification of HNE-modified proteasome subunits

The 20S proteasome purified from both rat liver and heart were subjected to 2D gel electrophoresis (Figs. 2 and 4) before and after treatment with HNE and identification of all protein spots was achieved by mass spectrometry (Table 1). It should be noted that the 20S proteasome 2D gel electrophoresis patterns exhibited a slightly greater number of protein spots than the 17 expected from mammalian proteasome (including the 14 constitutive subunits and the 3 inducible ones). As previously reported, individual proteasome subunits may appear as several protein spots at the same apparent molecular weight but at differing p*I* values [46]. This phenomenon has been attributed to post-translational modifications such as phosphorylation, glycosylation, or acetylation that alter the global protein charge. Identification of protein spots by mass spectrometry did confirm the existence of more than one spot for certain of the proteasome subunits (Table 1). From the comparison of the patterns of HNE treated 20S proteasome with those of the controls, spots observed in purified proteasome preparations were also seen in the patterns of HNE treated proteasomes. As already mentioned, treatment of 20S proteasome with HNE resulted in the appearance of new protein spots. As reported in Table 1, identification of these spots by mass spectrometry indicated these spots originated from the modification of a restricted number of proteasome subunits. The HNE modified

Table 1
Identification of liver and heart proteasome subunits by mass spectrometry prior to and following HNE treatment

20S subunit[a]	Liver[b]				Heart[c]			
	Spot number		# peptide	Coverage[d] (%)	Spot number		# peptide	Coverage[d] (%)
	Without HNE	500 μM HNE			Without HNE	500 μM HNE		
α1	8	NM	3	10	8	8, 8′	11	44
α2	12	12, 12′	9	34.2	12	12, 12′	15	46
α3	10–10″	10–10‴	8	18.8	10, 10′	NM	11	40
α4	11–11″	11–11‴	3	10	11–11″	11–11‴	10	36
α5	1–1′	1–1⁗	3	20.8	1–1″	1–1‴	NI	NI
α6	9, 9′	NM	9	21.3	9, 9′	9–9″	15	60
α7	4, 4′	NM	5	22.5	4–4″	NM	NI	NI
β1	3	NM	2	10.5	3, 3′	NM	4	18
β2	6	NM	4	8	NI	NI	NI	NI
β3	7	7, 7′	6	26.6	7	NM	1	7
β4	13	13, 13′	4	18	13	NM	19	52
β5	16	NM	3	15	NI	NI	NI	NI
β6	15	NM	4	13.8	15	15, 15′		
β7	5	NM	1	5	5	NM	8	29
iβ1	2	2, 2′	2	15.5				
iβ5	14	NM	1	5				

20S proteasome subunits of liver and heart identified by mass spectrometry before and after HNE treatment.
NM, non-modified; NI, non-identified.
[a] Proteasome subunits were named according to Groll et al. [33].
[b] Liver 20S proteasome subunits were identified by LC-MS/MS.
[c] Heart 20S proteasome subunits were identified by MALDI-TOF–TOF MS.
[d] Coverage referred to the non modified subunit.

subunits exhibited no change in their apparent molecular mass, whereas their p*I* was shifted to a more acidic one. Liver 20S proteasome treated with 50 μM HNE were primarily modified on subunits α2, α3, α4, α5, and β4. Upon treatment with 500 μM HNE, the number of modified subunit increased, and subunits β3 and iβ1 were also modified. Conversely, heart 20S proteasome appeared to be modified by HNE on subunits α1, α2, α4, α5, α6, and β6. Differences between liver and heart were evident with α3, iβ1, β3, and β4 subunits modified exclusively in proteasome from liver while α1, α6, and β6 subunits were modified exclusively in proteasome purified form heart. Thus, it appears that differences in subunit composition confer differential susceptibility to HNE modification and peptidase inactivation. Indeed, incorporation of inducible subunits into the core particle provokes changes in core structure and may modify HNE accessibility.

Discussion

Modification of the proteasome by the lipid peroxidation product 4-hydroxy-2-nonenal (HNE) has been observed in several pathophysiological situations including cardiac ischemia/reperfusion, cerebral ischemia, and aging [6,14,47]. Since these modifications have been associated with inactivation of the proteasome, the aim of the study was to characterize at both structural and functional levels the effects of HNE on proteasome purified from two different tissues, heart and liver. Trypsin-like activity was more susceptible to inactivation in the case of heart proteasome, while the chymotrypsin-like activity was primarily inactivated in the case of liver proteasome. Inactivation of proteasome from both liver and heart was associated with the modification of a restricted number of specific proteasome subunits that exhibited different patterns of modification depending on the proteasome source. Upon HNE modification, the 2D gel electrophoresis pattern was modified for seven liver proteasome subunits that have been identified as α2, α3, α4, α5, β3, β4, and iβ1 and six heart proteasome subunits that have been identified as α1, α2, α4, α5, α6, and β6. These findings are most likely due to the fact that, as evidenced by 2D gel electrophoresis of the purified proteasome preparations, the proteasome subtype composition is different in heart and liver. In a previous study [29], Ferrington and Kapphahn working with rat liver proteasome have reported by means of immunochemical detection of HNE adducts that the α6 subunit was the primary target of HNE modification and that this modification was associated with the rapid inactivation of the chymotrypsin-like peptidase activity. Our results are in agreement that the chymotrypsin-like activity is most sensitive to HNE inactivation for proteasome from liver. Nevertheless, the current study did not detect modification to the α6 subunit but a shift of isoelectric point of seven of the subunits was observed indicating modifications. As pointed out in the earlier study [29], the immunochemical detection may have missed modification on other subunits while the presence of Hsp 90 in their proteasome preparation may also have prevented modification of certain subunits as well as inactivation of trypsin-like peptidase activity. Indeed, we have previously reported that Hsp 90 can protect trypsin-like activity from inactivation upon both incubation with HNE and oxidative treatment with iron and ascorbate [45,48]. In addition, Hsp 90 binding to the proteasome may render the chymotrypsin-like activity more prone to HNE inactivation.

In the present study, although the modification of more than one subunit was observed, only a restricted number of subunits were found to be modified by HNE, indicating that HNE modification is not a random process and that specific subunits are targets for HNE. With the noticeable exception of the liver proteasome iβ1 subunit which is modified at 500 μM HNE, the subunits targeted by HNE were either α or non-catalytic β subunits suggesting an indirect mechanism for the observed inactivation of proteasome peptidase activities rather than a direct modification of catalytic β subunits. The proteasome peptidase activity inhibition profile was different depending on the tissue from which the proteasome had been purified indicating that incorporation of inducible subunits into the proteasome particle may provoke changes in its structure, and may modify HNE accessibility and hence the relative susceptibility of the different peptidase activities to inactivation by HNE. As observed for α5, two or three new spots representing HNE modified isoforms were observed following HNE treatment suggesting that this subunit is particularly sensitive to HNE, and possess several modification sites that reacted with HNE.

The proteasome has been implicated in the removal of oxidatively modified proteins following oxidative stress [10,11,49]. Therefore, impairment of proteasome function through inactivation of its peptidase activities by HNE may contribute to the build-up of not only oxidized but also ubiquitinated proteins and impact ubiquitin-dependent intracellular signalling pathway such as degradation of IκB and subsequent activation of NFκB [50,51]. Moreover, proteasome inhibition has been shown to promote induction of apoptosis [52–54]. In a previous study, we have shown that proteasome peptidase activities are decreased during cardiac ischemia/reperfusion and that, after purification of the proteasome, only the trypsin-like activity remained significantly reduced while peptidylglutamyl peptide hydrolase and chymotrypsin-like activities recovered [6]. This recovery is indicative of the presence in the cytosolic fraction of inhibitory and/or competing substrate proteins that are removed during purification. Indeed, heavily damaged proteins such as HNE cross-linked proteins have been shown to be resistant to proteolysis and to act as potent inhibitors of the proteasome [55,56]. That trypsin-like peptidase activity is the sole activity found to be impaired after proteasome purification following ischemia/reperfusion is consistent with our observation that this activity is the most susceptible to HNE inactivation of heart proteasome in vitro. In addition, three (α1, α2, and α4) of the six subunits modified

upon HNE treatment of heart proteasome (α1, α2, α4, α5, α6, and β6) exhibited HNE modification after ischemia/reperfusion [6]. Taken together, these results indicate that the observed inactivation of the trypsin-like peptidase activity of the proteasome during cardiac/ischemia reperfusion is most likely the consequence of the modification of specific proteasome subunits by HNE.

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