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Comparison of rat liver and brain proteasomes for oxidative stress-induced inactivation: Influence of ageing and dietary restriction

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Abstract

The present study examined brain and liver derived proteasome complexes to elucidate if there is a differential susceptibility in proteasome complexes from these tissues to undergo inactivation following exposure to oxidative stressors. It then examined the influence of ageing and dietary restriction (DR) on the observed proteasome inactivation. Studies used a filtration based methodology that allows for enrichment of proteasome complexes with less tissue than is required for traditional chromatography procedures. The results indicate that the brain has much lower levels of overall proteasome activity and exhibits increased sensitivity to hydrogen peroxide mediated inactivation as compared to proteasome complexes derived from the liver. Interestingly, the brain proteasome complexes did not appear to have increased susceptibility to 4-hydroxynonenal (HNE)-induced inactivation. Surprisingly, ageing and DR induced minimal effects on oxidative stress mediated proteasome inhibition. These results indicate that the brain not only has lower levels of proteasome activity compared to the liver, but is also more susceptible to inactivation following exposure to some (but certainly not all) oxidative stressors. This data also suggest that ageing and DR may not significantly modulate the resistance of the proteasome to inactivation in some experimental settings.

Keywords: *4-hydroxynonenal, ageing, brain, liver, oxidative stress, proteasome*

Abbreviations: *AL,* ad libitum*; DR, dietary restriction; NIA, National Institute of Aging; ROS, reactive oxygen species*

Introduction

The proteasome proteolytic system is responsible for degradation of oxidized and ubiquitinated proteins in both the nucleus and cytoplasm [1–3]. Structurally the proteasome is a multiprotein complex that consists of a catalytic core, the 20S proteasome, with additional cap-like structures capable of binding to the 20S to form a 26S proteasome complex. The 19S (PA700) and 11S cap-like structures are the best characterized of the 26S proteasome cap structures [4–8]. The beta sub-units of eukaryotic proteasome exhibit multiple endopeptidase activities including chymotrypsin-like (ChT-L), trypsin-like (T-L) and peptidylglutamyl-peptide hydrolase (PGPH) activities. These peptidase activities mediate the hydrolysis of proteins at hydrophobic, basic and acidic residues, respectively [1,9,10].

The proteasome is involved in a variety of cellular processes including regulating the level of misfolded

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and damaged proteins [11,12], regulating cell cycle progression [13,14], transcriptional regulation [15,16], modulating apoptosis [17] and contributing to the regulation of both immune and stress responses [18–20]. Inhibition of the proteasome complexes has been shown to occur in ageing tissues and age-related neurodegerative diseases [21–26]. The observed age-related decreases in proteasome activity, in both *in vitro* and *in vivo* settings, are believed to be mediated by several individual and/or co-ordinated effects of ageing. For example, studies have demonstrated a potential role for proteasome inhibition occurring as the result of increased levels of oxidized and/or aggregated proteins [27], increased oxidative modifications to the proteasome complexes [23,28–36] and deleterious changes in proteasome composition [25,29]. Inhibition of proteasome in both ageing and age-related diseases are believed to contribute to cellular dysfunction and further cellular deterioration [26,37,38]. This is based largely on the fact that proteasome inhibition is sufficient to recapitulate many aspects of ageing and age-related disease [39–41] and the observation that increases in proteasome levels is sufficient to extend cellular lifespan and increase resistance to a variety of stressors [42–46]. Understanding how the proteasome is modulated in conditions known to increase life span, including dietary restriction (DR), remains a critical and poorly defined area of research on the proteasome and ageing.

DR has been shown to increase life span and retard the age-related decline of several physiological functions and age-related pathologies [47]. DR was found to reduce the levels of oxidatively damaged proteins in liver [48,49] and increase the resistance of neurons to age-related stressors [50]. DR has been linked to the regulation of proteasome function, most notably increasing the basal levels of overall proteasome activity in the ageing rat liver [47] and muscle [51]. At present, it is unclear whether DR modulates stress-induced inactivation of proteasome complexes, which could potentially serve as a mechanism by which DR promotes beneficial effects on proteasome function. Cumulatively our data indicate that the brain has much lower levels of proteasome activity as compared to the liver and is much more vulnerable to inactivation following exposure to pro-oxidative stress environment, which together may have important implications to understanding the relative vulnerabilities of the brain and liver to toxicity following exposure to stressors. Because ageing and DR had minimal effects on each of these analyses, our data raise interesting questions with regards to how ageing and DR may promote changes in proteasome-mediated protein degradation in the brain and liver.

Materials

The substrates Boc-Leu-Arg-Arg-AMC (PGPH activity) and Suc-Leu-Leu-Val-Tyr-AMC (ChT-L activity) were purchased from Bachem America's Inc., (Torrance, CA). The substrate Boc-Leu-Ser-Thr-Arg-7-Amido-4-methylcoumarin (T-L activity) was purchased from Sigma Aldrich Company (St Louis, MO). The YM-100 centricon filters were purchased from Millipore Corporation (Bedford, MA). 4-Hydroxy-2-nonenal (HNE) was purchased from Cayman Chemical Company (Ann Arbor, MI). All other items were purchased from Sigma Aldrich Inc. (St. Louis, MO).

Animal tissues

Male *Helicobacter*-free F344/Brown Norway (F344 x BN F1) rats were obtained from the NIA Dietary Restriction (DR) colony. The rats in this study consisted of six 3-month old *ad libitum* (AL), six 25-month-old AL and six 25-month-old DR rodents. The DR and AL rodents from the present study were individually housed. DR was initiated at 14 weeks of age at 10% restriction, increased to 25% restriction at 15 weeks and to 40% restriction at 16 weeks, where it was maintained throughout the life of the animal. All animals were handled and euthanized in accordance with IACUC approved protocols at the Pennington Biomedical Research Center.

Filtration of tissue lysates to remove low molecular weight proteases

Liver and brain tissues were homogenized in lysis buffer containing 20 mm Tris-HCl pH-7.4, 5 mm Magnesium chloride, 2 mm ATP, 100 mm NaCl and 0.5 mm DTT, as described previously [52]. Lysates were centrifuged at 25 000 x *g* for 45 min. The supernatant obtained was diluted with 1 volume of lysis buffer and filtered through the YM-100 centricon filters to remove low molecular weight proteases. The proteins remaining on the filter were collected (here forth referred to as retentate), subjected to protein assays and then utilized for further proteasome activity assays.

In-gel proteasome activity assay

The activity of the proteasome in the retentate was analysed using 4% Native-PAGE, essentially as described by Glickman et al. [53]. The protein samples were run on Native PAGE at 120 V for $\sim$ 3 h at 4°C. The proteasome activity was visualized by incubating the gels in activity buffer containing 20 mm Tris-Cl pH-7.8, 2 mm ATP, 5 mm KCl, 5 mm Magnesium chloride, 0.5 mm DTT and 100 μm of Suc-Leu-Leu-Val-Tyr-AMC (substrate for ChT-L enzyme activity). Following 30 min incubation the 26S proteasome

bands were visualized under a UV light source at a wavelength of 360 nm. To visualize the 20S proteasome activity, the same gel was incubated in the activity buffer (without ATP) containing 0.02% SDS and visualized under a UV light source.

Assays for proteasome activity and composition

Assays for proteasome activity were performed as described previously by our laboratory [52] using the fluorogenic substrates for PGPH, ChT-L and T-L activities. The reaction was conducted in 250 μl of activity assay buffer within a 96-well black assay plate with clear bottom containing 50 μg/ml of liver or brain retentate, 20 mm Tris–HCl, pH 7.8, 1 mm EDTA, 0.5 mm DTT and 5 mm $MgCl_2$, 2 mm ATP and 50 μm of the corresponding substrate. Samples were then administered the indicated concentrations of H_2O_2 or HNE. The reaction mixture was incubated for 1 h at 37°C and the fluorescence of the released AMC product was measured in Molecular Devices plate reader at an emission wavelength of 355 nm and an excitation wavelength of 460 nm. The background fluorescence values obtained by incubating the lysates with MG132 were subtracted from activity values as described previously by our laboratory [52]. The proteasome activity per mg of the brain or liver protein per hour was calculated from the fluorescence values and all subsequent data expressed as percentage control values. All the values in the figures were represented as the average of six sets of tissues from each age group. Western blotting was used to determine potential changes in proteasome composition. These analyses were conducted using the anti-20S core antibody from Calbiochem which recognizes multiple sub-units in the core 20S complex (α5, α7, β1, β5i, β7). Equal loading was confirmed using Coomassie staining.

Results

Analysis of 26S and 20S proteasome activity present in 100 kDa cut-off filtrate

Because of the limited availability of tissues to utilize for ageing and DR studies, we sought to establish a method that allowed for the analysis of proteasome activity in small amounts of tissue, which currently is not possible with chromatography procedures that are commonly utilized by our laboratory and others. Additionally, we wanted to develop an assay that allowed for the monitoring of proteasome complexes without the presence of free-proteasome sub-units, which are capable of generating background peptidase activity in commonly utilized peptide-based assays of proteasome activity. To this end we conducted studies in which liver lysates were generated and filtered using filter sets that had a 100 kDa cut-off and thus allowed for free proteasome sub-units (< 40 kDa) to be separated from proteasome complexes. When we subjected the crude lysate, 100 kDa retentate or filtrate to in-gel analysis of proteasome activity and observed the presence of significant 26S (ATP stimulated) and 20S (SDS stimulated) proteasome activity in the crude lysate and retentate (Figure 1), that was not observed in the filtrate. Analysis of retentates from liver and brain revealed that the majority peptidase activity was inhibited by the proteasome inhibitor MG-132 (Figure 2), consistent with the majority of peptidase activity being mediated by proteasome complexes in the retentate. Interestingly, the liver was observed to have nearly twice the amount of proteasome activity as compared to the brain (Figure 2A). Additionally, Western blot analysis of the 20S core proteasome from the liver retentate was observed to have a different profile of proteasome sub-units, as compared to the brain retentate. These data are consistent with a difference in composition of the proteasome complexes from these different tissues.

Effects of hydrogen peroxide on liver and brain proteasome activity

Incubation of both liver and brain proteasome complexes with hydrogen peroxide induced a dose-dependent inhibition of ChT-L, T-L and PGPH activities of the proteasome (Figure 3). Brain proteasome

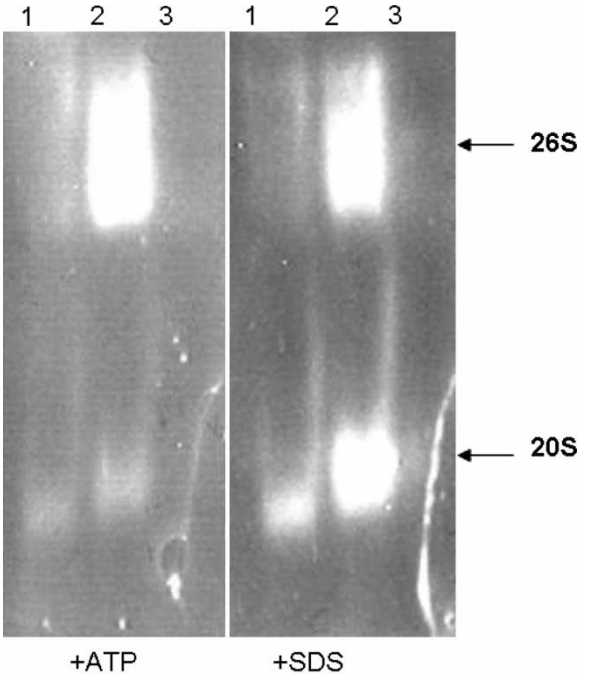

Figure 1. In-gel assay for peptidase activity of the proteasome. Liver lysates from 3 month-old rats were analysed for proteasome activity presence in the crude homogenate (1), > 100 kDa retentate (2) and filtrate (3). Equal concentrations of the samples were separated on a 4% native PAGE gel and assayed for the presence of 26S (ATP stimulated) and 20S (SDS stimulated) proteasome complexes via visualization of ChT-L activity of the proteasome. These data show an enrichment of proteasome complexes in the retentate relative to the homogenate or filtrate.

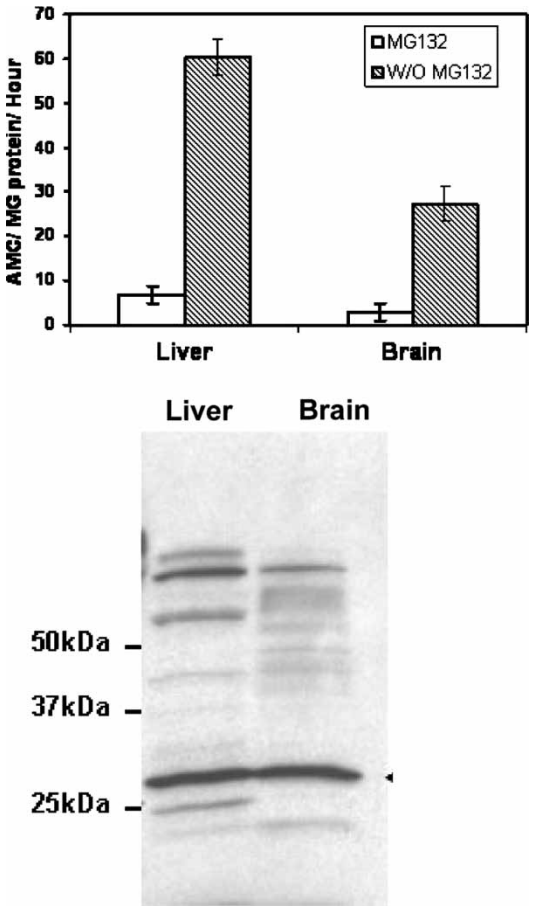

Figure 2. Analysis of brain and liver proteasome activity in the retentate fractions. (A) The liver and brain retentates from 3 month-old rats were analysed for the rates of ChT-L activity in the presence and absence of the proteasome inhibitor MG132. These data demonstrate that the liver possesses significantly higher levels of proteasome activity relative to the brain and that MG132 suppresses the majority of observed peptidase activity consistent with the involvement of the proteasome in observed peptidase activity. (B) Western blot analysis of equal amounts of liver and brain retentates using 4–20% SDS-PAGE and an antibody which recognizes the core 20S complex (Calbiochem Inc.) demonstrates that the liver retentate has a more diverse profile of proteasome sub-units as compared to the brain retentate. All the values in the graphs represent the average of six sets of tissues from each age group.

complexes exhibited dramatic sensitivities to hydrogen peroxide mediated inactivation of T-L, ChT-L and PGPH activities (Figure 3), as compared to the liver. In the presence of hydrogen peroxide, liver proteasomes showed relative IC_{50} values of 0.729, 0.429 and 0.644 for T-L, ChT-L and PGPH, respectively. In the presence of hydrogen peroxide, brain proteasomes showed relative IC_{50} values of 0.006, 0.184 and 0.015 for T-L, ChT-L and PGPH activities, respectively. Interestingly, extremely low levels of hydrogen peroxide were observed to stimulate chymotrypsin-like activity selectively in proteasome complexes from the brains of 3- and 25-month old rodents (Figure 3). In our overall analyses, ageing and DR did not exhibit significant effects on the amount of hydrogen peroxide-induced inactivation of the proteasome peptidase activities.

Effects of HNE on liver and brain proteasome activity

Incubation with the lipid peroxidation product HNE inhibited each of the peptidase activities of both liver and brain proteasome complexes (Figure 4) and did so in a dose-dependent manner. Interestingly, brain ChT-L activity was observed to exhibit increased resistance to HNE-induced inactivation (Figure 4) as compared to the liver. Brain and liver proteasome complexes exhibited similar levels of HNE-induced inactivation of T-L-like and PGPH activities (Figure 4). In the presence of HNE, Liver proteasomes showed relative IC_{50} values of 129, 215, 217 for T-L, ChT-L and PGPH activities, respectively. Brain proteasomes showed approximate relative IC_{50} values of 72, 280 and 290 for T-L, ChT-L and PGPH activities, respectively. Extremely low levels of HNE were observed to selectively stimulate PGPH activity in proteasome complexes from the brain (Figure 4). In our overall analyses, ageing and DR did not exhibit significant effects on the overall effects of HNE on proteasome peptidase activities.

Discussion

The proteasome contributes to multiple cellular processes ranging from proliferation, differentiation and apoptosis [1,3,18,54]. Accumulation of abnormal (oxidized, unfolded, cross-linked, glycated) proteins is known to occur in mammalian cells during both *in vitro* and *in vivo* ageing due a toxic combination of elevated levels of reactive oxygen species (ROS), decreased removal of and repair of damaged molecules and decreased replacement of essential molecules [30,35,55,56]. Cumulatively, these events promote the development of oxidative stress. Oxidatively damaged proteins may cross-link each other, form aggregates which can disrupt cellular homeostasis and accumulate in cells and tissues resulting in impaired cellular functioning [57]. The brain contains high levels of polyunsaturated fatty acids, which upon oxidation can form neurotoxic lipid peroxidation products including HNE [58,59]. Post-mitotic neurons injured by oxidative stress cannot be readily replaced, potentially compounding the effects of oxidative injury over a lifetime and potentially leading to a number of neurological disorders. Chronic alcohol use induces the oxidative stress in liver via the inflammatory response of Kupffer cells and other types (macrophages, neutrophils, lymphocytes) in response to elevated gut-derived endotoxin plasma levels [60]. This leads to amplified formation of ROS and cell-toxic or profibrogenic cytokines including TNF and TGF which promote fibrogenesis, hepatocellular carcinoma and liver injury [61,62]. However

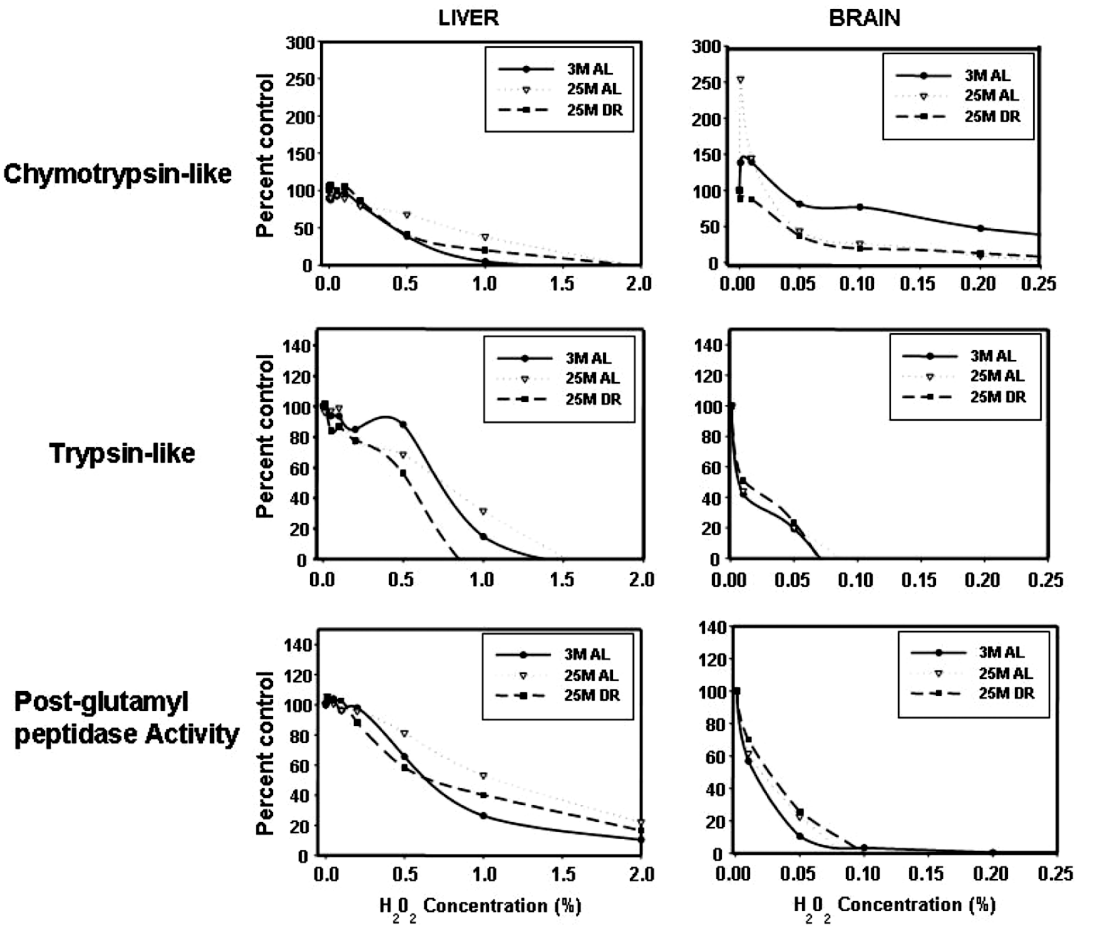

Figure 3. Effect of H_2O_2 on ChT-L, T-L and PGPH activities of the proteasome. The liver and brain retentates from 3 month-old AL, 25 month-old AL and 25 month-old DR rats were analysed for proteasome inactivation following treatment with increasing concentrations of H_2O_2. The effect of H_2O_2 on ChT-L, T-L and PGPH proteasome activities were analysed. All the values in the graphs represent the average of six sets of tissues from each age group.

it is clear that for the liver to undergo such toxicity there must be both high levels of ROS and a chronic exposure to ROS. It appears from current studies that the brain is much less capable of withstanding such prolonged periods of oxidative stress. As such, while oxidative stress is linked to ageing and toxicity in the liver and brain, it appears that the liver is much more resistant to the toxicity of oxidative stress as compared to the brain. This may be due to the high mitotic capacity of the liver relative to the brain, which allows it to regenerate damaged cells, but may also be due to other factors. Our data suggest that elevated levels of proteasome activity in the liver (relative to the brain) may potentially play a role in promoting the ability of the liver to withstand periods of exposure to oxidative stress relative to the brain. By having increased levels of proteasome activity the liver could potentially be able to remove deleterious proteins which are generated as the result of exposure to oxidative stressors. In addition to having increased levels of proteasome activity, our studies indicate that liver proteasomes are able to sustain their proteolytic activity in the presence of elevated levels of hydrogen peroxide, consistent with a model whereby enhanced preservation of proteasome activity contributes to viability of liver following exposure to stressors.

Our data may have important implications for understanding why the brain is so susceptible to cell loss and dysfunction in response to oxidative stress injury and age-related diseases of the nervous system. Having a predisposition to have proteasome activity lowered to a toxic level, via reduced basal levels of activity and increased sensitivity to hydrogen peroxide, the brain is extremely vulnerable to the toxic effects of some endogenous proteasome inhibitors. For example, hydrogen peroxide rapidly inactivates brain proteasomes relative to the proteasomes found in liver. Such inhibition of the proteasome could thereby promote further disturbances in cellular and tissue homeostasis and contribute to the ageing and cell dysfunction. However, the fact that brain proteasomes are not more vulnerable to HNE-induced inactivation suggests that brain proteasomes may be able to perform at a higher level in the face of some selective forms of ROS or lipid peroxidation products. It is interesting to note that previous studies from our laboratory demonstrated that neural cells were able to maintain viability and proteasome function following

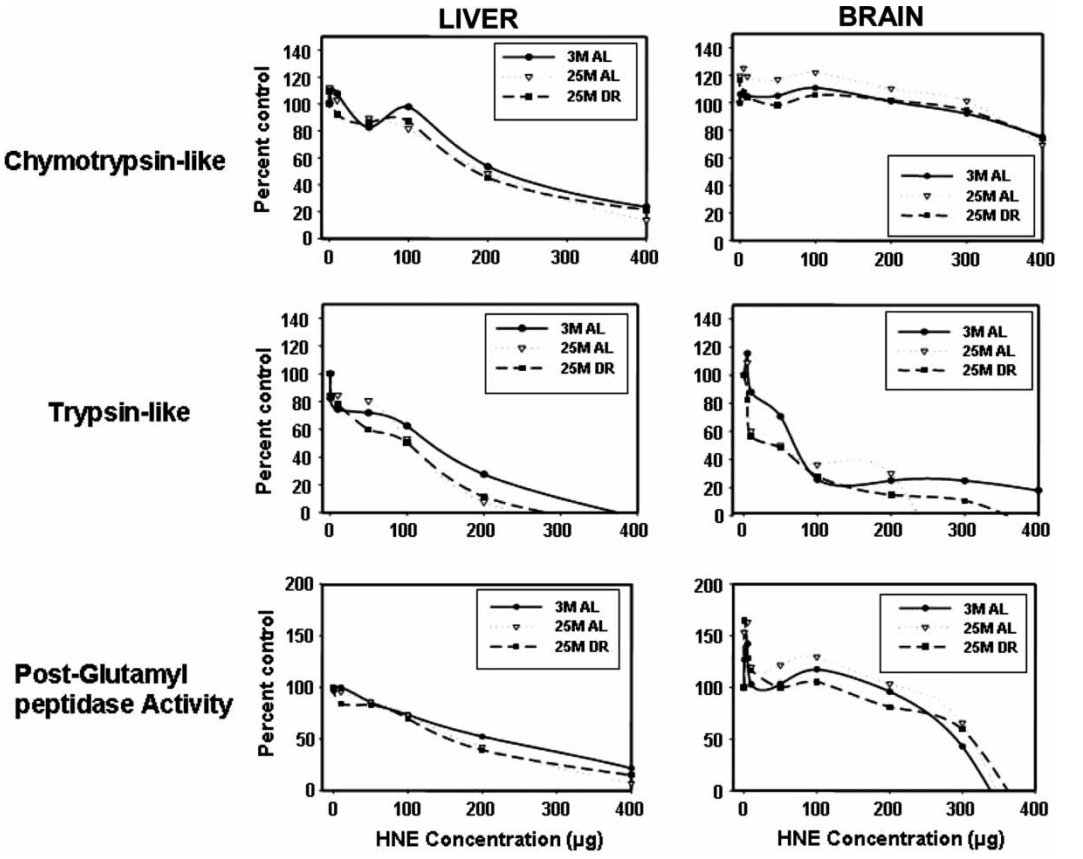

Figure 4. Effect of HNE on ChT-L, T-L and PGPH activities of the proteasome. The liver and brain retentates from 3 month-old AL, 25 month-old AL and 25 month-old DR rats were analysed for proteasome inactivation following treatment with increasing concentrations of HNE. The effect of HNE on ChT-L, T-L and PGPH proteasome activities were analysed. All the values in the graphs represent the average of six sets of tissues from each age group.

low level exposure to chronic ROS [63], which was associated with increased expression of selective proteasome sub-units. These data suggest that the cells of the brain have both compositional alterations in the proteasome and potential translational alterations in proteasome sub-unit expression, which can modulate the ability of brain proteasomes (and presumably proteasomes from other tissues) to successfully respond to the presence of some oxidative stressors. Consistent with this hypothesis we observed in Western blot analysis of the retentates that the liver exhibited a markedly different profile of proteasome sub-units as compared to the brain. Such compositional differences may contribute to the ability of the proteasome complexes from the brain to selectively exhibit increased activity in response to exposure to low level oxidative stressors. Such increases may be particularly important to the concept of hormesis, with low level stressors providing increased resistance to subsequent stressors through their ability to stimulate proteasome activity in some tissues.

Declines in proteasome activity are observed with age [22,25,39,64], with some studies reporting the ability of DR to rescue the proteasome activity in ageing animals [48,49,51]. The levels of proteasome were found to be similar during ageing in these previous studies, with the observed decline in proteasome activity mainly attributed to the changes in the proteasome sub-unit composition and/or the presence of deleterious post-translational modifications (including oxidative modifications). The inhibition of the proteasome has also been linked through a variety of experiments to play a role in ageing [25,39,48]. In previous studies DR was found to increase the levels of heat shock proteins and increase the 20S proteasome activity in Fisher 344 rat muscle [65]. The amount of the proteasome present in brain tissues was found to be lower than that observed in the muscle and to differ in composition as compared to the muscle [66,67]. Interestingly, 2D PAGE analysis and immunoblotting analysis indicated that brain and liver samples from young rodents have similar composition of proteasome sub-units as well as the same substrate specificity [38,66,67]. However, interferon-induced immunoproteasome sub-units like LMP7, LMP2 and MECL were observed to be elevated in the liver and lung, as compared to the brain [38,68]. Genetic depletion of the LMP2 and LMP7 sub-units in the brain and liver exacerbate age-related decreases in proteasome activities and promoted age-related increases in oxidative stress [69]. The differences in the susceptibility of the brain and liver proteasomes to

oxidative stress could be due to the presence of different proteasome sub-types (constitutive vs immunoproteasome) or due in part to different levels of 26S vs 20S proteasomes in these different tissues [70]. Together, these studies highlight the importance of understanding how different sub-types of proteasomes are affected by ageing and DR in the brain and other tissues. Additionally, in the present study we only utilized male rodents and thus there is the potential that female rodents may exhibit different responses following both DR and ageing. This experimental issue will need to be addressed in future studies. Similarly, studies are needed to determine the full relevance of these rodent studies to human ageing. While rodent studies have been immensely useful in understanding some aspects of human ageing, there remains a need to repeat key experimental aspects using human tissue and such studies are planned in our laboratory.

While chromatography techniques have been invaluable in establishing the biochemistry of the 20S and 26S proteasomes, the fact that they require the use of large amounts of tissue has made it difficult to study proteasome biology in settings where the amount of tissue available is extremely limited. In the present study we demonstrated that a filtration-based assay allows for rapid enrichment of proteasome complexes from both liver and brain lysates. This methodology may be extremely important to opening up the exploration of proteasome biology in experimental conditions where the amount of tissue available is very limited (as in the current study). Continuing to expand our methodologies for studying the biology and biochemistry of the proteasome are essential to accurately understanding the role this important enzyme plays in both biological and pathological conditions.

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