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Upregulation of PSMB8 and Cathepsins in the Human Brains of Dementia with Lewy Bodies

Qunxing Ding* and Haiyan Zhu

¹Department of Biological Sciences, Kent State University at East Liverpool, East Liverpool, OH 43920, USA

Abstract

Proteasome and lysosome are responsible for the homeostasis of proteins, lipids and carbohydrates in cells. Numerous reports indicate the proteolytic pathways have altered functions during neurodegeneration and aging. Dementia with Lewy bodies (DLB) is one of the leading forms of dementia, and the proteolytic alteration in DLB has not yet been fully investigated. This study shows that the components of proteasome and lysosome had selectively altered gene expression and enzymatic functions. Specifically, PSMB8, an inducible proteasomal β subunit, had elevated mRNA level and protein level in DLB brain compared with age-matched controls. The proteasomal caspase-like peptidase showed significant decreased activity in DLB brains and the trypsin-like/chemotrypsin-like activities did not reach statistical significance. Lysosomal cathepsin B and D had elevated mRNA levels while only cathepsin B showed elevated enzymatic activity in DLB brains. This data indicate that the alteration of proteolytic pathways is highly selective and comprehensive. Further study to elucidate the correlation between neurodegenerative development and the alteration of proteolytic pathways would be important for therapeutic development.

Keywords

Proteasome; lysosome; Dementia with Lewy bodies; immune proteasome; cathepsin; neurodegeneration

Introduction

Dementia with Lewy bodies (DLB) is a neurodegenerative disorder declined in thinking, reasoning and other cognition functions, and is the second most common dementia after Alzheimer's disease (AD) [1]. The pathological hallmark of DLB is the intracellular protein aggregates mainly formed by α -synuclein (SNCA), called Lewy bodies (LB) and Lewy neurites (LN) [2]. SNCA may associate with the presynaptic vehicle transport but its actual physiological role is not yet fully understood [3,4]. Protein aggregation is a common feature of many neurodegenerative disorders, including DLB, AD, Parkinson's disease (PD) and

*Corresponding author, qding@kent.edu.

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Huntington disease (HD). The wide presence of the aggregated structures in neurodegenerative disorders suggests that they may play an important role in etiology of neurodegeneration. For example, some reports indicated that aggregated proteins were toxic, other reports showed evidence that they might be protective during pathological development in multiple neurodegenerative processes, due to the fact that protein aggregation is a reversible process and regulated by multiple factors [4–8].

Cells constantly synthesize and degrade proteins to maintain cellular homeostasis, and the degradation of proteins relies on the function of the proteasome and lysosome [6], which are involved in the formation of protein aggregation. The proteasome is an intracellular proteolytic complex composed of multiple subunits, and possesses multiple proteolytic activities. The enzymatic functions of proteasome may be inhibited in pathological conditions to induce protein aggregation and even cell death [6]. For example, the inhibition of proteasome inhibition was identified in multiple neurodegenerative conditions to suggest the involvement of proteasome in neurodegeneration [7][8]. Meanwhile, selective upregulation of the lysosome have been demonstrated in a variety of neurodegenerative conditions, and a specialized form of elevated lysosomal activity, termed autophagy, is known to occur in a variety of neurodegenerative conditions [8][9].

In regarding to DLB, it was believed that proteasome and lysosome were involved in the formation of LB [10], and the SNCA in LB were ubiquitinated [11]. In fact, more than 90 different proteins were in the structure of LB in some co-localization studies, including some proteasome subunits, ubiquitin and ubiquitin-activating enzyme E1, and components of autophagy [12][13] [14]. It was also reported that the inhibition of proteasome lead to the formation of LB [15], and autophagy was coupled with the formation of LB [9,16,17].

In summary, tremendous progress has been made in the understanding of LB formation, as well as the involvement of proteasomal and lysosomal systems in LB formation. However, it is still not clear that how the gene expression pattern as well as the enzymatic functions are altered in DLB, and the correlation between gene expression and protein function is not yet fully investigated especially in human specimen. This study reports that the proteasomal subunits PSMB8, and lysosomal cathepsin B and D were upregulated in both RNA and protein levels in DLB brains. Meanwhile, the proteasomal caspase-like activity was decreased and the lysosomal cathepsin B activity was increased in DLB brains. Our finding supports the view that proteasome and lysosome are involved in the pathogenesis of DLB, and modulation of proteasomal and lysosomal systems may become potential therapeutic targets.

Methods and materials

Human brain tissues

Data were obtained from 5 control (3 males and 2 females) and 5 DLB cases (4 males and 1 female). The related clinic data were summarized in Table 1. The brain sections and frozen tissue were from brain region superior middle temporal gyri (SMTG) due to the facts that significant LB and LN are present in SMTG regions. The neuropathology and

neurophysiological diagnosis for each subject was conducted according to revised criteria [18] in the Sanders-Brown Center on Aging, University of Kentucky.

Real-Time RT-PCR

Total RNA was isolated with TriReagent (Sigma-Aldrich, St. Louis, MO, USA) method as described previously [19]. The concentration of each RNA sample was determined by OD260 after Qiagen RNA column purification (Cat No.: 74104, Qiagen, Hilden, Germany), and 800 ng total RNA were loaded in each reaction (CYBR® Green system was used). The ABI PRISM® 7000 Sequence Detection System and related reagents were used for real-time quantitative RT-PCR according to manufacturer's manuals (Applied Biosystems, CA).

Western blotting

Brain tissues were homogenized with lysis buffer (0.3% DTT, 0.8% Triton X-100, 7.5% glycerol, 50 mM HEPES in TBS, pH 7.4). The total proteins were then purified and quantified by OD280 method, and 30 µg total proteins were used in analysis for each subject. Briefly, total proteins were separated in 10% SDS-PAGE, and then transferred to nitrocellulose membrane. The membranes were blocked in 10% milk suspended in TBS, and probed with designed antibodies according to the manufacturer's procedure. Primary antibodies against the followed proteins were purchased from Santa Cruz Biotechnology Inc. These proteins are: PSMA1 (antibody cat# sc-166073, used at 1:500 dilution), PSMB3 (antibody cat# MCP102, used at 1:1000 dilution), PSMB8 (antibody cat# sc-100284, used at 1:1,000 dilution), cathepsin B (antibody cat# sc-365558, used at 1:1,000 dilution), cathepsin D (antibody cat# sc-136282, used at 1:500 dilution) and actin (antibody cat# sc-70319, used at 1:2,000 dilution as calibration control). The second antibody (donkey anti-mouse IgG-B: sc-2098, 1:400 dilution) was biotininated and detected by streptavidin-biotin complex according to the manufacturer's procedure (Santa Cruz, CA, USA).

Immunohistochemistry and *In-Situ* hybridization

Immunostaining of tissue sections (SMTG) was conducted with protocol as described previously [20]. Briefly, tissue sections were deparaffinized and rehydrated, then blocked in TBS with 2% donor horse serum for 5 minutes, incubated with primary antibody (PSMB8 antibody cat# sc-100284, 1:1,000 dilution) overnight at 4°C. After washing, tissue sections were sequentially incubated with biotinylated secondary antibody (donkey anti-mouse IgG-B: sc-2098, 1:200 dilution) for 1 hour, followed incubation with streptavidin-biotin complex for 1 hour. Bound antibody complexes were visualized by incubating sections in analyzing solution (0.1% (v/v) Triton X-100, 1.4 mM 3,3'-diaminobenzidine, 10 mM imidazole, and 8.8 mM H₂O₂ in 100 mM Tris, pH 7.6). Sections were then lightly counterstained with hematoxylin in the same solution. RNA *in-situ* detection followed similar procedure with the first antibody replaced with biotininated DNA probes (the forward primer labeled with biotin). All the antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) if not noted otherwise.

Analysis of protease activity

Total cell lysate was purified with the lysis buffer as described above, and used for the detection of enzymatic activities. The activities of the proteasome were quantified by monitoring the cleavage of substrates specific for chymotrypsin-like, trypsin-like, and caspase-like peptidase activities of the proteasome (Suc-LLVY-AMC, Bz-VGR-AMC, and Z-LLE-AMC respectively)[21][22][23] according to the manufacturer's protocol (Enzo Life Sciences International, Inc. PA, USA). The activities of cathepsin B and D were quantified by monitoring the cleavage of peptide substrates specific for cathepsin-activity [24] with kits from Creative BioMart (Kit-0181: Cathepsin B Activity Fluorometric Assay Kit, Kit-0182: Cathepsin D Activity Fluorometric Assay Kit, NY, USA) according to the manufacturer's protocol.

Separation of insoluble proteins and image analysis

Total proteins from control and DLB brain tissues were isolated in buffer contain 2.2% SDS and then run through 10% precast PAGE gel (5 µg total protein loading), after Coomassie staining the gels were scanned for image analysis according to manufacturer's procedure (Bio-RAD, CA, USA). The protein portion that could not run into the separation gel was quantified as SDS-insoluble proteins after scanning with program Image J (nih.gov). For quantifying the western Blot, related bands were measured in Image J, and the density of the bands were analyzed statistically. For *in-situ* immunohistochemistry results, 15 cells were randomly selected and measured with Image J in each section and the obtained data were analyzed statistically.

Statistical analysis

Data for correlation analyses were normalized by logarithmic transformation and subsequently analyzed by non-parametric Mann-Whitney *U test*. Student's *t-test* was also conducted as reference. The *p* values below 0.05 were considered statistically significant.

Results

Analysis was conducted using age-matched brain tissues from five control and five DLB patients. The subjects were limited to those that had short PMI (< three hours) to maintain the quality of RNA. Total RNA from SMTG regions were isolated and purified for pilot screen and the Ub, PSMA1, PSMA2, PSMB1, PSMB3, PSMB8, PSMB9, cathepsin B and cathepsin D showed obvious difference and these genes were measured with quantitative RT-PCR. The results showed in Figure 1. All these genes had higher mRNA levels in DLB subjects compared with control subjects, but only Ub, PSMB8, cathepsin B and cathepsin D reached statistical significance (*p* values were 0.004, 0.008, 0.009 and 0.035 respectively).

To validate the gene expression change, the protein levels of these genes were measured by Western blot method. PSMB8, cathepsin B and cathepsin D had significant higher protein levels in DLB than the control subjects, which confirmed the observation in their mRNA levels (Figure 2 showed two subjects in each group and the supplementary Figure 1 showed the other two subjects in each group).

To investigate the cellular distribution of PSMB8, the brain slices from the brain region (SMTG) were used for *in-situ* staining. One set of staining was conducted with cDNA probes, and the results indicated that neuronal cells from DLB subjects had higher level PSMB8 mRNA, strong signals were detected in nuclei of DLB SMTG section (Figure 3B). The antibody against human PSMB8 protein was used in brain SMTG slice as well, and the detected signals were mainly located in cytosol, higher in DLB subjects than control (Figure 3C, 3D).

The enzymatic activities of proteasome and cathepsins were measured with fluorescent substrates. The trypsin-like and chemotrypsin-like activities of proteasome did not show significant difference, but the caspase-like activity (also called postglutamyl activity) was dramatically inhibited in DLB subjects than control subjects (Figure 4A). In addition, the cathepsin B activity was increased in DLB subjects than control subjects, and the activity of cathepsin D did not show significant difference between DLB and control subjects (Figure 4B). Meanwhile, the insoluble portion of total proteins were separated with electrophoresis and the subjects with DLB had significant higher level of insoluble proteins (Figure 4C), which was correlated with the enzymatic change.

Discussion

Dysfunction of proteasome activities was reported in different neurodegenerative disorders, including AD [25], PD [26], and HD [27]. In DLB although ubiquitination of SNCA were detected but impairment of proteasome was not found [11], a deubiquitin enzyme, ubiquitin C-terminal hydrolase-1 (UCHL-1), had decreased mRNA and protein levels in DLB compared with age-matched control [28]. A 19S proteasome regulatory subunit RPT6 had reduced expression in DLB brain regions [29]. Nevertheless, selective alteration of proteasome subunits is a characteristic feature of DLB [30]. This study found that selected proteasome subunits had increased gene expression levels: ubiquitin and PASM8 (Figure 1). PSMB8 is an inducible proteasome subunit, a required component of immunoproteasome, which is a special form of proteasome to participate in a wide range of cellular activities, including MHC-I antigen presentation, pro-inflammatory cytokine production, T cell differentiation and proliferation, reduction of oxidative stress, and regulation of tumor development [31]. The immunoproteasome is also involved in protein degradation especially in pathological conditions [32], and formation of altered proteasome complexes could be an integral part of the cellular response to impaired constitutive proteasome function and altered proteostasis [33]. Since the constitutive activity of proteasome was reduced in DLB subjects compared with age-matched controls in this study (Figure 4), the increased expression of PSMB8 in DLB may be an indicator and compensation response resulted from the inhibition of the constitutive proteasome. This observation is also coincident with the upregulation of immunoproteasome in AD patients [34]. Proteasome complexes have three catalytic activities: chemotrypsin-like, trypsin-like and caspase-like peptidases. Different catalytic active sites are determined by different β -subunits [35], affected by different patterns of ubiquitination [36]. In this study, only caspase-like activity was reduced significantly (Figure 4A), which may be associated with the selective alteration of the expression levels of different subunits and other factors like ubiquitin and ubiquitin related enzymes (Figure 1). Obviously, reduced proteasomal activity is associated with increased

protein aggregation in DLB subjects (Figure 4C). Selective alteration of proteasome activities were reported in other neurodegenerative disorders, such as decreased trypsin-like activity in AD patients [34], and decreased chymotrypsin-like and caspase-like activities in HD cells [37]. These observations may indicate that multiple factors contribute to the different patterns of the alteration of proteasome activities, revealing the comprehensive pathways for pathogenesis in different diseases.

Cathepsins are important enzymes associated with lysosome, involved in protein degradation, autophagy and immune response, and in cancer development [38]. Overexpression of cathepsin D reduced SNCA aggregation, and the deficiency of cathepsin D exacerbated the accumulation of SNCA [39]. Recent studies have shown that reduced Cathepsin D expression resulted in SNCA accumulation and the degeneration of the dopaminergic system in experimental models and in patients with PD [40]. Cathepsin D is now considered one of the main lysosomal enzymes involved in SNCA degradation [41] and overexpression of Cathepsin D reduces the pathology associated with SNCA accumulation [42]. Although both the mRNA and protein levels of cathepsin B and D are elevated (Figure 1, 2), only cathepsin B showed increased enzymatic activity in this study (Figure 4B). It is clear that increased cathepsin B activity could not amend the elevation of aggregated proteins in DLB subjects (Figure 4C). Ubiquitin was another molecule measured in this study, which had much higher RNA level in DLB patients than control subjects (Figure 1). Ubiquitin is an important component in the function of proteasome, especially for ATP-dependent activities. Expression alteration of ubiquitin and related enzymes is very common in pathological conditions. For example, ubiquitin protein pool was reduced by proteasome inhibitor [43], overexpression of ubiquitin could reduce the protein aggregates and extend the lifespan of HD transgenic mice [44]. It is reported that higher level of ubiquitin may be used as a diagnostic marker of AD, which is comparable to other identified markers like A β 42 and tau, or apolipoprotein E ϵ 4 [45].

It is still under debate whether the impairment of proteasome is the cause or the result of neurodegeneration, one thing is clear is that proteasome/lysosome is highly involved in neurodegeneration [8]. The crosstalk between proteasome and lysosome was also reported previously [46], the results in this study also indicated the interaction between proteasome and lysosome, especially immunoproteasome and lysosome (Figure 1,2,4,5). However, this study only covers ten subjects for both DLB and control groups, so the data might not reveal overall propensity (if any) of the disease. It has been found that each individual patient really has a specific pattern of pathological development and readout (Figure 1, 2, 3), and the statistic average could not show the individual difference. Similar conditions were also found in enzymatic analysis (Figure 4). Due to the large diversity of genetic background, lifestyles and living conditions for each patient, we predict that the therapeutic strategy for different patient might be adapted individually based on their personal clinic profiles in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Dementia with Lewy bodies (DLB) is one of the leading forms of dementia
- Aggregated proteins are a pathological hallmark of DLB
- Proteasome has declined activity in the development of DLB
- PSMB8, an inducible beta subunit of proteasome has elevated expression in DLB
- Lysosomal cathepsin B and D have elevated expression
- Elevated cathepsin B activity cannot amend the decrease of proteasome activity, leading to protein aggregation.

Figure 1. Altered gene expression level of proteasomal and lysosomal components

Total RNA from DLB and control brain tissues (superior middle temporal gyri (SMTG) region, n=5) was purified and analyzed by real-time RT-PCR. Ub, PSMB8, cathepsin B and D showed statistical significant difference. Ub: ubiquitin; PSMA1 & 2: proteasome 20S α subunits; PSMB1 & 3: proteasome 20S β subunits; PSMB8 & 9: proteasome inducible β subunits; cathep B: lysosome cathepsin B; cathep D: lysosome cathepsin D. The vertical axis is the relative expression level after calibration with 18S rRNA gene in each subjects. **: $p < 0.01$, *: $p < 0.05$.

Figure 2. Protein level changes in human brain

Total proteins from DLB and control brain tissues (SMTG region, n=4) was isolated and analyzed by Western Blot. About 30 μ g total proteins were loaded in electrophoresis and related antibodies were used to detect the relative level of each protein as indicated in left side. Actin was used as internal control to calibrate the protein levels. Data shows two representative subjects in each group (left panel), the rest subjects were showed in supplementary Figure 2. The Statistical bar figure (right panel) covered all four subjects in each group (DLB and control). PSMB8, cathepsin B and D had higher protein levels in DLB subjects than control subjects. Ub: ubiquitin; PSMA1 & 2: proteasome 20S α subunits; PSMB1 & 3: proteasome 20S β subunits; PSMB8 & 9: proteasome inducible β subunits; Cathep B: lysosome cathepsin B; Cathep D: lysosome cathepsin D. The density of the Western Blot signals were measured by Image J and calibrated by the signal density of actin in each subject to show the relative difference. * $p < 0.05$.

Figure 3. *In situ* hybridization of PSMB8 RNA and protein in human brain

Brain sections from the SMTG region of control and DLB patients was analyzed for PSMB8. The brain sections were deparaffinized and rehydrated and hybridized with biotin-labelled DNA probe for mRNA level (panel A: control; panel B: DLB), and antibodies for protein level (panel C: control; panel D: DLB). The empty arrowheads indicated the countstained nuclei, the black-filled arrowheads indicated the stained signals of PSMD8. (panel A and B: mRNA; panel C and D: protein). Data were representative of results from three control subjects and four DLB subjects. (100x). Scale Bar: 20 μ m. 15 cells in each

image were randomly selected and measured with Image J and the numerous data were summarized in the panel E: significant higher signal levels of mRNA and protein of PSMB8 were detected in DLB. A: control mRNA level; B: DLB mRNA level; C: control protein level; D: DLB protein level. **: $p < 0.01$.

Figure 4. Enzymatic activity alteration and the correlation to SDS-insoluble protein level
Cell lysate was isolated from the SMTG tissue of each subject and the activity of each enzyme was analyzed. Significant higher activities of proteasomal caspase-like and cathepsin B were detected in DLB than control. The SDS-insoluble proteins were detected by Coomassie staining, which had higher level in DLB than control subjects, indicated the possible correlation between decreased proteasome activity and higher level protein aggregation (panel C). Five subjects in each group (control and DLB) were analyzed. *: $p < 0.05$, **: $p < 0.01$.

Figure 5. Schematic graph summarizing the involvement of PSMB8 and cathepsins in DLB pathogenesis

During neurodegeneration the dysfunction of protein metabolic processes occurs, which leads to the formation of LB. The dysfunction of protein degradation may also contribute to the pathological development of DLB, and the inhibition of institutive proteasome could lead to the induction of immunoproteasome (contains PSMB8) and lysosomal function (contains cathepsins). The arrows indicate the order of signal cascades and the question mark indicates the uncertainty of the direction.

Table 1

Human subjects and related clinic data

	Age (yrs)	Gender	PMI (hr)	CDR	MMSE
Control	79.5 ± 5.5	3M/2F	2.9 ± 0.76	0.14 ± 0.26	25.11 ± 1.49
DLB	78.8 ± 4.7	4M/1F	2.7 ± 0.60	1.75 ± 0.67	12.62 ± 2.51

PMI: postmortem interval; CDR: clinical dementia rating; MMSE: mini-mental state examination. Values are average ± standard deviation.

Table 2

Primers used in this study

Gene	Sense primer (5'to 3')	Antisense primer (5' to 3')
Ub	GTC AAA ATG CAG ATC TTC GTG AA	CAC CTC TCA GAC GCA GGA CC
PSMA 1	CGA AAT CAG TAT GAC AAT GAT GTC	ATG TCT CTC CAA GTA AGT AC
PSMA 2	TCG CTG ACT ACA TTC AGC C	TCG AGC TCT GTG CAC AAG CAC TC
PSMB 1	CTC CTG GCA GAG ACT TGG GGA	GTA GAC AGC ATT GCA GCA ATT G
PSMB 3	GAT GGT GAC CAC GGA CTT C	GCA GGT GCC ACT GAC CAC A
PSMB 8	GGG TGA ACA AGG TGA TTG AGA *	TTG ACA ACG CCT CCA GAA TAG
PSMB 9	CAT GGG ATA GAA CTG GAG GAA C	CAC ACC GGC AGC TGT AAT A
Cathep B	ACT TCT ACA ACG TGG ACA TGA G	CCA TGA TGT CCT TCT CGC TAT T
Cathep D	GTG GCA CAG ACT CCA AGT ATT A	ACG GTC AAA CAC AGT GTA GTA G
18S rRNA	GAC AGG ATT GAC AGA TTG ATA G	CAC TTG TCC CTC TAA GAA GTT G

Ub: ubiquitin;

* used as probe for *in-situ* mRNA detection after biotin labelling