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Increased Protein Hydrophobicity in Response to Aging and Alzheimer's disease

Kalavathi Dasuri^a, Philip Ebenezer^a, Le Zhang^a, Sun Ok Fernandez-Kim^a, Annadora J. Bruce-Keller^a, William R. Markesbery^b, and Jeffrey N. Keller^{a,c}

^aPennington Biomedical Research Center/Louisiana State University System, Baton Rouge, LA, USA.

^bDepartments of Pathology and Laboratory Medicine, and Neurology, Sanders-Brown Center on Aging, and Alzheimer's Disease Center, University of Kentucky College of Medicine, University of Kentucky, Lexington, Kentucky, USA

Abstract

Increased levels of misfolded and damaged proteins occur in response to brain aging and Alzheimer's disease (AD), which presumably increases the amount of aggregation prone proteins via elevations in hydrophobicity. The proteasome is an intracellular protease which degrades oxidized and ubiquitinated proteins, with the function of the proteasome known to be impaired in response to both aging and AD. In the present study we sought to determine the potential for increased levels of protein hydrophobicity occurring in response to aging and AD, identify the contribution of proteasome inhibition to increased protein hydrophobicity, and lastly to identify the contribution of ubiquitinated and oxidized proteins to the pool of hydrophobic proteins. In our studies we identified that aging and AD exhibited increases in protein hydrophobicity as detected using Bis-ANS, with dietary restriction (DR) significantly decreasing age-related increases in protein hydrophobicity. Affinity chromatography purification of hydrophobic proteins from aging and AD brains identified increased levels of oxidized and ubiquitinated proteins in the pool of hydrophobic proteins. Pharmacological inhibition of the proteasome in neurons, but not astrocytes, resulted in an increase in protein hydrophobicity. Taken together, these data indicate that there is a relationship between increased protein oxidation and protein ubiquitination and elevations in protein hydrophobicity within the aging and AD brain, which may be mediated in part by impaired proteasome activity in neurons. Our studies also suggest a potential role for decreased oxidized and hydrophobic proteins in mediating the beneficial effects of DR.

Introduction

Aging is associated with a number of deleterious changes in cellular proteins, including increases in protein oxidation and aggregation (1–3), while dietary restriction (DR) is known to decrease both of these aspects of protein homeostasis (4–8). Alzheimer's disease (AD) is a fatal age-related neurodegenerative disease associated with increases in both protein oxidation and protein aggregation (8–10). Mild cognitive impairment (MCI) is associated with cognitive decline, with amnesic MCI believed to be a precursor for the development of

^c**Corresponding author:** Dr Jeffrey N. Keller, Pennington Biomedical Research Center/LSU System, 6400 Perkins Road, Baton Rouge, LA 70808-4124 (P): 225-763-3190; jeffrey.keller@pbrc.edu.

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AD (8, 11). Numerous lines of evidence suggest a role for increased levels of protein hydrophobicity serving as a principle mediator of increased protein aggregation (12–14), with increased protein hydrophobicity also known to occur in some instances following exposure to oxidative stressors (15).

Currently, it is not known whether the levels of protein hydrophobicity increase in the brain in response to aging or AD, nor have studies outlined whether DR has any effect on the levels of protein hydrophobicity. Increases in protein hydrophobicity in response to aging or AD would be expected to be deleterious due to the fact that such hydrophobicity would be expected to disrupt protein function and lead to an intracellular environment which favors the accumulation and aggregation of oxidized and unfolded proteins. The relationships between oxidized protein levels and the amount protein hydrophobicity in the brain remains to be determined, but is important for an accurate understanding of how changes in the proteome may contribute to oxidative stress and alterations in neuronal homeostasis.

Protein degradation is essential for life, with impairments in proteolysis sufficient to promote deleterious effects on cellular homeostasis (16, 17). The proteasome is a large intracellular protease responsible for the majority of non-lysosomal mediated intracellular protein turnover (18–20) including the degradation of ubiquitinated (21–22) and oxidized proteins (23–27). Increases in protein hydrophobicity are believed to play a role in targeting of oxidized proteins to the proteasome for protein degradation. Impairment of proteasome activity occurs in the aging and AD brain (16, 29–31), and presumably contributes to deleterious changes in the proteome. Currently, it is not known what role the proteasome plays in regulating the levels of hydrophobic proteins in tissues such as the brain, even though increases in protein hydrophobicity are believed to play a role in targeting proteins to the proteasome for protein degradation.

In the present study, we demonstrate for the first time that aging and AD brains exhibit increased levels of protein hydrophobicity, with DR significantly ameliorating age-related increase in protein hydrophobicity. Increased levels of oxidized, and ubiquitinated proteins were observed in the pool of hydrophobic proteins from the brain, suggesting that there is a relationship between increased protein oxidation and ubiquitination, with elevations in protein hydrophobicity. Furthermore, we identified a potential role for proteasome inhibition mediating increases in protein hydrophobicity in neurons but not astrocytes. Together, these data contribute to our understanding of protein oxidation, proteasome inhibition, and protein hydrophobicity in the context of aging and AD.

Materials and Methods

Materials

Bis ANS was purchased from Sigma-Aldrich, Corp. (St. Louis, MO, USA). The antibodies to β -actin (SC-47778) and ubiquitin (SC - 8017) were purchased from Santa Cruz Biotechnology Company (Santa Cruz, CA, USA). MG132 was purchased from EMD Chemicals (Gibbstown, NJ, USA). The phenyl sepharose fast flow column material was purchased from GE Life Sciences (Piscataway, NJ, USA). The BCA reagent, Gelcode blue solution and snap cap spin columns were purchased from Thermo Scientific, Inc. (Waltham, Illinois, USA). Oxyblot kit was purchased from Millipore Company (Billerica, MA, USA). All electrophoresis and immunoblot reagents were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All cell culture supplies were obtained from GIBCO Life Sciences (Gaithersburg, MD, USA). All other items and chemicals were purchased from Sigma-Aldrich, Corp. (St. Louis, MO, USA).

Animal and Human tissues—Male Helicobacter-free F344/Brown Norway (F344 × BN F1) rats were obtained from the NIA Dietary Restriction (DR) colony. The rats in this study consisted of three month-old ad libitum (AL), twenty five month-old AL, and twenty five month-old DR rodents. All animals were handled and euthanatized in accordance with IACUC approved protocols at the Pennington Biomedical Research Center. A total of 3 animals were used for each condition, with brain homogenates comprised of the entire brain minus the cerebellum. The human brain tissues from control, MCI (Mild cognitive impairment) and AD (Alzheimer's disease) subjects were obtained from the Sander's Brown Center on Aging, University of Kentucky, KY, USA. The human brain tissue samples were matched for sex, age, and postmortem interval. Tissues from two females and one male were used in each group. Mean age for control, MCI, and AD subjects used in this study were 89.7 ($\pm$ 2.37), 89.3 ($\pm$ 3.59), and 77.6 ($\pm$ 7.18) years respectively. The mean post mortem interval for control, MCI, and AD subjects was 2.94 ($\pm$ 0.24), 3.1 ($\pm$ 0.294), and 3.0 ($\pm$ 0.44) hours respectively.

Primary rat neuron and astrocyte cultures and treatment with MG132—

Neuronal cultures were established as described previously by our laboratory (32). Briefly, primary rat cortical neuronal cells were cultured from E18 Sprague-Dawley rats and maintained in 5% CO₂ at 37°C in MEM/Neurobasal medium containing 5% fetal bovine serum (heat inactivated), N2 supplement, B27 supplement, and 1% antibiotic. Cells were used in experiments between days 5–8 post plating. Rat astrocyte cultures were established from E18 Sprague-Dawley rats as described previously by our laboratory (32). Astrocytes were maintained in 5% CO₂ at 37°C in MEM medium containing 5% fetal bovine serum (heat inactivated), N2 supplement and 1% antibiotic. The primary cultures of neurons and astrocytes were treated with MG132 (1 or 10 μ M) for 15 hours and then collected by pelleting the cells. All animals were utilized in accordance with IACUC approved protocols at the Pennington Biomedical Research Center.

Measurement of the changes in protein confirmation using UV induced photo incorporation of Bis ANS—

The photo incorporation of Bis ANS was carried out as described by Pierce et al., 2006 (33). Briefly, the clear cell lysates were prepared in buffer containing 50mM Tris-HCl (pH 7.4), 10 mM magnesium sulfate, 0.1% Triton X-100 and protease inhibitor mix. The Bis ANS photo incorporation was performed in 96 well clear plates. Samples were diluted to 1mg/ml and then 1mM Bis ANS was added to the samples. The plate was incubated on ice under the direct exposure of 115V 0.16 Amp hand held long wave UV lamp (365nm, model UVL-21; UV products Inc.). After the photo incorporation of the Bis ANS, protein lysates were boiled in Laemmli sample buffer for 5 min and loaded on to the BioRad precast 10–20% SDS-PAGE gels. After the gel electrophoresis, the fluorescence image was captured under UV light. For the equal loading of the protein samples, the same gel was later stained in the gel code blue solution (Thermo Scientific) and imaged under white light source.

Isolation of hydrophobic proteins from rat brain crude lysate—

Brain tissues were homogenized in 50 mM phosphate buffer (pH-7.0) containing 0.1% Triton X-100 and protease inhibitors and crude lysates were obtained by centrifugation at 18,000×g for 30 min. Equal amounts of protein lysates (6 mg) were taken and then lysates were made in the equilibration buffer of hydrophobic chromatography column by diluting the lysates with equal volume of buffer containing 50 mM phosphate buffer (pH 7.0), 3.4 M ammonium sulfate to get a final concentration of ammonium sulfate to 1.7 M. The resulting precipitate was removed by centrifugation and the supernatant was loaded on to the Phenyl Sepharose Fast Flow spin column (~150 μ l) that was previously equilibrated with 50mM phosphate buffer containing 1.7 M ammonium sulfate. After loading the sample, the column was

washed with the equilibration buffer until no proteins were observed in flow through fractions. Then the column was eluted with two column volumes of 50 mM phosphate buffer (pH 7.0). The levels of protein carbonyls and ubiquitinated proteins in the column bound fractions containing the hydrophobic proteins (we refer them as hydrophobic pool of proteins) were measured using Western blotting analysis.

Detection of the protein carbonyls and ubiquitinated protein—Protein carbonyl levels were analyzed using Oxyblot kit (Millipore) as described by the manufacturer. Briefly, 5 µg of crude lysates or 10 µl of HIC column bound fractions were derivatized with DNP and then the derivatized products were detected by the Western blot analysis as described by the manufacturer. Amounts of ubiquitylated protein in 10 µg of crude lysates or 40 µl of HIC column bound fractions were detected by Western blotting analysis using antibodies against ubiquitin.

Protein estimation—Protein concentration of the cell or tissue lysates was estimated using BCA (Thermo Scientific) reagent as described by the manufacturer.

Western blotting—The protein samples were boiled in Laemmli sample buffer and loaded on to BioRad Precast Criterion gels and immunoblotted with specified antibodies as described previously by our laboratory (34).

Results

Analysis of protein hydrophobicity in aging and DR brain

In order to determine whether protein hydrophobicity is increased in the brain in response to aging we analyzed the levels of protein hydrophobicity in the brains of 3 month-old and 25 month-old ad libitum fed rodents. A total of 3 animals were used for each condition, with brain homogenates comprised of the entire brain minus the cerebellum. In this analysis, we observed that the 25 month-old rodent brain exhibited an increase in Bis-ANS staining (Fig 1), consistent with increased protein hydrophobicity (12, 35–36). Surprisingly, the levels of protein hydrophobicity were observed to be decreased in 25 month-old rodent brains from animals subject to DR (Fig 1). Equal loading of samples was confirmed by Coomassie staining (Fig 1).

Analysis of protein ubiquitination and protein oxidation in aging and DR brain

We next sought to elucidate whether there was a difference in the amount of ubiquitinated and oxidized protein in aging and DR brain. In these studies we observed that in analysis of crude lysates there was a significant increase in both ubiquitinated (Fig 2) and oxidized protein (Fig 3), which is significantly reduced in response to DR (Fig 2 & 3). While crude lysates exhibited a marked increase in ubiquitinated protein, only a modest increase in ubiquitinated protein was observed in the hydrophobic protein pool of 25 month-old rodents (Fig 2). A robust increase in oxidized proteins was observed in the pool of hydrophobic proteins from aged rodents, which was significantly decreased in response to DR (Fig 3). It should be noted that low levels of ubiquitinated proteins were detected in the column bound hydrophobic fractions of rat brain lysates as compared to oxidized proteins using Western blot analysis.

Analysis of protein hydrophobicity, ubiquitinated protein, and oxidized protein in MCI and AD brain

In the next set of experimentation we analyzed protein hydrophobicity in the superior middle temporal gyrus (SMTG) of control, MCI and AD subjects. In this analysis we observed that AD had a higher level of protein hydrophobicity as compared to the control brain (Fig 4).

Analysis of ubiquitinated protein in crude lysates revealed that AD and MCI had elevated levels of ubiquitinated protein (Fig 5), as compared to control subjects. The increase in ubiquitinated protein in AD was significantly blunted in the pool of hydrophobic proteins as compared to crude lysates (Fig 5), yet was still observed to be elevated in AD subjects as compared to control or MCI subjects. Analysis of oxidized protein in crude lysates revealed that MCI and AD had the increased levels of oxidized protein (Fig 6), with oxidized proteins in hydrophobic protein pool being selectively increased in AD subjects as compared to all other experimental groups (Fig 6).

Analysis of protein hydrophobicity in neurons and astrocytes following proteasome inhibitor treatment

We next conducted experimentation to elucidate whether proteasome inhibition is sufficient to increase protein hydrophobicity in neurons and astrocytes. In these studies we observed a small but significant increase in protein hydrophobicity in primary rat neurons in response to proteasome inhibitor treatment (Fig 7). In contrast no increase in protein hydrophobicity was observed in astrocytes treated with proteasome inhibitors (Fig 8).

Discussion

The current study shows for the first time that there is an increase in protein hydrophobicity within the brain of aging rodents and MCI/AD subjects. The increase in protein hydrophobicity using the BiS ANS methodology suggest that there is a broad spectrum of proteins exhibiting increased hydrophobicity, and the data from BiS ANS is supported by work with our chromatography methodologies which demonstrate an increase amounts of hydrophobic proteins (data not shown). Protein conformations are known to play a role in altering the function and activity of proteins (12,35–38) and changes in conformation are sufficient to cause the initiation of protein aggregation (12,13). The increase in protein hydrophobicity observed in the current study could therefore contribute to numerous deleterious proteome alterations in aging and AD (39–42) including decreased protein function and increased protein aggregation. The decrease in hydrophobicity observed in DR brains in the present study provides a link between decreases in hydrophobicity and the presence of preserved brain homeostasis, based on the fact DR known to have many beneficial effects on multiple aspects of brain homeostasis (43–48). In future studies it will be important to identify the specific proteins which exhibit increased hydrophobicity, and elucidate whether such increases in hydrophobicity are a cause or a consequence of pathogenesis in the aging and AD brain.

Our current study illustrates that there is a relationship between the increase in protein oxidation and elevated levels of protein hydrophobicity within the brain. This is based on the observation that conditions associated with increased protein hydrophobicity were observed to have increased protein oxidation, and the fact that increased levels of oxidized protein were observed to be present in the hydrophobic pool of proteins from both aged brain and AD brain. These studies strengthen the potential for oxidative damage to proteins being an important inducer of increased protein hydrophobicity within the brain. While most studies on protein oxidation in the context of aging and AD have focused on the inactivation of proteins via the presence of oxidative modifications (16, 49–53), our studies raise the possibility that oxidation of proteins may contribute to pathogenesis by increasing the levels of aggregation prone proteins with elevated levels of hydrophobicity.

Curiously, the partial unfolding and denaturation of proteins which occurs in response to oxidation is believed to be a signal for targeted degradation by the proteasome or other proteases (26, 54–56). What is unclear is whether the observed increase in oxidized and hydrophobic proteins in the present study is due to increased levels of reactive oxygen

species, or due to decreased proteolysis of oxidized and hydrophobic proteins. The fact that there is an established link between decreased proteasome function and the increase in oxidized protein levels (35, 56–57), and observations that proteasome function is impaired in both aging and AD (16, 58–59), raises the specter for impaired proteasome function potentially serving as a primary mediator of increased protein oxidation within the hydrophobic pool of proteins.

In the present study, the levels of ubiquitinated and oxidized proteins were increased in both MCI and AD lysates (Fig 5 and 6). These studies are consistent with previous studies demonstrating increased oxidative stress and neuronal damage in both MCI and AD. What is particularly interesting in the present study is the observation that the greatest difference between AD and the MCI and control groups observed to be in the analysis of the hydrophobic proteins. In the hydrophobic pool of proteins, the AD brain selectively exhibits increased levels of ubiquitinated and oxidized protein (Fig 5 & 6). These data raise possibility for AD having unique changes in regards to protein hydrophobicity which may potentially be important in AD pathogenesis.

While only modestly increasing protein hydrophobicity, we observed that the ability of proteasome inhibition to increase protein hydrophobicity levels was selective for neurons. Surprisingly little is known with regards to the effects of proteasome inhibition on astrocytes, although we have consistently observed in our experimentation that astrocytes are much more resistant to the effects of proteasome inhibitors as compared to neurons (J.N. Keller, unpublished observations). These data raise the possibility that astrocytes have a greater dependence upon non-proteasomal pathways to regulate the turnover of hydrophobic proteins, or alternatively that the basal rates of protein oxidation and protein damage (which are contributors to protein hydrophobicity) are lower in astrocytes as compared to neurons. Regardless, our studies raise the potential for increases in protein hydrophobicity following proteasome inhibition to preferentially increase protein hydrophobicity in neurons during brain aging and AD.

One of the difficulties in understanding the basis by which oxidative stress may contribute to brain aging and AD is derived from the fact that oxidative damage, while increased in each of these conditions (8–10, 60), does not serve as a true representative biomarker for either aging or AD. For example, there is tremendous overlap in the amount of gross oxidative damage which is observed in the aged and AD brain, as compared to the brains from younger animals or control subjects. However, our studies suggest that it may not necessarily be the gross amount of oxidative damage which may contribute to the development of brain aging and AD, but rather it may be the amount of oxidized proteins within the hydrophobic pool of proteins which is most important to the potential promotion of brain dysfunction, in response to aging and AD. The robust level, and somewhat selectivity for increased protein oxidation within the hydrophobic pool of proteins of aging and AD brain, may highlight an important role for this specific pool of oxidized proteins playing a role in both aging and AD. Identifying which proteins exhibit increased oxidation in selective pool, and the pathways which contribute to their elevated levels of hydrophobicity in both aging and AD, would be expected to provide new insight as to the potential role of oxidative stress contributing to both brain aging and AD.

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Figure 1. Protein hydrophobicity increases with age in rat brain tissues

(A) SDS-PAGE analysis showing the Bis ANS incorporation in rat brain lysates, and Coomassie blue staining (B). (C) Relative levels of protein hydrophobicity in different tissue lysates of rat brain. Graph represents the mean and S.E.M for density of Bis ANS labeling. Results represent the 3 sets of experiments done under similar conditions. A total of 3 animals were used for each condition, with brain homogenates comprised of the entire brain minus the cerebellum. Hydrophobicity of 3M AL brain lysates is taken as percent control. * $p < 0.05$ compared to 3 month old animals. 3M: 3 month-old ; 25M: 25 month-old; AL: Ad Libitum; DR: Dietary restriction.

Figure 2. Levels of protein ubiquitination increases with aging in crude and HIC column bound fractions of rat brain lysates

(A) Western blot analysis showing levels of ubiquitination and relative beta actin levels in crude lysates of rat brain. (B) Graph representing the relative levels of ubiquitination in the crude lysates of rat brain. (C) Western blot analysis showing the levels of ubiquitinated proteins in HIC column bound fractions (obtained as described in methods) of rat brain lysates. (D) Graph representing the relative levels of ubiquitination in HIC column bound fractions of rat brain lysates. Results represent the three sets of experiments done under similar conditions. A total of 3 animals were used for each condition, with brain

homogenates comprised of the entire brain minus the cerebellum. Density with 3M AL brain lysates is taken as percent control. $*p < 0.05$ compared to 3 month old animals.

Figure 3. Protein oxidation levels increases with aging in crude and HIC column bound fractions or hydrophobic pool fraction of rat brain lysates

(A) Western blot analysis showing the levels of protein carbonyls (anti-DNP) and relative beta actin levels in crude lysates of rat brain. (B) Graph representing the relative levels of protein carbonyls in crude lysates of rat brain. (C) Western blot analysis showing the levels of protein carbonyls in HIC column bound fractions (obtained as described in methods) of rat brain lysates. (D) Graph representing the relative levels of protein carbonyls in HIC column bound fractions of rat brain lysates. Results represent the three sets of experiments done under similar conditions. A total of 3 animals were used for each condition, with brain

homogenates comprised of the entire brain minus the cerebellum. $*p < 0.05$ compared to 3 month old animals.

Figure 4. Increased protein hydrophobicity observed in human brain (SMTG) lysates of MCI and AD subjects

(A) SDS-PAGE analysis showing the Bis ANS incorporation in different human brain (SMTG) tissue lysates and the Coomassie blue staining (B). Relative levels of protein hydrophobicity in different tissue lysates of human brain (C). Graph represents the mean and S.E.M for density for protein hydrophobicity. Results represent the three sets of experiments done under similar conditions. Hydrophobicity of the control human brain lysates is taken as percent control. * $p < 0.05$ compared to control human brain lysates.

Crude lysates

HIC Elutions

Figure 5. Levels of protein ubiquitination in crude and HIC column bound fractions of human brain (SMTG) lysates of Control, MCI and AD subjects

(A) Western blot analysis showing the levels of ubiquitylation and relative beta actin levels in crude lysates of human brain lysates (B). Graph representing the relative levels of ubiquitination in crude lysates of human brain lysates (C). Western blot analysis showing the levels of ubiquitinated proteins in HIC column bound fractions (obtained as described in methods) of human brain lysates. (D) Graph representing the relative levels of ubiquitination in HIC column bound fractions of human brain lysates. Results represent the three sets of experiments done under similar conditions. Density of control brain is taken as percent control. * $p < 0.05$ compared to control human brain lysates.

Figure 6. Levels of protein oxidation in crude and HIC column bound fractions of human brain (SMTG) lysates of Control, MCI and AD subjects

(A) Western blot analysis showing the levels of protein carbonyls (anti-DNP) and relative beta actin levels in crude lysates of human brain lysates (B). Graph representing the relative levels of protein carbonyls in crude lysates of human brain lysates. (C) Western blot analysis showing the levels of protein carbonyls in HIC column bound fractions of human brain lysates. (D) Graph representing the relative levels of protein carbonyls in HIC column bound fractions of human brain lysates. Results represent the three sets of experiments done under similar conditions. Density of control brain is taken as percent control. $*p < 0.05$ compared to control human brain lysates.

Figure 7. Effect of proteasome inhibition on protein hydrophobicity in primary neurons
(A) SDS-PAGE analysis showing the Bis ANS incorporation in primary neuronal cell lysates treated with or without the proteasome inhibitor MG132, and the corresponding Coomassie blue staining **(B)** of the gel. **(C)** Relative levels of hydrophobicity in different neuronal lysates. Graph represents the mean and S.E.M for density for protein hydrophobicity. Results represent the three sets of experiments done under similar conditions. Hydrophobicity of neuronal cell lysates not treated with MG132 is taken as percent control.* $p < 0.05$ compared to cell lysates not treated with MG132.

Figure 8. Effect of proteasome inhibition on protein hydrophobicity in primary astrocytes (A) SDS-PAGE analysis showing the Bis ANS incorporation in primary astrocytes cell lysates treated with or without the proteasome inhibitor MG132, and the corresponding Coomassie blue staining (B) of the gel. (C) Relative levels of hydrophobicity in different astrocytes cell lysates. Graph represents the mean and S.E.M for density for protein hydrophobicity. Results represent the three sets of experiments done under similar conditions. Hydrophobicity of astrocytes cell lysates not treated with MG132 is taken as percent control. $*p < 0.05$ compared to cell lysates not treated with MG132.