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Increased Protein and Lipid Oxidative Damage in Mitochondria Isolated from Lymphocytes from Patients with Alzheimer's Disease: Insights into the Role of Oxidative Stress in Alzheimer's Disease and Initial Investigations into a Potential Biomarker for this Dementing Disorder

Rukhsana Sultana[a], Patrizia Mecocci[b], Francesca Mangialasche[b], Roberta Cecchetti[b], Mauro Baglioni[b] and D. Allan Butterfield[a,*]
[a]*Department of Chemistry, Center of Membrane Sciences, and Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY, USA*
[b]*Department of Clinical and Experimental Medicine, Institute of Gerontology and Geriatrics, University of Perugia, Perugia, Italy*

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Abstract. Alzheimer's disease (AD) is histopathologically characterized by the presence of senile plaques, neurofibrillary tangles, and synapse loss. The main component of senile plaques is amyloid β-peptide (Aβ), which has been shown to induce oxidative stress in *in vitro* and *in vivo* studies. AD is associated with elevated levels of oxidative damage in brain and peripheral lymphocytes. Further Aβ has been found to be accumulated in mitochondria, which might contribute to the reported alterations in the mitochondrial morphology, and impaired mitochondrial energy metabolism in AD brain. Biomarkers are desperately needed for earlier diagnosis of AD and to monitor efficacy of new therapies. Hence, in the present study we show that markers of oxidative damage are elevated in mitochondria isolated from AD lymphocytes suggesting that these oxidative stress indices potentially could serve as a viable biomarker for AD.

Keywords: Alzheimer's disease, lymphocytes, mitochondria, 3-nitrotyrosine, oxidative stress, protein-bound 4 hydroxy-2 *trans* nonenal, protein carbonyls

INTRODUCTION

Free radicals are produced in a low amount in the body as a process of natural metabolism. Free radicals are also formed by a tightly coupled and regulated process during oxidative phosphorylation, thereby playing

*Correspondence to: Professor D. Allan Butterfield, Department of Chemistry, Center of Membrane Sciences, and Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40506-0055, USA. Tel.: +1 859 257 3184; Fax: +1 859 257 5876; E-mail: dabcns@uky.edu.

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an important role in energy (ATP) production. Some of the free radicals, like nitric oxide, are important in regulating signaling processes within the cells, and some free radicals are used by the immune system to destroy invading pathogens. Hence, balanced levels of free radicals are important for normal cellular function, and they are normally checked in the body by the antioxidant defense system. When the levels of the free radicals exceed the limit that can be combated by cellular defense mechanisms, a phenomenon referred to as "oxidative stress" ensues. Oxidative stress occurs due to an imbalance in the levels of oxidants and antioxidant systems that could result from either an increase in the levels of oxidants or a decrease in the levels of antioxidant enzymes.

Mitochondria represent a main source of free radicals, particularly of the superoxide radical that can be dismutated by Mn superoxide dismutase (SOD) to produce hydrogen peroxide. In its protonated, non-charged form, HO_2 can diffuse freely across the membrane and is a likely candidate that can attack and damage biomolecules at a distant site. Moreover, hydrogen peroxide in the presence of redox-active metals like iron or copper may lead to the production of hydroxyl radicals that are highly reactive with a short half-life of seconds. Hence •OH radical can affect adjacent biomolecules inside mitochondria. Further, the degradation of the damaged mitochondria by autophagy and consequent release of heme iron into the cytosol also can contribute to free radical elevation [1], which can attack proteins, lipids, carbohydrates, and nucleic acids leading to altered cellular function and consequent cell death.

Alzheimer's disease (AD) is histopathologically characterized by the presence of senile plaques, neurofibrillary tangles, and synapse loss [2]. The main component of senile plaques is amyloid-β peptide (Aβ), which is generated by the cleavage of amyloid-β protein precursor (AβPP) by β- and γ-secretases. Aβ has been shown to induce oxidative stress in a number of *in vitro* and *in vivo* studies [3–6] and, on the other hand, oxidative stress can increase production of Aβ [7]. A large number of studies have shown increased levels of oxidative markers of biomolecules (protein, lipids, carbohydrates, and nucleic acids) in AD brain and peripheral systems [8–17]. Further, increasing evidence implicates Aβ accumulation in mitochondria in AD [18–21].

Biomarkers are desperately needed for earlier diagnosis of AD and to monitor efficacy of new therapies [22]. Currently, there are no agreed upon biomarkers that serve these purposes, especially in the earliest form of AD, i.e., mild cognitive impairment (MCI).

Although most of the studies on oxidative biomarkers have been performed in brain, several studies have demonstrated that oxidative stress phenomena can also be detected in peripheral tissues [23, 24]. In the present study, in order to begin to evaluate potential biomarkers for AD, we have measured indices of protein oxidation and lipid peroxidation in mitochondria isolated from lymphocytes of cognitively healthy and AD subjects.

MATERIALS AND METHODS

Subjects

Subjects were enrolled at the Memory Clinic of the Institute of Gerontology and Geritatrics, University of Perugia. Nine subjects (2 males, 7 females, mean age 80.1 ± 8.1) with a Mini-mental state examination (MMSE) score [25] ranging from 16 to 22 fulfilled the NINCDS-ADRDA criteria for AD [26]. Eleven (3 males, 8 females, mean age 80.1 ± 4.7) were cognitively normal subjects (MMSE 29–30).

All subjects were evaluated according to a standard protocol including a detailed anamnesis, clinical, and neuropsychological evaluation. Subjects with a history of having a smoking habit and/or alcohol abuse, major organ failure, dyslipidemia, or metabolic alterations were not included. After giving informed consent, patients and controls underwent a 20 ml blood sample withdrawal. Samples were immediately processed for mitochondria isolation.

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. The Oxyblot oxidized protein kit was obtained from Intergen, Inc. (Purchase, NY). Primary antibodies for 4-hydroxynonenal (HNE) and 3-nitrotyrosine (3-NT) were obtained from Chemicon (Temecula, CA).

Isolation of mitochondria

Freshly obtained blood was layered on Lymphoprep (Gibco, BRL, Bethesda, MD), centrifuged and washed twice. The pellet was resuspended in 400 μl of ice-cold PBS. Eight μl of 2.5% digitonin were added and kept on ice for 5 min inverting gently every 30 s. Each sample was sonicated for 1 min and then centrifuged at 600*g* for 10 min at 4°C to eliminate nuclei and unbro-

ken cells. The supernatant was centrifuged at 14,000*g* for 10 min at 4°C, the pellet resuspended in 400 μl ice cold PBS and centrifuged at 7,000*g* for 10 min at 4°C. The pellet was resuspended in 400 μl ice cold PBS and centrifuged at 3,500*g* for 10 min at 4°C. The pelleted purified mitochondria were immediately frozen and kept at −80°C until analyses.

Protein carbonyl measurement

Protein oxidation was determined by an oxidized protein detection kit (Oxyblot; Chemicon) [27]. Briefly, 5 μl of mitochondria (4 mg/ml) were incubated for 20 min with 12% sodium dodecyl sulfate (SDS) and 2,4-dinitrophenylhydrazine (DNPH) followed by addition of 7.5 μl of neutralization solution. The derivatized proteins (250 ng) were transferred onto nitrocellulose membrane by the slot blot technique. Membranes were incubated with blocking buffer for 60 min at 27°C and incubated with rabbit antibodies to DNPH (diluted 1 : 150) for 90 min, then by anti-rabbit IgG coupled to alkaline phosphatase (1 : 10,000) for 1 h at 27°C. After being washed and developed with SigmaFast chromogen (Sigma), blots were scanned into Adobe Photoshop (Adobe Systems, Inc., Mountain View, CA) and quantitated with Scion Image (PC version of Macintosh-compatible NIH Image).

3-Nitrotyrosine levels

Protein-bound 3-NT was determined by incubating the samples (5 μl) with Laemmli buffer (10 μl) (0.125 M Trizma base, pH 6.8, 4% SDS, 20% glycerol) for 20 min. Samples (250 ng of protein) were blotted onto nitrocellulose membranes, and immunochemical methods were performed [27]. The rabbit anti-3-NT primary antibody was incubated 1 : 200 in blocking buffer [bovine serum albumin (BSA) 3% in TBS-T] for 2 h. The membranes were washed three times with TBS-T and incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (1 : 10,000). Densitometric analysis of bands in images of the blots was used to calculate levels of 3-NT.

Lipid peroxidation

4-Hydroxy-2-nonenal (HNE), a lipid peroxidation marker, was measured as an index of lipid peroxidation [27]. The samples (5 μl) were incubated with 10 μl Laemmli buffer for 20 min at room temperature, and 250 ng of protein samples was loaded into each well on nitrocellulose membrane in a slot blot apparatus under vacuum. The membranes were incubated with anti-HNE rabbit polyclonal antibody (1 : 5,000) for 2 h, washed three times with TBS-T, and then incubated with an anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody (1 : 10,000). Blots were developed with SigmaFast tablets (BCIP/NBT), dried, and quantified in Scion Image.

Statistical analysis

Two-tailed, Student's *t*-tests were used to analyze differences in oxidative stress markers between AD and age-matched controls samples. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Reactive oxygen and nitrogen species can attack polyunsaturated fatty acids of lipid bilayers leading to the formation of reactive aldehydes, among which one of the most toxic products is HNE [15, 28]. HNE can react with proteins leading to the formation of stable covalent adducts with histidine, lysine, and cysteine residues via Michael addition [29–30]. Figure 1A shows the HNE-bound protein levels in mitochondria isolated from AD lymphocytes are significantly increased compared to control ($^{**}p < 0.001$). Further, the levels of protein-bound HNE did not show any significant correlation with MMSE score (Fig. 1B), although we observed a trend towards a negative correlation with MMSE score ($^{*}p < 0.07$, Table 1), suggesting that the increase of protein-bound HNE might reflect the decrease in cognitive functions.

Protein carbonyls and 3-NT levels were measured as markers of protein oxidation [31]. Protein carbonyl groups are generated by direct oxidation of certain amino acid side chains, peptide backbone scission, by Michael addition reactions with products of lipid peroxidation, or glycoxidation [29–31]. Oxidative stress also could stimulate additional damage via the over expression of inducibile nitric oxide synthase (iNOS) and the action of constitutive neuronal NOS (nNOS), both of which lead to increased levels of 3-NT. Levels of protein carbonyls ($p < 0.02$) (Fig. 2A), and 3-NT ($p < 0.03$) (Fig. 3A) were significantly increased in AD lymphocyte mitochondria compared to controls. The levels of protein carbonyls, and protein-bound 3-NT showed significant ($p < 0.05$, Figs 2B, 3B) negative correlation with MMSE score, suggesting that the increase

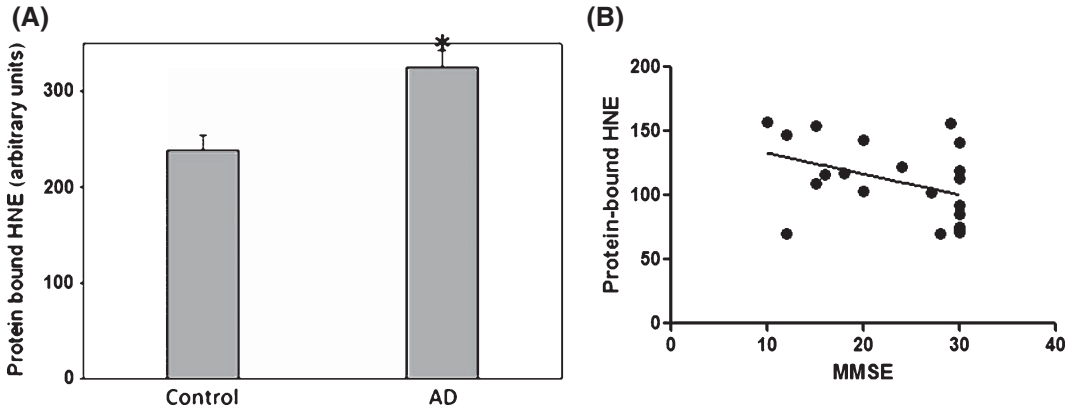

Fig. 1. Increased levels of protein-bound HNE were observed in mitochondria isolated from lymphocytes from AD patients compared to those of respective controls (A). Protein-bound HNE data are represented as arbitrary units, $^{*}p < 0.001$. Linear correlation analysis between protein-bound HNE and MMSE did not show any significant correlation (B) ($p < 0.07$), though a trend toward a significant negative correlation was observed (see text).

Table 1
Correlation analysis between oxidative stress markers and MMSE score (all 20 subjects included): for oxidative stress parameter, we report the R^2 value and the corresponding p value

Oxidative stress markers	R^2	p-value
Protein-bound HNE	−0.39	0.07*
Protein carbonyls	−0.5	0.02
Protein bound 3-NT	−0.49	0.02

*No significant correlation was found (p threshold set at 0.05).

of protein carbonyls and protein-bound HNE might reflect the decrease in cognitive functions. The 'R^2 values' and 'p values' for the linear correlation analysis are shown in Table 1.

DISCUSSION

This work provides the first report of increased levels of oxidative stress in the mitochondria isolated from lymphocytes of AD subjects compared to those of controls as indexed by elevated levels of protein carbonyls, protein-bound HNE and protein-resident 3-nitrotyrosine. Elevation of oxidative stress markers in AD lymphocyte mitochondria is consistent with the decreased plasma levels of GSH and increased levels of GSSG in AD lymphocytes, and consequent lower ratio of GSH/GSSG previously reported [32]. Further, this same study also showed that the levels of heme oxygenase-1 and activity and the levels

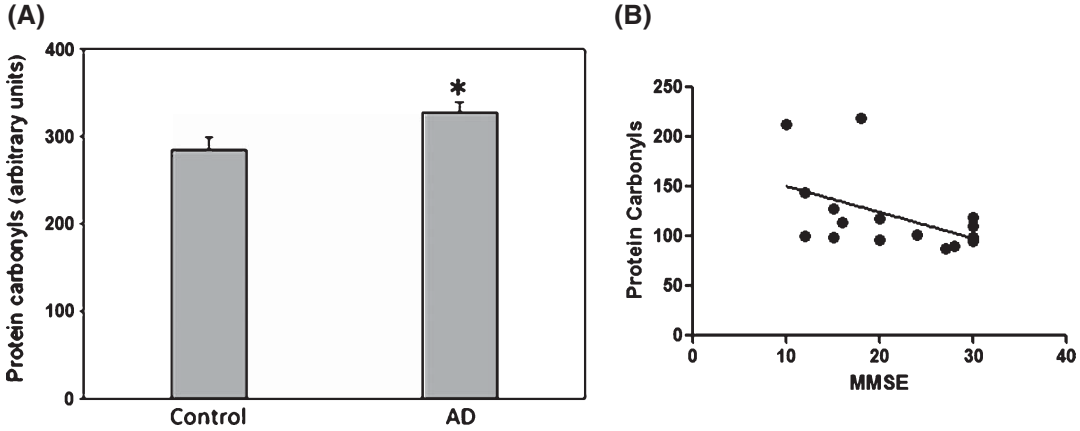

Fig. 2. Increased levels of protein carbonyls were observed in mitochondria isolated from AD compared to respective controls. A) Protein carbonyl data are represented as arbitrary units, $^{*}p < 0.02$. B) A significant negative correlation was observed between the protein carbonyl levels and MMSE scores ($p < 0.02$).

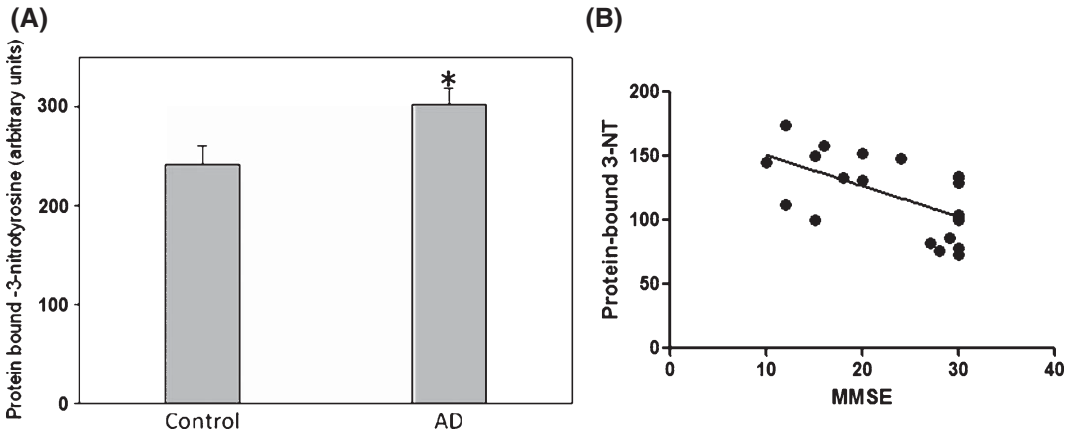

Fig. 3. Increased levels of protein bound-3 nitrotyrosine (3-NT) were observed in mitochondria isolated from AD compared to respective controls. A) Protein bound 3-NT data are represented as as arbitrary units, $^{*}p < 0.03$. B) A significant negative correlation was observed between the protein bound 3-NT levels and MMSE scores ($p < 0.02$).

of NOS-2, HSP72, HSP60, and thioredoxin reductase were elevated in AD lymphocytes [32], consistent with an oxidative environment. Using HPLC analysis, AD patients were shown to have significantly higher lymphocyte concentrations of the oxidized purine 8OHdG than do controls [33]. Moreover, the levels of plasma antioxidants such as lycopene, lutein, α-carotene, and β-carotene, were found to be significantly lower in patients with AD compared with controls and showed an inverse relationship to lymphocyte DNA 8-OHdG content [10]. The imbalance in the levels of antioxidants and increase levels of oxidative insult conceivably could be due to Aβ in the plasma of AD subjects. However, studies related with plasma Aβ levels showed contradictory results. A study conducted by Xu and colleagues [34] showed that the $A\beta_{1\text{-}40}$ level is increased and $A\beta_{1\text{-}42}$ is decreased, in contrast to others who showed no differences in Aβ levels [35, 36] or increased $A\beta_{42}$ in AD patients [37]. These differences could be due to variation of experimental conditions.

As noted above, Aβ is produced from the proteolytic cleavage of a transmembrane AβPP by the sequential actions of the aspartate proteases β- and γ-secretase [2]. A number of *in vitro* and *in vivo* studies showed that Aβ can induce oxidative stress [4–6]. Further, Aβ has been shown to exist in various aggregated states, among which are monomers, oligomers, protofibrils, and fibrils. The oligomeric form of Aβ is considered a highly toxic species of Aβ [38]. Recent studies reported the presence of Aβ in mitochondrial membranes [19, 20]. The localization of Aβ in the mitochondria suggest that it may initiate lipid peroxidation in the mitochondrial membrane by similar processes as discussed above, leading to alterations in components of the membrane such as lipid and proteins, including the electron transport system. These alterations, in turn, can lead to alterations in membrane fluidity, leakage of apoptosis-inducing molecules such as cytochrome C and apoptosis inducing factor from the mitochondria, all of which may contribute to decreased cellular energetics, neuronal apoptosis, and generation of reactive oxygen species in AD. In contrast, a previous study showed that the activities of respiratory chain enzymes in mitochondria isolated from lymphocytes of AD patients did not demonstrate any significant differences [39].

There is ample evidence of increased oxidative stress in AD brain and in fibroblasts from sporadic AD subjects [7, 15, 20, 40]. Alterations in the mitochondrial morphology and impaired mitochondrial energy metabolism have been well documented in AD brain [18, 20, 41]. Further, a number of studies suggest that there is decreased rate of cerebral metabolism in AD brain. The reduced level of cerebral metabolism has been also found in pre-AD stages, such as MCI and early AD (EAD), as revealed by PET studies [42]. Previous studies from our laboratory using proteomics have identified a number of proteins associated with glucose metabolism that are oxidatively modified and dysfunctional proteins [20, 43–49]. The decrease in the cerebral energy reported in AD brain by PET studies correlated with the altered expression and decreased activity of mitochondrial energy-related proteins such as pyruvate dehydrogenase complex (PDHC), α-ketoglutarate dehydrogenase complex (KGDHC), and isocitrate dehydrogenase [50]. Further, decreased glucose utilization has been reported in skin fibroblasts

of AD patients [51], and *in vitro* studies reported that incubation of isolated mitochondria with Aβ peptides decreased the activity of KGDHC and PDHC [52].

The assessment of a subject's cognitive function is used as one criterion for clinical diagnosis of AD. Thus, a better understanding of how the MMSE score relates to mitochondrial oxidative stress markers in the lymphocytes isolated from control and AD was obtained by linear correlation analysis. Our finding of a negative correlation of oxidative stress markers, such as protein carbonyls and protein-bound 3-NT, to MMSE score suggests that low MMSE scores characteristic of AD correlate with increased oxidative stress markers in the peripheral system reflecting oxidative damage to the central nervous system. A previous study by Montine and coworkers [53] showed no significant difference in the product of lipid peroxidation such as iso-prostanes and neuroprostanes in plasma; however, data presented in this study showed increased levels of protein-bound HNE in the mitochondria isolated from lymphocyte of AD and shows a trend toward negative correlation to the MMSE score, consistent with our findings reported in the present study. In the plasma, certain proteins like albumin are present in relatively high abundance which can mask the precise determination of oxidative stress markers in plasma, thereby limiting the discovery of biomarker in the plasma. However, by focusing on the mitochondria isolated from lymphocytes we may be able to overcome this limitation.

Taken together, the results of this study suggest that the elevated oxidative stress markers in the mitochondria from lymphocytes may potentially reflect the brain damage in AD and may potentially serve as a marker for AD diagnosis or treatment.

In conclusion, our study demonstrates that AD is associated with elevated levels of oxidative damage in mitochondria from peripheral lymphocytes, as well as the previously-reported neuronal damage, and suggests a mitochondrial functional impairment in AD lymphocytes. We also demonstrated that peripheral tissue, such as lymphocytes, could potentially offer a simple alternative method to detect increased free radical damage in AD, and demonstrated that oxidative stress in AD is present not only in neuronal tissues but also in peripheral lymphocytes. Further, an inverse correlation of the oxidative markers in mitochondria from peripheral lymphocytes with MMSE scores suggests that peripheral lymphocytes could serve as a tool to index the cognitive alteration in AD. Investigation of the protein expression differences between control and AD mitochondria isolated from lymphocytes, with the purpose to further elucidate the mechanisms of Aβ-induced oxidative damage, is being pursued in our laboratory. The present studies suggest that oxidative stress in mitochondria from peripheral lymphocytes from subjects with AD may represent a viable biomarker for AD. Of course, additional studies with MCI and with other neurodegenerative disorders will be necessary to validate this notion. However, the present studies represent a baseline from which other conditions can be compared for this purpose.

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