Lymphocyte mitochondria: toward identification of peripheral biomarkers in the progression of Alzheimer disease

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A B S T R A C T

Alzheimer disease (AD) is an age-related neurodegenerative condition. AD is histopathologically characterized by the presence of three main hallmarks: senile plaques (rich in amyloid-β peptide), neuronal fibrillary tangles (rich in phosphorylated tau protein), and synapse loss. However, definitive biomarkers for this devastating disease in living people are still lacking. In this study, we show that levels of oxidative stress markers are significantly increased in the mitochondria isolated from lymphocytes of subjects with mild cognitive impairment (MCI) compared to cognitively normal individuals. Further, an increase in mitochondrial oxidative stress in MCI is associated with MMSE score, vitamin E components, and β-carotene. Further, a proteomics approach showed that alterations in the levels of thioredoxin-reduced peroxide reductase, myosin light polypeptide 6, and ATP synthase subunit β might be important in the progression and pathogenesis of AD. Increased understanding of oxidative stress and protein alterations in easily obtainable peripheral tissues will be helpful in developing biomarkers to combat this devastating disorder.

Biomarkers are helpful for diagnosis of and monitoring the progression of a disease in addition to serving as a tool for testing the therapeutic efficacy of drugs [1]. Since its first description in 1906, the underlying mechanism(s) for Alzheimer disease (AD) so far is not clearly understood, nor has a therapeutic approach been developed that could be useful in dealing with this devastating pathology. Currently AD is the sixth leading cause of death in the United States and is the most common form of dementia. This disorder is currently estimated to affect 5.5 million people age 65 and older in the United States. With approximately 80 million people in the “Baby Boomer” population, it is expected that the number of AD patients will increase to as many as 16–20 million by 2050 unless a means to delay the onset or progression of the disease is developed [2]. Hence, there is an urgent need to develop biomarkers for AD, which if not achieved will lead to a significant public health crisis worldwide.

Currently AD is diagnosed by the presence of memory and cognitive impairment, early brain atrophy in several brain regions detected by structural MRI, a characteristic pattern of decreased glucose metabolism in parietal–temporal association cortices shown by FDG-PET analysis [3,4], and abnormal cerebrospinal fluid (CSF) markers such as elevated tau and phospho-tau levels and reduced levels of amyloid β-peptide (Aβ). Up to now definite diagnosis of AD is achieved histopathologically at autopsy and is characterized by the presence of abnormal protein deposits, including senile plaques (the main component of which is Aβ) and neurofibrillary tangles (the main component of which is hyperphosphorylated tau protein), and synaptic loss. Aβ [5], generated by the cleavage of amyloid precursor protein by β- and γ-secretases, has been shown to induce oxidative stress in a number of in vitro and in vivo studies [6–9] and, conversely, oxidative stress can increase production of Aβ [10]. A large number of studies have shown increased levels of markers of oxidative modification in various biomolecules (protein, lipids, carbohydrates, and nucleic acids) in AD brain and brain from subjects with arguably its earliest form, mild cognitive impairment (MCI), as well as in peripheral systems [11–20]. Further, increasing evidence implicates Aβ accumulation in mitochondria in the early stages of AD [21–24]. Mitochondria are the main source of energy, and impairment of mitochondrial function leads to decreased energetics and increased production of free radicals that can in turn have impact on cellular functions with consequent neuronal loss.

Previously, we demonstrated that elevated levels of indices of oxidation and lipid peroxidation were elevated in mitochondria isolated from lymphocytes obtained from AD patients [25]. To test the hypothesis that such changes occur early in the progression of
AD, in the present study we investigated the levels of oxidative stress markers in mitochondria isolated from lymphocytes of MCI individuals and showed an inverse correlation between elevated indices of protein oxidation and lipid peroxidation with Mini Mental State Examination (MMSE) score, vitamin E components, and β-carotene. Further, proteomics analysis identified that thioredoxin-dependent peroxide reductase, myosin light polypeptide 6, and ATP synthase subunit β might be important in the progression and pathogenesis of AD.

Materials and methods

Subjects

Subjects were enrolled at the Memory Clinic of the Institute of Gerontology and Geriatrics, University of Perugia. Demographic information of the MCI and control patients used for the measurement of oxidative stress parameters is presented in Table 1. Demographic information of the AD, MCI, and control patients used for the identification of proteins with altered protein levels is presented in Table 2.

All subjects were evaluated according to a standard protocol including a detailed anamnesis, clinical evaluation, 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 as described previously [25,26].

Materials

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated. The OxyBlot oxidized protein kit was obtained from Intergen (Purchase, NY, USA). Primary antibodies for 4-hydroxy-2-nonenal (HNE) and 3-nitrotyrosine (3-NT) were obtained from Chemicon (Temecula, CA, USA).

Freshly obtained blood was layered on Lymphoprep (Gibco BRL, Bethesda, MD, USA), centrifuged, and washed twice. The pellet was resuspended in 400 μl of ice-cold phosphate-buffered saline (PBS). Eight microliters of 2.5% digitonin was added and kept on ice for 10 min at 4°C and centrifuged at 3500 g, C. The pelleted purified mitochondria were immediately frozen and kept at –80°C until analyses. Mitochondria purity was determined spectrophotometrically after ferrocyanochrome c oxidation at 550 nm at 25°C [27].

Sample preparation

To the frozen mitochondria 100 μl of Media 1 buffer (0.32 M sucrose, 0.10 mM Tris–HCl, pH 8.0, 0.10 mM MgCl₂, 0.08 mM EDTA, 10 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 11.5 μg/ml aprotinin; pH 8.0) was added and the samples were vortexed followed by sonication for 10 s at 20% power with a Fisher 550 Sonic Dismembrator (Pittsburgh, PA, USA). Protein concentrations were determined according to the Pierce BCA method (Rockford, IL, USA).

Measurement of oxidation-derived protein and lipid damage markers in control and MCI samples

Protein carbonyl measurement

Protein carbonyl content in the samples was determined according to the manufacturer’s protocol (OxyBlot, Chemicon) and as described previously [28]. Briefly, 5 μl of control and MCI mitochondria samples (4 mg/ml) was incubated for 20 min with 10 mM dinitrophenylhydrazine (DNPH) and 5 μl of 12% sodium dodecyl sulfate (SDS). Samples were neutralized with 7.5 μl of neutralization solution (2 M Tris in 30% glycerol). 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 and then with anti-rabbit IgG coupled to alkaline phosphatase (1:10,000) for 1 h at 27°C. After being washed and developed with ScionFast chromogen (Sigma), blots were scanned into Adobe Photoshop (Adobe Systems, Inc., Mountain View, CA, USA) and quantitated with Scion Image (PC version of Macintosh-compatible NIH Image). To validate the protein carbonyl detection approach, protein carbonyls were reduced by sodium borohydride or incubated with a secondary antibody, which showed no staining on the blots (data not shown), as described previously [29].

3-Nitrotyrosine levels

To determine protein-bound 3-NT levels, 5 μl (10 μg) of control and MCI mitochondria samples were incubated with Laemmli buffer (10 μl; 0.125 M Trizma base, pH 6.8, 4% SDS, 20% glycerol)

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>MCI</th>
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<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>M/F</td>
<td>1/4</td>
<td>5/0</td>
</tr>
<tr>
<td>Age</td>
<td>76.6 ± 7.4</td>
<td>79.6 ± 7.5</td>
</tr>
<tr>
<td>MMSE</td>
<td>29.0 ± 1.4</td>
<td>24.8 ± 1.1</td>
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<tr>
<td>ACE-R</td>
<td>87.5 ± 76</td>
<td>71.4 ± 9*</td>
</tr>
<tr>
<td>GDS</td>
<td>3.8 ± 13</td>
<td>4.4 ± 11</td>
</tr>
<tr>
<td>HIS</td>
<td>2.0 ± 14</td>
<td>3.0 ± 2.9</td>
</tr>
<tr>
<td>ADL</td>
<td>5.2 ± 0.4</td>
<td>5.4 ± 0.5</td>
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<tr>
<td>IADL</td>
<td>6.0 ± 1.9</td>
<td>4.4 ± 0.5*</td>
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MMSE, Mini Mental State Examination (range 0–30); ACE-R, Addenbrooke’s Cognitive Examination—Revised (range 0–100); GDS, Geriatric Depression Scale (range 0–15); HIS, Hachinski Ischemic Score (range 0–18); ADL, Activity of Daily Living (range 0–6); IADL, Instrumental Activity of Daily Living (range 0–8). *p < 0.001; Mann–Whitney test.

Table 2

<table>
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<th>AD</th>
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for 20 min. Samples (250 ng of protein) were blotted onto nitrocellulose membranes, and immunochemical methods were performed as described previously [28]. The rabbit anti-3-NT primary antibody was incubated 1:200 in blocking buffer (bovine serum albumin (BSA) 3% in Tris-buffered saline–Tween (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. To validate the protein nitration detection approach the nitrotyrosine was reduced by dithionite or incubated with secondary antibody alone, which showed no staining on the blot (data not shown), as described previously [30].

**Protein-bound 4-hydroxy-2-nonenal**

Protein-bound HNE levels were measured as a marker of lipid peroxidation [18,28]. The control and MCI mitochondrial 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 a nitrocellulose membrane in a slot-blot apparatus under vacuum. The membranes were incubated with anti-HNE rabbit polyclonal antibody (1:5000) 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.

**Protein alterations: proteomics approach**

To identify proteins with differential levels between control (n=5), MCI (n=5), and AD (n=5) we used a proteomics approach (see Table 2 for demographic information) [31].

**Isoelectric focusing (IEF)**

Fifty micrograms of mitochondrial protein from control, AD, and MCI samples was precipitated separately by addition of 15% ice-cold trichloroacetic acid for 10 min, followed by centrifugation at 14,000 rpm (23,700g) for 5 min at 4 °C. Pellets were washed in wash buffer (1:1 (v/v) ethanol:ethyl acetate) a total of four times to remove excess salts. After the final wash, 200 μl of rehydration buffer (8 M urea, 2 M thiourea, 50 mM dithiothreitol (DTT), 2.0% (w/v) Chaps, 0.2% Biolytes, bromophenol blue) was added to the samples and incubated for 2 h at room temperature (RT) and then sonicated for 10 s at 20% power. Samples (200 μl) were applied to 11-cm pH 3–10 ReadyStrip IPI strips and actively rehydrated at 20 °C for 18 h at 50 V, followed by isoelectrofocusing at a constant temperature of 20 °C beginning at 300 V for 2 h and then 500 V for 2 h, 1000 V for 2 h, 8000 V for 8 h, and finishing at 8000 V for 10 h, rapidly. IEF strips were stored at −80 °C until the second dimension of analysis was performed.

**2D–PAGE**

2D–PAGE was performed to further separate proteins previously separated on IEF strips. IEF strips were thawed and equilibrated for 10 min in equilibration buffer A (50 mM Tris–HCl, pH 6.8, 6 M urea, 1% (w/v) SDS, 30% (v/v) glycerol, and 0.5% DTT) and then reequilibrated for 10 min in equilibration buffer B (50 mM Tris–HCl, pH 6.8, 6 M urea, 1% (w/v) SDS, 30% (v/v) glycerol, and 4.5% iodoacetamide (IA)). All strips were rinsed in a 1× dilution of Tris–glycine–saline (TGS) running buffer before being placed into Criterion precast linear gradient (8–16%) Tris–HCl polyacrylamide gels. Precision Plus Protein standards and samples were run at a constant voltage of 200 V for 65 min in a 1× dilution of TGS running buffer.

**SYPRO ruby staining**

After 2D–PAGE, gels were incubated in a Fixing solution (7% (v/v) acetic acid, 10% (v/v) methanol) for 20 min at RT. SYPRO ruby protein gel stain (~50 ml) was added to gels and allowed to stain overnight at RT on a gently rocking platform, followed by scanning of the gels with a Molecular Dynamics STORM phosphoimager (λex/λem, 470/618 nm).

**Image analysis**

Spot intensities from SYPRO ruby-stained 2D-gel images of cognitively normal, MCI, and AD samples were quantified densitometrically according to the total spot density using PDQuest analysis software from Bio-Rad (Hercules, CA, USA). Intensities were normalized to total gel densities and/or densities of all valid spots on the gels. For the determination of spots with increased or decreased levels, we normalized spot density in AD/MCI samples compared to MCI/control samples. Only protein spots with a statistically significant difference based on a Student t test at 95% confidence (i.e., p < 0.05) were considered for MS analysis.

**In-gel trypsin digestion**

Protein spots identified as significantly altered were excised from 2D gels with a clean, sterilized blade and transferred to Eppendorf microcentrifuge tubes. Gel plugs were then washed with 0.1 M ammonium bicarbonate (NH₄HCO₃) at RT for 15 min, followed by incubation with 100% acetonitrile at RT for 15 min. After solvent removal, gel plugs were dried in their respective tubes under a flow hood at RT. Plugs were incubated for 45 min in 20 μl of 20 mM DTT in 0.1 M NH₄HCO₃ at 56 °C. The DTT/NH₄HCO₃ solution was then removed and replaced with 20 μl of 55 mM IA in 0.1 M NH₄HCO₃ and incubated with gentle agitation at RT in the dark for 30 min. Excess IA solution was removed and plugs were incubated for 15 min with 200 μl of 50 mM NH₄HCO₃ at RT. A volume of 200 μl of 100% acetonitrile was added to this solution and incubated for 15 min at RT. Solvent was removed and gel plugs were allowed to dry for 30 min at RT under a flow hood. Plugs were rehydrated with 20 ng/μl of modified trypsin (Promega, Madison, WI, USA) in 50 mM NH₄HCO₃ in a shaking incubator overnight at 37 °C. Enough trypsin solution was added to completely submerge the gel plugs.

**Mass spectrometry**

Salts and contaminants were removed from tryptic peptide solutions using C18 ZipTips (Sigma–Aldrich), reconstituted to a volume of ~15 μl in a 50:50 (water:acetonitrile) solution containing 0.1% formic acid. Tryptic peptides were analyzed by MS/MS with an automated Nanomate electrospray ionization (ESI; Advin Bioscience, Ithaca, NY, USA) Orbitrap XL MS (Thermo Scientific, Waltham, MA, USA) platform. The Orbitrap MS was operated in a data-dependent mode whereby the eight most intense parent ions were filtered with the following criteria: two trypsin miscleavages, fixed carbamidomethyl modification, variable methionine oxidation, parent tolerance 10 ppm, and fragment tolerance of 25 mmu or 0.01 Da. Results were filtered with the following criteria: Δcorr > 1.5, 2.0, 2.5, and 3.0 for +1, +2, +3, and +4 charge states, respectively, ΔCN > 0.1, and p (protein and peptide) < 0.01. Accession numbers
from IPI were cross-correlated with SwissProt accession numbers for final protein identification. It should be noted that proteins identified with a single peptide were kept for further analyses if multiple spectral counts (number of observed MS/MS spectra) were observed in a single analysis or if the peptide was identified in a separate analysis and workup of the same protein spot.

Vitamin E and \( \beta \)-carotene measurement methods

Analyses were performed as described previously [26]. Blood samples were taken from subjects after a minimum of 2 h fasting; serum and plasma samples were aliquotted and frozen at \(-80^\circ\text{C}\). Plasma tocopherols, tocotrienols, \( \alpha \)-tocopherolquinone, 5-NO\( _2 \)-\( \gamma \)-tocopherol, and \( \beta \)-carotene were measured with reverse-phase high-performance liquid chromatography (HPLC) using an electro-chemical CoulArray system (ESA, Chelmsford, MA, USA). Aliquots of 200 \( \mu \text{l} \) were mixed and extracted three times with a 1:2 ratio of ethanol to hexane, concentrated to dryness with high-purity nitrogen gas, and reconstituted in 300 \( \mu \text{l} \) mobile phase. \( \beta \)-Tocopherol (Supercrome, Milan, Italy); \( \alpha \)-, \( \gamma \)-, and \( \delta \)-tocopherol; \( \beta \)-carotene (Sigma–Aldrich); \( \alpha \)-, \( \gamma \)-, and \( \delta \)-tocotrienol (LGC-Promochem, Milan, Italy); \( \beta \)-tocotrienol (Matreya-DBA, Pleasant–Gap, PA, USA); \( \alpha \)-tocopherolquinone (Research Organics, Rome, Italy); and 5-NO\( _2 \)-\( \gamma \)-tocopherol (gift from Professor K. Hensley, University of Toledo Health Sciences Center, Toledo, OH, USA) were used as standards. After filtration, analyte separation was conducted at room temperature on a Discovery C18 column (Sigma–Aldrich). The mobile phase (30 mmol lithium acetate/L, 83\% HPLC-grade acetonitrile, 12\% HPLC-grade methanol, and 0.2\% HPLC-grade acetic acid, pH 6.5) was delivered at 1 ml/min.

2D Western blot

Proteins were first separated by 2D-gel electrophoresis followed by protein transfer to nitrocellulose membrane. The membrane was blocked with 3\% BSA in PBS-T for 1 h at 4 \( ^\circ \text{C} \). The membrane was incubated with anti-peroxiredoxin polyclonal antibody (Abcam, Cambridge, MA, USA) (1:1000) for 2 h in 1\% BSA for 2 h at room temperature, and then the membrane was washed three times in PBS-T for 5 min each. An anti-rabbit IgG alkaline phosphatase secondary antibody was diluted 1:3000 in PBS-T and incubated with the membranes for 1 h at room temperature. The membranes were washed in PBS-T three times for 5 min and developed using Sigmafast tablets (BCIP/NBT substrate).

Statistical analysis

Two-tailed Student \( t \) tests were used to analyze differences in oxidative stress markers between MCI and age-matched controls samples. A \( p \) value of less than 0.05 was considered statistically significant. Correlation analyses were assessed for significance using GraphPad Prism 5.

Results

Oxidative stress markers

In our laboratory we measured the amount of protein carbonyls and 3-NT levels in biological samples to assess the oxidation status of protein [32]. The levels of protein carbonyls \((p < 0.05; \text{Fig. 1A})\) were significantly increased in MCI lymphocyte mitochondria compared to controls. \text{Fig. 1B} shows that levels of HNE-bound protein, a product of lipid peroxidation, are significantly increased in mitochondria isolated from MCI lymphocytes compared to controls \((p < 0.05)\). \text{Fig. 1C} shows that levels of 3-NT \((p < 0.05)\) are significantly increased in MCI lymphocyte mitochondria compared to controls.

Correlation analysis of oxidative stress markers and MMSE

The levels of protein carbonyls showed an inverse correlation \((r^2 = -0.22)\) with MMSE score \((\text{Fig. 2A})\) of MCI patients; however, this correlation is not statistically significant \((p=0.3)\). In contrast, protein-bound HNE showed a significant \((p=0.02)\) negative correlation \((r^2 = -0.58)\) with MMSE score \((\text{Fig. 2B})\). The levels of protein-bound 3-NT did not show any significant \((p=0.07)\) correlation with MMSE score \((\text{Fig. 2C})\), although we observed a trend toward a negative correlation with MMSE score \((r^2 = -0.45)\). The
correlation analysis of oxidative stress with MMSE suggests that the increased protein-bound HNE is related to the decrease in cognitive functions in MCI and further support the hypothesis that lipid peroxidation is an early event in the pathogenesis of AD [33].

Correlation analysis of vitamin E components and β-carotene with oxidative stress markers

The vitamin E and β-carotene data used in this study were part of a recently published paper by Meccoci’s laboratory [26] and showed that alterations in the levels of vitamin E have an effect on MMSE score. In the current study, we carried out a correlation analysis between vitamin E components and β-carotene with the markers of oxidative stress, and only those components that showed a significant correlation are reported here. Fig. 3 shows that the amounts of tocopheryl quinone ($r^2 = -0.48, p=0.02$), δ-tocopherol ($r^2 = -0.52, p=0.01$), and β-carotene ($r^2 = -0.47, p=0.03$) showed significant inverse correlations with protein carbonyls. Further, the levels of γ-tocotrienol ($r^2 = -0.55, p=0.02$), α-tocotrienol ($r^2 = -0.65, p=0.01$), δ-tocopherol ($r^2 = -0.64, p=0.008$), γ-tocopherol ($r^2 = -0.68, p=0.004$), and β-carotene ($r^2 = -0.62, p=0.01$) showed a significant inverse correlation with protein-bound HNE. A significant negative correlation was observed between protein-bound 3-NT and γ-tocotrienol ($r^2 = -0.52, p=0.04$), δ-tocopherol ($r^2 = -0.52, p=0.03$), tocopheryl quinone ($r^2 = -0.51, p=0.04$), γ-tocopherol ($r^2 = -0.69, p=0.003$), and β-carotene ($r^2 = -0.64, p=0.007$) (Fig. 4). The levels of 5-NO$_2$-γ-tocopherol, a product of reaction between γ-tocopherol and reactive nitrogen species (RNS) [34], showed a significant positive correlation with protein carbonyls ($r^2 = 0.54, p=0.01$), protein-bound HNE ($r^2 = 0.79, p=0.0003$), and protein-bound 3-NT ($r^2 = -0.75, p=0.001$) (Fig. 5).

Alterations in protein levels in mitochondria

Two-dimensional gel-based proteomic analyses were used to identify mitochondrial proteins that showed altered levels in MCI and AD compared to age-matched controls. From these experiments we present results from three comparisons: control vs MCI, control vs AD, and MCI vs AD. The comparison of mitochondrial proteins between control and MCI provides insight into which specific protein alteration(s) might be critical in the transition from MCI to AD. The proteins with altered levels between control and MCI are shown in Fig. 6. Table 3 lists the characteristics of the proteins including the number of peptide sequences, score, molecular weight, isoelectric point, p value, and fold-change level. All the proteins from lymphocyte mitochondria that were identified as altered between controls and MCI show increased levels and are grouped into four categories: cellular energetics, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase B chain (LDH), and ATP synthase subunit β; structural proteins, including annexin, β-centractin, and myosin light polypeptide 6; cell signaling, including Rho GDP-dissociation inhibitor 2 (RhoGDI); and cellular defense, including thioredoxin-dependent peroxide reductase/peroxiredoxin III (PRDX3).

Fig. 7 shows a representative 2D-gel image of spots observed to statistically change in density in control vs AD. The characteristics of the identified proteins are listed in Table 4. The levels of most of the proteins are significantly decreased in AD compared to controls, except for ATP synthase subunit β and Ras suppressor protein 1. The proteins with differential levels between control and AD are grouped into four categories: cellular energetics, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase B chain (LDH), and ATP synthase subunit β; structural proteins, including annexin, β-centractin, and myosin light polypeptide 6; cell signaling, including Rho GDP-dissociation inhibitor 2 (RhoGDI); and cellular defense, including thioredoxin-dependent peroxide reductase/peroxiredoxin III (PRDX3).

Fig. 8 shows a representative 2D-gel image of mitochondrial protein spots observed to statistically change in density in MCI vs AD. The characteristics of the identified proteins are listed in Table 5. The levels of all the identified proteins were significantly decreased in AD compared to MCI. The proteins with differential levels between MCI and AD are grouped into two categories: structural proteins, actin-related protein 2/3 complex subunit 2; and cellular defense, heat shock protein 70 and thioredoxin.

To validate the proteomics results, 2D Western blot analysis was performed for peroxiredoxin III (Fig. 9). The spot corresponding to peroxiredoxin III appeared at the same location on the gel from which we excised and submitted the spot for MS identification of the protein, thereby validating the correct identification of this protein and providing confidence in the identification of all proteins.

Discussion

Mitochondria are one of the main sources of production of reactive oxygen species. Oligomeric Aβ has been shown to appear in the mitochondria during the early stage of MCI [23,35]. Oligomeric
Aβ has been suggested to be highly reactive and is capable of inserting itself into the lipid bilayer consequently inducing free radical production because of the presence of methionine as the 35th amino acid residue [9]. Met in Aβ is capable of forming a sulfuranyl radical and subsequently abstracting an allylic hydrogen atom from acyl chains of unsaturated lipids present in the lipid bilayer [36]. As evidenced by increased markers of protein oxidation, peripheral mitochondria are highly vulnerable to oxidative stress, demonstrating early oxidative stress in the progression of AD, similar to what is observed in brain.

Previous studies from our laboratory showed that the mitochondria isolated from AD lymphocytes showed increased markers of oxidative stress, and the oxidative stress inversely correlated with MMSE score [25]. MCI samples in this study showed a significant relationship between an increase in protein-bound HNE and MMSE score. This observation is consistent with lipid peroxidation as a process that is an early event in the progression of disease and the reported cognitive decline in patients suffering from this devastating disorder [37].

β-Carotene is a precursor of vitamin A and has been reported to have the ability to scavenge peroxy radicals. Further, in vitro studies showed that β-carotene can inhibit Aβ aggregation in a dose-dependent manner. The levels of β-carotene were reported to be decreased in brain, serum, and plasma of AD and MCI subjects [26,38,39]. Recently published data on levels of β-carotene using the same sample used in the current study showed a significant decrease in MCI plasma compared to control and it correlated inversely with MMSE score [26]. In the present study, we found that the levels of β-carotene were inversely proportional to the markers of oxidative stress. Decreased levels of β-carotene might lead to increased aggregation of Aβ and consequently to oxidative stress-mediated damage and be important in AD pathogenesis.

Vitamin E is a major lipid-soluble vitamin known to be good chain-breaking antioxidant [40,41]. Vitamin E includes a group of eight compounds referred to as tocopherols and tocotrienols [40]. Previous studies have reported reduced levels of α-tocopherol in the CSF and plasma of subjects with AD or MCI [26,42–45].
The exact role of vitamin E in AD pathogenesis is still controversial. A number of clinical trials using α-tocopherol failed to show any protective effect as a therapeutic in clinical studies involving late-stage AD or MCI patients [42,46,47]. In vitro and in vivo studies showed that other forms of vitamin E, i.e., γ-tocopherol and tocotrienols, were more effective than α-tocopherol in reducing oxidative stress [26,48]. In the present study, we showed that with the increased oxidative stress in mitochondria isolated from lymphocytes in patients with MCI the levels of δ-tocopherol decreased, suggesting that the alteration in the levels of δ-tocopherol might be an early event in the progression of AD.

Two markers of vitamin E damage, i.e., 5-NO2-γ-tocopherol and α-tocopherylquinone, showed a significant difference with respect to oxidative stress markers. γ-Tocopherol has been shown to protect against peroxynitrite-induced lipid peroxidation [49]. A previous study showed increased levels of 5-NO2-γ-tocopherol in AD brain [34]. In this study, we found a direct correlation between the levels of 5-NO2-γ-tocopherol and the oxidative stress markers. The increased nitration of γ-tocopherol could be due to increased nitration observed in the MCI samples, which could potentially serve as a marker for monitoring the nitrosative stress in AD pathogenesis. α-Tocopherylquinone is a metabolite of α-tocopherol, which upon reduction to tocopheroylhydroquinone has been shown to have antioxidant activity [50]. The decrease in the levels of α-tocopherylquinone with concomitant increase in oxidative stress markers reported here suggests one factor important in the elevated oxidative stress.

In addition to changes in oxidative stress, analysis of the mitochondrial proteome showed altered protein levels in MCI and AD mitochondria compared to controls. A large number of proteins were found to have altered expression during the progression of AD, which could be functionally categorized as energetics, structural, antioxidant, and cell signaling. Interestingly, we found that proteins involved in energetics, i.e., GAPDH, lactate dehydrogenase B chain, and ATP synthase subunit β, are significantly increased in the MCI samples compared to control, indicating that cells might be experiencing stress and there is an energy demand to overcome this stress. Previous studies from our laboratory and others showed alteration of these proteins in AD and MCI brain [51–56]. It is widely accepted that early in the progression of AD glucose metabolism is affected as shown by PET analysis [3]. We earlier identified a number of proteins in the glycolytic pathway as oxidatively modified [56]. GAPDH is not just a glycolytic enzyme, but has multifunctional roles and is found in various subcellular locations in brain [57]. One role of GAPDH is to capture nitric oxide. Hence, the increase in GAPDH we found here could also be due to increased nitric oxide formation in MCI lymphocytes as indexed by the observed increased protein-bound 3-NT levels.

Fig. 4. A correlative analysis between protein-bound HNE and γ-tocotrienol/α-tocotrienol/δ-tocopherol/γ-tocopherol and β-carotene showed a significant negative correlation, whereas 5-NO2-γ-tocopherol showed a significant positive correlation with protein-bound HNE (see text).
Decreased levels of heat shock cognate 71 protein and protein disulfide-isomerase A3 were found in mitochondria isolated from the lymphocytes during the transition of the disease from MCI to AD. Heat shock cognate 71 is also decreased in AD mitochondria compared to controls. Both protein disulfide-isomerase A3 and heat shock cognate protein have a Cys residue (at position 5 and 267, respectively) that is sensitive to oxidation. The observation of increased oxidative stress together with decreased levels of these proteins is consistent with increased accumulation of damaged protein reported in AD.

Vimentin is an intermediate filament that supports the morphology, organization, and function of mitochondria. The protein ARPC2/3 is important in the regulation of actin filament polymerization. Both vimentin and ARPC2/3 levels were decreased in AD compared to controls, which is consistent with cytoskeletal reorganization and may contribute to fragmentation of mitochondria [59]; such changes would consequently increase ROS/RNS production and decrease cellular energetics.

PRDX3 and myosin light polypeptide 6 levels were significantly increased in MCI mitochondria compared to control; however, during the transition of MCI to AD the levels of both these proteins were significantly decreased in the mitochondria, suggesting that altered levels of these proteins might be important in the progression of AD.

PRDX3 is found exclusively in the mitochondrion and comprises 5% of the total mitochondrial matrix. PRDX3 is a two-cysteine peroxidase of the mammalian peroxiredoxins (Prx’s), a family of six isoforms that is capable of catalyzing H₂O₂ reduction [60,61]. During the reduction of H₂O₂ the active NH₂-terminal Cys of one subunit in the PRDX3 homodimer is oxidized to cystine sulfenic acid (Cys–SOH), which forms a disulfide linkage with the C-terminal Cys–SH of the other homodimer subunit. Thioredoxin (Trx) then reduces the thiolate form of the active cysteine that lowers the active site pKₐ and preserves the anionic charged state of NH₂-terminal Cys–SH. Recent studies suggest that the mammalian Trx/Prx redox system is critical for antioxidant regeneration.

Fig. 5. A correlative analysis between protein-bound 3-NT and γ-tocotrienol/δ-tocopherol/tocopheryl quinone/γ-tocopherol and β-carotene showed a significant negative correlation, whereas 5-NO₂-γ-tocopherol showed a significant positive correlation with protein-bound 3-NT (see text).
and regulation of intracellular ROS levels generated by respiration and metabolism of dopamine [62,63].

As discussed earlier Aβ is found in the mitochondria early during the progression of the disease, which can initiate the process of lipid peroxidation in the mitochondrial membrane leading to alterations in the function and composition of mitochondria [22,23], eventually leading to impairment of the electron transport chain and consequently increased formation of ROS and oxidative stress. The increased levels of PRDX3 during the early stages of the disease suggest that oxidative stress occurs during the early stages of disease progression. Hence, increased levels of PRDX3 at the MCI stage of AD suggest a compensatory mechanism to protect against elevated oxidative stress as observed by the increased markers of oxidative stress in the MCI samples. Elevated levels of PRDX3 were induced by hypoxia, which protects the cells against excitotoxic cell death and apoptosis. Lubec and co-workers [64] showed decreased levels of PRDX3 in AD and Down syndrome brain.

Interestingly, the levels of both PRDX3 and thioredoxin are decreased in mitochondria isolated from AD lymphocytes. The reduced levels of PRDX3 lead to increased formation of ROS, including H₂O₂, that could accelerate apoptosis via increased production of oxidative stress by-products such as HNE, with increased rates of mitochondrial membrane potential collapse, release of cytochrome c, and activation of caspase [65]. A previous

![Fig. 6. Identification of the proteins with differential levels between mitochondria isolated from control and MCI lymphocytes. Representative 2D-gel images of the proteins isolated from the mitochondria of (top) control and (bottom) MCI lymphocytes. Proteins showing altered levels were indicated by spot ID, and their identification was from nanospray ESI–MS/MS analyses. n=5 for control and MCI.](image)

![Fig. 7. Identification of the proteins with differential levels in mitochondria isolated from control and AD lymphocytes. Representative 2D-gel images of the proteins isolated from the mitochondria of (top) control and (bottom) AD lymphocytes. Proteins showing altered levels were indicated by spot ID, and their identification was from nanospray ESI–MS/MS analyses. n=5 for control and AD.](image)

<table>
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<tr>
<th>Spot</th>
<th>Protein identified</th>
<th>Accession No.</th>
<th>Coverage</th>
<th>No. of peptides</th>
<th>Score</th>
<th>MW (kDa)</th>
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MW, molecular weight; pI, isoelectric point. (i) indicates increase.
study showed increased levels of oxidative stress markers, altered antioxidant enzyme levels and activities, and decreased plasma levels of reduced glutathione and increased levels of oxidized glutathione [66] in the lymphocytes of AD patients, consistent with an elevated oxidative environment in AD. Further, others showed that the levels of plasma antioxidants such as α-carotene and β-carotene were significantly lower in patients with AD compared with controls [13]. The imbalance in the levels of antioxidants and increased levels of oxidative insult conceivably could be due to Aβ in the plasma of AD subjects. However, studies related to plasma Aβ levels showed contradictory results.

Myosin light polypeptide 6 levels is increased in MCI mitochondria from lymphocytes compared to the control. However, during the transition from MCI to AD the level of this protein is significantly decreased. Myosin light polypeptide is a regulatory light chain of myosin and hence important for the function of myosin. Decreased levels of myosin during the progression of AD may compromise mitochondrial structure and consequently the function, leading to loss of cellular energetics, increased production of free radicals, and eventually cell death.

In conclusion, the mitochondria isolated from MCI lymphocytes showed increased oxidative stress that correlated with altered levels of a number of vitamin E components, suggesting that increased oxidative stress markers in the peripheral system may potentially reflect brain damage and could potentially serve as a biomarker for progression, diagnosis, or treatment of AD. Further, identification of affected mitochondrial proteins in common during the progression of AD suggests these proteins might be instrumental in progression, diagnosis, or treatment early in the disease. Further, identification of proteins that are targets of oxidation could be helpful in understanding better the mechanism of disease progression and pathogenesis. Such studies are currently under way in our laboratory.

**Table 4**

<table>
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MW, molecular weight; pl, isoelectric point. (i) indicates increase and (d) indicates decrease.

**Fig. 8.** Identification of the proteins with differential levels in mitochondria isolated from MCI and AD lymphocytes. Representative 2D-gel images of the proteins isolated from the mitochondria of (top) MCI and (bottom) AD lymphocytes. Proteins showing altered levels were indicated by spot ID, and their identification was from nanospray ESI–MS/MS analyses. n = 6 for MCI and AD.

**Fig. 9.** A representative blot validating the location of peroxiredoxin III on 2D Western blot. Spot 5202 on gel (A) corresponding to peroxiredoxin III was validated by 2D Western blot (B), which was probed with anti-peroxiredoxin III antibody, consistent with the molecular weight, pl, and location of peroxiredoxin III, thereby confirming its identity after proteomics analysis.
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Acknowledgments

References


MW, molecular weight; pl, isoelectric point. (d) indicates decrease.


