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# Redox proteomics identification of oxidatively modified hippocampal proteins in mild cognitive impairment: Insights into the development of Alzheimer's disease

D. Allan Butterfield,[a,b,c,*] H. Fai Poon,[a] Daret St. Clair,[d] Jeffery N. Keller,[b,e] William M. Pierce,[f] Jon B. Klein,[g] and William R. Markesbery[b,h]

[a]*Department of Chemistry, University of Kentucky, Lexington, KY 40506-0055, USA*
[b]*Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40506, USA*
[c]*Center of Membrane Sciences, University of Kentucky, Lexington, KY 40506, USA*
[d]*Graduate Center for Toxicology, University of Kentucky, Lexington, KY 40506, USA*
[e]*Department of Anatomy and Neurobiology, University of Kentucky, Lexington, KY 40506, USA*
[f]*Department of Pharmacology and Core Proteomics Laboratory, University of Louisville, Louisville, KY 40208, USA*
[g]*Kidney Disease Program and Core Proteomics Laboratory, University of Louisville, Louisville, KY 40208, USA*
[h]*Departments of Pathology and Neurology, University of Kentucky, Lexington, KY 40506, USA*

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**Mild cognitive impairment (MCI) is generally referred to the transitional zone between normal cognitive function and early dementia or clinically probable Alzheimer's disease (AD). Oxidative stress plays a significant role in AD and is increased in the superior/middle temporal gyri of MCI subjects. Because AD involves hippocampal-resident memory dysfunction, we determined protein oxidation and identified the oxidized proteins in the hippocampi of MCI subjects. We found that protein oxidation is significantly increased in the hippocampi of MCI subjects when compared to age- and sex-matched controls. By using redox proteomics, we determined the oxidatively modified proteins in MCI hippocampus to be α-enolase (ENO1), glutamine synthetase (GLUL), pyruvate kinase M2 (PKM2) and peptidyl-prolyl *cis*/*trans* isomerase 1 (PIN1). The interacteome of these proteins revealed that these proteins functionally interact with SRC, hypoxia-inducible factor 1, plasminogen (PLG), MYC, tissue plasminogen activator (PLAT) and BCL2L1. Moreover, the interacteome indicates the functional involvement of energy metabolism, synaptic plasticity and mitogenesis/proliferation. Therefore, oxidative inactivation of ENO1, GLUL and PIN1 may alter these cellular processes and lead to the development of AD from MCI. We conclude that protein oxidation plays a significant role in the development of AD from MCI and that the oxidative inactivation of ENO1, GLUL, PKM2 and PIN1 is involved in the progression of AD from MCI. The current study provides a framework for future studies on the development of AD from MCI relevant to oxidative stress.**


## Introduction

Mild cognitive impairment (MCI) is generally referred to the transitional zone between normal cognitive aging and early dementia or clinically probable Alzheimer's disease (AD) (Winblad et al., 2004). Most individuals with MCI eventually develop AD, which suggests that MCI may be the earliest phase of the AD (Almkvist et al., 1998; Flicker et al., 1991; Luis et al., 2003; Morris et al., 2001). Although a variety of criteria are used to define MCI, they have in common that (a) MCI is referred to as the measurable cognitive deficits of non-demented persons and (b) MCI represents a clinical syndrome that does not fulfil a diagnosis of dementia but leads to a high risk of progressing to a dementia disorder (Winblad et al., 2004). When persons are diagnosed with MCI, most progress to AD and other dementia types, but some are stable or even recover (Winblad et al., 2004). Moreover, neuroimaging studies by magnetic resonance imaging (MRI) demonstrate the atrophy of the hippocampus or entorhinal cortex in MCI patients, indicating the relationships with transition of normal aging to MCI then later to clinical AD (de Leon et al., 2004).

Oxidative stress plays a significant role in AD (Butterfield et al., 2001; Butterfield and Lauderback, 2002; Giasson et al., 2002; Markesbery, 1997; Zhu et al., 2004). Manifested by elevated levels of nucleic acid oxidation, protein oxidation and lipid peroxidation, oxidative damage is most severe in AD hippocampus, a brain

* Corresponding author. Department of Chemistry, Center of Membrane Sciences, and Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40506, USA. Fax: +1 859 257 5876.
*E-mail address:* dabcns@uky.edu (D.A. Butterfield).
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region that is responsible for memory processing and cognitive function (Butterfield et al., 2001; Butterfield and Lauderback, 2002). Although the role oxidative stress plays in AD is now well recognized, it remains unclear whether oxidative stress is a primary event or an epiphenomenon of AD progression. The highest level of oxidative damage is correlated to the brain regions that exhibit high levels of amyloid β-peptide (Aβ) and the most neuronal denegation in AD (Butterfield et al., 2001; Butterfield and Kanski, 2001; Butterfield and Lauderback, 2002; Giasson et al., 2002; Hensley et al., 1995a; Markesbery, 1997; Markesbery and Carney, 1999; Zhu et al., 2004). Moreover, oxidative stress-mediating entities per se induce neuronal death in vitro; and protein oxidation and lipid peroxidation in the superior and middle temporal gyri (SMTG) of MCI patients are increased (Keller et al., 2005). All of these studies strongly suggest that oxidative stress is indeed a primary event in the development of AD. To gain insight into the primary role of oxidative stress in the development of MCI to AD, we used redox proteomics to identify the proteins that are oxidatively modified in the hippocampus of MCI. We found that α-enolase (ENO1), glutamine synthetase (GLUL), pyruvate kinase M2 (PKM2) and peptidyl-prolyl *cis*/*trans* isomerase 1 (PIN1) are significantly oxidized in the hippocampi of MCI subjects when compared to that in control.

## Methods and materials

### *Subjects*

The normal control subjects in this study were four females and two males, and the average age was 82 ± 2.6 years. The amnestic MCI patients were four females and two males, and the average age was 88 ± 1.5 years (Table 1). All subjects came from our longitudinally followed normal control group that has annual neuropsychological testing and neurological and physical examinations every 2 years. Control subjects had: (a) no cognitive complaints; (b) normal cognitive test scores, especially objective memory test scores; (c) intact ADLs; and (d) normal neurologic examinations. Amnestic MCI patients met the criteria described by Petersen (Petersen, R.C., (ed) *Mild Cognitive Impairment, Aging to Alzheimer's Disease*, Oxford University Press: New York 2003) which included: (a) a memory complaint corroborated by an informant, (b) objective memory test impairment (age- and education-adjusted), (c) general normal global intellectual function, (d) intact ADLs, (e) Clinical Dementia Rating score of 0.0 to 0.5, (f) no dementia and (g) a clinical evaluation that revealed no other cause for memory decline.

Table 1
Profile of subjects used in this study

| | Age (years) | Gender | Brain weight (g) | PMI (h) | Braak |
|---|---|---|---|---|---|
| Control 1 | 86 | Female | 1300 | 3.75 | I |
| Control 2 | 74 | Male | 1400 | 4 | I |
| Control 3 | 86 | Female | 1150 | 1.75 | I |
| Control 4 | 90 | Female | 1110 | 4 | II |
| Control 5 | 76 | Female | 1315 | 2 | I |
| Control 6 | 79 | Male | 1240 | 1.75 | II |
| Average | 82 ± 2.6 | | 1253 ± 44 | 2.88 ± 0.5 | |
| MCI 1 | 91 | Female | 1155 | 5 | III |
| MCI 2 | 93 | Female | 1050 | 2.75 | III |
| MCI 3 | 87 | Male | 1200 | 3.5 | VI |
| MCI 4 | 87 | Male | 1170 | 2.25 | III |
| MCI 5 | 88 | Female | 1080 | 2.25 | V |
| MCI 6 | 82 | Female | 1075 | 3 | III |
| Average | 88 ± 1.5 | | 1122 ± 25[a] | 3.13 ± 0.4 | |

[a] The average brain weight of MCI subjects is statistically lower than that of control subjects, $P < 0.05$.

### *Neuropathology evaluation*

Hippocampal specimens were taken immediately at the time of autopsy and immersed in liquid nitrogen. Immediately adjacent specimens were immersed in formalin for histological and immunohistochemical evaluation. Sections also were taken from the middle frontal gyrus (Brodmann area 9), superior and middle temporal gyri (areas 21/22), inferior parietal lobule (areas 39/40), medial occipital lobe (areas 17/18), anterior cingulate gyrus (area 24), posterior cingulate gyrus (area 23), hippocampus, entorhinal cortex, amygdala, basal ganglia, thalamus, midbrain, pons, medulla and cerebellum. Sections were stained with H&E, the modified Bielschowsky method and neocortical and medial temporal lobe structures were immunostained with 10D-5 (for beta amyloid peptide) and alpha-synuclein using standard methods.

Neurofibrillary tangles (NFT), diffuse plaques and neuritic plaques (NP) were counted in the five most involved fields using Bielschowsky-stained sections of middle frontal gyrus, middle temporal gyrus, inferior parietal lobule and the posterior cingulate gyrus. Similar counts were performed with Gallyas-stained sections of hippocampal CA1 and subiculum, amygdala and entorhinal cortex. The details of the findings of 10 MCI cases (6 of the present series) are given in Markesbery et al. (2005).

Control subjects had Braak stage scores of II or less and only modest numbers of diffuse and NP. In essence, in MCI patients, NP were significantly elevated in all four neocortical areas and the amygdala compared to controls. There was a significant increase in NFT in amygdala, entorhinal cortex and hippocampal CA1 and subiculum compared to controls. None of the MCI or control subjects had Lewy bodies. Two of the MCI patients had tiny microscopic infarcts—one had five microinfarcts and another had one. One patient had a single lacunar infarct. All of these were quite small and had no role in the patient's symptoms.

### *Sample preparation*

The hippocampal samples were homogenized in a lysis buffer (10 mm HEPES, 137 mm NaCl, 4.6 mm KCl, 1.1 mm $KH_2PO_4$, 0.6 mm $MgSO_4$) containing the protease inhibitors leupeptin (0.5 mg/ml), pepstatin (0.7 μg/ml), trypsin inhibitor (0.5 μg/ml) and PMSF (40 μg/ml). Homogenates were centrifuged at 15,800×*g* for 10 min to remove debris. The supernatant was extracted to determine the total protein concentration by the BCA method (Pierce, Rockford, IL).

### *Total protein carbonyl levels of MCI hippocampus*

Levels of total protein carbonyls were determined immunochemically. Protein carbonyl levels were determined as adducts of 2,4-dinitrophenylhydrazine (DNPH) (Butterfield and Stadtman, 1997; Stadtman, 1992). Hippocampus samples (5 μl) were treated with an equal volume of 12% SDS. The hippocampal proteins were then derivatized with 10 μl of 20 mM 2,4-DNPH for 20 min. The

reaction was stopped by addition of neutralizing reagent (7.5 μl of 2 M Tris/30% glycerol buffer, pH 8.0). Levels of protein carbonyls in hippocampus of MCI and control patients were measured by using the slot-blot technique with 250 ng of protein loaded per slot. The 2,4-dinitrophenyl hydrazone (DNP) adduct of the carbonyls is detected on nitrocellulose paper using a primary rabbit antibody (Chemicon) specific for DNP–protein adducts (1:100) followed by a secondary goat anti-rabbit IgG (Sigma) antibody. The resultant stain was developed by application of Sigma-Fast BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablets; and the densities, which are proportional to the total protein carbonyl level, were determined by Scion-Image software package.

*Two-dimensional gel electrophoresis*

Samples of the hippocampal proteins were prepared as previously described (Poon et al., 2004). Briefly, 200 μg of protein from the hippocampi of six control and six MCI subjects was each applied to 12 pH 3–10 ReadyStrip™ IPG strips (Bio-Rad, Hercules, CA) for isoelectric focusing (IEF). After focusing, the IEF strips were stored at −80°C until second dimension electrophoresis was performed.

For second dimension electrophoresis, 12 Linear Gradient (8–16%) Precast criterion Tris–HCl gels (Bio-Rad) were used to separate proteins according to their molecular weight (MrW) after IEF. Precision Protein™ Standards (Bio-Rad) were run along with the samples. After electrophoresis, the 12 separate gels were incubated in fixing solution for 20 min. The gels were stained with SYPRO Ruby for 2 h, after which the gels were placed in deionized water overnight for destaining.

*Western blotting*

Western blotting of the 2D gels was performed as previously described (Poon et al., 2004). Protein (200 μg) from each of the six control and six MCI subjects was incubated with 10 mM 2,4-dinitrophenyl hydrazine (DNPH) solution (2 N HCl) at room temperature for 20 min. The gels were prepared in the same manner as for 2D electrophoresis as described above. The proteins from the 2D electrophoresis gels were transferred onto nitrocellulose paper using a Transblot-Blot® SD semi-Dry Transfer Cell (Bio-Rad) at 15 V for 2 h. The DNP adducts of the carbonyls of the brain proteins were detected immunochemically as described above.

*Image analysis*

The gels and nitrocellulose blots were scanned and saved in TIF format using a Storm 860 Scanner (Molecular Dynamics) and a Scanjet 3300C (Hewlett Packard), respectively. PDQuest software (Bio-Rad) was used for matching and analysis of visualized protein spots among different gels and oxyblots. The principles of measuring intensity values by 2D analysis software were similar to those of densitometric measurement. The average mode of background subtraction was used to normalize intensity values, which represent the amount of protein (total protein on gel or oxidized protein on oxyblot) per spot. After completion of spot matching, the average normalized intensity of six individual gels (or oxyblots) from the six controls was compared to the average normalized intensity of six individual gels (or oxyblots) from the MCI subjects using Student's t test. Only those spots that were considered statistically significant were selected for identification. Similar statistical analysis is usually used for proteomics data analysis (Castegna et al., 2002a; Korolainen et al., 2002; Maurer et al., 2005). As discussed in detail previously (Boguski and McIntosh, 2003; Maurer et al., 2005), sophisticated statistical analyses for microarray data are not applicable for proteomics data.

*Trypsin digestion*

Samples were digested using the techniques previously described (Poon et al., 2004). Briefly, the selected protein spots were excised and washed with ammonium bicarbonate ($NH_4HCO_3$) then acetonitrile at room temperature. The protein spots were incubated with dithiothreitol then iodoacetamide solutions. The gel pieces were digested with 20 ng/μl modified trypsin (Promega, Madison, WI) using 25 mM $NH_4HCO_3$ with the minimum volume to cover the gel pieces. The gel pieces were chopped into smaller pieces and incubated at 37°C overnight in a shaking incubator.

*Mass spectrometry*

Peptide digests (1 μl) were mixed with 1 μl α-cyano-4-hydroxy-*trans*-cinnamic acid (10 mg/ml in 0.1% TFA:ACN, 1:1, v/v). The mixture (1 μl) was deposited onto a fast evaporation nitrocellulose matrix surface, washed twice with 2 μl 5% formic acid and analyzed with a TofSpec 2E (Micromass, Manchester, UK) MALDI-TOF mass spectrometer in reflectron mode. The mass axis was adjusted with trypsin autohydrolysis peaks (*m*/*z* 2239.14, 2211.10 or 842.51) as lock masses. The MALDI spectra used for protein identification from tryptic fragments were searched against the NCBI protein databases using the MASCOT search engine (http://www.matrixscience.com). Peptide mass fingerprinting used the assumption that peptides are monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues. Up to 1 missed trypsin cleavage was allowed. Mass tolerance of 100 ppm was the window of error allowed for matching the peptide mass values. To assign a level of confidence to the identification of specific proteins from the mass spectra, we used the probability-based Mowse score, which indicates the probability that the match between the database and a spectrum is a random event. This probability equals $10^{(-\text{Mowse score}/10)}$. Mowse scores greater than 62 were considered significant.

*Immunoprecipitation of ENO1, GLUL, PKM2 and PIN1*

The antibodies against ENO1, GLUL, PKM2 and PIN1 are commercially available. The anti-ENO1 antibody (Santa Cruz Biotech. Inc., Santa Cruz, CA) is raised against a peptide mapped near the C-terminus of human ENO1. The anti-PKM2 antibody (Abgent, San Diego, CA) is generated from rabbits immunized with a synthetic peptide selected within amino acid 100–200 of human PKM2. The anti-PIN1 antibody is raised against amino acid 41–163 of human PIN1. The anti-GLUL antibody is generated in mice with purified GLUL from sheep brain, but reactivity against human is previously reported (Hensley et al., 1995b). The immunoprecipitation was performed as described previously (Lauderback et al., 2001). The antibodies were added directly to the hippocampal homogenate, and the mixture was incubated on a rotary mixer overnight at 4°C. The antigen/antibody complexes were precipitated with protein-A-conjugated agarose beads if the

antibodies were raised in rabbit; protein G-conjugated agarose beads are used if the antibodies were raised in goat or mouse. Agarose beads were added in 50 μl aliquots from a stock of 300 mg/ml in PBS and mixed on a rotary mixer for 1 h at room temperature. Beads were then centrifuged and washed with the washing buffer (pH 8, 50 mM Tris HCl, 150 mM NaCl, 0.1% Tween 20) three times. The ENO1, GLUL, PKM2 and PIN1 from each control and MCI subject (12 total) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA).

*Post-Western blot immunochemical detection of protein carbonyl levels: ENO1, GLUL, PKM2 and PIN1*

The carbonyl levels of ENO1, GLUL, PKM2 and PIN1 were detected by post-Western blot derivatization after immunoprecipitation (Conrad et al., 2000). Following the electroblotting procedure, the nitrocellulose membranes were equilibrated in 20% methanol for 5 min. Membranes were then incubated in 2 N HCl for 5 min. The membranes were next incubated in 0.5 mM DNPH solution exactly 5 min. The membranes were washed three times in 2 N HCl and five times in 50% methanol (5 min each wash). After post-Western blot derivatization, the immunochemical detection and measurement of carbonyl levels of GLUL, PKM2 and PIN1 were similar to that for total protein carbonyl level detection described above.

*Enzyme activities of enolase, glutamine synthetase, pyruvate kinase and peptidyl-prolyl cis/trans isomerase*

Enolase activity assay was performed as described previously (Wagner et al., 2000) with modification (Poon et al., 2005). Enolase was added to 100 μl of assay mixture (20 mM $Na_2HPO_4$, pH 7.4, 400 mM KCl, 0.01 mM EDTA, 2 mM 2-phospho-D-glycerate) in a UV-transparent microtiter plate (Corning, NY). The enzymatic activity was determined by the change of absorption at $A_{240}$ for 5 min.

Glutamine synthetase was determined by the method of Rowe et al. (1970) as modified by Miller et al. (1978). The absorbance was recorded at 505 nm as described above.

Pyruvate kinase activity is determined according to previously described (Mildvan and Cohn, 1965). Briefly, 95 μl of assay mixture (1.66 mM of ADP, 0.22 mM NADH, 0.1 mM of phosphoenolpyruvate and 5 units lactate dehydrogenase) was added to 5 μl of the hippocampal protein (2 μg/μl). The enzymatic activity was determined by the change of absorption at $A_{340}$ for 15 min.

Peptidyl-prolyl *cis/trans* isomerase activity was measured by a protocol from Kullertz et al. (1998). Typically, the 100 μl assay mixture consisted of 97 μl of HEPES buffer (32 mM, pH 7.8), 1 μl (25 mg/ml) Suc-AEPF-pNA (Bachem, Switzerland) and 1 μl (60 mg/ml) of chymotrypsin solution (Sigma, MO). The reaction was started by addition of 2 μl of brain homogenate (2 mg/ml) to the assay mixture, and the absorbance of *p*-nitroaniline was followed at 395 nm for 10 min (Sultana et al., in press-a).

*Protein interacteome*

The functional protein interacteome was obtained by using Interaction Explorer™ Software PathwayAssist™ software package (Stratagene, La Jolla, CA). PathwayAssist is a software for functional interaction analysis. It allows for the identification and visualization of pathways, gene regulation networks and protein interaction maps (Donninger et al., 2004). The proteins are first imported as the gene symbols as a set of data. This data set is then searched against ResNet, a database containing over 500,000 biological interactions built by applying the MedScan text-mining algorithms to all PubMed abstracts. These interactions are then visualized by building interaction networks with shortest-path algorithms. This process can graphically identify all known interactions among the proteins. The information of the function of these proteins and their relevance to diseases are then obtained by using the BIOBASE's Proteome BioKnowledge Library form Incyte Corporation (Incyte, Wilmington, DE) (Hodges et al., 2002).

*Statistics*

The data were analyzed by Student's *t* tests. A value of $P < 0.05$ was considered statistically significant.

## Results

To determine if protein oxidation occurs in MCI subjects, the total protein carbonyl level of hippocampi of MCI patients was compared to control subjects. The average age and postmortem interval (PMI) of the control and MCI subjects were not statistically different (Table 1). However, the mean brain weight of MCI subjects was significantly lower (10%) than that of the controls (Table 1). Moreover, the Braak stage of control subjects ranged between I and II, whereas the MCI subjects ranged between III and V (Table 1). These results are consistent with previous findings that cognitive impairment is associated with lower brain weight and more severe neurofibrillary pathology (Riley et al., 2005). Therefore, these control subjects were age- and sex-matched (4 females and 2 male) to the MCI subjects (Table 1).

When compared to control, the hippocampus in MCI demonstrated increased protein carbonyl levels (Fig. 1). By using redox proteomics, the oxidized proteins were separated and identified. Fig. 2 shows the representative 2D gels of the hippocampi of control and MCI subjects. The corresponding 2D Western blots of oxidized proteins in the hippocampi of control and MCI patients are shown in Fig. 3. Comparing the densitometric intensities of individual spots, we determined that four proteins were oxidatively

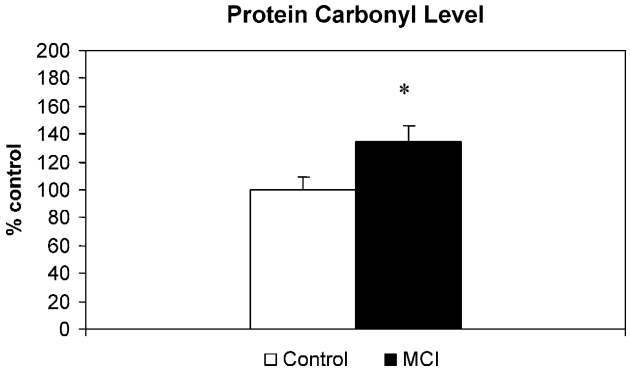


Fig. 1. Total protein carbonyl levels of control and MCI subjects. Data represent the average mean total protein carbonyl of MCI and control subjects. Error bar indicates SEM for 6 subjects in each group. Measured value is normalized with the mean of the control subjects. *$P < 0.05$.

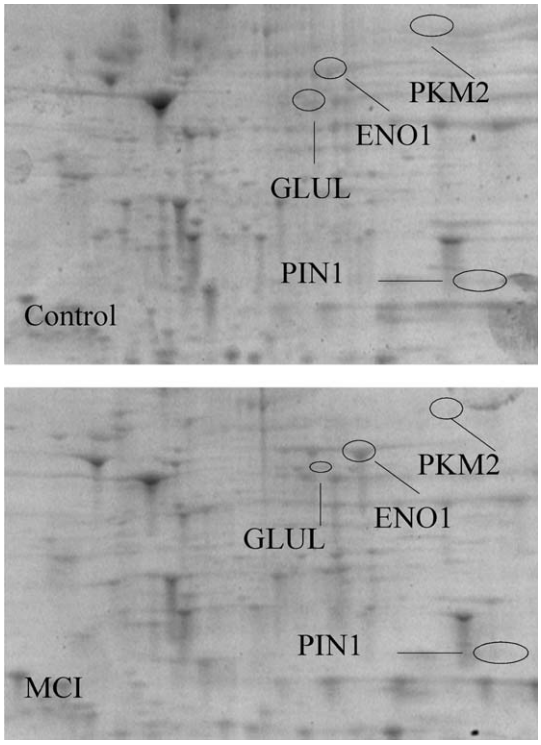


Fig. 2. Representative 2D gels show proteins from the hippocampus of a control subject (top) and a MCI subject (bottom).

modified in the hippocampi of MCI subjects when compared to that of the controls, indicated by the increased carbonyl level of these proteins (Table 2).

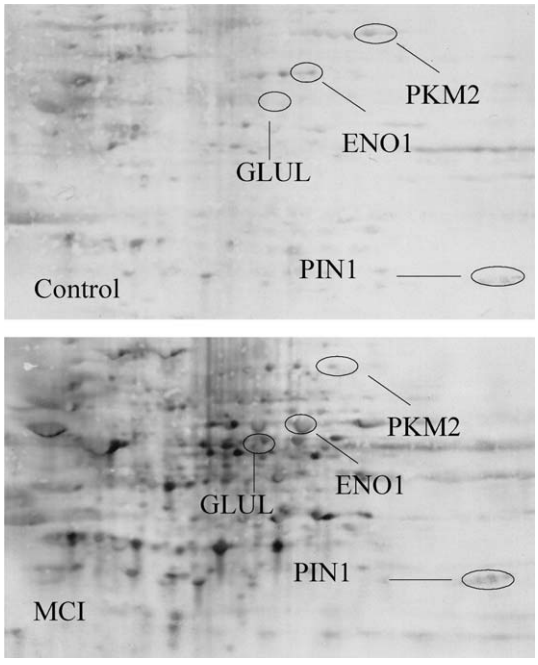


Fig. 3. Representative 2D oxyblots show oxidized proteins from the hippocampus of a control subject (top) and an MCI subject (bottom). The average specific carbonyl levels of each protein spots in six MCI hippocampi were compared to those of six control hippocampi. Only spots that showed statistically significant different average specific carbonyl levels were identified by mass spectrometry.

Table 2
Carbonyl level of protein in MCI brain

| Identification | % Control | *P* value |
|---|---|---|
| Enolase 1 (ENO1) | 367 ± 109 | <0.05 |
| Glutamine synthetase (GLUL) | 770 ± 261 | <0.05 |
| Pyruvate kinase M2 (PKM2) | 883 ± 346 | <0.05 |
| Peptidyl-prolyl *cis*/*trans* isomerase (PIN1) | 434 ± 129 | <0.05 |

The four excessively oxidized proteins in MCI hippocampus identified by mass spectrometry were ENO1, GLUL, PKM2 and PIN1. The mass spectra of these proteins used for identification are shown in Fig. 4A, and the database search for these proteins results in a single identification (Fig. 4B). The parameters for the identification of the oxidized proteins by mass spectrometry are summarized in Table 3; these protein identifications agreed with the expected MrW and p*I* range based on their positions on the 2D gels.

To validate the proteomics results, we used traditional immunochemistry to detect the oxidized hippocampal protein in MCI subjects. Consistent with the proteomics results, the carbonyl levels of ENO1, GLUL, PKM2 and PIN1 were significantly increased by about 35%, 40%, 30% and 50%, respectively, in the hippocampi of the MCI subjects compared to controls (Fig. 5A). The increased carbonyl levels of ENO1, GLUL, PKM2 and PIN1 in the hippocampi of MCI subjects were more robust when detected by proteomics method. The differences in the magnitude of fold changes of carbonyl levels between the two techniques are likely because proteomics measures the carbonyl level per unit of protein, whereas Western blotting measures the carbonyl level of total protein. Clearly, both techniques showed that ENO1, GLUL, PKM2 and PIN1 are oxidatively modified in the hippocampi of MCI subjects, thus validating our proteomics results.

The oxidized proteins are not only oxidatively modified, but also oxidatively inhibited, demonstrated by the decreased activity of ENO1 (48%), GLUL (23%), PKM2 (26%) and PIN1 (11%) in hippocampi of MCI subjects when compared to controls (Fig. 5B). These results are consistent with the notion that oxidative modification of proteins generally leads to its inactivation (Aksenova et al., 1998; Babior, 1999; Butterfield and Stadtman, 1997; Hensley et al., 1995a; Poon et al., 2005; Sultana and Butterfield, 2004).

Using Pathway explorer™ to examine the functional interaction of ENO1, GLUL, PKM2 and PIN1 (Fig. 6), we found that these proteins are important in energy metabolism, synaptic plasticity and mitogenesis/proliferation. Moreover, they functionally interact with SRC, hypoxia-inducible factor 1, plasminogen (PLG), MYC, tissue plasminogen activator (PLAT) and BCL2L1. The information of the function of these proteins and their relevance to diseases is stated in Fig. 7.

## Discussion

Because hippocampal dysfunction is involved early in AD (Barnes, 1988) and since MCI precedes AD (Petersen, 2003; Winblad et al., 2004), investigation of oxidative stress in hippocampi of MCI subjects can determine if oxidative stress is a primary event or an epiphenomenon in the development of AD. The current study demonstrates that protein oxidation is signifi-

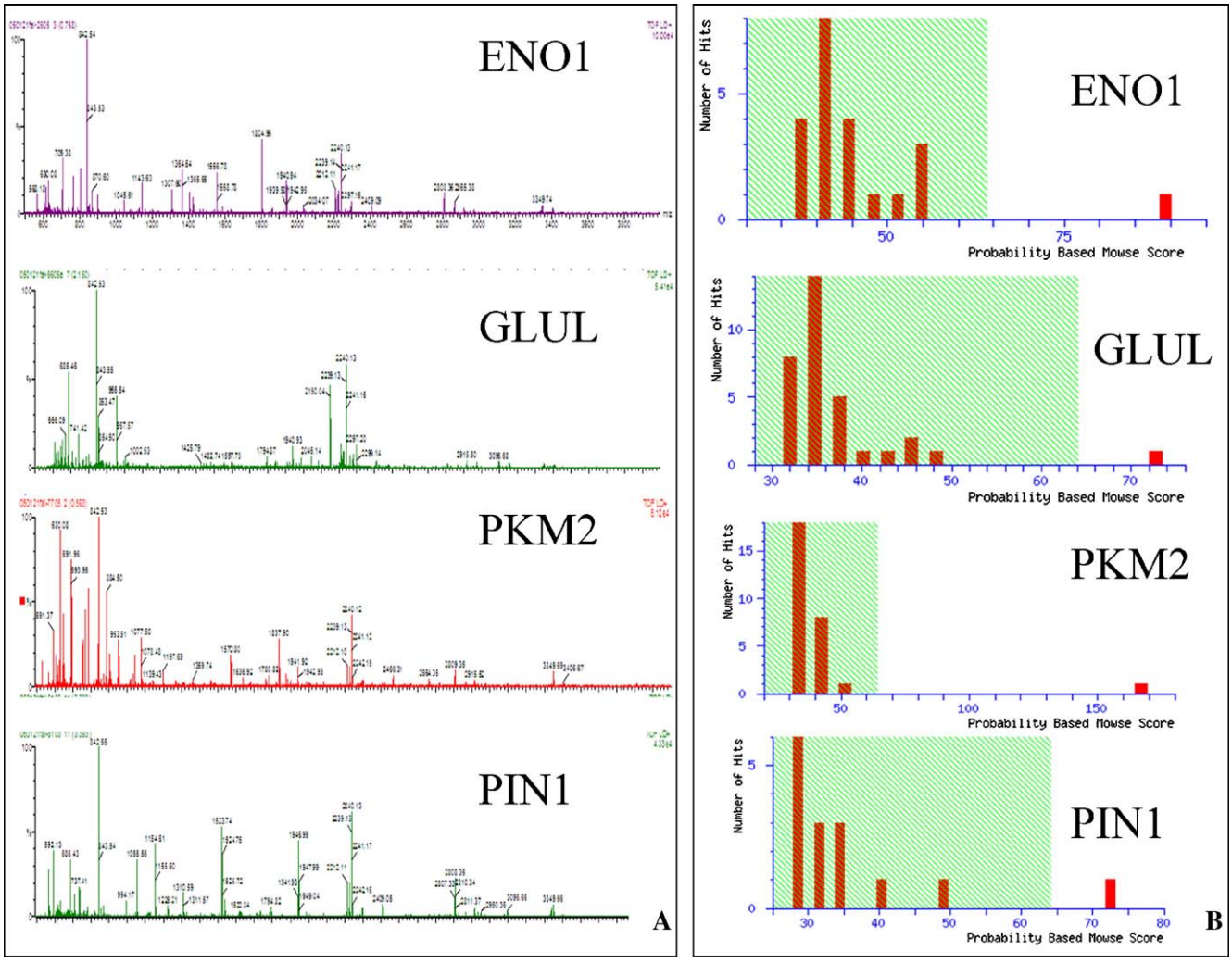


Fig. 4. Mass spectrometry and peptide mass fingerprinting. (A) Spectral masses (in mass per charge unit, $m/z$) of ENO1, GLUL, PKM2 and PIN1 obtained by MALDI-TOF mass spectrometry. (B) Possible matched proteins to the spectral masses of ENO1, GLUL, PKM2 and PIN1 are presented as multiple bars with differential probability-based MOWSE scores ($x$ axis). Only proteins with MOWSE scores greater than 62 (outside shaded area) were considered significantly matched. All of our spectra result in a single significant match, indicating high confidence of the identifications.

cantly increased in the hippocampi of MCI subjects when compared to age- and sex-matched controls (Table 1), which suggests that protein oxidation is important in the development of AD. These results are consistent with our previous study suggesting that oxidative stress is increased in the SMTG of MCI subjects (Keller et al., 2005). Others (Moreira et al., 2005) concur that oxidative damage is a major, early feature of AD, driving the deposition of Aβ, but suggest that deposited Aβ and hyperphosphorylated tau serve neuroprotective roles.

It is well established that protein oxidation is not a random event but rather is associated with increased oxidative modification of specific proteins (Castegna et al., 2002a, 2002b; Perluigi et al., 2005; Poon et al., 2004, 2005, in press). Therefore, we further investigated the specific proteins that are oxidatively modified in hippocampi of the MCI subjects by using redox proteomics. The proteins that are significantly oxidized in the hippocampi of MCI subjects are ENO1, GLUL, PKM2 and PIN1 (Table 2). Moreover, each of these oxidized proteins shows activity decline in the hippocampi of MCI subjects (Fig. 5B).

ENO1 is a subunit of enolase, which interconverts 2-phosphoglycerate to phosphoenolpyruvate. A decline of enolase activity resulted in abnormal growth and reduced metabolism in brain (Tholey et al., 1982). PKM2 is also a glycolytic enzyme that

Table 3
Summary of the identification of the proteins by mass spectrometry

| Identification | p*I* | MW (Da) | % Coverage | # Peptide match | MOWSE score | *P* value |
|---|---|---|---|---|---|---|
| Enolase 1 (ENO1) | 7.19 | 47,168 | 26 | 10 | 89 | $1.3 \times 10^{-09}$ |
| Glutamine synthetase (GLUL) | 6.67 | 42,061 | 17 | 8 | 73 | $5.0 \times 10^{-08}$ |
| Pyruvate kinase M2 (PKM2) | 7.79 | 58,062 | 41 | 21 | 199 | $1.3 \times 10^{-20}$ |
| Peptidyl-prolyl *cis*/*trans* isomerase (PIN1) | 9.05 | 18,246 | 32 | 5 | 71 | $7.9 \times 10^{-08}$ |

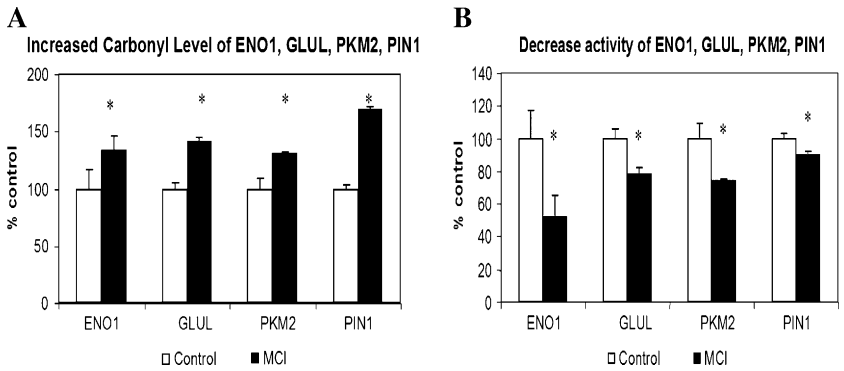


Fig. 5. (A) Increased carbonyl level of ENO1, GLUL, PKM2 and PIN1. Data represent the alteration of the ENO1, GLUL, PKM2 and PIN1 carbonyl levels in MCI subjects compared to control subjects using traditional immunochemical detection. Error bars indicate SEM for 6 subjects in each group. Measured values are normalized with the mean of the control subjects. $*P < 0.05$. (B) Decreased enzyme activities of ENO1, GLUL, PKM2 and PIN1. Data represent the alteration of the ENO1, GLUL, PKM2 and PIN1 activity in MCI subjects compared to control subjects. Error bars indicate SEM for 5 to 6 subjects in each group. Measured values are normalized with the mean of the control subjects. $*P < 0.05$.

converts phosphoenolpyruvate to pyruvate with phosphorylation of ADP to ATP. Therefore, the oxidative inactivation of ENO1 and PKM2 in the hippocampi of MCI subjects could conceivably result in the reduced ATP production and alter ATP-dependent processes, such as signal transduction and cell potential maintenance, thereby leading to altered $Ca^{2+}$ homeostasis and neuronal dysfunction.

It is well documented that GLUL activity declines in AD (Aksenov et al., 1996; Butterfield et al., 1997; Hensley et al., 1995b; Howard et al., 1996). GLUL catalyzes the rapid amination

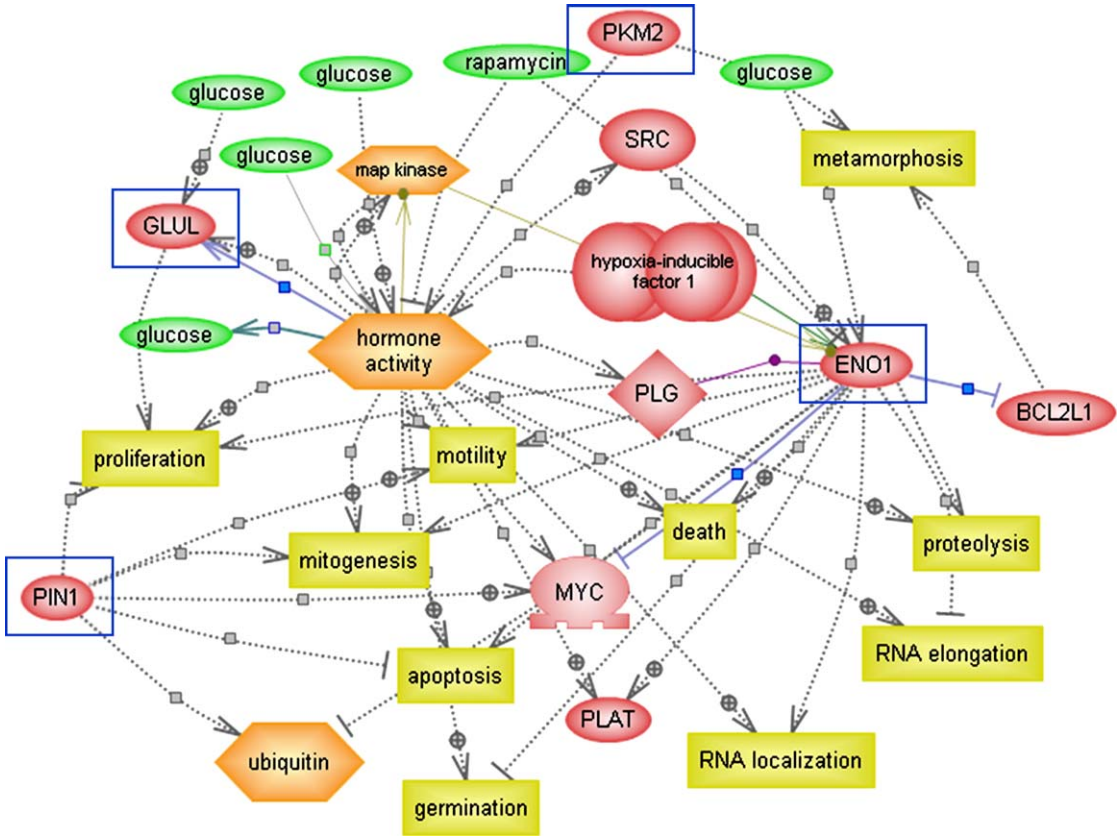


Fig. 6. Interacteome of ENO1, GLUL, PKM2 and PIN1 indicates that these proteins are involved in energy metabolism, mitogenesis/proliferation and neuroplasticity. Functions of the proteins that interact with ENO1, GLUL, PKM2 and PIN1 are obtained from BIOBASE's Proteome BioKnowledge Library: (a) SRC, a tyrosine kinase that is involved in cell proliferation, cell adhesion and cytoskeletal organization; (b) hypoxia-inducible factor 1, a receptor that mediates transcriptional responses to oxidative stress inducing process hypoxia; (c) PLG, a fibrinolytic protein that plays a role in Aβ degradation by forming plasmin, also involved in cell migration and extracellular matrix degradation; (d) MYC, a transcription factor that activates or represses gene expression, regulates cell proliferation and cell cycle progression; (e) PLAT, a serine protease that converts inactive plasminogen to plasmin; and (f) BCL2L1, an alternative form of bcl-xL that inhibits apoptosis and prevents mitochondrial depolarization by closing the mitochondrial voltage-dependent anion channel, abnormally regulates apoptosis.

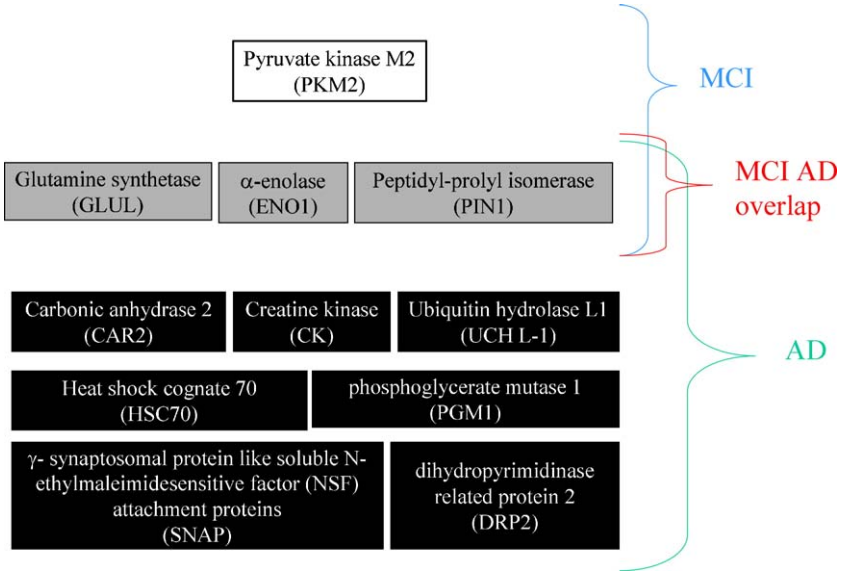


Fig. 7. Comparison between the oxidized protein profile of MCI subjects and AD subjects. ENO1, GLUL and PIN1 overlap these two oxidized protein profiles, indicating the significance of oxidative modification of these proteins in the development of MCI to AD.

of glutamate to form the non-neurotoxic amino acid glutamine. This reaction maintains the optimal level of glutamate and ammonia in neurons and modulates excitotoxicity. Together with the action of glutamate receptors and glutamate transporters, this process is important to neuroplasticity (Lamprecht and LeDoux, 2004). Therefore, oxidative inactivation of GLUL suggests that the glutamate–glutamine cycle in hippocampi of MCI subjects is impaired, which may contribute to glutamate dysregulation and impairment of neuroplasticity during the development of AD (Lee et al., 2002).

PIN1 is a chaperone enzyme that reversibly alters the conformation of proteins from *cis* to *trans* between a given amino acid and proline (Schutkowski et al., 1998). PIN1 recognizes phosphorylated Ser–Pro and phosphorylated Thr–Pro motifs in proteins and thereby binds to many cell cycle regulating proteins and tau protein. PIN1 is colocalized with phosphorylated tau and also shows an inverse relationship to the expression of tau in AD brains (Holzer et al., 2002; Kurt et al., 2003; Ramakrishnan et al., 2003). Moreover, PIN1 is oxidatively inhibited in AD brain (Sultana et al., in press-a) and is able to restore the function of tau protein in AD models (Lu et al., 1999). Therefore, the oxidative inactivation of PIN1 in the hippocampi of MCI subjects could be one of the initial events that trigger tangle formation, oxidative damage, cell cycle alterations and eventual neuronal death in AD brains.

Examination of the interacteome of ENO1, GLUL, PKM2 and PIN1 indicates that they functionally interact with SRC, PLG, MYC, PLAT BCL2L1 (Fig. 6). ENO1, PKM2 and GLUL are involved in energy metabolism because they interact with glucose during glycolysis and glutamine metabolism. Therefore, alteration of these proteins could eventually lead to impaired brain energy metabolism, a common feature that is shared by both AD and MCI subjects (Cao et al., 2003; Messier and Gagnon, 1996). ENO1, PIN1, GLUL and BCL2L1 are involved in mitogenesis, proliferation and apoptosis. Therefore, the alteration of these proteins in our study supports the notion that cell cycle events initiate apoptosis in neuronal cells (Herrup et al., 2004). Moreover, ENO1, PKM2, SRC, MYC and hypoxia-inducible factor 1 are involved in RNA elongation and the MAP kinase pathway. These processes are important to signal transduction and protein synthesis, two essential processes of neuroplasticity and memory formation (Lamprecht and LeDoux, 2004). Therefore, our current study suggests that alteration of these proteins may contribute to the cognitive disturbance in MCI subjects. Two other proteins that interact with ENO1, GLUL, PKM2 and PIN1 are PLG and PLAT. PLAT converts inactive PLG to plasmin that degrades amyloid β (Aβ), the major component of senile plaques (Tucker et al., 2000). Because PLG and PLAT interact with the proteins identified in this study, one can speculate that the oxidative modification of these proteins in the hippocampi of MCI subjects may contribute to Aβ accumulation, thereby leading to the development of AD.

Comparing the oxidized protein profiles of hippocampus in MCI subjects to inferior parietal lobule and hippocampus in AD (Castegna et al., 2002a, 2002b; Sultana et al., in press-b), we see ENO1, GLUL and PIN1 overlap in two of these oxidized protein profiles (Fig. 7). This suggests that oxidative inactivation of ENO1, GLUL and PIN1 alter the cellular processes in which these proteins are involved, such as energy metabolism, mitogenesis/proliferation and neuroplasticity. Impaired brain energy metabolism is reported in MCI and AD subjects (Cao et al., 2003; Messier and Gagnon, 1996), mitogenesis-mediated cell cycle events in MCI subjects precede the neuronal death in AD (Yang et al., 2003), and the decline of neuroplasticity is suggested to correlate with the development of AD from MCI (Ikonomovic et al., 2003). Therefore, this study not only demonstrates that protein oxidation plays a significant role in the development of MCI to AD, but also indicates that the oxidative inactivation of ENO1, GLUL, PKM2 and PIN1 is likely involved in the progression of MCI to AD, possibly through impaired energy metabolism, triggering abnormal cell cycle events and the decline in neuroplasticity. Therefore, this study provides a framework for future studies for the development of AD from MCI at the molecular level.

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