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Oxidatively Modified GST and MRP1 in Alzheimer's Disease Brain: Implications for Accumulation of Reactive Lipid Peroxidation Products

Rukhsana Sultana[1,3] and D. Allan Butterfield [1,2,3,4]

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Alzheimer disease (AD) is a neurodegenerative disorder characterized pathologically by intracellular inclusions including neurofibrillary tangles (NFT) and senile plaques. Several lines of evidence implicate oxidative stress with the progression of AD. 4-hydroxy-2-trans-nonenal (HNE), an aldehydic product of membrane lipid peroxidation, is increased in AD brain. The alpha class of glutathione S-transferase (GST) can detoxify HNE and plays an important role in cellular protection against oxidative stress. The export of the glutathione conjugate of HNE is required to fully potentiate the GST-mediated protection. The multi-drug resistance protein-1 (MRP1) and GST proteins may act in synergy to confer cellular protection. In the present study, we studied oxidative modification of GST and MRP1 in AD brain by immunoprecipitation of GST and MRP1 proteins followed by Western blot analysis using anti-HNE antibody. The results suggested that HNE is covalently bound to GST and MRP1 proteins in excess in AD brain. Collectively, the data suggest that HNE may be an important mediator of oxidative stress-induced impairment of this detoxifying system and may thereby play a role in promoting neuronal cell death. The results from this study also imply that augmenting endogenous oxidative defense capacity through dietary or pharmacological intake of antioxidants may slow down the progression of neurodegenerative processes in AD.

KEY WORDS: Alzheimer's disease; hippocampus; oxidative stress; 4-hydroxy-2-trans-nonenal (HNE); Glutathione S-transferases (GSTs); Multidrug resistance protein1 (MRP1).

INTRODUCTION

Alzheimer disease (AD) is a progressive, age-related neurodegenerative disorder characterized pathologically by neurofibrillary tangles (NFT), senile plaques and synapse loss (1). The major component of senile plaque is a 40- to 42- amino acid peptide, Amyloid beta-peptide (Aβ) and considered to have a casual role in the development and progress of AD (2). Amyloid beta peptide is proteolytically produced by beta- and gamma-secretase cleavage of an integral membrane protein known as amyloid precursor protein (APP) (3). Several lines of evidence suggest that enhanced oxidative stress is associated with the pathogenesis and/or progression of AD (4,5). This damage is characterized by oxidative modification of a number of cellular macromolecular targets, including proteins, lipids, DNA and

[1] Department of Chemistry, University of Kentucky, Lexington, KY 40506, USA.
[2] Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40506, USA.
[3] Center of Membrane Sciences, University of Kentucky, Lexington, KY 40506, USA.
[4] Address reprint requests to: Department of Chemistry, Center of Membrane Sciences, and Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40506, USA. Tel: 859-257-3184; Fax: 859-257-5876; E-mail: dabcns@uky.edu

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carbohydrates. Amongst the earliest of these changes following an oxidative insult are increases in toxic carbonyls, nitrotyrosine and 4-hydroxy-2-trans-nonenal (HNE) (6–8). HNE is an α,β-unsaturated aldehyde that is generated during the peroxidation of polyunsaturated fatty acid (PUFA), particularly arachidonic acid. There are numerous findings supporting important an role of HNE and acrolein in the development of AD (4,7,9). Thus, significant increase of free HNE in cerebrospinal fluid (10), amygdala, hippocampus and parahippocampal gyrus was detected in brain of AD patients when compared with control subjects (11).

Glutathione transferases (GST) (E.C. 2.5.1.18) are enzymes, which inactivate the toxic products of oxygen metabolism including HNE (12). A significant decrease of glutathione transferase activity and of other antioxidative enzymes was described in amygdala, hippocampus and inferior parietal lobule in patients with AD (13). This could lead to more pronounced effects of HNE in these brain regions.

The glutathione S-transferases (GSTs) catalyze the conjugation of electrophilic substrates to glutathione (GSH). These enzymes also carry out a range of other functions. Cytosolic GSTs of mammals have been particularly well characterized, and were originally classified into Alpha, Mu, Pi and Theta classes on the basis of a substrate/inhibitor specificity, primary and tertiary structure similarities and immunological identity (14). The alpha class of GST plays an important role in cellular protection against oxidative stress (15,16). In this view, export of the glutathione conjugate is required to fully potentiate the GST-mediated protection. The multidrug resistance protein-1 (MRP-1) exports HNE-GSH conjugates from the cells, and MRP1and GST proteins may act in synergy to confer cellular protection (17).

In this study, we applied immunoprecipitation followed by Western blot analysis to quantify GST/MRP1 adducts of HNE in postmortem AD and control brain. Modification of these proteins may alter their normal cellular function. The measurement of HNE adducts may prove useful for the quantitation of protein modifications linked to oxidative stress in AD. Thus, we determined HNE adducts of GST and MRP-1 in the hippocampus, a brain area that is severely affected in early stages of AD and shows pronounced neuritic AD related changes. To our knowledge the formation of HNE adduct with GST and MRP1 in human AD brain sample has not been reported. In the present study, we evaluated the expression and level of bound HNE to GST and MRP1 proteins to gain insight into the contribution of these proteins in the progression of AD.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich (St. Loius,MO) unless otherwise stated. MRP1 antibody was purchased from Alexis, anti-HNE antibody was purchased from Alpha diagnostic International, while anti-GST antibody and alkaline phosphate conjugated secondary antibody were purchased from Calbiochem.

Frozen Hippocampal samples were obtained from eight AD patients and from eight age matched controls for the present study. The Rapid Autopsy Program of the University of Kentucky Alzheimer's Disease Research Center (UK ADRC) resulted in extremely short postmortem intervals (PMIs). All AD subjects displayed progressive intellectual decline and met NINCDS-AD-RDA Workgroup criteria for the clinical diagnosis of probable AD. All AD subjects met accepted guidelines for the histopathologic diagnosis of AD.

Subcellular Fractionation

Hippocampal tissue was processed for isolation of membrane protein and cytosolic fractions as described by Pinkett and Andersons method (18). Briefly, tissue was homogenized in ice-cold lysis buffer (0.32 M sucrose, 10 mM Tris–HCl (pH 8.0), 0.1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF). The homogenate was centrifuged at 1000×g for 10 min and supernatant was centrifuged again at $16,000 \times g$ for 15 min to obtain crude membrane pellet and the resulting supernatant (cytosolic fraction) was stored at −70 °C until used for further experiments.

GST Assay

GST activity was measured essentially as described by Habig et al. (19) using 1,chloro 2,4-dinitrobenzene as substrate. The standard assay mixture contain 1 mM CDNB (1,chloro 2,4-dinitrobenzene), 1 mM reduced glutathione (GSH) and 100mM potassium phosphate buffer (pH 6.5) in a volume of 1 ml. The reaction was followed at 340 nm.

Protein Estimation

The protein concentration was determined by using the BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

Immunoprecipitation and Western Blotting

Five hundred micrograms of cytosolic or membrane proteins were immunoprecipitated with polyclonal anti-alpha GST antibody or monoclonal anti-MRP1 antibody for 24 h at 4°C, and then the antibody–antigen complexes were collected with protein A/G–Sepharose-conjugated beads. After the addition of sample loading buffer, protein samples were denatured and electrophoresed on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane at 160 mA/gel for 2 h. The blots were blocked for 1 h at room temperature in fresh blocking buffer

(10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20, pH 7.4, containing 5% nonfat dried milk). Dilutions (1:1000) of primary antiHNE /anti-MRP1/anti-GST were made in wash blot with 3% nonfat dry milk. Following three washes with wash blot, the blots were incubated with alkaline phosphotase-conjugated secondary antibodies in wash blot for 1 h at room temperature. The blots were washed again three times in wash blot, the bands were visualized using Sigma fast tablets (5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) and Nitro Blue Tetrazolium (NBT)) as substrates.

To determine the specificity of antibody 1 μg of purified protein was incubated with 5 μl of primary antibody at room temperature for 2 h, followed by pelleting down of antigen–antibody complexes at 14,000 rpm for 10 min. No positive immunoreactivity was observed corresponding to protein of interest using the 14,000 rpm supernatant implies its specificity (data not shown).

Statistical Analysis

The results are presented as means ± SD. Statistical evaluation was done by a Student's *t*-test for analysis of significance. Differences were considered to be significant at $P < 0.05$.

RESULTS

Immunoblot probed with anti-GST (Fig. 1a) and anti-HNE (Fig. 1c) antibodies detected a band of 27 kDa from anti-GST immunoprecipitated proteins suggesting the presence of bound HNE to GST. 250% increase in immunoreactivity was detected for HNE in AD samples compared to that of control. The amount of HNE bound to GST was significantly higher ($P < 0.001$) in AD compared to that of the control. GST protein levels were elevated by 91% in AD brain compared to that of control ($P < 0.001$). The ratio of GST protein level to that of bound HNE was 1.83 in AD brain and 1.00 in control, significantly increased in AD brain compared to that of the control ($P < 0.001$). The level of GST activity was significantly decreased in AD hippocampus (820 ± 80 nmol/min/mg protein) compared to that of control (1310 ± 120 nmol/min/mg protein). These results are consistent with and confirm previous findings (13). Figure 2a and 2c shows the western blots probed with anti-MRP1 and anti-HNE antibody that shows 34.3 and 19.9% increase in levels of HNE ($P < 0.05$) and MRP1 in the AD samples compared to that of control ($P < 0.05$). The ratio of MRP1 protein level to that of bound HNE was 1.11 in AD brain and 1.00 in control, significantly increased in AD brain compared to that of the control ($P < 0.05$).

DISCUSSION

Our results indicate that HNE, a lipid peroxidation product, is bound to α-GST and MRP1 protein in the AD hippocampal tissue to a greater extent than

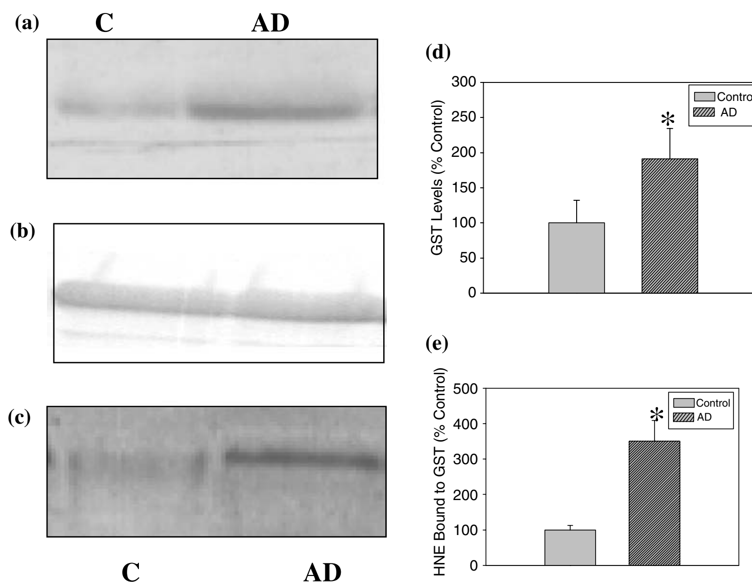

Fig. 1. HNE binds to alpha class GST in human AD hippocampal cytosolic fraction. Alpha class of GST is immunoprecipitated as described in materials and methods followed by transfer to a nitrocellulose membrane and immunoreacted with anti-GST (a), anti-actin (b) and anti-HNE antibody (c). Alpha GST protein was observed as a single band of 27 kDa. (d) and (e) are representative densitometry representation of blot probed with Anti-GST and anti-HNE antibody. The data points are mean ± SEM (bars) values of eight separate experiments with similar result. $*P < 0.001$.

in control brain. There is a growing body of evidence indicating that the magnitude of lipid peroxidation in the brains of AD patients examined postmortem exceeds that in age-matched control individuals, particularly in medial temporal lobe structures: hippocampus, pyriform cortex and amygdala (4,7,9,20). These studies are supported by more recent publications, which report increased F_2-isoprostanes in the brains of patients with AD relative to age-matched controls (21). Indeed, many of the cytotoxic effects of lipid peroxidation can be reproduced directly by electrophilic lipid peroxidation products such as HNE. These include depletion of glutathione, dysfunction of structural proteins, reduction in enzyme activities, and induction of cell death (22). HNE covalently binds to cysteine, lysine, and histidine residues of proteins via Michael addition; HNE also interacts with proteins through Schiff-based chemistry (23). HNE can impair the functions of different proteins, and recent findings suggest that HNE mediates oxidative stress-induced impairment of ion-motive ATPases (24), glucose transport in cultured rat hippocampal neurons, and impaired glutamate transport in rat neocortical synaptosomes (25,26), and altered guanosine triphosphate (GTP)-binding proteins (27) in neurons.

Numerous antioxidant mechanisms have evolved, including several metabolic routes to detoxify products of lipid peroxidation. The diffusible reactive aldehydes generated from lipid peroxidation are excellent substrates for glutathione transferases (GSTs) and a number of oxidoreductases that act to detoxify these molecules. The alpha class of GST plays an important role in cellular protection against oxidative stress. The tripeptide glutathione (GSH), which contains a cysteine residue, appears to play an important role in detoxifying HNE in cells. The activity of some of the major metabolizing enzymes for HNE and related aldehydes is altered in brains of AD patients. We observed a significant decrease in GST activity consistent with the previous studies that showed significant decrease of glutathione transferase activity in brain and CSF of patients with AD (13). Interestingly, we observed a significant increase in alpha-class of GST protein in AD brain. The observed increase in GST protein levels with no change in activity could be attributed to one of the following: increase in mRNA, stabilization of mRNA, or defect in protein clearance from the cell. A previous study by Keller et al. showed the altered function of proteosome (28). Xie et al. (29) showed that the addition of GST to cell culture protects neuronal cells against HNE toxicity.

The MRP1 is a member of the ATP-binding cassette (ABC) transporter super family. Like other ABC transporters, MRP1 is a membrane-bound transport protein that mediates the extrusion of its substrates at the expense of ATP. MRP1 has a

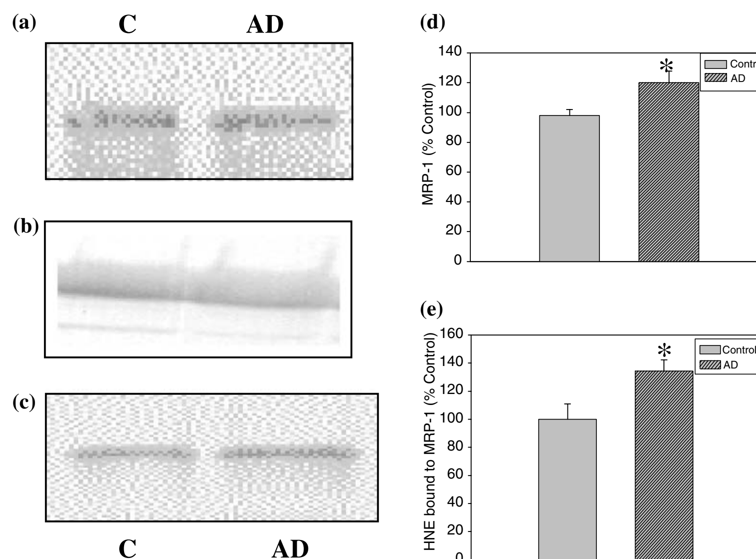

Fig. 2. HNE binds to MRP1 in AD hippocampus. MRP1 is immunoprecipitated as described in materials and methods followed by transfer to a nitrocellulose membrane and immunoreacted with anti-MRP1 (a), anti-Actin (b), and (c) anti-HNE antibody. MRP1 protein was observed as a single band of 160 kDa . (d) and (e) are representative densitometry representation of blot probed with Anti-MRP1 and anti-HNE antibody. The data points are mean ± SEM (bars) values of eight separate experiments with similar result. * $P < 0.05$.

broad specificity for glutathione S-conjugates, most notably cysteinyl leukotrienes, and for anionic conjugates of bile salts and steroid hormones (30). In addition, MRP1 is able to extrude natural product drugs that are used in chemotherapeutic strategies, such as daunorubicin and vincristine, in cotransport with reduced glutathione (31). In the present study, HNE-adduct formation was greater in the AD hippocampal region compared to that of control. This Michael addition adduct may contribute to alteration of protein structure leading to loss of function (32). Previous studies from our laboratory and others have shown oxidative modification of Creatine kinase (CKBB), glutamate synthase (GS) and Glt-1 resulting in loss of activity of these enzymes in AD brain (33,34). It is well known that transcriptional factors, such as NF-κB and AP-1, are activated by the product of lipid oxidation 4-hydroxynonenal (HNE), whose harmful role has been recognized in covalently binding biomolecules, such as GLT-1 and recently, in participating in cell modulation (35). In addition, the mechanism of transcription is strictly dependent on the intracellular redox balance, which might be affected by protein oxidation.

In addition, the notion that HNE is bound to the GST and MRP1 proteins, suggest an accumulation of glutathione-conjugated substrates inside the cells, and decrease in the endogenous GSH antioxidant system. Taken together we speculate that oxidative stress may alter the functions of structural and enzymatic proteins in AD (4).

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