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Original Contribution

Hydroxynonenal-generated crosslinking fluorophore accumulation in Alzheimer disease reveals a dichotomy of protein turnover

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ABSTRACT

Lipid peroxidation generates reactive aldehydes, most notably hydroxynonenal (HNE), which covalently bind amino acid residue side chains leading to protein inactivation and insolubility. Specific adducts of lipid peroxidation have been demonstrated in intimate association with the pathological lesions of Alzheimer disease (AD), suggesting that oxidative stress is a major component of AD pathogenesis. Some HNE-protein products result in protein crosslinking through a fluorescent compound similar to lipofuscin, linking lipid peroxidation and the lipofuscin accumulation that commonly occurs in post-mitotic cells such as neurons. In this study, brain tissue from AD and control patients was examined by immunocytochemistry and immunoelectron microscopy for evidence of HNE-crosslinking modifications of the type that should accumulate in the lipofuscin pathway. Strong labeling of granulovacuolar degeneration (GVD) and Hirano bodies was noted but lipofuscin did not contain this specific HNE-fluorophore. These findings directly implicate lipid crosslinking peroxidation products as accumulating not in the lesions or the lipofuscin pathways, but instead in a distinct pathway, GVD, that accumulates cytosolic proteins.

Introduction

Oxidative stress is intimately associated with the aging process and Alzheimer disease (AD), causing damage to every category of macromolecule [1]. We and others have demonstrated protein carbonyl modifications [2], adducts of advanced glycation [3–5], nucleic acid modifications [6–8], and lipid peroxidation [9], all directly attributable to oxidative stress. Cellular cytopathological effects include, among others, intra- and intermolecular crosslinks involving the reaction of lipid peroxidation products with proteins [10].

Lipid peroxidation may be particularly important in AD and aging given the extensive turnover of membranes made of highly unsaturated membranes in the central nervous system, the post-mitotic nature of the vast majority of central neurons, and the accumulation of lipofuscin in central neurons that is synonymous with aging. The process of lipid peroxidation generates highly reactive aldehydes, most notably hydroxynonenal (HNE) and acrolein [11,12]. HNE modifications have been identified in neurofibrillary pathology of AD and represent a potential mechanism for insolubility and accumulation of neurofibrillary tangles and other inclusions [9,13].

Among the modifications, protein crosslinking, intra-and interpeptide links can greatly increase insolubility and resistance to degradation [14,15]. The one described crosslink modification stemming from lipid peroxidation is two lysines joined by HNE [16]. A specific reagent to this modification was generated by reacting Nα-acetyllysine (NAL) and HNE and preparing an antiserum specific to the NAL-HNE epitope. This study was based on previous work demonstrating the effects of HNE on the model protein glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* [17]. Exposure of this enzyme to HNE led to enzyme inactivation because of reaction of the epsilon-amino group of an active site lysine residue with the double bond (C3) of HNE, forming a 1:1-HNE Michael adduct [18]. Interestingly, crosslinks of HNE with

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glucose-6-phosphate dehydrogenase, and later with NAL, were found to generate a fluorophore that has the physical and chemical properties described for lipofuscin [14].

Since crosslinking modifications may play a role in neurofibrillary tangle insolubility [2,10] and the manner in which neurons deal with highly modified proteins, we examined brains from patients with the anti-fluorophore antibody [16] to evaluate the process of lipid peroxidation adduct accumulation and metabolism in normal brain and in AD.

Methods

Tissue

10 cases (ages 60 to 87 years, postmortem interval (PMI) ranging from 4 to 14 hours), which met CERAD criteria for AD [19] and corresponded to Braak stage V-VI [20], were used. In addition, 2 young control cases (ages 17, 31 years), and 7 age-matched controls (ages 53–86, PMI ranging from 9 to 17 hours) were used. Hippocampal and adjacent neocortical tissue as well as cerebellum was obtained at autopsy under an approved IRB protocol and fixed in methacarn (methanol: chloroform: acetic acid; 6:3:1) for 16 hours embedded in paraffin and 6 μm sections cut.

Antibodies

Affinity purified rabbit polyclonal antisera to anti-fluorophore HNE modifications was used in this study [16]. The NAL-HNE antibody has been previously characterized [16] and was shown to have no reactivity with Nα-acetylhistidine, Nα-acetylcysteine, or other non-fluorescent NAL-HNE adducts. Additionally, antisera to tau (AT8; Thermo-Scientific) was used to localize neurofibrillary pathology.

Immunocytochemistry

Tissue sections were deparaffinized in xylene and rehydrated through graded ethanol followed by the elimination of endogenous peroxidase activity with 30-min incubation in 3% H_2O_2 in methanol. After incubating the sections in 10% normal goat serum (NGS), the primary antibodies were applied for 16 hours at 4 °C. Using the peroxidase-anti-peroxidase method, the immunostain was developed with 3-3'-diaminobenzidine (Dako). Omission of primary antibody was used as a negative control. To confirm the specificity of the immunostain, the antibody was diluted in a solution of the immunizing antigen [16] and incubated for 16 hour at 4 °C. The adsorbed antibody solution was applied to a tissue section of AD hippocampus and unadsorbed antibody was applied on the adjacent serial section.

Immunoelectron microscopy

Vibratome sections (60 μm) were cut from a case of AD aged 69 with a 3 hour PMI that was fixed in glutaraldehyde/paraformaldehyde. Sections were washed with TBS (50 mM Tris–HCl, pH 7.6, 150 mM NaCl), incubated in 10% NGS for 1 hour followed by incubation in primary antibody diluted in 1% NGS overnight. An adjacent section was incubated in 1% NGS overnight to serve as a negative control. The sections were then rinsed in 10% NGS and gold-conjugated antibody to rabbit IgG (17 mn) was applied. After immunoreaction, the sections were thoroughly rinsed in PBS, post-fixed in 2.5% glutaraldehyde for 1 hour and thoroughly rinsed again. After treating with 1% osmium tetroxide for 1 hour, the sections were rinsed, dehydrated through acetone and embedded in Spurr's media. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed in a JEOL 100CX electron microscope at 80 kV. The same area of the CA1 region of the hippocampus from a serial section in which the primary antibody omitted was also analyzed.

Alternatively, tissue fixed in methacarn was embedded in LR Gold resin as previously described [21] and 60 nm sections placed on

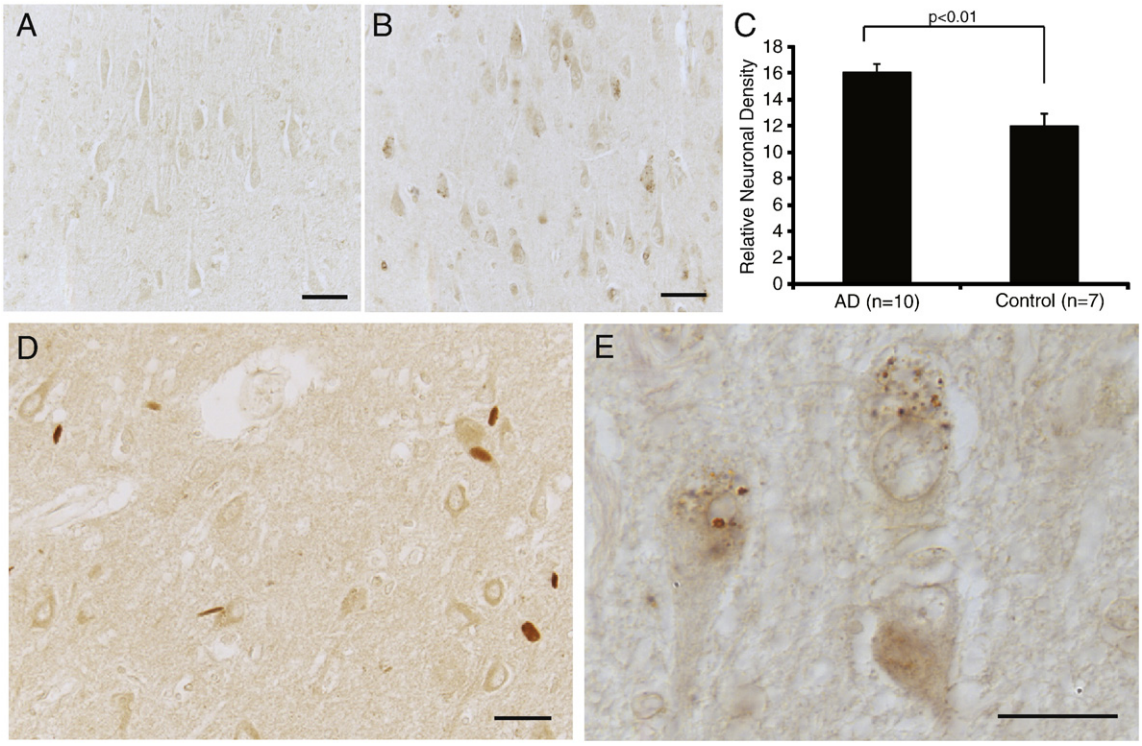

Fig. 1. Localization of fluorophore immunoreactivity is increased in AD pyramidal neurons (B) as compared to an age-matched control (A). Densitometric analysis of the pyramidal neuron levels of fluorophore, shows significantly higher levels in the AD cases (n = 10) compared to age-matched controls (n = 7) ($p<0.01$, C). In the AD cases, many neurons contained intensely labeled GVD structures (B, and higher magnification in E) and some cases demonstrated labeling of Hirano bodies (D). Scale bar for A,B,D = 50 μm; E = 20 μm.