Elevated levels of 4-hydroxynonenal-histidine Michael adduct in the hippocampi of patients with Alzheimer’s disease

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ABSTRACT

Alzheimer’s disease (AD) is among the most common causes of progressive cognitive impairment in humans and is characterized by neurodegeneration in the brain. Lipid peroxidation is thought to play a role in the pathogenesis of AD. 4-hydroxynonenal (HNE) results from peroxidation of polyunsaturated fatty acids and it in turn gives evidence of lipid peroxidation in vivo. HNE reacts with protein histidine residue to form a stable HNE-histidine Michael adduct. To clarify the influence of lipid peroxidation on the pathogenesis of AD, we measured HNE-histidine Michael adduct in hippocampi from four AD patients and four age-matched controls by means of semiquantitative immunohistochemistry using a specific antibody to cyclic hemiacetal type of HNE-histidine Michael adduct. This antibody does not react with the ring-opened form of HNE-histidine Michael adduct and the pyrrole form of HNE-lysine Michael adduct. The HNE adduct was detected in the hippocampi of both AD and control donors, especially in the CA2, CA3 and CA4 sectors. Immunoreactive intensity of HNE adduct in these sectors were significantly higher in AD patients than in the controls. The HNE adduct was found in the perikarya of pyramidal cells in the hippocampus. These results show that the hippocampi of patients with AD undergo lipid peroxidation and imply that this activity underlies the production of cytotoxic products such as HNE that are responsible for the pathogenesis of AD.

The progressive cognitive impairment of Alzheimer’s disease (AD) is associated with neuronal loss as well as the formation of neurofibrillary tangles (NFTs) and senile plaques in the brain (25). Free radical-mediated oxidative damage, energy depletion, deposition of amyloids and NFTs, excitotoxicity, and vascular endothelial cell damage are all thought to participate in the pathogenesis of AD (13). Oxygen-derived free radicals, byproducts of respiration, cause oxidative damage to cellular biomolecules including lipids, proteins and nucleic acids. The brain seems to be especially vulnerable to lipid peroxidation by free radicals, because it consumes approximately one-fifth of humans’ oxygen intake, has a relative paucity of antioxidant systems and contains high concentrations of polyunsaturated fatty acids (PUFAs) (11). Lipid peroxidation results in structural damage to membranes and generation of secondary products such as reactive aldehydes.

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Abbreviations used: AD, Alzheimer’s disease; DAB, 3,3'-diaminobenzidine tetrahydrochloride; HNE, 4-hydroxynonenal; MAP2, microtubule-associated protein 2; NFT, neurofibrillary tangle; PBS, phosphate buffered saline; PUFAs, polyunsaturated fatty acids.
that modify proteins and nucleic acids (5). Therefore, as reported, lipid peroxidation in the brains of AD patients not only harms cells but increases levels of such peroxidation products as thiobarbituric acid-reactive substances (10), acrolein-deoxyguanosine adducts (9), and cytotoxic compounds such as acrolein (12) and 4-hydroxynonenal (HNE) (17).

Lipid peroxidation propagates itself by autoxidation initiated by free radicals and produces considerable amounts of secondary products before the process terminates (21). These well-known secondary products include reactive aldehydes, such as acrolein, malondialdehyde, HNE, and 4-hydroxynonenal (5). HNE is product that results from the lipid peroxidation of n-6 PUFAs, e.g., linoleic acid and arachidonic acid (5). Because the brain is rich in arachidonic acid (29), lipid peroxidation tends to produce HNE at that site. These substances then react with proteins, nucleic acids and small molecules such as glutathione in cells and eventually impair normal biological functions of the essential components (5). Indeed, HNE is cytotoxic for cultured neuronal cells (2, 5, 8, 15). Therefore, HNE might induce neuronal cell death and neurodegeneration in patients with AD.

Measuring the exact quantity of HNE in vivo is difficult, since they are rapidly consumed when their chemically active aldehyde reacts with such cellular components as glutathione, proteins and nucleic acids (5). HNE reacts with lysine, histidine and cysteine residues in proteins to form Michael adducts and also Schiff base products (lysine ε-NH$_2$). When HNE reacts with proteins, HNE-histidine Michael adduct is a major product that develops in the cyclic hemiacetal form (3, 27). Because the cyclic hemiacetal form of HNE is relatively stable (5), the detection of HNE Michael adduct is considered a reliable index of lipid peroxidation. This property enabled us to measure HNE-histidine Michael adduct using the specific antibody to cyclic hemiacetal type of HNE-histidine Michael adduct.

To clarify the influence of lipid peroxidation on the pathogenesis of AD, we directly assessed the cyclic hemiacetal type of HNE-histidine Michael adduct in brain specimens from AD subjects and age-matched controls. As a result, the HNE adduct was detected in the hippocampi of both groups, especially the CA2, CA3 and CA4 sectors. The important difference was significantly higher levels of the HNE adduct in the brains of patients with AD. This is the first report of specific antibody usage for direct detection of HNE-histidine Michael adduct in the cyclic hemiacetal form within hippocampi from humans with AD.

**MATERIALS AND METHODS**

*Human subjects.* All clinical data from patients and information at autopsies from four patients with AD (two women and two men) and four (one woman and three men) normal (no AD), age-matched subjects who died during the last several decades were retrieved from the autopsy database of the Department of Tokyo Metropolitan Geriatric Hospital, Tokyo, Japan. Brain specimens were registered in the Brain Bank for Aging Research (BBAR) organized by Tokyo Metropolitan Geriatric Hospital and Tokyo Metropolitan Institute of Gerontology (TMIG). Brain specimens used in this study were from patients clinically diagnosed as having AD-positive and control subjects without any sign of AD. All AD patients met accepted criteria for the neuropathologic diagnosis of AD based on the National Institute of Aging (NIA)-Reagan Institute Criteria for the Neuropathological Diagnosis of AD (1997) (1), combining abundant neuritic plaques in the neocortex (definite AD with consortium to establish a registry for AD criteria) and a profusion of NFTs in the limbic and neocortical areas (Braak and Braak staging, VI). Normal subjects used as controls were individuals with no history of dementia or other neurological disorders. Neuropathologic evaluation of control brains revealed only age-associated gross and histopathologic alterations (Braak and Braak NFT staging, I and SP stage, 0 or A). The subjects’ demographic data are summarized in Table 1. The human studies were approved by Ethics Committees of TMIG and the Tokyo Metropolitan Geriatric Hospital.

*Immunohistochemistry.* Specimens were taken from the hippocampus and fixed with 4% paraformaldehyde in this study. Paraffin-embedded hippocampal sections were deparaffinized, rehydrated with xylene, alcohol and phosphate buffered saline (PBS), microwaved for 5 min in boiling 10 mM citrate buffer, pH 6.0, and immersed in 3% H$_2$O$_2$ in methanol for 15 min to reduce endogenous peroxidase activity. After blocking treatment with 10% non-immune goat serum in PBS (blocking solution) for 60 min at room temperature, the specimens were incubated with the primary antibodies overnight at 4°C and then for 60 min at room temperature. Mouse monoclonal antibody against HNE-histidine Michael adduct which was specific for their cyclic hemiacetal form was purchased from NOF Corporation (Tokyo,
Japan), and was used at a dilution of 1:100 with a blocking solution. After adequate washing with PBS, specimens were incubated with the secondary antibody (goat anti-mouse IgG conjugated with horseradish peroxidase, Simplestain MAX-PO (M); Nichirei Biosciences Inc., Tokyo, Japan) for 60 min at room temperature. Thorough washing with PBS and incubation with 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Wako Pure Chemical Industries, Osaka, Japan) followed for 10 min at room temperature to visualize HNE-adduct. For double-labeling immunohistochemistry, two primary antibodies were used: anti-microtubule-associated protein 2 (MAP2) and the anti-HNE-histidine adduct antibodies. Tissue specimens were incubated with the anti-MAP2 antibody (1:500 dilution; Chemicon AB5622, rabbit polyclonal antibody; Billerica, MA, USA) to confirm pyramidal neurons, and then with Simplestain AP (R) as a secondary antibody conjugated with alkaline phosphatase. The alkaline phosphatase activity was visualized with Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories, Burlingame, CA, USA). Subsequently, the specimens were incubated with the anti-HNE adduct antibody as mentioned above. Hematoxylin was used for counter staining. The antibody used against HNE-histidine Michael adduct is specific for their cyclic hemiacetal form and does not react with the ring-opened form of HNE-histidine Michael adduct and the pyrrole form of HNE-lysine Michael adduct.

The four hippocampal sectors (CA1 through CA4) were delineated and cells that had a large nucleus containing a clearly visible nucleolus in these sectors were referred to pyramidal cells according to the description of Mani et al. (14). Immunoreactive intensity of the HNE-Michael adduct was assessed by measuring 20 fields of hippocampal sectors in the specimens under a 40× objective microscopic field. The extent of staining intensity in pyramidal cells was classified into the following two grades: Pyr-small (small staining size less than half of the pyramidal cell nucleus) and Pyr-large (large staining size more than half of the pyramidal cell nucleus).

The extent of staining intensity in non-pyramidal cells was also classified into the following two grades: Non-small (small staining size less than the cell nuclei) and Non-large (large staining size more than the cell nuclei). The percent ratio of these grades in each sector was calculated by following formulation: percent of Pyr-small = (numbers of Pyr-small grade / (numbers of Pyr-small grade + Pyr-large grade)) × 100; percent of Pyr-large = (numbers of Pyr-large grade / (numbers of Pyr-small grade + Pyr-large grade)) × 100; percent of Non-small = (numbers of Non-small grade / (numbers of Non-small grade + Non-large grade)) × 100; and percent of Non-large = (numbers of Non-large grade / (numbers of Non-small grade + Non-large grade)) × 100.

Statistical analysis. The results are expressed as mean ± SEM. Statistical analyses were conducted by using Graphpad Prism 4 software (version 4.0, Graphpad Software Inc., San Diego, CA, USA). Significance was defined as a P value less than 0.05.

RESULTS

Clinical features of subjects
The subjects’ demographic data are summarized in Table I. The mean ages of the AD group and the control group were 86 and 78 years, respectively, and there was no significant difference between these two groups (t-test, two-sided). Neither brain weight, gender, nor postmortem interval differed significantly between the two groups according to a t-test (two-sided), Fisher’s exact test, or t-test (two-sided), respectively.

HNE Michael adduct in the hippocampi from AD patients and non-AD controls
HNE Michael adduct in the hippocampi was detected in all specimens from both AD and controls. Especially, CA2–4 sectors contained an abundance of the HNE adduct compared with the adjacent CA1 sector (Fig. 1B and Table 2).

HNE Michael adduct immunoreactivity was seen

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Demographic data for AD subjects and controls</th>
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<tr>
<td></td>
<td>Age (yr)</td>
</tr>
<tr>
<td>AD</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>Controls</td>
<td>78 ± 2</td>
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</tbody>
</table>

Data are expressed as mean ± SEM. Parentheses indicate the number of subject. AD, Alzheimer disease; PMI, postmortem interval; h, hour; BW, brain weight; g, gram; yr, year; NFT, neurofibrillary tangle; SP, senile plaque
M. Fukuda et al.

Notably, the staining intensity for HNE Michael adduct was far stronger in these sectors of the AD brain samples than in those of the controls (Table 2, Fisher’s exact test: $P < 0.0001$). Similarly, the ratio of the severe grade indicated as Pyr-large was relatively higher in the CA2/3 and CA4 sectors of AD patients compared with controls (Fig. 2A).

Additionally, a large amount of amorphous HNE as intracellular accumulations within pyramidal cells in the hippocampi of AD and controls (indicated in brown in Fig. 1C and D). In addition to the criteria of pyramidal cells in Materials and Methods, pyramidal cells were also confirmed by anti-MAP2 antibody as shown in Fig. 1D. The HNE adduct seemed to be composed of a mass of granular substances localized in the perikaryon of pyramidal cells in the hippocampus, especially at the CA2, CA3 and CA4 sectors (Fig. 1C and D).
Michael adduct was seen around the nuclei of non-pyramidal cells other than the pyramidal cells in the hippocampus, and this material was referred to as non-pyramidal cell deposits (abbreviated as Non-small and Non-large in Fig. 1C). Numerous non-pyramidal cell deposits were found in the CA2, CA3 and CA4 sectors of the hippocampi from AD and controls (Table 2), but the staining intensity of these deposits again differed significantly in the two groups (Table 2, Fisher’s exact test: \( P < 0.02 \)). That is, the ratio of large amorphous deposits (Non-large grade in Table 2) to total deposits was relatively higher in these sectors of the AD tissue compared with that from age-matched controls (Fig. 2B).

DISCUSSION

Here, we found an intracellular accumulation of HNE-histidine Michael adduct in pyramidal cells of the hippocampus, most notably at the CA2, CA3 and CA4 sectors by using a specific antibody to cyclic hemiacetal type of HNE-histidine Michael adduct. This antibody does not react with the ring-opened form of HNE-histidine Michael adduct and the pyrrole form of HNE-lysine Michael adduct (26). Although the HNE adduct occupied hippocampi from both AD and age-matched, non-AD controls, significantly larger quantities of the HNE adduct were present in the CA2–4 sectors of AD patients than those of controls. These results show that pyramidal neurons in these sectors within hippocampi of persons with AD are prone to undergo lipid peroxidation. Consequently, increased lipid peroxidation might be responsible for the neuronal degeneration and death characteristic of AD. In support is the fact that HNE, a lipid peroxidation product is cytotoxic to cultured neuronal cells via impairment of Na\(^+\)-K\(^+\)-ATPase (15), disruptions of microtubule structure (7, 20), caspase-3 activation, and cytochrome c release (6).

The distribution of the HNE adduct differed among CA sectors in the hippocampus. Although a few pyramidal cells in the CA1 sector contained the HNE adduct, a great number of cells in the CA2–4 sectors contained massive amounts of the HNE adduct. A similar spatial distribution pattern was found for malondialdehyde-conjugated proteins identified in persons with AD and age-matched controls (4). Thus, these results suggest that pyramidal cells in the CA1 sector might resist lipid peroxidation. On the other hand, after sodium borohydride treatment, the ring-opened form of HNE-histidine Michael adduct was seen frequently in hippocampi of AD patients but rarely in age-matched controls, and in the former case the percentage of immunoreactive pyramidal cells in the CA1 sector was comparable to that of the CA2–4 sectors (19). That result disagrees with the outcome presented here, possibly because antibodies of differing specificities were used in the two studies. That is, the previously used antibody against the ring-opened form of HNE-histidine Michael adduct rarely stained hippocampi from control subjects, whereas the antibody used in this study stained hippocampal tissues from controls and AD patients to comparable extents.

We also showed that amorphous deposits immunoreactive to the anti-HNE adduct antibody in non-pyramidal cells were significantly increased in the CA2–4 sectors of hippocampal samples from AD patients compared with age-matched control. These deposits were found around small nuclei in the CA2–4 sectors of hippocampal samples. Although the all cell types containing these small nuclei were
not identified, some cells were positively stained with glial fibrillary acidic protein which is an astrocyte marker protein but not all cells. Immunoreactivity similar to the amorphous staining described here was seen in astrocytes when using antibody against the ring-opened form of HNE-histidine Michael adduct (19) and antibody against malondialdehyde-conjugated proteins (4). Therefore, the cells containing the small nuclei we viewed around amorphous deposits could be astrocytes.

In addition to the cyclic hemiacetal form and the ring-opened form of HNE-histidine Michael adduct, the pyrrole form of HNE-lysine Michael adduct (18, 24) was detected in the hippocampus (24) and entorhinal cortex (18) of samples from AD patients by using specific antibodies. Other than the immunohistochemical detection of HNE-conjugated proteins, elevated levels of HNE itself (17, 28) and isoprostanes (16, 22, 23), stable products derived from lipid peroxidation of PUFAs, these reports confirm increased lipid peroxidation in the brains of AD compared with control individuals. Thus, our results and those studies provide firm evidence of increased lipid peroxidation in brains that manifest AD.

Here we have documented strong evidence of lipid peroxidation in the hippocampi of both AD patients and age-matched, non-AD controls by identifying the presence of the HNE adduct. Presumably, the increased levels of HNE adduct in the hippocampi of AD patients signifies that the brains of such patients with AD tend to be more sensitive to lipid peroxidation than normal brains. In that environment, accelerated lipid peroxidation might play a pivotal role in the pathogenesis of AD by producing HNE and/or other cytotoxic products.

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REFERENCES


