Journal of Neuropathology and Experimental Neurology

Vol. 56, No. 8
August, 1997
pp. 866–871

4-Hydroxy-2-Nonenal Pyrrole Adducts in Human Neurodegenerative Disease

K. S. Montine, P. J. Kim, S. J. Olson, W. R. Markesbery, and T. J. Montine

Abstract. Increasing age and inheritance of the ε4 allele of apolipoprotein E (APOE4) are significant risk factors for sporadic and late onset familial Alzheimer disease (AD); however, the mechanisms by which either leads to AD are unknown. Numerous studies have associated advancing age with increased indices of oxidative challenge to brain, and with still further increased oxidative damage to relevant brain regions in AD patients. A major consequence of oxidative damage to brain is lipid peroxidation with production of the neurotoxic metabolite 4-hydroxy-2-nonenal (HNE). HNE reacts with protein to yield several adducts, including a pyrrole adduct that forms irreversibly in biological systems. Previously, we have shown in a small number of AD and control patients that HNE pyrrole adduct antiserum is immunoreactive with neurofibrillary tangles (NFT), and that this reactivity was significantly associated with inheritance of APOE4. Others have confirmed this pattern of immunoreactivity in AD brain but did not observe an association with APOE4. Herein, we have expanded the study group to 19 AD patients homozygous for APOE4 or APOE3, as well as 30 patients with other neurodegenerative diseases, including diffuse Lewy body disease, Pick's disease, progressive supranuclear palsy, Parkinson's disease, and human immunodeficiency virus-1 encephalitis. HNE pyrrole adduct immunoreactivity on NFT in AD patients was strongly associated with APOE4 homozygosity. With the exception of rare immunoreactive Pick bodies in one case of Pick's disease, no other structure was recognized by HNE pyrrole adduct antiserum in this series of patients. We propose that there is a significant difference between the interaction of apoE3 and apoE4 with lipid peroxidation in the brains of AD patients.

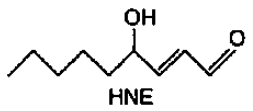
INTRODUCTION

Alzheimer disease (AD) is the most common dementing illness in this country, occurring in an estimated 4 million adults (1). Genetic causes of some of the dominantly inherited forms of AD are known, but these represent less than 10% of patients with AD (2). In contrast, the vast majority of patients with AD suffer from sporadic or late-onset familial forms of the disease. The etiology of this latter form of AD is not yet known; however, increasing age and apolipoprotein E genotype (APOE) are significant risk factors. It has been repeatedly demonstrated that inheritance of the ε4 allele of APOE (APOE4) carries a significantly increased risk of late-onset familial and sporadic AD (3). Others have suggested that the other common APOE genotypes, APOE3 and especially APOE2, may be associated with protective effects rather than APOE4 conferring a deleterious trait (4). Despite these epidemiological associations, the mechanisms by which aging and APOE genotype may contribute to the pathogenesis of AD have not been elucidated.

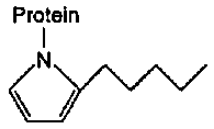
Substantial indirect evidence suggests that a prominent part of the aging process is increased oxidative challenge (5, 6). The aging brain displays several features of increased oxidative damage, with lipid peroxidation being prominent (7, 8). Furthermore, several indices of oxidative damage, including lipid peroxidation, are significantly elevated in tissue ravaged by AD as compared with age-matched patients without dementia (9–17). Many of the deleterious effects of lipid peroxidation are due to production of 4-hydroxy-2-nonenal (HNE) (18). Our laboratory has proposed that different apoE isoforms may influence the metabolism of HNE, a potent neurotoxic product of lipid peroxidation (19, 20). Others have presented evidence that apoE isoforms may have different potencies as antioxidants (21).

HNE reacts with a variety of cellular nucleophiles, including low molecular weight species and macromolecules (22). HNE forms a number of different protein adducts, the most abundant of which are Michael and imine adducts in aqueous solutions; however, both are potentially reversible (22, 23). Another product of HNE with protein is the pyrrole adduct (see diagram) that is formed by HNE reacting with protein-bound amino groups (24–26). Protein-bound pyrrole adducts are essentially irreversible in the central nervous system (27), making HNE pyrrole adducts potential candidates for immunochemical detection.

Several laboratories have associated neurofibrillary tangles (NFT) in AD brain with oxidative stress (28–32). We and others have demonstrated HNE pyrrole adduct immunoreactivity on NFT and neuropil threads in AD



HNE



Pyrrole Adduct

From the Department of Pathology, Vanderbilt University Medical Center, Nashville, Tennessee (KSM, PJK, TJM) and the Sanders-Brown Center on Aging and Department of Pathology, University of Kentucky, Lexington, Kentucky (WRM).

Correspondence to: Thomas J. Montine, MD, PhD, Department of Pathology, Vanderbilt University Medical Center, C3321A Medical Center North, Nashville, TN 37232.

TABLE 1

Pathologically confirmed disease	Number of patients	Age in years Mean (range)	Male:Female	Mean duration of disease (years)	Number of patients with HNE pyrrole adduct immunoreactivity
Alzheimer disease	12	78 (59–96)	1:2	8	7
Pick's disease	5	70 (67–78)	3:2	14	1
Diffuse lewy body disease	6	77 (72–85)	6:0	7	0
HIV encephalitis	8	42 (25–58)	8:0	2	0
Parkinson's disease	7	77 (68–89)	4:3	10	0
Progressive supranuclear palsy	4	70 (64–79)	1:1	7	0

Age, gender, and duration of disease for the 42 patients included in this study. Duration of disease was measured as time from the onset of neurological impairment recorded in the patients' medical charts for all disease categories except HIV encephalitis, where duration was measured as the time from a positive test for HIV infection. HNE pyrrole adduct immunoreactivity with any structure other than atherosclerotic plaque or scattered monocytes was regarded as positive.

patients and to a lesser extent in age-matched controls; however, an association between HNE pyrrole adduct immunoreactivity and APOE was not consistently observed (26, 33). In addition, neither study addressed the important issue of whether HNE pyrrole adduct immunoreactivity is specific to AD. Herein, we present data on HNE pyrrole adduct immunoreactivity in a larger number of AD patients, as well as patients with other cortical and subcortical degenerative diseases.

MATERIALS AND METHODS

Cases were selected from autopsies performed at Vanderbilt University Medical Center and the University of Kentucky Medical Center. Inclusion criteria were pathologically confirmed neurodegenerative disease, homozygosity for APOE, and a postmortem interval less than 12 hours. Standard histopathological criteria were used for the diagnosis of AD, Parkinson's disease (PD), diffuse Lewy body disease (DLBD), Pick's disease, progressive supranuclear palsy (PSP), and human immunodeficiency virus-1 (HIV) encephalitis (34–39). Cases with coincident histopathological changes of AD and PD were excluded. Four cases of DLBD had coincident histopathological changes of AD, while the 2 remaining cases did not. The number of patients within each disease category, age, gender, and duration of disease are presented in Table 1.

The dates of postmortem examinations ranged from 1987 to 1995, with overlapping dates among all categories of disease. Brains were removed at autopsy, immediately placed in 10% buffered formalin for 7 to 10 days, and then dissected. Tissue blocks were embedded in paraffin and 8-μm sections were prepared for histological and immunohistochemical analysis. The tissue blocks examined for immunohistochemistry were hippocampus and entorhinal cortex for AD, midbrain for PD, hippocampus and superior temporal gyrus for Pick's disease, temporal pole for DLBD, cerebral cortex and subcortical white matter for HIV encephalitis, and midbrain, dentate and subthalamic nucleus for PSP.

HNE pyrrole adduct immunohistochemistry exactly followed published methods (26). Anti-ubiquitin immunohistochemistry was performed according to the manufacturer's specifications (DAKO, Carpinteria, CA). Consecutive histologic sections were immunostained with preimmune serum, HNE pyrrole adduct antiserum, and ubiquitin antiserum. Ubiquitin and HNE pyrrole adduct immunoreactive NFT were quantified as previously described (26) by averaging the number of immunoreactive NFT in 4 contiguous ×100 microscopic fields of entorhinal cortex that extended from the pial surface to the white matter. APOE genotype was determined according to established methods (40).

RESULTS

Regardless of disease category, HNE pyrrole adduct antiserum was reactive with scattered monocytes and blood vessels involved by atherosclerosis, but not histologically normal vessel walls. This pattern of general immunoreactivity is identical to that observed previously (26).

Seven of the 12 patients with AD demonstrated HNE pyrrole adduct immunoreactivity that was confined to most NFT and occasional neuropil threads (Fig. 1). Consecutive tissue sections were immunostained with anti-ubiquitin antiserum to quantify NFT. The proportion of NFT immunoreactive for HNE pyrrole adducts varied from 58% to 96% with an overall mean for the 7 cases of 71%, similar to previous results (26). Numerous senile plaques were highlighted by anti-ubiquitin antiserum in each case; however, no HNE pyrrole adduct immunoreactive plaque was observed despite the fact that rare adjacent neuropil threads were immunoreactive.

The group of 12 AD patients was evenly divided into APOE3 and APOE4 homozygotes. Patients homozygous for APOE genotype were deliberately chosen to clearly discern any potential association between HNE pyrrole adduct immunoreactivity and APOE genotype. Homozygosity for APOE4 was significantly associated with anti-HNE pyrrole adduct immunoreactivity in the group of 12 patients with AD presented in this study (chi-square test, $P < 0.01$). These data were combined with earlier

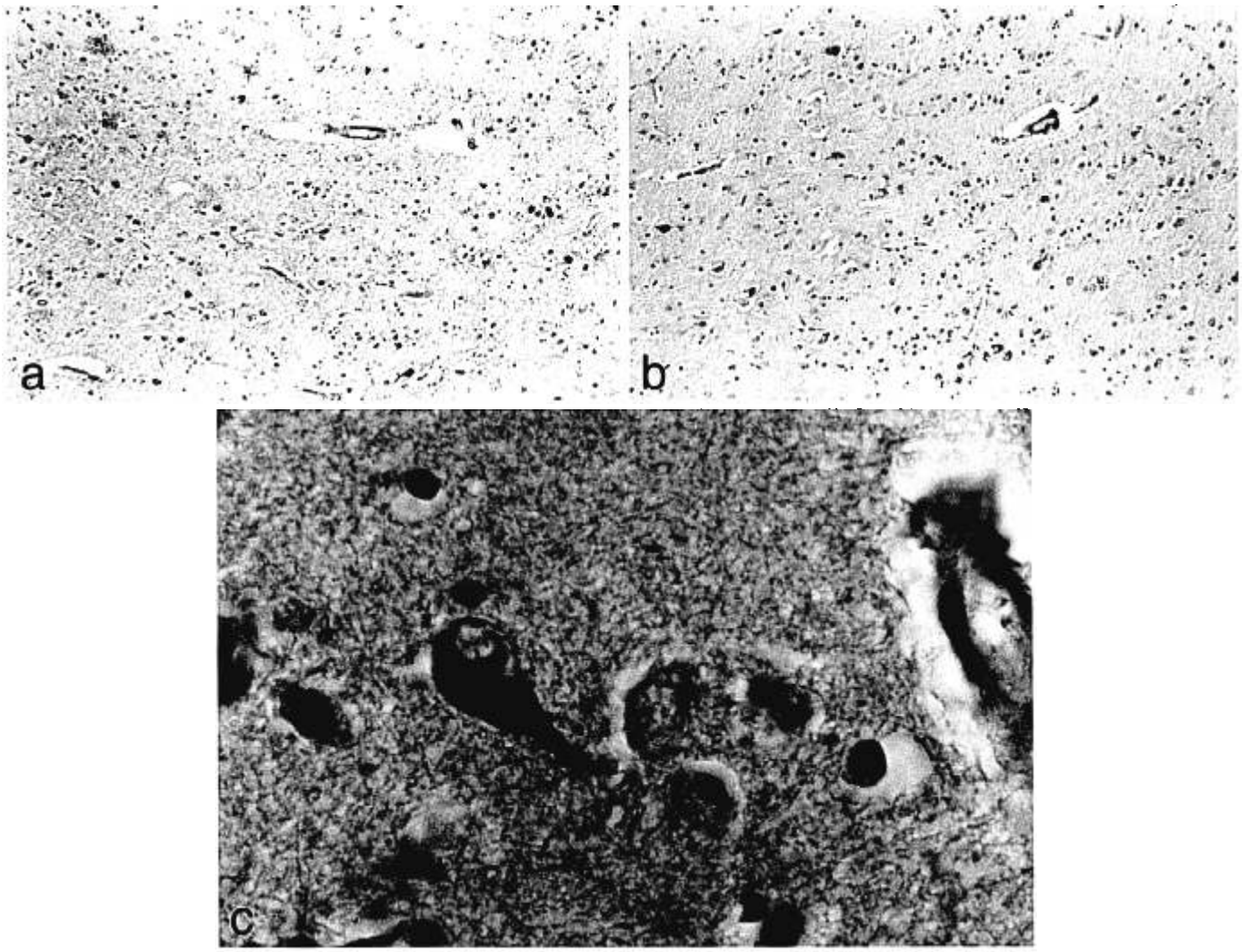


Fig. 1. Photomicrographs of tissue sections from entorhinal cortex of an AD patient homozygous for APOE4 that were reacted with ubiquitin (A) or HNE pyrrole adduct antiserum (B and C, hematoxylin counterstain). A. Ubiquitin immunohistochemistry showing senile plaques and NFT (×100). B. The same region of cortex in the consecutive tissue section reacted with HNE pyrrole adduct antiserum (×100). Note immunoreactivity of NFT and absence of plaque staining. C. NFT stained with HNE pyrrole adduct antiserum (×1,000).

results from 7 AD patients who were homozygous for APOE3 or APOE4 (26). The resulting contingency table (Table 2) demonstrates a highly significant association between homozygosity for APOE4 and anti-HNE pyrrole adduct immunoreactivity on NFT in patients with AD. Consonant with the results of a larger series of patients (41), APOE4 homozygous AD patients tended to have a higher density of NFT (25 ± 4 NFT/100× microscopic field [mean ± SEM]) compared with APOE3 homozygotes (19 ± 5 NFT/100× microscopic field); however, this difference was not statistically significantly. Age at death was younger for homozygous APOE4 AD patients (70.1 ± 2.9 years [mean ± SEM]) in comparison with AD patients homozygous for APOE3 (85.3 ± 3.4 years). No homozygous APOE2 AD patients were identified for study.

These results confirmed, in almost twice as many patients as our previous study, the significant association between HNE pyrrole adduct immunoreactivity in AD patients and homozygosity for APOE4. The next goal was to assess specificity of anti-HNE pyrrole adduct immunoreactivity in a group of neurodegenerative diseases other than AD. Thirty additional patients with 5 different neurodegenerative diseases were examined (Table 1). HNE pyrrole adduct immunoreactivity was present only in a single case of Pick's disease other than in the 7 AD patients already described. Thus, 88% of all patients with HNE pyrrole adduct immunoreactivity had AD.

TABLE 2
Contingency table comparing homozygosity for APOE3 or APOE4 with HNE pyrrole adduct immunoreactivity in the 12 AD patients from this study and 7 additional patients from an earlier study (26). A chi-square test yielded $p < 0.001$ in these 19 patients.

		APOE4/4	APOE3/3	Total
HNE-pyrrole adduct Immunoreactive	Positive	10	1	11
	Negative	0	8	8
	Total	10	9	19

In the 5 patients with Pick's disease, the APOE genotypes were APOE3/3 in 4 patients and APOE3/4 in the remaining case. Only one patient (APOE3/3) displayed 3 Pick bodies that were reactive with HNE pyrrole adduct antiserum. Ubiquitin immunohistochemistry confirmed that the reactive structures were Pick bodies and that only a small minority of Pick bodies were immunoreactive with HNE pyrrole adduct antiserum. No other structure was reactive with HNE pyrrole adduct antiserum in this or the remaining cases of Pick's disease.

No cortical Lewy bodies were immunoreactive with HNE pyrrole adduct antiserum in the 6 patients with DLBD. APOE genotypes for these 6 patients included 2 patients homozygous for APOE3, 2 homozygous for APOE4, and 2 APOE3-4 heterozygotes. In 2 APOE4 homozygotes who had coincident histopathological changes of AD and DLBD, hippocampal NFT were immunoreactive with HNE pyrrole adduct antiserum, while cortical Lewy bodies were not.

Midbrain histologic sections from patients with a clinical history of parkinsonism and histopathological changes of idiopathic PD failed to display immunoreactivity for HNE pyrrole adducts. Specifically, classical Lewy bodies in the substantia nigra, highlighted by ubiquitin immunoreactivity, were not immunoreactive with HNE pyrrole adduct antiserum in consecutive tissue sections. Histopathological changes of AD and PD were coincident in 3 patients with at least one APOE4 allele who were not included in the study group. Even in these patients with hippocampal NFT immunoreactive for HNE pyrrole adducts, no midbrain structure (including brainstem Lewy bodies) was immunoreactive for HNE pyrrole adducts.

Patients with PSP were included in the study group to compare AD with another neurodegenerative disease characterized by tau-containing intraneuronal tangles, although globose NFT of PSP are different from the NFT of AD (38). No HNE pyrrole adduct immunoreactivity was identified in tissue sections from the midbrain, dentate and subthalamic nucleus of these 4 patients despite the demonstration of globose tangles by ubiquitin immunohistochemistry.

The last type of dementing illness examined was HIV encephalitis, a disease that has been associated with a pro-oxidative state (42, 43). No HNE pyrrole adduct immunoreactivity was present in lesioned or histopathologically normal tissue sections of cerebral cortical gray and white matter from patients with HIV encephalitis.

DISCUSSION

Results from this study confirm the association between HNE pyrrole adduct immunoreactivity of NFT and inheritance of APOE4 in AD patients. Furthermore, this study demonstrates that HNE pyrrole adduct immunoreactivity is highly specific to AD when compared with other cortical and subcortical neurodegenerative diseases.

Previously, we demonstrated a significant association between HNE-pyrrole adduct immunoreactivity on NFT and inheritance of APOE4 in a small number of AD patients consisting of both APOE homo- and heterozygotes (26). Subsequently, another laboratory, using very similar methods, also demonstrated HNE-pyrrole adduct immunoreactivity on NFT in AD patients, but without significant association with APOE genotype (33). These investigators examined 19 AD patients; however, only 2 patients were APOE4 homozygotes. In the present study, we decided to examine a larger group of AD patients homozygous for APOE in an attempt to accentuate potential differences between apoE3 and apoE4 and to limit potentially confounding effects from APOE heterozygosity. This difference in patient groups most likely underlies the opposing conclusions reached by these studies with respect to the association between APOE genotype and HNE pyrrole adduct immunoreactivity on NFT.

Only a subset of NFT within a given patient with AD were immunoreactive for HNE pyrrole adducts, similar to earlier results (26, 33). There are several possible explanations for this outcome, one being potential differences in the molecular pathogenesis among NFT. Regardless of the reason for this difference in immunoreactivity among NFT, our data suggest that HNE pyrrole adduction is not necessary for NFT formation, although this does not negate the possible contribution of HNE pyrrole adducts to NFT formation in some instances. Even if HNE pyrrole adducts are completely unrelated to NFT formation, the major conclusion from our study is still valid, i.e. that APOE genotype strongly influences the accumulation of HNE pyrrole adducts in brains of patients with AD.

We have shown that HNE preferentially reacts with purified apoE3 as compared with apoE4 (20). The present study demonstrates an association between APOE4 and the accumulation of HNE pyrrole adducts in vivo. This apparent disparity between in vitro and in vivo results may be explained at two levels. First, the difference between HNE reactivity with purified apoE3 and apoE4 derives primarily from increased Michael adduct formation

with the cysteinyl-containing apoE3, whereas the present study has concentrated on another type of HNE adduct, the pyrrole adduct. Second, although we have shown that apoE is a sensitive target for covalent modification by HNE in neuroglial culture (20), the situation in vivo is undoubtedly more complex, and it seems likely that apoE isoforms may also indirectly modify the apparent reactivity of HNE, perhaps through their central role in brain lipid metabolism. Nevertheless, since pyrrole adducts are known to damage the neuronal cytoskeleton (27), our results may explain in part the stratification of risk for AD with inheritance of APOE4.

Another major focus of this work was to investigate the distribution of HNE pyrrole adducts in neurodegenerative diseases other than AD. We chose a number of different diseases that share clinical or pathological features with AD (44–51). Of the 30 non-AD patients studied, HNE pyrrole adduct immunoreactivity was found in only 1 case of Pick's disease (APOE 3/3), evidenced by only 3 Pick bodies. The significance of this trace amount of reactivity in Pick's disease is not clear because such minimal HNE pyrrole adduct immunoreactivity may be observed in aged nondemented patients (26, 33).

Several neurodegenerative diseases have been associated with increased indices of oxidative damage to the relevant regions of brain. This association is strongest for PD and AD, but also has been observed in Pick's disease and PSP, and has been suggested for HIV encephalitis. Indeed, others have demonstrated immunoreactivity for the Michael and imine adducts of HNE with protein in nigral neurons of patients with PD (52); however, these investigators did not probe for HNE pyrrole adducts. In our series, the majority of patients with diseases associated with increased oxidative damage did not show immunoreactivity for HNE pyrrole adducts, demonstrating that HNE pyrrole adduct accumulation is not a general product of oxidative damage to brain. In addition, our data show that HNE pyrrole adduct accumulation was not simply a manifestation of apoE4 metabolism in degenerating brain because DLBD cases homozygous for APOE4 lacked immunoreactivity and a single AD APOE3 homozygote was immunoreactive. Finally, HNE pyrrole adduct accumulation cannot be dismissed as a nonspecific outcome of neuron cytoskeleton alteration and inclusion formation because cortical Lewy bodies in DLBD, classical Lewy bodies in PD, and globose NFT in PSP all failed to show immunoreactivity. In summary, HNE pyrrole adduct immunoreactivity cannot be ascribed simply to idiosyncracies of apoE4 metabolism alone, neurodegeneration, oxidative damage to brain, or intraneuronal inclusion formation; rather, it is a relatively specific event that probably results from a particular combination of these and other variables.

Enhanced oxidative challenge with damage to cellular macromolecules is an emerging leitmotif among neurodegenerative diseases. Our results indicate that oxidative damage to the brain is not uniform across neurodegenerative diseases, even among those diseases associated with increased biochemical indices of oxidative stress. Indeed, our results suggest that even within a particular neurodegenerative disease, the mode of oxidative damage may be different and strongly influenced by the patient's APOE genotype. These findings are consistent with the hypothesis that a component of AD pathogenesis may involve a specific interaction between different apoE isoforms and lipid peroxidation.

ACKNOWLEDGMENTS

We wish to thank Dr A. Saunders of the Joseph and Kathleen Bryan Alzheimer's Disease Research Center at Duke University for determining APOE genotypes, and Ms Tracie Moss for her expert technical assistance.

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Received February 26, 1997
Revision received April 30, 1997
Accepted May 2, 1997