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Oxidatively modified nucleic acids in preclinical Alzheimer's disease (PCAD) brain

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ABSTRACT

Previous studies show increased oxidative DNA and RNA damage and diminished 8-oxoguanine glycosylase (OGG1) mediated base excision repair in vulnerable brain regions of mild cognitive impairment and late-stage Alzheimer's disease (LAD) subjects compared to normal control (NC) subjects. Recently, a preclinical stage of AD (PCAD) has been described in which subjects show no overt clinical manifestations of AD but demonstrate significant AD pathology at autopsy. To determine if DNA or RNA oxidation are significantly elevated in PCAD brain we quantified 8-hydroxyguanine (8-OHG) in sections of hippocampus/parahippocamapal gyri in PCAD and NC subjects using immunohistochemistry and confocal microscopy and in superior and middle temporal gyri (SMTG) using gas chromatography/mass spectrometry. To determine if increased DNA oxidation is associated with altered repair capacity, levels of OGG1 protein in HPG were measured by immunohistochemistry and levels of OGG1 mRNA were measured in SMTG using quantitative PCR. Results show significantly increased ($p < 0.05$) 8-OHG immunostaining in DNA and RNA of PCAD HPG and significantly increased 8-OHG in PCAD SMTG. Quantification of OGG1 showed significantly elevated mRNA in PCAD SMTG and a trend toward elevated protein immunostaining in PCAD HPG. Overall, the data suggest oxidative damage to nucleic acids and a compensatory increase in OGG1 expression occur early in the pathogenesis of AD.

1. Introduction

Increasing evidence supports a role for oxidative damage in the pathogenesis of Alzheimer's disease (AD). Multiple studies demonstrate increased lipid peroxidation (Markesbery and Lovell, 1998; Lovell et al., 2001; McGarth et al., 2001; Reich et al., 2001; Markesbery et al., 2005; Williams et al., 2006), protein oxidation (reviewed in Sultana et al., 2006; Sultana and Butterfield, 2010) and DNA (reviewed in Lovell and Markesbery, 2007) and RNA oxidation (Nunomura et al., 2001; Shan et al., 2003; Ding et al., 2006; Shan and Lin, 2006) in vulnerable regions of brain in late-stage AD (LAD). In addition, more recent studies show increased levels of markers of oxidative damage in the brain and cerebrospinal fluid (CSF) of subjects with mild cognitive impairment (MCI), the earliest clinical manifestation of AD. Increased levels of lipid peroxidation markers including the α,β-unsaturated aldehydes acrolein, 4-hydroxynonenal (HNE) and 4-hydroxyhexenal (HHE) have been reported in vulnerable regions of MCI brain (Williams et al., 2006; Sultana and Butterfield, 2010; Bradley et al., 2010a). Other studies of levels of neuroprostanes and isoprostanes, non-enzymatic by products of lipid peroxidation, demonstrate increased levels in CSF of MCI subjects (Markesbery et al., 2005). Global levels of protein oxidation as well as multiple specific oxidatively modified proteins are elevated in MCI brain (reviewed in Sultana and Butterfield, 2010) and markers of DNA and RNA oxidation are also significantly elevated in MCI brain (Ding et al., 2005, 2006; Wang et al., 2006; Lovell and Markesbery, 2008; Shao et al., 2008). Although increased levels of oxidative stress present in MCI and LAD brain are thought to contribute to the accumulation of DNA oxidative damage, it is possible that decreased DNA repair capacity may also be important. Studies of the oxoguanine glycosylase 1 (OGG1), a critical member of the predominant DNA repair pathway in brain show significantly decreased OGG1 activity in LAD and MCI brain (Lovell et al., 2000; Weissman et al., 2007; Shao et al., 2008). Our studies of OGG1 in MCI brain showed no significant differences in OGG1 protein but a significant decrease in enzyme activity that was associated with increased post translational modification of the protein by 4-HNE (Shao et al., 2008).

Although considerable evidence suggests DNA and RNA oxidation is present in MCI and LAD brain as well as Parkinson's disease (Zhang et al., 1999), and dementia with Lewy bodies (Nunomura et al., 2002) there have been no reported studies of DNA or RNA oxidation in subjects with preclinical AD (PCAD). Subjects with PCAD are characterized as those who demonstrate normal antemortem neuropsychological test scores but who

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exhibit significant AD pathology at autopsy. Recent studies of PCAD brain show by-products of lipid peroxidation are significantly elevated in PCAD HPG but not in superior and middle temporal gyrus (Bradley et al., 2010a,b). In contrast, measures of protein oxidation did not show significant alterations in PCAD brain (Aluise et al., 2010; Bradley et al., 2010a,b). Because patterns of oxidative damage to lipids and proteins in PCAD brain were somewhat different compared to MCI and LAD brain the current study was carried out to determine if DNA and RNA oxidation occurs in vulnerable brain regions in PCAD using immunohistochemistry and confocal microscopy to quantify levels of 8-hydroxyguanine (8-OHG) in sections of hippocampus/parahippocampal gyri (HPG) and gas chromatorgraphy/mass spectrometry (GC/MS) with stable labeled internal standards to quantify levels of 8-OHG in nuclear DNA from superior and middle temporal gyrus (SMTG). Levels of 8-OHG in RNA were quantified using sections pretreated with RNase free DNase whereas DNA associated 8-OHG was quantified in sections pretreated with DNase free RNase. To determine if decreased levels of OGG1 protein were associated with increased 8-OHG immunostaining, we also subjected sections of HPG to double labeling for total 8-OHG and OGG1. To verify our immunohistochemical data for OGG1 we also quantified OGG1 mRNA and protein levels in PCAD and NC SMTG using quantitative PCR (qPCR) and Western blot analysis.

2. Materials and methods

2.1. Subject selection and neuropathologic examination

Sections (10 μm) of paraffin embedded HPG were obtained from short postmortem interval (PMI) autopsies of 10 subjects with PCAD (2 men, 8 women) and 8 age-matched normal control (NC) subjects (1 man, 7 women) through the Neuropathology Core of the University of Kentucky Alzheimer's Disease Center (UK-ADC). All subjects were followed longitudinally in the UK-ADC where they underwent neuropsychological testing annually and physical examinations biannually. Both NC and PCAD subjects had antemortem Mini Mental Status Examination (MMSE) scores in the normal range (NC = 28.1 ± 0.5; PCAD = 28.5 ± 0.4) and showed no evidence of memory decline. These studies were carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and were approved by the University of Kentucky Institutional Review Board.

Histopathologic examination of multiple sections of neocortex, hippocampus, entorhinal cortex, amygdala, basal ganglia, thalamus, nucleus basalis of Meynert, midbrain, pons, medulla, and cerebellum using hematoxylin and eosin and the modified Bielschowsky stains along with 10D-5 (for Aβ) and α-synuclein immunochemistry was carried out on all subjects. Braak staging scores were determined using the Gallyas stain on sections of entorhinal cortex, hippocampus, and amygdala, and the Bielschowsky stain on neocortex. Normal control subjects showed only age-associated changes and Braak staging scores of 0–II and met the National Institute on Aging-Reagan Institute (NIA-RI Working Group, 1997) low likelihood criteria for the histopathologic diagnosis of AD. In contrast, subjects identified with PCAD had moderate or frequent neuritic plaque scores, Braak staging scores from III to V and met intermediate or high NIA-RI criteria for the histopathologic diagnosis of AD. Demographic data for all subjects in the study are shown in Table 1.

To verify our immunohistochemical results we used gas chromatorgraphy mass spectrometry (GC/MS) with stable labeled internal standards to quantify 8-OHG in nuclear DNA and quantitative real time PCR (q-PCR) to quantify mRNA levels of OGG1 in the superior and middle temporal gyrus (SMTG) of a subset of the subjects analyzed by immunohistochemistry. The SMTG was chosen for these studies because of the limited availability of frozen HPG specimens and because SMTG shows considerable AD pathology.

2.2. Immunohistochemistry and antibodies

To visualize the cellular distribution of DNA and RNA oxidation, 10-μm sections of paraffin-embedded HPG from NC and PCAD subjects were cut using a Shandon Finesse microtome (Thermo Fisher Scientific, Waltham, MA), placed on Plus-slides, and rehydrated through xylene, descending alcohols, and water. Following rehydration, sections were digested with 10 μg/mL proteinase K (Boehringer Mannheim, Indianapolis, IN) in phosphate buffered saline (PBS, pH 7.4) for 40 min at 37 °C. Sections were then washed in Tris-buffered saline (TBS; 150 mM Tris–HCl, 150 mM NaCl, pH 7.6) and pretreated with RNase free DNase-I (10 U/μl in PBS, Roche, Mannheim, Germany) or DNase-I free RNase (0.5 μg/μl PBS, Roche, Mannheim, Germany) for 2 h at 37 °C. Sections were then blocked for 2 h in 1.5% normal goat serum in TBS at room temperature and incubated overnight in a 1:100 dilution of mouse anti-8-OHG (QED Biosciences, San Diego CA) in 1.5% goat serum/TBS at 4 °C. Following thorough washing in TBS, sections were incubated in a 1:1000 dilution of Alexa-488 conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) for 2 h at room temperature. After 5 rinses in TBS and distilled/deionized water, sections were coverslipped using fluorescent anti-fade (Molecular Probes) and imaged using a Leica DM IRBE confocal microscope equipped with argon, krypton and HeNe lasers and a 40× oil objective. Confocal images were captured from a single *z* plane without optical sectioning and were the average of 3 scans. Images were captured from 10 fields/section with an average of 10–20 cells per field without knowledge of subject diagnosis. All sections were analyzed on the same day with the same instrument settings to allow comparison of fluorescence intensities between sections. Sections were analyzed by subject number without knowledge of diagnoses. The first section analyzed was reimaged at the midpoint and end of the experiment to verify that instrumental parameters had not diminished. Fluorescence intensities of all cells in each field were quantified using Leica image analysis software. Mean fluorescence intensities were calculated for 8-OHG in DNA or RNA for each slide. Results are reported as mean ± SEM% control staining.

To verify 8-OHG immunostaining was uniquely associated with neurons and not astrocytes, representative sections of HPG were triple labeled for 8-OHG, neuron specific β-tubulin (β-tubulin-III; Tuj-1), and GFAP, an astrocyte specific marker. The sections were labeled for 8-OHG as described above, blocked 1 h in 1.5% normal goat serum/TBS followed by incubation overnight in a 1:100 dilution of monoclonal anti-tubulin-III (Tuj-1; Covance, Denver, PA) and a 1:100 dilution of rabbit anti-GFAP (Dako, Capinteria, CA). The sections were washed three times in TBS and incubated in 1.5% normal goat serum/TBS containing Alexa-568 labeled goat anti-rabbit IgG (1:1000) and Alexa-633 labeled goat anti-mouse IgG (1:1000) for 1 h. Following three washes in TBS the sections were coverslipped using fluorescent anti-fade and imaged as described above using a 100× objective.

Oxoguanine glycosylase-1 immunostaining was carried out by subjecting rehydrated sections to antigen retrieval using 1 mM EDTA (pH 8.0) and an IHC Antigen Retireval system (Thermo Fisher, Fremont, CA). Following antigen retrieval, sections were blocked in 1.5% goat serum/TBS for 2 h and total 8-OHG (DNA + RNA) immunolabeled by incubation overnight in a 1:100 dilution of anti-8-OHG. Treatment with proteinase K and DNase or RNase was omitted because if led to diminished OGG1 immunostaining. After thorough washing in TBS sections were incubated in a 1:1000 dilution of Alexa-488 labeled goat anti-mouse IgG as described above. The sections were then double labeled using a mouse antibody against OGG-1 by incubation overnight at 4 °C in a 1:100 dilution of anti-OGG1 (Abcam; Cambridge, MA). Sections were washed three times in TBS and then incubated in a 1:1000 dilution of Alexa-565 labeled goat anti-mouse IgG for 2 h at room temperature. The sections were rinsed 5 times with TBS, twice with distilled/deionized water and coverslipped and imaged as described above.

2.3. GC/MS quantification of 8-OHG in DNA

Nuclear DNA was purified as described by Mecocci et al. (1994) with minor modifications as described by Wang et al. (2006). Briefly, nuclear isolates were resuspended in digestion buffer (DB) containing SDS (0.5%), Tris (0.05 M), EDTA (0.1 M) with proteinase K (0.5 mg/mL) and incubated overnight at 55 °C for nDNA extraction. NaCl (160 μL/10 mL DB) was added and the solution extracted 3× with tris-buffered phenol contained 8-hydroxyquinoline (5.5 mM) and 3× with isoamyl alchohol/chloroform (4% (v/v)). nDNA was precipitated from the aqueous layer by addition of 800 μl NaCl (5 M)/10 mL DB and an equal volume of absolute ethanol overnight at −20 °C. Pellets were washed 3× with 60% (v/v), air dried, and resuspended in autoclaved distilled/deionized water. The concentration and purity of DNA samples were determined by NanoDrop 1000 Spectrophotometey (NanoDrop, Wilmington, DE, USA). Samples were stored at −80 °C until analysis.

For GC/MS analyses nDNA (200 μg) samples were prepared placed in 5 mL conical glass tubes along with 10 nmol stable labeled 8-OHG (8-[8-^{13}C,7,9-$^{15}N_2$] hydroxyguanine). The samples were lyophilized and the tubes evacuated. Samples

Table 1
Subject demographic data.

	Mean ± SEM Age (years)	Sex	Mean ± SEM PMI (h)	Median Braak Staging Score	Mean ± SEM MMSE score
NC	83.9 ± 1.9	1M/7W	2.4 ± 0.2	I	28.1 ± 0.5
PCAD	86.0 ± 2.1	2M/8W	2.9 ± 0.2	IV*	28.5 ± 0.4

* $p < 0.05$.