Acta Neuropathol (2003) 106 : 552–556
DOI 10.1007/s00401-003-0761-7

REGULAR PAPER

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Oxidative damage in the olfactory system in Alzheimer's disease

Received: 15 January 2003 / Revised: 18 July 2003 / Accepted: 22 July 2003 / Published online: 29 August 2003

Abstract Increased oxidative damage is a prominent and early feature of vulnerable neurons in Alzheimer's disease (AD). However, while damage to proteins, sugars, lipids, nucleic acids and organelles such as lysosomes, mitochondria, and endoplasmic reticulum are evident, the source of increased reactive oxygen species has not been determined. Furthermore, a major limitation in further determining the source, as well as finding a means to arrest damage, is the paucity of cellular models directly homologous to AD since the vulnerable neurons of the brain in AD cannot be studied in vitro. Here, we examined the olfactory epithelium in situ to see if neurons there exhibit a similar pathological oxidative balance to vulnerable neurons in AD. In biopsy specimens, (eight AD and three controls) we found that neurons, and also the surrounding epithelial cells, show an increase in oxidative damage for a subset of the markers increased in the brain of cases of AD. Lipid peroxidation and heme oxygenase-1, a stress response protein, were increased, while nucleic acid or protein oxidation, demonstrated in vulnerable neurons in AD, were not increased. These findings highlight the systemic nature of oxidative abnormalities in AD, but that different cell types may express this abnormality by a different array of oxidative stress markers, supporting the potential for using olfactory neurons or other cells derived from AD patients in culture to understand the mechanistic basis for increased oxidative damage in AD and as a model to screen compounds for therapeutic intervention.

Keywords Alzheimer's disease · Heme oxygenase · Lipid peroxidation · Olfactory neurons · Oxidative stress

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Introduction

Oxidative damage and antioxidant response are demonstrated features of vulnerable neurons in Alzheimer's disease (AD) [14, 24]. Studies show increased damage to a wide array of biological macromolecules seen in neurons vulnerable to death in AD [3, 7, 8, 18, 28, 33, 34]. Additionally, a number of lines of evidence now indicate that oxidative damage and antioxidant responses precede clinical manifestations of the disease, and may be a major predisposing factor for the development of AD [19, 20].

The human olfactory system is also affected at an early stage of AD. In a community-based longitudinal study, impaired odor identification was associated with an increased likelihood of subsequent cognitive decline, especially in patients with an apolipoprotein ε 4 allele [9]. Moreover, olfactory loss or impairment was shown to parallel cognitive decline and predict clinical deterioration in AD [10, 29], with olfactory identification deficits appearing in the early stage of the disease [6, 15]. Accumulation of tau as neurofibrillary tangles also initially affects structures that receive connections from the olfactory bulb [2, 22, 26] and involves the olfactory bulb [11, 12].

Olfactory neurons can regenerate, be grown in culture, and be sampled from living patients [38]. This study investigates the cellular expression of oxidative markers in the human olfactory epithelium from biopsy samples of AD and control patients. We found oxidative abnormali-

ties in lipid peroxidation and heme oxygenase pathways similar to those found in AD, while sugar, nucleic acid and protein oxidation, known to be increased in the brain in AD, were little changed. Neurons and epithelial cells displayed these changes. These findings provide feasibility for mechanistic studies using cells in culture to address both the mechanism responsible for oxidative damage in AD as well as evaluate therapeutic interventions to reduce it.

Materials and methods

Tissue from the upper nasal cavity was taken from eight patients with probable AD, four under the age of 65 years (ages 59–64 years) and four above the age of 65 (ages 66–74 years), and from three control patients 65 and older (ages 65–75 years). AD cases were not pathologically confirmed, therefore, probable AD cases were selected based on a history of progressive cognitive deficit of 3 years or more, following NINCDS-ADRDA recommendations [16]. Controls were neurologically normal patients undergoing upper nasal surgery. Consent was given by either the patient or, in the case of probable AD patients, by a relative of the patient in agreement with the Ethical Committee at the Institute of Neurology in the University of Genoa. Biopsies were performed by an otorhinolaringologist (G.C.). Local anesthesia was applied with a cotton swab saturated with neoblucaine and naphazoline, and 3- to 4-mm squares of olfactory mucosa was sampled. At the time of biopsy, no patients had viral or allergic rhinitis. All controls were neurologically normal subjects. Following fixation in Bouin's solution, tissues were embedded in paraffin, and 6-μm sections were placed on silane-coated slides (Sigma). After deparaffinization with xylene, the sections were hydrated with ethanol. Endogenous peroxidase activity in the tissue was eliminated by a 30-min incubation with 3% H_2O_2 in methanol, and nonspecific binding sites were blocked with a 30-min incubation in 10% normal goat serum in TRIS-buffered saline (50 mM TRIS-HCl and 150 mM NaCl, pH 7.6). All incubations were conducted at room temperature unless otherwise indicated.

Antibodies to markers of well-described oxidative changes of AD were used, including rabbit antiserum to heme oxygenase-1, 1:100, [31]; antiserum to N^{ε}-(carboxymethyl) lysine (CML), 1:100 [5]; antiserum to the pyrrole formed by the reaction of the lipid peroxidation product hydroxynonenal (HNE-pyrrole) with protein, 1:100 [27]; and antiserum to the advanced glycation end product (AGE), pentosidine, 1:100 [32], and antiserum to 8-hydroxyguanosine, 1:100 [18]. Omission of the primary antibody or absorption with 100 μg/ml antigen was also performed to determine specificity. In addition, sections for CML were treated with 70% formic acid for 5 min prior to immunostaining. Antisera against τ, 1:1,000, was also used [23]. Olfactory neurons were identified with rabbit antiserum to neuronal specific protein gene product 9.5 (PGP 9.5, mixed marker of sympathetic and sensory nerve fibers used to define olfactory neurons), 1:1,000 (Ultraclone, Wellow, UK). Immunostaining was visualized using the peroxidase-antiperoxidase procedure by using 3,3'-diaminobenzidine (DAB; Dako, Carpenteria, CA) as the cosubstrate [35].

AD and control samples were stained simultaneously with the DAB solution for 2 min for each antibody used.

Quantitative assessment of immunoreaction

Immunoreaction was quantitated with an Axiophot microscope equipped with an Axiocam and associated Image Analysis system (Zeiss, Thornwood, NY). Three adjacent fields (each field 460 μm× 428 μm) of olfactory and/or respiratory epithelium were selected starting from the outermost edge of the sample. The intensity of immunoreaction was evaluated by measuring the average optical density (OD) of an area comprising the mucosal layer, which included the olfactory neurons, sustinticular and basal cells, as we described previously [18]. Areas containing goblet cells were omitted. The average OD measurement was obtained for each of the three fields and then averaged. Finally, the OD value was corrected

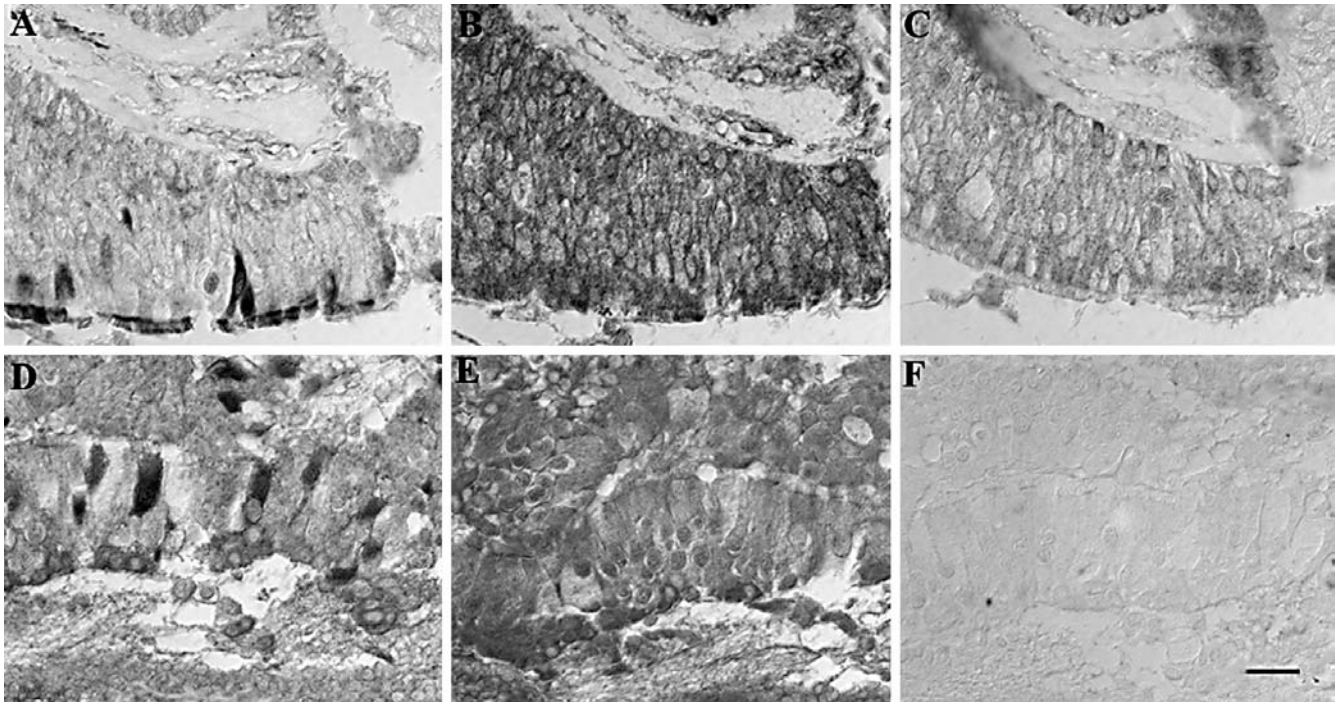

Fig. 1 Serial sections of AD (**A**–**C**) and control (**D**–**F**) nasal biopsy stained with PGP9.5, a marker used to define olfactory neurons (**A**, **D**) and two markers for oxidative stress: protein adducts of lipid peroxidation (HNE-pyrrole) (**B**, **E**) and heme oxygenase-1 (oxidative stress response) (**C**, **F**) (*AD* Alzheimer's disease, *HNE* hydroxynonenal). *Bar* 20 μm

for background by subtracting the OD of the same cell layer in which the tissue was processed but the primary antibody omitted. All measurements were done under the same optical and light conditions. Significance was tested using nested two-way ANOVA with fixed effects [17] for both HNE and heme oxygenase-1.

Results

Organization of olfactory mucosa

The olfactory sensory neuroepithelium is embedded in the respiratory mucosa in the upper nasal septum, and is the only region of the central nervous system directly exposed to the environment. Within this layer, olfactory neurons are identified as ciliated, slender bipolar neurons with cell bodies in the lower to mid-portions of the epithelium, and are recognized by the neuronal marker PGP9.5 (Fig. 1A, D).

Oxidative markers

Immunoreaction for markers of oxidative stress was not only prominent diffusely in the apical cytoplasm of olfactory neurons, but was also seen in all cells, including those of pure respiratory epithelia (Fig. 1). Quantitative evaluation (Table 1) showed that, while there was a general trend of increase in AD compared to controls, significance was only reached for HNE-pyrrole ($P<0.0001$) (with relative values of 45–79 for controls and 62–95 for AD respectively with a 95% confidence limits 13.7 and 22.8) and heme oxygenase-1 ($P<0.0001$) (with values 46–65 for controls and 76–105 for AD respectively with 95% confidence limits of 26.2 and 34.6) for those AD cases over the age of 65 (Fig. 2). For those AD cases under 65 years of age, the results were similar to the controls, with no significant difference obtained (data not shown). However, samples from younger controls were not available to determine whether controls age-matched for the 59- to 64-year AD group would show a difference. Tau was observed only in dystrophic neurites of AD patients as previously reported [13, 36, 37].

Table 1 Average values obtained from each experiment shown as relative intensity of immunoreaction, see methods (*HNE* hydroxynonenal, *HO-1* heme oxygenase-1)

Control			Alzheimer's disease		
Age	HNE	HO-1	Age	HNE	HO-1
65	44.54	65.13	66	71.90	77.21
	46.50	52.84		78.74	76.44
	49.85	62.77		64.55	77.69
70	57.32	54.32	67	83.41	82.92
	60.27	46.07		86.28	79.91
	61.38	47.59		78.20	77.39
75	72.08	58.26	67	85.07	104.77
	72.03	54.14		92.24	99.23
	78.77	55.26		94.70	94.30
			74	69.75	
				75.53	
				62.22	

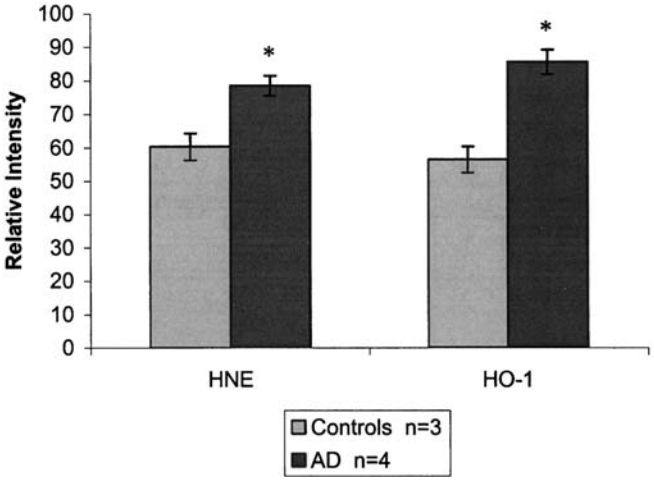

Fig. 2 Olfactory biopsy sections from four probable AD patients (ages 66–74 years) show increased HNE-pyrrole and heme oxygenase-1 when compared to three controls (ages 65–75 years), $P<0.002$ and $P<0.0001$, respectively

Discussion

In this study, we demonstrate oxidative damage and response in olfactory epithelium, which is increased in AD. However, only those AD cases over the age of 65 showed a significant difference compared to aged-matched controls. This raises the possibility that olfactory epithelium that encounters oxidative insults physiologically shows age-dependent as well as AD-related changes. Unfortunately, samples from younger controls were not available to evaluate this possibility. Previous studies with other stress-related markers show similar age-related differences [39]. That neurons and epithelial cells demonstrate similar levels of oxidative stress markers contrasts with the finding in brain where neurons show the most marked damage. Yet with closer examination, endothelial cells, even in brain [1], showed marked oxidative damage in AD. In addition, we confirmed earlier studies by our group that noted increased tau-positive neurites in AD but not controls.

Seen in entirety, these findings suggest that AD pathogenesis is not limited to neurons and is consistent with prior work [4, 30] showing that non-neuronal cells also display metabolic abnormalities in AD. The findings support systemic alterations in AD. This aspect has important implications for mechanistic modeling studies since cells derived from non-brain sources may be informative to AD. In preliminary studies, we have shown that cultured olfactory neurons and fibroblasts show oxidative imbalance for the same markers shown here. This aspect opens the opportunity of evaluating potential therapeutic agents to restore oxidative balance to cells displaying bona fide AD abnormalities.

Another important aspect of our findings is that oxidative imbalance was expressed as increased lipid peroxidation and heme oxygenase-1, not the full range of changes noted in vulnerable neurons in the cortex [24]. This suggests that lipid peroxidation/heme oxygenase-1 increase can occur in the relative absence of other oxidative abnormalities. We have seen the same restriction of increase to lipid peroxidation/heme oxygenase-1 in progressive supranuclear palsy [21]. These findings raise the possibility that various mechanisms are responsible for oxidative imbalance, and that those seen in the olfactory epithelium are only a portion of the full range of characteristics seen in AD. Further studies such as those we plan for olfactory neurons and other AD cells in culture are necessary to uncover the mechanistic basis for oxidative damage in AD.

Acknowledgements This work was supported by the National Institutes of Health, the Alzheimer's Association, and Panacea Pharmaceuticals. Mark A. Smith and George Perry are compensated consultants and own equity in Panacea Pharmaceuticals.

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