Pergamon

www.elsevier.com/locate/neuroscience

PII: S0306-4522(01)00115-4

Neuroscience Vol. 104, No. 3, pp. 609–613, 2001

0306-4522/01 $20.00+0.00

Letter to Neuroscience

FIBRILLAR β-AMYLOID EVOKES OXIDATIVE DAMAGE IN A TRANSGENIC MOUSE MODEL OF ALZHEIMER'S DISEASE

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Key words: oxidative stress, Alzheimer's disease, amyloid β protein, fibrillar β-amyloid, animal model.

β-Amyloid is one of the most significant features of Alzheimer's disease, and has been considered to play a pivotal role in neurodegeneration through an unknown mechanism. However, it has been noted that β-amyloid accumulation is associated with markers of oxidative stress including protein oxidation (Smith et al., 1997), lipid peroxidation (Mark et al., 1997; Sayre et al., 1997), advanced glycation end products (Smith et al., 1994), and oxidation of nucleic acids (Nunomura et al., 1999). Furthermore, studies from cultured cells have shown that β-amyloid leads to an increase in hydrogen peroxide levels (Behl et al., 1994), and the production of reactive oxygen intermediates (Harris et al., 1995). Taken together, this evidence supports the idea that β-amyloid plays a key role in oxidative stress-evoked neuropathology. In this study, we examined the induction of oxidative stress in response to amyloid load in a mouse model of Alzheimer's disease. The mice carrying mutant amyloid precursor protein and presenilins-1 (Goate et al., 1991; Hardy, 1997), develops β-amyloid deposits at 10–12 weeks of age and show several features of the human disease (Holcomb et al., 1998; Matsuoka et al., 2001; McGowan et al., 1999; Takeuchi et al., 2000; Wong et al., 1999). Both 3-nitrotyrosine and 4-hydroxy-2-nonenal (protein and lipid oxidative stress markers, respectively) associate strongly with fibrillar β-amyloid, but not with diffuse (thioflavine S negative) β-amyloid, and the levels increase in relation to the age-associated increase in fibrillar amyloid load.

From these data we suggest that fibrillar β-amyloid is associated with oxidative damage which may influence disease progression in the Alzheimer's disease brain. © 2001 **IBRO. Published by Elsevier Science Ltd. All rights reserved.**

β-Amyloid (Aβ) is initially deposited in the cingulate cortex in the mice carrying mutant amyloid precursor protein (APP) and mutant presenilins (PS1) (PS/APP) at 10–12 weeks of age, but it rapidly encroaches on the entire cerebral cortex and hippocampus. As β-amyloid is mainly observed in the frontal brain in young adult mice, we focused our studies on this region (Fig. 1A–C). At 7 months of age, Aβ immunoreactivity was extensive (Fig. 1A), but immunoreactivity for 3-nitrotyrosine (NT) and 4-hydroxy-2-nonenal (HNE) was limited (Fig. 1B,C). In the brains of 2.5-year-old PS/APP mice, NT and HNE immunoreactivity was markedly increased compared to that seen in younger brain (Fig. 1B vs. C and Fig. 1E vs. F). To examine whether NT and HNE were associated with both fibrillar and non-fibrillar Aβ, serial sections were immunolabeled using primary antibodies against either Aβ, NT or HNE (Fig. 2A–C), followed by thioflavine S. Some of the 4G8 immunoreactive Aβ deposits were associated with NT and HNE immunoreactivity (indicated by closed arrow in Fig. 2A–C) but others were not (indicated by open arrow in Fig. 2A). Thioflavine S staining was used to identify fibrillar Aβ deposits on sections previously stained with anti NT or HNE, thus the two panels (Fig. 2D-a/b, 2E-a/b, 2F-a/b, and 2G-a/b) show the same section in each case. Both NT and HNE immunoreactivity was associated with thioflavine S/4G8 positive deposits (Fig. 2D,E), whereas thioflavine S negative/4G8 positive deposits were devoid of NT or HNE immunoreactivity (Fig. 2A–C). Positive immunoreactivity completely disappeared following either omission of the primary antibody (data not shown), or pre-incubation with NT and HNE-modified protein (Fig. 2F and G).

It has been reported that HNE is primarily detected in neurofilament tangles and dystrophic neurites in human Alzheimer's disease (AD) (Sayre et al., 1997). Another AD mouse model, APP single transgenic mouse, showed HNE in dystrophic neurites (Smith et al., 1998a). To

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Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; APP, amyloid precursor protein; HNE, 4-hydroxy-2-nonenal; iNOS, inducible nitric oxide synthase; NT, 3-nitrotyrosine; PS, presenilin; PS/APP, transgenic mice overexpressing APP and PS-1 mutant genes.

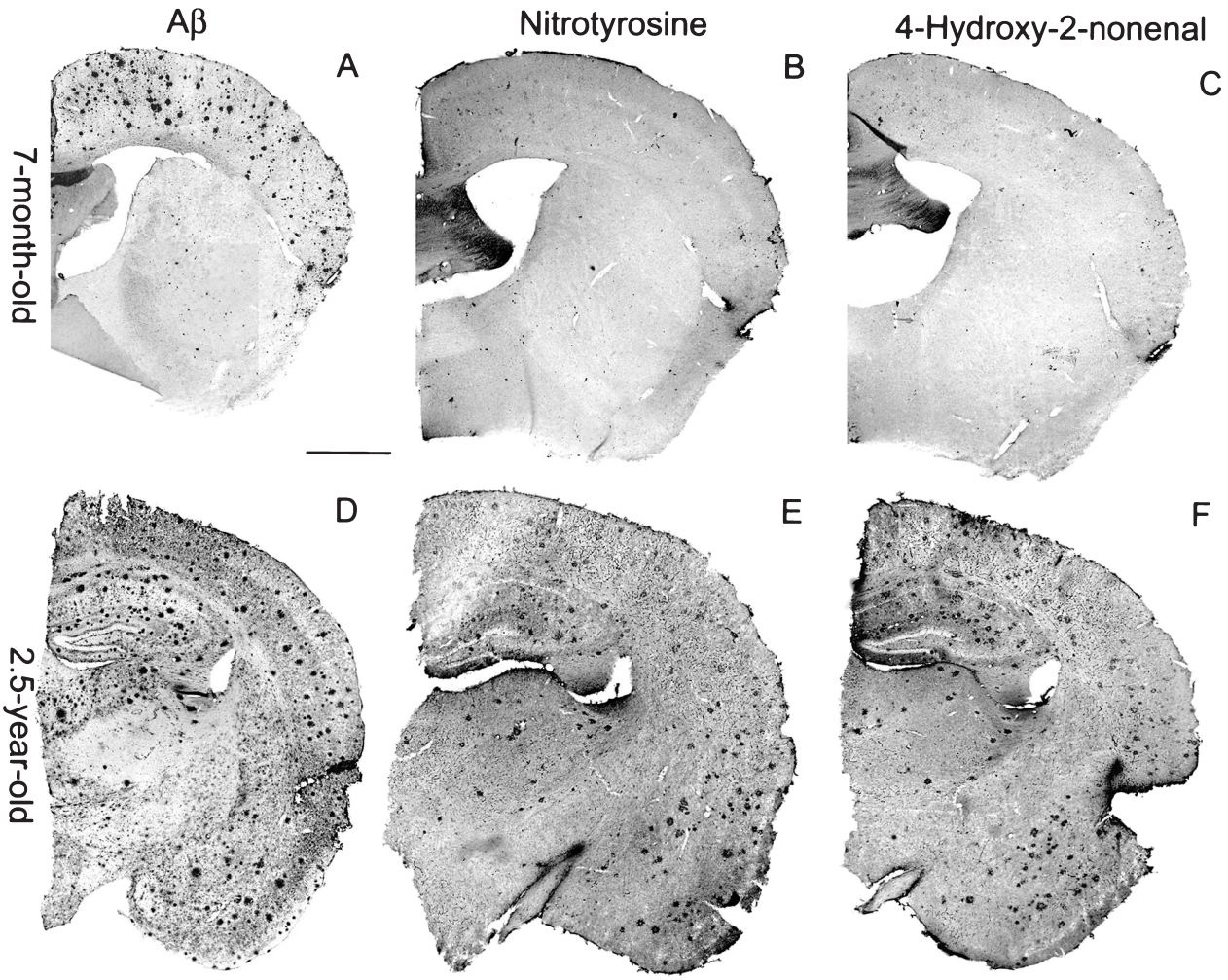

Fig. 1. Aβ and increase of oxidative stress markers with aging. Aβ and oxidative stress markers in 7 month (A–C) and 2.5-year-old (D–F) PS/APP mouse brain. Immunoreactivity for NT and HNE increased markedly between these ages (B and C vs. E and F). Scale bar = 500 μm.

assess whether this was also the case in the PS/APP mice, we compared immunoreactivity for HNE and the neurite markers, tau and APP (Fig. 3). HNE immunoreactivity gave a purple product (Fig. 3A), whereas co-localization of tau and HNE resulted in a darker (black) product (Fig. 3A, indicated by arrowheads). To improve clarity, co-localization of HNE with neurite markers was also compared on consecutive sections (Fig. 3B–D). HNE, tau and APP immunoreactivity (Fig. 3B) were all observed in plaque-associated neurites (Fig. 3C,D, respectively). However, as the neurites are fine and cover a limited area of the 30 μm section, it was not possible to identify regions of the neurite with overlapping signal for both HNE- and tau/APP on consecutive sections.

In this study, we have found that oxidative stress markers co-localize with thioflavine S positive (fibrillar) Aβ, but not thioflavine S negative (diffuse) Aβ. Previous studies have shown that amyloid plaques are associated with immunoreactivity for HNE, NT, superoxide dismutase, hemeoxygenase, and iron regulatory proteins in other amyloid mouse models, as well as in the human AD brain (Good et al., 1996; Pappolla et al., 1998; Smith et al., 1998a,b,c, Smith et al., 1997). The human AD brain and most of the amyloid mouse models contain both fibrillar and diffuse plaques, but no distinction has been made previously between Aβ conformation and degree of association with oxidative damage, *in vivo*. Although our studies clearly demonstrate the relative toxicity of fibrillar vs. diffuse deposits in the PS/APP mouse model, it is likely that in the human AD brain, several factors including age, length of exposure to elevated Aβ levels and the formation of tangles contribute to oxidative damage which probably makes it appear more widespread.

Reactive oxygen intermediates can react with nitric oxide (NO) to form peroxynitrite. The peroxidation of membrane lipids can result in the release of HNE which is known to conjugate primarily to the histidine residue of proteins (Uchida and Stadtman, 1992). There is ample evidence that Aβ can induce the production of reactive oxygen intermediates (Behl et al., 1994), which may have profound effects on cell function or viability. Aβ treatment of rat hippocampal neurons has been shown to lead to a large induction of both free and protein-bound HNE, and both Aβ-induced HNE production or HNE

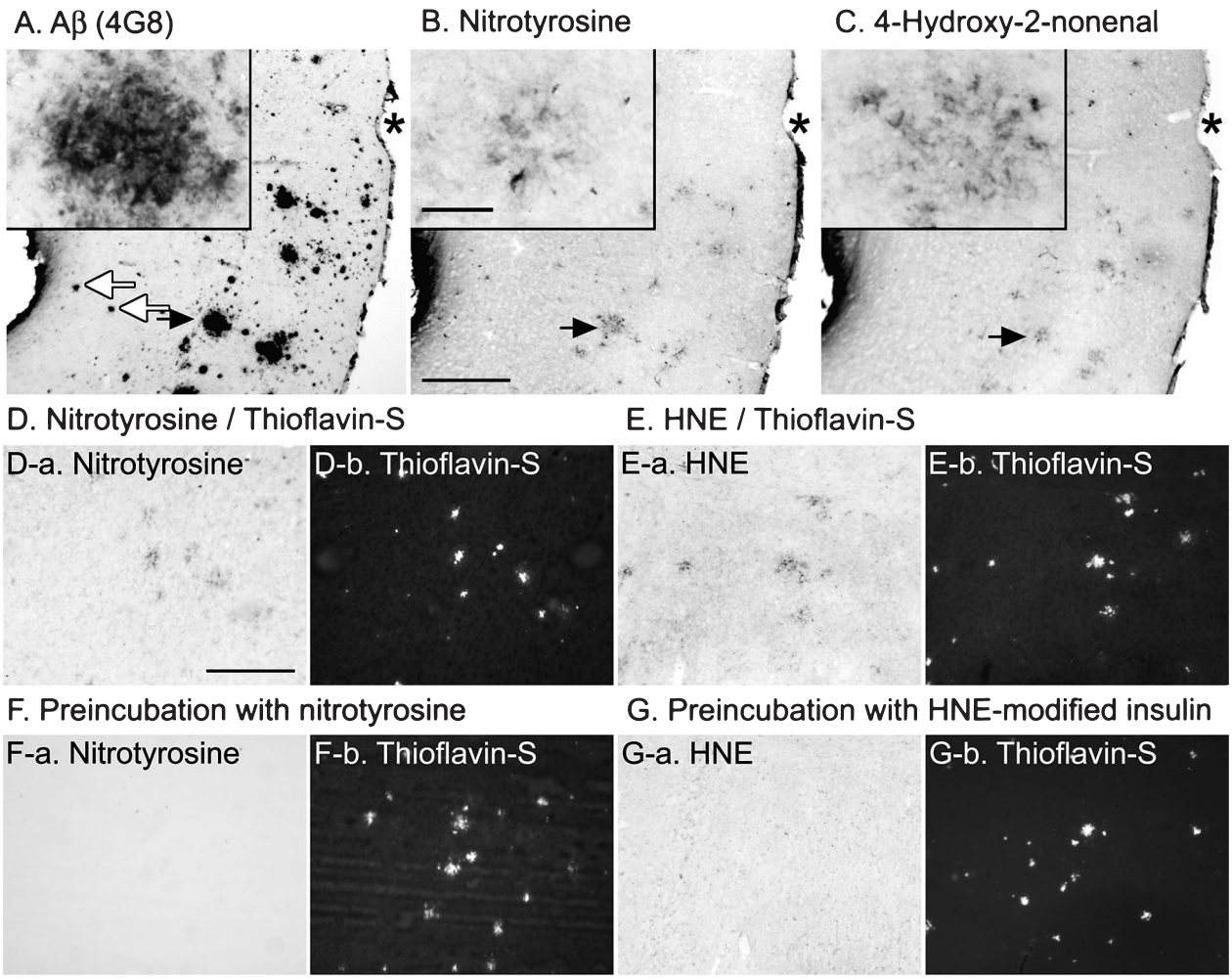

Fig. 2. Co-localization of Aβ and oxidative stress markers. (A–C) Serial sections were incubated with primary antibodies against Aβ, NT, and HNE. Closed and open arrows indicate 4G8 immunoreactive Aβ with, or without NT- and HNE-immunoreactivity, respectively. The asterisk indicates the same point on the sections. (D, E) Co-localization of NT and HNE with thioflavine S positive fibrillar Aβ. After immunodetection with either anti-NT or anti-HNE ('a' in each panel, D-a and E-a), sections were counter-stained with thioflavine S to show fibrillar Aβ deposits ('b' in each panel, D-b and E-b). (F, G) Absorption tests for NT and HNE immunostaining. Primary antibodies were pre-incubated with 1 μM of either NT or HNE-modified insulin, and sections were incubated with pre-incubated primary antibodies. After immunodetection ('a' in each panel, F-a and G-a), sections were counter-stained with thioflavine S to confirm the distribution of fibrillar Aβ deposits ('b' in each panel, F-b and G-b). Scale bars = 300 μm (B; also applies to A, C), 50 μm (B inset; also applies to A, C insets), 250 μm (D-a; also applies to D–G).

treatment directly, is concomitant with impaired calcium homeostasis in treated cells (Mark et al., 1997). In addition, Aβ treatment has been shown to exacerbate mitochondrial reactive oxygen species formation leading to loss of mitochondrial membrane potential (Keller et al., 1999). It is perhaps of note then, that older PS/APP show cognitive impairment at an age when the fibrillar amyloid load is severe (Morgan et al., 2000).

NT is generated following the nitrosylation of tyrosine residues by NO-derived peroxynitrite (Yi et al., 1997). Aβ has been shown to stimulate the production of NO in glial cells (Akama et al., 1998). In human AD brain, inducible nitric oxide synthase (iNOS) is associated with senile plaques and intracellular neurofibrillary tangles (Lee et al., 1999; Vodovotz et al., 1996), although it has not been possible to visualize plaque-associated iNOS in the mouse model.

Although oxidative damage has been linked with amyloidosis in transgenic mice, there has, in general, been a negative correlation between amyloid load and neuronal cell death (Dickson, 1997). Recent studies on the PS/APP mouse model have shown however, that while amyloid deposition is not associated with global cell loss (Takeuchi et al., 2000), focal loss of neurons is associated with close proximity to fibrillar, but not diffuse, Aβ deposits (Brad Hyman, Harvard Medical School, personal communication). This is in accord with *in vitro* studies that show that fibrillar Aβ is more neurotoxic than non-fibrillar Aβ (Pike et al., 1993). Our finding that markers of oxidative damage are co-localized with the degenerating neurites surrounding them suggests that the accumulation of fibrillar Aβ is linked to neurodegeneration, and that oxidative damage intermediaries may contribute to the neurotoxicity.

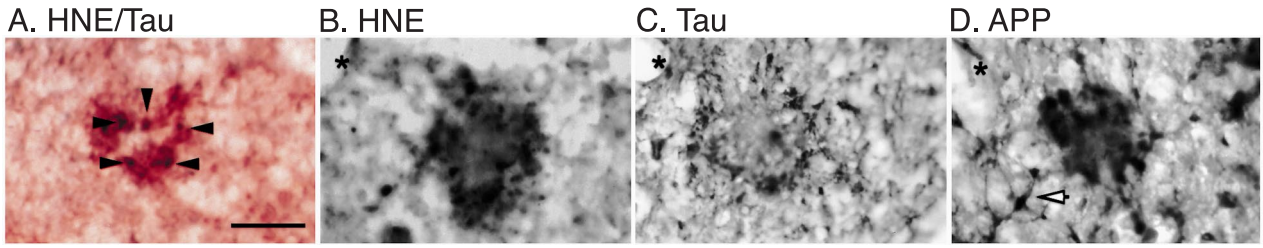

Fig. 3. Localization of HNE and neurite markers in 7-month-old PS/APP mice brain. (A) HNE- and tau-immunoreactivity were detected by 3,3′-diaminobenzidine with, or without nickel enhancement to obtain a blue or brown product, respectively. Overlapping co-localization is shown as black, and is indicated by arrow heads. (B–D) Localization of HNE, tau and APP were visualized in consecutive sections. The anti-APP antibody also detects neuronal staining as these mice overexpress APP (indicated by open arrow head). The asterisk in B–D indicates the same point on the sections. Scale bar = 50 μm (A–D).

EXPERIMENTAL PROCEDURES

We crossed mice expressing mutant (K670N, M671L) *APP* (line Tg2576) (Hsiao et al., 1996) with mutant (M146L) *PS-1* mice (line 6.2) (Duff et al., 1996) to generate PS/APP offspring and their littermate controls. Animals were maintained according to NIH guidelines, and the experimental protocol has been approved by the Animal Experimental Committee at Nathan Kline Institute. All efforts were made to minimize the number of animals used and their suffering. The animals were perfused through the aorta with 10 mM phosphate-buffered saline, pH 7.4, under deep anesthesia, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After overnight post-fixation, brains were dehydrated with sucrose and cut into 30 μm sections. Prior to incubation with primary antibodies, sections were treated with hydrogen peroxide and incubated with normal goat serum to block non-specific signals. Sections were incubated with primary antibodies against NT (clone 1A6, 4 μg IgG/ml, Upstate Biotechnology, Lake Placid, NY, USA), HNE (1 μg IgG/ml, Japan Institute for the Control of Aging, Shizuoka, Japan) (Toyokuni et al., 1997), tau (PHF-1, 1:1000 dilution, gift from Dr. Peter Davies, Department of Pathology, Albert Einstein College of Medicine) (Greenberg and Davies, 1990; Vincent et al., 1994) APP (C1/6.1, 5 μg IgG/ml, gift from Dr. Paul Mathews, Nathan Kline Institute/New York University Medical Center) (Janus et al., 2000) and Aβ (clone 4G8, 333 ng IgG/ml, Senetek, Maryland Heights, MO, USA) overnight at room temperature. Antibodies were detected using an ABC kit (Vector Laboratories, Burlingame, CA, USA).

For double immunolabeling, the immunostaining process was cycled twice. In the second cycle, the primary antibody was detected by 3,3′-diaminobenzidine without nickel enhancement to obtain a brown product. Fibrillar Aβ was identified by incubation of the sections with thioflavine S (10 μg/ml in 70% ethanol for 20 min) followed by observation through a fluorescent filter (excitation: 405–445 nm, and emission: 515–565 nm).

To confirm the specificity of the primary antibodies, absorption tests were performed as follows. HNE-modified insulin was prepared according to Uchida and Stadtman (1992). In brief, bovine insulin (0.5 mg, Sigma, St. Louis, MO, USA) and 2 mM HNE (Alexis, San Diego, CA, USA) in 50 mM phosphate buffer, pH 7.2 were incubated at 37°C for 2 h, and the reaction was terminated following the addition of sodium hydroxide and dithiothreitol. The reaction mixture was lyophilized and re-dissolved in phosphate buffer. After pre-incubation of the primary antibodies with 0.1, 1.0 or 10 μM of NT (Sigma) or HNE-modified insulin overnight at 4°C, sections were incubated with primary antibodies and detected as described previously.

Acknowledgements—The authors thank Drs. Peter Davies (Albert Einstein Medical College) and Paul Mathews (Nathan Kline Institute/New York University Medical Center) for the anti-tau and anti-APP antibodies, respectively, and Dr. Carl Nathan (Weill Medical College of Cornell University) for critical reading of manuscript. This study was supported in part by NIA (AG 172116 to K.D.).

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(*Accepted* 12 *March* 2001)