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A Possible Link Between Astrocyte Activation and Tau Nitration in Alzheimer's disease

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Abstract

Alzheimer's disease (AD) pathology has been characterized, in part, by the self-assembly of the tau molecule into neurofibrillary tangles (NFT). While different post-translational modifications have been identified that accelerate tau aggregation, nitration at tyrosine residues prevents or slows tau filament formation in vitro. Of the five tyrosine residues within the molecule, nitration at the first tyrosine residue (Tyr 18) results in a profound inhibition of filament self-assembly. To determine whether nitration at Tyr 18 occurs in AD pathology, monoclonal antibodies were raised against a synthetic tau peptide nitrated at Tyr 18. A clone, termed Tau-nY18, reacts specifically with tau proteins nitrated at Tyr 18 and fails to cross-react with other nitrated tyrosine residues spanning the length of the molecule or with other proteins known to be nitrated in neurodegenerative diseases. In situ, Tau-nY18 sparsely labels the neuronal pathological hallmarks of the disease, including NFT and dystrophic neurites. Surprisingly however, Tau-nY18 robustly labels nitrated tau within activated, GFAP positive astrocytes intimately associated with amyloid plaques. Furthermore, this antibody detects nitrated tau in soluble preparations from both severe AD brains (Braak stage V, VI) and age-matched controls. Collectively, these findings suggest that nitration at Tyr 18 may be linked to astrocyte activation, an early event associated with amyloid plaque formation.

Keywords

Tau; Nitration; Alzheimer's disease; Astrocytes; Monoclonal antibody; Glial fibrillary acidic protein; Tyrosine

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder pathologically characterized by the formation of neurofibrillary tangles (NFT) and amyloid plaques, in which the major protein constituents are aggregated tau proteins and beta-amyloid peptides (A β),

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respectively (Glenner and Wong, 1984; Grundke-Iqbal et al., 1986a; Kosik et al., 1986; Goedert et al., 1988). Tau is a microtubule-associated protein (MAP) expressed within neurons and various glial cell types, including astrocytes and oligodendrocytes, although at much lower levels than neurons (Papasozomenos and Binder, 1987; Shin et al., 1991; Couchie et al., 1992; LoPresti et al., 1995). Under normal physiological conditions, tau is a highly soluble protein (Schweers et al., 1994) primarily involved in promoting microtubule assembly and stability through the specific interaction between the tau tandem repeat region and tubulin proteins (Lee et al., 1988). While these repeats localize to the C-terminal one-third region of the molecule and are highly conserved among different species, the N-terminal region has the greatest variability. For example, tau from human and non-human primates contains a unique sequence of 10 amino acids, which includes a tyrosine residue at position 18 (Tyr 18) not found in other species (Himmler et al., 1989; Alonso et al., 1995).

Tyrosine residues have been identified as specific sites for oxidative and/or nitrative modifications following exposure to peroxynitrite (Beckman et al., 1994a; Ischiropoulos and al-Mehdi, 1995; Beckman JS, 1996). Although a limited number of proteins appear to be susceptible to peroxynitrite-mediated nitration (Giasson et al., 2000; Horiguchi et al., 2003), tau is selectively vulnerable at tyrosine residues within the most N-terminal region of the molecule (Tyr 18 and 29). Notably, nitration at Tyr 18 largely prevents or slows tau filament self-assembly, an inhibitory mechanism not observed by either oxidation or pseudophosphorylation at the same tyrosine site (Reynolds et al., 2005b, a).

Although nitration prevents tau filament formation *in vitro*, nitrated proteins are selectively localized to tau deposits in various neurodegenerative diseases, as detected by different nitro-tyrosine antibodies (Giasson et al., 2000; Souza et al., 2000; Horiguchi et al., 2003). However, these antibodies lack protein and site-specificity, a limitation that prevents studying site-directed modifications during disease progression. To examine site-directed tyrosine nitration, we previously developed the monoclonal antibody Tau-nY29, an immunological probe that reacts specifically with tau nitrated at Tyr 29 (Reynolds et al., 2006). Tau-nY29 labels a subset of soluble tau and insoluble paired helical filaments (PHF-tau) purified from pathologically severe AD brains (Braak stages V and VI), but fails to label tau from non-demented age-matched controls (Reynolds et al., 2006). These data suggest that nitration at Tyr 29 is a disease-related event during AD pathogenesis. It remains unclear whether other tyrosine-nitration events occur in AD.

In the present study, we report a novel site-directed nitration-specific monoclonal antibody, termed Tau-nY18, which recognizes tau nitrated at Tyr 18. Unlike Tau-nY29, Tau-nY18 recognizes nitrated soluble tau isolated from age-matched controls (Braak stages I-III), as well as nitrated soluble tau from severely affected AD brains (Braak stages V and VI). *In situ*, Tau-nY18 sparsely labels the neuronal pathological hallmarks of the disease, including the NFT and neuritic plaques. In contrast to its intermittent reactivity with the neuronal pathology, Tau-nY18 robustly labels nitrated tau within activated, glial fibrillary acidic protein (GFAP) positive astrocytes from both AD brains and age-matched controls. In most instances, Tau-nY18-labeled astrocytes are associated with or in close proximity to amyloid plaques. Collectively, our results suggest that nitration of tau at Tyr 18 occurs in soluble tau from normal controls (Braak stage I-III), as well as soluble and insoluble PHF-tau preparations from severely affected AD brains (Braak stages V and VI), an event that appears to be associated with astrocyte activation and/or amyloid deposition.

MATERIALS AND METHODS

Tau-nY18 antibody production

Mouse monoclonal antibodies were raised against a synthetic peptide ($^{12}\text{EDHAGTY}^{\text{NO}_2}\text{GLGDRK}^{24}$) containing the nitrated Tyr 18 residue (Cell Essentials, Boston, MA), prepared as previously described (Reynolds et al., 2006). Briefly, female Balb/c mice were immunized subcutaneously with KLH-conjugated peptides every 14 days over a twelve month period (Pierce, Rockford, IL); spleens were then removed and fused to SP2/o myeloma cells (Binder et al., 1985). Positive clones were selected for their ability to bind tau singly nitrated at Tyr 18.

Recombinant tau proteins

Mutant and wild type proteins were expressed in *E. coli* strain BL21 (DE3) using the pT7C-ht40 plasmid as previously described (Goedert et al., 1989). This vector contains a histidine affinity tag fused to the N-terminus of the full length human cDNA (441 residues). Tyrosine residues were mutated to phenylalanine using a Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and verified by DNA sequencing. Five mutant proteins were created, each containing a single tyrosine residue within the protein (Tyr 18, 29, 197, 310 or 394) (Reynolds et al., 2005a). These proteins were then exposed to peroxynitrite treatment, resulting in protein nitration at a single tyrosine site.

Peroxynitrite treatment

Peroxynitrite was prepared as previously described (Beckman et al., 1994b). Proteins were buffer exchanged into nitration buffer (100mM potassium phosphate, 25mM sodium bicarbonate and 0.1mM diethylenetriaminepentaacetic acid) (Ischiropoulos and al-Mehdi, 1995) and then treated with 100-fold molar excess of peroxynitrite in two boluses at room temperature with constant stirring. Proteins were then buffer exchanged in S300 buffer (250mM NaCl, 10mM HEPES, 0.1mM EGTA, pH 7.4) and stored at -80°C until further use (Reynolds et al., 2005b).

Solid phase assays

Enzyme-linked immunosorbent assays (ELISA) were performed by attaching either 100 ng of total full length tau (ht40), bovine wild type GFAP (Fitzgerald Inc., Concord, MA), nitrated tau (nht40), nitrated GFAP (nGFAP), or singly nitrated mutant tau proteins (prepared as described above) to the solid phase. Proteins were first diluted in borate saline buffer and immobilized in 96-well microtiter plates (Corning Inc., Corning NY). After blocking for 1 hour in 5% non-fat dry milk, plates were incubated overnight at 4°C in primary antibody solution. The 3-nitrotyrosine (3-NT, 1mg/ml) and GFAP (0.5 $\mu\text{g}/\text{ml}$) antibodies were obtained from Chemicon (Temecula, CA) and Sigma-Aldrich (St. Louis, MO), respectively. Secondary antibody incubation was performed with either peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Vector Laboratories, Burlingame, CA). Conjugated antibodies were then reacted with a 3',3',5,5'-tetramethylbenzidine liquid substrate solution for 10 minutes at room temperature (Sigma-Aldrich, St. Louis, MO). The reaction was stopped using 3% (v/v) H_2SO_4 solution, and the absorbance was determined at 450 nm.

Tau enrichment from human brain

Soluble and PHF-tau from human brains were enriched from four pathologically severe AD cases (Braak stages V and VI) and seven normal age-matched controls (Braak stages I-III, Table 1), obtained from Rush University Medical Center and prepared as described elsewhere (Ksiezak-Reding et al., 1992; Hanger et al., 1998; Reynolds et al., 2006). Briefly, gray matter from frontal cortex was homogenized in five volumes (v/w) of ice cold homogenization buffer

(100 mM 2-(N-MES), 0.5mM MgCl₂, 1mM EGTA, 1M NaCl, 50mM D-N-acetylglucosamine, 50mM imidazole, 25mM β -glycerophosphate, 20mM NaF, 10mM sodium pyrophosphate, 0.5mM PMSF, pH 6.5) and centrifuged at 27,000 $\times$ g for 30 minutes to remove nuclear and membrane bound material, followed by centrifugation at 95,000 $\times$ g for two hours to separate PHF-tau from soluble tau.

Western blot analysis

Proteins were loaded into a 10% Tris-HCl SDS polyacrylamide gel and separated by electrophoresis. Then proteins were transferred to a nitrocellulose membrane (Trans-Blot, Bio-Rad). Blots containing immobilized proteins were blocked with 5% non-fat dry milk in Tris buffered saline prior to incubation with primary antibodies, Tau-nY18 (1 μ g/ml), Tau-nY29 (1 μ g/ml), Tau-5 (1 μ g/ml), β -tubulin (1 μ g/ml, A. Frankfurter), or α -synuclein (2.0mg/ml, Chemicon, Temecula, CA) at 4°C for 24 hours. Secondary antibody incubation was performed with peroxidase-conjugated anti-mouse antibodies (Vector Laboratories, Burlingame, CA). Blots were then reacted with ECL-western blotting substrate (Pierce, Rockford, IL). Membranes were developed on a Alphatek AX390SE (Broadview, IL) using a Kodak Bio-max XAR film (VWR International, Batavia, IL).

Immunohistochemistry

Control and AD post-mortem brain tissue fixed in 4% paraformaldehyde was obtained as 40- μ m thick sections from the Northwestern Alzheimer's Disease Center Brain Bank. For AD and controls, cases included tissue sections from the inferior temporal gyrus (ITG), hippocampus (CA1), and entorhinal cortex from eight severe AD cases (Braak stages V-VI); five cases from Braak stages I-III were used as age-matched controls (Braak and Braak, 1991). For non-AD tauopathy cases, three specimens with pathologically confirmed CBD and three cases with PSP were obtained from the Mayo clinic Brain Bank (Jacksonville, FL). In all six cases, sections from the hippocampus and frontal cortex were analyzed. Immunohistochemistry was performed on floating tissue sections as described previously (Ghoshal et al., 2002; Garcia-Sierra et al., 2003). Briefly, sections were incubated for 24 hours at 4 °C with monoclonal antibody Tau-nY18 (1 μ g/ml) or AD2 (.02 μ g/ml) (Buee-Scherrer et al., 1996), or with a rabbit polyclonal antibody, R1 (1.5 μ g/ml) (Berry et al., 2004). A biotinylated goat-anti mouse or anti-rabbit antibody was used at a concentration of 1:5000 (Jackson ImmunoResearch), followed by a one hour incubation with avidin-biotin complex (Vectastain ABC-Elite, Vector Laboratories, Burlingame, CA). Sections were developed with metal enhanced 3,3'-diaminobenzine (Pierce, Rockford, IL.) and mounted onto glass slides. Sections were dried, dehydrated through graded alcohols, treated with xylenes and coverslipped with Permaslip (Alban Scientific Inc., St. Louis, MO). Primary antibody dilutions were predetermined by testing serial dilutions on similar tissue. When indicated, the citric acid epitope retrieval method was performed prior to incubation with the primary antibody as previously described (Pellicer and Sundblad, 1994).

Triple-label immunofluorescence

Immunofluorescence was performed as previously described (Garcia-Sierra et al., 2003). Sections were incubated with Tau-nY18 (1 μ g/ml), Alz-50 (1 μ g/ml), R1 (1.5 μ g/ml) (Hyman et al., 1988; Garcia-Sierra et al., 2003) and/or a primary rabbit polyclonal antibody against GFAP (Sigma Aldrich, St. Louis, MO) (0.5 μ g/ml) for 24 hours at 4°C. Anti-fluorescein (FITC)-conjugated goat anti-mouse, Texas-red conjugated goat anti-rabbit (Jackson ImmunoResearch), and/or Cy-5 conjugated anti-rabbit antibodies were used as secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ). Sections were counterstained with Thiazin Red (TR, 0.002%) and then treated with Sudan Black (0.05%) to reduce background lipofuscin autofluorescence. Sections were visualized using a 510-Zeiss laser scanning

confocal microscope (Thornwood, NY). All confocal images were acquired as z-stacks of single optical sections.

RESULTS

Tau-nY18 reacts with site-specificity against nitrated tau at Tyr 18

Due to the selective nitration at Tyr 18 and Tyr 29, as compared to other tyrosine residues within the molecule (Reynolds et al., 2005b, a), we were interested in determining whether nitration at Tyr 18 occurs in AD pathogenesis. Therefore, monoclonal antibodies were raised against a synthetic tau peptide containing the nitro-group on Tyr 18. All antibodies were screened for site specificity by ELISA using full-length human recombinant tau (ht40), nitrated tau (nht40) and singly nitrated tau proteins containing the nitro-group on Tyr 18, 29, 197, or 394. An IgG2b clone, termed Tau-nY18, showed remarkable specificity to tau nitrated at Tyr 18 (Figure 1A). Conversely, a generic nitro-tyrosine antibody (3-NT), failed to react in a site-selective fashion (Figure 1B), recognizing each nitrated tyrosine residue within the tau molecule.

Although tau is evolutionarily conserved, Tyr 18 is primate specific (Figure 2A), and is present in all six canonical isoforms spliced from the tau transcript. Due to this evolutionary difference, we suspected no cross-reactivity between the Tau-nY18 antibody and different species of tau or other MAPs. To test the specificity of this antibody to human tau and nonhuman MAPs, MAP fractions (porcine and bovine MAP fractions highly enriched for tau, MAP1A, MAP1B, and MAP2) were exposed either to vehicle or peroxynitrite treatment then blotted with 3-NT and Tau-nY18 antibodies. The 3-NT antibody recognized porcine and bovine MAP fractions following peroxynitrite treatment (Figure 2B, C), confirming the addition of nitro-groups to tyrosine residues. In contrast, Tau-nY18 failed to cross-react with either nitrated bovine or porcine MAP preparations, labeling only human recombinant tau proteins selectively nitrated at Tyr 18 (Figure 2B, C). To confirm that Tau-nY18 does not cross-react with other proteins known to be nitrated in neurodegenerative disease (Giasson et al., 2000; Horiguchi et al., 2003), we exposed recombinant α -synuclein, and tubulin proteins (α and β) isolated from bovine brain preparations to either vehicle or peroxynitrite treatment. Using specific antibodies against these proteins (α -synuclein and β -tubulin antibodies, respectively), formation of SDS-stable oligomers were detected following peroxynitrite treatment, a result that was further confirmed with the generic 3-NT antibody (Figure 3A, B). On the contrary, Tau-nY18 failed to cross-react with either α -synuclein or tubulin proteins following nitration (Figure 3A, B).

Nitration of tau at Tyr 18 occurs in AD and age-matched controls

After establishing the specificity of the Tau-nY18 antibody to tau nitrated at Tyr 18 *in vitro*, frontal cortex samples from AD brains (Braak stages V and VI) were homogenized and probed with Tau-nY18 to determine whether nitration of this residue occurs in AD pathogenesis. Tau-nY18 recognized a protein that migrates at the same electrophoretic mobility as nitrated tau immunoblotted with the Tau-nY29 antibody (Figure 4A). In contrast, using the 3-NT antibody, a smear of nitrated proteins was detected within these samples (Figure 4A). These results further demonstrate the remarkable specificity of the Tau-nY18 antibody against tau nitrated at Tyr 18. To determine whether nitration at Tyr 18 is a disease-related event, soluble tau biochemically enriched from AD and age-matched control brains (Table 1) was resolved by SDS-PAGE and immunoblotted with Tau-nY18. Nitrated tau was detected in soluble preparations from severe AD and age-matched control brains (Figure 4B). Total tau within these samples was observed using Tau-5, a tau monoclonal antibody that recognizes all six tau isoforms before or after nitration (Figure 4C). In addition to soluble tau, insoluble PHF-tau preparations isolated from pathologically severe AD brains (Braak stage V and VI) were also labeled (Figure 4D). Collectively, these results suggest that nitration of tau at Try 18 is a

modification that possibly occurs prior to AD onset and persists throughout the progression of the disease.

Tau-nY18 labels the NFTs, neuritic plaques and nitrated tau within activated astrocytes

In the ITG, CA1 region, and entorhinal cortex of age-matched control tissue, Tau-nY18 labeled nitrated tau within activated astrocytes. These astrocytes were largely embedded in or in close proximity to amyloid plaques. Comparable morphological structures were readily observed in cases with severe AD pathology; although, in a few instances no obvious plaque association could be seen. The representative photomicrographs in Figure 5 illustrate Tau-nY18-positive astrocytes associated with (*) or in close proximity to (arrow) amyloid plaques in the ITG of control (Figure 5A) and AD cases (Figure 5B). Additionally, some labeling of the hallmark neuronal pathology of the disease, including a few NFTs, neuritic plaques and dystrophic neurites (Figure 5C, D), were also observed.

Previous reports have shown that pretreatment with citric acid can enhance staining by unmasking specific epitopes (Shin et al., 1991; Pellicer and Sundblad, 1994; Kanai et al., 1998). Indeed, pretreatment of tissue sections with citric acid enhanced the staining of the dystrophic neurites surrounding amyloid plaques, NFTs, and neuropil threads (Figure 5E). However, this method diminished but did not abolish the staining of astrocytes when compared to untreated tissue. To verify that Tau-nY18 reactivity within activated astrocytes *in situ* was not due to cross-reactivity with GFAP, ELISA experiments were performed using tau proteins singly nitrated at Try 18 (nY18), GFAP exposed to peroxynitrite (nGFAP) and untreated GFAP controls. Tau-nY18 failed to cross-react with either nGFAP or GFAP controls (Figure 6A). The presence of GFAP and nGFAP within these samples was confirmed by reaction with GFAP and 3-NT antibodies, respectively (Figure 6B, C).

The robust labeling of activated astrocytes by Tau-nY18 was further investigated by triple-label immunofluorescence. To examine the association between Tau-nY18-positive astrocytes, GFAP and amyloid plaques, tissue sections from the ITG from age-matched controls and cases with severe AD pathology were triple-labeled with Tau-nY18 and an anti-GFAP antibody, followed by TR counterstaining. In all control cases analyzed, Tau-nY18 co-localized extensively with GFAP in astrocytes associated with or in close proximity to amyloid plaques (Figure 7, A-D). Similarly, in cases with severe AD pathology, analogous morphological structures were readily observed (Figure 7, E-H). However, only in severe AD cases, a subset of Tau-nY18-positive dystrophic neurites which lack GFAP immunoreactivity were observed (Figure 7 E-H and I-L, arrows). Furthermore, Tau-nY18 also recognized some TR-positive neuropil threads, neuritic plaques, NFTs and, in some instances, astrocytes that were not associated with amyloid plaques (Figure 7, M-P). These results suggest that nitration at Try 18 can occur in both neuronal and astrocytic manifestations of AD pathology.

To determine whether nitrated tau within astrocytes could be labeled with other tau antibodies, sections from the ITG (Braak stage VI) were co-labeled with Tau-nY18 and a rabbit polyclonal antibody against total tau (R1). In all AD cases analyzed, Tau-nY18 and R1 co-localization was observed only in the neuropil threads, dystrophic neurites within the neuritic plaques and NFT. However, R1-positive astrocytes were not observed (Figure 8, A-C). To determine whether the Tau-nY18 epitope prevents the binding of the R1 antibody, adjacent sections were single labeled with R1 and GFAP antibodies followed by TR counterstaining. In the sections analyzed, no R1 positive astrocytes were observed (Data not shown). Additional sections were also co-labeled with Tau-nY18 and Alz-50, a monoclonal antibody known to recognize a conformation-dependent tau epitope that emerges during the early stages of AD pathology (Carmel et al., 1996; Guillozet-Bongaarts et al., 2005). In some instances, co-localization was observed within dystrophic neurites, neuropil threads and NFT (Figure 8, D-F). Occasionally, Tau-nY18-positive astrocytes also co-localized with the Alz-50 epitope (Figure 8, G-I).

although this was a somewhat rare observation. Still, these results suggest that some astrocytic tau may undergo conformational changes similar to neuronal tau in AD.

To test the hypothesis that nitration of Tyr 18 is a unifying feature in other pathological astrocytes, Tau-nY18 was used to probe sections from the frontal cortex and hippocampus in cases with cortical basal degeneration (CBD) and progressive supranuclear palsy (PSP). CBD and PSP are diseases with abundant glial and neuronal tau pathology, but little amyloid plaque deposition (Spillantini et al., 1997; Lee et al., 2001; Berry et al., 2004). Tau-nY18 failed to label any glial or neuronal lesions in either CBD or PSP (Figure 9A and D), while adjacent sections labeled with R1 or AD2 antibodies (Buee-Scherrer et al., 1996; Berry et al., 2004) revealed the pathological glial and neuronal tau lesions characteristic of these diseases (Figure 9B, C, E, F). These results suggest that tau in astrocytes associated with amyloid plaques is different from that in the pathological astrocytes of PSP and CBD.

DISCUSSION

Tau nitration at Tyr 18 occurs in conjunction with or as a result of astrocyte activation in AD

This is the first report identifying the presence of soluble and PHF-tau nitrated at Tyr 18 in AD brains. Our immunohistochemical observations in normal control brains revealed a limited number of Tau-nY18-positive astrocytes which, in most instances, were associated with or in close proximity to amyloid plaques. Analogous morphological structures were observed in cases with severe AD pathology, which may suggest a possible relationship between amyloid deposition and tau nitration. In our Western analysis of soluble tau, it was not clear whether the amounts of nitrated tau increased with the overall pathology of the disease. This discrepancy can be explained by the fact that we also observed nitrated tau in insoluble PHF-tau preparations from severe AD cases. Alternatively, nitrated tau in reactive astrocytes labeled by Tau-nY18 is predominantly associated with amyloid plaques and, as such, might not correlate with disease progression since plaque number does not correlate well with cognitive decline in AD (Braak and Braak, 1991; Arriagada et al., 1992).

In some cases with severe AD pathology (Braak stages V and VI), Tau-nY18 labeled a subset of GFAP-positive astrocytes in areas lacking visible plaques. These results are consistent with an increase in the inflammatory response in AD and suggest that other factors (e.g. pro-inflammatory cytokines or soluble amyloid aggregates) may contribute to tau nitration (Landolfi et al., 1998; Hu and Van Eldik, 1999; Akiyama et al., 2000). For example, following neuropathological insults, astrocyte activation induces the release of nitric oxide (NO) and inflammatory molecules, which can potentially lead to neuronal injury (Tani et al., 1996; Akama et al., 1998; Pawate et al., 2004). It is thought that nitric oxide can mediate cytotoxicity by interacting with oxygen radicals to form peroxynitrite, which presumably would nitrate tyrosines *in vivo* (Ischiropoulos et al., 1992; Beckman, 1996) in much the same manner as it does *in vitro*. Furthermore, a possible correlation between GFAP over-expression and tau nitration may exist, since NO is necessary and sufficient to induce the synthesis of GFAP, a pathological hallmark of inflammation (Eng and Ghirnikar, 1994; Nagele et al., 2004; Brahmachari et al., 2006). *In vitro*, nitration of Tyr 18 appears to prevent aggregation of tau into filaments, and it is tempting to suggest that nitration at this site may prevent tau filament formation in reactive astrocytes (Reynolds et al., 2005a, b). Alternatively, it is also possible that nitration at this residue may only be indicative of oxidative damage and inflammation, an event induced by glial activation (Nunomura et al., 2001). However, much work still remains to clarify the role of site-specific tau nitration in reactive glia and its association with AD.

Tau-nY18 localizes nitrated tau in both neuronal and astrocytic compartments

While tau is readily found within neurons, previous studies have reported tau expression within various glial cell types including oligodendrocytes and astrocytes (Papazosomenos and Binder, 1987; Shin et al., 1991; Couchie et al., 1992; LoPresti et al., 1995). These findings are consistent with the identification of abnormal tau inclusions within both glial and neuronal cell types in various neurodegenerative disorders, referred to collectively as non-AD tauopathies (Spillantini et al., 1997; Schofield et al., 2003; Berry et al., 2004; Reynolds et al., 2006). In AD, the presence of select tau-positive astrocytes, even in brain regions which lack neuronal pathology, has been established using the Tau-2 antibody and two different polyclonal antibodies (anti-PHF/tau and anti-human tau) (Papazosomenos, 1989a, b; Shin et al., 1991; Shin et al., 1992). Based on these observations, several attempts were made to co-label astrocytes using Tau-nY18 and different antibodies against tau with only limited success. These results may be explained by technical differences in tissue processing protocols used by different laboratories. For example, Papazosomenos et al. (1989a) and Shin et al. (1992), performed immunohistochemistry on tissue sections fixed in 10% formalin for 6-10 hours or 2-3 weeks, respectively, followed by paraffin embedding. Shin and colleagues further performed a hydrated autoclaving method to enhanced tau immunoreactivity. In contrast, immunohistochemistry in our laboratory was performed on free floating sections fixed in 4% paraformaldehyde for 30 hours at 4°C. These differences suggest that paraffin embedding or freezing the tissue can differentially affect tau immunoreactivity. Indeed, freezing samples can change the underlying immunogen structure adversely impacting antibody affinity (Munger, 1958). Furthermore, previous reports have demonstrated dramatic differences in tau immunoreactivity using different fixatives (Papazosomenos, 1989b).

The relative sensitivity of antibodies to a specific tau conformation may also play a role in antibody binding. For example, in our solid phase assays using recombinant soluble tau protein, Tau-nY18 was more sensitive than Tau-nY29. Similarly, in our western blot analysis, PHF-tau nitrated at tyrosine 18 was readily observed. In contrast, Tau-nY18 did not readily label PHF-tau in tissue sections as was obvious from the sparse staining of neuropil threads. Various epitope retrieval methods exist that augment tau immunoreactivity in tissue (Shin et al., 1991; Pellicer and Sundblad, 1994; Kanai et al., 1998). We found that these methods diminished Tau-nY18 labeling of activated astrocytes but increased neuronal labeling. These results further support the possibility that the Tau-nY18 epitope within neurons might be masked and/or inaccessible to this antibody.

In some instances, we found a few Tau-nY18-positive astrocytes that co-localized with the Alz-50 antibody, a marker of pathologically folded tau in neurons. Although the data presented here provide further evidence for tau alterations in astrocytes, it should be noted that the Tau-nY18-positive astrocytes represent only a subset of the reactive glia in aged and AD brain. Numerous other reactive astrocytes, not associated with A β -containing plaques are not stained with Tau-nY18 or other tau antibodies. Although different factors may contribute to tau nitration, our data suggest the possibility that tau nitration in AD may be associated with amyloid deposition.

The extensive characterization and high degree of specificity of Tau-nY18 provides strong evidence that this antibody reacts only with nitrated tau. First, in AD brain homogenates, Tau-nY18 reacted with the same molecular weight bands as Tau-nY29. Second, Tau-nY18 did not react with non-nitrated tau or tau nitrated at other tyrosine sites. Third, Tau-nY18 did not react with other nitrated proteins known to be modified in neurodegenerative diseases. Finally, it should be noted that neurons lacking tau pathology exist throughout the human brain in AD and age-matched controls alike. These neurons do not react with any of the known tau antibodies in tissue sections even though they contain abundant soluble tau. Hence, the majority

of evidence indicates that Tau-nY18 labeling of reactive astrocytes in AD and age-matched controls is due to its binding to nitrated tau.

Nitration at tyrosine residues occurs differentially in AD and other non-AD tauopathies

Previously, we reported the nitrative modifications at tyrosine 29 in AD and non-AD tauopathies using Tau-nY29 (Reynolds et al., 2006). Tau-nY29 labeled the characteristic tau lesions in AD, but did not label nitrated tau in astrocytes. In non-AD tauopathies, a small number of inclusions were stained. For instance, Tau-nY29 labeled the globose tangles in PSP and the dystrophic neurites and perinuclear inclusions in CBD, but it did not label the astrocytic pathology in either of these disease states. In AD, Tau-nY18 labeled numerous astrocytes but failed to label the glial or neuronal tau lesions in CBD and PSP. Antigen retrieval methods, used to rule out the possibility of a masked nY18 epitope in these diseases (Pellicer and Sundblad, 1994), did not reveal Tau-nY18 immunoreactivity (Data not shown). The dissimilarities in tau nitration between AD and other non-AD tauopathies suggest that disease-specific factors may mediate this process. For instance, expression of different tau isoforms, conformational differences in tau, and/or the degree of amyloid plaque burden are but a few of the disease-related events that could account for the observed differences in tau nitration. Future studies are required to elucidate the mechanisms that contribute to the site and cell-type specificity of tyrosine nitration in AD and non-AD tauopathies.

Selective tyrosine nitration: Implications for Alzheimer's disease

To date, various post-translational modifications of tau have been reported that can potentially induce or prevent tau filament formation (Grundke-Iqbal et al., 1986b; Ghoshal et al., 2002; Gamblin et al., 2003; Garcia-Sierra et al., 2003; Horowitz et al., 2004). Of these modifications, phosphorylation has been most commonly associated with tau aggregation. In fact, PHF-tau isolated from AD brains is extensively phosphorylated (Shapiro et al., 1991; Derkinderen et al., 2005). Fyn, a member of the *src* family of tyrosine kinases, has been shown to mediate site-specific tau phosphorylation at Tyr 18, a modification known to occur in AD (Lee et al., 2004). Therefore, it is possible that nitration at this residue is an event that specifically regulates Fyn phosphorylation, thereby preventing tau aggregation.

In our previous report, we showed that nitration at Tyr 29 occurs in the major pathological hallmarks of AD including the NFT and neuritic plaques (Reynolds et al., 2006). However, nitration at Tyr 29 within glia was not observed. In this study, we demonstrate that nitration at Tyr 18 occurs mainly within activated, GFAP-positive astrocytes in aged-matched control and AD brains. These reactive glia are associated with amyloid plaque formation and, as such, occur earlier than the NFT, neuropil threads, and plaque-invasive dystrophic neurites. Indeed, our western blot analysis suggests that nitration at Tyr 18 occurs prior to nitration at Tyr 29, since the Tyr 18 nitration is detected in soluble tau prior to the pathological onset of AD.

It remains unclear, however, why site-specific nitration occurs preferentially in different cell types. Theoretically, peroxynitrite is a molecule capable of free diffusion and, as such, would seemingly impact all cellular compartments equally. It is possible that differential tyrosine nitration is driven by the existence of specific isoforms and/or different conformational states of tau in different compartments, allowing nitration to occur predominantly at a specific site or sites. Another possibility is the existence of specific nitro-transferases, putative enzymes hypothesized to be capable of catalyzing the nitro-tyrosine modification of proteins (Kamisaki et al., 1998). Nitro-transferases are thought to promote the formation of neuronal processes via tau nitration during neuronal differentiation (Cappelletti et al., 2004). Similarly, nitration of tau within astrocytes could be required to induce the formation of astrocytic processes during activation.

Overall, our findings suggest that nitration at tyrosine residues are events which occur prior to, or during the early stages of AD pathogenesis (Giasson et al., 2000; Nunomura et al., 2001; Horiguchi et al., 2003; Reynolds et al., 2006). Therefore, it will be important to determine whether nitration at Tyr 18 is a modification that serves as a protective or toxic mechanism in cells. Although it prevents tau-filament formation *in vitro*, its role *in vivo* remains to be determined.

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Figure 1.

Nitration site specificity of the Tau-nY18 antibody. (A) The affinity of the Tau-nY18 antibody against full length human recombinant tau (ht40), full length nitrated tau (nht40), and tau proteins singly nitrated at Tyr 18, 29, 197, and 394, was determined by ELISA. Note that Tau-nY18 reacts specifically with tau proteins nitrated at Tyr 18. (B) The 3-NT antibody reacts with all nitrated tyrosine residues within the molecule. Each titer represents an average of five independent measurements.

Figure 2.

The Tau-nY18 antibody is species specific. (A) A sequence alignment diagram of the N-terminal region of tau protein from different species. Note that the alignment identifies Tyr 18 to be primate specific. Porcine and bovine MAP fractions (B and C, respectively) highly enriched for tau, MAP1A, MAP1B and MAP2 were exposed to either vehicle (-) or peroxynitrite (ONOO⁻) (+). MAP fractions (1 μ g) were resolved electrophoretically and blotted with 3-NT and Tau-nY18 antibodies. Fractions exposed to peroxynitrite are detected by the 3-NT antibody but fail to react with Tau-nY18. For each experiment, recombinant human tau proteins (50 ng) singly nitrated at Tyr 18 were included as a positive control (Con). Results are representative of five independent experiments.

Figure 3.

Tau-nY18 antibody does not cross-react with other nitrated proteins known to undergo nitration in human diseases. Recombinant α -synuclein (A) and β -tubulin (B) proteins were exposed to either vehicle (-) or peroxynitrite (ONOO⁻) (+), then resolved by SDS-PAGE and probed with the indicated antibodies. Antibodies against α -synuclein and β -tubulin identify the presence of monomers in vehicle treated samples. Insoluble oligomers were detected following ONOO⁻ treatment using 3-NT or antibodies against the native proteins. Within these experiments, Tau-nY18 did not label other nitrated proteins but did label tau nitrated at Tyr 18 (Con). In all cases, 50 ng of proteins were loaded per lane. Results are representative of five independent experiments.

Figure 4.

Nitration at Tyr 18 occurs in age-matched controls and in AD brains. (A) Gray matter from the frontal cortex of two AD cases (Braak stages V and VI) was homogenized and subjected to western blot analysis. Immunodetection was performed using the antibodies Tau-nY18, Tau-nY29 and 3-NT (1 μ g/ml) as indicated. To investigate whether nitration at Tyr 18 was an event associated with AD, soluble tau from pathologically severe AD brains (Braak stages V and VI) and normal age-matched controls (Braak stages I-III,) was biochemically enriched, resolved by SDS-PAGE, and resulting immunoblots probed with Tau-nY18 (1 μ g/ml) and Tau-5 (1 μ g/ml) antibodies. (B) Tau-nY18 labels soluble tau isolated from both AD brains and age-matched controls. (C) Total tau within these samples was detected with the Tau-5 antibody which recognizes all forms of tau. (D) PHF-tau from severe AD brains (Braak stages V and VI) was also detected with Tau-nY18 (1 μ g/ml) and Tau-nY29 (1 μ g/ml) antibodies as indicated. In all cases 10 μ g of total protein were loaded per lane.

Figure 5.

Tau-nY18 labels reactive astrocytes, NFT, and neuritic plaques in the ITG of aged-matched control and AD brains. Activated astrocytes found associated with (*) or in close proximity to (arrow) amyloid plaques are labeled with Tau-nY18 in all controls (A) and all pathologically severe AD cases analyzed (Braak stages V and VI) (B). Staining of the hallmark AD pathology including the NFT (C) and neuritic plaques (D) are also observed, but these structures are labeled to a lesser extent than reactive astrocytes. (E) Epitope retrieval (citric acid treatment method) of tissue sections unveils the epitope and increases the staining intensity of hallmark AD deposits, but reduces the number of activated astrocytes labeled. Calibration bars represent 50 μm.

Figure 6.

Tau-nY18 does not cross-react with GFAP or nitrated GFAP (nGFAP) molecules. ELISA experiments were performed to determine binding of Tau-nY18 (1 μ g/ml), anti-GFAP (1 μ g/ml) and 3-NT (1 μ g/ml) to GFAP and nGFAP proteins. (A) The Tau-nY18 antibody does not cross-react with either GFAP or nGFAP, but labels full length nitrated tau and tau singly nitrated at Tyr 18 (nY18). (B) An anti-GFAP antibody was used to demonstrate the presence of GFAP proteins. (C) Nitration of GFAP was confirmed using the nitration specific 3-NT antibody. In all experiments, 100 ng of protein was attached per well. Each titering curve represents the average of five independent experiments.

Figure 7.

Tau-nY18 partially co-localizes with GFAP. Triple-label immunofluorescence was performed using laser scanning confocal microscopy to determine the localization of nitrated tau using Tau-nY18 (green), GFAP (blue), and Thiazin Red (red) in the ITG of age-matched control (Braak stage III) and severe AD brains (Braak stage V and VI). (A-D) Activated, GFAP positive astrocytes surround the amyloid plaques and co-localize extensively with nitrated tau at Tyr 18 in age-matched controls. In AD brains, comparable morphological structures were readily observed (E-H). Tau-nY18 also labels a subset of dystrophic neurites within plaques, which co-localize with TR but not with GFAP (arrows) (E-H and I-L). Neurofibrillary tangles co-localize extensively with Thiazin red but not with GFAP (M-P). In all panels calibration bars represent 50 μ m.

Figure 8.

Tau-nY18 co-localizes with the Alz-50 epitope in astrocytes. Double-label immunofluorescence was performed using laser scanning confocal microscopy to determine if Tau-nY18-positive astrocytes also label with other tau antibodies. (A-C) Sections from ITG (Braak stage VI) were labeled with Tau-nY18 (green) and R1 (red). Co-localization was observed within dystrophic neurites invading amyloid plaques, neuropil threads and NFT. However, R1-positive tau within astrocytes was not observed. (D-F) Adjacent sections labeled with Tau-nY18 and the conformation-specific Alz-50 antibody show partial co-localization within the dystrophic neurites in amyloid plaques and NFT (G-I). Occasional astrocytes co-labeled with Tau-nY18 and Alz-50 were also observed. In panels A-C calibration bars represent 50 μ m. In panels D-F and G-I, calibration bars represent 20 μ m.

Figure 9.

Tau-nY18 fails to label the pathological lesions in two non-AD tauopathies. In areas from the frontal cortex, immunohistochemical analysis was performed in cases with cortical basal degeneration (CBD) and progressive supranuclear palsy (PSP). Sections were labeled with Tau-nY18 and counterstained with hematoxylin. Tau-nY18 did not label the glial or neuronal tau lesions in either CBD (A) or PSP (D). In contrast, R1 and AD2 antibodies label the characteristic inclusions including the dystrophic neurites (B), perinuclear inclusions (C, asterisk), and astrocytic plaques in CBD (C). Similarly, in PSP, the globose tangles (E, arrow), the tufted astrocytes (F) and coiled bodies (asterisk) were observed. In all panels, Calibration bars represent 50 μ m

Table 1

Cases used for enrichment of soluble and PHF-tau.

Case #	Gender	Age	Braak stage
BB98-003	M	75	-
BB00-002	M	69	-
15196262	M	81	I
15115927	M	77	I
11072071	M	88	II
20610726	F	87	III
20800682	F	84	III
09740777	M	77	V
62749390	F	84	V
73257978	F	87	V
98939847	M	81	VI

Seven non-demented, aged-matched controls (Braak stages I-III), and four severe AD cases (Braak stages V and VI) were used to determine the presence of tau nitrated at Tyr 18 within soluble and insoluble (PHF) tau preparations.