Nitration and oligomerization of tau induced by peroxynitrite inhibit its microtubule-binding activity

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Abstract Abnormally nitrated tau has been found recently in the neurofibrillary tangles of Alzheimer’s disease (AD). However, neither the biochemical nature of nitrated tau nor the basis of nitrated tau aggregation is known. Herein, we found that in vitro incubation of peroxynitrite with recombinant tau resulted in nitration and oligomerization of tau in a dose-dependent manner. Moreover, the nitrated tau showed a significantly decreased binding activity to taxol-stabilized microtubules in vitro. Further study demonstrated that peroxynitrite also induced tau nitration in neuroblastoma N2a cell line, and the nitrated tau was accumulated in the cells. We conclude that abnormal nitration of tau contributes to the impaired biological activity of tau in binding to the microtubules and the aggregation of tau, implying a novel mechanism responsible for the neurodegeneration seen in AD brain.

Keywords: Tau; Peroxynitrite; Nitration; Oligomerization; Microtubule binding; Alzheimer’s disease

1. Introduction

Neurofibrillary tangle (NFT) and β-amyloid (Aβ) plaques are the two defining features of Alzheimer’s disease (AD). NFT are composed of bundles of paired helical filament (PHF), whose major protein subunit is abnormally hyperphosphorylated microtubule-associated protein tau [1–3]. By binding to microtubule, tau functions to promote tubulin polymerization and maintains microtubule stability [4,5]. When tau is abnormally hyperphosphorylated both in vitro and in vivo, its binding affinity for microtubule is decreased, thus leading to the disruption of the neuronal cytoskeleton [6–8]. In addition, several tau gene mutations have been identified in frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17), providing further genetic evidence for tau dysfunction in neurodegeneration [9–11]. All these studies prove that tau abnormalities impair its functions and thus play an important role in pathogenesis of neurodegenerative disease.

Studies demonstrated that there existed widely nitrative lesions in AD brain. The levels of 3-nitrotyrosine and dityrosine, two biomarkers of nitration injury, were elevated consistently in the hippocampus, neocortical regions and cerebrospinal fluid (CSF) of AD patients [12]. Moreover, both the 3-nitrotyrosine concentration and the 3-nitrotyrosine/tyrosine ratio in the CSF increased significantly and this elevation correlates positively with decreased cognitive functions [13]. While the increased protein nitration was found in neurons, including but certainly not restricted to those containing NFT [14]. Proteomic identification of nitrated proteins in AD brain has detected six target proteins including enolase and triosephosphate isomerase, both of which are important enzymes in glucose metabolism and nitration may decrease their activities that may account for the metabolic impairment in AD [15]. Recently, it was reported that tau was nitrated and co-localized with NFT in AD brain [16]. However, how nitrated tau may contribute to AD pathology is not understood.

Peroxynitrite, the product of the rapid reaction between superoxide anions (O2·−) and nitric oxide (NO), is a potent nitration mediator and strong oxidant implicated in AD [12,14,17]. At physiological pH, peroxynitrite becomes peroxynitrous acid, a species that rapidly undergoes hemolytic cleavage to yield OH· and NO2· that firstly oxidize tyrosine to form tyrosyl radical (Tyr). Then, NO2· nitrates Tyr to form 3-nitrotyrosine and Tyr’ also interacts with each other to generate dityrosine [18,19]. It has been manifested that peroxynitrite may nitrate various proteins both in vitro and in vivo [20–27].

In the present study, we demonstrated that peroxynitrite treatment causes tau nitration and oligomerization; such abnormal modifications inhibit microtubule-binding activity of tau. We propose that tau nitration may contribute to AD pathogenesis through impairing normal functions of tau.

2. Materials and methods

2.1. Materials

Dithiothreitol (DTT), iodoacetamide, GTP, paclitaxel and Hoechst 33258 were purchased from Sigma (St. Louis, MO, USA). P11 cellulose phosphate was from Whatman Inc. (Clifton, NJ, USA), sephacryl S-300 was from Pharmacia Biotech (Uppula, Sweden). Peroxynitrite and 3-nitrotyrosine monoclonal antibody were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Anti-tau monoclonal antibody tau5 was purchased from Lab Vision Corporation (Fermont, CA, USA). Anti-α-tubulin mAb DM1A and rabbit polyclonal antibody R134d against total tau were gifts from Dr. K Iqbal and I Grundke-Iqbal (New York State Institute for Basic Research, Staten Island, NY, USA). Anti-nitrated tau monoclonal antibody n847 was a gift from Dr. John Q. Trojanowski (Center for Neurodegenerative Disease Research, Department of Pathology and Laboratory Medicine, Hospital of The University of Pennsylvania, Philadelphia, PA, USA). Rhodamine Red-X-conjugated goat

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anti-rabbit IgG (H + L) and Oregon Green 488-conjugated goat anti-mouse IgG (H + L) were from Molecular Probes (Eugene, OR, USA). Goat anti-rabbit and anti-mouse peroxidase-conjugated secondary antibody, chemiluminescent substrate kit were from Pierce Chemical Company (Rockford, IL, USA).

2.2. Purification of tau

Human 3R tau ( htt39) that has three tandem C-terminal repeats plus two 58-amino acid N-terminal insertions was expressed in Escherichia coli strain BL21 and tau was isolated from the bacterial lysate as described [28,29]. Briefly, the bacterial pellet was lysed in homogenate buffer (50 mM Mes, 5 mM DTT, and 5 mM PMSF, pH 6.8), then centrifuged at 4 °C, 38,000 × g for 30 min to collect supernatant. After adding equal volume solution containing 1 mM MgCl2 and 0.2 mM EDTA, the supernatant was loaded onto a phosphocellulose column. The column was washed thoroughly and proteins were eluted with gradient 0.4-1 M NaCl in PC buffer (25 mM Mes, 1 mM DTT, 0.5 mM MgCl2, and 0.1 mM EDTA, pH 6.8). The positive fractions identified by dot blot were bathed in boiled water for 10 min. After centrifuging at 4 °C, 48,000 × g for 30 min, the supernatant containing tau was concentrated by ultrafiltration and loaded onto Sephacryl S-300 Gel filtration column prewashed with PC buffer. The collected fractions were analyzed by Western blot and then purified tau fractions were desalted and concentrated by ultrafiltration for further study.

2.3. Treatment of tau with peroxynitrite

The reaction of peroxynitrite with tau was carried out as described previously [20] with minor modifications. Briefly, tau (1 mg/ml) dissolved in reaction buffer (100 mM potassium phosphate, 25 mM sodium bicarbonate, and 0.1 mM diethylenetriamine pentaacetic acid, pH 6.4) was reacted with or without peroxynitrite at 37 °C for 10 min. The concentration of peroxynitrite stock solution was determined by measuring the absorbance at 302 nm (ε102 = 1670 M−1 cm−1) and the final concentration peroxynitrite used in the reaction was 0.025, 0.125, 0.25, 1.25, and 2.5 mM, respectively. At the end of incubation, the complete decomposition of peroxynitrite was determined by the above method. DTT and iodoacetamide are agents prevented formation of disulfide bond. After addition of 10 mM DTT in reaction buffer, tau was treated with peroxynitrite as indicated concentrations. Iodoacetamide (15 mM) firstly alkylated sulfhydryls of cysteine residue of tau in reaction buffer at 37 °C for 30 min, and then tau was reacted with different concentrations of peroxynitrite as described above. The reaction was terminated by addition of one-third volume of sample buffer containing 200 mM Tris–HCl, pH 7.6, 8% SDS, 40% glycerol, 40 mM DTT, 0.025% bromophenol blue and boiled for 10 min. The equal amounts of protein were used for Western blot analysis.

2.4. Purification of porcine tubulin

Tubulin was isolated from porcine brain by two temperature-dependent polymerization–depolymerization cycles as described [30], followed by the removal of the microtubule-associated proteins (MAPs) through phosphocellulose chromatography in PM buffer (100 mM Pipes, pH 6.9, 2 mM EGTA, 1 mM MgSO4, and 2 mM DTT). Aliquots were dropped in liquid nitrogen and stored at −80 °C. Protein concentrations were determined by Bradford method [31].

2.5. Microtubule-binding assay

Taxol-stabilized microtubules were prepared as described [32]. Briefly, MAPs-free tubulin (2 mg/ml) was incubated with 1 mM MgSO4, 1 mM GTP and 5% DMSO at 37 °C for 45 min. Then taxol was added to the mixture at a final concentration of 60 μM for further 10 min incubation at 37 °C. The microtubules in pellets were collected by centrifugation at 22 °C, 96000 × g for 20 min. The binding ability of tau to microtubules was determined as described [6]. Briefly, tau (50 μg/ml) or peroxynitrite-treated tau (50 μg/ml) was incubated with taxol-stabilized microtubules (0.5 μg/ml) at 37 °C for 30 min in a buffer containing 50 mM Pipes (pH 6.9), 1 mM EGTA, 1 mM MgSO4 and 1 mM GTP. Bound tau in pellets and unbound tau in supernatants was separated by centrifugation at 22 °C, 50000 × g for 20 min. Both supernatant and pellet were recovered for Western blot analysis with anti-tau monoclonal antibody tau5.

2.6. Western blot

The equal amounts of protein were separated by a 10% SDS-polyacrylamide gel, and then transferred on to nitrocellulose membranes. Membranes were blocked with 5% defatted milk dissolved in TBS–TWEEN (TBS–T) (50 mM Tris, pH 7.6, 150 mM NaCl, and 0.2% Tween 20) for 1 h at 37 °C. The membrane was then incubated with anti-tau monoclonal antibody tau5 [1(2000)] or anti-α-tubulin mAb DMIA (1:500) or mAb n847 (1:500) overnight at 4 °C. The membrane was washed three times with TBS-T and then incubated with anti-mouse IgG conjugated to horseradish peroxidase (1:3000) for 1 h at 37 °C. The blot was washed three times with TBS-T and then visualized using enhanced chemiluminescence method. The protein bands were quantitatively analyzed by Kodak Digital Science 1D software (Eastman Kodak Company, New Haven, CT, USA), and the amount of protein was expressed as sum optical density.

2.7. Cell culture, protein extraction and immunofluorescence staining

N2a/Peuht39 cells, a Neuroblastoma N2awt cell line (a kind gift from Dr. HX Xu, The Burnham Institute, San Diego, USA) stably transfected with the human tau cDNA (Tau39), were grown in 6-well or 24-well plates in DMEM: OPTI-MEM (1:1) in the presence of 200 μg/ml G418 with 5% FBS (vol/vol) in a humidified atmosphere of 5% CO2 at 37 °C. Cells in 70% of confluence were treated with 0.02, 0.05, 0.1, 0.25 or 0.5 mM of peroxynitrite. As obvious nitration of tau was only observed by 0.5 mM of peroxynitrite, we used this concentration for the cell study. After 24 h of peroxynitrite treatment, the cells were lysed for protein extraction with buffer containing 50 mM Tris–Cl, pH 8.0, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.02% sodium azide, 100 μg/ml PMSF, and 10 μg/ml protease inhibitors (leupeptin, aprotinin, and pepstatin) followed by probe sonicate for 5 s on ice. The cell lysates were then centrifuged at 12000 × g for 10 min at 4 °C; aliquots of supernatants were added to one-third volume of sample buffer, boiled for 10 min, and then used for Western blot analysis. For immunofluorescence staining, cells were fixed with methanol at −20 °C for 5 min and permeabalized with PBS-0.5% Triton X-100 for 5 min. After blocking with 5% BSA for 1 h at 37 °C, the cultures were incubated overnight at 4 °C with mAb n847 (1:500) which specially recognized nitrated tau [16] or with rabbit polyclonal anti-tau R134d (1:1500). After washing, cells were incubated with the secondary antibody (Rhodamine Red-X-conjugated goat anti-rabbit IgG (1:2000) for R134d, Oregon Green 488-conjugated goat anti-mouse IgG (1:2000) for n847) at 37 °C for 1 h. Finally, Hoechst 33258 (1 μg/ml) was used to stain the nuclei. Fluorescence was observed using a fluorescence microscope (BX60; Olympus, Tokyo, Japan) with appropriate filter sets.

2.8. Statistical analysis

Data were expressed as means ± S.E. and analyzed using SPSS 11.5 statistical software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Peroxynitrite induces tau nitration and oligomerization

Purified human recombinant tau was treated with different concentrations of peroxynitrite in the presence of CO2 in vitro and the products were analyzed by Western blot with 3-nitrotyrosine mAb (Fig. 1A and D) or mAb tau-5 to tau (Fig. 1B and C). We observed that exposure of tau to peroxynitrite/CO2 led to tau nitration and oligomerization in a dosage-dependent manner (Fig. 1A and B). Although nitrated tau was mainly monomers, distinct nitrated tau dimers, trimers and oligomers were also detected (Fig. 1A), and the decrease of tau monomer was consistent to the increase of tau oligomers upon peroxynitrite treatment (Fig. 1B). We also observed that tau nitration and oligomerization were completely abolished by addition of 10 mM DTT to the reaction buffer (Fig. 1A and B), however, peroxynitrite-induced nitration and oligomerization of tau was still seen when iodoacet-
Amide was employed simultaneously to block the formation of disulfide bond (Fig. 1C and D). These data demonstrate that peroxynitrite induces efficiently tau nitration and oligomerization in vitro. And it is also suggested that formation of disulfide bond may only play minor role in tau oligomerization.

3.2. Peroxynitrite inhibits the binding of tau to microtubules

To investigate the effect of tau nitration and oligomerization on its biological activity, we measured the in vitro microtubule-binding activity of tau by using taxol-stabilized microtubules. We found that microtubule-bound tau monomer in the pellet (Fig. 2A) was decreased whereas unbound tau monomer in the supernatant (Fig. 2B) was increased dose-dependently after peroxynitrite treatment. Although tau oligomers were seen in both microtubule-bound and unbound fractions, the amount of microtubule-bound tau oligomers was much less than that of unbound tau oligomers (Fig. 2A and B), and tau oligomers by themselves did not precipitate at the condition used (data not shown). Quantitative data showed that the ratio of microtubule-bound to unbound tau was decreased 36%, 58% and 64% of the normal control level after treatment of peroxynitrite at concentrations of 0.125, 0.625 and 1.25 mM, respectively (Fig. 2C). These data suggest that nitration and oligomerization of tau induced by peroxynitrite inhibits its microtubule-binding activity.

3.3. Peroxynitrite induces tau nitration and aggregation in N2a/Peuht39 cells

To study whether peroxynitrite could also nitrate tau in the cell, we treated N2a/Peuht39 cell line with 0.5 mM peroxynitrite for 24 h and nitration of tau in the cell was measured by mAb n847, an antibody specifically reacts with nitrated tau. Immunoblotting results confirmed the presence of nitrated tau monomer and oligomers in lysates of N2a/Peuht39 cells treated with...
microtubule assembly and in stabilizing the already formed glycosylation of tau inhibits its biological activity in promoting microtubule assembly and in stabilizing the already formed microtubule, whereas glycosylation of tau stabilizes the structure of neurofibrillary tangles [34]. In addition to these post-translational modifications, it is found recently that tau isolated from paired helical filament in AD brains is nitrated and the extent of nitration is well correlated with the evolution of the disease [16]. However, the nature of tau nitration in AD pathology is not known.

In the present study, we have demonstrated that peroxynitrite induces both nitration and oligomerization of tau in vitro. The extent of nitration and formation of oligomers were concentration dependent. Since the oligomers were resistant to denaturation by SDS and boiling (conditions used to handle the samples for electrophoresis), the covalent bonds must be involved. To this point, it was reported previously that peroxynitrite resulted in formation of dityrosine bond in α-synuclein [22]. Additionally, peroxynitrite could also oxidize –SH group in cysteine residue of tau to form disulfide bond [35]. As human tau39 (htau39) we used in the present study contains only one cystine (i.e., Cys-291), the disulfide bond formed from this cystine should only lead to formation of tau dimmers rather than oligomers. To verify the role of dityrosine bond in stabilizing tau oligomers, we treated tau with iodoacetamide, an alkylating agent that blocks formation of disulfides, prior to addition of peroxynitrite. We found that tau was still oligomerized at this condition (Fig. 1C), suggesting the major role of dityrosine bond in stabilizing nitrated tau in the present study. Supporting results was reported in α-synuclein, a neuronal protein shown similar biological and biophysical properties to tau both in normal and pathologic states [36].

We also found that addition of disulfide reducing agent DTT into the reaction system not only completely abolished the oligomerization of tau, but also completely inhibited tau nitration. DTT is a strong reducing agent. One of the possible reasons for the prevention of nitration of tau by DTT may be due to the inactivation of the peroxynitrite by reduction.

Studies have demonstrated that tyrosine nitration of proteins alters theirs biophysical properties and biological activities [22,24,37–39]. Thus, we tested whether the microtubule-binding activity of htau39, i.e., Y18, Y29, Y197, Y279 and Y363. Among them Y279 is located in microtubule-binding domain. Thus, it is reasonable to speculate that nitration of Y279 may play major role in the inhibition of microtubule-binding activity. However, site-specific mutation of the above five tyrosine is needed to confirm the speculation.

We further investigated the role of tau nitration in N2a/Peuht39 cell line. Abnormal posttranslational modifications including hyperphosphorylation, glycosylation, glycation, ubiquitination and truncation of microtubule-associated protein tau have been previously reported to play different roles in the development of AD pathology [33], such as hyperphosphorylation but not glycosylation of tau inhibits its biological activity in promoting microtubule assembly and in stabilizing the already formed microtubule.
oxynitrite-treated cells, tau was accumulated in cell cytoplasm, and the cells appeared round and the neurite were retracted. These results imply that nitration of tau may disrupt cytoskeleton of the cells. The relationship of tau nitration with other posttranslational modifications, such as hyperphosphorylation, needs further investigation.

Fig. 3. Peroxynitrite induces tau nitration and aggregation in N2a/Peuht39 cells. N2a/Peuht39 cells were treated with 0.5 mM peroxynitrite and analyzed after 24 h. (A) Cell lysates were subjected to Western blot analysis with mAb n847 which specially recognized nitrated tau. (B) Control (a, b, c, f) or treated cells (c, d, g, h) were studied by immunofluorescence staining with mAb n847 (b, d) or rabbit polyclonal antibody R134d to total tau (f, h) and the nuclei of the cells were counterstained by Hoechst 33258 (a, c, e, g). Arrowheads indicated distribution of nitrated tau (d) or total tau (f, h). Scale bar = 10 μm.
Taken together, we have found in the present study that tau can be nitrated and oligomerized by peroxynitrite. Nitration of tau impairs its microtubule-binding activity and accumulates in neuroblastoma N2a cells.

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References


