Biochimica et Biophysica Acta 1863 (2017) 414–427

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Pathological concentration of zinc dramatically accelerates abnormal aggregation of full-length human Tau and thereby significantly increases Tau toxicity in neuronal cells

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ARTICLE INFO

Article history:
Received 19 July 2016
Received in revised form 1 November 2016
Accepted 22 November 2016
Available online 23 November 2016

Keywords:
Tau protein
Alzheimer disease
Zinc
Protein aggregation
Tau toxicity

ABSTRACT

A pathological hallmark of Alzheimer disease and other tauopathies is the formation of neurofibrillary tangles mainly composed of bundles of fibrils formed by microtubule-associated protein Tau. Here we study the effects of Zn^{2+} on abnormal aggregation and cytotoxicity of a pathological mutant ΔK280 of full-length human Tau. As revealed by Congo red binding assays, transmission electron microscopy, immunofluorescence, Western blot, and immunogold electron microscopy, pathological concentration of Zn^{2+} dramatically accelerates the fibrillization of ΔK280 both *in vitro* and in SH-SY5Y neuroblastoma cells. As evidenced by annexin V-FITC apoptosis detection assay and MTT reduction assay, pathological concentration of Zn^{2+} remarkably enhances ΔK280 fibrillization-induced apoptosis and toxicity in SH-SY5Y cells. Substitution of Cys-291 and Cys-322 with Ala, however, essentially eliminates such enhancing effects of Zn^{2+} on the fibrillization and the consequent cytotoxicity of ΔK280. Furthermore, Zn^{2+} is co-localized with and highly enriched in amyloid fibrils formed by ΔK280 in SH-SY5Y cells. The results from isothermal titration calorimetry show that Zn^{2+} binds to full-length human Tau by interacting with Cys-291 and Cys-322, forming a 1:1 Zn^{2+}-Tau complex. Our data demonstrate that zinc dramatically accelerates abnormal aggregation of human Tau and significantly increases Tau toxicity in neuronal cells mainly via bridging Cys-291 and Cys-322. Our findings could explain how pathological zinc regulates Tau aggregation and toxicity associated with Alzheimer disease.

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1. Introduction

Amyloid fibrils which result from the misfolding of more than 20 amyloidogenic proteins in cells can lead to serious neurodegenerative diseases, such as human Tau protein and human amyloid β peptide in Alzheimer disease (AD), human copper, zinc superoxide dismutase and human TAR DNA-binding protein of 43 kDa (TDP-43) in amyotrophic lateral sclerosis, and human prion protein in prion diseases [1–4].

A pathological hallmark of AD, the most common neurodegenerative disease in the elderly (age ≥65 years), and other tauopathies, is the formation of neurofibrillary tangles, which are mainly composed of bundles of fibrils formed by microtubule-associated full-length human Tau [5–9]. Although AD has posed a serious threat to human health, there are no satisfying treatments for it so far [10]. Amyloid fibril formation of Tau protein has been widely investigated during the last two decades, in order to understand the pathology of AD and other tauopathies associated with Tau fibrillization [11–20]. The Lee lab has investigated Tau fibrillization through a battery of experiments [14,20], and has demonstrated that introduction of minute quantities of Tau fibrils into Tau-expressing cells rapidly recruit large amounts of soluble Tau into filamentous inclusions resembling neurofibrillary tangles [14]. By using immunocytochemistry, imaging, and Western blot, the Lee lab has successfully demonstrated that Tau fibrils induced in cells with regulated expression of full-length human Tau can be gradually cleared when soluble Tau expression is suppressed, but the presence of even a minute amount of residual Tau fibrils is sufficient to rapidly restore Tau fibrillization once Tau expression is turned on again [20]. The Bose lab has indicated that a range of filamentous Tau aggregates are the major species that underlie the spreading of Tau pathology in P301S transgenic mice [19]. Very recently, it has been reported that compounds containing 1,2-dihydroxybenzene inhibit Tau oligomerization by modifying two cysteine residues in Tau, Cys-291 and Cys-322, thereby reducing Sarkosyl-insoluble Tau levels, neuronal death, and brain

Abbreviations: AD, Alzheimer disease; TEM, transmission electron microscopy; ThS, thioflavin S; PI, propidium iodide; DAPI, 4′,6′-diamino-2-phenylindole dihydrochloride; IAPP, islet amyloid polypeptide; TDP-43, TAR DNA-binding protein of 43 kDa; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ITC, isothermal titration calorimetry; GSK-3β, glycogen synthase kinase-3β.

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http://dx.doi.org/10.1016/j.bbadis.2016.11.022
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dysfunction in P301L transgenic mice [18]. Although many efforts have been made [8,10–20], the mechanism for amyloid fibril formation of Tau protein is not well understood so far. Thus the characterization of factors regulating Tau fibrillization is very important to elucidate the pathology of AD and related tauopathies and to help the establishment of medical treatment [11–17].

Zinc, present in and essential to all forms of life, is one of the most abundant transition metals in the brain [21–26]. Although the intracellular concentration of free zinc is normally very low and tightly regulated because of its multiple functions in cellular process of neurons [27], there is a dramatic increase in the level of cytosolic zinc when it is released from synaptic vesicles or metallothionein under pathological oxidative conditions [28,29]. Physiological concentration of free Zn^{2+} in cells is between 1 nM and 10 nM [29], and pathological concentration of Zn^{2+} in cells is in the 10–300 μM range [21,22,24,27]. Therefore, we want to know whether and how pathological concentration of Zn^{2+} affects the fibrillization and cytotoxicity of a pathological mutant ΔK280 of full-length human Tau, associating with a tauopathy called frontotemporal dementia and parkinsonism linked to chromosome 17 [5]. Such a deletion mutation ΔK280 has been reported to strongly promote Tau aggregation by enhancing the propensity of Tau to form β-structures, thereby leading to synaptic loss and cognitive defects in inducible mice expressing ΔK280 [30,31]. It has been reported that Zn^{2+}-Tau interactions not only dramatically accelerate the fibrillization of human Tau fragment $Tau_{244\text{-}372}$ *in vitro* [12] but also fundamentally determine Tau toxicity independent of Tau hyperphosphorylation in a *Drosophila* tauopathy model expressing a human Tau mutant R406W [32]. Clearly, Tau protein is involved in neuronal zinc homeostasis [24,26,33]. In addition, Zn^{2+} has been found to stimulate Tau hyperphosphorylation not only by activating glycogen synthase kinase-3β (GSK-3β) or Raf/mitogen-activated protein kinase-kinase/extracellular signal-regulated kinase pathway [34–36], but also by inhibiting the activity of protein phosphatase 2A [29,37]. The ability of Zn^{2+} to interact with Tau and influence Tau hyperphosphorylation and aggregation directly has widespread implications for understanding the pathogenesis of tauopathies [12,24–26,32,33]. Zn^{2+} also plays a significant role in the fibrillization of other amyloidogenic proteins/peptides such as islet amyloid polypeptide (IAPP) [38], amyloid β [39,40], prion protein [41,42], and TAR DNA-binding protein 43 kDa (TDP-43) [43]. However, how Zn^{2+} regulates Tau aggregation and toxicity in neuronal cells remains elusive.

In this paper, by using Congo red binding assays, confocal laser scanning microscopy, transmission electron microscopy (TEM), Western blot, immunogold electron microscopy, and annexin V-FITC apoptosis detection assay, we investigated the effects of Zn^{2+} on abnormal aggregation and cytotoxicity of the pathogenic mutant ΔK280 of full-length human Tau. Our results indicate that pathological concentration of Zn^{2+} dramatically accelerated the fibrillization of ΔK280 in SH-SY5Y neuroblastoma cells, thereby remarkably enhancing apoptosis and Tau toxicity in SH-SY5Y cells. Substitution of the two cysteine residues in Tau, however, essentially eliminated such enhancing effects of Zn^{2+} on the fibrillization and the consequent cytotoxicity of ΔK280. Our findings link Tau aggregation and toxicity regulated by zinc to the pathogenesis of AD and other tauopathies.

2. Materials and methods

2.1. Materials

Congo red (fresh molecular weight of 696.67), thioflavin S (ThS), thioflavin T (ThT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were from Sigma-Aldrich (St. Louis, MO). Dithiothreitol (DTT), Triton X-100 and Sarkosyl were obtained from Amresco (Solon, OH). DNA polymerase Kod-plus-Neo was from Toyobo (Tokyo, Japan). SP Sepharose Fast Flow was Amersham Biosciences products (Uppsala, Sweden). Mouse/rabbit anti-FLAG monoclonal antibody, 10-nm gold-labeled anti-mouse antibody, rabbit anti-β-actin antibody and mouse anti-β-tubulin antibody were also purchased from Sigma-Aldrich (St. Louis, MO). Alexa 546/488-conjugated secondary antibody was from Invitrogen (Carlsbad, CA). Other chemicals used were made in China and of analytical grade.

2.2. Plasmids and proteins

The construction of prokaryotic plasmids expressing wild-type full-length human Tau and its pathogenic mutant ΔK280 and the cysteine mutants (single cysteine mutants C291A and C322A and double cysteine mutant C291A/C322A) of ΔK280 as well as ΔK280 of Tau fragment $Tau_{244\text{-}372}$, and Tau protein purification were carried as described [11–13,16,17]. Purified Tau protein was analyzed by SDS-PAGE with one band. The concentration of human Tau was determined according to its absorbance at 214 nm with a standard calibration curve drawn by bovine serum albumin.

2.3. Cell culture and stable cell line construction

SH-SY5Y neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin in 5% CO_2 at 37 °C.

SH-SY5Y cell line stably expressing FLAG-tagged wild-type full-length human Tau or its ΔK280 variant or the cysteine mutants of ΔK280 (stable ΔK280-FLAG cells) was constructed with lentiviral vector construction system. All of the target DNA fragments were cloned into a lentiviral vector pHAGE-puro by Mlu I and BamH I restriction sites. Lentiviral vector construction system constructed with CMV promoter was packaged in HEK293T cells by various combinations of plasmids and liposome, the three plasmids pHAGE-ΔK280 (or other three target plasmids), pVSVG, and pLP were mixed at a ratio of 2:1:1 and the ratio of liposome to DNA was 2:1. After 36 h of transfection, the virus were harvested and filtered, then SH-SY5Y cells were infected by the packaged lentivirus with a high infection efficiency, and the expression of each protein was detected by Western blot.

2.4. Congo red binding assays

A stock solution of 1 mM Congo red was freshly prepared in 10 mM HEPS buffer (pH 7.4) containing 100 mM NaCl and filtered through a 0.22 μm pore size filter before use. The DPPC lipid vesicles were prepared in 10 mM HEPES buffer (pH 7.4) containing 100 mM NaCl and 1 mM DTT as described [44]. The polymerization induced by Congo red for ΔK280 of full-length human Tau and its cysteine mutants in 96-well plates was set up by a mixture of 10 μM Tau protein incubated with 50 μM Congo red and 0–100 μM Zn^{2+}/Al^{3+}/Ca^{2+} or the mixture of Zn^{2+} with Al^{3+} or Ca^{2+} or DPPC in 10 mM HEPES buffer containing 1 mM DTT and 100 mM NaCl (pH 7.4). To not only mimic the reducing environment present in normal neuronal cells but also block the formation of an intramolecular disulfide bond in Tau protein, 1 mM DTT was added into the buffer. The reaction components were mixed quickly and immediately read for 10 h at 37 °C in SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA) using absorbance at 550 nm. All kinetic experiments were repeated three times.

Kinetic parameters were determined by fitting the absorbance at 550 nm *versus* time to the empirical Hill equation [11],

$$A = A(\infty)\frac{(t/t_{50})^n}{1+(t/t_{50})^n} \quad (1)$$

where A is the absorbance at 550 nm, $A(\infty)$ is the absorbance at 550 nm in the long time limit, t_{50} is the elapsed time at which A is equal to one-half of $A(\infty)$, and n is a cooperativity parameter.

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