Zinc induces tau hyperphosphorylation through extracellular signal-regulated kinase 1/2 (ERK1/2) pathway

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Zinc induces tau hyperphosphorylation through extracellular signal-regulated kinase 1/2 (ERK1/2) pathway

Directed by Professor Young Soo Ahn

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The pathologic hallmarks of Alzheimer's disease (AD) include senile plaque and neurofibrillary tangles (NFTs) which are mainly composed of Aβ and microtubule associated protein, tau, respectively. The tau from NFT is hyperphosphorylated compared to the soluble form tau. Therefore, the factors regulating tau phosphorylation may be crucial to the pathogenesis in AD. Zinc has been implicated as a possible pathogenic agent due to its effect of increasing Aβ precipitation. The purpose of this study was to investigate the effects of zinc on the tau phosphorylation. We constructed the tetracycline-off regulatory system controlling the expression of wild-type $tau_{1\text{-}441}$ in SH-SY5Y cells. In the absence of tetracycline, the level of tau increased and reached its maximum in 5 days in SH-SY5Y cells expressing wild-type $tau_{1\text{-}441}$. To assay the phosphorylation of tau, three kinds of phospho-specific antibodies were used in Western blot including pSer202, pSer214 and pSer396 antibodies which recognize phosphorylation sites that are known to be related to NFT formation. We measured the changes in the level of tau phosphorylation as a ratio of the level of phospho-tau of each phosphorylation site to that of total

tau. Zinc increased the phosphorylation of Ser202 and Ser214 in SH-SY5Y cells expressing wild-type tau_{1-441}. Therefore, we investigated possible mechanisms to be involved in these changes of tau phosphorylation by PDTC plus zinc treatment in SH-SY5Y cells expressing wild-tau_{1-441}. We found that zinc did not affect CDK5, GSK3 α/β and PKA activity but increased ERK activity. Reduced levels ERK prepared from siRNA-transfected cells inhibited hyperphosphorylation of tau at Ser214 site. To functionally assay the effect of zinc-induced tau hyperphosphorylation, we produced tau mutants that have mimicked phosphorylation of serine202/214 residues by site-directed mutagenesis to alanine. The Ser202Ala and Ser214Ala-tau exhibited stable microtubule polymerization and decreased insoluble tau aggregation. From these findings, it can be concluded that zinc induces tau hyperphosphorylation through ERK activation pathway, leading to microtubule depolymerization and insoluble tau aggregation.

Key words: tau hyperphosphorylation, zinc, extracellular signal-regulated kinase (ERK), microtubule polymerization, tau aggregation

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I. INTRODUCTION

Alzheimer's disease (AD) is the most usual neurodegenerative disorder leading to dementia in the aged human population. It is characterized by the presence of two main brain pathological hallmarks: senile plaque and neurofibrillary tangles (NFTs). NFTs are composed of fibrillar polymers of the abnormally phosphorylated cytoskeletal protein tau[1]. Tau filaments accumulate in dystropic neurites as fine neuropil threads or as bundles forming the NFTs which become extracellular ghost tangles after the death of the neuron.

Tau is a microtubule-associated protein predominantly expressed in nerve cells that promote microtubule assembly and microtubule stabilization[2]. Tau protein is cytosolic and mainly present in axons although it can be also found associated to the cell membrane. In AD, aggregated tau was found to differ from monomeric tau by increased phosphorylation that results in reduced electrophoretic mobility[3]. This hyperphosphorylated tau shows a very low capacity to bind microtubules.

The function of tau and its association with other proteins are affected by post-translational modifications. Phosphorylation of tau has been shown to decrease the rate of tau turnover, and reduce the ability of tau to bind microtubules, actin filaments and plasma membrane[4]. To date, at least 30 phosphorylation sites have been identified in paired helical filament (PHF)-tau. It is believed that several protein kinases are involved in the phosphorylation of tau, and no single kinase can phosphorylate all these sites. Among more than ten protein kinases that have been shown to phosphorylate tau *in vitro*, glycogen synthase kinase 3 (GSK-3), cyclin-dependent kinases 5 (CDK5), calcium- and calmodulin-dependent protein kinases II (CaMK II), cAMP-dependent protein kinase (PKA) and mitogen-activated protein kinases (MAPKs) are most implicated in the regulation of tau phosphorylation and in the abnormal hyperphosphorylation of tau in AD brain[5].

The mitogen-activated protein (MAP) kinase family belongs to the proline-directed protein kinases that receive attention with respect to tau hyperphosphorylation. The MAPs include extracellular signal-regulated kinases (ERKs), the stress-activated protein kinase/C-jun amino terminal kinase (SAPK/JNK) and p38 kinase. Furthermore, there is an evidence for increased activation of ERK in AD brain. The ERKs include p44 ERK1 and p42 ERK2, and PK40ERK, all of which have been shown capable of phosphorylating recombinant tau at several of the same as PHF-tau[6]. Despite in vitro data implicating MAP kinase in hyperphosphorylation of tau protein, studies with intact cells have provided conflicting results. Lu et al.[7] demonstrated that microinjection of rat hippocampal neurons with purified sea star p44 ERK1 resulted in PHF-like tau hyperphosphorylation associated with compromised microtubule assembly. Phosphorylation of proline-rich regions of tau induces conformational changes[8], which cause global structural and functional transformations of tau from its less phosphorylated,

microtubule-bound form to its hyperphosphorylated, aggregated form. Tau hyperphosphorylation cascade, which culminates in NFT formation, the AT8 epitopes (pSer202 and pThr205) were visible prior to the generation of NFTs[9]. It has been reported that phosphorylation at Ser202 enhances tau polymerization and that phosphorylation at both Ser202 and Thr205 not only induces polymerization, but also facilitates filament formation[10]. Increased ERK expression results in increased tau phosphorylation at pathological sites, including AT8 epitope (Ser202/Thr205) and AT100 (Thr212/Ser214).

Zinc is widely distributed in brain. During synaptic activity, vesicular zinc is released into synaptic clefts, but recycled into the synapses via a transporter mechanism. Zinc can be released in excessive amount, as much as several hundred micromolar, from excited presynaptic neurons into synaptic clefts in stroke, trauma and seizure, to be cytotoxic to neighboring neurons[11]. Recently several studied have shown that zinc levels are elevated in brain regions such as hippocampus and amygdala that are heavily affected by AD pathology. In AD, both calcium and zinc have been implicated in the amyloid toxicity pathway[12]. Zinc, as well as copper, is believed to accelerate the formation of amyloid fibrils[13]. Amyloid is implicated as a potential membrane protein that may promote the influx of calcium across the plasma membrane. Zinc does not normally appear in the cell as a free or unbounded form. It is believed to be toxic in this state. This may be related to the ability of free zinc to enter via AMPA channels[14], promoting excitotoxicity. Dysregulated metal metabolism occurs in neurodegenerative disorders[15]. In recent years a metal chelating therapy has emerged as a promising tool to attenuate abnormal metal-protein interactions. The natural metal chelator metallothionein-3 (MT-3) also known as a small non-inducible cysteine- and metal-rich protein mainly expressed in the brain[16]. In the brain its expression was found in zinc-enriched neurons. The decrease of metallothionein-3 in AD may contribute to

the shuttle of these metals to points of interaction, thereby accelerating the pathogenic process[17]. Given the previous reports, although zinc may have important roles in the pathogenesis of AD, the molecular mechanisms that underlie its cellular effects remain to be characterized in detail. The present study investigated the role of zinc on tau hyperphosphorylation and demonstrated that zinc-induced hyperphosphorylation was mediated by extracellular signal-regulated kinase 1/2 (ERK1/2).

Ⅱ. MATERIALS AND METHODS

1. Antibodies and chemicals

Monoclonal Tau-5 antibody against total tau and rabbit polyclonal antibodies against pSer202, pSer214, pSer396 and GSK 3α/β were purchased from Biosource International (Camarillo, CA, USA). Rabbit antibodies against CDK5 and pTyr15-CDK5 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Polyclonal phosphorylation state-specific ERK (pThr202/Tyr204) antibody, polyclonal ERK antibody against total ERK, and the ERK pathway inhibitor U0126 were from Cell Signaling (Beverly, MA, USA). Tablets of Complete ™ Mini protease inhibitors were purchased from Roche (Manheim, Germany). All fine chemicals were purchased from Sigma (St. Louis, MO, USA). The ECL detection system was obtained from Amersham Biosciences (Piscataway, NJ, USA). All cell culture products and lipofectamine were purchased from Invitrogen (Grand Island, NY, USA).

2. Cloning and purification of human wild-type tau_{1-441}

Total RNA was isolated form cultured human neuroblastoma SK-N-MC (ATCC HTB-10) cells according to the protocol supplied with TRI REAGENT ™ from Molecular Research (Cincinnati, OH, USA) as described previously[18]. The yield of total RNA was determined by UV absorbance at 260 nm and its purity was estimated by the absorbance ratio A_{260}/A_{280} nm. In addition, RNA integrity was confirmed by ethidium bromide staining of ribosomal RNA following electrophoresis. Human wild-type tau was amplified by PCR with the following primer pairs: forward primer is 5′-CGGGATCCGCCACCATGGCTGAGCCCCGCCAGGAGTTCGAAGT

GA-3′ and reverse primer is 5′-ACGCGTCGACATCACAAACCCTGCTTGGCCAG-3′. PCR products were subcloned into pcDNA3.1 vector (Invitrogen, USA). The constructed plasmids were transformed into *E.coli* (DH5α), positive colonies were selected and the DNA sequence was analyzed using a DNA sequencer (ABI PRISM, 3100).

3. Bacterial expression and purification of recombinant human tau proteins and establishment of N-terminal tau_{1-48} antibody

Full-length cDNA clones encoding human tau protein was subcloned into pET-15b plasmid (Novagen, USA) and transformed into *Escherichia coli* BL21(DE3) strain for expression. Mutant Ser202Ala- and Ser214Ala-tau were made from the wild-type tau cDNA using a QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA). All mutations were verified by sequencing of separate clones from each mutant. Transformed cells were grown at 37 ℃ in Luria-Bertani medium with 50 μg/ml ampicillin at 300 rpm. *E. coli* cells were grown to optical density of 0.6-1.0 at 600 nm. Expression was induced by adding IPTG to a final concentration of 0.4 mM. After further incubation at 37 °C for 2 hr, the cells were collected by centrifugation at 3000g for 15min at 4 °C. The cell pellet was resuspended in lysis buffer and broken by sonication on ice with Ultrasonic Cell Disrupter. The homogenate was clarified by centrifugation. Supernatants were filtered through a 0.45 μm membrane filter. The filtered lysate was loaded onto CM Sepharose column (Amersham Biosciences) and washed extensively with 50 mM PIPES, pH6.8. Tau protein was eluted using a gradient 50-400 mM NaCl in 50 mM PIPES buffer. Column fractions were screened by gel electrophoresis,

the peak tau fractions were dialyzed against 50 mM PIPES, pH 6.8 containing protease inhibitor and reconstituted at a concentration 5 mg/ml, then aliquoted and stored at -20 °C until use.

N-terminal tau fragment (amino acids 1-48) cDNA clones from human recombinant wild-type tau (amino acids 1-441) was subcloned into pRSET plasmid (Novagen, USA) and transformed into *Escherichia coli* BL21(DE3) strain for expression. This fragment has 6xHis-tag and purified using nickel-NTA resin. Purified 1 mg of antigen, 6xHis-N-terminal tau_{1-48}, was combined with Freund's adjuvant. Subcutaneous injection of antigen to rabbit (New Zealand White) was performed at intervals of two weeks, three times. After 6 weeks, we tested the antibody using ELISA tests and Western blot.

4. **Generation of SH-SY5Y cells stably expressing human wild-type tau_{1-441}**

Human neuroblastoma SH-SY5Y (ATCC CRL-2266) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin from Invitrogen (Grand Island, NY, USA). SH-SY5Y cells were transfected with pTet-Off regulator plasmid (BD Biosciences), using the Lipofectamin (Invitrogen), and stable clones were selected with 500 μg/ml G418. After selection, the cells were stably transfected with pRevTre-wild-type tau_{1-441} and isolated with 200 μg/ml hygromycin B (A.G. Scientific) for 3 weeks. The cells were screened for tau expression by Western blot.

5. **Western blot analysis**

Cell lysed in buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1%

Triton X-100, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM sodium fluoride and complete protease inhibitor cocktail tablet (Roche Diagnostics). Cell lysates were centrifuged at 13,000 g for 15 min. The protein content in the supernatants was measured using BCA Protein Assay kit (Pierce). The phosphorylation of tau at various sites was determined by phospho-specific antibodies.

6. **Protein kinase activity assay**

The CDK5 and GSK 3α/β activities were measured using immunoprecipitation of CDK5, and GSK 3α/β antibody, respectively. Briefly, 1 mg of protein was mixed with 5 μg of antibody. After incubation at 4 °C overnight, 50 μl of protein A sepharose was added to the reaction mixture, and it was constantly mixed for 2 hr. The immunoprecipitated samples were washed twice with lysis buffer and twice with a kinase buffer (20 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 10 mM β-glycerophosphate, 10 mM sodium fluoride, 200 μM sodium orthovanadate, 1 mM dithiothreitol). Kinase activities was measured by mixing the immunoprecipitates with kinase buffer containing 5 μCi [γ-^{32}P]ATP and purified recombinant tau protein. The samples were incubated at 30°C for 30 min. The reactions were terminated by the addition of 2x SDS sample buffer. The samples were boiled and loaded on 10% SDS-polyacrylamide gels.

The PKA activity was assessed by using a PKA assay kit according to the protocol provided by the manufacturer (Upstate Biotechnology). The catalytic activity of PKA was measured using Kemptide as a substrate, as described previously. Briefly, 50 μg of cell lysate was incubated for 10 min at 30 °C with Kemptide, 5 μM cAMP and 100 μCi [γ-^{32}P]ATP (3000

Ci/mmol) in Assay Dilution Buffer (20 mM MOPS, pH7.2, 20 mM $MgCl_2$, 25 mM β-glycerophosphate, 5 mM EGTA, 1mM sodium orthovanadate, 1mM DTT). The reaction mixture was stopped by spotting onto p81 phosphocellulose filter paper. The filters were washed three times with 0.75 % o-phosphoric acid and rinsed in acetone prior to scintillation counting.

7. **Microtubule assembly assay**

Purified human recombinant wild-type, Ser202Ala and Ser214Ala tau proteins were respectively incubated with 1 mM GTP, 20 μM tubulin (Cytoskeleton) in assembly buffer (80 mM PIPES, pH6.9, 0.5 mM EGTA, 1 mM DTT, 2 mM $MgCl_2$) for 20 min at 37°C. The assembly of tubulin into microtubules was monitored over time by a change in turbidity at 350 nm using a spectrophotometer.

8. **RNA interference**

Cells were grown to 30-50% confluence before transfection. We used Validated Stealth™ RNAi duplex MAPK1 (Invitrogen) and transfected it into cells using Lipofectamine 2000 (Invitrogen). Two days after transfection, cells were harvested in lysis buffer for immunoblotting. The target sequence is as following.

#1: 5'-GCUGUUCCCAAAUGCUGACUCCAAA-3'

#2: 5'- CCGAAGCACCAUUCAAGUUCGACAU -3'

9. **Isolation of sarkosyl-insoluble tau from cultured cells**

SH-SY5Y cells were transfected with wild-type and Ser214Ala tau for 48 hr and extracted with RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl,

1% Triton X-100, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 0.5% sodium deoxycholate, 0.1% SDS, pH8.0 and Complete protease inhibitor cocktail tablet) and centrifuged to generate soluble-tau. The RIPA-insoluble pellets are rinsed and centrifuged twice with RIPA buffer to remove soluble fractions. Finally, the RIPA-insoluble pellets were re-extracted with sonication to recover the most insoluble cytoskeletal aggregates. Quantitative Western blot analyses were used to determine tau level in each fraction.

III. RESULTS

1. Induction of human wild-type tau_{1-441} expression in SH-SY5Y cells

Tetracycline-regulated gene expression systems have gained a wide use since they allow selective and stringent regulation of transgene expression *in vitro* and *in vivo*[19,20]. The Tet-Off system is based on a tetracycline-regulated transactivator (tTA) that in the absence of tetracycline induces gene transcription through binding to a tetracycline-response element (TRE) located upstream of a CMV minimal promoter followed by the transgene. The pRev-TRE system is based on two vectors (pRev-Tet-Off and pRev-TRE). First, we transfected SH-SY5Y cells with pTet-off and selected with 500 μg/ml G418 at 48 hr after transfection. We have screened as many as 20 clones to obtain one that exhibits suitably high induction and low background. Clones were isolated, expanded and checked for inducibility by transient transfection with wild-type tau plasmid DNA. The established Tet-Off cell lines were transfected with pRev-TRE-human wild-type tau_{1-441} constructs and incubated with 200 μg/ml hygromycin B. We selected to hygromycin-resistant colonies after 4 weeks. The stably transfected SH-SY5Y cells were routinely grown in the presence of tetracycline to suppress expression of the wild-type tau_{1-441}. As it can be seen from Fig. 1A, we identified to tau expression using tau-5 antibody, which recognizes total tau. Expression was induced by thoroughly removing tetracycline. SH-SY5Y cells maintained in media containing tetracycline were used as control. In the presence of tetracycline, tau was not detectable by Western blotting (Fig. 1A). In contrast, tau was detected within 48 hours of tetracycline removal. The level of tau appeared to reach its maximum by day 5 (Fig. 1B). These results demonstrate the tight regulation of tau expression by tetracycline in the SH-SY5Y cells.

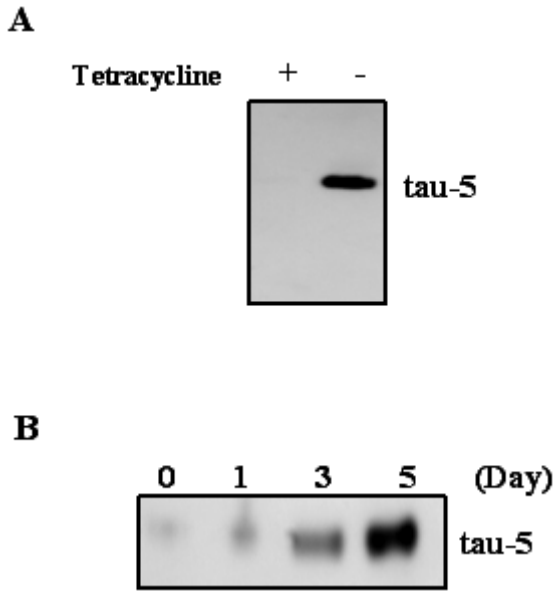

Figure 1. Induction of human wild-type tau_{1-441} expression in SH-SY5Y cells. Cells were grown in medium supplemented with 2 μg/ml tetracycline to suppress expression. Human wild-type tau_{1-441} expression in SH-SY5Y cells was induced by thoroughly removing tetracycline and determined by immunoblotting against tau-5 antibody.

2. Effect of PDTC plus zinc treatment on tau phosphorylation levels in SH-SY5Y cells expressing human wild-type tau_{1-441}

In our earlier studies, pyrrolidine dithiocarbamate (PDTC) increased the intracellular zinc, reaching plateau within 30 min both in BCECs and HeLa cells[21, 22]. Inhibition of NF-κB activity by PDTC was prevented by various metal-saturated EDTAs, but not by zinc-saturated EDTA, indicative of zinc-mediated PDTC action. Therefore, we used PDTC as zinc ionophore. To identify the effects zinc has on tau phosphorylation, we studied tau

phosphorylation at various sites. Changes in the phosphorylation of tau at several sites were examined by Western blots using phosphorylation site-specific tau antibodies. For each phosphorylation-epitope, the phosphorylation-specific immunoreactivity was normalized to the total tau. SH-SY5Y cells stably expressing human wild-type tau_{1-441} were treated with 100 μM PDTC plus 0.5 μM zinc for 0, 15, 30 and 120 min, respectively. We found that the phosphorylation of tau at Ser202 and Ser214 sites was significantly increased time-dependently compared to the control level (Fig. 2). However, under same conditions, we observed that the phosphorylation of tau at Ser-396 site was not. It has been well documented by many that the development of epitopes unique to AD tau filaments involves such as Ser202[23] and Ser214[24].

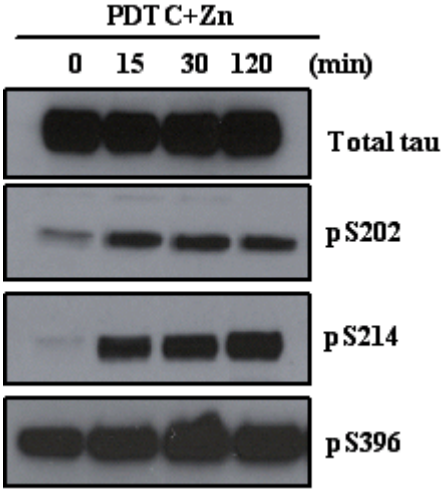

Figure 2. Effect of PDTC plus zinc treatment on tau phosphorylation levels in SH-SY5Y cells expressing human wild-type tau_{1-441}. SH-SY5Y cells stably expressing human wild-type tau_{1-441} were treated with 100 μM PDTC plus 0.5 μM zinc for 0, 15, 30 and 120 min, respectively. Cell lysates

were subjected to SDS-PAGE, followed by immunoblot analyses with phospho-Ser202-tau (pS202), phospho-Ser214-tau (pS214), phospho-Ser396-tau (pS396) and total tau antibody (Tau-5).

3. Effects of PDTC plus zinc treatment on CDK5, GSK 3α/β, and PKA activities in SH-SY5Y cells expressing human wild-type tau_{1-441}

Tau has been reported to be phosphorylated by several protein kinases (e.g. CDK5, GSK 3α/β, PKA and MAPK). To identify the protein kinases that can be involved in the zinc-induced hyperphosphorylation of tau, we investigated changes in the activities of various protein kinases following the treatment of PDTC plus zinc (Fig. 3). Cyclin-dependent kinase 5 (CDK5) is found in its active form only in neuronal cells. Like other members of the CDK family, CDK5 catalytic activity is influenced by both p35 binding and phosphorylation. Phosphorylation of either serine 159 or tyrosine 15 dramatically increases CDK5 activation. We observed that levels of pTyr15-CDK5 were decreased by PDTC plus zinc treatment (Fig. 3A). However, no difference in total CDK5 levels between untreated control and PDTC plus zinc treatment group. The GSK 3α/β activity was decreased by phosphorylation of Ser9 on GSK 3β and increased by phosphorylation of Tyr216 and Tyr279 on GSK 3α/β . In mammalian cells, on stimulation with insulin or outgrowth factors, GSK-3 is rapidly phosphorylated at serine21 in GSK3α or serine 9 in GSK3β, resulting in inhibition of GSK3 kinase activity[25,26]. We found that PDTC plus zinc treatment not changed the level of pTyr216 and pTyr279 on GSK 3α/β and increased the phosphorylation level of Ser9 on GSK 3β (Fig. 3B). The same lysates used for the kinase assay, we also observed the decreased activity of GSK 3α/β (Fig. 3C). The cAMP-

dependent protein kinase A (PKA) has been long been considered a candidate for *in vivo* tau phosphorylation. Tau that is phosphorylated *in vitro* with PKA has reduced microtubule binding. But we found that zinc had no effect on PKA activity (Fig. 3D). Theses results indicated that tau phosphorylation might be more favored by kinases other than CDK5, GSK 3α/β and PKA.

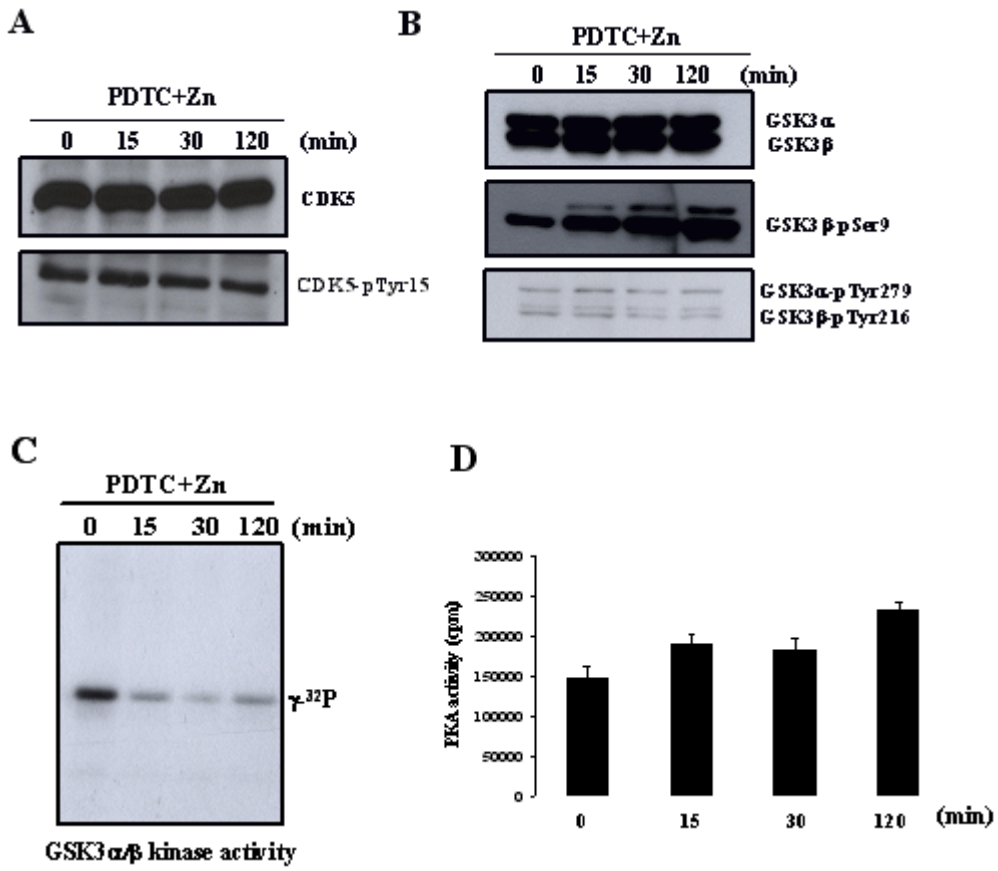

Figure 3. Effects PDTC plus zinc treatment on CDK5, GSK 3α/β and PKA activities in SH-SY5Y cells expressing human wild-type tau$_{1\text{-}441}$. SH-SY5Y cells expressing wild-type tau$_{1\text{-}441}$ were treated with 100 μM PDTC plus

0.5 μM zinc for 0, 15, 30 and 120 min, respectively. The samples were analyzed by immunoblotting with antibody against of CDK5, phospho-Tyr15 CDK5 (A), GSK 3α/β and phospho Ser9-, Tyr279-, Tyr216-GSK3β (B). Alternatively, GSK 3α/β and PKA activities were measured using recombinant human tau (C), and Kemptide as a substrate, respectively (D).

4. Effect of PDTC plus zinc treatment on mitogen-activated protein kinases (MAPKs) activity in SH-SY5Y cells expressing human wild-type tau_{1-441}

MAP kinases have been shown to phosphorylate tau *in vitro*[27]. Given the important role that MAPKs play in oxidative stress signaling and cell cycle, especially in neuronal survival and death in vitro and in brain, there interest in understanding the role that MAPKs may play in the pathogenesis of AD. While total ERK1/2 levels are slightly decreased[28] or little changed[29] between controls and AD, strong evidence demonstrated that active ERK is markedly increased in AD[30]. The involvement of ERK in phosphorylation of tau in cells, however, is not clear. Thus we examined whether MAP kinases are involved in tau phosphorylation during PDTC plus zinc treatment in SH-SY5Y cells expressing wild-type tau_{1-441}. As shown in Fig. 4, total ERK1/2, JNK/SAPK and p38 levels were unchanged after PDTC plus zinc treatment. Also, the levels of phospho-p38 and phospho-JNK/SAPK were not changed. In contrast, the level of phospho-ERK1/2 was increased from 15 min. These findings suggest that activation of ERK may be involved in wild-type tau phosphorylation.

Next, we examined whether ERK1/2 mediates tau phosphorylation by zinc. Activating forms of ERK1/2 and upstream activating kinases MEK1/2 were shown to co-distribute with the progressive neurofibrillary changes in

Alzheimer's disease[31]. To investigate the effect of ERK1/2 activation on zinc-induced tau hyperphosphorylation, SH-SY5Y cells were pre-treated with 10 μM U0126, specific inhibitor of the upstream ERK kinase (MEK), for 1 hour before PDTC plus zinc treatment. As shown in Fig. 5, addition of U0126 inhibited the tau phosphorylation at Ser214 site. These results suggest that ERK hyperphosporylates Ser214 site of tau.

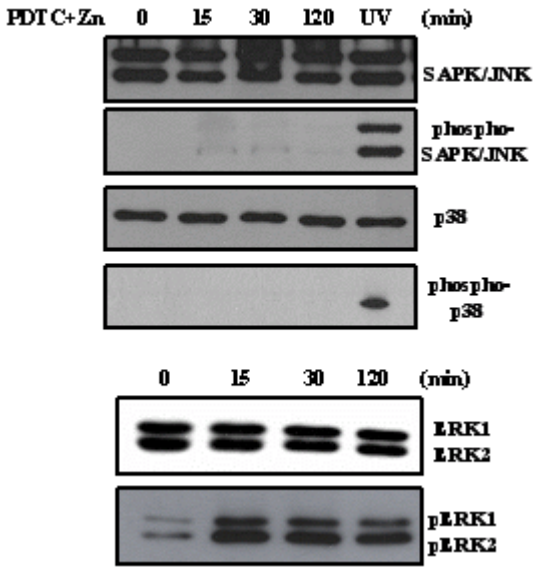

Figure 4. Effect of PDTC plus zinc treatment on mitogen-activated protein kinase (MAPKs) activity in SH-SY5Y cells expressing human wild-type tau$_{1\text{-}441}$. SH-SY5Y cells expressing wild-type tau$_{1\text{-}441}$were treated with 100 μM PDTC plus 0.5 μM zinc for 0, 15, 30 and 120 min, respectively. The samples were analyzed by immunoblotting with antibody against SAPK/JNK, phospho-SAPK/JNK, P38, phospho-P38, ERK and phospho-ERK.

In the ERK pathway, the MAPKKK, which are called Raf kinases, phosphorylated and activate MAPK/ERK kinase (MEK1/2), which in turn phosphorylates and activates ERK1/2. Raf kinases are tightly regulated by multiple mechanisms, and as such, they represent a key regulatory point for signal transduction via the MAPK pathway[32]. Given that Ras, the upstream activator of Raf-1, is activated in Alzheimer's disease[33] and that both MEK1/2 and ERK are activated in perinuclear and nuclear region in neurons of the hippocampus of patient with Alzheimer's disease[34].

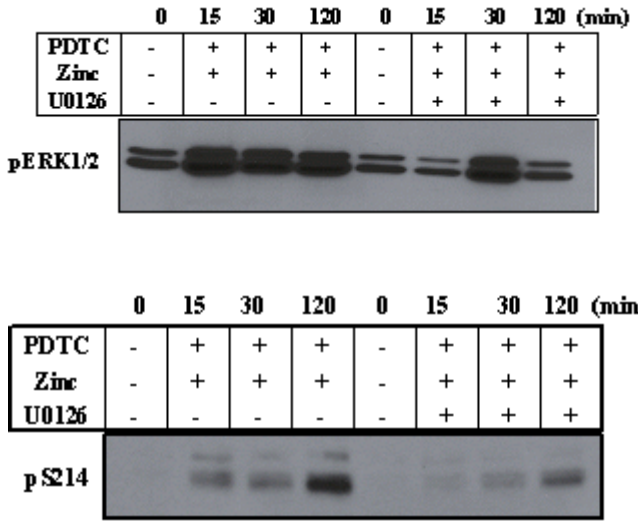

Figure 5. Effect of U0126, a MEK inhibitor, on zinc-induced tau hyperphosphorylation in SH-SY5Y cells expressing wild-type tau_{1-441}. SH-SY5Y cells expressing wild-type tau_{1-441} were pretreated with 10 μM U0126(MEK inhibitor) and then added with PDTC plus zinc. The samples were analyzed by immunoblotting with antibody against ERK and pSer214-tau.

To further confirm whether or not ERK1/2-Ras-Raf signaling pathways can directly activate on the tau phosphorylation cascades, we used Ras- and Raf-

dominant negative plasmid: The pCMV-Ras 17 contains a mutated from of Ras with serine to asparagine mutation at residue 17 and the pCMV-Raf 621 contains a mutated from of Raf with serine to alanine mutation at residue 621. Expression in the SH-SY5Y cells of a dominant-negative Ras (N17) or Raf (S621A) blocked the zinc-induced tau hyperphosphorylation at Ser214 site (Fig. 6). These results suggest that tau hyperphosphorylation associates the Ras/Raf/ERK signaling pathway.

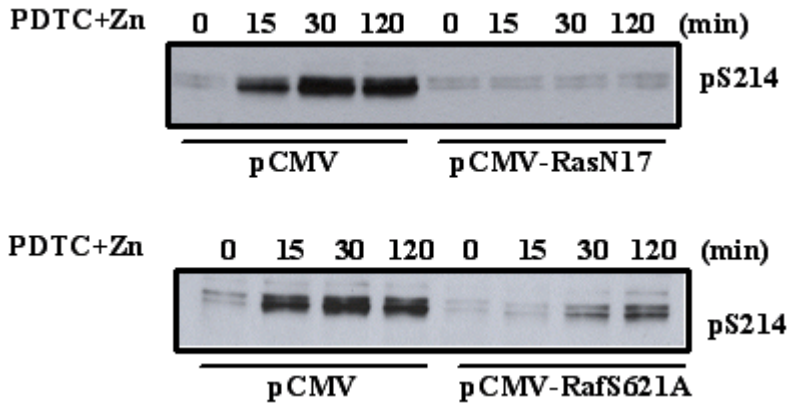

Figure 6. Effects of dominant-negative Ras (pCMV-RasN17) and Raf (pCMV-RafS621A) on PDTC plus zinc-induced tau hyperphosphorylation in SH-SY5Y cells expressing wild-type tau$_{1-441}$. Dominant-negative Ras (pCMV-RasN17) or Raf (pCMV-RafS621A) were transfected in SH-SY5Y cells expressing wild-type tau$_{1-441}$, and, 48 hr after transfection, cell lysates were prepared. The extracts were analyzed by immunoblotting with antibody against pSer214-tau.

5. Effect of ERK siRNA on zinc-induced tau hyperphosphorylation

We next sought to determine if tau hyperphosphorylation was triggered by increased ERK activity in SH-SY5Y cells expressing wild-type tau$_{1-441}$. To

determine any physiological relevance ERK might have on tau hyperphosphorylation, RNA interference technique was used to knock-down ERK expression in SH-SY5Y cells. RNA interference experiments were performed with MAPK1 siRNA in order to prevent zinc-induced ERK activation. After 2 days of transient transfection, we observed the down-regulation of total ERK. Reduced levels ERK prepared from siRNA-transfected cells inhibited hyperphosphorylation of tau at Ser214 site (Fig. 7). All these experiments demonstrate that zinc-induced tau hyperphosphorylation id dependent on the ERK pathway in SH-SY5Y cells expressing wild-type $tau_{1-441.}$

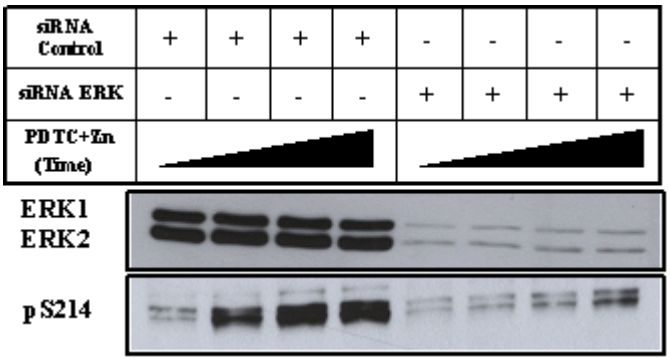

Figure 7. Effect of ERK siRNA on zinc-induced tau hyperphosphorylation in SH-SY5Y cells expressing wild-type tau_{1-441}. SH-SY5Y cells were transfected with siRNA ERK for 48 hr and PDTC plus zinc treated for 0, 15, 30 and 120 min. The samples were analyzed by immunoblotting with antibody against ERK and pSer214-tau.

6. Effects of Ser202Ala- and Ser214Ala-tau mutation on microtubule polymerization

One physiological role of tau is to aid in the polymerization of tubulin to

form stable microtubules. The intracellular neurofibrillary lesions consist of paired helical filaments and straight filaments, which are made of the microtubule-associated protein tau in a hyperphosphorylated state[35]. When hyperphosphorylated, tau can no longer efficiently bind to microtubules, thereby forms fibrillar aggregates. To determine if this function of tau is compromised in the presence of activated ERK, we performed a microtubule polymerization assay in a cell-free system. Recombinant human wild-type tau

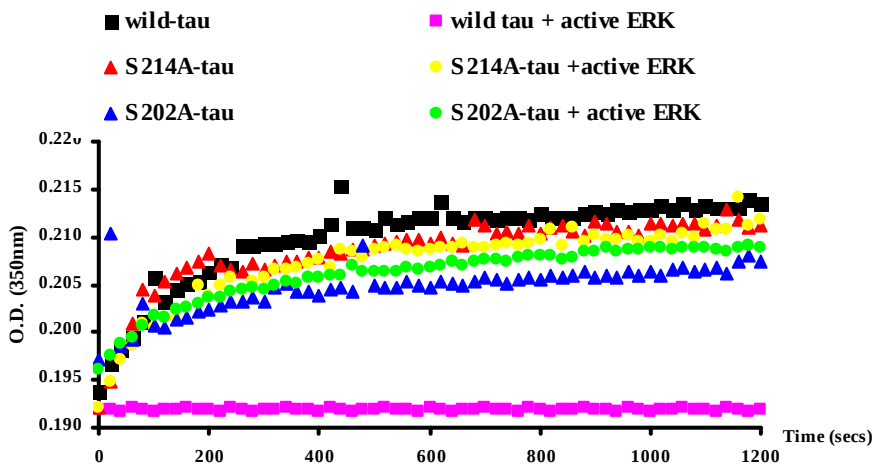

Figure 8. Effects of Ser202Ala- and Ser214Ala-tau mutation on microtubule polymerization. Following the human recombinant tau protein were incubated in the presence or absence of purified active ERK for 5 min at 37℃, and then was initiated by addition of tubulin The time-course of tubulin polymerization was followed over 20 min by the change in turbidity at 350 nm.

protein at 2 μM (0.1 mg/ml) and tubulin (20 μM) were incubated with activated ERK in assembly buffer (as described previously). Over time, recombinant human wild-type tau alone, without activated recombinant ERK, did efficiently polymerize into microtubules (Fig. 8). Following the addition of activated recombinant ERK, microtubule polymerization by wild-type tau was decreased whereas mutant Ser202Ala-tau and Ser214Ala-tau were normally polymerized into microtubules (Fig. 8). These results suggest that tau hyperphosphorylation at Ser202 and Ser214 sites interferes with stable microtubule polymerization.

7. Effect of Ser214Ala-tau mutation on sarkosyl-insoluble tau

Interestingly, neuropil threads thought to be the earliest manifestation of tau pathology[10]. Some of the sites in tau become phosphorylated in both normal and aggregated tau derived from diseased human brain. Aggregated, neurofibrillary tangle (NFT)-like tau derived from overexpression of human tau has been shown to be enriched in sarkosyl-insoluble fractions[36-38]. Sarkosyl-insoluble fractions from AD contain tau-paired helical filaments capable of binding thioflavin S. We examined if tau can form tau aggregates in the presence of zinc. Lysates were prepared from cells expressing the wild-type tau and Ser214Ala-tau in the presence of zinc and then separated into sarkosyl-soluble and sarkosyl-insoluble fractions. The level of tau aggregation was measured by tau-5 antibody (epitope 210-230 amino acids) but the tau-5 antibody not recognizes insoluble fraction (data not shown). Interestingly, sarkosyl-insoluble fraction was detected by N-terminal tau antibody (epitope 1-48 amino acids). As shown in Fig. 9A, zinc increased tau aggregate in the sarkosyl-insoluble fractions. The result shows that tau-5 epitopes (amino acids 210-230) does not necessarily lead to zinc-induced tau aggregation, whereas the aggregates were observed with truncated tau (N-terminal tau). We next

investigated whether Ser214Ala-tau affects tau aggregation by zinc. In the presence of Ser214Ala-tau, the level of tau aggregation was considerably decreased compared to wild-type tau (Fig. 9B). These observations demonstrate that Ser214Ala-tau inhibits zinc-induced tau aggregation.

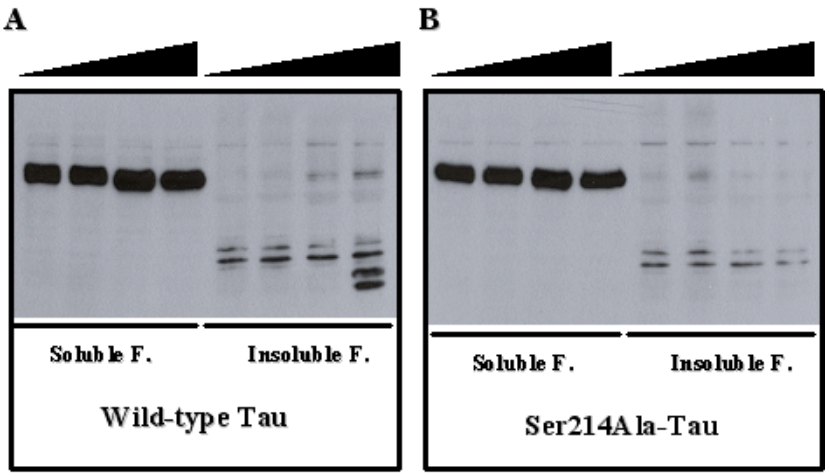

Figure 9. Effect of Ser214Ala-tau mutation on sarkosyl-insoluble tau. Lysates were extracted from SH-SY5Y cells expressing wild-type tau_{1-441} and Ser214Ala-tau with RIPA buffer. Supernatant (soluble fraction) and pellet (Insoluble fraction) were separated by centrifugation. The RIPA-insoluble pellets were re-extracted with sonication to recover the most insoluble cytoskeletal aggregates. All samples were analyzed by immunoblotting with antibody against N-terminal tau_{1-48}.

IV. DISCUSSION

Alzheimer's disease process is apparently accompanied by a substantial change in the levels of zinc in the different areas of the brain. It has already been reported that the regions of the control brains revealed an even distribution of zinc as opposed to autopsies from AD group where the zinc level generally differs significantly between the regions[39]. The notion that zinc might be involved in the etiology of AD has been proposed repeatedly over the last two cascades, and specific suggestions of a possible role of zinc in the etiology of AD have been proposed in recent years. Several factors could increase the regional variability of zinc in AD brains. Therefore, our findings suggest that zinc is an important metal that can regulate tau hyperphosphorylation through stimulating ERK, leading to microtubule depolymerization and insoluble tau aggregation.

We incorporate the strategy of inducible tau expression to develop cellular models tauropathy, because self-assembly of tau is concentration dependent and stable transfectants are unlikely to express high level of tau, which would otherwise lead to microtubule bundling in cell, thus impeding a successful mitosis essential for the establishment of stable transfectants. Moreover, the reliance of tau filament assembly on putative inducers would be markedly diminished following tau overexpression. We established conditional tau transfectants from cultured human neuroblastoma SH-SY5Y cells. Because we are concerned with the relevance of their utility of the investigation of sequence of events leading to neurofibrillary degeneration in AD and other tauropathies. The tetracycline off (TetOff) mechanism was used to regulate the expression of wild-type tau. We identified to tau expression for 5 days. Interestingly, based in the findings from this study, extension of the induction period beyond 5 to 7 days led to generation of small amounts of truncated tau

fragments that were smaller in molecular weight than recombinant tau $_{1\text{-}441}$ (data not shown). It is still not clear how the truncated tau fragments are affecting the tau function, but it will be further examined.

Tau hyperphosphorylation has been extensively studied because it could be toxic *in vivo*[27], a toxicity that could be manifested before tau aggregation. In our studies show that zinc induces hyperphosphorylation of the tau. Furthermore, these results are consistent with other reports. Harris et al.[40] showed in N2a cells that 24h of 200 μM zinc treatment was shown to induce a dramatic increase of AT8 (Ser202/Thr205) immunoreactivity. Many kinases may be involved in tau phosphorylation *in vitro* and *in vivo*, including glycogen synthase kinase 3β (GSK 3β), cyclin-dependent kinase-5 (CDK5), extracellular signal-regulated kinase (ERK), microtubule-affinity regulating kinase and fyn kinase[27,41]. Our findings suggest zinc activates ERK and subsequently cause tau hyperphosphorylation in SH-SY5Y cells expressing wild-type tau$_{1\text{-}441}$ and may be relevant to the pathogenesis of AD. Several lines of evidence suggest that ERK is involved in tau phosphorylation[30, 31]. In the brain, ERKs have been shown to mediate long-term potentiation, which is the proposed molecular mechanism that underlies learning and memory; impaired memory is prominent in AD[42]. ERK is associated with neuronal microtubules in the rodent and bovine brains, and phospho-ERK is increased in a subpopulation of neurons containing phospho-tau and NFTs and in dystrophic neuritis in senile plaques in AD brains. In addition, stress-induced tau phosphorylation is associated with increased phosphor-ERK immunoreactivity in neurons. Finally, phospho-ERK phosphorylates serines in Lys-Ser-Pro repeats in tau *in vitro*. In human and rhesus monkey brains, anti-ERK immunostaining showed that ERK levels are highest in the CA3 region, the mossy fiber zone, and the granule cell layer of the dentate gyrus. These are areas where observed the greatest increases in phosphor-ERK and phospho-

tau in NSE-apoE4 mice, supporting anatomically the relationship between ERK activation and tau phosphorylation[43]. There have been many reports suggesting the activated forms of ERK1 and ERK2 colocalize with the neurofibrillary lesions in postmortem AD brains[27, 28], also is compatible with a role for ERKs in the pathological hyperphosphorylation of tau.

In the upstream events of ERK activation, both B-Raf and Raf-1 (also known as C-Raf) is highly expressed in neurons in several areas of the brain including hippocampus, with the former being restricted to the neuronal soma and the latter to the neuritic extensions[34]. Raf-1 activation is tightly regulated process that occurs at the plasma membrane and involves several steps including Ras-mediated membrane recruitment, conformational changes and subsequent phosphorylation/dephosphorylation. Given that both Ras/Raf and MEK/ERK are activated in Alzheimer's disease, these data provide a missing link between upstream events and downstream ERK activation in AD and give further credence to our study that the entire ERK pathway is abnormally activated in AD. For a better understanding of its role on ERK activity, endogeneous ERK was knocked-down in SH-SY5Ycells by siRNA (small interfering RNA) directed against ERK, which resulted about 90% decrease in protein level detected by immunoblotting. Decreased ERK protein level remarkably diminished tau hyperphosphorylation at Ser214 site. In 1986, Microtubule associated protein tau was discovered to be abnormally hyperphosphorylated in AD brain and to be the major protein subunit PHF/tangles[45]. Since this discovery, the hyperphosphorylation of tau in the brain of patients with AD and with other tauropathies[46, 47], and a dysregulation of the phosphorylation system is believed to be involved in this tau pathology. Therefore, the identification of protein kinases involved in the abnormal hyperphosphorylation and self assembly of tau into PHF/tangles has been a major goal of research on the pathogenesis of AD and other tauropathies.

Intracellular protein aggregates underlie a variety of neurodegenerative disease, such as Parkinson's disease, Huntington's disease, prion disease, FTDs, and AD. All of these present with intracellular protein aggregates, each dominated by a specific protein species. One species in particular, the microtubule-binding protein tau, characterizes aggregates in two diseases, AD and FTDs. The protein forms insoluble NFTs that accompany and may promote neurodegeneration in these brain diseases. Based on numerous pathological studies, it is generally assumed that abnormal hyperphosphorylation of tau is more or less directly related to its aggregation and the biochemically defined sarkosyl-insoluble pathological tau pool reflects the histopathologically defined NFTs and NTs then NFTs and NTs are direct correlates of synaptic deficits and neurodegeneration. *In vitro* studies have demonstrated that small tau aggregates, termed paired helical filaments, precipitate from solution and undergo fibrillization to form larger aggregates, which may be related to NFTs found in neurons[48], but the mechanisms underlying formation of NFTs from aggregates have not been elucidated. Based on the findings form this study (Fig. 9), it can be postulated that zinc-induced insoluble tau aggregates are composed of N-terminal fraction of tau protein, which is otherwise soluble. However, much work needs to be done to clarify the possibility that N-terminal tau fraction might be involved in NFTs. Up to now, in contrast to C-terminal truncation of tau, little is known about its N-terminal processing. The N-terminal 15 amino acids of tau are required for the formation of the Alz-50[49] conformation of tau, a conformation known to be a marker for early tangles[10]. Recently, it was reported that early N-terminal changes of tau could play integral in tangle maturation in AD. This observation further supports our finding.

In conclusion, the present study demonstrates that zinc induces tau hyperphosphorylation through Ras/Raf/ERK cascades and interferes with

microtubule polymerization, leading to tau aggregation. The findings of this study offer new insight into the possible relationship between Alzheimer's disease (AD) pathology and brain zinc.

V. CONCLUSION

The present study demonstrates that zinc activates extracellular signal-regulated kinases 1/2 (ERK1/2) and activated ERK induces tau hyperphosphorylation, leading to tau aggregation. These results are summarized to:

1. Zinc induces hyperphosphorylation of tau at Ser202 and Ser214 sites.

2. Zinc activates tau hyperphosphorylation through extracellular signal-regulated kinase 1/2 (ERK1/2) pathway.

3. Knock-down of ERK expression decreases zinc-induced tau hyperphosphorylation.

4. Zinc-induced tau hyperphosphorylation interferes with stable microtubule polymerization.

5. Zinc-induced tau hyperphosphorylation and N-terminal truncation tau play an important role in a process of aggregate formation of tau.

From these results, it can be concluded that zinc is an important metal that can regulate tau hyperphosphorylation through stimulating ERK, leading to microtubule depolymerization and insoluble tau aggregation.

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ABSTRACT (IN KOREAN)

아연이 extracellular signal-regulated kinase 1/2을 통한 tau hyperphosphorylation의 유도

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알쯔하이머병은 치매를 일으키는 가장 흔한 원인으로 알려져 있다. 이 질환의 발생은 노화와 직접적인 관련이 있으며, 인구의 노령화 현상으로 매년 환자수가 증가하고 있는 추세이다. 현재 알쯔하이머 발병의 중요한 원인인자로 세포외의 amyloid와 세포내의 tau 축적이 대표적인 가설로 들 수 있다. 아연은 중추신경계에 glutamate를 포함하는 시냅스전 소포체에 고농도로 농축되어 있으며, 일부는 시냅스후 신경세포로 glutamate와 같이 유리되거나 국소적으로 많은 양이 세포내에 유리되게 되는데, 이때 아연이 세포내의 tau 단백의 응집 원인으로 추정하고 있다. 따라서 본 연구에서는 아연이 시냅스후 신경세포로의 유입이 tau 단백 기능에 어떠한 영향을 주는지를 탐구하였다. 이를 위해 우선 세포모델에서 tau단백을 발현하는 SH-SY5Y세포주를 구축하였으며, 세포내로의 아연의 효과를 보기 위해 아연의 이온투과체 (ionophore)인 pyrrolidine dithiocarbamate (PDTC)를 사용하였다. 세포내로 아연을 처리하였을 때 tau단백의 serine 202번과 214

번의 과인산화가 유도되었다. 따라서 아연이 유도하는 tau 과인산화 과정이 어떠한 kinase 단백 활성을 통해 일어나는지를 조사하였다. 그 중 아연이 세포내로 유입되었을 때 extracellular signal-regulated kinase 1/2 (ERK1/2)가 증가되는 것을 확인하였으며, 이는 small interference RNA ERK를 처리하였을 때 tau 단백의 과인산화가 억제되는 것으로 보아 아연이 ERK1/2를 매개한다고 알 수 있었다. 또한 MEK 억제제인 U0126을 이용한 실험에서 tau 단백의 과인산화가 ERK의 upstream인 Ras/Raf/MEK 의 일련의 과정을 통해 일어나는 것을 확인하였다. 일련의 실험을 통해 아연이 tau의 과인산화를 유도하였을 때 tau기능에 어떠한 영향을 주는지도 살펴보았다. Tau 기능 중 대표적인 microtubule polymerization은 아연에 의해 과인산화 된 부위를 mutation시켰을 때 정상적인 polymerization이 일어나는 것을 확인하였고, 비 이상적인 tau 단백의 축적도 감소하는 것을 볼 수 있었다. 따라서 이상의 결과들을 종합하여 볼 때 아연이 tau pathology에 중요한 역할을 담당할 것으로 사료된다.

핵심되는 말: tau hyperphosphorylation, zinc, extracellular signal-regulated kinase (ERK), microtubule polymerization, tau aggregation

PUBLICATION LIST

Ryoo SR, Jeong HK, Radnaabazar C, Yoo JJ, Cho HJ, Lee HW, Kim IS, Cheon YH, Ahn YS, Chung SH, Song WJ. DYRK1A-mediated hyperphosphorylation of Tau: A functional link between Down syndrome and Alzheimer's disease. J Biol Chem. 2007;282(48):34850-7