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TPPU Protects Tau from H_2O_2-induced Hyperphosphorylation in HEK293/tau Cells by Regulating PI3K/AKT/GSK-3β Pathway*

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Summary: Neurofibrillary pathology of abnormally hyperphosphorylated tau is a hallmark of Alzheimer's disease (AD) and other tauopathies. Phosphatidylinositol 3-kinase (PI3K)/Akt/glycogen synthase kinase-3 beta (GSK-3β) signaling pathway is pivotal for tau phosphorylation. Inhibition of soluble epoxide hydrolase (sEH) metabolism has been shown to effectively increase the accumulation of epoxyeicosatrienoic acids (EETs), which are cytochrome P450 metabolites of arachidonic acid and have been demonstrated to have neuroprotective effects. However, little is known about the role of sEH in tau phosphorylation. The present study investigated the role of a sEH inhibitor, 1-(1-propanoylpiperidin-4-yl)-3-[4-(trifluoromethoxy)phenyl] urea (TPPU), on H_2O_2-induced tau phosphorylation and the underlying signaling pathway in human embryonic kidney 293 (HEK293)/Tau cells. We found that the cell viability was increased after TPPU treatment compared to control in oxidative stress. Western blotting and immunofluorescence results showed that the levels of phosphorylated tau at Thr231 and Ser396 sites were increased in H_2O_2-treated cells but dropped to normal levels after TPPU administration. H_2O_2 induced an obvious decreased phosphorylation of GSK-3β at Ser9, an inactive form of GSK-3β, while there were no changes of phosphorylation of GSK-3β at Tyr216. TPPU pretreatment maintained GSK-3β Ser 9 phosphorylation. Moreover, Western blotting results showed that TPPU upregulated the expression of p-Akt. The protective effects of TPPU were found to be inhibited by wortmannin (WT, a specific PI3K inhibitor). In conclusion, these results suggested that the protective effect of TPPU on H_2O_2-induced oxidative stress is associated with PI3K/Akt/GSK-3β pathway.
Key words: TPPU; tau; GSK-3β; Alzheimer's disease

Neuronal microtubule-associated protein tau was reported to disconnect from microtubules and abnormally hyperphosphorylate in the brain of patients with Alzheimer's disease (AD)[1]. Aberrant tau aggregates into paired helical filaments and straight filaments, forming neurofibrillary tangles, which is considered to be responsible for the loss of biological activity of tau and gain of its toxic function[2].

Phosphatidylinositol 3-kinase (PI3K)/Akt/glycogen synthase kinase-3 beta (GSK-3β) pathway plays an important role in regulating tau phosphorylation. As one of the major tau kinases, GSK-3β, also known as a serine/threonine protein kinase, phosphorylates tau at many sites[3]. The activity of GSK-3β is regulated by phosphorylation at Ser 9 and Tyr 216[4]. Ser 9 phosphorylation inhibits the enzymatic activity of GSK-3β, whereas Tyr 216 phosphorylation is required for its full activity. Numerous studies have demonstrated that GSK-3β-induced tau hyperphosphorylation is associated with AD-like amnesia[5, 6]. Administration of wortmannin (WT, a specific PI3K inhibitor) induces tau hyperphosphorylation by activating GSK-3β in rat brain, murine neuroblastoma N2a cells and hippocampus slices, which results in hippocampus-related synaptic disorder and AD-like spatial memory deficits[6–8].

Epoxyeicosatrienoic acids (EETs) are cytochrome P450 metabolites of arachidonic acid and rapidly hydrolyzed to dihydroxyeicosatrienoic acids (DiHETs) by soluble epoxide hydrolase (sEH)[9]. Inhibition of sEH metabolism through pharmacological inhibition, or genetic disruption, has been shown to effectively increase accumulation of EETs and has been demonstrated to have neuroprotective and anti-oxidative effects[10]. Additionally, the protective effects of sEH inhibition are found associated with PI3K/Akt pathway[11].

The purpose of the present study was to address the potential role and underlying mechanism of sEH in the pathophysiology of hydrogen peroxide (H_2O_2)-induced tau hyperphosphorylation using a sEH inhibitor TPPU. We found that TPPU suppressed H_2O_2-induced increased GSK-3β activity and protected tau from hyperphosphorylation by regulating the PI3K/Akt/GSK-3β pathway.

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1 MATERIALS AND METHODS

1.1 Cell Culture

HEK293 cells stably transfected with the longest human tau cDNA (HEK293/Tau) were kindly provided by Professor Xiao-chuan WANG of Tongji Medical College, Huazhong University of Science and Technology, China. They were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1 μg/mL G418 (Invitrogen Co., USA) in a humidified chamber at 37°C in 5% CO_2. Cells were grown to approximately 80% confluence on six-well culture plates for cell lysates preparation or on 96-well culture plates for measurement of cell viability.

1.2 Drugs Administration and Cell Viability Analysis

For cell viability analysis, cells were grown on 96-well culture plates at a density of 8000 cells/100 μL, and then the cells were exposed to different concentrations (0, 0.1, 1, 10 μmol/L) of TPPU for 24 h at 37°C, which was followed by H_2O_2 (100 μmol/L) incubation for another 24 h. Following the treatment, the cell viability was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

For Western blotting and immunofluorescence, cells were grown on 6-well or 24-well culture plates, and then the cells were exposed to 1 μmol/L TPPU for 24 h and followed by 100 μmol/L H_2O_2 for 24 h or 100 μmol/L WT for 1 h at 37°C.

1.3 MTT assay

MTT was dissolved in PBS to give a concentration of 5 mg/mL. MTT solution (20 μL) was then added into each well to yield a final volume of 100 μL/well. Plates were incubated for 4 h at 37°C. The media were replaced with 100 μL DMSO to dissolve formazan crystals during 20-min shaking on the orbital shaker. The absorbance of the formazan product was measured using a Synergy™ H4 Hybrid Microplate Reader (BioTek Inc., USA) at a wavelength of 570 nm. Assays were repeated independently three times and cell viability was presented as the percentage of the treated cells against the vehicle control.

1.4 Measurement of SOD and MDA

Cells were treated with 1 μmol/L of TPPU or medium for 24 h at 37°C, which was followed by H_2O_2 (100 μmol/L) incubation for another 24 h. At the end of the treatment, cells were washed and homogenized by three sonication cycles, and centrifuged at 12 000 r/min for 15 min at 4°C. The protein concentration was quantified by BCA reagent kit (Boster, China). MDA and SOD were determined with a commercial kit from Jiancheng Bioengineering Institute (China). MDA, a metabolite of lipid peroxides, was used as an indicator of lipid peroxidation. The level of MDA in the supernatant was determined based on measuring the malondialdehyde formed by the thiobarbituric acid reaction. N-butyl alcohol was used for extraction during this process. Four mL n-butyl alcohol was added into each tube of mixed solution prepared according to the instruction, centrifuged at 3 000 *g* for 10 min, put at 4°C overnight, and finally, the supernatants were detected at excitation wavelength of 485 nm and emission wavelength of 528 nm next day.

Total SOD activity in the supernatant was determined based on its ability to inhibit the oxidation of hydroxylamine by the xanthine-xanthine oxidase system. The mauve product (nitrite) produced by the oxidation of hydroxylamine has an absorbance at 550 nm. One unit of SOD activity was defined as the amount that reduced the absorbance at 550 nm by 50%.

1.5 Western Blotting

Western blotting was used to analyze the protein levels of GSK3β (12456, 1:1000 dilution, Cell Signaling Technology, USA), phospho-Ser9-GSK-3β (5558, 1:1000 dilution, Cell Signaling Technology, USA), phospho-Tyr216-GSK-3β (ab75745, 1:1000 dilution, Abcam, USA), phospho-Ser473-Akt (5012, 1:500 dilution, Cell Signaling Technology, USA), Akt (4685, 1:1000 dilution, Cell Signaling Technology, USA), β-actin (3700, 1:1000 dilution, Cell Signaling Technology, USA), phospho-Ser396-Tau (9632, 1:1000 dilution, Cell Signaling Technology, USA), and phospho-Thr231-Tau (AB9668, 1:1000 dilution, Millipore, USA).

Cells were rinsed in ice-cold phosphate buffer saline (PBS) and solubilized in lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 2 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100, 0.5 mmol/L PMSF, 1:1000 cocktail) and sonicated on ice for 30 min. After centrifugation at 10 000 *g* for 15 min at 4°C, supernatants were removed and one third volume of 4× sample buffer (200 mmol/L Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 10% β-mercaptoethanol, and 0.05% bromophenol blue) was added, boiled for 10 min, and then stored at –20°C. Samples were run on 10% polyacrylamide SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Non-specific binding was blocked with 5% fat-free milk in PBS with 0.1% Tween 20 at room temperature for 1 h. The membranes were then incubated with primary antibodies, followed by anti-rabbit or anti-mouse IgG antibody conjugated to IRDyeTM (800CW; Licor Biosciences, USA) for 1 h at room temperature, and visualized using the Odyssey Infrared Imaging System (Licor Biosciences). Western blotting results were quantified using ImageJ software.

1.6 Immunofluorescence

HEK293/tau cells were grown on cover slips. After drug treatment, the cells were fixed with 4% PFA for 20 min, permeabilized with 1% Triton X-100 for 10 min and blocked with 3% BSA in PBS for 1 h at room temperature. Next, primary phospho-Thr231-Tau (1:200) antibody prepared in PBS was added, and incubated on the cover slips overnight at 4°C. After washing with PBS, fluorescent Texas Red tag conjugated goat-anti-rat IgG (1:200) secondary antibody was added, and the slides were incubated at room temperature for 1 h. Slides were further stained for nuclei with DAPI (Sigma-Aldrich, Germany). Images were acquired at room temperature with an Olympus fluorescence microscope fitted with filters for Texas Red and DAPI. The digital images were captured with a CCD camera.

1.7 Statistical Analysis

Data were presented as $\bar{x}\pm s$ and analyzed by the unpaired 2-tailed Student *t* test for 2-population com-

parison and by one way analysis of variance with Dunnett post hoc test for multiple comparisons. Statistical significant differences in the mean values were defined as $P<0.05$.

2 RESULTS

2.1 Effect of TPPU on H_2O_2-induced Cytotoxicity in HEK293/tau Cells

MTT assay showed that only 43.40%±0.77% of the cells survived after treatment with 100 μmol/L H_2O_2 in the absence of TPPU, and the cell viability was increased to 50.50%±1.72% and 67.60%±7.50 % after pretreatment with TPPU at a concentration of 100 nmol/L and 1 μmol/L, respectively (fig. 1A). Meanwhile, TPPU pretreatment was not found to cause any significant cytotoxic effect at the concentration of 0.1 μmol/L and 1 μmol/L (fig. 1A). However, the cell viability decreased significantly after 10 μmol/L TPPU treatment (data not shown), and therefore we chose 1 μmol/L TPPU in the following experiments.

Additionally, it was found that the MDA levels were significantly increased and the SOD activities profoundly decreased after treatment with 100 μmol/L H_2O_2, which could be reversed by pretreatment with 1 μmol/L TPPU (fig. 1B and 1C).

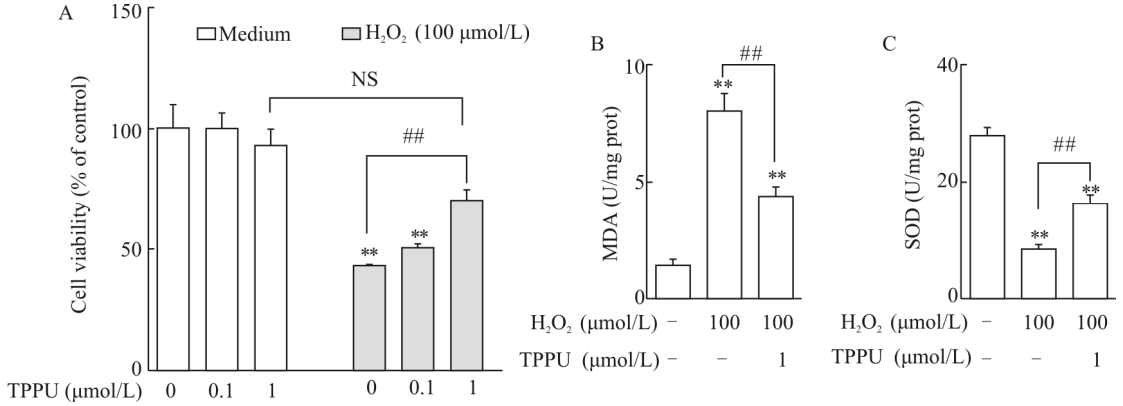

Fig. 1 TPPU reduced H_2O_2-induced cytotoxicity in HEK293/tau cells
A: cell viability after TPPU and H_2O_2 treatment (n=5 per group), $^{*}P<0.05$, $^{**}P<0.01$ *vs*. medium; $^{\#\#}P<0.01$; B, C: MDA levels (B) and SOD activities (C) of cells treated with TPPU and H_2O_2 (n=3 per group). $^{*}P<0.05$, $^{**}P<0.01$ *vs*. medium; $^{\#\#}P<0.01$

2.2 TPPU Protected Tau from H_2O_2-induced Hyperphosphorylation in HEK293/tau Cells

Oxidative stress has been implicated to play a crucial role in the pathogenesis of AD and has been observed in the AD brain[12–15]. It was found that treatment with 100 μmol/L H_2O_2 significantly increased the levels of hyperphosphorylation of tau at the sites Thr231 and Ser396, with no change in total tau immunoreactivity (fig. 2A and 2B). Immunofluorescence staining also revealed the similar results (fig. 3A and 3B).

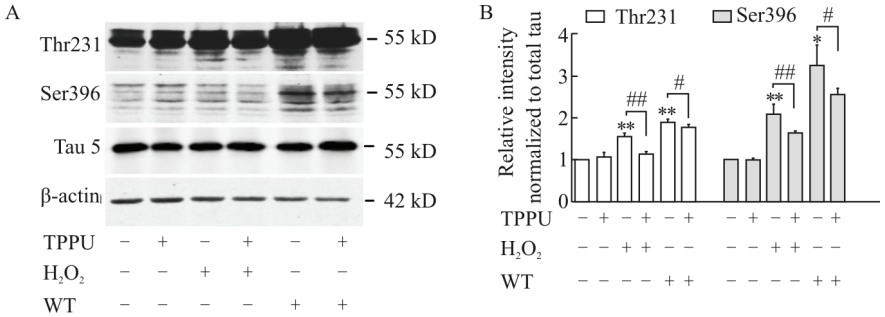

Fig. 2 TPPU protected Tau from H_2O_2-induced hyperphosphorylation in HEK293/tau cells
A: Western blot analysis of Tau posphorylation after treatment with H_2O_2, TPPU and WT; B: the statistical graph (n=3 per group). $^{*}P<0.05$, $^{**}P<0.01$ *vs*. medium; $^{\#}P<0.05$, $^{\#\#}P<0.01$

2.3 TPPU Inactivated GSK-3β by Phosphorylating Its Ser 9 Residue in H_2O_2-induced Oxidation

GSK-3β is a key signaling molecule highly expressed in the CNS and has been suggested to play an important role in tau hyperphosphorylation[3]. To verify the effects of TPPU on GSK-3β, we measured the total GSK-3β (tGSK-3β) level, the activity-dependent phosphorylation of GSK-3β at Ser 9 (pS9GSK-3β, inactive form) and phosphorylation of GSK-3β at Tyr 216 (pT216GSK-3β, active form) in the cell lysate by Western blotting. We found that the level of pS9GSK-3β decreased significantly in H_2O_2-treatment group, whereas the level of total GSK-3β was not changed and quantitative analysis showed that the ratio of pS9GSK-3β/GSK-3β decreased to 77.5%±3.5% of the control level (fig. 4A and 4B). Supplement of TPPU restored the ratio of pS9GSK-3β/GSK-3β to normal level, which might prevent tau hyperphosphorylation.

Additionally, we found that the level of pT216GSK-3β was not changed after TPPU or H_2O_2

treatment, which suggested that the decreased activity of GSK-3β after TPPU supplementation mainly depended on its Ser9 phosphorylation (fig. 4A and 4B).

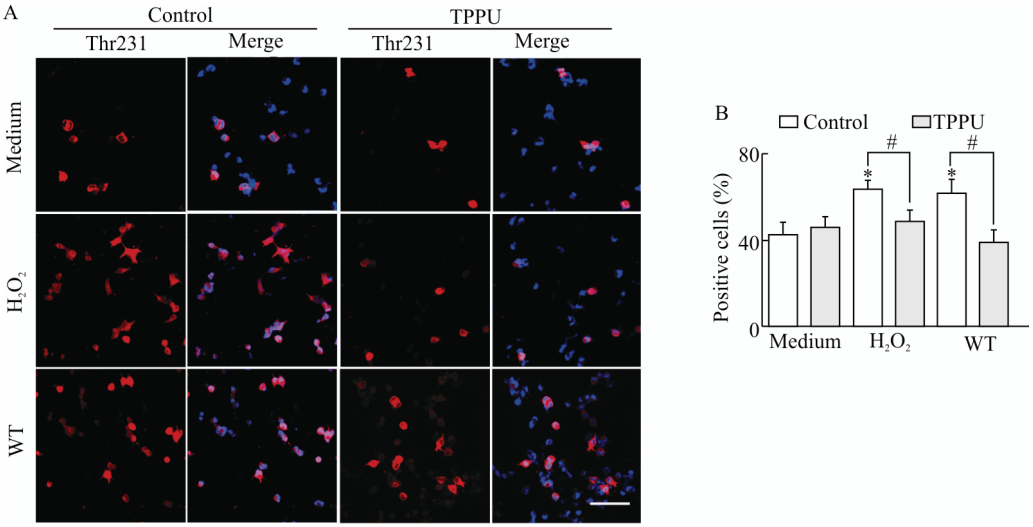

Fig. 3 Immunofluorescence results of tau posphorylation in HEK293/tau cells treated with H_2O_2 and TPPU

A: immunofuoresence images (scale bar=100 μm); B: the statistical graph (n=3 slices per group). $^{*}P$<0.05 *vs*. medium, $^{\#}P$<0.05

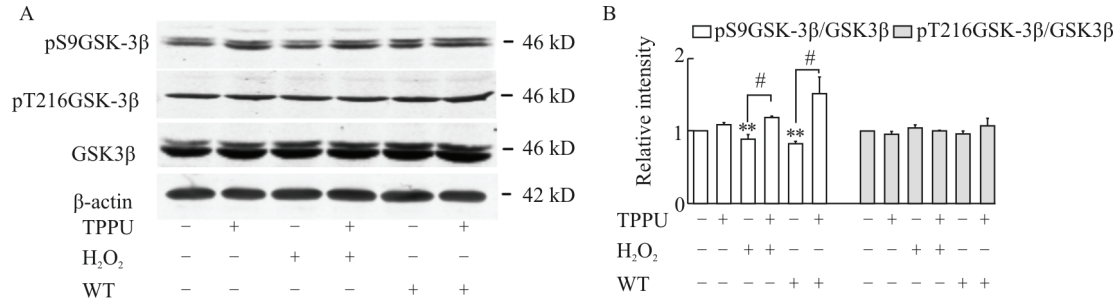

Fig. 4 TPPU inactivated GSK-3β by phosphorylating its Ser9 residue in H_2O_2-induced oxidation.

A: Western blot analysis of pS9GSK-3β, pT216GSK-3β, total GSK-3β after TPPU, H_2O_2 or WT treatment; B: the statistical graph (n=3 per group). $^{**}P$<0.01 *vs*. medium; $^{\#}P$<0.05

2.4 TPPU Protected Tau from H_2O_2-induced Hyperphosphorylation in HEK293/tau Cells by Inactivating PI3K/AKT/GSK-3β Pathway

The PI3K pathway plays a critical role in the tau phosphorylation. PI3K activates Akt, which in turn inactivates GSK-3β by inhibition of phosphorylation of its Ser9 residue[16]. Thus, to further explore the potential signaling pathways contributing to the anti-hyperphosphorylation effect of TPPU, we examined Akt activation. We found that H_2O_2 treatment decreased Akt phosphorylation at the site Ser473 compared to the control, but the decreased phosphorylation of Akt was recovered by TPPU pretreatment (fig. 5A and 5B).

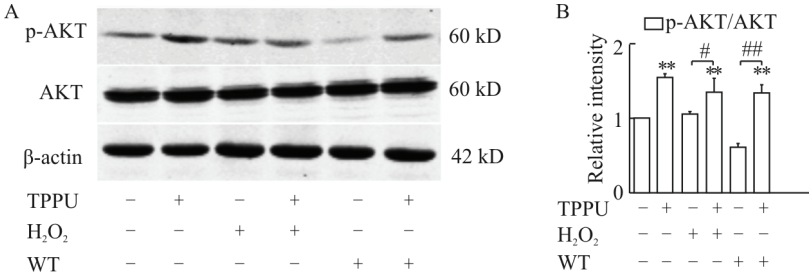

Fig. 5 TPPU protected Tau from H_2O_2-induced hyperphosphorylation in HEK293/tau cells by inactivating PI3K/AKT/GSK-3β pathway.

A: Western blot analysis of phosphorylated Akt at the site Ser473 after TPPU, H_2O_2 or WT treatment; B: the statistical graph (n=3 per group). $^{**}P$<0.01 *vs*. medium; $^{\#}P$<0.05, $^{\#\#}P$<0.01

To confirm that the maintenance phosphorylation of Akt/pS9GSK-3β pathway contributes to the protective effect of TPPU, we pretreated cells with TPPU and applied WT for 1 h, and measured tau hyperphosphorylation using Western blot analysis. We found that treatment with WT significantly increased the levels of hyperphosphorylation of tau at Thr231 and Ser396 (fig. 2A and 2B, fig. 3). However, the increased hyperphosphorylation of tau was significantly inhibited by treatment with TPPU (fig. 2A and 2B, fig. 3). In line with expectations, the levels of p-AKT and pS9GSK-3β decreased by WT treatment, whereas TPPU recovered the phosphorylation of these sites (fig. 4 and fig. 5). These results suggested that the cytoprotective effect of TPPU on the H_2O_2-induced tau hyperphosphorylation of HEK/293 cells is mediated at least in part via the PI3K/Akt/GSK-3β signaling pathways.

3 DISCUSSION

In the present study, we investigated the anti-oxidative effects of TPPU on H_2O_2-induced tau hyperphosphorylation and showed that pretreated TPPU decreased the levels of tau phosphorylation after H_2O_2-induced oxidative stress. Moreover, TPPU maintained phosphorylation of AKT at Ser473 and phosphorylation of GSK-3β at Ser9, which was blocked by the PI3K/AKT inhibitor WT, suggesting that PI3K/AKT/GSK-3β signaling pathway is involved in the inhibitory effects of TPPU on tau hyperphosphorylation.

EETs are synthesized by cytochrome P450 epoxygenases from arachidonic acid and terminated primarily by sEH to the vicinal DiHETs[17]. It has been reported that EETs regulate anti-apoptotic[18, 19], anti-inflammatory[20], angiogenic[21, 22], and anti-oxidant activities[10] and so on. EETs act their cellular effects by multiple signaling pathways including the cAMP/protein kinase A (PKA), PI3K-Akt and mitogen-activated protein kinase cascades[17]. However, delivering EETs directly to injured organs or tissues is impractical because they can be metabolized rapidly by sEH into their less active DiHETs[23, 24]. Inhibition of sEH activity through pharmacological blockade or genetic deletion maintains EETs level by reducing the degradation of EETs and promoting the accumulation of EETs in blood and tissue[10]. Here, we applied a sEH inhibitor TPPU to HEK293/tau cells and found that it exhibited anti-oxidative effects and protected cells from tau hyperphosphorylation by regulating PI3K/AKT/GSK-3β signaling pathway.

Oxidative stress plays various roles in cell signaling, including apoptosis, cell proliferation, gene expression, and the activation of cell signaling cascades[25, 26]. Employing 100 μmol/L H_2O_2 to create the oxidative stress condition, we found that the level of phosphoylated GSK-3β Ser9 residue in H_2O_2-treated HEK293/tau cells was significantly decreased compared with that in control cells. Moreover, there were no obvious changes of phosphorylation of GSK-3β at Tyr216 after H_2O_2 administration, which suggested that regulation of GSK-3β activity via phosphorylation at Tyr216 may not contribute much to oxidative stress.

An increased phosphorylation of tau at Thr231 has been proposed to be an early event in the pathogenic processes of AD because the hyperphosphorylation of tau at this site in cerebrospinal fluid (CSF) is strongly associated with future development of AD in patients with mild cognitive impairment[27]. Thr231 phosphorylation is involved in the impeding of microtubule-binding of tau[28].

GSK-3β is a major tau kinase playing a crucial role in AD-like tau hyperphosphorylation[3] and its activity can be inhibited by AKT phosphorylated at Ser9 site. When phosphorylation at Ser9 is increased, the signaling activity of GSK-3β is inhibited, and when phosphorylation at Ser9 is decreased, its activity is enhanced[29]. In our study, we found that TPPU maintained GSK-3β Ser9 site phosphorylation but not Tyr216 and protected tau from aberrant hyperphosphorylation in oxidative stress.

The PI3K/AKT/GSK-3β pathway is pivotal to maintenance of the neuronal network, cell survival, and longevity. Dysregulation of this signaling pathway is the major mechanism underlying the pathology of AD[8, 30]. The involvement of TPPU in the PI3K/Akt signaling pathway was further examined in the present study and it was observed that treatment with TPPU increased Akt Ser473 phosphorylation, thereby resulting in an increased pAkt/Akt ratio. Western blot analysis demonstrated that administration of TPPU suppressed WT-induced Akt Ser473 phosphorylation, thus indicating that TPPU exerts its cytoprotective via PI3K/AKT/GSK-3β pathway.

In conclusion, our research demonstrated a protective effect of TPPU on H_2O_2-induced hyperphosphorylation of tau, and this protective role is associated with PI3K/Akt/GSK-3β pathway, which may provide an alternative for the treatment of AD and other tauopathies.

Conflict of Interest Statement

The authors declare no conflict of interest.

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