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Knockdown of Phosphotyrosyl phosphatase activator (PTPA) induces apoptosis via mitochondrial pathway and the attenuation by simultaneous tau hyperphosphorylation

Dan-Ju Luo, Qiong Feng, Zhi-Hao Wang, Dong-Sheng Sun, Qun Wang, Jian-Zhi Wang*, Gong-Ping Liu*

Department of Pathophysiology, Key Laboratory of Chinese Ministry of Education for Neurological Diseases, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, PR China

*Correspondence to: Gong-Ping Liu, E-mail: liugp111@mail.hust.edu.cn; Jian-Zhi Wang, E-mail: wangjz@mails.tjmu.edu.cn. Department of Pathophysiology, Key Laboratory of Neurological Diseases of Education Committee of China, Tongji Medical College, Huazhong University of Science and Technology, 13 Hangkong Road, 430030 Wuhan, PR China. Tel.: +86 27 83692625; Fax: +86 02783692625;

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Abstract

Phosphotyrosyl phosphatase activator (PTPA) is decreased in the brains of Alzheimer's disease (AD) and the AD transgenic mouse models. Here, we investigated whether downregulation of PTPA affects cell viability and the underlying mechanisms. We found that PTPA was located in the integral membrane of mitochondria, and knockdown of PTPA induced cell apoptosis in HEK293 and N2a cell lines. PTPA knockdown decreased mitochondrial membrane potential and induced Bax translocation into the mitochondria with a simultaneous release of Cyt C, activation of caspase-3, cleavage of PARP, and decrease of Bcl-xl and Bcl-2 protein levels. Overexpression of PP2A catalytic subunit ($PP2A_C$) did not rescue the apoptosis induced by PTPA knockdown, and PTPA knockdown did not affect the level of and their phosphorylation of MAPKs, indicating that PP2A and MAPKs were not involved in the apoptosis induced by PTPA knockdown. In the cells with overexpression of tau, PTPA knockdown induced PP2A inhibition and tau hyperphosphorylation but did not cause significant cell death. These data suggest that PTPA deficit causes apoptotic cell death through mitochondrial pathway and simultaneous tau hyperphosphorylation attenuates the PTPA-induced cell death.

Keywords: PTPA; Apoptosis; Alzheimer's disease; mitochondrial pathway; Cyt C; tau hyperphosphorylation

Introduction

Protein phosphatase 2A (PP2A), one of the four major classes of eukaryotic serine/threonine protein phosphatases (Wera & Hemmings 1995), plays important roles in the regulation of a wide range of cellular processes including metabolism, motility, cell division, proliferation, signaling, and gene expression (Mumby & Walter 1993). The core structure is composed of a constant 65 kDa structural subunit A and

a 36 kDa catalytic subunit C, which associates with a variable regulatory subunit B to form the PP2A holoenzyme. Regulatory subunit B has three subfamilies, B, B' and B'' regulatory subunit. These different regulatory subunits confer distinct enzymatic specificities and intracellular localizations to the holoenzyme (Cohen 1989).

Peptidyl prolyl cis/trans isomerases (PPIases) are ubiquitous proteins that catalyze the cis-trans isomerization of proline residues in polypeptide chains. Phosphotyrosyl phosphatase activator (PTPA), which encoded by the PPP2R4 gene in humans, has been reported to possess an *in vitro* PPIase activity on model substrates (Jordens *et al.* 2006). PTPA was initially reported to increase the weak phosphotyrosyl phosphatase activity of Ser/Thr phosphatase PP2A (Cayla *et al.* 1990). Recent findings suggest that its physiological function is more likely to reactivate the Ser/Thr phosphatase activity of an inactive form of PP2A (PP2Ai) which can be isolated as a complex with protein phosphatase methylesterase (PME1), which specifically demethylates PP2A(Longin *et al.* 2004). Our recent finding indicated that PTPA activates PP2A through reducing the level of phosphorylated at tyrosine-307 of $PP2A_C$ by activating protein tyrosine phosphotase 1B (PTP1B) (Luo *et al.* 2013). PTPA was found to be a highly conserved protein during evolution, which has been found from yeast to human (Van Hoof *et al.* 2000), suggesting an important biological function of this protein. Deletion of YPA1 and YPA2, the homologous gene of PTPA in yeast, is lethal (Van Hoof *et al.* 2001), and deletion of YPA1 leads to an aberrant bud morphology, abnormal actin distribution, and growth defects (Van Hoof *et al*. 2000). The PTPA gene is located on chromosome 9q34 which has been revealed as a hot spot for melanoma and leukemia (Van Hoof *et al.* 1995).

Recently, some other physiological functions of PTPA were gradually revealed. Deletion of the RRD1 (rapamycin resistant deletion) gene, the homologous gene of PTPA in yeast reduces the stability of PP2A and alters its substrate specificity (Fellner *et al.* 2003). RRD1 is a transcription elongation factor that is associated with RNA polymerase II (RNAPII) on actively transcribed genes, and is involved in regulating the expression of ribosomal protein genes and starvation genes in response to

rapamycin exposure (Douville *et al.* 2006). RRD1 is reported to interact with and isomerize RNAPII, more specifically with the C-terminal domain of RNAPII, altering the structure of this C-terminal domain in response to rapamycin (Jouvet *et al.* 2010, Poschmann *et al.* 2011). This phenomenon was confirmed to be directly mediated by RRD1 *in vitro*, presumably through its peptidyl prolyl isomerase activity (Jouvet *et al*. 2010). PTPA ovexpression induced tumor cell lines HCT116 apoptosis with manifest hallmarks of apoptosis including chromatin condensation, membrane blebbing, positive staining with annexin V, dephosphorylation of Bad, and caspase-3 cleavage (Azam *et al.* 2007).

Alzheimer's disease (AD) is the most common human neurodegenerative disorder characterized by the presence of extracellular senile plaques, intracellular neurofibrillary tangles, and massive loss of neurons, which is accompanied with a progressive loss of memory and deterioration of cognitive functions. Studies suggest that the number of tangles is positively correlated with the degree of dementia in AD patients (Arriagada *et al.* 1992), suggesting a crucial role for tau hypephosphorylation in neurodegeneration and cognitive impairments. However, the upstream factors leading to tau hyperphosphorylation and the mechanisns underlying tau-involved neurodegeneration are not fully understood. Recently, we found that PTPA is decreased in brains of AD patients and tg2576 mice (Luo *et al*. 2013). In this study, we explored the effects of PTPA knockdown on cell viability. We found that PTPA knockdown induced cell apoptosis via mitochondrial apoptotic pathway, which involves Cyt C release, Bax translocation and caspase-3 activation, with decreasing anti-apoptotic Bcl-2 and Bcl-xl proteins levels in HEK293 or N2a cell lines without or only with very little tau expression (~0.1 ng/10^6 cells) which therefore does no interfere with the analysis (Li *et al.* 2007a, Khlistunova *et al.* 2006, Biernat *et al.* 2002). We also found that the PTPA knockdown-induced apoptosis is independent of $PP2A_C$ and MAPKs. However, knockdown of PTPA in HEK293/tau cells induced tau phosphorylation to resist the apoptosis induced by PTPA knockdown.

Materials and methods

Chemicals and antibodies

Cytoplasmic and nucleus extraction kit, mitochondrial membrane potential (MMP) detection kit, cell counting kit-8 (CCK-8) reagent and ATP assay kit were purchased from Beyotime (China). BCA Protein Assay kit was purchased from Pierce (Rockford, IL, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was purchased from Invitrogen. The PTPA knockdown plasmid (siPTPA) and its control plasmid (scrambled siPTPA, ssiPTPA) were synthesized by NeuronBiotech company (Shanghai, China), the shRNA sequences for siPTPA were the same as previously used (Luo *et al*. 2013):
5'-AGCTTCGTTCCCTGTGATCCAGCACTTCAAGAGAGTG

CTGGATCACAGGGAACTTTTTTGGAAC-3',
5'-TCGAGTTCCAAAAAAGTTCCCTGTGATCCAGCACTCTCTTGAAGTGCTGGATCACAGGGA ACGA-3'. The non-sense sequences for ssiPTPA is:
5'-AGCTTCATACCGCTCAGTAGCGACATTCAAGAGATGTCGCTACTGAGCGGTATTTTTTTG GAAC-3',
5'-TCGAGTTCCAAAAAAATACCGCTCAGTAGCGACATCTCTTGAATGTCGCTACTGAGCGGT ATGA-3'. PcDNA3.0-wt-PP2AC was a gift of Dr. Haendeler (University of Frankfurt, Frankfurt, Germany). The antibodies used were listed in Table 1.

Table 1

Name	Species	Application	dilution ratio	source
PTPA	Mouse	WB	1:500	upstate
Lamin B1	Rabbit	WB	1:1000	abcam
caspase-3	Rabbit	WB	1:1000	cell signaling
cleaved caspase-3	Rabbit	WB/IF	1:1000/1:50	cell signaling

cleaved PARP	Rabbit	WB/IF	1:1000/1:50	cell signaling
PARP	Rabbit	WB	1:1000	cell signaling
Cyt C	Mouse	WB/IF	1:500/1:50	RD
Cox IV	Mouse	WB	1:500	abcam
Tom40	Rabbit	WB	1:500	abcam
PDI	Rabbit	WB	1:500	abcam
GM130	Mouse	WB	1:500	BD Transduction
LC3	Rabbit	WB	1:500	cell signaling
Bax	Rabbit	WB/IF	1:500/1:50	chemicon
Bad	Rabbit	WB	1:500	stressgen
p-Bad	Mouse	WB	1:500	santa cruz
Bcl-2	Rabbit	WB	1:1000	abcam
Bcl-xl	Rabbit	WB	1:1000	abcam
PP2Ac	Mouse	WB	1:1000	Millipore
ERK1/2	Rabbit	WB	1:500	cell signaling
p- ERK1/2	Rabbit	WB	1:500	cell signaling
p38	Rabbit	WB	1:500	cell signaling
p-p38	Rabbit	WB	1:500	cell signaling
JNK	Mouse	WB	1:500	sigma
p-JNK	Rabbit	WB	1:500	cell signaling
pS396	Rabbit	WB	1:1000	SAB
pS404	Rabbit	WB	1:1000	SAB
tau1	Mouse	WB	1:1000	Millipore
tau5	Mouse	WB	1:1000	Millipore

p-PP2Ac(Tyr 307)	Rabbit	WB	1:500	abcam
M-PP2Ac(Leu 309)	Mouse	WB	1:500	Upstate
DM-PP2Ac(Leu 309)	Mouse	WB	1:500	Millipore
GFP	Mouse	WB	1:1000	abcam
GAPDH	Mouse	WB	1:1000	abcam
DM1A	Mouse	WB	1:1000	sigma

Cell culture and transfection

Wild-type mouse neuroblastoma N2a cells (N2a/wt) were maintained in a medium containing 50% Dulbecco's modified Eagle's medium (DMEM) and 50% Opti-MEM, supplemented with 5% fetal bovine serum (GibcoBRL, Grand Island, NY, USA). Wild-type human embryonic kidney 293 (HEK293/wt) or HEK293 cells with stable expression of the longest human tau (tau441) cDNA (HEK293/tau) were cultured in a medium containing 90% DMEM and 10% fetal bovine serum. The cells were maintained at 37 ºC in humidified atmosphere containing 5% CO_2.

HEK293/wt, N2a/wt and HEK293/tau cells were plated into six-well plates overnight to 70% saturation and plasmids were transfected using Lipofectamine 2000 according to the manufacturer's instruction.

Preparation of cytoplasmic and nuclear extracts

Cytoplasmic and nuclear extracts were prepared using the cytoplasmic and nuclear extraction kit according to the manufacturer's instructions (Beyotime, China). Briefly, HEK293/wt cells were gathered by centrifugation, medium was discarded and the pellets were washed in ice-cold PBS and resuspended by pipetting

up and down ten times in 200 µl of ice-cold cell lysis buffer. After maintained on ice for 15 min, cell lysis were centrifuged in a refrigerated centrifuge (Thermo Scientific) at 12,000 rpm for 5 min at 4 ºC and supernatants were aliquoted and stored at -20 ºC for western blotting analysis. The pellets were then washed in 500 µl of ice-cold cell lysis buffer and resuspended in 100 µl of nuclear extraction buffer. After vigorously shaking at 4 ºC for 30 min, nucleus extracts were aliquoted and stored at -20 ºC until use.

Preparation of mitochondrial fractionation

Mitochondrial fractionation of HEK293/wt cells was carried out as described (Parone *et al.* 2006). Briefly, for cells, HEK293/wt cells were collected in ice-cold mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.5) with 1 mM PMSF and cocktail (Roche). Cells were broken by 15 passages through a 25-gauge needle, and the suspension was centrifuged for 5 min at 1,500 g at 4 ºC. For rat cortex, 100~200 mg cortex was washed with PBS buffer or normal saline (NS), dried with filter paper, then cut the tissue into pieces and added them into a small capacity glass homogenizer. Add 1 ml of ice-cold mitochondrial buffer with 1 mM PMSF and cocktail (Roche), Grinding tissue 20 times in ice bath and the suspension was centrifuged for 5 min at 1,500 g at 4 ºC. The supernatant (whole cell lysate) was further centrifuged at 16,000 g for 30 min. The supernatant (cytosolic fraction) was separated from the pellet (mitochondrial fraction). For analysis of membrane protein, mitochondrial pellets were resuspended in 100 µl of mitochondrial buffer, or mitochondrial buffer containing 0.1 M Na_2CO_3 (pH 11.5) and incubated on ice for 30 min. The insoluble membrane fractions were centrifuged at 16,000 g for 15 min, and the supernatants were precipitated with 10% (v/v) trichloroacetic acid.

Western blotting

Proteins were separated by 10% or 12% (for cleaved caspase-3 and Cyt C) SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, NJ, USA). The blots were incubated overnight with corresponding primary antibodies at 4 ºC, and then the blots were detected using anti-rabbit or anti-mouse IgG secondary antibody conjugated to IRDyeTM (800CW, Li-Cor Biosciences, Lincoln, NE) at room temperature for 1 h and visualized by infrared fluorescence imaging. The optical density of bands was quantified by Odyssey system (Li-Cor Bioscience, Lincoln, NE). The alterations of detected proteins were normalized against DM1A.

CCK-8 and MTT assay

HEK293/wt or N2a/wt cells were plated onto 96-well plates, using a hemocytometer to make sure the number of cells in each hole was consistent and no more than $5\times10^3/cm^2$ and ssiPTPA or siPTPA plasmid was transfected using Lipofectamine 2000 according to the manufacturer's instruction. For CCK-8 assay, media were removed and 90 µl fresh media plus 10 µl kit reagent were added into the cells and incubated in cell incubator for 1 h protected from light, cell viability was assessed using the ELISA plate reader at 450 nm immediately. For MTT assay, media were discarded and 200 µl fresh medium plus 50 µl MTT (5 mg/ml) reagent were added into the cells and incubated in cell incubator for 4 h protected from light, then the mixture were removed and 200 µl DMSO were added to dissolve the formazan, cell viability was assessed using the ELISA plate reader at 450 nm immediately.

MMP assay

The HEK293/wt or N2a/wt were seeded into 12-well plates, at a density of 2×10^4 cells per well and then transfected with ssiPTPA or siPTPA plasmid for 48 h as

described above, the cells were incubated with the mixture of 250 µl JC-1 staining fluid and 250 µl cell culture medium in the dark at 37ºC for 20 min. Subsequently, the cells were washed twice with the staining buffer preserved in 4 ºC. Lastly, 500 µl cell culture medium was added into each well and the fluorescence intensity of both mitochondrial JC-1 monomers (λex 514 nm, λem 529 nm) and aggregates (λex 585 nm, λem 590 nm) were detected using a monochromator microplate reader. The values of MMP staining from each sample were expressed as ratio of red fluorescence (i.e. aggregates) to green fluorescence (i.e. monomers).

ATP assay

The level of ATP in HEK293/wt cell lines was determined using the ATP Assay Kit. Briefly, cells were harvested and lysed with the lysis buffer, followed by centrifugation at 10,000×g for 2 min at 4 ºC, then 50 µl supernatant and 50 µl luciferase reagents were mixed to catalyze the light production from ATP and luciferin. The emitted light was measured using a microplate luminometer and linearly related to the ATP concentration. Total ATP levels were expressed as nmol/mg protein

Statistical analysis

All experiments were repeated at least three times. Experimental values were obtained from three independent experiments with a similar pattern and expressed as means ± SD. Statistical analyses were performed using SPSS software package 12.0 (SPSS, Chicago, IL, USA). Statistical significance between two groups was determined by Student's two-tailed t-test with 95% confidence.

Results

Localization of PTPA

Firstly, we detected the expression of PTPA in cell lines (HEK293/wt and N2a/wt), primary rat neurons and rat cortex by Western blotting, and we found that PTPA exists in not only cell lines but also rat primary neuron cultures and rat cortex (Fig. 1A, B).

To clarify the intracellular distribution of PTPA, we detected the subcellular localization of PTPA. We found that PTPA existed in the cytoplasm, nucleus (Fig. 1C) and mitochondrial fraction of HEK293/wt cells (Fig. 1D). We also found that PTPA expressed in the mitochondrial fraction of rat cortex (Fig. 1E). Prudently, we also confirmed the purification of mitochondrial fraction by some specific antibodies - PDI (protein disulfide isomerase), GM130 and LC3 which were used as the marker of endoplasmic reticulum (ER), Golgi apparatus and lysosome respectively. The result showed that the mitochondria fraction were not been contaminated (Fig. S1). In order to determine the topology of PTPA in mitochondria, we treated the purified mitochondria with a high-pH wash (Na_2CO_3) that can release non-specific membrane-associated proteins (Zhao *et al.* 2009). We found that PTPA were largely precipitated in control buffer. Na_2CO_3 treatment dissolved Cyt C, a protein marker of mitochondrial intermembrane space, into the supernatant fraction, however, PTPA and the integral membrane proteins, Tom40 and Cox IV, were still retained in the pellet after treatment with Na_2CO_3. These results suggested that PTPA, like Tom40 and Cox IV, is an integral mitochondrial membrane protein (Fig. 1F).

PTPA knockdown induced apoptosis in cell lines without or with litter tau expression

Previously we have found that PTPA is decreased in the brains of AD and the AD transgenic mouse models (Luo *et al*. 2013), so we detected whether downregulation

of PTPA affects cell viability, we firstly investigated the effects of PTPA knockdown in HEK293/wt or N2a/wt cells. Routinely, we detected the PTPA protein level after overexpression of siPTPA plasmid for 48 h in HEK293/wt or N2a/wt cells; we found that PTPA protein level decreased to ~47% or ~55% of the control level in HEK293/wt (Fig. 2A, B) or N2a/wt cells (Fig. 2C, D). Then we detected cell viability after overexpression of siPTPA for 48 h by cell counting kit-8 (CCK-8) and the results showed the cell viability of HEK293/wt or N2a/wt cells decreased by 25% or 20% compared with the control group while PTPA knockdown (Fig. 2E, F).

To further confirm the results that PTPA knockdown decreased cell survival, we measured the cell viability after siPTPA overexpression by MTT assay. We found that PTPA knockdown decreased the cell viability to ~82% or ~73% of the original level at 24 h or 48 h in HEK293/wt cells, respectively (Fig. 2G). We also got similar results in N2a/wt cells (Fig. 2H).

Caspase-3 is a frequently activated death protease among the caspases which are crucial mediators of apoptosis (Porter & Jänicke 1999), it is synthesized as an inactive proenzyme and cleaved to the active form in cells undergoing apoptosis (Cohen 1997). On the other hand, the activation of effector caspases such as caspase-3 leads to downstream cleavage of various cytoplasmic or nuclear substrates including PARP(Vaux & Strasser 1996, Li *et al.* 1998), so cleaved PARP was considered to be an indicator of apoptosis. Then, we detected the level of cleaved caspase-3 and cleaved PARP after PTPA knockdown in HEK293/wt cells. We found that PTPA knockdown significantly increased cleaved caspase-3 and cleaved PARP levels, though total caspase-3 or PARP had no significant changes (Fig. 2I). Taken together, the results suggested that knockdown of PTPA induced cell apoptosis in HEK293/wt or N2a/wt cell lines without or with litter tau expression.

PTPA knockdown decreased MMP and induced Bax translocation and Cyt C release

We have previously detected PTPA exists in not only cytoplasm but also mitochondria membrane, taking into account the special distribution pattern of PTPA, we predicted that there is some correlation between its downregulation-induced apoptosis and mitochondria.

To investigate the underlying mechanisms of PTPA knockdown induced apoptosis, we firstly detected the effects of PTPA knockdown on MMP. JC-1 could aggregate in mitochondria matrix to form polymers and present red fluorescence in normal cells, and disperse in the mitochondrial matrix to form monomers when the MMP is low, showing a green fluorescence. Therefore, the ratio of red fluorescence to green fluorescence, that is the ratio of polymers to monomers, can reflect the level of the MMP. We found that PTPA knockdown decreased the MMP (Fig. 3A). ATP levels were found to be decreased simultaneously while PTPA knockdown (Fig. S2).

It is reported that Bax translocates to mitochondria and leads to subsequent loss of MMP (Smaili *et al.* 2001) and there is evidence suggesting that Bax is involved in the pathway leading to the release of Cyt C (Arnoult *et al.* 2002), a molecular trigger of apoptosis. We next detected the alterations of Bax localization, and the result showed that Bax level in mitochondrial fraction was significantly increased after PTPA knockdown (Fig. 3B, C).

Cyt C was identified to be an apoptosis-related protein (Liu *et al.* 1996). Release of Cyt C from mitochondria to cytoplasm has been observed in mammalian cell lines exposed to various apoptotic stimuli (Arnoult *et al*. 2002) and is closely related to the decreasing of MMP (Gottlieb *et al.* 2003). We found that Cyt C protein level in cytoplasmic fraction increased significantly during PTPA knockdown (Fig. 3D, E).

We also detected the levels of anti-apoptotic proteins Bcl-2 and Bcl-xl (Adams & Cory 1998), and a pro-apoptotic protein Bad (Henshall *et al.* 2002). We found that

PTPA knockdown decreased Bcl-2 and Bcl-xl levels, though the levels of total and phosphorylated Bad had no significant changes (Fig. 3F, G).

$PP2A_C$ and MAPKs were not involved in PTPA knockdown induced-apoptosis

As previously described, PTPA can activate $PP2A_C$, and $PP2A_C$ has been reported to be important to maintain cell survival (Valdiglesias *et al.* 2011, Janzen *et al.* 2011). Inhibition of PP2A by okadaic acid (OA) induced apoptosis (Valdiglesias *et al*. 2011, Bøe *et al.* 1991). To investigate the role of $PP2A_C$ in PTPA knockdown induced-apoptosis, we detected cell survival while cotransfection with siPTPA and wild type $PP2A_C$ plasmids (wt-$PP2A_C$). We found that overexpression of $PP2A_C$ did not attenuate the cell apoptosis induced by PTPA knockdown (Fig. 4A, B).

As it is well known, MAPKs pathway plays an important role in apoptosis (Gupta *et al.* 1999, Yang *et al.* 2000), we also investigated whether MAPKs pathway was involved in the PTPA knockdown-induced apoptosis. We found the levels of total ERK, JNK, p38, and their phosphorylation forms had no significant changes during PTPA knockdown (Fig. 4C, D). These results suggested that $PP2A_C$ and MARKs were not involved in the cell apoptosis induced by PTPA knockdown.

PTPA knockdown had no effects on cell viability in HEK293/tau cells

Finally, we detected the effects of PTPA knockdown on cell viability in HEK293/tau cells. We found that cell viability had no significant difference compared with the control (Fig. 5A), and there were no positive cleaved caspase-3 or PARP staining band while overexpression of siPTPA (Fig. 5B). We also found that PTPA knockdown inactivated PP2A by increasing its phosphorylation level of tyrosine 307, and decreasing its methylation level of leucine 309, inducing tau phosphorylation at Ser396 (pS396), Ser404 (pS404) and 198/202 (tau-1) epitopes (Fig. 5C-E).

Discussion

Alzheimer's disease is an age-related neurodegenerative disorder that is characterized by progressive damage of cognitive functions and loss of memory. The brain of an individual with AD exhibits extracellular senile plaques composed of Aβ protein, and intracellular neurofibrillary tangles composed of hyperphosphorylated tau proteins (Barinaga 1998). Caspase activation-mediated events that trigger cytochrome c release and apoptosis have been identified in the affected brain region of AD (Sleegers & Van Duijn 2002). However, the neurons bearing hyperphosphorylated tau and tangles undergo chronic degeneration rather than acute apoptosis during the development of AD, suggesting at least a temporal neuroprotective effect of tau hyperphosphorylation and the formation of tau filaments (Li *et al*. 2007a). In the present study, we found that PTPA knockdown induces cell apoptosis through decreasing MMP and translocating Bax into mitochondria to release Cyt C in cell lines with or without litter tau expression. However, knockdown of PTPA induced tau phosphorylation through inactivation of PP2A to resist PTPA knockdown induced-apoptosis in HEK293/tau cells with robust tau expression.

Mitochondrial apoptotic pathway is the main apoptotic pathway and is currently a research hotspot. The mitochondrial pathway of apoptosis can be summarized as follows: mitochondrial upstream signaling molecules acting on the mitochondrial membrane and causing Bax to shift to the outer mitochondrial membrane to form protein channel, and mitochondrial permeability transition pore opening to release apoptotic effectors such as Cyt C, Smac and other specific proteins to activate the caspase family proteins to act on the corresponding substrate and induce apoptosis (Mohamad *et al.* 2005). In the present study, we found that knockdown of PTPA by siPTPA decreased MMP, and then released Cyt C into the cytoplasm to activate capsease-3 and PARP. We also detected the translocation of Bax, which is a requisite

process for mitochondrial apoptotic pathway (Wei *et al.* 2001). The mechanisms for MMP decrease induced by PTPA knockdown is still not clear now, however, the results that PTPA distributes in the integral membrane of mitochondria, suggest that PTPA protein may also contribute to the constitute of MMP, which requires further study.

Bcl-2 and Bcl-xl are two known anti-apoptotic genes most studied (Kroemer 1997, Muchmore *et al.* 1996), they were reported to block loss of MMP and the subsequent apoptosis (Breckenridge & Xue 2004). In the present study, we found that PTPA knockdown decreased Bcl-2 and Bcl-xl levels, which is also contributed to the decline of MMP.

PP2A is an important phosphatase for cell survival (Mumby & Walter 1993). PTPA is known to activate PP2A and knockdown of PTPA induced PP2A inactivation. To exclude the involvement of PP2A in the cell apoptosis induced by PTPA knockdown, we detected cell viability during PTPA knockdown and $PP2A_C$ overexpression simultaneously. We found that overexpression of $PP2A_C$ could not rescue the decrease of cell viability induced by PTPA knockdown.

Our previous studies have found that phosphorylation of tau antagonizes apoptosis by stabilizing beta-catenin (Li *et al.* 2007b) and tau hyperphosphorylation can attenuate the ER stress- and death-associated protein kinase-induced apoptosis (Liu *et al.* 2012, Duan *et al.* 2013). Here, we found that PTPA knockdown induced cell apoptosis in cell lines with or without litter tau expression. However, in HEK293/tau cell lines which has stable tau expression, PTPA knockdown decreased $PP2A_C$ activity through increasing its phosphorylation level in Tyr-307 site and decreasing its methylation level in Leu-309 site, which is consistent with our previous study (Luo *et al*. 2013), to induce tau phosphorylation attenuating PTPA knockdown-induced cell apoptosis. Since PTPA is constitutively expressed in the brain of aged and AD patients (Van Hoof *et al*. 1995), and the protein level is decreased in AD brain and AD-like transgenic mice models (Luo *et al*. 2013), we speculate that tau

phosphorylation at an early stage of AD may be an active attempt to avoid rapid cell loss induced by decreased PTPA.

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Conflicts of interest

The authors declare that they have no potential conflicts of interest to disclose.

Figure legends

Fig.1 PTPA localizes in mitochondria.

(A-B) The level of PTPA protein was detected in the homogenate from HEK293/wt, N2a/wt (A), rat primary neuron cultures and rat cortex (B) by western blotting. (C) PTPA level in the cytoplasmic and nuclear fractions of HEK293/wt cells were detected by western blotting, Lamin B1 and GAPDH were used as marker of nucleus and cytoplasm respectively. (D-E) PTPA level in the whole cell lysates, cytoplasm or mitochondria fraction of HEK293/wt cells (D) or cortex of rat brain (E) was detected by western blotting; Cox IV and GAPDH were used as mitochondrial and cytoplasmic marker respectively. (F) The mitochondrial fractions prepared from HEK293/wt cells were re-suspended in buffers with or without Na_2CO_3 (0.1 mol/L, pH 11.5) followed by centrifugation, and the supernatants (S) or membrane pellets (P) were detected with the indicated antibodies by Western blot.

Fig.2 PTPA knockdown induced apoptosis in cell lines without or with little endogenous tau.

(A-D) HEK293/wt (A, B) or N2a/wt (C, D) cells were transfected with ssiPTPA or siPTPA plasmid for 48 h, PTPA level was detected by western blotting (A, C) and quantitative analysis (B, D). Anti-a-tubulin (DM1A) was used as a loading control. (E-H) HEK293/wt (E, G) or N2a/wt (F, H) cells were transfected with ssiPTPA or siPTPA plasmid for 48 h, Cell viability was measured by cell counting kit-8 (CCK-8) (E, F) or MTT assay (G, H). (I) HEK293/wt cells were transfected with ssiPTPA or siPTPA plasmid for 48 h, cleaved caspase-3, caspase-3, cleaved PARP, PARP levels were detected by western blotting. DM1A was used as a loading control. The data were expressed as mean±SD. *, $p<0.05$ *vs* ssiPTPA. **, $p<0.01$ *vs* ssiPTPA.

Fig. 3 PTPA knockdown decreased MMP, induced Bax translocation and Cyt C release.

(A) MMP was detected after HEK293/wt cells was transfected with ssiPTPA or siPTPA plasmid for 48 h. (B-E) HEK293/wt cells were transfected with ssiPTPA or siPTPA plasmid for 48 h, Bax (B, C) or Cyt C (D, E) level in mitochondria and cytoplasm fractions were detected by western blotting (B, D) and quantitative analysis (C, E). GAPDH and Cox IV were used as cytoplasmic and mitochondrial marker respectively. (F, G) HEK293/wt cells were transfected with ssiPTPA or siPTPA plasmid for 48 h, Bcl-xl, Bcl-2, phosphorylated Bad at Ser112 (p-Bad) and Bad levels were detected by western blotting (F) and quantitative analysis (G). DM1A was used as a loading control. The data were expressed as mean±SD. *, $p<0.05$ *vs* ssiPTPA, **, $p<0.01$ *vs* ssiPTPA.

Fig. 4 $PP2A_C$ or MAPKs did not involve in the apoptosis induced by PTPA knockdown.

(A, B) HEK293/wt cells were transfected with ssiPTPA, siPTPA or wt$PP2A_C$ plasmid, cell viability was detected at 0 h, 24 h and 48 h by MTT assay (A), $PP2A_C$, PTPA (B), p-ERK, ERK, p-JNK, JNK, p-p38 and p38 levels (C, D) at 48 h were detected by western

blotting (B, C) and quantitative analysis (D). DM1A was used as a loading control. The data were expressed as mean±SD. *, p<0.05 *vs* ssiPTPA.

Fig. 5 PTPA knockdown did not induce apoptosis in HEK293/tau cells

HEK293/tau cells were transfected with ssiPTPA or siPTPA plasmid for 48 h, cell viability was measured by cell counting kit-8 (CCK-8) (A). Cleaved caspase-3, cleaved PARP, caspase-3 and PARP levels were detected by western blotting (B). PTPA, pS396 (phosphorylation of Ser-396), pS404 (phosphorylation of Ser-404), tau 1 (unphosphorylated tau), tau5 (total tau), P-PP2A$_C$ (phosphorylation level of PP2A$_C$ at Tyroine307), M-PP2A$_C$ (methylation level of PP2A$_C$ at leucine309), DM-PP2A$_C$ (demethylation level of PP2A$_C$ at leucine309) and PP2A$_C$ levels were detected by western blotting (C) and quantitative analysis (D, E). DM1A was used as a loading control. The data were expressed as mean±SD. *, p<0.05 *vs* ssiPTPA.

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