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Presenilins regulate the cellular level of the tumor suppressor PTEN

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Abstract

Alzheimer's disease (AD) is characterized by amyloid plaques consisting of β -amyloid ($A\beta$) peptides and neurofibrillary tangles consisting of hyperphosphorylated tau protein. $A\beta$ is proteolytically derived from its precursor protein through cleavages by β -secretase and γ -secretase complex comprising presenilins (PS, PS1/PS2), nicastrin, APH-1 and PEN-2. PS1 is also known to activate the PI3K/Akt cell survival pathway in a γ -secretase-independent manner. The tumor suppressor PTEN, which antagonizes the PI3K/Akt pathway, has increasingly been recognized to play a key role in neural functions and its level found reduced in AD brains. Here, we demonstrate that the protein level of PTEN is dramatically reduced in cultured cells and embryonic tissues deficient of PS, and in the cortical neurons of PS1/PS2 conditional double knockout mice. Restoration of PS in PS deficient cells reverses the reduction of PTEN. Regulation of PTEN by PS is independent of the PS/ γ -secretase activity since impaired γ -secretase by the γ -secretase inhibitor treatment or due to nicastrin deficiency has little effect on the protein level of PTEN. Our data suggest an important role for PS in signaling pathways involving PI3K/Akt and PTEN that are crucial for physiological functions and the pathogenesis of multiple diseases.

Keywords

Akt; Alzheimer's disease; phosphatase and tensin homologue deleted on chromosome 10; phosphoinositide 3-kinase; presenilin

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1. Introduction

Alzheimer's Disease (AD) has two hallmark lesions: the extracellular β -amyloid ($A\beta$) plaques and the intracellular hyperphosphorylated tau fibrillary tangles [12,15,45]. Mutations in the *PSEN1* and *PSEN2* genes account for the majority of cases of early-onset familial AD (FAD) [21,35,38]. *PSEN* genes encode polytopic membrane proteins termed presenilins (PS1 and PS2), which function as the catalytic subunit of γ -secretase, an intramembrane protease consisting of at least three other components: nicastrin (Nct), anterior pharynx-defective-1 (APH-1), and presenilin enhancer-2 (PEN-2). γ -secretase has a wide spectrum of type I membrane protein substrates including Notch, ErbB4 receptor tyrosine kinase, CD 44, nectin-1 α , E-cadherin, and low density lipoprotein receptor-related protein (LRP) (for review, see Refs. [7,47]). Sequential cleavages of amyloid precursor protein (APP) by β -secretase (BACE) and γ -secretase release highly fibrillogenic $A\beta$ peptides which accumulate in the brains of aged individuals and patients with AD [9,13]. FAD-associated presenilin variants are thought to exert their pathogenic function by selectively elevating the levels of highly amyloidogenic $A\beta$ 42 peptides [5,12,15]. PS null mice are embryonic lethal and show severe malformation resembling that of Notch deficiency [10,52].

In addition to its roles in $A\beta$ production and Notch cleavage, PS1 has been reported to play multiple physiological roles such as those in intracellular trafficking of membrane proteins, calcium homeostasis, neuronal development, neurite outgrowth, apoptosis, synaptic plasticity, and tumorigenesis [39,44,47,53]. Recently, several studies have suggested that PS1 regulates the phosphoinositide 3-kinase (PI3K) signaling that governs a variety of crucial cellular functions including cell proliferation, migration and apoptosis [3,16,51]. PI3K phosphorylates phosphatidylinositol (4, 5) – diphosphate (PIP2) to generate phosphatidylinositol (3, 4, 5) – triphosphate (PIP3). Elevated PIP3 levels result in Akt activation by promoting its phosphorylation at residues serine 473 and threonine 308. Activated Akt in turn inactivates downstream substrate glycogen synthase kinase-3 β (GSK-3 β), which is strongly implicated in tau hyperphosphorylation [1,2,22,24,29]. PS1 can positively regulate PI3K/Akt activation in a γ -secretase-independent manner, hence inactivating GSK-3 β and reducing tau phosphorylation. FAD-linked mutations in PS1 conversely down-regulate the PI3k/Akt signaling [3,16,51].

Pten (phosphatase and tensin homologue deleted on chromosome 10) is a tumor suppressor gene that mutates frequently in many sporadic and hereditary cancers [41,42]. *Pten*-null mice die at early embryonic stages, and heterozygous knockout mice develop a number of tumors [8,33]. PTEN contains a tyrosine phosphatase functional domain, exhibiting both protein and lipid phosphatase activity *in vitro* [23]. PTEN dephosphorylates the 3' position of PIP3 to generate PIP2, thus antagonizing the activity of PI3K/Akt [23,30,41,42]. In addition to its tumor suppressing function, PTEN has been found necessary for normal cerebellar architecture and for proper migration of neurons and glia [26]. Mouse brains with conditionally inactivated *Pten* showed an increased soma size of neurons without altering proliferation [11,19]. Mutations in PTEN-induced kinase 1 (PINK1) have been linked to hereditary early-onset Parkinson's disease [46], implying the importance of PTEN signaling in neurodegenerative diseases. Recent studies showed decreased levels and altered

distribution of PTEN along with elevated PI3K signaling in AD patient brains [14,55]. In addition, our previous study demonstrated that PTEN affects the phosphorylation and aggregation of tau [55,56]. These results suggest that a loss of PTEN function may contribute to neurodegeneration in AD. In the present study, we explored the effects of PS deficiency on PTEN and revealed a significant modulation of the cellular level of PTEN by PS.

2. Materials and Methods

2.1. Cell lines

PS1 single knockout (PS1 KO), PS1/PS2 double knockout (PS DKO), and nicastrin knockout (Nct KO) mouse embryonic fibroblast cells, as well as the wild type cells derived from the respective control mice, were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin (Hyclone, Logan, UT, USA). Nct KO cells stably expressing human nicastrin were kindly provided by Dr. G. Thinakaran and cultured in media supplemented with 0.4 mg/ml hygromycin (Roche, Indianapolis, IN, USA). Mouse embryonic stem cells isolated from PS1/PS2 double knockout (PS DKO) as well as wild type mouse (PS wt) were maintained as previously described [57]. Mouse neuroblastoma N2a cells stably coexpressing the human APP Swedish mutant (N2a Swe) and one of the human PS1 variants (which includes a single amino acid substitution, D385A, in PS1 transmembrane domain 7 [17], a deletion of the first two PS1 transmembranes Delta 1–2 [20], and a deletion of PS1 exon 9 Delta 9 [5]) were maintained in medium containing 50% DMEM and 50% Opti-MEM (Invitrogen, Carlsbad, CA, USA), supplemented with 5% FBS, penicillin/streptomycin and 0.4 mg/ml G418 (Invitrogen, Carlsbad, CA, USA).

2.2. Transfection and stable cell line establishment

Wild type fibroblast cells were transiently transfected with pcDNA (as control) or PS1 cDNA constructs using FuGENE 6 (Roche, Indianapolis, IN, USA), following the manufacturer's protocol. To establish stable cell lines, PS1 cDNA (or pcDNA) and pcDNA3/hygro vectors were co-transfected into PS1 KO cells; and transfected cells were selected with 0.4 mg/ml hygromycin. PS DKO cells were co-transfected with PS1 (or PS2) cDNA and pAG3zeo vectors, and selected with 0.2 mg/ml zeocin (InvivoGen, San Diego, CA, USA).

2.3. Immunoblotting and data analysis

Cells were lysed in lysis buffer (0.5% IGEPAL CA-630 and 0.5% deoxycholic acid in phosphate buffered saline, supplemented with protease inhibitors). Lysates of PS1 KO and PS1 heterozygous mouse embryos collected on embryonic day 15 (E15) [52] were kindly provided by Dr. G. Thinakaran. Total brain lysates of PS1/PS2 conditional double knockout (PS cDKO) mice [36] and littermate controls at 2 months of age were kindly provided by Dr. J. Shen. Equal amounts of proteins were separated by SDS-PAGE, transferred onto PVDF membranes, and immunoblotted with different antibodies. Monoclonal antibodies against PTEN and phospho-Akt and polyclonal antibody against PS2 carboxy-terminus were from Cell Signaling (Danvers, MA, USA). Monoclonal antibody against α -tubulin was from Sigma (St. Louis, MO, USA). Polyclonal antibodies against PS1 amino-terminus (Ab14) and

against APP carboxy-terminus (369) have previously been described [6,43,54]. Protein levels were quantified by densitometry using Scion Image (Scion Corporation, Frederick, MD, USA). Three independent samples were studied. The protein level of PTEN was normalized to that of α -tubulin and statistically compared to that of control using Student's t-test.

2.4. Immunohistochemistry

Paraffin-embedded sagittal brain sections (10 μ m) of PS1/PS2 conditional double knockout (PS cDKO) mice [36] and littermate controls at 5 months of age were kindly provided by Dr. J. Shen. Sections were deparaffinized, hydrated and then immunostained with monoclonal anti-PTEN antibody overnight at 4°C. After this, sections were incubated with biotinylated goat anti-mouse antibody for 1h at room temperature and then incubated in ABC-Elite (HRP) reagent (Vector Laboratories, Burlingame, CA, USA) for another 1h. Reactions were visualized by developing in DAB substrates (Vector Laboratories, Burlingame, CA, USA). All samples were visualized under light microscope.

2.5. γ -secretase inhibitor treatment

γ -secretase inhibitor L685, 458 was a kind gift from Dr. Y. Li and dissolved in DMSO. To study the effects of γ -secretase activity on the PTEN level, mouse neuroblastoma cells stably expressing human APP Swedish mutant (N2a Swe) were treated with 500nM L685, 458 for 24 hrs.

2.6. A β assay

To confirm the inhibition of γ -secretase activity by L685, 458, the conditioned media of L685, 458-treated N2a Swe cells were incubated with the monoclonal antibody 4G8 that recognizes the amino acids 17–24 of A β (Signet Laboratories, Dedham, MA, USA) and the immunoprecipitated A β were immunoblotted with monoclonal antibody 6E10 that recognizes the amino acids 1–17 of A β (Signet Laboratories, Dedham, MA, USA) [48,49,54].

3. Results

3.1. PS deficiency results in a reduced level of PTEN

It has been reported that PS deficiency impairs the PI3K/Akt pathway [3,16]. Since PTEN functions as an antagonist of the PI3K/Akt pathway [23,30,41,42], we investigated whether PS also regulates the level of PTEN in mouse embryonic fibroblast cells by western blot analysis. Consistent with previous reports, we found that the level of phosphorylated Akt was significantly reduced in cells lacking PS1 (PS1 KO) or both PS1 and PS2 (PS DKO) compared to that in wild type (PS wt) cells, indicating that the PI3K/Akt pathway was compromised by PS deficiency (Fig 1A). Interestingly, we found that in both PS1 KO and PS DKO cells, the level of PTEN was dramatically reduced compared to that in PS wt cells (Fig. 1A). To ascertain that this observation is not specific to fibroblasts, we further examined the level of PTEN in embryonic stem cells derived from PS1/2 double knockout mice (Fig. 1B, left panel), as well as in the total lysate of PS1 knockout mouse embryos

(Fig. 1B, right panel). The level of PTEN was found to be significantly reduced in all samples lacking PS.

Furthermore, to make sure that the phenomenon of reduced PTEN levels is also present in the brain deficient of PS, we examined the level of PTEN in the brain of conditional double knockout mice lacking both presenilins (PS cDKO) in the postnatal forebrain [36] using the immunohistochemical approach. Although the level of PTEN in the hippocampus of PS cDKO mice showed little difference compared to the littermate controls, a clear reduction in PTEN-positive immunoreactivity was observed in the neocortex of the PS cDKO mice compared to that in littermate controls (Fig. 2A). In addition, we compared the protein level of PTEN in the total brain lysate of PS cDKO mice and that of littermate controls. Although the difference was not significant ($P=0.07$) (Fig. 2B), the level of PTEN in PS cDKO mice was indeed lower than that in littermate control mice and the non-significant difference between them could be attributed to the incomplete deficiency of PS1 in the PS cDKO mouse brains.

3.2. Expression of exogenous PS reverses the reduced PTEN level in PS deficient cells

To further confirm that the reduction in PTEN level is indeed a direct consequence of PS deficiency and to exclude the possibility that such a change may result from variation of cell clones, we ascertained the reversibility of the phenotype by stably expressing PS into PS deficient cells. Consistent with the previous reports [3,16], restoring PS1 in PS1 KO cells and PS1 or PS2 in PS DKO cells to a level comparable to that in wild type cells increased the level of phosphorylated Akt (Fig. 3). Similarly, overexpression of exogenous PS was able to increase the level of PTEN in both PS1 KO and PS DKO cells, partially reversing the phenotype of PTEN reduction (Fig. 3).

3.3. PS rather than the γ -secretase activity regulates the level of PTEN

Since PS1 is crucial for the activity of γ -secretase and has been proposed to contain the catalytic domain(s) of the γ -secretase complex, it is conceivable that the enzymatic activity may be required for the regulation of the level of PTEN. To test this possibility, we treated cells with L685, 458 to inhibit γ -secretase activity. The inhibition of the γ -secretase activity was confirmed by the accumulation of APP CTFs and reduction of A β generation. However, we found that the inhibition of γ -secretase activity failed to mimic the effect of PS deficiency on the reduction of PTEN (Fig. 4A). Nicastrin deficiency is also known to abolish γ -secretase activity and cause an accumulation of APP CTFs. We further demonstrated that the level of PTEN was not changed in nicastrin KO fibroblast cells compared to the wild type control cells as well as the nicastrin KO cells stably expressing human nicastrin (Fig. 4B). In addition, we found that overexpression of wild type PS1 led to an increase (by approximately 70%) in PTEN level in mouse fibroblasts (Fig. 4C). Overexpression of a FAD variant PS1 (Delta 9) and two loss-of-function PS1 mutants (D385A and Delta 1–2) was also able to increase PTEN level by approximately 45% in mouse N2a cells. These results strongly suggest that the regulatory effect of PS on PTEN level requires the physical presence of the PS molecule but not the enzymatic activity of the γ -secretase.

4. Discussion

Presenilins are critical components in the γ -secretase complex and all PS FAD mutations enhance the ratio of A β 42/A β 40, which is regarded as crucial for AD pathogenesis [5,12,15]. Recently it has been shown that PS can modulate the PI3K/Akt pathway and FAD PS mutations might promote AD pathology by inhibiting the PI3K/Akt pathway [3,16]. The tumor suppressor PTEN antagonizes the PI3K/Akt pathway and PI3K/Akt-PTEN signaling modulates multiple vital cellular processes including growth, proliferation, and apoptosis [41,42]. In addition, several studies have shown that PI3K/Akt activation protected against A β neurotoxicity [27,40,50]. However, pathological studies showed elevated Akt activation in AD brains [14,32,34]. Since tau contains several Akt phosphorylation sites including Ser214, which together with its neighboring Thr212 forms the AT100 tau epitope that is highly specific in the paired helical filaments (PHFs) present in AD [18,28], elevated Akt activation may promote tau hyperphosphorylation in AD. On the other hand, it has been reported that the level of PTEN in AD brains is decreased [14,55]. We recently also found that overexpression of wild-type PTEN could reduce tau phosphorylation/aggregation and increase tau-microtubule association, whereas loss-of-function PTEN increased the aggregation of a mutant tau form that was associated with frontal temporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) [55,56].

In the present study, we investigated whether PS could regulate Akt activation through modulating the level of PTEN. Interestingly, although PTEN antagonizes the PI3K/Akt pathway and Akt activity is impaired by PS deficiency, our results showed that PS deficiency resulted in a dramatic reduction of the level of PTEN while overexpressing exogenous PS partially reversed the reduction of PTEN in PS deficient cells. These observations raise the possibility that an intrinsic mechanism may exist to balance the PI3K/Akt and the PTEN signaling pathways so that PS deficient cells do not undergo over-proliferation or over-apoptosis. Interestingly, PS1 knockout mice that are rescued through neuronal expression of human PS1 transgene develop spontaneous skin cancers [53]. Since PTEN is an important tumor suppressor, there is a possibility that reduced PTEN level due to PS1 deficiency may contribute to tumorigenesis in these mice.

PS deficiency dramatically impairs the γ -secretase activity and PS cDKO mice have a diminished level of the toxic A β species in the cerebral cortex compared to that in controls [4,36]. These mice, however, exhibit impairments in hippocampal memory and synaptic plasticity and in their late age develop neurodegeneration accompanied by increased tau hyperphosphorylation, indicating an essential role of PS in synaptic plasticity, learning and memory, and neuronal survival in the adult cerebral cortex [36]. Our study showed that the level of PTEN in the cortical neurons of PS cDKO mice were decreased comparing to that in control mice. Whether PTEN is involved in PS-modulated neural functions deserves further investigation.

Although PS1 FAD mutations still remain the PS1/ γ -secretase activity and increase the production ratio of A β 42/A β 40, PS1 FAD mutations failed to rescue the defective PI3K/Akt signaling pathway in PS1 deficient cells, suggesting that modulation of the PI3K/Akt pathway by PS1 is not dependent on the PS1/ γ -secretase activity [3,16]. In the present study,

we also found that the level of PTEN is affected by the physical presence of PS rather than the enzymatic activity of PS/ γ -secretase. Regarding the possible mechanisms underlying PS regulation of PI3K/Akt signaling, Baki et al. suggested that PS1 stimulates Akt activation by promoting cadherin/PI3K association [3]. Kang et al. proposed that PS mediated Akt and ERK signaling via selective signaling receptors [16]. We have tested whether PS1 could interact with PTEN to modulate its stability but the co-immunoprecipitation experiments failed to show any interaction between PS1 and PTEN (data not shown). It is well-known that the intracellular domain of Notch (NICD) released by PS/ γ -secretase cleavage regulates a variety of downstream gene expression involved in development [25,37]. Accumulating evidence begins to suggest that the intracellular domain of APP (AICD) may also be involved in gene expression regulation [25,31]. Since PS deficiency abolishes the PS/ γ -secretase enzymatic activity for NICD/AICD generation, it is therefore unlikely that PS deficiency reduces the level of PTEN through directly regulating the *PTEN* gene expression.

In summary, the current study revealed an important role for PS in regulating the PI3K/Akt and PTEN pathways independent of the PS/ γ -secretase activity. Since PTEN is one of the most important tumor suppressors [41,42] and has been found necessary for normal cerebellar architecture and proper migration of neurons and glia [26], it is likely that PS deficiency may influence both neurodegeneration and tumorigenesis through abnormally modulating the crucial PI3K/Akt and PTEN pathways.

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Fig. 1.

PS deficiency reduces the level of PTEN. (A) Equal amounts of proteins from wild type (PS wt), PS1 single knockout (PS1 KO), and PS1/PS2 double knockout (PS DKO) mouse embryonic fibroblast cells were analyzed by SDS-PAGE and immunoblotted with antibodies against PTEN, phospho-Akt, and α -tubulin (as loading control), respectively. (B) Equal amounts of lysate proteins were analyzed by SDS-PAGE and immunoblotted for PTEN and α -tubulin. Lysates were prepared from PS wt and PS DKO mouse embryonic stem (ES) cells (left panel) or from the whole tissues of PS1 heterozygous and PS1 KO embryos (collected on E15, right panel). All data represent means $\pm$ S.E. of 3 independent experiments. *, $p < 0.05$.

Fig. 2.

The level of PTEN was reduced in cortical neurons of PS1/PS2 conditional double knockout (cDKO) mice. (A) Paraffin-embedded brain sections from PS cDKO (lower panels) and littermate control (upper panels) mice were deparaffinized, sequentially incubated with monoclonal anti-PTEN antibody, biotinylated goat anti-mouse antibody, and ABC-Elite (HRP) reagent. Reactions were visualized by developing in DAB substrates. Right panels were higher magnification views of the boxed area in corresponding left panels. White arrows indicate PTEN-immunoreactive cells. Scale bar: 100 μ m. (B) Equal amounts of total brain lysates from PS cDKO and littermate control mice were analyzed by SDS-PAGE and

immunoblotted for PTEN, PS1 N-terminal fragment (NTF) and α -tubulin. Data represent means $\pm$ S.E. of 3 samples, $p=0.07$

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Fig. 3.

Reduction of PTEN level in PS deficient cells was partially reversed by overexpression of exogenous PSs. (A) PS1 KO fibroblast cells were stably transfected with human PS1 or pcDNA constructs. Equal amounts of protein were analyzed by SDS-PAGE and immunoblotted for PTEN, phospho-Akt, PS1 N-terminal fragment (NTF) and α-tubulin. (B) PS DKO fibroblast cells were stably transfected with human PS1, PS2 or PAG2zeo constructs. Equal amounts of protein were immunoblotted for PTEN, phospho-Akt, PS1-NTF, PS2 C-terminal fragment (CTF) and α-tubulin. All data represent means±S.E. from 3 independent experiments. *, $p < 0.05$.

Fig. 4.

The effect of PS on the level of PTEN is independent of the γ -secretase activity. (A) Mouse neuroblastoma N2a cells stably expressing Swedish mutant APP were treated with DMSO or 500nm L685, 458 for 24 hrs. Equal amounts of protein from cell lysates were analyzed by SDS-PAGE and immunoblotted for PTEN, APP C-terminal fragments (CTFs), and α -tubulin. A β in the conditioned media was immunoprecipitated by 4G8 followed immunoblotting with 6E10. (B) Equal amounts of cell lysate from wild type, nicastrin knockout (Nct KO), and Nct KO cells stably expressing human nicastrin (hNct) were analyzed by SDS-PAGE and immunoblotted for PTEN, APP CTFs, and α -tubulin. (C) Wild type fibroblast cells were transiently transfected with human PS1 or pcDNA constructs and the lysates were immunoblotted for PTEN and both endogenous (Endo) and exogenous (Exo) PS1 NTF. (D) Equal amounts of cell lysate from N2a cells stably co-expressing both

human APP Swedish mutant and one of PS1 variants (including the loss of function mutants D385A and Delta 1–2 and the FAD mutant Delta 9) were immunoblotted with PTEN and PS1 antibodies. NA: no exogenous PS1 overexpression. All data represent means $\pm$ S.E. from 3 independent experiments. *, $p<0.05$.

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