

RESEARCH

Open Access



Neuroprotective effects of donepezil against $A\beta_{25-35}$ -induced neurotoxicity

Bu-Lang Gao^{1*} , Ning-Ning Che², Xue Li² and Chun-Feng Ren³

Abstract

Purpose: The purpose of this study was to investigate the neuroprotective effect of donepezil against β -amyloid₂₅₋₃₅ ($A\beta_{25-35}$)-induced neurotoxicity and the possible mechanism.

Methods: PC12 cells were conventionally cultured. Serial concentrations of $A\beta_{25-35}$ and donepezil (0, 0.5, 1, 5, 10, 20 and 50 $\mu\text{mol/L}$) were added to the PC12 cells, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) staining was performed to detect the effects of these treatments on PC 12 viability. The PC 12 cells were pretreated with 1, 5, 10, 20 or 50 $\mu\text{mol/L}$ donepezil two hours before 20 $\mu\text{mol/L}$ $A\beta_{25-35}$ was added to pretreatment groups A, B, C, D and E. Normal control group I and the 20 $\mu\text{mol/L}$ $A\beta_{25-35}$ -treated group were selected. An MTT assay was used to detect PC12 cell viability, and the level of lactate dehydrogenase (LDH) was determined. PC12 cells were pretreated with 10 $\mu\text{mol/L}$ GF109203X (a protein kinase C [PKC] antagonist) 30 min before 10 $\mu\text{mol/L}$ donepezil was added to pretreatment group F, and normal control group II, the 10 $\mu\text{mol/L}$ GF109203X-treated group and the 10 $\mu\text{mol/L}$ donepezil-treated group were chosen. The expression of phosphorylation-PKC (P-PKC) and its major substrate phosphorylated myristoylated alanine-rich protein C kinase substrate (P-MARCKS) was measured by Western blotting. The effects of donepezil on the subcellular distribution of the PKC α and PKC ϵ isoforms were detected by immunofluorescence staining.

Results: Treatment with $A\beta_{25-35}$ (5, 10, 20 or 50 $\mu\text{mol/L}$) for 24 h significantly ($P < 0.05$) decreased PC 12 cell viability in a dose-dependent manner. Compared with the PC12 cells in the control group, those in the 20 $\mu\text{mol/L}$ $A\beta_{25-35}$ -treated group exhibited lower viability but higher LDH release. Compared with the 20 $\mu\text{mol/L}$ $A\beta_{25-35}$ -treated group, pretreatment groups B, C, D and E exhibited significantly ($P < 0.05$) increased cell viability but significantly ($P < 0.05$) decreased LDH release. Western blotting demonstrated that compared with control, 10 $\mu\text{mol/L}$ donepezil promoted PKC and MARCKS phosphorylation and that the expression of P-PKC and P-MARCKS in pretreatment group F was significantly ($P < 0.05$) lower than that in the donepezil-treated group. Immunofluorescence staining revealed that the PKC α and PKC ϵ isoforms were located mainly in the cytoplasm of PC12 control cells, whereas donepezil increased the expression of the PKC α and PKC ϵ isoforms in the membrane fraction. The Western blot results showed that donepezil altered the subcellular distribution of the PKC α and PKC ϵ isoforms by decreasing their expression in the cytosolic fraction but increasing their expression in the membrane fraction.

Conclusion: Donepezil can antagonize $A\beta_{25-35}$ -induced neurotoxicity in PC 12 cells, and PKC activation may account for the neuroprotective effect of donepezil.

Keywords: Donepezil, Beta amyloid 25–35, Alzheimer’s disease, Protein kinase C, Neuroprotection

*Correspondence: browngao@163.com

¹ Department of Medical Research, Shijiazhuang People’s Hospital, 9 Fangbei Road, Shijiazhuang 050011, Hebei Province, China
Full list of author information is available at the end of the article

Introduction

As a neurodegenerative disease characterized by progressive loss of memory and cognitive impairment, Alzheimer’s disease (AD) is the most common form of



© The Author(s) 2022. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

dementia in the elderly [1, 2]. There are two pathological hallmarks of AD: extracellular deposits of amyloid- β ($A\beta$) protein and intracellular phosphorylated tau protein [3–6]. Abnormal extracellular deposition of the $A\beta$ peptide results in the formation of senile plaques in the hippocampus, which is the pathological feature of AD associated with neurodegeneration [7]. The $A\beta$ protein may trigger the hyperphosphorylation of the tau protein and cause impairment of axonal transport and destabilization of microtubules, leading to neuronal apoptosis. This neurotoxicity of the $A\beta$ protein is one of the most important mechanisms of AD, and inhibition of the neurotoxicity of this protein is thus one approach for treating this disease. Currently, the predominant agents for treating AD are acetylcholinesterase inhibitors, which can enhance cholinergic neurotransmission in the synaptic cleft by inhibiting acetylcholine degeneration [8]. As a classical inhibitor of acetylcholinesterase approved by the FDA, donepezil can provide symptomatic relief and neuroprotection against $A\beta$ toxicity in cell and animal models [9–11] and halt progressive atrophy in the brains of AD patients [12]. However, the specific pharmaceutical mechanism of donepezil is not clear, especially regarding its effects on neuroprotection against exogenous $A\beta$; however, some research has indicated that donepezil may be able to alter the metabolism of the amyloid precursor protein, leading to decreased secretion of $A\beta$ [13]. This study investigated the effect of donepezil on rat pheochromocytoma cells (PC 12) exposed to $A\beta_{25-35}$ and the possible mechanism.

Materials and methods

Reagents

Donepezil was provided by Eisai China Inc. (Suzhou, China). $A\beta_{25-35}$, dimethyl sulfoxide (DMSO), a mouse monoclonal anti- β -actin antibody and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were provided by Sigma-Aldrich (St. Louis, MO, USA). The protein kinase C (PKC) inhibitor GF109203X was provided by Calbiochem (San Diego, CA, USA). A rabbit polyclonal anti-phospho-PKC (P-PKC) antibody and a rabbit polyclonal anti-phospho-myristoylated alanine-rich C kinase (MARCKS) antibody were provided by Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal anti-PKC α and PKC ϵ antibodies were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). A Dulbecco's modified Eagle medium (DMEM), foetal bovine serum (FBS), penicillin, streptomycin and pancreatin-EDTA (ethylene diamine tetraacetic acid) mixture was provided by Gibco (Carlsbad, CA, USA). RIPA cell lysis buffer was provided by Shenneng Bocai (Shanghai, China). A BCA protein assay kit was provided by Pierce (Pierce Biotechnology, Rockford, IL, USA). An

ECL Western Blotting Detection kit was provided by Amersham-Pharmacia Biotech (GE Healthcare, Little Chalfont, UK).

Cell culture

PC12 cells were routinely plated into 100 mm culture dishes in DMEM containing 10% foetal bovine serum, 1% streptomycin and 1% penicillin. The cells were cultured in 100% humidity and 5% CO₂ at 37 °C. When the cells reached 80% cell fusion, the medium was discarded, and the cells were lysed with pancreatin for passage. Cells in logarithmic growth were taken for experiments.

MTT assay

The MTT assay was used to assess cell viability. The cells were plated in a 96-well plate at a density of 1×10^4 cells per well. Twenty-four hours later, donepezil or $A\beta_{25-35}$ at 0, 0.5, 1, 5, 10, 20 or 50 μ mol was added to the cells, with six wells for each concentration. At the same time, medium without cells was used as the blank control. Twenty-four hours after treatment, the medium was replaced, and 180 μ L of medium and 20 μ L of 5 g/L MTT were added to each well for further incubation for 4 h. Then, the medium was discarded, and 200 μ L of DMSO was added to each well for incubation for 30 min at 37 °C. The absorption of the cells at 570 nm was evaluated and compared with that of the cells in the control group to determine the relative viability of the cells.

Effect of donepezil pretreatment on cell viability

The cells were plated at a density of 1×10^4 cells per well in a 96-well plate and were divided 24 h later into the normal control group, the $A\beta_{25-35}$ -treated group, and the $A\beta_{25-35}$ plus 1, 5, 10, 20, or 50 μ mol donepezil-treated groups. For the $A\beta_{25-35}$ plus donepezil-treated groups, donepezil was added at different concentrations and then 20 μ mol/L $A\beta_{25-35}$ was added two hours later for further incubation for 24 h. Then, cell viability was evaluated by the MTT assay (as described above).

Lactate dehydrogenase (LDH) release

The manufacturer's protocol was used to assay LDH released from damaged cells. The treatment and classification of the groups were the same as those described above. The supernatant was collected from each group, and protein inhibitors were added on ice. Then, 120 μ L of supernatant was transferred to an enzyme-labelling plate. After the reaction, working fluid was added and incubated for 30 min at room temperature, and the absorbance at 500 nm was measured.

Western blotting for P-PKC and P-MARCKS expression

The cells were inoculated at a density of 10^5 cells/mL in a 10-cm culture dish. The cells were divided into the normal control group, the donepezil-treated group, the GF109203X plus donepezil-treated group and the GF109203X-treated group. Thirty minutes after 10 μ mol/L GF109203X was added to the GF109203X plus donepezil-treated group and the GF109203X-treated group, 10 μ mol/L donepezil was added to the donepezil-treated group and the GF109203X plus donepezil-treated group, and an equivalent amount of solvent was added to the normal control group. After the protein concentration was measured in each group, 20 μ g of protein was taken for sodium dodecyl sulphate (SDS)-polyacrylamide gel (PAGE) electrophoresis. Electrophoresis was performed at 100 v for 20 min and then at 150 v for 60 min. Then, the proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA), which were saturated with 5% non-fat milk and incubated with β -actin (1:10,000), phospho-PKC (1:1000) and P-MARCKS (1:1000) primary antibodies at 4 °C overnight. After washing with Tris-buffered saline (TBS) for 30 min, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit/mouse IgG secondary antibodies (1:1000) for one hour at room temperature. TBS was used to wash the membranes, and ABC compound was added at room temperature for 30 min. The signals were developed using an ECL Western Blotting Detection kit.

Immunofluorescence for PKC α and PKC ϵ expression

The cells were inoculated at a density of 10^5 cells/mL in 6-well plates, and the cells were divided into the control and donepezil-treated groups. In the donepezil-treated group, 10 μ mol/L donepezil was added after the cells were inoculated, while the inoculated cells of the normal group were not treated with donepezil. Two hours later, the medium was discarded, and PBS was used to wash the cells twice. Thirty minutes after 40 g/L paraformaldehyde fixation, PBS was used to wash the cells three times for 5 min each time. Then, the cells were treated with PBS containing 3% H₂O₂ and 0.1% Triton X-100 for 20 min at room temperature, and PBS was used to wash the cells. The cells were incubated with PBS containing 3% bovine serum albumin (BSA) for 30 min at room temperature, and a mouse monoclonal anti-PKC α (1:100) or anti-PKC ϵ (1:100) antibody was added at 4 °C overnight. After washing with PBS, a FITC-conjugated anti-mouse IgG secondary antibody was added and incubated for 90 min. After washing with PBS three times, fluorescent glycerol was used for mounting, and the cells were observed under a microscope.

PKC translocation

A PKC translocation assay was conducted according to the procedure described by Etcheberrigaray et al. with some modifications [14]. In brief, after cell lysis in lysis buffer (20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2 mM EGTA, 5 mM DTT, 0.32 M sucrose and 2 mM PMSF), the extracts were centrifuged at $12,000 \times g$ for 20 min at 4 °C. The resulting supernatants were regarded as the cytosolic fractions. The pellets were incubated with the same lysis buffer, to which 1% Triton X-100 was added on ice for 45 min. After centrifugation at $12,000 \times g$ for 20 min at 4 °C, the supernatants were collected for use as the membrane fractions. After BCA (bicinchoninic acid) quantification, 20 mg of protein mixed with $5 \times$ loading buffer and $20 \times$ reducing agent was boiled for 5 min and loaded onto a 7.5% SDS-PAGE gel. The following procedure was the same as that described above, except that the primary antibodies were mouse monoclonal anti-PKC α and anti-PKC ϵ antibodies (1:1000 in BSA).

Statistical analysis

SPSS 19.0 software (IBM, Chicago, IL, USA) was used for statistical analysis. The data are presented as the mean \pm standard deviation (SD). Comparisons between multiple groups were performed using one-way ANOVA, and comparisons between two groups were performed using LSD *t*-test. $P < 0.05$ was considered statistically significant.

Results

Effects of A β_{25-35} and donepezil on cell viability

Treatment with A β_{25-35} at 5, 10, 20 or 50 μ mol/L for 24 h significantly ($P < 0.05$) decreased the cell viability from 85.26 ± 8.03 to 40.84 ± 4.28 , whereas donepezil had no significant effects on the cell viability at any of the concentrations (from 0.5 μ mol/L to 50 μ mol/L) (Table 1).

Table 1 Effects of A β_{25-35} and donepezil on cell viability ($n = 6$, mean \pm SD)

Concentration (μ mol)	Cell viability (A β_{25-35})	Cell viability (donepezil)
Control	100	100
0.5	97.53 ± 8.54	99.28 ± 6.76
1	85.26 ± 8.03	98.29 ± 8.78
5	$78.94 \pm 7.67^*$	97.94 ± 5.45
10	$70.69 \pm 7.32^*$	99.78 ± 5.74
20	$56.65 \pm 4.15^*$	97.48 ± 5.56
50	$40.84 \pm 4.28^*$	95.56 ± 7.85
<i>P</i>	0.00	0.23

* $P < 0.05$ compared with the control group

Table 2 Effects of donepezil on Aβ₂₅₋₃₅-induced cell viability and LDH release (n = 6, mean ± SD)

Groups	Cell viability	LDH
Control	100	100
Aβ ₂₅₋₃₅	57.35 ± 3.95*	164.57 ± 14.52*
Aβ ₂₅₋₃₅ + 1 μmol donepezil	71.83 ± 6.01	152.63 ± 11.35
Aβ ₂₅₋₃₅ + 5 μmol donepezil	79.61 ± 7.72**	129.26 ± 11.56**
Aβ ₂₅₋₃₅ + 10 μmol donepezil	86.37 ± 7.45**	124.21 ± 7.44**
Aβ ₂₅₋₃₅ + 20 μmol donepezil	88.98 ± 9.36**	129.66 ± 5.27**
Aβ ₂₅₋₃₅ + 50 μmol donepezil	87.35 ± 7.42**	138.25 ± 5.93**
P	0.004	0.000

*P < 0.05 compared with the control group

**P < 0.05 compared with the Aβ₂₅₋₃₅ group. The concentration of Aβ₂₅₋₃₅ administered to each group was 20 μmol/L

Effects of donepezil on Aβ₂₅₋₃₅-induced cell viability and LDH release

Compared with the normal control, 20 μmol/L Aβ₂₅₋₃₅ significantly (P < 0.05) decreased cell viability but increased LDH release. Compared with the Aβ₂₅₋₃₅, Aβ₂₅₋₃₅ + 5, 10, 20 or 50 μmol donepezil significantly (P < 0.05) increased the cell viability from 57.35 ± 3.95 to 87.35 ± 7.42 but decreased LDH release from 164.57 ± 14.52 to 138.25 ± 5.93, indicating that 5–20 μmol donepezil antagonized the decrease in cell viability induced by 20 μmol Aβ₂₅₋₃₅ (Table 2).

P-PKC and P-MARCKS expression

Compared with that in the normal control group, P-PKC and P-MARCKS expression was significantly (P < 0.05) increased in the 10 μmol donepezil-treated group (158.86 ± 11.62 and 147.56 ± 12.05, respectively). Compared with that in the donepezil-treated group, P-PKC and P-MARCKS expression was significantly (P < 0.05) decreased in the GF109203X + donepezil-treated group (99.67 ± 10.36 and 99.64 ± 8.86, respectively, Fig. 1 and Table 3).

Effects of donepezil on PKC subtype distribution

Immunocytochemical fluorescence staining demonstrated that the PKCα and PKCε proteins were mostly evenly distributed in the cytoplasm in the normal control group but exhibited higher expression in the cytomembrane in the 10 μmol donepezil-treated group (Fig. 2). Additionally, the expression of the PKCα and PKCε isoforms in the cytosolic and membrane fractions was measured by Western blot separately to further confirm the subcellular distribution after donepezil treatment. The results showed that donepezil decreased the expression of PKCα (from 100 to 74.83 ± 9.41, P < 0.01)

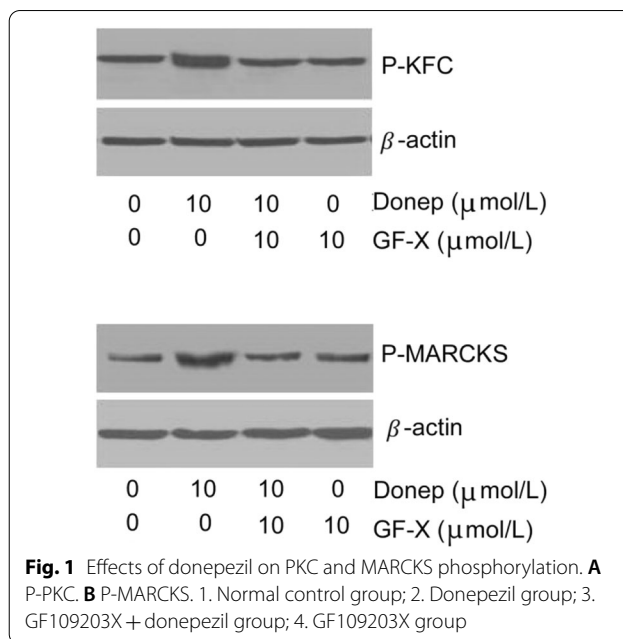


Fig. 1 Effects of donepezil on PKC and MARCKS phosphorylation. **A** P-PKC. **B** P-MARCKS. 1. Normal control group; 2. Donepezil group; 3. GF109203X + donepezil group; 4. GF109203X group

Table 3 P-PKC and P-MARCKS expression

Groups	P-PKC	P-MARCKS
Control	100	100
Donepezil	158.86 ± 11.62*	147.56 ± 12.05*
GF109203X + donepezil	99.67 ± 10.36**	99.64 ± 8.86**
GF109203X	95.47 ± 8.53	93.58 ± 7.36
P	0.001	0.015

P-PKC phosphorylated protein kinase C; P-MARCKS phospho-myristoylated alanine-rich C kinase substrate

*P < 0.05 compared with the control group, and **P < 0.05 compared with the donepezil group

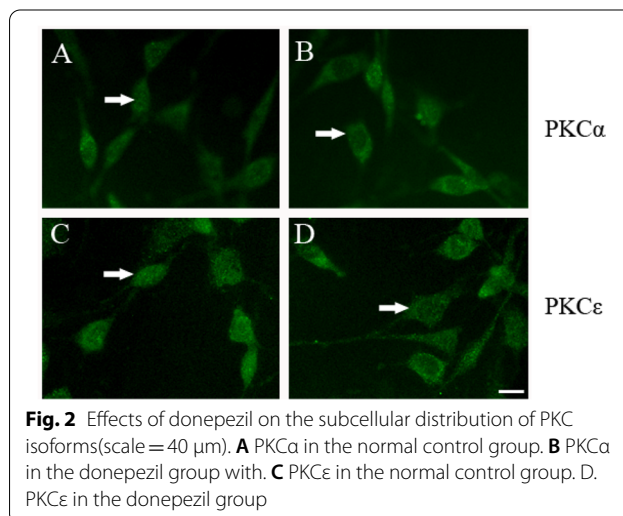


Fig. 2 Effects of donepezil on the subcellular distribution of PKC isoforms (scale = 40 μm). **A** PKCα in the normal control group. **B** PKCα in the donepezil group with. **C** PKCε in the normal control group. **D**. PKCε in the donepezil group

and PKC ϵ (from 100 to 79.75 ± 8.16 , $P < 0.05$) in the cytosolic fraction but increased their expression (PKC α : from 100 to 130.89 ± 12.45 , $P < 0.01$; PKC ϵ : from 100 to 148.39 ± 15.58 , $P < 0.001$) in the membrane fraction (Fig. 3). This implies that donepezil can induce the translocation of the PKC α and PKC ϵ isoforms from the cytosol to the membrane, indicating that donepezil administration can activate the PKC signalling pathway.

Discussion

Our study demonstrated that the acetylcholinesterase inhibitor donepezil antagonizes the ability of A β_{25-35} to decrease cell viability. This protective effect of donepezil was partially offset by the PKC inhibitor GF109203X, indicating that the PKC pathway participates in the cellular protective effect of donepezil. Donepezil increased the expression of P-PKC and P-MARCKS and altered the distribution of PKC α and PKC ϵ , suggesting that donepezil has an activating effect on PKC, which may be the mechanism by which donepezil protects against A β_{25-35} .

Twenty-four hours after treatment with 5–50 $\mu\text{mol/L}$ A β_{25-35} , the viability of PC 12 cells was decreased in a dose-dependent manner; however, donepezil at the same concentration did not significantly affect cell viability. In contrast, 1–50 $\mu\text{mol/L}$ donepezil antagonized the neurotoxicity of 20 μmol A β_{25-35} at. Compared with the control, treatment with A β_{25-35} led to marked changes in PC

12 cells, including cell shrinkage, diminished cell bodies, reduced protrusions and decreased adherence ability. These cell changes were protective responses that resulted from exposure to foreign toxic substances, and pretreatment with donepezil significantly antagonized the toxic effects. However, the specific proteins that participate in the morphological changes of cells and the detailed mechanism remain to be explored.

PKC plays a very important regulatory role in the initiation and development of AD, and PKC α and PKC ϵ are thought to be closely related to the nosogenesis of AD. Normally, PKC is located in the cytoplasm, and when it is activated, it is transported to the membrane serosa [15] through a process called protein trafficking. PKC ϵ has also been found to modulate the activity of α secretase by participating in M receptor-mediated sAPP α secretion [16]. In the fibroblasts of AD patients, the function of PKC has been found to be lacking, leading to decreased responses of neurons to various growth factors and neurotransmitters; additionally, decreased PKC activity together with decreased trafficking ability of PKC subtypes is closely related to decreased memory and cognitive function [16, 17]. The downstream substrate of PKC, MARCKS, is expressed in the mouse brain and is associated with the spatial learning ability of animals. In senile plaques in AD, MARCKS coexists with PKC and A β , and the phosphorylation of MARCKS is considered to be a marker of PKC activation, which is associated with neuroprotection [18]. PKC ϵ is often associated with preconditioning neuroprotection and mediates ischaemic tolerance by activating ERK in brain slices [19, 20]. PKC α has also been implicated in both cell survival and cell death signalling pathways and is activated in the rat brain upon ischaemic preconditioning [21–23]. The activation of PKC is neuroprotective and can increase neuronal strength against apoptosis and maintain cellular homeostasis through the phosphorylation of HSP27 [24, 25]. PKC ϵ induces neuroprotection against ischaemia by regulating many pathways, including the phosphorylation of the mitochondria K $^{+}$ ATP channel [20], increased synaptosomal mitochondrial respiration [26] and the activation of the extracellular signal-regulated kinase (ERK) pathway [19], via N-methyl-d-aspartate (NMDA) receptors [20], and by regulating gamma-aminobutyric acid (GABA) synapses [27]. Our study demonstrated that donepezil is able to activate PKC and MARCKS by increasing their phosphorylation, which may be the molecular basis for its effects on cell viability and neuroprotection.

MARCKS phosphorylation is regarded as a marker of PKC activation. In their inactivated states, PKC isoforms are localized in the cytosolic fraction, but they translocate to the membrane fraction after activation. To

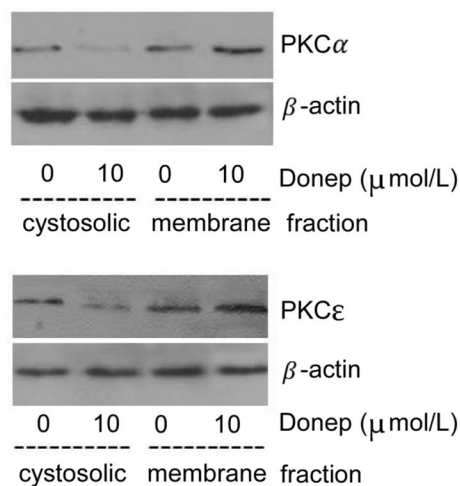


Fig. 3 Effects of donepezil (Donep) on the subcellular distribution of the PKC α and PKC ϵ isoforms. PC 12 cells were treated with vehicle alone or with 10 μmol donepezil for 2 h. Cytosolic and membrane proteins were then extracted separately for Western blot analysis. Donepezil decreased the expression of PKC α (from 100 to 74.83 ± 9.41 , $P < 0.01$) and PKC ϵ (from 100 to 79.75 ± 8.16 , $P < 0.05$) in the cytosolic fraction but increased their expression (PKC α from 100 to 130.89 ± 12.45 , $P < 0.01$; PKC ϵ : from 100 to 148.39 ± 15.58 , $P < 0.001$) in the membrane fraction

determine whether donepezil can affect the subcellular distribution of PKC isoforms, PKC α and PKC ϵ , the two main isoforms involved in the pathogenesis of AD, were evaluated after donepezil treatment. Since STED analysis technology is not available in our lab, we performed further experiments to support our point of view. To detect the subcellular distribution of the PKC isoforms, immunocytochemical staining was performed, and the subcellular distribution of the PKC α and PKC ϵ proteins in the cytosolic and membrane fractions was also measured by Western blotting, as in our previous study [28]. The results showed that donepezil decreased PKC α and PKC ϵ expression in the cytosolic fraction but increased the expression of these two isoforms in the membrane fraction (Fig. 3). This implies that donepezil can induce the translocation of the PKC α and PKC ϵ isoforms from the cytosolic fraction to the membrane fraction. Higher levels of the PKC α and PKC ϵ isoforms were found in the membrane fraction in the donepezil-treated groups than in the vehicle-treated groups, supporting our conclusion that donepezil can activate the PKC signalling pathway. Activation of the PKC signalling pathway may thus become a key to the development of new drugs for the treatment of dementia in Alzheimer's disease, which may indicate the clinical significance of our study.

Apoptosis and subsequent loss of neuronal function induced by various pathological and physiological factors may be the common pathological changes in neurodegenerative diseases, and protecting the function of the remaining neurons is one approach to treatment. As a specific and potent acetylcholinesterase inhibitor, donepezil is able to improve cognitive function in mild and moderate AD patients by increasing cholinergic nerve function and activating PKC to antagonize apoptosis. Donepezil can also increase the secretion of amyloid precursor protein α to support neurons and consequently exert its neuroprotective effect [13].

In our previous study, two cell lines were used in one experiment. When we investigated the effects of the protein kinase C activator TPPB on amyloid precursor protein (APP) processing, we used PC12 cells and SH-SY5Y^{APP695} cells, human neuroblastoma SH-SY5Y cells stably transfected with human wild-type APP695 cDNA [29]. The same cells were used to study PMS777, a new cholinesterase inhibitor with anti-platelet activated factor activity [30]. We used human neuroblastoma SK-N-SH cells and PC12 cells to study the effect of deprenyl on APP processing [28]. These three experiments showed similar results, including the effects of these agents on APP expression, sAPP α secretion, and A β secretion, in the two cell lines [28–30]. In neuroprotective studies, we investigated the effects of erythropoietin on A β -induced neurotoxicity in SH-SY5Y cells and PC 12 cells, and the

results showed the same trend [31, 32]. Although the two cell lines come from two different species, the experiments showed similar results. Thus, in the present study, we exposed PC12 cells to A β as an in vitro model for investigating the neuroprotective effects of donepezil.

Our study is clinically significant because extracellular deposits of A β protein is able to result in formation of senile plaques in the hippocampus, which is the pathological characteristics of AD associated with neurodegeneration. Among the fragments studied thus far, A β ₂₅₋₃₅ represents the shortest fragment of A β , which is processed in vivo by brain proteases. A β ₂₅₋₃₅ is the functional domain of A β required for its neurotoxic effect in dementia clinically, and it retains the toxicity of the full-length peptide [33]. It is highly cytotoxic to neuronal cells and widely used both in vitro and in vivo in neuroscience research [34], including our own previous studies [2, 31, 32, 35]. Thus, A β ₂₅₋₃₅ was chosen for this experiment. The doses of A β ₂₅₋₃₅ in our study were used according to relevant studies [36, 37]. We firstly applied 5, 10, 20 or 50 μ mol/L of A β ₂₅₋₃₅ to test the neurotoxicity of A β ₂₅₋₃₅ in the PC 12 cells. After this test, we chose the concentration of 20 μ mol/L of A β ₂₅₋₃₅ for the protective experiment of donepezil. At the concentration of 20 μ mol/L of A β ₂₅₋₃₅ to test the protective effect of donepezil, the cell viability was 56% (as determined by the MTT assay). If donepezil is added after A β administration, there may not be enough cells left to be rescued by donepezil, and the neuroprotective effect of donepezil would consequently be difficult to show. If a lower concentration of A β ₂₅₋₃₅ (5 or 10 μ mol/L, for instance) is used, the neuroprotection afforded by donepezil may not be necessary, and thus its potential neuroprotective ability would also be difficult to show. Thus, we used A β ₂₅₋₃₅ at a concentration of 20 μ mol/L (which damaged a portion of the cells) and added donepezil before A β administration to show its neuroprotective effect against A β .

Pretreating cells with donepezil before adding A β is a prophylactic approach that may not mimic the in vivo situation. Before treatment with A β ₂₅₋₃₅ in our study, the effect of donepezil on cell viability was tested with the concentration of 0.5, 1, 5, 10, 20 and 50 μ mol/L, none of which had a negative effect on the PC 12 cells. Then, donepezil at 5, 10, 20 and 50 μ mol/L was used to test its neuroprotective effect on the PC 12 cells after treatment with A β ₂₅₋₃₅. The protective effect of donepezil was dose dependent when used in the doses range of 5, 10, 20 and 50 μ mol/L for the treatment of PC 12 cells. In the treatment of cells, we used the concentration μ mol/L of the donepezil. When used in human for the treatment of AD, the dose of donepezil is mg/d and the highest dose of donepezil is 10 mg per day, which is probably to prevent possible side effects caused by larger-dose donepezil.

In conclusion, donepezil can antagonize A β ₂₅₋₃₅-induced neurotoxicity in PC 12 cells, and the activation of PKC may account for the neuroprotective effect of donepezil. Activation of PKC may become a key to develop novel approaches for the treatment of dementia in Alzheimer's disease.

Acknowledgements

None.

Author contributions

BLG contributed to study design. BLG and NNC conducted experiment. BLG, NNC, XL and CFR collected data. CFR supervised this study. BLG was involved in writing and revision of the paper. All the authors read and approved the final manuscript.

Funding

None.

Availability of data and materials

All data and materials will be available from the corresponding author.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shijiazhuang People's Hospital. This is cell experiment and no patients have been enrolled.

Consent for publication

The authors agreed to the publication of all the data and materials.

Competing interests

None.

Author details

¹Department of Medical Research, Shijiazhuang People's Hospital, 9 Fangbei Road, Shijiazhuang 050011, Hebei Province, China. ²Neurology, Henan Provincial People's Hospital, School of Clinical Medicine, Henan University, Zhengzhou 450003, China. ³Department of Laboratory Test, First Affiliated Hospital, Zhengzhou University, Zhengzhou 450003, China.

Received: 10 October 2021 Accepted: 20 October 2022

Published online: 28 October 2022

References

- Cai P, Fang SQ, Yang XL, et al. Rational design and multibiological profiling of novel donepezil-trolox hybrids against alzheimer's disease, with cholinergic, antioxidant, neuroprotective, and cognition enhancing properties. *ACS Chem Neurosci*. 2017;8(11):2496–511. <https://doi.org/10.1021/acscchemneuro.7b00257>.
- Yang HQ, Li X, Yang WM, et al. Neuroprotective effects of new protein kinase C activator TPPB against Abeta(2)(5)(-)(3)(5) induced neurotoxicity in PC12 cells. *Neurochem Res*. 2012;37(10):2213–21. <https://doi.org/10.1007/s11064-012-0846-6>.
- Gandy S, DeKosky ST. Toward the treatment and prevention of Alzheimer's disease: rational strategies and recent progress. *Annu Rev Med*. 2013;64:367–83. <https://doi.org/10.1146/annurev-med-092611-084441>.
- Risacher SL, Saykin AJ. Neuroimaging and other biomarkers for Alzheimer's disease: the changing landscape of early detection. *Annu Rev Clin Psychol*. 2013;9:621–48. <https://doi.org/10.1146/annurev-clinpsy-050212-185535>.
- Selkoe DJ. Altered structural proteins in plaques and tangles: what do they tell us about the biology of Alzheimer's disease? *Neurobiol Aging*. 1986;7(6):425–32.
- Ye CY, Lei Y, Tang XC, et al. Donepezil attenuates Abeta-associated mitochondrial dysfunction and reduces mitochondrial Abeta accumulation in vivo and in vitro. *Neuropharmacology*. 2015;95:29–36. <https://doi.org/10.1016/j.neuropharm.2015.02.020>.
- Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*. 2002;297(5580):353–6. <https://doi.org/10.1126/science.1072994>.
- Klafki HW, Staufenbiel M, Kornhuber J, et al. Therapeutic approaches to Alzheimer's disease. *Brain*. 2006;129(Pt 11):2840–55. <https://doi.org/10.1093/brain/awl280>.
- Noh MY, Koh SH, Kim Y, et al. Neuroprotective effects of donepezil through inhibition of GSK-3 activity in amyloid-beta-induced neuronal cell death. *J Neurochem*. 2009;108(5):1116–25. <https://doi.org/10.1111/j.1471-4159.2008.05837.x>.
- Romberg C, Mattson MP, Mughal MR, et al. Impaired attention in the 3xTgAD mouse model of Alzheimer's disease: rescue by donepezil (Ari-cept). *J Neurosci*. 2011;31(9):3500–7. <https://doi.org/10.1523/JNEUROSCI.5242-10.2011>.
- Tang SS, Hong H, Chen L, et al. Involvement of cysteinyl leukotriene receptor 1 in Abeta1-42-induced neurotoxicity in vitro and in vivo. *Neurobiol Aging*. 2014;35(3):590–9. <https://doi.org/10.1016/j.neurobiolaging.2013.09.036>.
- Hashimoto M, Kazui H, Matsumoto K, et al. Does donepezil treatment slow the progression of hippocampal atrophy in patients with Alzheimer's disease? *Am J Psychiatry*. 2005;162(4):676–82. <https://doi.org/10.1176/appi.ajp.162.4.676>.
- Zimmermann M, Gardoni F, Marcello E, et al. Acetylcholinesterase inhibitors increase ADAM10 activity by promoting its trafficking in neuroblastoma cell lines. *J Neurochem*. 2004;90(6):1489–99. <https://doi.org/10.1111/j.1471-4159.2004.02680.x>.
- Etcheberrigaray R, Tan M, Dewachter I, et al. Therapeutic effects of PKC activators in Alzheimer's disease transgenic mice. *Proc Natl Acad Sci U S A*. 2004;101(30):11141–6. <https://doi.org/10.1073/pnas.0403921101>.
- Yang HQ, Sun ZK, Ba MW, et al. Involvement of protein trafficking in deprenyl-induced alpha-secretase activity regulation in PC12 cells. *Eur J Pharmacol*. 2009;610(1–3):37–41. <https://doi.org/10.1016/j.ejphar.2009.03.047>.
- Cisse M, Braun U, Leitges M, et al. ERK1-independent alpha-secretase cut of beta-amyloid precursor protein via M1 muscarinic receptors and PKCalpha/epsilon. *Mol Cell Neurosci*. 2011;47(3):223–32. <https://doi.org/10.1016/j.mcn.2011.04.008>.
- Hongpaisan J, Sun MK, Alkon DL. PKC epsilon activation prevents synaptic loss, Abeta elevation, and cognitive deficits in Alzheimer's disease transgenic mice. *J Neurosci*. 2011;31(2):630–43. <https://doi.org/10.1523/JNEUROSCI.5209-10.2011>.
- Su R, Han ZY, Fan JP, et al. A possible role of myristoylated alanine-rich C kinase substrate in endocytic pathway of Alzheimer's disease. *Neurosci Bull*. 2010;26(4):338–44. <https://doi.org/10.1007/s12264-010-0131-0>.
- Lange-Asschenfeldt C, Raval AP, Dave KR, et al. Epsilon protein kinase C mediated ischemic tolerance requires activation of the extracellular regulated kinase pathway in the organotypic hippocampal slice. *J Cereb Blood Flow Metab*. 2004;24(6):636–45. <https://doi.org/10.1097/01.WCB.0000121235.42748.BF>.
- Raval AP, Dave KR, Mochly-Rosen D, et al. Epsilon PKC is required for the induction of tolerance by ischemic and NMDA-mediated preconditioning in the organotypic hippocampal slice. *J Neurosci*. 2003;23(2):384–91.
- Cui XY, Li JF, Han S, et al. Hypoxic preconditioning increases cPKCgamma membrane translocation in murine brain. *Sheng Li Xue Bao*. 2004;56(4):461–5.
- Kurkinen K, Busto R, Goldsteins G, et al. Isoform-specific membrane translocation of protein kinase C after ischemic preconditioning. *Neurochem Res*. 2001;26(10):1139–44.
- Zhang D, Anantharam V, Kanthasamy A, et al. Neuroprotective effect of protein kinase C delta inhibitor rottlerin in cell culture and animal models of Parkinson's disease. *J Pharmacol Exp Ther*. 2007;322(3):913–22. <https://doi.org/10.1124/jpet.107.124669>.
- Lambert H, Charette SJ, Bernier AF, et al. HSP27 multimerization mediated by phosphorylation-sensitive intermolecular interactions at the amino terminus. *J Biol Chem*. 1999;274(14):9378–85.
- Rogalla T, Ehrnsperger M, Preville X, et al. Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative

- stress/tumor necrosis factor alpha by phosphorylation. *J Biol Chem.* 1999;274(27):18947–56.
26. Dave KR, DeFazio RA, Raval AP, et al. Ischemic preconditioning targets the respiration of synaptic mitochondria via protein kinase C epsilon. *J Neurosci.* 2008;28(16):4172–82. <https://doi.org/10.1523/JNEUROSCI.5471-07.2008>.
 27. DeFazio RA, Raval AP, Lin HW, et al. GABA synapses mediate neuroprotection after ischemic and epsilonPKC preconditioning in rat hippocampal slice cultures. *J Cereb Blood Flow Metab.* 2009;29(2):375–84. <https://doi.org/10.1038/jcbfm.2008.126>.
 28. Yang HQ, Ba MW, Ren RJ, et al. Mitogen activated protein kinase and protein kinase C activation mediate promotion of sAPPalpha secretion by deprenyl. *Neurochem Int.* 2007;50(1):74–82. <https://doi.org/10.1016/j.neuint.2006.07.016>.
 29. Yang HQ, Pan J, Ba MW, et al. New protein kinase C activator regulates amyloid precursor protein processing in vitro by increasing alpha-secretase activity. *Eur J Neurosci.* 2007;26(2):381–91. <https://doi.org/10.1111/j.1460-9568.2007.05648.x>.
 30. Yang HQ, Sun ZK, Zhao YX, et al. PMS777, a new cholinesterase inhibitor with anti-platelet activated factor activity, regulates amyloid precursor protein processing in vitro. *Neurochem Res.* 2009;34(3):528–35. <https://doi.org/10.1007/s11064-008-9816-4>.
 31. Sun ZKYH, Lu GQ. Effects of erythropoietin on cell apoptosis induced by beta-amyloid peptide. *J Shanghai Jiaotong Univ (Chin Ed).* 2007;27(10):1177–80.
 32. Zhi-Kun S, Hong-Qi Y, Zhi-Quan W, et al. Erythropoietin prevents PC12 cells from beta-amyloid-induced apoptosis via PI3KAkt pathway. *Transl Neurodegener.* 2012;1(1):7. <https://doi.org/10.1186/2047-9158-1-7>.
 33. Pike CJ, Walencewicz-Wasserman AJ, Kosmoski J, et al. Structure-activity analyses of beta-amyloid peptides: contributions of the beta 25–35 region to aggregation and neurotoxicity. *J Neurochem.* 1995;64(1):253–65. <https://doi.org/10.1046/j.1471-4159.1995.64010253.x>.
 34. Giunta S, Galeazzi R, Valli MB, et al. Transferrin neutralization of amyloid beta 25–35 cytotoxicity. *Clin Chim Acta.* 2004;350(1–2):129–36. <https://doi.org/10.1016/j.cccn.2004.07.025>.
 35. Sun ZK, Yang HQ, Pan J, et al. Protective effects of erythropoietin on tau phosphorylation induced by beta-amyloid. *J Neurosci Res.* 2008;86(13):3018–27. <https://doi.org/10.1002/jnr.21745>.
 36. Choi SLJ, Kim H. Korean Red Ginseng Inhibits Amyloid-beta-Induced Apoptosis and Nucling Expression in Human Neuronal Cells. *Pharmacology.* 2020;105(9–10):586–97.
 37. Song NYH, Pang W, Qie Z, Lu H, Tan L, Li H, Sun S, Lian F, Qin C, Jiang Y. Mulberry extracts alleviate aβ 25–35-induced injury and change the gene expression profile in PC12 cells. *Evid Based Complement Alternat Med.* 2014. <https://doi.org/10.1155/2014/150617>.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

