Original Paper

Wnt / β -Catenin Signaling Pathway Against $A\beta$ Toxicity in PC12 Cells

Yaping Zheng^a Jin Wang^a Deheng Li^a Meixia Guo^b Minghui Zhen^c
Quanzhong Chang^a

^aDepartment of Basic Medicine, Luohe medical College, Luohe, ^bSanQuan medical College, Xinxiang,

^cChildren's hospital of Zhengzhou, Zhengzhou, China

Key Words

PC12 cell • Wnt signaling pathway • $A\beta_{25-35}$

Abstract

Background/Aims: Alzheimer's disease (AD) is characterized by accumulation of β -amyloid ($A\beta$). However, the mechanism of how $A\beta$ affects neuronal cell death remains elusive. The balance of pro- and anti-apoptotic Bcl-2 family proteins (e.g., Bcl-2 and Bax) has been known to play a pivotal role in neuronal cell death. Of note, expression levels of these proteins are changed in the neurons in AD. To date no study has elucidated the relationship between $A\beta$ and Bax. **Methods:** The present study explored the role of Wnt/ β -catenin pathway in the neurotoxic effect of $A\beta_{25-35}$. Flow cytometry was employed to determine the apoptosis, western blotting to assess the protein abundance of Bcl-2 and BAX, MTT assay to decipher the cells viability. **Results:** As a result, the addition of Wnt3a significantly prevented oligomeric $A\beta$ -induced neuronal cell death and viability. Furthermore, treatment with $A\beta_{25-35}$ increased Bax and Bcl-2 protein abundance and mRNA levels, an effect significantly blocked by Wnt3a (100 ng/ml) and GSK3 β inhibitor TWS119 (10 μ M). **Conclusion:** These findings are first to demonstrate that Wnt/ β -catenin signaling pathway regulates $A\beta_{25-35}$ -induced apoptosis.

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Published by S. Karger AG, Basel

Introduction

Accumulation of β -amyloid ($A\beta$) in the brain is the primary influence driving AD pathogenesis [1, 2]. Recent studies has shown that oligomeric $A\beta$ induces apoptotic neuronal death in the rat and mouse neurons *in vitro* and *in vivo* [2, 3]. However, the molecular mechanism of $A\beta$ -induced apoptosis remains elusive.

Y. Zheng and J. Wang contributed equally and thus share first authorship

Quanzhong Chang

Department of Basic Medicine, LuoHe medical College,
450053 Luohe, (China)
Tel.+8615890233618, E-Mail cqzchang@sina.com

Bcl-2 is specifically considered an important anti-apoptotic protein and is thus classified as an oncogene [4] whereas Bax belongs to pro-apoptotic subfamily, which promotes apoptosis by translocating into the mitochondrial membrane and facilitating cytochrome release [5]. Moreover, expression levels of Bcl-2 family proteins, such as Bax, Bak, Bad, Bcl-2 and Bcl-x are altered in the vulnerable neurons in AD [6].

Wnt signaling pathways associates not only with formation of the embryonic dorsoventral axis, but also in a number of other developmental events according to establishment of cell polarity or determination of cell fate [7]. The Wnt proteins are a diverse family of secreted lipid-modified glycoproteins that have 350-400 amino acids [8]. In the previous study we have found that A β_{25-35} could regulate phosphorylated-sites of tau protein and GSK-3 through Wnt/ β -catenin pathway in PC12 cells [9]. However, whether Wnt/ β -catenin pathway affects A β -induced apoptosis remains unclear. In this study we have established an AD model from the adrenal pheochromocytoma PC12 cell line, and show that the apoptosis induced by A β_{25-35} is modulated by Wnt/ β -catenin signaling pathway.

Materials and Methods

Ethics Statement

All procedures and assays were approved by the Institutional Animal Care and Use Committee of Luohe medical College.

Cell culture

PC cells were cultured in DMEM medium supplemented with 10% FCS and 1% penicillin/streptomycin under standard conditions. Cells grown on the coverslips were treated in 30 μ M A β_{25-35} for 24h; before and after the treatment, cells were also applied with agonist of Wnt/ β -catenin signaling (Wnt3a, 50ng/ml/100ng/ml) for 2h.

Primary Rat Hippocampal Neuronal Cultures

Primary hippocampus neurons were isolated from the bilateral hippocampus of the newborn Sprague-Dawley (SD) rats (provided by laboratory animal center in henan province license SCXK 2010-0002) as described previously [10]. Briefly, collected neurons were seeded in a concentration of 1×10^6 cells/cm². Cells were cultured for 6 d in medium, consisting of Eagle's minimum essential medium containing 10 mm sodium bicarbonate, 1% glucose, 1 mm l-glutamine, 20 mm KCl, 1 mm sodium pyruvate, and 10% (v/v) heat-inactivated fetal bovine serum (Sigma). After a 4–6-h period to allow cell attachment to the substrate, the culture medium was replaced with Neurobasal medium containing B27 supplements (Invitrogen) in a humidified atmosphere (6% CO₂, 94% room air) at 37 °C. All experiments were performed on cells that had been in culture for 5–8 days.

Cell viability assay

PC12 cells were grown in DMEM medium supplemented with 10% FCS and 1% penicillin/streptomycin. The cells were incubated in a stable environment with 5% CO₂ at 37°C in a humidified incubator. The medium was replaced every 24 hours. Cells were grown to about 80% confluence prior to A β_{25-35} treatment, and then exposed to A β_{25-35} at different concentrations (10, 20, 30, 40, 50 μ M). Cells grown in a medium without A β_{25-35} served as control.

To measure the effects of Wnt3a upon A β_{25-35} -induced apoptosis in PC12 cells, cells were treated with A β_{25-35} in the presence of Wnt-3a at different concentrations (50, 100, 200, 250 ng/ml). Each group of cells were seeded in 96-well microtiter plates and incubated for 24 hours. At different points, 20 μ l of MTT was added to each well followed by 4 hours incubation.

The medium was discarded and 150 μ l of DMSO was added into each well, and incubated for 20min. The OD(optical density) 492nm was measured. The proliferation inhibition rate was calculated as: (1-the OD of the experimental group/the OD of the control group) $\times 100\%$. Each experiment was repeated three times.

Determination of apoptosis

PC12 cells were collected and centrifuged at 1,000 $\times$ g for 5 min and re-suspended in fresh DMEM medium at a density of 2×10^5 cells/ml. Apoptotic and necrotic cells were evaluated by Annexin V(AV) binding and propidium iodide(PI) uptake. Samples were analyzed by flow cytometry. Each of the concentrations was repeated three times, the final result is expression as the mean of three times.

Western blotting

Protein abundance was determined in PC12 cells and hippocampal neurons. The cells were washed in ice-cold PBS. RIPA lysis buffer containing phosphatase and protease inhibitor cocktail tablet was added to the washed cells or the bone powder. The samples were incubated on ice for 30 min and then centrifuged at 14,000 rpm and 4°C for 20 min. The supernatant was removed and used for Western blotting. Total protein (40-60 μ g) was separated by SDS-PAGE, thereafter transferred to PVDF membranes and blocked in 5% non-fat milk/Tris-buffered saline/Tween-20 (TBST) at room temperature for 1 hour. Membranes were probed overnight at 4°C with polyclonal rabbit anti-Bcl-2 antibody and polyclonal rabbit anti-Bax antibody (1:700 in 5% fat free milk in TBST). After incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Sigma, United States, 1:2000) for 1 hour at room temperature, the bands were visualized with enhanced chemiluminescence reagents (Sigma, United States). Membranes were also probed with ACTIN antibody as loading control. Densitometric analysis was performed using quantity One software (Abbotec, United States).

Quantative Real Time-PCR (qRT-PCR)

Total RNA was extracted from hippocampal neurons in TriFast (Peqlab, Erlangen, Germany) according to the manufacturer's instructions [11]. After DNase digestion reverse transcription of total RNA was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Penzberg, Germany). Real-time polymerase chain reaction (RT-PCR) of the respective genes were set up in a total volume of 20 μ l using 40 ng of cDNA, 500 nM forward and reverse primer and 2x GoTaq® qPCR Master Mix (Promega, Mannheim, Germany) according to the manufacturer's protocol. Cycling conditions were as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 10 sec, 58°C for 15 sec and 68°C for 30 sec. For amplification of the following primers were used (5'>3' orientation):

for Bcl-2: fw: TCAGGTACTCAGTCATCCA;

rev: GGAGATCGTGATGAAGTAC;

for BAX: fw: CCAAGAAGCTGAGCGAGTGTCTC;

rev: AGTTGCCATCAGCAAACATGTCA

for GAPDH: fw: CGGAGTCAACGGATTTGGTCGTAT;

rev: AGCCTTCTCCATGGTGGTGAAGAC

Specificity of PCR products was confirmed by analysis of a melting curve. Real-time PCR amplifications were performed on a CFX96 Real-Time System (Bio-Rad, Munich, Germany) and all experiments were done in duplicate. GAPDH was amplified to standardize the amount of sample RNA. Relative quantification of gene expression was achieved using the Δ CT method as described [12].

Statistics

Data are provided as means $\pm$ SEM, n represents the number of independent experiments. All data were tested for significance using unpaired Student t -test. Only results with $p < 0.05$ were considered statistically significant.

Results

In order to identify the effect of Wnt/ β -catenin signaling upon the A β_{25-35} -induced cytotoxicity in PC12 cells, cells were treated with A β_{25-35} applied either in 10 μ M, 20 μ M, 30 μ M and 40 μ M prior to MTT assay. As illustrated in Fig. 1, exposure of cells to different concentrations of A β_{25-35} for 24 hours led to a decrease of the cell viability, an effect being concentration dependent. The results in Fig. 2 showed that the effect of A β_{25-35} was abolished by Wnt3a(100 ng/ml). These data indicate that Wnt/ β -catenin signaling pathway can

Fig. 1. A β ₂₅₋₃₅ neurotoxicity in PC12 cells. Original tracing showing the cell viability of PC12 cells with A β ₂₅₋₃₅ treatment at different concentrations (10, 20, 30, 40 and 50 μ M). Cell viability was evaluated by the MTT assay.

Fig. 2. Wnt-3a protects survival of hippocampal neurons from A β ₂₅₋₃₅ neurotoxicity. Original tracing showing the cell viability of PC12 cells with A β ₂₅₋₃₅ (30 μ M) treatment in the presence of Wnt3 α at different concentrations (50, 100, 200 and 250 ng/ml). Cell viability was evaluated by the MTT assay.

Fig. 3. The role of Wnt3a in the A β ₂₅₋₃₅-induced-apoptosis of PC12 cells. A. Control group; B. A β ₂₅₋₃₅-treated (30 μ mol/L) group; C. A β ₂₅₋₃₅ treated in the presence of Wnt3a (50 ng/ml); D. A β ₂₅₋₃₅ treated in the presence of Wnt3a (100 ng/ml). E. Arithmetic means $\pm$ SEM (n = 3 independent experiments) of A β ₂₅₋₃₅-induced apoptosis in PC12 cells without (white bar) or with A β ₂₅₋₃₅ (black bar) in the absence or presence of Wnt3a either in 50 ng/ml (light grey bar), or in 100 ng/ml (dark grey bar). * (p < 0.05), ** (p < 0.01) indicate statistically significant difference.

alleviate cellular damage caused by A β ₂₅₋₃₅.

In order to test whether Wnt/ β -catenin signaling pathway associates with the A β ₂₅₋₃₅-mediated apoptosis in PC12 cells, immunocytochemistry flow cytometry was applied. As illustrated in Fig. 3, incubation with A β ₂₅₋₃₅ for 24 hours significantly increased the apoptotic rates of cells, an effect blocked by Wnt3a. The results suggest that Wnt signaling pathway is involved in the A β ₂₅₋₃₅-induced cellular apoptosis.

Fig. 4. Effect of A β_{25-35} on Bax and Bcl-2 protein abundance in PC12 cells in the absence and presence of Wnt3a. A. Original western blot showing the protein abundance of Bcl-2 and Bax as well as respective actin in PC12 cells without or with A β_{25-35} in the presence or absence of Wnt3a(50ng/ml, 100ng/ml). B. Arithmetic means $\pm$ SEM (n = 3 independent experiments) of Bcl-2 and Bax as well as respective actin protein abundance in PC12 cells without(white bar) or with A β_{25-35} (black bar) in the absence or presence of Wnt3a either in 50ng/ml(light grey bar), or in 100ng/ml(dark grey bar). *(p<0.05), **(p<0.01) indicate statistically significant difference.

Fig. 5. Effect of A β_{25-35} on Bax and Bcl-2 protein abundance in hippocampal neurons in the absence and presence of Wnt3a. A. Original western blot showing the protein abundance of Bcl-2 and Bax as well as respective actin in hippocampal neurons without or with A β_{25-35} in the presence or absence of Wnt3a(50ng/ml, 100ng/ml). B. Arithmetic means $\pm$ SEM (n = 3 independent experiments) of Bcl-2 and Bax as well as respective actin protein abundance in hippocampal neurons without(white bar) or with A β_{25-35} (black bar) in the absence or presence of Wnt3a either in 50ng/ml(light grey bar), or in 100ng/ml(dark grey bar). *(p<0.05), **(p<0.01) indicate statistically significant difference.

In order to elucidate whether Wnt/ β -catenin signaling pathway associates with the apoptosis-related protein abundance in pretreated A β_{25-35} PC12 cells and hippocampal neurons. Western Blot was applied to detect the expression of Bcl-2 and Bax protein in PC12 cells. As shown in Fig. 4A, the expression level of Bax protein in control group was less than that in A β_{25-35} group, while the expression level of Bcl-2 protein in the negative control group was more than that in A β_{25-35} group. As shown in Fig. 4B, Wnt3a blocked A β_{25-35} -mediated decline of the ratio of Bcl-2/Bax in PC12 cells. Furthermore, as illustrated in Fig. 5, results in hippocampal neurons were consistent with that in PC12 cells. Thus, Wnt3a alleviates A β_{25-35} -mediated decline of Bcl-2/Bax.

In order to determine whether Wnt/ β -catenin signaling pathway associates with the mRNA levels of Bcl-2 and Bax, qRT-PCR was utilized. As illustrated in Fig. 6 and Fig.7,

Fig. 6. Effect of A β_{25-35} on Bax and Bcl-2 mRNA levels in hippocampal neurons in the absence and presence of Wnt3a. A. Arithmetic means $\pm$ SEM (n = 6 independent experiments) of Bcl-2 transcriptional level in untreated (white bar) and A β_{25-35} treated (black bar) hippocampal neurons in the presence of Wnt3a (100ng/ml) (dark grey bar). B. Arithmetic means $\pm$ SEM (n = 6 independent experiments) of Bax transcriptional level in untreated (white bar) and A β_{25-35} treated (black bar) hippocampal neurons in the presence of Wnt3a (100ng/ml) (dark grey bar).

Fig. 7. Effect of A β_{25-35} on Bax and Bcl-2 mRNA levels in hippocampal neurons in the absence and presence of TWS119. A. Arithmetic means $\pm$ SEM (n = 5 independent experiments) of Bcl-2 in untreated (white bar) and A β_{25-35} treated (black bar) hippocampal neurons in the presence of Wnt3a (100ng/ml) (dark grey bar) or TWS119 (10 μ M) (light grey bar). B. Arithmetic means $\pm$ SEM (n = 5 independent experiments) of Bax in untreated (white bar) and A β_{25-35} treated (black bar) hippocampal neurons in the presence of Wnt3a (100ng/ml) (dark grey bar) or TWS119 (10 μ M) (light grey bar).

Wnt3a (100 ng/ml) and GSK3 β inhibitor TWS119 (10 μ M) alleviated A β_{25-35} -mediated increase of Bax and decline of Bcl-2.

Discussion

The present study addressed that the activation of Wnt/ β -catenin signaling cascade by Wnt3a overcame A β_{25-35} neurotoxic effects in PC12 cells. To this end, protein and mRNA levels of Bcl-2 and Bax were determined utilizing western blotting and qRT-PCR, apoptosis by flow cytometry, cells viability by MTT. As a result, the agonist of Wnt/ β -catenin signaling (Wnt3a) and GSK3 β inhibitor (TWS119) blunted A β_{25-35} -mediated inhibition upon Bcl-2/Bax ratio.

Moreover, A β is the main component of senile plaques, the deposits of which in the brain can cause loss or death of neurons. As the major segment of A β [13, 14], A β_{25-35} decreases the activity of PC12 cells whereas increases the intracellular calcium concentration as well as the phosphorylation of tau [15]. In the previous study, we found that A β_{25-35} regulated phosphorylation of tau protein and GSK-3 through Wnt/ β -catenin pathway in PC12 cells [9].

It has been reported that Wnt signaling modulates cell survival [16, 17], but to the best of our knowledge, the mechanism remains elusive. GSK3- β is a downstream effector of Wnt signaling pathway, over-expression of which induces apoptosis in PC12 cells [18]. Moreover, neuronal apoptosis induced by trophic withdrawal is associated with an increased activity of GSK-3, and the increase in GSK-3 β activity facilitates the induction of apoptosis [19]. De Ferrari et al. found that A β -dependent neurotoxicity induced a loss of function of Wnt signaling components, and lithium or compounds that mimic this signaling cascade may be putative candidates for therapeutic intervention in Alzheimer's patients [20]. Our findings are consistent with the work of Alvarez et al. [21], in which Wnt3a overcomes β -amyloid toxicity in rat hippocampal neurons. Moreover, Wnt-7a ligand protects against cytoplasmic calcium disturbances induced by A β [22]. Several studies point to a possible role for Wnt in AD [20, 23, 24].

The expression of Bcl-2 and Bax expression is mediated by A β_{25-35} at protein and transcriptional levels [21]. Moreover, A β_{25-35} affects Bcl-2 and Bax by influencing Bcl-9, CBP and TCF/LEF [6, 25, 26]. The previous study of our lab has shown that A β_{25-35} regulates phosphorylation of Tau protein through Wnt/ β -catenin signalling in PC12 cells.

In conclusion, Wnt/ β -catenin pathway is involved in A β_{25-35} -induced cellular damage caused in PC12 cells and hippocampal neurons.

Disclosure Statement

All authors disclose that they have not any potential conflict of interest (e.g., consultancies, stock ownership, equity interests, patent-licensing arrangements, lack of access to data, or lack of control of the decision to publish).

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