Overexpression of estrogen receptor beta alleviates the toxic effects of beta-amyloid protein on PC12 cells via non-hormonal ligands

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Abstract

After binding to the estrogen receptor, estrogen can alleviate the toxic effects of beta-amyloid protein, and thereby exert a therapeutic effect on Alzheimer’s disease patients. Estrogen can increase the incidence of breast carcinoma and endometrial cancer in post-menopausal women, so it is not suitable for clinical treatment of Alzheimer’s disease. There is recent evidence that the estrogen receptor can exert its neuroprotective effects without estrogen dependence. Real-time quantitative PCR and flow cytometry results showed that, compared with non-transfected PC12 cells, adenovirus-mediated estrogen receptor β gene-transfected PC12 cells exhibited lower expression of tumor necrosis factor α and interleukin 1β under stimulation with beta-amyloid protein and stronger protection from apoptosis. The Akt-specific inhibitor Abi-2 decreased the anti-inflammatory and anti-apoptotic effects of estrogen receptor β gene-transfection. These findings suggest that overexpression of estrogen receptor β can alleviate the toxic effect of beta-amyloid protein on PC12 cells, without estrogen dependence. The Akt pathway is one of the potential means for the anti-inflammatory and anti-apoptotic effects of the estrogen receptor.

Key Words

estrogen; estrogen receptor β; Alzheimer's disease; beta-amyloid protein; adenovirus; neural regeneration

Abbreviations

ER: estrogen receptor; AD: Alzheimer’s disease; Ad: adenovirus; Aβ: beta-amyloid protein; EGFP: enhanced green fluorescent protein

INTRODUCTION

The incidence rate of Alzheimer’s disease (AD) significantly increases in females versus males after 80–85 years of age. Estrogen has thus been considered to play an important role in the incidence and development of AD⁵⁻⁷. Estrogen is a liposoluble steroid hormone. It is produced mainly by the ovary and testis in the periphery and by neural cells in the hypothalamus and limbic system in the brain⁹. There is evidence that estrogen binds to the estrogen receptor (ER) on the neural cell membrane and in the neural cell nucleus to exert anti-inflammatory, anti-oxidative, and anti-apoptotic effects, antagonize the toxic effects of beta-amyloid protein (Aβ), and thereby postpone the progression of AD⁴⁻⁸. Estrogen replacement therapy increases the risk for breast
carcinoma and endometrial cancer in postmenopausal women\textsuperscript{9-10}, and therefore its clinical application for treatment of AD is limited.

The ER subtypes, ER\(\alpha\) and ER\(\beta\), are expressed differently. Both of them are expressed in the nervous system, cardiovascular system, immune system and reproductive system. However, ER\(\alpha\) is predominantly expressed in the reproductive system and ER\(\beta\) in the nervous system\textsuperscript{11-12}. ER\(\beta\) exerts biological effects in a ligand-independent manner as well as through the classic estrogen-ER pathway. Without binding to estrogen, ER\(\beta\) exerts biological effects by phosphorylation of its N-terminal activation domain in the cellular membrane by mitogen-activated protein kinases (MAPK) and subsequent activation of intracellular pathways (such as steroid receptor coactivator-1) in a ligand-independent manner\textsuperscript{13}. There is also evidence that without binding to estrogen, ER\(\beta\) inhibits the activity of death promoters related to Bcl-2/Bcl-xl, exerting anti-apoptotic effects\textsuperscript{14}. It remains poorly understood whether simply regulating ER\(\beta\) expression can have neuroprotective effects on AD without the application of estrogen.

PC12 cells are cloned from a rat adrenal pheochromocytoma cell line. They can differentiate into cells with the features of sympathetic neurons, are often used for studying the molecular mechanism of nerve diseases, and also widely used for establishing AD cell models\textsuperscript{4, 9}. In this study, the ER\(\beta\) gene was delivered into PC12 cells via an adenovirus vector and allowed to overexpress. Using common PC12 cells as a control, we investigated the effects of ER\(\beta\) overexpression on the anti-inflammatory and anti-apoptotic capabilities of PC12 cells under stimulation with A\(\beta\) in the absence of estrogen.

RESULTS

**Ad-ER\(\beta\)-EGFP effectively transfected PC12 cells**
Enhanced green fluorescent protein (EGFP) expression appeared after transfecting PC12 cells with an adenovirus bearing the ER\(\beta\) gene for 24 hours and peaked after 48 hours. EGFP expression differed at different virus concentrations. EGFP expression increased with increasing virus particle concentration. EGFP expression peaked at a virus particle concentration of \(5 \times 10^7\)/well, \(1 \times 10^8\)/well, \(5 \times 10^8\)/well, \(1 \times 10^9\)/well, respectively. The successfully transfected PC12 cells exhibit green fluorescence, with the highest transfection rate appearing at virus particle concentrations of \(5 \times 10^7\)/well and \(1 \times 10^8\)/well.

**ER\(\beta\) was highly expressed in transfected PC12 cells**
Three groups were used: a control group (non-transfected PC12 cells), a blank group (Ad-EGFP blank plasmid-transfected PC12 cells) and a transfection group (Ad-ER\(\beta\)-EGFP-transfected PC12 cells). Western blot results showed that ER\(\beta\) protein expression was detected in the PC12 cells in each group, and ER\(\beta\) protein expression was significantly higher in the transfection group than in the control and blank groups \((P < 0.01)\). There was no significant difference in ER\(\beta\) protein expression between the control group and the blank group \((P > 0.05)\) (Figure 2).

![Figure 1](image1.png) Ad-ER\(\beta\)-EGFP plasmid-transfected PC12 cells. A-D are PC12 cells transfected at a virus particle concentration of \(5 \times 10^7\)/well, \(1 \times 10^8\)/well, \(5 \times 10^8\)/well, \(1 \times 10^9\)/well, respectively.

![Figure 2](image2.png) Estrogen receptor beta expression in PC12 cells. a\(P < 0.01\), vs. blank group, b\(P < 0.01\), vs. control group (one-way analysis of variance, Student-Newman-Keuls test). Quantitative results are expressed as gray value ratio of target protein to \(\beta\)-actin.

Experimental results are expressed as mean ± SD of six wells from each group. The experiment was performed in triplicate.
These findings suggest that the ERβ gene was successfully transduced into PC12 cells, and the Ad-EGFP blank plasmid did not produce effects on ERβ expression in PC12 cells.

**Overexpressed ERβ alleviated the pro-inflammatory effects of Aβ on PC12 cells**

Non-transfected PC12 cells treated with Aβ were included in the control group. ERβ-transfected PC12 cells were divided into an Aβ group, in which Aβ was added, and an Abi-2 group, in which the Akt-specific inhibitor Abi-2 was added together with Aβ. After co-incubation for 24 hours, real-time quantitative PCR results showed that tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) mRNA expression in the ERβ-transfected Aβ group was significantly lower than in the control group (P < 0.01, P < 0.05) and in the ERβ-transfected Abi-2 group (P < 0.01, P < 0.05) (Table 1).

| Group               | TNF-α mRNA | IL-1 mRNA |
|---------------------|------------|-----------|
| Control             | 13.01±4.17 | 4.12±1.04 |
| Beta-amyloid protein| 6.11±2.40^ac | 2.74±0.78^bd |
| Abi-2               | 8.72±2.31  | 3.66±0.81 |

*P < 0.01, ^P < 0.05, vs. control group; ^c P < 0.01, ^d P < 0.05, vs. Abi-2 group (one-way analysis of variance, Student-Newman-Keuls test). Quantitative results are expressed as the fluorescence intensity ratio of the target gene to β-actin. Data are expressed as mean ± SD of six wells per group. The experiment was performed in triplicate.

**Overexpressed ERβ alleviated Aβ-induced PC12 cell apoptosis (Figure 3)**

Flow cytometry with Annexin V/propidium iodide (PI) double staining showed that the rate of apoptosis in PC12 cells in the Aβ group (11.27 ± 2.14%) was significantly lower than in the control group and in the Abi-2 group (21.14 ± 4.13%, 15.33 ± 4.21%, P < 0.01, P < 0.05).

**DISCUSSION**

Adenovirus vectors can transfect different types of eukaryotic cells, with no limitation as to whether the target cells are dividing cells, high transgene efficiency, nearly 100% transfection efficiency in in vitro experiments, and it is easy to prepare high titer viral vector[15-16]. In addition, Ad cannot be integrated into the host cell genome, and is only expressed transiently with high safety[17]. For this reason, this study used Ad as a vector to transfect the ERβ gene into PC12 cells. PC12 cells are from rat pheochromocytoma cells, can be transplanted, and they have been widely used for in vitro studies of nervous system diseases including AD[18-20]. Results from this study showed that ERβ expression was extremely low in common PC12 cells. The ERβ gene could be introduced into PC12 cells with an Ad vector with a high transfection rate but no obvious cytotoxicity. The introduced ERβ gene was overexpressed successfully. Compared with non-transfected PC12 cells, an empty Ad vector did not influence ERβ expression in PC12 cells and therefore this vector can be used for further experiments.

AD is pathologically characterized by reduced neuron numbers, intracellular neurofibrillary tangles, and extracellular Aβ deposition[21]. Therefore, Aβ has been considered an important substance in the incidence and development of AD[22]. In vivo and in vitro studies have demonstrated that Aβ can stimulate glial cells to release inflammatory factors, evoke oxidative stress, damage cholinergic nerves and induce apoptosis[23-26]. In the present study, real-time quantitative PCR results showed that after addition of Aβ, TNF-α and IL-1 expression in the PC12 cells was significantly increased, and flow cytometry results showed that the rate of apoptosis of PC12 cells was also significantly increased. In the absence of estrogen, ERβ-transfected PC12 cells exhibited significantly lower expression of inflammatory factors and a lower rate of apoptosis than common PC12 cells. This suggests that ERβ can alleviate the pro-inflammatory and pro-apoptotic effects of Aβ in an estrogen-independent manner. Akt, also known as Protein Kinase B, is a serine/threonine-specific protein kinase with a molecular weight of about 60 kDa that plays a key role in regulating cellular growth, differentiation and apoptosis[27-29]. There
is evidence that Akt activity and phosphorylation levels in the frontal lobe and temporal lobe are significantly decreased in AD patients and that activation of Akt can alleviate the toxic effect of Aβ on cells in vitro[30-31]. In addition, after binding to its receptor, estrogen can exert its biological effects via the Akt pathway[32]. However, there have been no reports regarding whether ERβ interacts with the Akt pathway in a ligand-independent manner. Results from this study showed that the anti-inflammatory and anti-apoptotic effects of ERβ were inhibited after the Akt-specific inhibitor Abi-2 was added into ERβ-transfected PC12 cells in the absence of estrogen. This finding suggests that ERβ exerts its anti-inflammatory and anti-apoptotic effects and alleviates the cytotoxic effects of Aβ in an estrogen-independent manner through the Akt pathway. Future studies are needed to investigate whether there are other ligand-independent ERβ pathways.

**MATERIALS AND METHODS**

**Design**
A molecular biological contrast observation.

**Time and setting**
This study was performed at the Laboratory of Neurology, Translational Medicine Center, Norman Bethune Hospital, Jilin University, China between September and December 2011.

**Materials**
PC12 cells were purchased from Beijing Dingguo Changsheng Biotech Co., Ltd., Changchun, Jilin Province, China. Adenovirus gene vector Ad-ERβ-EGFP was constructed by Shanghai GeneChem Co., Ltd, Shanghai, China.

**Methods**

**PC12 cell transfection with Ad-ERβ-EGFP**
According to the method from Gollapudi and Oblinger[33], PC12 cells were added into a 96-well plate at a density of 2 × 10^5/well. Ad-ERβ-EGFP virus particles were prepared in four different concentrations: 5 × 10^5/well, 1 × 10^6/well, 5 × 10^6/well, 1 × 10^7/well. Ten wells were treated with each virus particle concentration. PC12 cells were incubated at 37 °C in a 5% CO2 incubator for 48 hours, and EGFP expression was observed under a fluorescence microscope (Olympus, Japan). At the same time, PC12 cells were transfected with an Ad-EGFP empty plasmid constructed using the same method. Cells with the highest EGFP expression were selected using a fluorescence microscope and further cultured and amplified.

**Detection of ERβ expression in PC12 cells by western blot method**
Untransfected PC12 cells were selected as controls. The PC12 cells transfected with Ad-EGFP empty plasmid served as the blank group. The PC12 cells transfected with Ad-ERβ-EGFP served as the transfection group. ERβ protein expression in the three groups was detected as follows. After centrifugation, cells were collected, lysed for 30 minutes with a lysis buffer containing proteinase inhibitor (Sigma, St. Louis, MO, USA), homogenized for 30 seconds, and centrifuged at 12 000 × g for 20 minutes. The supernatant was diluted with lysis buffer. Total protein concentration was determined with the BCA method[34]. 15 μg total protein from each sample was mixed with 4 μL 6 × loading buffer. Following denaturation in boiling water for 5 minutes, the protein sample was subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis at 80 V for 40 minutes and at 110 V for 90 minutes, and transferred onto a nitrocellulose membrane (GE healthcare, Bethesda, MD, USA) for 60–90 minutes at 200 mA using a standard wet transfer protocol. The protein sample was blocked with 10% bovine serum albumin solution containing nonfat milk for 2 hours, washed six times with tris-buffered saline with 0.05% Tween-20 (Boster, Wuhan, China), 10 minutes each, incubated with primary rabbit anti-ERβ polyclonal antibody IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:2 000) at 4°C overnight. On the following day, the membrane was washed six times with tris-buffered saline with 0.05% Tween-20, 10 minutes each, incubated with horseradish peroxidase-labeled secondary goat anti-rabbit polyclonal antibody IgG (Santa Cruz Biotechnology; 1:200) for 2 hours at room temperature, and developed in the dark with enhanced chemiluminescent reagent (Promega, Madison, WI, USA). β-actin was used as the internal reference for the experiment.

**AD cell model construction and intervention**
According to the method from Li et al[35], we created an AD cell model. The precise procedure was as follows: non-transfected PC12 cells were randomly selected as the controls (control group) and cultured with culture medium containing 20 μM Aβ (Santa Cruz Biotechnology). Randomly selected ERβ gene-transfected PC12 cells were divided into Aβ and Abi-2 groups. In the Aβ group, culture medium containing 20 μM Aβ was added. In the Abi-2 group, 20 μM Akt-specific inhibitor Abi-2 was added into the culture medium already containing 20 μM Aβ. All PC12 cells in each group were incubated at 37°C in a 5% CO2 incubator for 24 hours.
Real-time quantitative PCR detection of TNF-α and IL-1β mRNA
A cell suspension from each group was centrifuged at 800 × g for 5 minutes and then the supernatant was discarded. According to the method from Romero-Ramirez et al. [34], PCR was performed for detection of TNF-α and IL-1β mRNA expression as follows. After addition of Trizol (Invitrogen, Carlsbad, CA, USA), total RNA was extracted from the cells. RNA level was determined using a UV-240 ultraviolet spectrophotometer (Shimadzu Corporation, Japan). Total RNA A₂₆₀/A₂₈₀ ratio ranged between 1.80-2.00. In strict accordance with the introductions provided in the Superscript III Reverse Transcriptase kit (Invitrogen Life Technologies, Carlsbad, CA, USA), 2 µg total RNA was used for cDNA synthesis. According to the instructions provided in the SYBR Green PCR kit (Beijing TransGen Biotech Co., Ltd., Beijing, China), real-time quantitative PCR of the synthesized cDNA was performed as follows. The PCR reaction system contained 1 µL specific primer (0.5 µL upstream primer and 0.5 µL 10 µM downstream primer), 12.5 µL 2 × SYBR Green QPCR Master Mix, 2.5 µL diluted cDNA and 9 µL nuclease-free PCR-grade water. PCR reaction conditions were at 95°C for 5 minutes, followed by 40 cycles each of 95°C for 30 seconds, 60°C for 1 minute and 72°C for 30 seconds. The green fluorescence of the PCR product was determined using a PCR instrument (Labnet International, Inc., Woodbridge, NJ, USA), and β-actin was used as an internal reference. TNF-α and IL-1β mRNA specific primer reference sequences were from GeneBank, designed using Prime primer 5.0 software (PREMIER Biosoft International, CA, USA) and synthesized by Shanghai Bioengineering Institute, China. The precise sequence is as follows.

TNF-α Upstream primer: 5'-CTC CAG CTG GAA GAC TCC TCC CAG-3'
Downstream primer: 5'-CCC GAC TAC GTG CTC CTC ACC-3'

IL-1β Upstream primer: 5'-GAC CTG CTT CTT TGA GGC TGA C-3'
Downstream primer: 5'-TTC ATC TCG AAG CCT GCA GTG-3'

Flow cytometry detection of PC12 cell apoptosis
PC12 cells from each group were prepared as a single cell suspension. Cell apoptosis was determined by flow cytometry as follows. Following trypsin digestion for a few minutes, PC12 cells were re-suspended in phosphate buffered saline and prepared as a single cell suspension. After addition of 5 µL AnnexinV FITC (KeyGen Biotech, Nanjing, China) and 5 µL Propidium Iodide (KeyGen Biotech), the mixture was thoroughly mixed for 5–15 minutes at room temperature in the dark. Thereafter, the apoptosis of labeled PC12 cells (%) was determined by flow cytometry using a BD FACSCalibur flow cytometer (Becton Dickinson Medical Devices Co., Ltd., Shanghai, China). An argon-ion laser near a threshold at 488 nm was used. Results were analyzed using Cellquest software (Becton Dickinson Medical Devices Co., Ltd., Shanghai, China).

Statistical analysis
All data were statistically processed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Measurement data were expressed as mean ± SD. One-way analysis of variance was used for data comparison among groups. Student-Newman-Keuls test was used for data comparison between groups. A level of alpha = 0.05 was considered significant.

Author contributions: This study was designed and evaluated by Lihui Si, Zhu Tian and Manhua Cui, and performed by all authors.

Conflicts of interest: None declared.

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