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Estrogen Receptor Alpha 36 Gene Knockdown Promote the Expression of NF-κB in PC12 Cells*

Ping Zou[1], Chao Qu[2], Yihui Xu[2,3], Hongyan Li[2], Dannv Han[2], Dan Shi[2], Wei Zou[2,4#]
[1]Teachers College, Dalian University, Dalian, China
[2]College of Life Science, Liaoning Normal University, Dalian, China
[3]School of Life Science, Shandong University, Jinan, China
[4]The Research Center of Developmental and Educational Psychology, Liaoning Normal University, Dalian, China
Email: #weizou60@hotmail.com

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ABSTRACT

The nuclear transcription factors κB (NF-κB) is widely existed in various kinds of cell types in the nervous system and plays an important role in neuron apoptosis and neurodegenerative diseases. Estrogen receptor alpha 36 (ER-*α*36) is a novel variant of ER*α* (as known ER-*α*66) which can transduce both estrogen- and antiestrogen-dependent activation of MAPK signal pathway and stimulate cell growth. Here, we aimed to detect the effect of ER-*α*36 gene silencing on the expression of NF-κB in normal cultured PC12 cells and to provide an experimental foundation for understanding the function of ER-*α*36 in nerve cells. PC12 cells with ER-*α*36 expression knocked down by the shRNA method. Then Western blot and immunocytochemical staining were performed to detect the expression and translocation of NF-κB after transfection. The results showed that NF-κB expression was significantly higher comparing with the control group after transfection ($P < 0.01$). Also, NF-κB subunit entered nuclear after transfection; Immunofluorescence staining and immunocytochemical staining of PC12 cells demonstrated that ER-*α*36 was expressed mainly on the plasma membrane and on the cell nucleus membrane. These data indicate that ER-*α*36 gene silencing can increase the expression of NF-κB and promote its nuclear translocation in PC12 cells.

Keywords: NF-κB; Estrogen Receptor Alpha 36; PC12 Cells

1. Introduction

Nuclear factor kappa B (NF-κB), as a dimeric transcription factor, is widely existing in neurons of central nervous system. Activated (NF-κB) controls the expression of genes that regulate a broad range of biological processes through canonical and non-canonical pathways, such as synaptic plasticity, cell injury, and the adjustment of the immune and inflammatory response factors expressions, such as cell adhesion molecules and cytokines. In the central nervous system, NF-κB controls inflammatory reactions and the apoptotic cell death following nerve injury [1], which plays a regulating role in the course of inflammation and immunoreaction during neuron apoptosis and neurodegenerative diseases and the change of NF-κB expression caused the neuron death and astrocyte activation. It is also reported that the activation of NF-κB and CREB is involved in the protection of chromaffin cells and the sympathoadrenal PC12 cells (an established model for the study of neuronal cell apoptosis and survival) against serum deprivation-induced apoptosis by the neuroactive steroids dehydroepiandrosterone (DHEA), its sulfate ester DHEAS and allopregnanolone (Allo) [2]. However, NF-κB is essential for neurosurvival as well. NF-κB activation is a part of recovery process that may protect neurons against oxidative-stresses or brain ischemia-induced apoptosis and neurodegeneration [3].

Recent studies show that estrogen receptor-*α* mediates the brain anti-inflammatory activity of estradiol. It has also been reported that estradiol-induced enhancement of object memory consolidation involves hippocampal extracellular signal regulated kinase activation and membrane-bound estrogen receptors [4]. ER-*α*36, as a newly discovered estrogen receptor subtype, lacks both transactivation domains and functions as a dominant-negative effector of transactivation activities of the full-length

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#Corresponding author.

ER-*α*66 and ER*β* [5]. ER-*α*36 primarily localizes to the cytoplasm and plasma membrane, it can transduce both estrogen-and antiestrogen-dependent activation of MAPK signal pathway, stimulating proliferation of breast cancer cells [6].

The interaction between ER-*α*66 and NF-κB has been studied in many kinds of cell types. ER can affect the NF-κB transcript activity by several aspects. It has been reported that the ER protects against debilitating effects of the inflammatory response by inhibiting the NF-κB in the MCF-7 breast cancer cell line. Tamoxifen treatment in ER-positive breast cancer up regulate NF-κB gene [7]. And the activity of NF-κB relies on the expression of ER in MCF-7 and HER2 cell line: the more ER expressed, the less NF-κB there was [8]. The activity of the NFκB signaling cascade is associated with mammary carcinogenesis, especially tumors with an aggressive and ER-negative phenotype [9]. ER-*α*36 expression is regulated differently from ER-*α*66, consistent with the findings that ER-*α*36 is expressed in specimens from ER-negative breast cancer patients and established ER-negative breast cancer cells that lack ER-*α*66 expression [10,11]. Although the function of ER-*α*36 has been studied in cancer, to our knowledge, it has not yet been known in the nervous system. As ER-*α*36 may play important roles in these progresses, it is of great importance to detect the function of ER-*α*36 and its interaction with NF-κB in neuron apoptosis and neurodegenerative diseases.

2. Results

2.1. The Transfection of ER-*α*36 shRNA down Regulate the Expression of ER-*α*36 in PC12 Cells (Figure 1)

The transfection efficiency of ER-*α*36 shRNA plasmid was detected by observing the fluorescence 48 hours after transfection and it was nearly 87%. ER-*α*36 expression was also detected by Western blot and the expression level of ER-*α*36 protein was suppressed by up to 25% which shows that there was high interference efficiency.

2.2. The Down Regulation of ER-*α*36 Promote the Expressions of NF-κB and Related Proteins in PC12 Cells (Figure 2)

NF-κB is widely existed in various kinds of cell types in nervous system. After transfection in PC12 cells for 48 hours, NF-κB was activated and had translocated from plasma to the nucleus. Compared with the control group, the expression level of NF-κB increased significantly after transfection. These date indicate that ER-*α*36 gene-silencing can increase the expression of NF-κB and promote its nuclear translocation in PC12 cells. The expression of p-P38 and P38 were also detected by Western blot and there is a markerable increase after transfection.

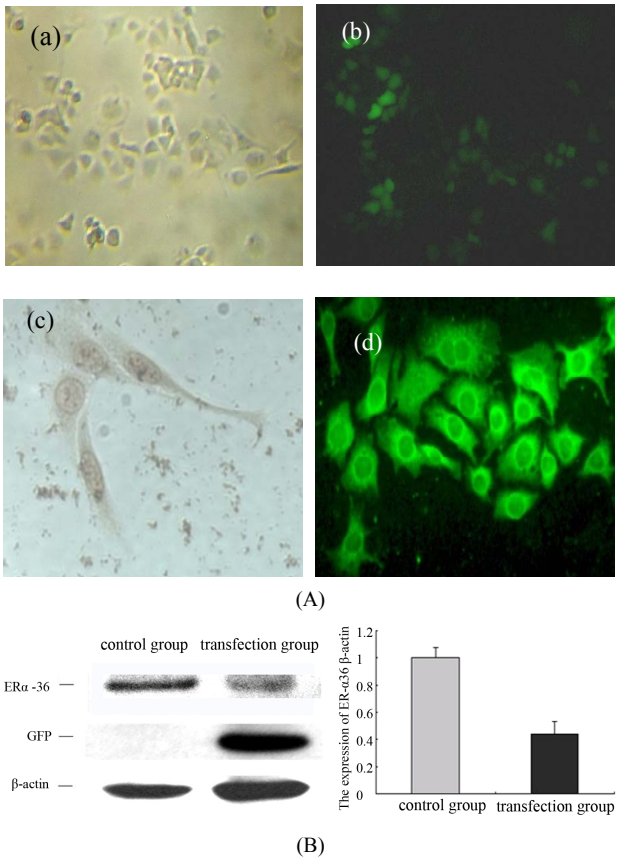

Figure 1. The transfection of ER-α36 shRNA down regulate the expression of ER-*α*36 in PC12 cells. (A) Control group (a) ×100). GFP protein expressions in the PC12 cells after transfection for 48 hours (b) ×100) immunocytochemical staining detection ER-α36 location in PC12 (c) ×400). Immunofluorescence staining detection ER-*α*36 location in PC12 (d) ×400). (B) Western blot detection on ER-*α*36 expression after transfection for 48 hours in PC12 cells. Densitometric analysis of ER-*α*36/*β*-actin. The results are presented as means ± SEMs of three independent experiments.

3. Discussion

ER-*α*36 has been increasingly implicated in breast cancer cells, however, the role of ER-*α*36 in the brain has not been reported. ER-*α*36 as a newly discovered estrogen receptor subtype lacks both transcriptional activation domains of ER*α* (AF1 and AF2), but retains the DNA-binding domain and partial dimerization and ligand-binding domains. It is predominantly localizes to the cytoplasm and plasma membrane and mediates the membrane-initiated estrogen signaling in breast cancer cells. We show here that ER-*α*36 expressed on the plasma membrane and cell nuclear envelope in the PC12 cells. Thus, our results reveal the difference of ER-*α*36 between PC12 cells and breast cancer cells.

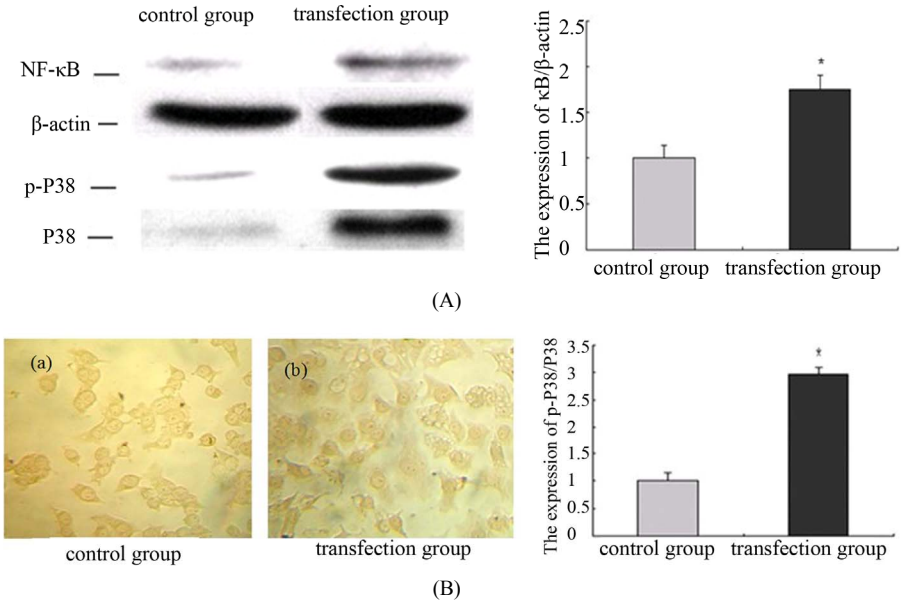

Figure 2. The down regulation of ER-α36 promote the expressions of NF-κB and related proteins in PC12 cells. (A) The expressions of NF-κB, p-P38 and P38 were detected by western blot after transfection for 48 hours in PC12 cells. Densitometric analysis of NF-κB/β-actin; Densitometric analysis of p-P38/P38. The results are presented as means ± SEMs of three independent experiments (*significantly different from the control group); (B) Immunocytochemical staining showed that NF-κB subunit expression and location in PC12 cells after transfection for 48 hours.

NF-κB is a transcription factor that regulates the expression of a large number of genes that are critical for the regulation of cellular process, such as inflammatory responses, apoptosis, and cell proliferation [12]. NF-κB pathways may promote central nervous system (CNS) cell survival through the inhibition of caspase-1 and -3 activity [13], and NF-κB as key mediators of the neuroprotective against inflammatory stress in nigral dopaminergic (DA) neurons [14].

In recent studies, NF-κB transrepression of steroid hormone receptors has been found [15]. 17 beta-estradiol (E2*β*) treatment produced strong protective effects by reducing infarct volume, neuronal apoptosis, and inflammatory responses caused by NF-κB activation through estrogen receptors in transient cerebral ischemia model [16]. Several groups have reported the direct interaction between ER*α* and NF-κB in the nucleus of living cells [17] and a reciprocal transcription inhibition between agonist-bound ER*α* and activated NF-κB [18]. Previous work demonstrated that the NF-κB transcription factor promotes survival and chemoresistance in human breast cancer [19]. The NF-κB signaling pathways have been implicated as mediators of breast cancer drug resistance. The literature reported that ER-*α*36 existed in breast cancer patient samples and found that its expression levels were high in ER-negative tumors and low in ER-positive tumors [20,21], in addition, ER-*α*36 mediates nongenomic antiestrogen signaling in ER-negative breast cancer cells such as activation of the MAPK/ERK signaling in these cells [22,23], is involved in the resistance of breast cancer to endocrine therapy, for example, tamoxifen [24]. It is also been demonstrated that the nuclear translocation and activation of NF-κB was significantly blocked by p38 MAP kinases inhibitor SB 203580 [25]. In our study, the expression of NF-κB in transfection group was increased together with the increase of p-P38 expression after ER-*α*36 gene was silenced 48 hours later. These results demonstrate that p38 MAP kinase might be upstream of NF-κB which plays an important role in nervous system.

In summary, we reported the function of ER-*α*36 might be associated with NF-κB transcription factor and showed that ER-*α*36 gene silencing promoted the expression of NF-κB in ER-negative PC12 cells. It's suggesting that non-genomic estrogen signaling mediated by ER-*α*36 contributes to development and progression of PC12 cells that express NF-κB. This is the first report to our knowledge that demonstrates that ER-*α*36 gene silencing promote the expression of NF-κB in PC12 cells, but which kind of cross-talk is involves in this process remains to be determined.

4. Materials and Methods

4.1. Chemicals and Antibodies

All chemicals used in this study were purchased from Sigma Chemical Company (St Louis, MO). Antibodies of NF-κB, GFP, p-P38 and P38 were purchased from cell signaling Company. Rabbit polyclonal anti-ER-*α*36 antibody was a gift from Dr Zhao-Yi Wang (Creighton

University, CaliforniaPlaza, USA).

4.2. Cell Culture and Transfections

PC12 cells were grown in RPMI medium 1640 (Sigma) supplemented with 5% FBS and 10% HS at 37˚C under a humidified 5% CO_2 atmosphere. For transient transfection experiments, 6×10^5 cells per well were seeded in 6-well plates in 2ml of RPMI medium 1640 without antibiotics. Transient transfections were performed with lipid-Lipofectamine™ 2000 for 48 hours. Each experiment was performed on triplicate samples and repeated at least three times.

4.3. Western Blot Analysis

The cells were collected in ice-cold PBS, and then the cells extracts were prepared in RIPA buffer with proteinase inhibitor cocktail from Sigma (St. Louis, MO). Cell lysates were boiled with gel-loading buffer for 5 min at 100˚C, resolved on 10% SDS-PAGE, transferred to PVDF membrane, probed with appropriate antibodies and visualized with enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

4.4. Immunocytochemistry

Cells were fixed in 4% (w/v) paraformaldehydein PBS (pH 7.4) followed by Permeabilization in 0.2% (v/v) Triton X-100 in PBS (PBST). Background staining was minimalized by incubating these ctionsins 50% (v/v) ethanol, 0.9 (v/v) hydrogen peroxide in PBS for 30 min to block endogenous peroxidaseactivity. 0.9% followed by 4% (w/v) bovine serum albumin in PBS for 1 h at room temperature. 1:200 dilution of the anti-NF-κB antibody was added to the slides and incubated for 1 hour at 37˚C. After removing unbound antibody with PBST washes, immuno reactivity was detected with a biotinylated secondary antibody followed by an avidin horseradish peroxidase complex. Immunoreactivity was visualized using a diaminobenzidine staining kit for 30 min.

4.5. Immunofluorescent

Cells were plated onto laminin-coated coverslips whereas were plated onto poly-l-lysine-coated coverslips. Cells grown on cover glasses were washed twice with PBS and then fixed with 4% ice cold paraformaldehyde for 15 min. The cover slips were washed three times with 0.1% Triton X-100 in PBS and blocked with 5% bovine serum albumin (BSA) (Roche) in phosphate buffered saline solution for 1 h, and after that incubated with anti-ER-α36 antibodies (rabbit polyclonal, 1:100, Millipore). After washing (three times for 10 min in PBS), cells were incubated with Rhodamine-conjugated affinipure goat anti-rabbit antibodies (1:300, Zhongshan Goldenbridge Biotechnology, China). Nuclei were stained using DAPI (0.2 μg/mL, Sigma). Fluorescence was imaged with a Bio-Rad MRC 600 confocal imaging system. Images were acquired using a Leica TCS SP2 MultiPhoton confocal microscope.

4.6. Statistical Analysis

Statistical analyses were performed using SPSS statistical software. Treatment effects were analyzed using one-way analysis of variance. Significance was set at $P < 0.05$.

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