The Effects of Various Estrogen Doses on the Proliferation and Differentiation of Cultured Neural Stem Cells

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Abstract

The neuroprotective effects of estrogen in neurodegenerative diseases, such as Alzheimer’s disease, cerebral ischemia and Parkinson’s disease, are well documented and involve stimulating neurogenesis. However, the dosage and timing of estrogen treatment is controversial, and the underlying mechanism remains unclear. In this study, we tested the effects of various estrogen doses on the proliferation and differentiation of NSCs. First, we identified estrogen receptor α, β and GPR30 were highly expressed in NSCs. The results from a cell cycle analysis detected by flow cytometry revealed that 10 nM 17β-estradiol (E2) treatments for 3 days significantly increased the proliferation of neural stem cells (NSCs) and the expression level of p-ERK1/2, whereas 50 nM E2 exposures markedly decreased the proliferation of NSCs and the expression level of p-ERK1/2. According to immunofluorescence staining and Western blot analyses, 10 nM E2 treatment for 7 days stimulated NSCs to differentiate into neurons and inhibited their differentiation into astrocytes. These results demonstrate NSCs is definitely the target of estrogen and that an appropriate dose of E2 (10 nM) can significantly increase the proliferation of NSCs and significantly stimulate NSCs to differentiate into neurons, which supports the neuroprotective role of estrogen in neurodegenerative diseases.

Keywords: Estrogen; Neural stem cells (NSCs); Proliferation; Differentiation

Abbreviations: E2: 17β-Estradiol; NSCs: Neural Stem Cells; Tuj1: Neuronal Class III b-Tubulin; GFAP: Glial Fibrillary Acid Protein; CNPase: 30-Cyclic-Nucleotide 30-Phosphodiesterase; BrdU: Bromodeoxyuridine; ER: Estrogen Receptor

Introduction

For a long time, estrogen was thought of only as a “sex hormone” within the reproductive system. However, epidemiological and animal studies have demonstrated estrogen’s neuroprotective effects in neurodegenerative diseases, such as Alzheimer’s disease (AD) [1], cerebral ischemia [2] and Parkinson’s disease (PD) [3], which together lead to a high incidence of disability and mortality due to neuronal loss.

Neurogenesis in the adult brain, which can be stimulated by physiological factors, such as growth factors and environmental enrichment, and by pathological processes, including stroke and neurodegeneration [4], is considered to be the most effective strategy for these neurodegenerative diseases. Neurogenesis continues throughout life in specific regions of the mammalian brain, including the dentate gyrus (DG) of the hippocampus. These newborn cells can migrate into damaged brain regions and differentiate into neural cells to alleviate neural injury [5]. Therefore, neural stem cells (NSCs) have become the target of treatments for neurodegenerative diseases. NSCs have unique properties, including pluripotency, and hold promise for neurodevelopmental biology, regenerative medicine and drug discovery.

However, the factors governing the cell fate of NSCs are still poorly understood. Estrogen exhibits neuroprotective effects by promoting neurogenesis [6-8]. Thus, estrogen may play a profound role in the modulation of NSCs, and NSC transplantation combined with estrogen modulation may be a therapeutic approach for neurodegenerative diseases. However, there is some controversy between several studies, and the underlying mechanisms remain unclear.

The most important characteristics of NSCs are proliferation and controlled differentiation. Thus, in this study, we investigated the effects of different estrogen doses on the proliferation and differentiation of NSCs.

Materials and Methods

The experimental protocol was approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University (Xi’an, China) and performed in accordance with the guidelines for Animal Experimentation of the Fourth Military Medical University.

Isolation and culture of NSCs

NSCs were harvested from the brains of E14.5-E16.5 Sprague-Dawley rat embryos (from pregnant dams purchased from the Experimental Animal Center of the Fourth Military Medical University)

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as previously described [9]. Briefly, hippocampi were isolated in ice-cold dissection buffer (HBSS, Gibco, USA) under a stereomicroscope. After the meninges were removed, single cell suspensions were obtained by mechanical dissociation. Cells were washed, briefly centrifuged, re-suspended in fresh medium, and cultured at 5 × 10^4 cells/ml on 25 cm² cell culture flasks (Corning, USA) in serum-free Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium supplemented with 20 ng/ml basic fibroblast growth factor (bFGF, Peprotech, USA), 20 ng/ml epidermal growth factor (EGF, Peprotech), B-27 and N-2 supplements (Gibco), penicillin and streptomycin. The resultant neurospheres were harvested and mechanically dissociated to produce a single cell suspension for re-plating every 6-7 days. All experiments were performed on single cells after the second or third passage.

To estimate the rate of proliferation, single cell suspensions were plated in serum-free medium with bFGF. To induce differentiation, the medium was changed to (DMEM)/F12 containing 4% fetal bovine serum (Sigma-Aldrich, USA) without bFGF.

**Estrogen exposure**

Cells were seeded onto plates or poly-D-lysine-coated glass coverslips (1 mg/ml, Sigma) for 2 h. Then, the cells were exposed to 17β-estradiol (E2, Cayman, America) at different physiological concentrations (Con, 0 nM, 1 nM, 10 nM, 20 nM and 50 nM) and durations (3 and 7 days for cell cycle and differentiation analyses). Dimethyl sulfoxide (DMSO) alone was used as the vehicle control. E2 was dissolved in DMSO in 10 mM stock solutions. Further dilutions were made using culture medium. The final concentration of DMSO in the culture medium never exceeded 0.02%, a level that had no effect by itself.

**Cell cycle analysis**

Cell cycle was assessed by flow cytometry, as previously described [10]. After 3 days of E2 exposure, the cells were collected by trypsinization and centrifuged in phosphate buffered saline (PBS) twice. The cells were then fixed in pre-cooled 70% ethanol at -20°C, and stained with propidium iodide (PI) solution. DNA content was determined by flow cytometry using the CellQuest Software. A total of 10,000 events were counted for each sample (FACScalibur, Becton-Dickinson, USA). The percentage of cells in a particular cell cycle stage was calculated with the ModFit software (Becton-Dickinson, USA).

**Immunofluorescence staining and cell differentiation assays**

Hippocampal NSCs were phenotyped 7 days after E2 exposure according to their protein marker expression. Cells were seeded onto poly-D-lysine-coated coverslips at 3 × 10^4 cells/well in 24-well plates in DMEM/F12 medium containing 4% fetal bovine serum. The cells were exposed to E2 as described. 24 h after re-plating or seven days after E2 exposure, cells were fixed in 4% paraformaldehyde for 45 min, washed 3 times with PBS, and incubated for 30 min at room temperature in a blocking solution of PBS plus 1% BSA and 0.3% Triton. Cells were then incubated at 4°C overnight in rabbit anti-ERα (1:100, Abcam, UK), rabbit anti-ERβ (1:100, Abcam), rabbit anti-GPR30 (1:100, Abcam), mouse anti-neuronal class III b-Tubulin (TuJ1, 1:500, Sigma-Aldrich, USA), rabbit anti-glial fibrillary acidic protein (GFAP, 1:500, Millipore, USA), or mouse anti-20, 30-cyclic-nucleotide 30-phosphodiesterase (CNPase, 1:200, Abcam) diluted in the blocking solution. Cells were then incubated with secondary antibodies (1:500 Alexa Fluor 488 or Alexa Fluor 594, Invitrogen, USA) for 2 h at room temperature. Finally, cells were incubated with DAPI for 10 min at room temperature to stain the cell nuclei.

**Western blotting**

Cell samples were harvested from culture plates at 24 h after re-plating or 3 and 7 days after E2 exposure. Samples were lysed in sample buffer composed of 62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.1% w/v bromphenol blue. Insoluble materials were separated by centrifugation at 12,000 g for 10 min, and the supernatant was heated to 100°C for 10 min and then cooled on ice for 30 min. Electrophoresis was conducted by SDS-PAGE using 10% poly-acrylamide gels in accordance with routine protocols. Separated proteins were transferred onto nitrocellulose membranes, and the membranes were blocked (with gentle shaking) for 1 h at room temperature in a solution containing 5% non-fat milk powder and 0.1% Tween-20 in Tris-buffered saline (TBST). After washing 3 times in TBST, the membranes were incubated overnight at 4°C in blocking solution plus rabbit anti-ERα (1:500, Abcam), rabbit anti-ERβ (1:500, Abcam), rabbit anti-GPR30 (1:500, Abcam), rabbit anti-phosphorylated extracellular regulated kinases (pERK1/2, 1:2500, Cell Signaling, USA), rabbit anti-ERK1/2 (1:2500, Cell Signaling), rabbit anti-TuJ1 (1:1000, Sigma-Aldrich), or rabbit anti-GFAP (1:1000, Millipore). Mouse anti-β-actin (1:1000, Sigma-Aldrich) and rabbit anti-GAPDH antibody (1:1000, Sigma-Aldrich) served as a loading control. All membranes were then washed 3 times in PBS and incubated with peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG in TBST for 1 h. Immunoreactive bands were detected using enhanced chemiluminescence (ECL, Amersham, UK) and were quantified with Bio-Rad Quantity One software (Hercules, CA, USA).

**Statistical analysis and quantification**

Results are presented as means ± SDs. Control and treatment group means at each time point were compared using two-way ANOVAs, and concentration and exposure duration were the between-group factors. The Bonferroni correction was applied for multiple pair-wise comparisons. All statistical calculations were performed using the GraphPad Prism software (GraphPad, USA). A p-value >0.05 was considered significant.

**Results**

**Identification of cultured NSCs**

Using our isolation and culture methods, most cells (>98%) plated on poly-D-lysine coated coverslips were immunopositive for the stem cell marker nestin 24 h after plating, confirming that the vast majority of cells used for subsequent assays were indeed NSCs (Figure 1A).

**Estrogen receptors (ERs) are highly expressed in NSCs**

As shown in Figure 1B-1D, the results from the immunofluorescence staining reveal that ERα, ERβ and GPR30 are strongly expressed in identified NSCs. And the results of Western blot reveal that ERα, ERβ and GPR30 are highly expressed in identified NSCs (Figure 2).

**Effects of various E2 doses on NSC proliferation**

As shown in Figure 3, the results from the flow cytometric analysis revealed that the percentage of cells in S+G2 phase in the control, 0 nM, 1 nM and 20 nM groups were 26.4% ± 2.7%, 27.7% ± 2.0%, 28.1% ± 2.5% and 29.1% ± 2.6%, respectively. No significant differences were observed among these groups. 10 nM E2 treatment resulted in a significant increase in the percentage of the cells in the S+G2 phase (to 38.7% ± 3.5%, *p<0.05 vs. 0 nM group). Treatment with 50 nM E2 induced a marked decrease in the percentage of cells in the S+G2 phase (21.5% ± 2.6%, *p<0.05 vs. 0 nM group).
Effects of various E2 doses on p-ERK1/2 expression in NSCs

Next, we investigated the effects of various E2 doses on the ERK signaling cascade, which regulates proliferation. As shown in Figure 4 (Western blot), 10 nM E2 treatment induced a significant increase in the level of phosphorylated ERK1/2 (*p<0.05 vs. the 0 nM group). Treatment with 50 nM E2 markedly decreased the phosphorylation levels of ERK1/2 (*p<0.05 vs. 0 nM group).

Effects of various E2 doses on NSC differentiation

To investigate whether E2 treatment induced the differentiation of cultured NSCs, we examined cell phenotypes 7 days after E2 treatment using the neuronal marker Tuj1, the astrocytic marker GFAP, and the oligodendrocytic marker CNPase. As shown in Figure 5, immunofluorescence assays revealed that 1 nM, 20 nM and 50 nM E2 did not affect the percentages of Tuj1-positive or GFAP-positive cells in the culture compared to 0 nM. However, 10 nM E2 treatment significantly increased the percentage of Tuj1-positive cells and decreased the percentage of GFAP-positive cells in the culture.
The results from the Western blot analyses supported the immunofluorescence assay results. As shown in Figure 6, 1 nM, 20 nM and 50 nM of E2 did not affect the protein expression of Tuj1 or GFAP in culture compared to 0 nM. However, 10 nM E2 treatments significantly increased the protein expression of Tuj1 and decreased the protein expression of GFAP in culture. E2 treatment did not affect the protein expression of CNPase in the culture (data not shown).

Discussion

It has been widely recognized that estrogen exhibits greater functionality (e.g. in the brain) than just the regulation of reproduction. Several studies have demonstrated the neuroprotective effects of estrogen on neurodegenerative diseases, such as AD [16], cerebral ischemia [2,7] and PD [3,8], which involve promoting neurogenesis. These newborn cells can migrate into damaged brain regions and may differentiate into neurons and form new synapses to alleviate neural injury [5,11].

Several pieces of evidence suggest that estrogen plays a significant role in the promotion of neurogenesis. Gould et al. [12] found that female mice had significantly more newly generated cells in the dentate gyrus (DG) compared to males after injection with bromodeoxyuridine (BrdU). Additionally, within the estrous cycle, the number of newly generated cells in the DG was 50% higher in female mice during proestrus (estradiol levels high) versus estrus or diestrus (estradiol levels low). Furthermore, removal of the ovary diminished the number of newborn cells, and this effect was reversed by estrogen replacement [12]. However, some researchers have shown opposite results. In another study, acute treatment with a moderate (not low or high) dose of estrogen rapidly increased newborn cell proliferation in ovariectomized animals, and chronic estrogen treatment for 3 weeks did not stimulate neurogenesis [13]. In male rats, repeated estrogen administration did not significantly affect neurogenesis [14]. In another recent study, short term treatment with estrogen decreased the rate of newly generated cells in the subventricular zone (SVZ) and olfactory bulb (OB) of adult female ovariectomized mice [15]. These contrasting results indicate that the in vivo effects of estrogen on neurogenesis are complex. Several factors, such as the dosage and timing of estrogen treatment, probably influence the outcome.

Thus, many researchers have explored the effects and underlying mechanisms of estrogen on neurogenesis by using NSCs. As estrogen exerts its function by binding to ERα and ERβ and newly identified GPR30 receptor, we first detected whether our cultured NSCs expressed these estrogen receptors. The results of immunofluorescence staining and Western blot revealed that ERα, ERβ and GPR30 receptors were highly expressed in cultured NSCs. A previous study demonstrated that NSCs expressed both estrogen receptor α and β (ERα and ERβ) [16]. These results identified NSCs as a target for the actions of estrogen. Interestingly, we first found the newly identified estrogen receptor GPR30 was highly expressed in the cultured NSCs. The GPR30 receptor is reported to be a novel estrogen receptor uniquely localized to the endoplasmic reticulum [17], which mediated fast non-genomic effects of estrogen. This receptor is widely distributed and has numerous physiologic or pathologic functions in differentiated and matured brain cells [18,19]. However, the role of GPR30 receptors in NSCs has not been explored before, which warrants further study.

Next, we explored the role of different doses E2 on proliferation and differentiation of NSCs. The results from the cell cycle analysis (flow cytometry) showed that 10 nM E2 treatment for 3 days significantly increased the proliferation of NSCs, whereas 50 nM E2 markedly decreased the proliferation of NSCs. Brännvall K et al. [16] found that 10 nM E2 significantly increased the number of BrdU-labeled cells among NSCs by 7%, and this effect was inhibited by an ER antagonist. In another study, 10 nM E2 increased the number of generated neurons (over 50%) in mouse embryonic stem (ES) cells, with increased neurite branching [20].

To elucidate the underlying mechanism, we examined the effects of estrogen on the phospho-activation of ERK1/2 (p-ERK1/2). The ERK signaling cascade regulates the proliferation [21] of NSCs. In our study, 10 nM E2 treatments significantly increased p-ERK1/2 expression, whereas treatment with 50 nM E2 markedly decreased p-ERK1/2 expression. Thus, the effects of estrogen on NSC proliferation are mediated by the regulation of p-ERK1/2 expression.

NSCs from embryonic and adult brains can undergo differentiation into three major types of brain cells: neurons, astrocytes, and oligodendrocytes. The differentiation of NSCs into new neurons, which could be used in basic and translational studies for the treatment of neurodegenerative diseases, is particularly promising. However, control over their differentiation is still a critical obstacle.

In this work, by using immunofluorescence staining and Western blot analyses, we observed that 10 nM E2 significantly stimulated the differentiation of NSCs into neurons and significantly inhibited the differentiation of NSCs into astrocytes. The ratio of differentiated oligodendrocytes remained unchanged. In a previous study, 10 nM E2 increased the ratio of β-tubulin III-positive neurons to GFAP-positive glial cells in embryonic rat NSCs (determined by immunostaining), and this result demonstrated the influence of estrogen on neurogenesis during embryonic development [16]. Icaritin, which is an ER modulator that possesses neuroprotective effects [22], also facilitated the differentiation of mouse ES cells into the neuroectoderm and increased the proportion of β-tubulin III-positive phenotypes (the number of GFAP-positive phenotypes remained unchanged) [23]. Altogether, these results suggest that estrogen affects the ratio of differentiated neurons from NSCs.

Currently, human NSC-replacement therapy holds significant potential for treating neurodegenerative diseases. Human NSCs can give rise to neurons and glial cells in vitro, and survive to differentiate...
into neurons in the rat brain [24]. E2 increased the number of dopaminergic (DA) neurons derived from human NSCs in vivo when these cells were grafted into mouse brains; this result also supports the role of estrogen during the transplantation of human NSCs for Parkinson’s disease [25].

In conclusion, our study demonstrates that NSCs is definitely a target for the actions of estrogen and that an appropriate dose of E2 (10 nM) can significantly increase the proliferation of NSCs and significantly stimulate NSCs to differentiate into neurons, which lay the foundation for the role of estrogen in neuroprotection for neurodegenerative diseases.

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