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EFFECTS OF 17- β ESTRADIOL AND ESTRIOL ON NMDA-INDUCED TOXICITY AND APOPTOSIS IN PRIMARY CULTURES OF RAT CORTICAL NEURONS

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Estrogens possess neuroprotective and antiapoptotic properties, however, the issue of involvement of estrogen receptors (ER)-dependent genomic pathway in these effects still remains controversial. Moreover, the majority of data on antiapoptotic effects of estrogens concern non-neuronal cells. In the present study we compared effects of the potent ER agonist, estradiol-17 β (E2), and its metabolite with a weak affinity for ER, estriol, on the neurotoxicity induced by high (1 and 5 mM) NMDA concentrations and on the apoptosis induced by low (0.1 mM) concentration of NMDA in rat primary cortical neurons. The obtained data showed that 24-hour exposure of cortical neurons to NMDA (0.1–5 mM) resulted in a dose-dependent increase in LDH level. Twenty four-hour pretreatment with estriol (100 nM and 500 nM) reduced the NMDA (1 and 5 mM)-induced toxicity by 16–26%, while estradiol-17 β (500 nM) reduced NMDA (5 mM) — induced toxicity by 14%. Twenty four hour exposure of cortical neurons to NMDA (0.1 mM) resulted in decrease of the level of antiapoptotic protein — Bcl-2 by 60% and increased the number of apoptotic cells by 50% compared to the control. Twenty four hour pretreatment with estradiol-17 β or estriol (100 and 1000 nM) prevented the NMDA-induced apoptotic changes. The specific estrogen receptor antagonist ICI 182,780 (100 nM) had no effect alone and did not antagonize the effects of estrogens on NMDA-induced toxicity as well as on changes in Bcl-2 level. The higher efficacy of estriol, together with the fact that the specific ER receptor antagonist, ICI 182,780, did not inhibit the above-described effects support the hypothesis about a nongenomic mechanism of the anti-NMDA action of estrogens.

Key words: *estradiol-17 β , estriol, ICI 182, 780, NMDA, neurotoxicity, apoptosis, primary culture, cortical neurons.*

INTRODUCTION

Clinical and experimental data indicate, that estrogens have beneficial effects in neurodegenerative processes induced by variety of insults in vivo and in vitro. Thus estrogens show neuroprotective effects against hypoglycemia,

hypoxia, oxidants, β -amyloid peptide and excitatory amino acid agonists (7, 9, 19, 20). Increasing body of evidence indicate that apoptosis is crucial mechanism in neuronal damage in Alzheimer patients, a disease which development in woman is prevented by estrogen replacement therapy. Apoptosis is characterized by cascade of biochemical processes, associated with changes of expression of some regulatory proteins, leading to activation of specific endonucleases, proteases and ultimately to cell death. The family of apoptotic regulatory proteins include proapoptotic: Bax, Bcl-x_s, Bad, Bid, Bak; and antiapoptotic: Bcl-2, Bcl-x_L. In vivo estradiol-17 β increases expression of antiapoptotic Bcl-2 in cortex and hypothalamus of rats, and in vitro in NT2 neurons, and another antiapoptotic protein, Bclx_L in primary culture of hippocampal neurons (5, 6, 17, 20).

It is still unclear whether estrogens affect the neuronal survival by either their classic action, i.e. activation of the gene transcription *via* nuclear receptors, or nongenomic one, e.g. rapid activation of signal transduction pathways. A great many effects develop *via* the genomic pathway on which estrogens bind to the intracellular receptor — α or β and act through an estrogen response element (ERE). Estrogens can also act through other-than-ERE sequences, e.g. AP-1 and SRE sites (23). The genomic effects of estrogens that set in motion their neuroprotective mechanism involve enhancement of the expression of some neurotrophins and their receptors, induction of calcium-binding proteins and stabilization of microtubules (16, 22). The rapid nongenomic neuroprotective effects of estrogens are attributed to inhibition of the oxidation of lipids, lipoproteins and nucleic acids (2), inhibition of neuronal nitric oxide synthase (11) and, possibly, to their direct interaction with glutamate receptors (24). Recently we found that estrogens reduced kainate neurotoxicity in primary cortical neurons (12). Since the excitatory amino acid-induced toxicity is mainly mediated by NMDA receptors, in the present study we examined the effects of estrogens on the NMDA-induced toxicity in primary cultures of rat cortical neurons. Because, depending on concentration, the glutamate receptor agonists may lead to neuronal death via necrotic or apoptotic way and majority of data on antiapoptotic effects of estrogens concern peripheral, but not neuronal tissue, the effects of estrogens on the NMDA-induced apoptosis was also examined.

In the present study we compared the effects of estradiol and its metabolite estriol (with a weak affinity for ER) on the NMDA-induced neurotoxicity (estimated by increase in LDH level) and apoptosis (estimated by decrease of Bcl-2 level and by staining with acridine orange/ethidium bromide). In order to further corroborate the involvement of intracellular estrogen receptors, an effect of selective ER antagonist ICI 182, 780 has also been studied.

MATERIALS AND METHODS

Primary culture of cortical neurons

Primary cortical neurons were prepared as described previously (4, 13) from rat embryos at 16–17 days gestation. Briefly, cortices were removed under a dissecting microscope, washed, minced, and finally dissociated using a nylon mesh (120 μ M and 30 μ M in size). Cells were then placed on poly-L-lysine-coated plates with a density of 5×10^5 cells per 2 cm^2 well. They were cultured for 11 days prior to experimentation in Eagle's Minimum Essential Medium (Lab. Sera and Vaccines, Lublin, Poland) containing a 10% heat-inactivated foetal calf serum (Hungarpol, Hungary) and an estrogen-free insulin-transferrin-selenium supplement (Gibco). In order to discourage non-neuronal cell proliferation, cytosine β -D-arabino-furanoside (Sigma; 1 μ M) was applied for 48 h after plating. Thus the level of astrocytes, identified by their content of the intermediate filament protein GFAP (glial fibrillary acidic protein), did not exceed 10%. The cultures were kept at 37°C in a humidified atmosphere containing a 5% CO_2 .

Experimental treatment of cultures

After 11 days of culturing, the cells were pretreated with estradiol-17 β (Sigma; 100 nM, 500 nM or 1000 nM), estriol (Sigma; 100 nM, 500 nM or 1000 nM) and ICI 182, 780 (TOCRIS; 100 nM), a specific estrogen receptor antagonist. Twenty four hours later, the cells were exposed to NMDA (Sigma; 0.1 mM, 1 mM or 5 mM) for another 24 h in a medium containing 21 mM glucose instead of the serum. Both estrogens and ICI 182, 780 were dissolved in a water solution of a 20% 2-hydroxypropyl- β -cyclodextrin (Research Biochemicals International) and were added in a volume of 10 μ l per 1 ml of the culture medium.

Lactate dehydrogenase activity

Neurotoxicity was detected by an efflux of lactate dehydrogenase (LDH) into the culture media after 24-hour treatment with NMDA. A Sigma colorimetric method (Sigma Procedure No. 500) was used, according to which the amount of colored hydrazone, formed in a reaction of pyruvic acid with 2,4-dinitrophenylhydrazine, was inversely proportional to the LDH activity in the sample and could be quantified by measuring the wave-length absorbance at 400–550 nm. The data were normalized to the amount of LDH released from vehicle-treated cells receiving NMDA (100%), and were expressed as a percentage of LDH from 6–13 separate platings \pm S.E.M.

Analysis of Bcl-2 (Western blot)

Cells were washed with phosphate-buffered saline and lysed in sample buffer containing 4% sodium dodecyl sulfate (SDS) and 10% 2-mercaptoethanol in 0.125 M Tris-Cl pH 6.8. Collected lysates were boiled at 100°C for 3 min. and then quantified for protein content. Proteins were separated by SDS-PAGE (4% stacking gel, 12% resolving gel) under constant voltage (120 V) and transferred onto nitrocellulose membrane (Millipore). The membrane was processed using standard procedure and incubated with a polyclonal rabbit anti-rat/mouse Bcl-2 antibody (Pharmingen, Becton Dickinson Company; 1:1000) followed by peroxidase-labelled secondary antibody (Boehringer Mannheim; 40 mU/ml). Immunoblots were visualized with chemiluminescence detection kit (Boehringer Mannheim) and semiquantitative analysis of band intensity was performed using FujiLas 1000 and FujiGauge softwares. All studies were repeated in at least three independent culture preparations and data are expressed as the means \pm S.E.M.

Identification of apoptotic cells

Viable and apoptotic cells were identified by fluorescence microscopy using acridine orange and ethidium bromide uptake as described by Gorman *et al.* (8). The number of apoptotic cells and the number of total cells were determined at $\times 40$ magnification. Only cells with condensed or fragmented nuclei were counted as apoptotic cells. At least 150 cells were counted for each data point. The percentage of apoptotic cells per total number of cells was calculated for 4–5 samples per group.

Statistical analysis

The significance of differences between the means was evaluated by the Duncan test following a one-way or two-way analysis of variance, respectively.

RESULTS

Effects of estrogens on NMDA-induced increase in LDH release in rat primary cortical neurons

A twenty-four hour exposure of primary cortical neurons to NMDA resulted in a dose-dependent increase in cell damage, since 1 and 5 mM of NMDA increased LDH release by 26% and 40%, respectively (Fig. 1). Both estradiol-17 β (100 and 500 nM) and estriol (100 and 500 nM) given alone had no effect on LDH level (data not shown). Estriol at a concentration of 100 and 500 nM significantly attenuated the toxic effect of 1 and 5 mM of NMDA, whereas estradiol-17 β only at a concentration of 500 nM significantly decreased the toxicity induced by 5 mM NMDA (Fig. 2). ICI 182, 780, a selective ER antagonist, given alone did not affect LDH level in both control and NMDA-treated cultures (data not shown), nor did it modify protective effects of estrogens against the NMDA-induced neuronal cell damage (Fig. 2).

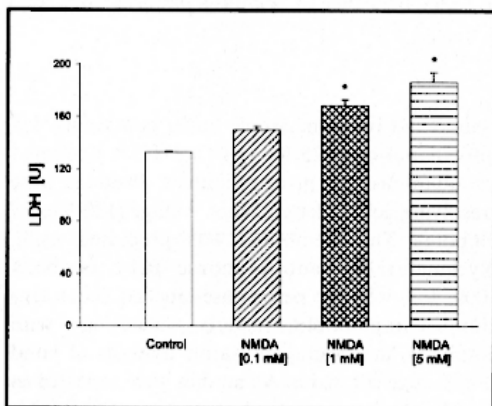


Fig. 1. The effect of NMDA on LDH efflux from rat cortical neurons in primary cultures. Results are shown as mean values from 6–8 separate platings (5×10^5 cells/ $2 \text{ cm}^2/\text{ml}$) \pm S.E.M. * $p < 0.05$ compared with control cultures by one-way ANOVA followed by Duncan's test.

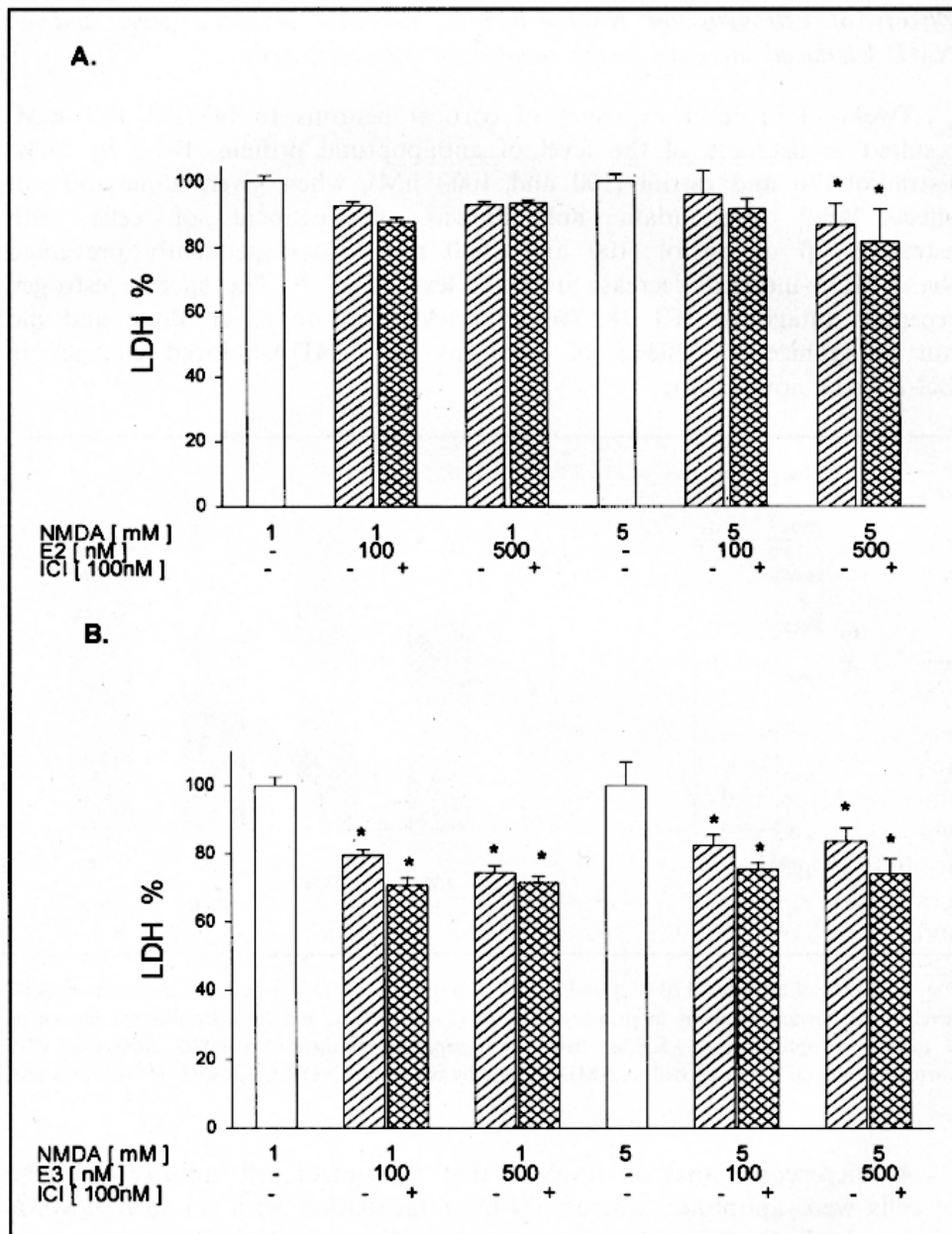


Fig. 2. Effects of estradiol-17 β (E2) [A] or estriol (E3) [B] and ICI 182,780 on the NMDA — induced toxicity in rat cortical neurons in primary cultures. The data were normalized to the amount of LDH released from vehicle-treated cells receiving NMDA (100%), and were expressed as a percentage of LDH from 6–13 separate platings (5×10^5 cells/2 cm²/ml) \pm S.E.M. * $p < 0.05$ compared with corresponding cultures exposed to NMDA alone by two-way ANOVA followed by Duncan's test.

Effects of estrogens on NMDA-induced decrease in Bcl-2 level and on NMDA-induced increase in the number of apoptotic cells

Twenty four hour exposure of cortical neurons to NMDA (0.1 mM) resulted in decrease of the level of antiapoptotic protein, Bcl-2 by 60%. Estradiol-17 β and estriol (100 and 1000 nM), when given alone did not affect Bcl-2 level (data not shown). Pretreatment of cells with estradiol-17 β or estriol (100 and 1000 nM) dose-dependently prevented the NMDA-induced decrease in Bcl-2 level (Fig. 3). The specific estrogen receptor antagonist ICI 182,780 (100 nM) had no effect alone and did not antagonize the effects of estrogens on NMDA-induced changes in Bcl-2 (data not shown).

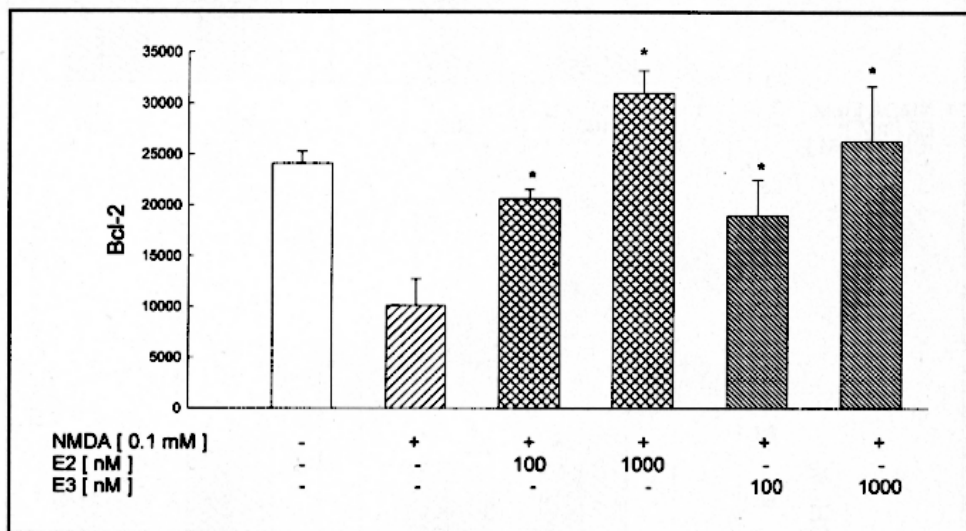


Fig. 3. Effects of estradiol-17 β (E2) and estriol (E3) on the NMDA — induced decrease in Bcl-2 level in rat cortical neurons in primary cultures (5×10^5 cells/2 cm²/ml). Results are shown as a means of optical units \pm S.E.M. from 4–6 separate platings. * $p < 0.05$ compared with corresponding cultures exposed to NMDA alone by two-way ANOVA followed by Duncan's test.

A microscopic analysis revealed that in control cell cultures ca. 23% of cells were apoptotic, whereas 24-hour incubation with 0.1 mM NMDA increased the percentage of apoptotic cells to 74%. Pretreatment of cells with estradiol-17 β (1000 nM) had no effect in control (data not shown), but attenuated the NMDA-induced apoptosis by 23% (Fig. 4). The specific estrogen receptor antagonist ICI 182,780 (100 nM) had no effect on the antiapoptotic changes evoked by estradiol-17 β (data not shown).

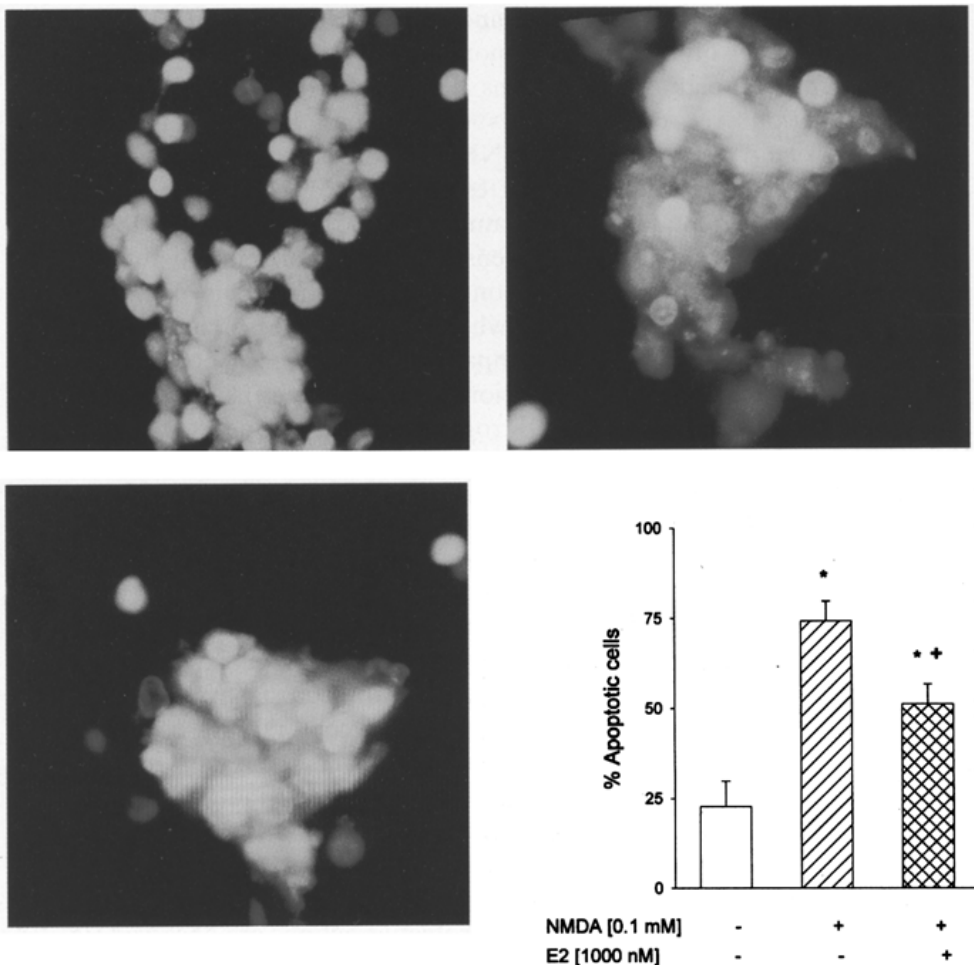


Fig. 4. Effect of estradiol-17 β (E2; 1000 nM) on the NMDA-induced increase in the number of apoptotic cells. A. control cells; B. cells treated with NMDA (0.1 mM); C. cells treated with estradiol-17 β (1000 nM) and NMDA (0.1 mM); D. Results (mean \pm S.E.M.) from 4–5 independent samples per group. * $p < 0.05$ compared with control; + $p < 0.05$ compared with NMDA group.

DISCUSSION

The NMDA receptor is crucially involved in the mechanism of neuronal damage following various insults, such as hypoxia, hypoglycemia or prolonged seizures. The measurement of the NMDA-evoked LDH release from neuronal cells *in vitro* is a reliable and widely used method of estimation of a putative neuroprotective drug efficacy. The relationship between NMDA concentration

and the percentage of LDH release found in this study is in general agreement with the reports of other authors (1), nonetheless, substantial neurotoxicity of NMDA at micromolar concentrations has also been reported (18).

Preincubation of cell cultures with equimolar concentrations of estradiol-17 β and estriol inhibited NMDA toxicity; however, estriol had a stronger effect despite the fact that estriol showed a lower affinity for ER receptors. Moreover, the selective ER antagonist, ICI 182, 780, had no effect on the neuroprotective activity of estrogens, which supports the hypothesis that estrogens affect NMDA toxicity in a non-genomic manner. Our data are in line with the study of Weaver *et al.* (24) who showed that estradiol inhibited the NMDA-induced toxicity in hippocampal cell cultures in a nongenomic way — possibly through a direct interaction between estrogens and the NMDA receptor. Indeed, an interaction of estrogens and membrane excitatory amino acid receptors has been reported. Regan and Guo (18) observed that estradiol, estrone and equiline at relatively high concentrations (10–30 μ M) reduced NMDA neurotoxicity in murine cortical cultures, that effect being only slightly antagonized by ICI 182, 780. Other nongenomic neuroprotective effects of estrogens have been suggested to be due to free radical scavenging (10) and to inhibition of vascular and neuronal L-type voltage-gated calcium channels (15). Since NMDA receptors and calcium channels are main gates for entry of calcium ions, which are critical for inducing both necrosis and apoptosis, it is likely that interference of estrogens with these receptors may be involved in nongenomic antiapoptotic estrogens effect. However, some data indicate that the neuroprotective effect of estrogens on the glutamate-induced toxicity may depend on ER receptors (17, 21). It should be mentioned here that genomic neuroprotective effects of estrogens occur when these steroids are used at low nanomolar concentrations; on the other hand, after micromolar concentrations of estrogens, mostly nongenomic, neuroprotective actions are observed (18, 19).

Glutamate receptor agonists, depending on concentration and type of cell culture, may induce neuronal cell damage via necrotic or apoptotic mechanism (3). In our study exposure of primary cortical neurons to 100 μ M NMDA for 24 hours decreased the level of the antiapoptotic Bcl-2 protein, suggesting induction of apoptotic processes. This is in agreement with other study, where 24 hour incubation of rat cortical neurons with 500 μ M of glutamate significantly decreased the level of Bcl-2 (14). Estrogens alone in concentration of 100 and 1000 nM had no effect on Bcl-2 level in primary cortical cells, but reversed effects of NMDA on this marker of apoptosis. The lack of effect of 24 hour exposure of estrogens alone on Bcl-2 is somewhat surprising, since Bcl-2 gene contains estrogen response elements and an enhancement of Bcl-2 gene expression following estrogen administration has been well documented in other cell cultures e.g. in neuronally differentiated NT2 cells (20). This difference may be due to the fact, that Singer *et al.* (20) used low nanomolar concentration of estrogen, which

is likely to act through genomic way, whereas higher doses of estrogen may induce nongenomic changes as well. Moreover, in the present experiment, the inhibitory effects of estrogens on NMDA-induced changes in Bcl-2 level were not antagonized by selective estrogen receptor antagonist, ICI 182, 780, excluding involvement of intracellular receptors. The hypothesis on nongenomic effects of estrogens on apoptosis in our study is also supported by observation, that both estradiol-17 β and its metabolite estriol, characterized by 20 fold lower affinity to intracellular estrogen receptors, inhibit the NMDA-evoked alterations to the same extent. Furthermore, estradiol-17 β simultaneously inhibited the NMDA-induced changes in Bcl-2 level and in the number of apoptotic cells, which suggests a functional role of this protein in the antiapoptotic action of estrogens.

In conclusion, our study shows that estradiol-17 β and estriol attenuate NMDA-induced neurotoxicity and apoptosis in primary cultures of rat cortical neurons. We suggest that the neuroprotective effect of estrogens on the NMDA-induced changes is not mediated by intracellular hormone receptors.

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REFERENCES

- Almeida A, Heales SJR, Bolanos JP, Medina JM. Glutamate neurotoxicity is associated with nitric oxide-mediated mitochondrial dysfunction and glutathione depletion. *Brain Res* 1998; 790: 209—216.
- Behl C, Skutella T, Lezoualch F, Post A, Widmann M, Newton CJ, Holsboer F. Neuroprotection against oxidative stress by estrogens: structure-activity relationship. *Mol Pharmacol* 1997; 51: 535—541.
- Cheung NS, Pascoe CJ, Giardina SF, John CA, Beart PM. Micromolar L-glutamate induces extensive apoptosis in an apoptotic- necrotic continuum of insult-dependent, excitotoxic injury in cultured cortical neurones. *Neuropharmacology* 1998; 37: 1419—1429.
- Dawson VL, Dawson TM, London ED, Brecht DS, Snyder SH. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc Natl Acad Sci USA* 1991; 88: 6368—6371.
- Dubal DB, Shughrue PJ, Wilson ME, Merchenthaler I, Wise PM. Estradiol modulates Bcl-2 in cerebral ischemia: A potential role for estrogen receptors. *J Neurosci* 1999; 19: 6385—6393.
- Garcia-Segura LM, Cardona-Gomez P, Naftolin F, Chowen JA. Estradiol upregulates Bcl-2 expression in adult brain neurons. *Neuroendocrinology* 1998; 9: 593—597.
- Goodman Y, Bruce A J, Cheng B, Mattson M P. Estrogens attenuate and corticosterone exacerbates excitotoxicity, oxidative injury, and amyloid beta-peptide toxicity in hippocampal neurons. *J Neurochem* 1996; 66: 1836—1844.
- Gorman A, McCarthy J, Finucane D, Reville W, Cotter T. Morphological assesment of apoptosis. In *Techniques in Apoptosis. A user's guide*, TG Cotter, SJ Martin (eds). New York, Portland Press, 1994, pp. 1—20.

9. Green PS, Gridley KE, Simpkins JW. Estradiol protects against β -amyloid (25–35)-induced toxicity in SK-N-SH human neuroblastoma cells. *Neurosci Lett* 1996; 218: 165–168.
10. Green PS, Gridley KE, Simpkins JW. Nuclear receptor-independent neuroprotection by estratrienes: a novel interaction with glutathione. *Neuroscience* 1998; 84: 7–10.
11. Hayashi T, Ishikawa T, Yamada K, Kuzuya M, Naito H, Hidaka A. Biphasic effect of estrogen on neuronal constitutive nitric oxide synthase via Ca^{2+} -calmodulin dependent mechanism. *Biochem Biophys Res Commun* 1994; 203: 1013–1019.
12. Kajta M, Lason W. Oestrogen effects on kainate-induced toxicity in primary cultures of rat cortical neurons. *Acta Neurobiol Exp* 2000; 60: 365–369.
13. Koch JY, Choi DW. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J Neurosci Methods* 1987; 20: 83–90.
14. Liu X, Zhu X-Z. Roles of p53, c-Myc, Bcl-2, Bax and caspases in glutamate-induced neuronal apoptosis and the possible neuroprotective mechanism of basic fibroblast growth factor. *Mol Brain Res* 1999; 71: 210–216.
15. Mermelstein PG, Becker JB, Surmeier DJ. Estradiol reduces calcium currents in rat neostriatal neurons via a membrane receptor. *J Neurosci* 1996; 16: 595–604.
16. Miranda RC, Sohrabji F, Toran-Allerand D. Interactions of estrogen with the neurotrophins and their receptors during neural development. *Horm Behav* 1994; 28: 367–375.
17. Pike CJ. Estrogen modulates neuronal Bcl- x_L expression and β -amyloid-induced apoptosis: relevance to Alzheimer's disease. *J Neurochem* 1999; 72: 1552–1563.
18. Regan RF, Guo Y. Estrogens attenuate neuronal injury due to hemoglobin, chemical hypoxia, and excitatory amino acids in murine cortical cultures. *Brain Res* 1997; 764: 133–140.
19. Singer CA, Figueroa-Masot XA, Batchelor RH, Dorsa DM. The mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. *J Neurosci* 1999; 19: 2455–2463.
20. Singer CA, Rogers KL, Dorsa DM. Modulation of Bcl-2 expression: a potential component of estrogen protection in NT2 neurons. *Neuropharmacology* 1998; 9: 2565–2568.
21. Singer CA, Rogers KL, Strickland TM, Dorsa DM. Estrogen protects primary cortical neurons from glutamate toxicity. *Neurosci Lett* 1996; 212: 13–16.
22. Singh M, Meyer EM, Simpkins JW. The effect of ovariectomy and estradiol replacement on brain-derived neurotrophic factor messenger ribonucleic acid expression in cortical and hippocampal brain regions of female Sprague-Dawley rats. *Endocrinology* 1996; 136: 2320–2324.
23. Watters JJ, Campbell JS, Cunningham MJ, Krebs EG, Dorsa DM. Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription. *Endocrinology* 1997; 138: 4030–4033.
24. Weaver CE, Park-Chung M, Gibbs TT, Farb DH. 17 β -estradiol protects against NMDA-induced excitotoxicity by direct inhibition of NMDA receptors. *Brain Res* 1997; 761: 338–341.

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