Endocr (2007) 31:44–51
DOI 10.1007/s12020-007-0010-2

ORIGINAL PAPER

The effects of glutamate can be attenuated by estradiol via estrogen receptor dependent pathway in rat adrenal pheochromocytoma cells

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Published online: 12 May 2007

Abstract Estrogens have been suggested to exhibit neuroprotective activities against several insults including beta-amyloid and glutamate, one of the excitatory neurotransmitters in the central nervous system. In the present study, we showed that exposure to glutamate not only inhibited the cell growth of exponentially growing rat pheochromocytoma PC12 cells in a time- and dose-dependent manner, but also influenced cell adherence capacity. Glutamate-induced growth inhibition was significantly attenuated by the co-administration of estradiol in PC12 cells. Pre-exposure of the PC12 cells to the estradiol was not required for protection against glutamate-induced growth inhibition. Administration of anti-estrogen ICI182,780 efficiently blocked the neuroprotective effects of estradiol. Glutamate-induced changes in cell adherence, on the other hand, were not significantly affected by estradiol. These data indicate that the neuroprotective effects of estradiol against glutamate-induced insults in PC12 cells, at least in part, involve estrogen receptor-dependent pathways.

Keywords Estrogen · Glutamate · Neuroprotection · PC12 cells

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Introduction

Glutamate is the major excitatory neurotransmitter in the central nervous system and produces its neurophysiologic effects by acting at three separate types of excitatory amino acid receptors [1]. Injury to the brain, such as trauma or ischemia, is associated with abnormal release of neurotransmitters, including glutamate [2]. The etiology of a range of acute and chronic neurological disorders including hypoxia, hypoglycemia, stroke and epilepsy, is believed to conform to uncontrolled release of some neurotransmitters [2, 3]. Besides its physiological role, previous studies also showed that glutamate is involved in several neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease and amyotrophic lateral sclerosis [1, 4–7]. Accumulation of excess extracellular glutamate causes a specific pattern of neurodegeneration in the brain of experimental animals and in differentiated and undifferentiated cultured neuronal cell lines, including rat pheochromocytoma PC12 cells [8, 9]. Nonetheless, it seems that acute excitation by glutamate is not able to fully explain the delayed and progressive neuronal degeneration observed in the pathologies in which glutamate is implicated [10, 11].

Estrogens play important roles in neuroprotective and neurotropic actions [6, 12–15] including a reduction in the incidence of Alzheimer's disease, Parkinson's disease and death from stroke [16–20]. The physiological functions of estrogens have been extensively studied and most of their actions, especially those involved in reproduction and cell growth are mediated through estrogen receptor (ER) proteins: ER-alpha or ER-beta. ER-alpha and ER-beta are differentially distributed in the brain and likely mediate different estrogen-dependent processes [21, 22]. Upon binding to its receptor, an

estrogen-ER complex is formed and can act as a transcription factor to regulate transcription of target genes [23]. Nonetheless, the role of ER-dependent transcription in estrogens' neuroprotective activity remains controversial [24]. The study of Green et al. [20] showed that the neuroprotective effects of estrogens can be disassociated from the ER in the HT-22 murine hippocampal cell line and in the SK–N–SH neuroblastoma cell line, using an enantiomer of 17-beta estradiol, which has identical chemical properties but interacts only weakly with known ERs. Some studies, on the other hand, reported that the ER antagonists were able to block the neuroprotective activity of estrogen in culture models, suggesting the involvement of ERs [5, 25]. In other models such as the mouse, the results are similarly inconclusive [26, 27].

The clinical importance of estrogens in neurodegenerative diseases and normal neuronal development has been investigated extensively, but the molecular mechanisms of their neuroprotective activities remain relatively poorly defined. The present study addresses the neuroprotection of estradiol against the effects of glutamate in rat pheochromocytoma PC12 cells and the potential involvement of the ER-dependent pathway for the neuroprotective effects of estrogen. The well-studied PC12 cells are of neural origin and have been used as a model system for studying the mechanisms of neuronal development, differentiation and survival in many laboratories.

Results

Estrogen receptor in PC12 cells

The presence of estrogen receptor-alpha (ER-alpha) was investigated in PC12 cells (Fig. 1). Western blot analysis showed a positive signal of ER-alpha protein in PC12 cell extracts (Fig. 1A). The presence of ER-alpha protein was also detected within PC12 cells based on the observation that antibody against ER-alpha protein exhibited a shift in fluorescence intensity compared to the control antibody as analyzed via flow cytometer (Fig. 1B) The ER-alpha mRNA was also detectable in PC12 cells with RT-PCR assay (data not shown).

When exponentially growing PC12 cells were exposed to glutamate, a dose- and time-dependent decrease in cell viability was observed (Fig. 2A, B). In Fig. 2B, at all the time points investigated, the number of viable cells in glutamate treated samples was higher or similar to the number of viable cells in control sample at 8 h. The number of viable cells at this time point was approximately the seeding cell number.

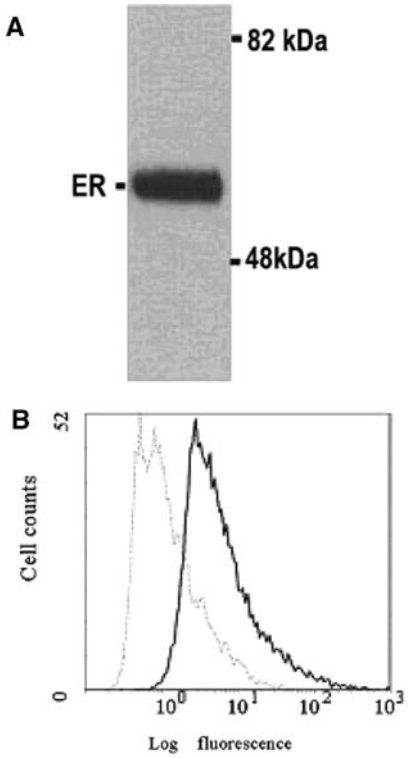

Fig. 1 Expression of estrogen receptor in PC12 cells. (**A**) Western blot analysis of estrogen receptor-alpha (ER-alpha) protein in PC12 cells. Total proteins were prepared and fractionated via 10% SDS-PAGE. The molecular weight markers are indicated on the right. (**B**) Flow cytometric analysis of the ER-alpha in PC12 cells. PC12 cells were collected and prepared for analysis as described in the materials and methods. The heavy solid and light dashed line indicates the cross-reactivity of ER antibody and control antibody, respectively

The cell density also played a role in the extent of the glutamate effect (Fig. 2C). At high cell densities, there were greater decreases of cell viability (up to 63%) induced by glutamate, while the cell viability of cells at low densities was inhibited to a lesser extent (26.2%). To analyze whether the effect of glutamate on PC12 cell viability was due solely to cytotoxicity, cells of different origins were treated with 10 mM glutamate for 24 h and the number of viable cells was determined (Fig. 3). SK–N–SH cells, a neuroblastoma cell line, were not affected by treatment with glutamate. Hela and MCF-7 cells, which are not neuronal cells, were very resistant to the insults of glutamate as well.

Estradiol effects on glutamate treated PC12 cells

Estrogens are implicated to play a neuroprotective role in several animal and cell culture models. To analyze how estradiol mediates its protective effects, glutamate treated PC12 cells were incubated with various concentrations of estradiol for 24 h and the numbers of viable cells were determined (Fig. 4A). Co-administration of estradiol effi-

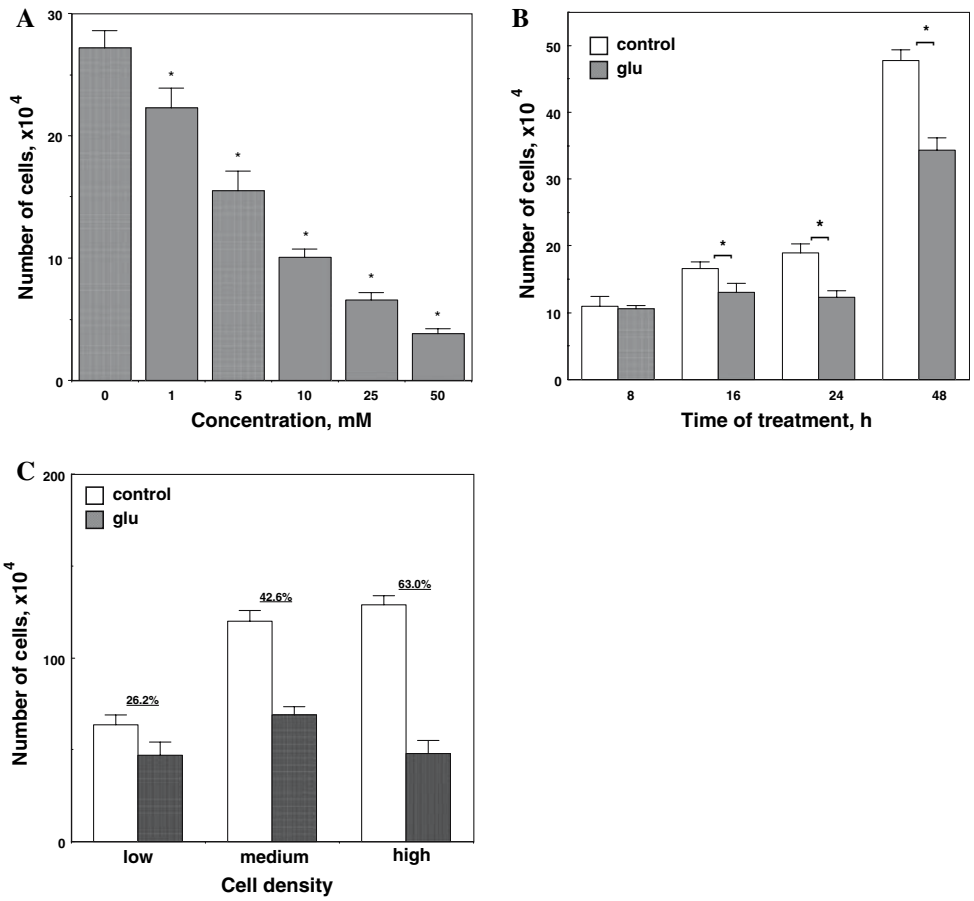

Fig. 2 Effects of glutamate on cell proliferation of PC12 cells. (**A**) PC12 cells were incubated with indicated concentration of glutamate (glu) for 24 h and the number of viable cells was determined. (**B**) PC12 cells were treated with 10 mM glutamate for the indicated time and the numbers of viable cells were determined. (**C**) PC 12 cells were seeded into three groups of culture plates and grown for 1, 2, or 3 days prior to the addition of 10 mM glutamate for 24 h, and the numbers of viable cells were determined. The groups of cells that grew for 1, 2, or 3 days were each designated low, medium and high cell density. The number above each set of columns represents the percentage of viable cells calculated as the number of viable cells grown in glutamate divided by the number of viable control cells. Values are mean ± SD of eight independent experiments, each performed in triplicate or quadruplicate

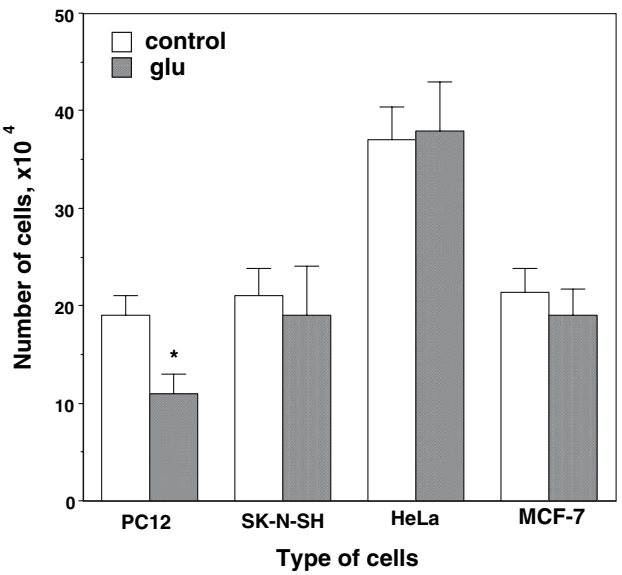

Fig. 3 Differential effects of glutamate on cell proliferation in different cell types. Cells were grown and incubated with 10 mM glutamate (glu) for 24 h and the numbers of viable cells were determined. Values are mean ± SD of three independent experiments, each performed in triplicate

ciently blocked the decrease of cell viability induced by glutamate in PC12 cells. The protective effect of estradiol was time-dependent (Fig. 4B). Estradiol started to exhibit protective effects at 24 h of treatment (Fig. 4C), and very little protection from glutamate was noted at earlier time points (16 h of treatment or less). Interestingly, cell adherence of PC12 cells appeared to be affected by the glutamate treatment as well (Fig. 4C). The percentage of cells that lost their adherence and became floating in the medium increased from 21% in the control sample to 45% in the glutamate treated sample. Although estradiol increased the number of viable cells from 54.7% in glutamate treated cells to 91.8%, it did not change the percentage of non-adherent cells in glutamate treated PC12 cells. The percentage of floating cells remained at approximately 42–45% of the total cell population.

To investigate whether pre-exposure to estradiol facilitated protection from glutamate, PC12 cells were incubated with 25 μM of estradiol for 24 h prior to the glutamate insults (Fig. 5). The results showed that the protective effects of estradiol did not require pre-exposure to estradiol. Co-administration of 25 μM of estradiol resulted in similar protection as with pre-exposed samples. Treating PC12

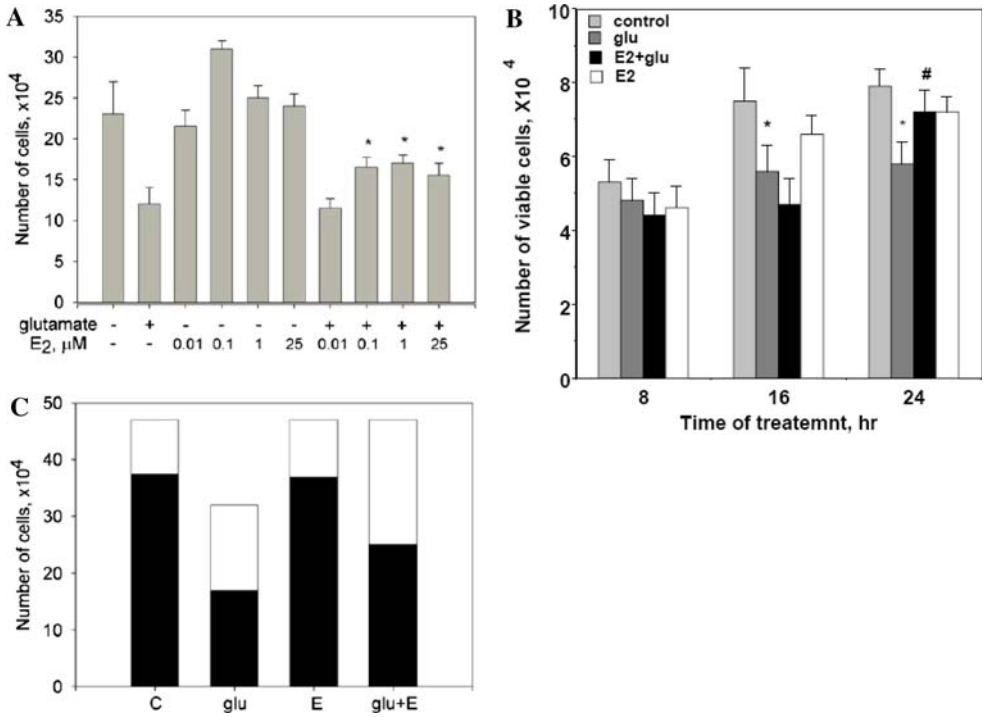

Fig. 4 Effects of estradiol on glutamate-induced changes in PC12 cells. (**A**) Cells were incubated with 10 mM glutamate alone or with estradiol at the indicated concentrations and the numbers of viable cells were determined. *$P < 0.05$ compared to glutamate treatment. (**B**) Cells were incubated with glutamate (glu), estradiol (E_2), glutamate plus estradiol (E_2 + glu), or neither (control) for the indicated times and the numbers of viable cells were determined. *$P < 0.05$ compared to the control. #$P < 0.05$ compared to glutamate treated cells. (**C**) Cells were incubated with glutamate alone or with estradiol as indicated and the numbers of adherent and non-adherent cells were determined. solid line: adherent cells; open line: floating cells. Values are mean ± SD of three independent experiments, each performed in triplicate

cells with estradiol for 48 h showed protection similar to the 24 h pre-exposure treatment (data not shown).

We examined the potential involvement of ER-alpha by analyzing the effects of co-administering a ''pure'' anti-estrogen, ICI182,780, to counteract the estradiol induced protection in glutamate treated PC12 cells (Fig. 6). The addition of ICI182,780 efficiently blocked the neuroprotective effects of estradiol against 10 mM of glutamate. Similar blockage was produced with higher concentrations of ICI182,780 at 0.2, 1 and 5 μM.

Previous reports show that the effects of glutamate were mediated through apoptosis and could be blocked by RNA and protein synthesis inhibitors [38]. We sought to check the correlation between apoptosis related gene expression and the effects of glutamate on decreased cell viability (Fig. 7). The results showed that the mRNA levels of BAX, BAD and c-jun were within similar range among all samples, including glutamate treated, estradiol treated, glutamate and estradiol co-treated and vehicle treated PC12 cells. The presence of another apoptosis related gene Bcl-XL was not detected in PC12 cells.

To determine whether glutamate treated PC12 cells undergo apoptosis, cells treated with 10 mM glutamate for

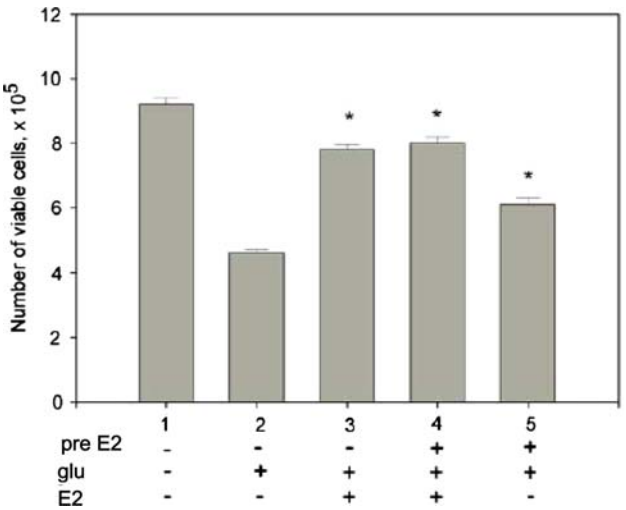

Fig. 5 The effects of pre-exposure to estradiol on glutamate-induced changes in cell viability. PC12 cells were either treated with 25 μM estradiol for 24 h prior to the addition of 10 mM glutamate (glu) or 25 μM estradiol (E2) was administered simultaneously with 10 mM glutamate. Cells were treated with glutamate for 24 h and the numbers of viable cells were determined. *$P < 0.05$ compared to glutamate treatment alone, column 2). Values are mean ± SD of five independent experiments, each performed in triplicate

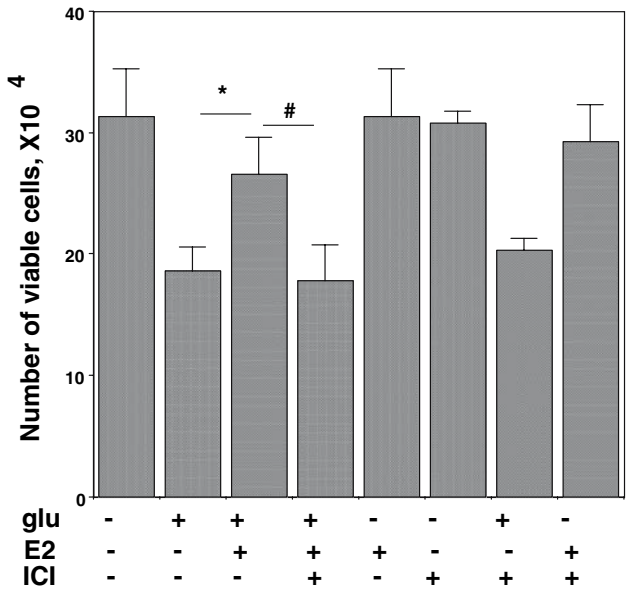

Fig. 6 Effects of anti-estrogen on glutamate-induced changes in cell viability. Cells were incubated with glutamate (glu), estradiol (E2) or anti-estrogen ICI182,780 (ICI) as indicated for 24 h and the numbers of viable cells were determined. *$P < 0.05$ compared to glutamate treatment alone. #$P < 0.05$ compared to glutamate and estradiol co-administration. Values are mean ± SD of four independent experiments, each performed in triplicate

24 h were stained with Annexin-V and then analyzed flow cytometrically. The percentage of cells that stained Annexin-V positive were 0.76% which was comparable to vehicle treated cells (0.96%). The majority of the cells remained annexin-V and PI double negative (92.5% for the vehicle control and 94.6% for glutamate treated). It thus indicated that at the time and concentration tested, apoptosis does not appear to be the main cause for observed decrease in cell viability induced by glutamate and cell death as indicated by positive PI staining was minimal.

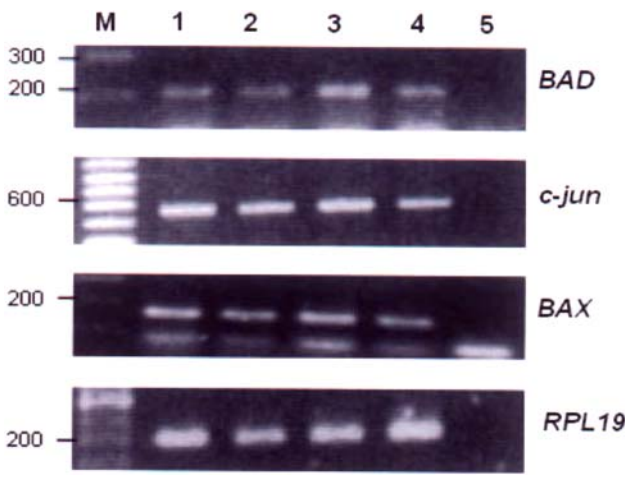

Fig. 7 Analysis of glutamate induced apoptosis in PC12 cells. PC12 cells were treated with glutamate alone or with estradiol for 24 h and the mRNA levels of apoptosis associated gene were determined using RT-PCR. Molecular weight markers are in base pairs (bp). Lane 1: control, lane 2: glutamate (glu), lane 3:estradiol (E2), lane 4:glu + E2, lane 5:H_2O substituted for RNA in the sample. Three independent experiments were performed

Discussion

The results showed that estradiol had a protective effect against glutamate insult and that the neuroprotective effect by estradiol depended on the concentration and the duration of exposure. Furthermore, the results suggest the mode of action of estradiol, at least partially, includes ER-dependent pathways, based on the observations that the neuroprotective effect of estradiol can be attenuated by the selective estrogen antagonist ICI182,780 in PC12 cells.

The effects of glutamate on PC12 cell viability showed time- and concentration-dependency and agreed with previous studies that evaluated the effects of glutamate on cells by the release of LDH [25]. Although glutamate significantly decreased cell viability, the exact mechanisms of its insult remain controversial. In this study, we observed that the number of trypan blue-stained cells did not increase after glutamate treatment, and number of viable cells was higher or at least similar to the number of cells seeded. It is thus logical to propose that the decreased cell number compared to control after glutamate treatment (<25 mM) were mainly due to inhibition of cell growth. However, the possibility of glutamate-induced cell death still remains and further investigation is required to determine whether glutamate cause any significant cell death.

Glutamate produces its neurophysiologic effects by acting at three types of specific membrane receptors; *N*-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isozazolepropionic acid (AMPA), and kainite [1, 35]. In PC12 cells, the debate on the presence of functional glutamate receptors remains unsettled. The mRNA of NMDA receptor subunits were expressed in PC12 cells, but the presence and the functionality of receptor proteins are rather contradictory [36, 37]. Reports in the literature show that only a very small amount of NMDA receptor protein was detected in PC12 cells, and no functional channels controlled by NMDA receptor proteins were found in this cell line [22, 38]. The studies of Froissard and Duval also indicated that the effects of glutamate on cell viability in PC12 cells did not involve binding to NMDA receptor proteins [39]. The effects of glutamate, thus, appear to involve pathways other than the classical receptor-mediated pathway since neither the NMDA receptor blockers nor NMDA itself showed any effects in PC12 cells [37]. However, some results have also been reported that PC12 cells indeed expressed functional NMDA receptor proteins

[40]. In addition, glutamate receptor antagonists were able to block the effects of glutamate, implicating a receptor-dependent pathway in PC12 cells [27]. Our results indicated that the effects of glutamate on cell viability are cell specific and may depend on the presence of NMDA receptors. We observed that the two NMDA receptor-lacking cell lines used in this study, SK–N–SH and Hela, were completely tolerant to the glutamate insult while the NMDA receptor-positive PC12 cells were sensitive to the same insult. The studies of Zaulyanov et al. [41] also reported that neither HT-22 nor SK–N–SH cell lines that lack NMDA receptors was influenced by glutamate while the NMDA receptor containing primary rat cortical neurons were sensitive to the same glutamate treatment. Kim et al. [42] reported similar observation in that SK–N–SH cells were less vulnerable to glutamate-induced insults than the rat primary cortical neurons.

The expression levels of BAX, BAD and c-jun in this study as determined by RT-PCR did not change by the glutamate treatment in PC12 cells. Although more detained examination should provide more conclusive results, it does appear that at the mRNA level these genes showed limited changes. Moreover, apoptosis analysis of PC12 cells also indicated that the percentage of cells undergo apoptosis was not significantly elevated by glutamate treatment compared to that of control cells.

Estrogen is regarded as a molecule that exhibits neuroprotective effects. Its mechanism of action appears to be complex and is mediated through either ER-dependent or ER-independent pathways. Via the ER-dependent pathway, estrogen binds to ERs and regulates the transcription of target genes, including genes involved in cell growth, survival and differentiation, such as the expression of anti-apoptotic genes *Bcl-XL* and *Bcl-2* and pro-apoptotic genes *BAD* and *c-jun* [5, 29, 43]. Estrogen can also function via ER-independent pathway by interacting with MAPK and PI3-K signaling cascades, bind to NMDA receptors and act as an antioxidant without the involvement of transcriptional regulation [6, 12, 14]. In this study, we observed that concomitant estradiol treatment was neuroprotective because the severity of the glutamate insult on cell viability decreased by 70%. Pre-treatment with estradiol exhibited similar protective effects against glutamate. Up to 27% protection against glutamate was still observed even if estradiol was withdrawn at the time of glutamate administration after the 24-h pre-treatment. In the study of Honda et al. [15], estrogen provided protection against the glutamate insult only when rat primary cortical neurons were pre-treated with estrogen for at least 24 h. Whether pre-treatment with estrogen is essential for its protection is still not fully settled and might be cell-type dependent since different types of cells were used in our and their systems.

The mechanism of how estradiol mediated its protection against glutamate insult appeared to involve ER-dependent pathways in that the protection by estradiol was blocked by the addition of ICI182,780, a ‘‘pure’’ anti-estrogen in our study. Traditionally, an ER-dependent pathway would be suggested if the effects of estrogen could be attenuated by an estrogen antagonist [44]. Moreover, we were able to detect the presence of ER-alpha protein and mRNA in PC12 cells. It thus indicated that the possibility of ER-alpha involved in the estrogen-induced protection in PC12 cells. However, some studies found that higher levels of ER-alpha were needed to be introduced into PC12 cells by various means including transfection of ER-alpha cDNA in order to study the effects of estrogen [29, 45]. To elucidate the potential involvement of ER-beta, the recently identified ER subtype, we measured the levels of ER-beta in PC12 cells via RT-PCR and found little expression. The ER-beta subtype has been suggested to exhibit different characteristics than the ER-alpha subtype. ER-beta controls neuropeptide gene expression, which is required for lactation, sexual behavior, maternal behavior and participation in the cellular functions mediated by phytoestrogens, estrogen-like chemicals derived from plants [46]. In conclusions, the results indicate that the protective effects of estrogens appear to be complex and might be mediated through multiple pathways; whether the classical ER-alpha pathway is the sole pathway or whether ER-beta plays any role requires further investigation.

One surprising finding was the apparent change in cell adhesion accompanying the decreased cell viability in response to glutamate treatment. A recent report [47] provides some insights in this observation. They showed that adhesion and/or neuron-substrate interactions could be one of the cellular responses to glutamate. By growing neurons on different substrates, they found that embryonic hippocampal neurons showed differential vulnerability to the insult of glutamate. Neurons that grew on an integrin’s ligand exhibited increased resistance to glutamate-induced insults compared with neurons grown on polylysin. Integrins are membrane proteins capable of mediating adhesive interactions of cells with extracellular matrix and with other cells, and are suggested to be involved in the intracellular signaling cascades of motility, proliferation and survival [48]. It thus appeared that the effects of glutamate were associated with the cell’s adhesion. It would be intriguing to understand how these changes in adhesion take place in response to glutamate and the roles that estradiol might play in this process.

Materials and methods

Chemicals and cell culture materials

Glutamate and water-soluble 17 beta-estradiol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). ICI182,780 was purchased form Tocris (Avonmouth, UK). The PC12 rat adrenal pheochromocytoma cells (CCRC 60048) were grown in phenol red free Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% 3-times-charcoal-stripped fetal bovine serum (FBS) and maintained at 37°C in a humidified atmosphere of 90% air and 10% CO_2. The human neuroblastoma cell line SK–N–SH, the human ovary carcinoma cell line Hela, and the human breast cancer cell line MCF-7 were obtained from the American Type Culture Collection (ATCC) and were cultured according to the provider's suggestions.

Cell viability assays

Cells were cultured in DMEM supplemented with 10% FBS until they reached near confluence, after which they were cultured for another 24 h in serum-free medium. Cells were then collected by trypsinization, counted and seeded into polylysin coated culture plates in medium containing 10% FBS. After culturing for 24 h to ensure attachment of the cells, the medium was removed and fresh medium supplemented with 10% FBS alone or with test chemicals was added. After the cells were allowed to grow for a predetermined period of time at 37°C, the numbers of viable and non-viable cells in the adherent and non-adherent cell populations in each sample were each determined using the trypan blue dye exclusion assay in which viable cells remained transparent while non-viable cells appeared blue under the microscope.

Determination of estrogen receptor in PC12 cells

The PC 12 cells were collected, washed twice with phosphate-buffered NaCl solution (PBS) and incubated with PBS containing antibodies against ER (Ab-10, NeoMarkers) for 1 h at 25°C. Following washing with PBS twice, cells were incubated with FITC-secondary antibodies for 1 h at 25°C. Finally, cells were treated with 1% formaldehyde for 10 min, collected by centrifugation, resuspended in PBS and analyzed with flow cytometer (PC500, Beckman-Coulter). Each analysis was repeated at least twice. For western blot analysis, cell lysate was prepared, fractionated via 10% SDS-PAGE, blotted and incubated with antibody against ER-alpha protein AB-10 by the procedure suggested by manufacturers.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was prepared from PC12 cells treated with glutamate alone or with 17 beta-estradiol for 24 h as previously described [28]. The RT reaction was performed using 0.1–0.2 μg of total RNA in a 20-μl reaction mixture, and a portion of the RT product containing an equal amount of cDNA from each sample was then subjected to PCR. The PCR reaction mixture contained 2 μl of 10X PCR buffer, 1.6 μl of 50 μM $MgCl_2$, 11.7 μl of diethylpyrocarbonate-treated water, 1.5 μl of RT-reaction mixture, 1 μl of 10 mM dNTPs, l unit of Taq DNA polymerase and 2 μl of test primers, including primers for *Bcl-XL*, *BAD*, *BAX* and *c-jun* [29] or ribosomal protein L19 (*RPL19*) primers (5′-CTGAAGGTCAAAGGGAATGTG-3′ and 5′-GGACAGAGTCTTGAT-GATCTC-3′; 125 μg/ml). *RPL19* is constitutively expressed and used as a control for the RT-PCR reaction [30]. The cDNA fragments were amplified using a standard PCR procedure (35 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min) and the amplified product was fractionated using agarose gel electrophoresis and stained with ethidium bromide. Data were semi-quantified by comparing the DNA intensity of interest to the intensity of RPL19.

Differential display RT-PCR

Total RNA was prepared from PC12 cells treated with 10 mM of glutamate alone or together with 25 μM of 17 beta estradiol for 16 h at 37°C as previously described [31]. The differentially expressed gene patterns were analyzed using the GenePhor Electrophoresis System according to manufacturer's suggestions (Amersham Pharmacia Biotech), follow by re-amplification and subcloning of these differentially expressed cDNA fragments into the pGEM-T vector in *Epicurian coli* according to previously reported protocols [31, 32]. Sequences of these cloned cDNA fragments were determined using standard methods and primers with sequences that were complementary to the sequenced cDNA fragments.

Apoptosis analysis of PC12 cells

The cells were cultured and treated with glutamate as described previously and subjected to the analysis of apoptosis according to the manufacture's suggestions (Roche). Briefly, PC12 cells treated with 10 mM glutamate for 24 h were harvested and stained with annexin-V-FLUOS and propidium iodine and analyzed with Cytomics FC500 (Beckman–Couter).

Statistical analysis

The method of least squares analysis of variance was used to analyze the data [33, 34]. The means of treated samples were compared using orthogonal contrast with the comparisons of each individual treatment to the control blank

Acknowledgments This work was partially supported by National Science Society grants 90-2311-B-031-004, 91-2311-B-031-001, 92-2311-B-031-001 and 93-2311-B-031-001 impartiality. There is no conflict of interest that prejudice this works.

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