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Endogenously Expressed Estrogen Receptors Mediate Neuroprotection in Hippocampal Cells (HT22)

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Abstract Discovery of estrogen receptors (ER) in the central nervous system and the ability of estrogens to modulate neural circuitry and act as neurotrophic factors, suggest a therapeutic role of this steroid. To gain better understanding of the specificity and cellular mechanisms involved in estrogen-mediated neuroprotection, a mouse hippocampal neuronal cell line (HT22) was evaluated. Earlier reports indicated this cell line was devoid of ERs. Contrary to these findings, characterization of HT22 cells using RT-PCR, immunoblot, immunocytochemical, and radioligand binding techniques revealed endogenous expression of ER. The predominant subtype appeared to be ERα with functional activity confirmed using an ERE-tk-luciferase assay. The ability of an ER antagonist, ICI-182780, to block the neuroprotective effects of estrogens confirmed ER was involved mechanistically in neuroprotection. In conclusion, HT22 cells express functional ERα or a closely related ER enabling this cell line to be used to profile estrogens for neuroprotective properties acting via an ER-dependent mechanism. J. Cell. Biochem. 95: 302–312, 2005.

Key words: estrogen receptors; genomic; neuronal; estrogen responsive element

The ability of estrogens to modulate physiological activities beyond those associated with reproduction has been well established. For example, in the central nervous system a growing body of literature suggests that estrogens can serve as important neurotrophic and neuroprotective factors [Wise, 2003]. Our understanding of estrogens' role in the brain is limited and so the mechanisms by which estrogens are involved in neuroprotection remain unclear. More than one mechanism is likely to be involved since estrogens act on a variety of systems in the central nervous system that impinge on multiple pathways involved in neuroprotection. Literature reports support estrogen action in the brain by both estrogen receptor (ER)-dependent [Dubal et al., 2001; Wilson et al., 2002; Yang et al., 2003] and ER-independent mechanisms [Behl et al., 1997; Green et al., 1998; Moosmann and Behl, 1999; Singh et al., 1999]. Action through ER-dependent mechanisms refers to the classical activity of the steroid hormone receptor family, functioning primarily as ligand-activated transcriptional factors [Beato and Sánchez-Pacheco, 1996]. The ability of estrogen, acting through its cognate receptor, to regulate gene activity in the central nervous system has been shown in a variety of paradigms including those associated with neurotrophic support [Sohrabji et al., 1995], apoptosis [Singer et al., 1998; Linford et al., 2001] as well as interaction with a variety of signal transduction pathways [Toran-Allerand et al., 1999; Wade et al., 2001]. The ability of estrogens to act independently of its receptor often referred to as nongenomic action, has been reported in a variety of testing scenarios. For example, estrogens have been shown to act as antioxidants [Behl et al., 1997; Green et al., 1997] as well as inhibit lipid peroxidation [Tang et al., 1996; Gridley et al., 1997]. In addition, a growing body of literature suggests that estrogens may directly influence rapid signal transduction events via a membrane-associated ER [Toran-Allerand et al., 2002; Deecher et al., 2003] or by direct interaction with proteins involved in signaling [Wong et al., 2002].

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Understanding normal brain physiology and the effects of hormone depletion on the neural circuitry will be important in determining the role of estrogens on maintaining normal brain function. Hence, defining a strategy to identify compounds that can mimic estrogens neuroprotective effects, elucidating estrogen-regulated genes as potential therapeutic targets and defining signaling pathways that estrogens influence are imperative to defining whether estrogen mimetics will have therapeutic utility. Numerous investigators have used cell lines as tools to identify the role of estrogens in neuroprotection. One of the cell lines used frequently in this approach is hippocampal cell line (HT22), a neuronal line derived from the mouse hippocampus. Several reports have failed to detect ER expression in HT22 cells leading to the conclusion that the neuroprotective action of estrogens is due to nongenomic mechanisms [Behl et al., 1995; Green et al., 1998]. Hence, this cell line has become a cell model to study nongenomic action of estrogens.

Recent reports demonstrating that activation of exogenous ER in the HT22 cells can result in neuroprotection [Mize et al., 2003] has prompted us to reevaluate the ER status of this cell line. A variety of techniques were used to identify ER expression at both the mRNA and protein level and confirm its functional activity. In this study, we demonstrate for the first time that HT22 cells express endogenous estrogen receptors. Additionally, these ERs appear to be involved with estrogensneuroprotective effects in HT22 cells following glutamate toxicity. Preliminary characterization of this ER suggests that this receptor is ERα or a closely related estrogen receptor.

MATERIALS AND METHODS

Compounds

The compounds 17-β-estradiol (E2) and progesterone (P4) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Estrone and Δ8,9-dehydroestrone were obtained from the Wyeth Research compound repository (Princeton, NJ). An ER antagonist, ICI-182780 (7-alpha,17-beta)-7-[9-[(4,4,5,5,5-pentafluoroopentyl)sulfinyl] nonyl-estra-1,3,5(10)-triene-3,17-diol, was purchased from Zeneca Pharmaceuticals (Mereside Alderley Park, MacCleffield Cheshire, England).

Cell Culture

A mouse hippocampal cell line (HT22) was licensed from David Schubert (Salk Institute, La Jolla, CA) and propagated in growth media [DMEM (high glucose, 25 mM HEPES; Gibco, Grand Island, NY) #12430-054] supplemented with 10% fetal calf serum (Irvine Scientific; #3000, Santa Ana, CA) sodium pyruvate and 1% GlutaMAX-1 (Life Technologies, Inc., Gaithersburg, MD). The cells were maintained at 35°C in a humidified chamber with 10% CO_2. Cell density was closely monitored to prevent excessive growth (i.e., over confluency) that resulted in neuronal differentiation and the loss of ER expression. The cell line was assessed for ER expression using radioligand binding assays. Maximal ER expression levels were identified after cells were removed from cryopreservation and passed three times in growth media. Cells were isolated from tissue culture flasks using pancreatin (Gibco #25720-020) and used in experiments up to cell passage 20. For evaluation of ER content, cytosolic and membrane preparations were used. These preparations were generated by plating 3×10^6 cells on 150 mm culture dishes in growth media for 24 h and the media was replaced with phenol red-free growth media containing 10% charcoal stripped fetal calf serum for an additional 24 h. The next day the cells were harvested as described below. This washout period is required to enhance the expression levels of cytosolic ER by reducing the steroid content of the media (data not shown).

Estrogen Receptor Preparations

A cell fractionation procedure was used to identify ER associated with plasma membranes from those localized in nuclear/cytosolic fractions as previously reported [Fitzpatrick et al., 1999; Deecher et al., 2003] with the following modifications. Cells were harvested in phosphate-buffered saline (PBS) and pelleted by centrifugation (1,500*g*, 5 min). The cell pellet was resuspended in 2 ml binding buffer (10 mM Tris HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4 at 37°C), homogenized (30 s, setting 3, PT1200 polytron, Kimematica; Lucerne, Switzerland) and centrifuged at 15,000*g* for 15 min to separate soluble protein. The supernatant was homogenized again and centrifuged at 100,000*g* for 1 h. The pellet (P2) containing membranes for evaluating membrane-associated ER and the supernatant (S2) for evaluation of cytosolic ERα

or ERβ were collected and used in radioligand binding assays. Preparations were evaluated for protein concentration using BCA assay (Pierce, Rockford, IL) with bovine serum albumin as the standard. The membrane and cytosol preparations used in experiments contained 60 ± 20 and 33 ± 9 μg protein/reaction, respectively.

RT-PCR

Cells were plated in growth media at 3×10^6 cells/150 mm culture dish and maintained for 24 h. On day 2, the media was replaced with phenol red free growth media and maintained overnight. On day 3, total RNA was extracted from HT22 cells using the RNAeasy Kit (Qiagen, Valencia, CA). Transcripts for ER were detected in RNA extracted from HT22 cells by PCR amplification using two different primer sets. Primers specific for ERα (forward: 5′-GCAGCTCAAGATGCCCAT-3′; reverse: 5′-GGCGGCGTTGAACTCGTA-3′) generated a product of approximately 108 bp while primers specific for ERβ (forward: 5′-CTGTAGCCAGTCCATCCTA-3′; reverse: GTTGGCCATTGCACATTT-3′) a product of approximately 194 bp. Samples were incubated in a thermocycler for 40 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 30 s) followed by a final extension at 72°C for 7 min.

Immunoblotting

Protein for Western blots was collected from HT22 cells using the same harvest procedure described in the radioligand binding assay protocol. Cytosolic preparations were diluted in a Laemmli sample buffer containing 0.71M β-mercaptoethanol and heated for 5 min at 95°C prior to loading on a 10% SDS–PAGE gel. Proteins were loaded on gels according to either protein levels or E2 binding activity determined from the radioligand binding assays. Fractionated proteins were then transferred to a PVDF membrane (Invitrogen, Grand Island, NY) for immunobloting according to the manufacturer's recommendations. Following transfer, membranes were blocked for 1 h at RT with blocking buffer (PBS, 5% milk and 0.03% Tween-20) and then incubated with primary antibody diluted in blocking buffer overnight at 4°C. The antibodies used in these analyses included a commercially available murine monoclonal antibody specific for epitopes in the hinge region of ERα (SRA1000, StressGen Corp., British Columbia, Canada) and a rabbit polyclonal antibody generated in-house that was specific for ERβ. Blots were washed the following morning in TPBS (PBS containing 0.3% Tween-20) and incubated at RT for 2 h with a 1/20,000 dilution of the appropriate secondary antibody conjugated with HRP (Bio-Rad Laboratories, Hercules, CA). Blots were washed sequentially in TPBS and PBS and then immunoreactive proteins were visualized with a chemiluminescent substrate (SuperSignal, Pierce). Molecular mass standards (Amersham, Buckinghamshire, UK) and purified recombinant human ERα or ERβ (rERα or rERβ) were included in each gel.

Fluorescence Immunocytochemistry (ICC)

Initial screening was done to determine which antibodies would be appropriate to visualize ER using ICC techniques. Evaluation of the SRA1000 antibody showed no specific ER labeling and therefore was not used in these studies (data not shown). Another antibody specific for ERα, MC20, a commercially available polyclonal antibody generated against an epitope mapping at the carboxy terminus of murine ERα (Santa Cruz Biotechnology, Santa Cruz, CA), showed specific labeling and was deemed appropriate for subcellular localization of ERα in HT22 cells. Cells were initially plated on cover slips, washed in PBS and fixed for 30 min at RT in 4% paraformaldehyde. Following fixation, cells were washed in PBS, incubated for 1 h in 50 mM NH_4Cl and then blocked in 10% BSA for a minimum of 3 h. Cells were subsequently incubated in primary antibody for 3 h at RT and then washed in PBS followed by incubation with a fluorescein isothiocyanate (FITC)-labeled secondary antibody for 1 h at room temperature. After incubation with the secondary antibody, cells were washed in PBS and the cells on cover slips were mounted on slides using VECTASHIELD® mounting medium with DAPI (Vector Laboratories, Burlingame, CA; nuclear marker). Fluorescent microscopy (Nikon PM2000) was used to visualize DAPI-stained nuclei (excitation max: 350 nm and emission max: 470 nm) and fluorescein isothiocyanate labeled ER (excitation max: 496 nm and emission max: 518 nm). Parallel staining was done on cells with or without primary antibody to define specific staining.

Radioligand Binding Assays

Experiments were performed using established ER radioligand binding assays [Shen et al., 1998; Fitzpatrick et al., 1999; Deecher et al., 2003] with some modifications. Assays evaluating the membrane preparations for

membrane-associated ER followed previous reported methods [Deecher et al., 2003] where separation of free radioligand was removed by ultracentrifugation. Binding reactions using cytosolic preparations for evaluation of ER were prepared in triplicate in 96-well V-bottom microtiter plates (Greiner, Orlando, FL; catalog #921222) and separation of free radioligand was done by charcoal precipitation. Briefly described, cytosolic preparation (50 μl) was added to each well followed by 25 μl binding buffer to establish maximum radioligand bound (total bound) or E2 (1 μM) to determine minimum radioligand bound (nonspecific bound, NSB). Reactions were initiated by the addition of 25 μl of 200 pM [^{125}I]16-α-iodo-E2 (NET-144; 2,200 Ci/mmol specific activity; Perkin Elmer, Boston, MA) in binding buffer for a final reaction volume of 100 μl. The reactions were incubated on an orbital shaker for 2 h (25°C). Ice-cold buffer (100 μl) containing 1% (w/v) Norit® A-activated carbon (Fisher Scientific, Fair Lawn, NJ) and 0.01% (w/v) dextran T500 (Pharmacia Biotech, Uppsala, Sweden) was added to each well to trap unbound [^{125}I]16-α-iodo-E2. The microtiter plates were centrifuged at 2,500*g* for 10 min and 150 μl of supernatant was removed from each well. The amount of radioactivity present in the supernatant was measured using a 10-channel gamma counter (ICN Micromedic Systems, Huntsville, AL).

Luciferase Reporter Assays

HT22 cells were plated in 96-well plates at a density of 10,000 cells/well in growth media with 10% fetal calf serum for 24 h to ensure adherence and appropriate growth rates. Cells were subsequently washed in phenol red free and serum-free media to allow for optimal uptake of virus. Fifty microliters of phenol red free serum-free media containing adenovirus with an ERE-tk-luc reporter was added directly to the cells. The cells were infected using a multiplicity of infection of 10 and incubated for 1 h at 37°C. The recombinant adenovirus is replication defective and contains two copies of the *Xenopus laevis* vitellogenin A2 ERE (5′-GGTCACAGTGACC-3′) linked to the thymidine kinase promoter (−110 to +10) and the luciferase gene (pGL3 basic-Promega) [Bodine et al., 1997]. Following infection, cells were washed in phenol red free media containing 2% stripped serum (reduced steroid content) and incubated at 37°C for at least 2 h. Cultures were then treated with either 0.5 μM E2, 1 μM of the ER antagonist ICI-182780 (ICI) or a combination of ICI-182780 and E2 and incubated at 37°C overnight. The next day, media was removed and the cells were lysed and processed for luciferase activity utilizing a Firefly luciferase assay kit (Promega).

Glutamate Toxicity Bioassay

On day 1 of assay, cells were plated in a 96-well flat bottom plate at 2,000 cells/well in a plating volume of 200 μl phenol red free growth medium. These cells require plating initially in growth media for appropriate adherence and to prevent cell differentiation. The plates containing the cells are incubated for 4 h (37°C) to allow for adherence and 5 μl of test compound (5, 50, 500, 5,000 nM) was added directly to each well containing cells and media. Extensive experimental testing was completed to determine appropriate pre-treatment time required for adequate neuroprotection by estrogens. Shorter pre-treatment times required increased concentration of estrogens to prevent cell toxicity. For experiments using ICI-182780 as an antagonist, the ICI (1 μM) was added first prior to the addition of E2. On day 2, 5 μl of glutamate (0.5–1.0 mM) in media was added to each well and the plates incubated 20–24 h at 35°C in a humidified chamber with 10% CO_2. Each batch of cells was initially tested to determine the appropriate glutamate concentration that would induce 40%–60% cell death by using the plating conditions described above. The typical glutamate concentration used for all studies ranged from 0.5 to 1.0 mM. For every compound tested, each concentration was tested in six wells of a 96-well plate alone and in combination with glutamate. On day 3, 100 μl of media is removed from each well and 15 μl formazan solution was added to each well [MTT assay kit #1465007 Roche Diagnostics, Basal, Switzerland]. The cells in the plate were incubated for an additional 4 h to allow for maximum uptake of the formazan. To terminate the formazan uptake, 100 μl solubilizing agent was added to each well and the plates incubated overnight in a cell incubator at 35°C (10% CO_2). On day 4, the plates were read at 595 nm on a spectrophotometer 96-well plate reader (Molecular Probes, Eugene, OR).

Statistical Analysis

Specific estrogen receptor protein labeling was determined by normalizing the counts per

minute (cpms) for the total reaction volume (200 μl) by subtracting the mean of NSB ligand from the total amount of bound ligand. The sum is then divided by the protein concentration in the reaction and the values are reported as cpm/fmoles bound/mg protein. Data generated from the ERE-tk-luciferase assays were analyzed using ANOVA followed by multiple comparisons test and then analyzed using least significant difference (pLSD). Data generated from the glutamate toxicity assay is collected from the Spectrometer 96-well plate reader. The data is downloaded to an Excel statistical application program and the estimated EC_{50} value with 95% confidence intervals determined. Determinations of EC_{50} values and 95% confidence limits are done using a logistic concentration response program written by Wyeth Biometrics Department (Princeton, NJ). The statistical program uses wells containing vehicle-treated as the maximal absorbance (λ) determinant and the wells containing glutamate challenge as the minimal absorbance (λ) determinant. Estimation of the EC_{50} value is completed on a log scale and the line is fit between the maximal and minimal absorbance values. Statistical significance ($P < 0.05$) is defined between EC_{50} values due to non-overlapping confidence intervals. The EC_{50} value is reported as the concentration of test compound that rescues 50% of the cell population under glutamate challenge. The glutamate challenge can be varied, but typically the concentration of glutamate used induces 40%–60% cell death. The percent toxicity of glutamate is reported for each experiment. The EC_{50} value reported for each test compound was determined based on percent glutamate toxicity for each individual experiment. A Dunnet's test was defining the vehicle as the control compared to all treatments. The standard reference compound used in all assays was 17-β-estradiol (E2).

RESULTS

Identification and Localization of Estrogen Receptors in HT22 Cells

RT-PCR and Western blot analyses were used to identity whether ER was expressed in HT22 cells. RT-PCR amplification of HT22 RNA with ER subtype specific primers, revealed the presence of ERα transcripts (Fig. 1, panel A). The expression of ERα was then confirmed at the protein level by immunoblotting using a monoclonal antibody (SRA1000) specific for the hinge region of ERα (Fig. 1, panel B). Another commercially available polyclonal antibody specific to the carboxyl terminus, MC20, identified a similar size band (data not shown).

Subcellular localization of ERα in HT22 cells was subsequently evaluated using ICC techniques. No specific cellular staining was observed with the monoclonal antibody (SRA1000) used in Western blots (data not shown). This antibody has been evaluated in other cell types that are known to express ERα and showed no specific ER labeling [Deecher et al., 2003]. Therefore, a commercially available polyclonal antibody generated against an epitope mapping at the carboxy terminus of murine ERα (MC-20, Santa Cruz Biotechnology) was used instead for the localization studies. This antibody has been used for ICC by other investigators to show localization of ERα in a variety of other cell types [Norfleet et al., 1999; Toran-Allerand et al., 2002]. Labeling with DAPI, a known nuclear marker, was shown as a control for specific labeling of the HT22 cell nucleus (Fig. 2, panel A).

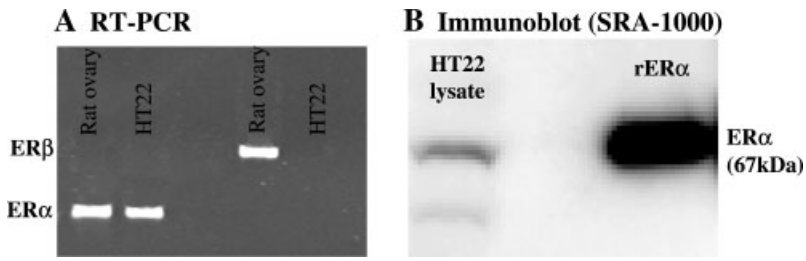


Fig. 1. Hippocampal cell line (HT22) cells express ERα but not ERβ using RT-PCR and Western blot analyses. ER transcripts were detected in RNA samples extracted from HT22 cells by RT-PCR using two different primer sets specific for either ERα or ERβ transcripts (**panel A**). RNA extracted from rat ovary was used as a control in these experiments and the approximate positions of ERα (108 bp) and ERβ (194 bp) transcripts are indicated. A monoclonal antibody specific for ERα, SRA1000, was used to confirm the expression of ERα in HT22 cell lysates (50 μg) (**panel B**). A recombinant form of human ERα (rERα) was used as a standard in these studies. These data are representative of two experiments done on extracts from cell passages 5 and 7.

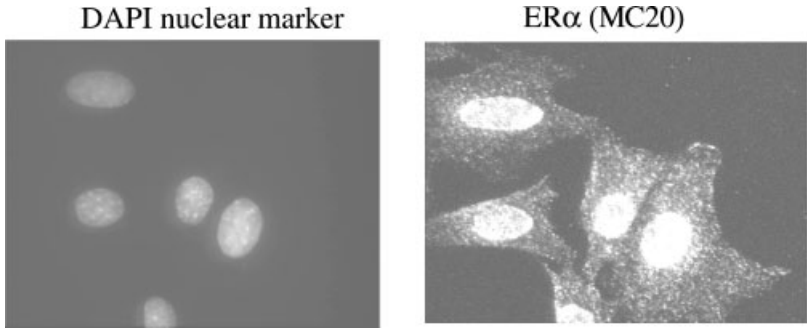


Fig. 2. Nuclear localization of estrogen receptorα (ERα) in a mouse HT22. Immunocytochemical analyses using an ERα specific antibody, MC20, show nuclear staining in permeabilized cells typical of ER labeling. Labeling with DAPI, a nuclear marker, was shown as a positive control for specific labeling of the nucleus. Localization studies were done on whole cells from passage 5 to 20. This experiment was from cell passage 7 and representative of three other experiments performed on cell passage 7 up to 14. The cellular staining was visualized for DAPI (excitation max: 350 nm and emission max: 470 nm) and fluorescein isothiocyanate (excitation max: 496 nm and emission max: 518 nm).

Specific staining was observed using the MC20 antibody in the cell nucleus of HT22 cells, indicating that ERα was localized primarily in the nucleus (Fig. 2, panel B). Additionally, some punctuate cytoplasmic staining was also observed. Cells stained without primary antibody showed no specific labeling (data not shown).

Radioligand Binding Assays

Radioligand binding assays were performed on membrane and cytosolic preparations from HT22 cells to quantify estrogen receptor levels. Specific labeling with [^{125}I]16-α-iodo-3,17-β-estradiol (200 pM) was noted only in binding reactions containing cytosolic preparations (Fig. 3). This radioligand was chosen for its high specific activity and ability to label ER in cells with low receptor expression. The estrogen receptor content ranged from 5 to 25 fmol/mg protein and the levels of ER were dependent on cell passage number. It should be noted that ER expression in the HT22 cells decreased significantly after cells are maintained beyond 20 cell passages (data not shown). Results of these assays revealed specific binding activity in cytosolic samples and acceptable protein expression for pharmacological characterization. No detectable specific labeling of membrane-associated ER was found in the binding reactions containing membrane preparations; hence no further evaluation was done.

Functional Activity of Estrogen Receptors in HT22 Cells Using Adenovirus ERE-tk-Luc Constructs

An adenovirus containing an ERE-tk-luc reporter was used to determine whether the ERs were functionally active in this cell line. Cells infected with the reporter virus and treated with 500 nM 17-β-estradiol (E2) showed a statistically significant increase in luciferase activity (Fig. 4). Cells incubated with 1 μM of the estrogen receptor antagonist, ICI-182780, alone showed a significant reduction in baseline luciferase activity. This ICI-182780 inhibition of baseline activity indicates endogenous ER activation due to residual levels of estrogens in stripped serum remaining in the culture medium [Tora et al., 1989]. Attempts to reduce

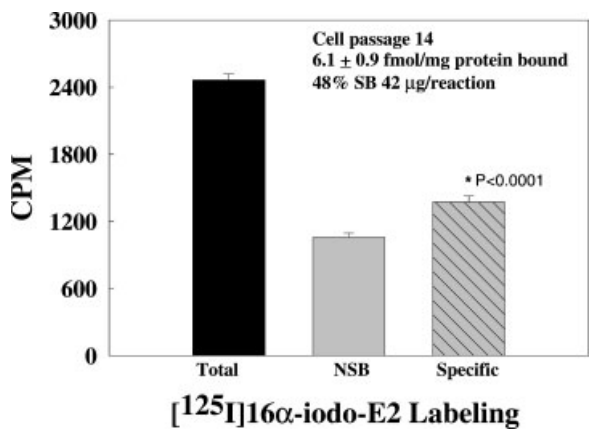


Fig. 3. Radioligand binding analyses of HT22 cytosolic fractions reveal specific radiolabeling. For the represented example, cytosolic preparations (42 μg protein/reaction) from HT22 cells were incubated with 200 pM of [^{125}I]16-α-iodo-E2 with E2 (1 μM) included as the nonspecific determinant (NSB). Values depicted in the graph are mean cpms ± SEM of triplicate determinations of cpms bound. The receptor content labeled in this experiment was 6.1 ± 0.9 fmol/mg protein. These experiments were repeated six times on HT22 cytosolic fractions taken from cell passages 5 to 20. The specific binding (SB) represents 48% of the specific labeling bound (*indicates $P < 0.0001$ compared to total bound).

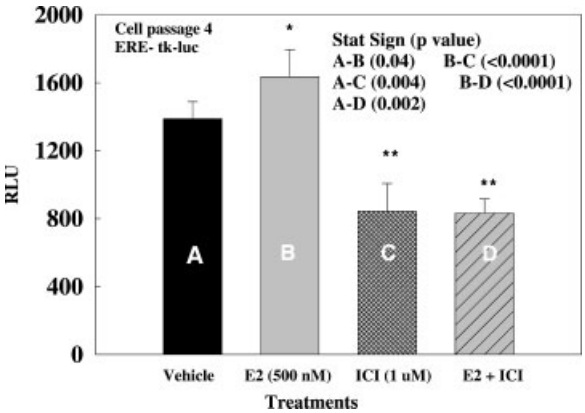
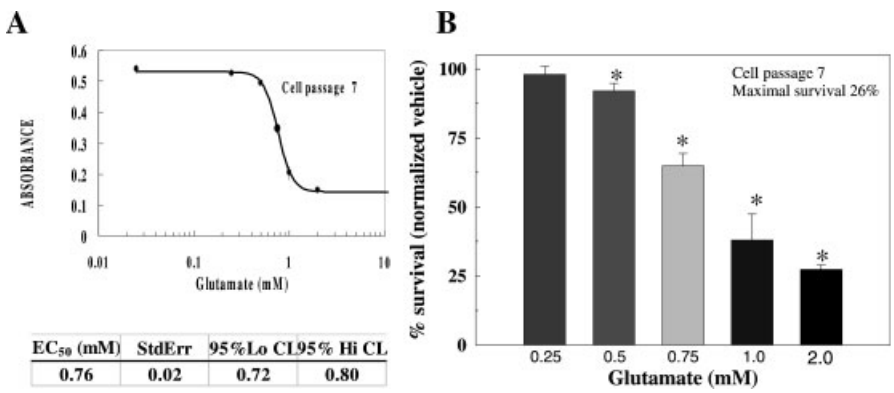


Fig. 4. Functional determination of estrogen receptor activation by E2 using an ERE-tk-luciferase assay. Activation of an ERE-tk-luc response was demonstrated with 500 nM E2. The estrogen specific antagonist, ICI-182780 (1 μM), blocked basal and E2-stimulated activation in the HT22 cells. Each treatment is the mean ± SEM of six wells. These experiments were performed 6 times using whole cells taken from cell passages 4 to 10. This representative experiment was done using cells from passage four. Statistical significance between groups was determined by Fischer's pLSD test.

endogenous basal activity by performing these assays in the absence of serum were unsuccessful due to neuronal cell differentiation. Therefore, the addition of ICI-182780 reflects the true basal level. Cells incubated with the combination of 500 nM E2 and ICI-182780 showed luciferase activity very similar to cells treated with ICI-182780 alone, suggesting that the ICI-182780 (1 μM) completely blocked the activation of ERE-tk-luc by 17-β-estradiol. These data demonstrate that functional ERs are present in this HT22 cell line. A lower concentration (50 nM) of E2 did not show statistically significant transactivation of luciferase reporter probably due to high basal activation and low signal to noise ratio (data not shown).

Glutamate-Induced Neurotoxicity and Neuroprotection by Estrogens

The toxic insult chosen for this measure of estrogen neuroprotection was a glutamate challenge following a 24-h pretreatment with each compound tested. The concentration of glutamate used for each experiment ranged between 0.5 and 1.0 mM with an expected range of 40–60% induced cell toxicity. An example of a concentration response curve by glutamate and level of cellular toxicity in the HT22 cells is depicted (Fig. 5). Glutamate induces a dose-dependent increase in cell toxicity with an EC_{50} value of 0.76 ± 0.26 mM. The maximal toxicity induced was 80–87% at the highest concentration tested (2 mM). All compounds tested in this assay are initially screened at 5 μM to determine if compound alone induces cellular toxicity (data not shown). The following compounds showed no statistical significant effect on cell viability at 5 μM, 17-β-estradiol (E2), estrone, Δ8,9-dehydroestrone (Δ8,9), or ICI-182780 (ICI). These compounds were further evaluated to determine if they could protect cells from glutamate-induced toxicity. Three of these



EC_{50} (mM)	StdErr	95%Lo CL	95% Hi CL
0.76	0.02	0.72	0.80

Fig. 5. Glutamate challenge is used to induce neurotoxicity in HT22. Optimization of the glutamate-induced toxicity was completed to define the effective concentration (EC_{50} value) that induces 40–60% cellular toxicity in the HT22 cells for the neuroprotection screening assays. A concentration range from 0.5 to 1.0 mM was used in all assays and cells were used from passage 4 to 20 in these assays. This representative example was completed on cells from passage seven. **Panel A** represents a typical signal to noise ratio for absorbance over a range of glutamate concentrations. **Panel B** depicts percentage of cell survival at each glutamate concentration tested. Each data point represents the mean and standard deviation of six test wells (*indicates $P < 0.05$ compared to vehicle treated wells using Dunnett's test).

compounds (E2, estrone, Δ8,9) dose-dependently reversed the effects of glutamate toxicity in the presence of 0.5 mM glutamate (Fig. 6, panel A) with varying protective effects against this neuronal insult with EC_{50} values denoted in the graph. The compound Δ8,9-dehydroestrone was the most effective of the estrogens evaluated with an EC_{50} value of 320 ± 53 nM and 91% protection from glutamate toxicity at the highest concentration tested. Compounds select for the ERβ receptor [Harris et al., 2003] were also tested in this bioassay and showed no neuroprotective properties at concentrations that are considered ERβ receptor selective (data not shown).

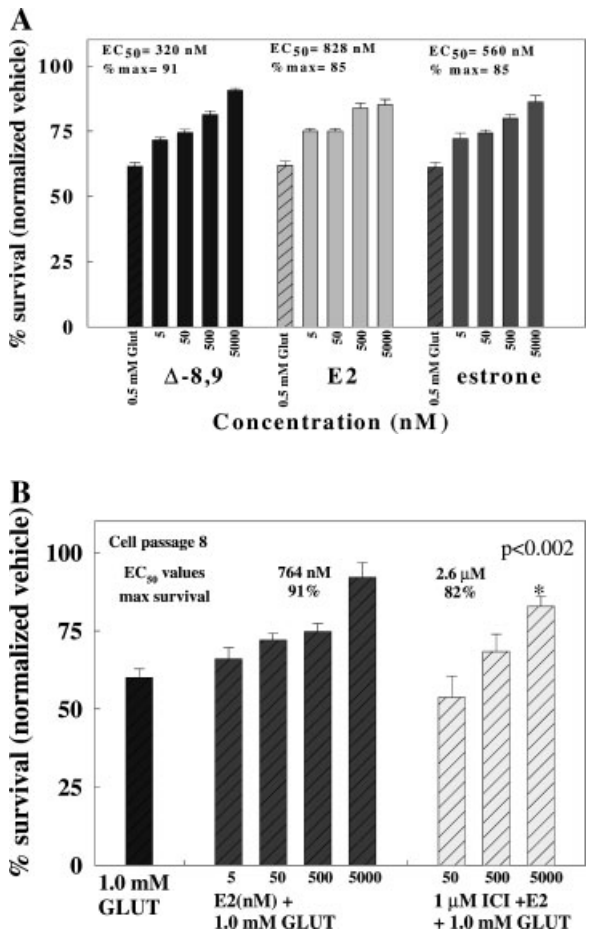


Fig. 6. Characterization of various steroids in a glutamate-induced neurotoxicity assay. Neuroprotection by Δ8,9-dehydroestrone, E2 and estrone in the HT22 cell line (**panel A**). The estrogen receptor antagonist, ICI-182780, blocks the neuroprotective effect of E2 (**panel B**) (*indicates $P < 0.002$ compared to 5 μM E2 with out 1 μM ICI). Data represented in these figures are from one experiment that has been replicated a minimum of four times using cells from cell passage 4 to 15. Comparison of 95% confidence limits was used to determine statistical significance. The percent maximum represents the percentage survival at the highest concentration tested.

To determine whether estrogens ability to maintain cell viability during toxic challenge was due in part to endogenously expressed ERs, experiments using the ER antagonist (ICI-182780) were evaluated. The ER antagonist was added 5 min prior to the addition of varying concentrations of E2. The cells were incubated overnight with the test compounds and glutamate (1.0 mM) was added to the test wells. The presence of ICI-182780 (1 μM) shifted the concentration response curve to the right with the EC_{50} values of 764 ± 78 nM and 2.6 ± 0.8 μM without or with ICI-182780, respectively (Fig. 6, panel B). Additionally, E2 at the highest concentration (5 μM) in combination with ICI-182780 showed a statistically significant diminished percent of maximal cell viability. The ER antagonist was tested alone at 5 μM to ensure no effect on cell survival (data not shown). These data demonstrate that estrogens have varying neuroprotective properties that are dependent on structural make-up. The fact that the ER antagonist, ICI-182780, can reverse the neuroprotective effects of estrogens further indicates that ERs play a role in neuroprotection in this cell line.

DISCUSSION

In the present study, we characterized ER expression in HT22 cells at the mRNA level using RT-PCR and at the protein level using immunoblotting, immunocytochemistry, and radioligand binding assays. Contrary to previous reports, ER was expressed by this cell line and receptor functionality was confirmed using an ERE-tk-luciferase reporter assay. A screening assay used to profile the neuroprotective properties of various estrogens to glutamate toxicity revealed that one of these ligands, Δ8,9-dehydroestrone, had improved protective activity compared with the others tested. In addition, this estrogen-induced neuroprotection was reversed by a known ER antagonist that blocks receptor activation. These data support the hypothesis that ERs play a role in the neuroprotective properties reported by estrogens. Our data is in direct contrast to other investigators who have reported a lack of ER in this cell line.

The initial study that proposed the use of the HT22 cell line as a model to study nongenomic

estrogen neuroprotection was based on the finding that this cell line lacked functional estrogen receptors [Behl et al., 1995]. Subsequent studies utilizing this cell line [Behl et al., 1997; Green et al., 1998; Moosmann and Behl, 1999; Gursoy et al., 2002] have cited this initial paper to support the nongenomic neuroprotective mechanisms noted for estrogens in this cell line. In this report, HT22 cells were transfected with a reporter plasmid containing estrogen responsive element (ERE) and no E2 activation of the ERE was noted [Behl et al., 1995]. Several reasons could be responsible for the lack of demonstration of functional ER in HT22 cells using this procedure. The culturing conditions for the cells at the time of transfection are critical for optimal ER expression. Based on our observations using the HT22 cells, factors that effect ER expression levels are passage number, cell confluency, and culture maintenance media. Additionally, transient transfections of reporter genes linked to multiple copies of ERE upstream of various promoters and ER expression plasmids has been extensively used to validate the presence of functional ER in cells. The plasmids are generally delivered through various transfection methods using calcium phosphate precipitation, DEAE dextran, lipophilic agents, or electroporation. The transfection efficiency varies widely among different conditions and is also cell type-dependent. In contrast, reporter gene delivery through replication defective adenovirus is very efficient and can be carried out using a variety of cell types and the reporter gene delivery can be optimized to approximately 80%–90% infection efficiency. The earlier report used a method of electroporation of cells with an ERE-MTV-luciferase reporter [Behl et al., 1995]. Subsequent treatment of these transfected cells with estradiol did not increase luciferase reporter activity and the result was interpreted as the lack of functional ER in these cells. In our study, replication defective adenovirus recombinants were used as a tool to deliver the ERE-tk-luciferase reporter gene into HT22 cells. Infection of HT22 cells with the reporter virus and subsequent treatment with E2 resulted in an increased reporter activity and the response depicted is completely blocked by treatment with ICI-182780, an ER antagonist. This finding suggests that the low levels of ER identified in the HT22 cells by our radioligand binding studies are functional. This ERE-tk-luciferase virus has also been successfully used to identify low levels of functional ER in human osteoblast cell lines [Bodine et al., 1997]. A report that supports our findings that genomic pathways are involved in neuroprotection in this cell line is a study using transfected ER into HT22 cells [Fitzpatrick et al., 2002]. The findings of this report demonstrate that the presence of ERs influence rapid signaling events that mediate neuroprotection by estrogens. Although these investigators needed to transfect in exogenous ER to demonstrate responsiveness, this study demonstrates that the machinery for genomic estrogen neuroprotection resides within the HT22 cells. Speculation as to why endogenous levels of ER were not sufficient to illicit a response in this report, could be that the cell culture conditions were not conducive for optimal ER expression and low levels of ER were not enough to maintain neuroprotection during toxic challenge. In the present study, we have identified the cell culture requirements necessary to maintain optimal ER expression at detectable functional levels. Loss of detectable ER in cell lines that are continuously maintained in culture is not unexpected [Shen et al., 1998; Deecher et al., 2003].

Having established expression of ER in HT22 cells, a glutamate toxicity bioassay was subsequently developed to profile the neuroprotective properties of various estrogens and to determine whether a known ER antagonist could block this protective effect. The data from these studies are consistent with the hypothesis that neuroprotection is mediated via ER in this cell line. The definitive piece of evidence that the neuroprotective action of estrogen has an ER-dependent component was from experiments demonstrating that the known ER antagonist, ICI-182780, blocked the protective capacity of various estrogens' neuroprotective activity following glutamate challenge. This genomic contribution to estrogens neuroprotection is not unexpected since other investigators reported that when HT22 cells were stably transfected with ER, neuroprotection could be achieved with lower doses of estrogens [Mize et al., 2003]. These results are also consistent with a recent study demonstrating that equine estrogens have differing neuroprotective potencies in HT22 cells [Bhavnani et al., 2003]. Additionally, other neuronal cell lines have been identified as containing endogenous low levels of ER [Shen et al., 1998] and have been used to evaluate ER

receptor-dependent mechanisms in neuroprotective paradigms [Yang et al., 2003].

The ability of estrogens to protect neural tissue from various central nervous system insults has been reported [Zhang et al., 1998; Azcoitia et al., 1999; Dubal et al., 1999, 2001]. Multiple approaches have been taken to identify the mechanisms by which estrogens protect from neuronal insults (for review [Wise, 2002]). Suggestions of genomic and non-genomic means have been proposed, all of which may be involved. The HT22 cell line has been used to study nongenomic action of estrogens neuroprotection. The findings in this study clearly identify endogenous ER, lack of membrane containing ER and reversal of estrogen neuroprotection by an ER antagonist in the HT22 cell line. We speculate that this ER subtype is ERα or ERα-related based on the data from the RT-PCR and immunblot experiments. Although the diminished responsiveness to E2 in the functional ERE-tk-luc assay and the neuroprotection assay may suggest an ERα-related subtype. This would not be an unexpected finding as others have suggested neuronal ER subtypes (for review [Toran-Allerand, 2004]). In addition, culture conditions can have significant influences on the neuroprotective capacity of estrogens in this cell line given their ability to modulate ER expression. Therefore, genomic mechanisms should not be ruled out when using this cell line as a model of estrogen neuroprotection.

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