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17α-Estradiol Exerts Neuroprotective Effects on SK-N-SH Cells

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Estradiol (E2) has been shown to exert organizational, neurotrophic, and neuroprotective effects in the CNS. The present study assessed the specificity of the neuroprotective effects of estradiol for the potent 17β-isomer. SK-N-SH cells from a human neuroblastoma cell line, which we have shown to be estrogen-responsive, were cultured at low or high plating density. Then cells were exposed to 17β-E2 (0.2 or 2 nM), 17α-E2 (0.2 or 2 nM), or cholesterol, testosterone, dihydrotestosterone, progesterone, or corticosterone (all at 2 nM). Cultures were insulted by serum deprivation, which caused a profound loss of cells. At 1 or 2 d of serum deprivation and steroid hormone replacement, the protection afforded cells by the steroid addition was assessed. Serum deprivation killed ~90% of cells cultured at both low and high plating density. Both 17α- and 17β-E2 provided protection of SK-N-SH cells at either plating density. Further, a 10-fold molar excess of tamoxifen antagonized only approximately one-third of the neuroprotective effects of either isomer of estradiol, and a 100-fold excess of tamoxifen had no additional effect on the neuroprotection by 17β-E2. By contrast, none of the other steroids tested protected cells from the insult of serum deprivation. These results indicate that the neuroprotective effects of estrogens are not attributable to the general steroid structure, and the majority of the neuroprotection may not be mediated via a tamoxifen-antagonized receptor mechanism.

Key words: estrogens; 17β-estradiol; 17α-estradiol; neuroprotection; SK-N-SH neuroblastoma; cell plating density; serum deprivation; tamoxifen

Estrogens have been shown to be important for the differentiation of certain nuclei of the brain (Gorski et al., 1980), and recent evidence suggests that estrogens may be important for normal brain function throughout life (Simpkins et al., 1994). In adult rats, estradiol (E2) has been shown to enhance sprouting of commissural association fibers in the hippocampal dentate gyrus after entorhinal cortex lesions (Morse et al., 1986). Estrogen environment influences the synaptology of the hippocampus, because changes in synaptic density in the CA1 region are associated with endogenous (Wooley and McEwen, 1992) and exogenous (Wooley et al., 1990) levels of 17β-estradiol (17β-E2). Recently, we have shown that 17β-E2 induced the expression of the neurotrophic factors, nerve growth factor, and brain-derived neurotrophic factor (Singh et al., 1993, 1995). We and others have observed an increase in the high affinity uptake of choline (O'Malley et al., 1987; Singh et al., 1994), in the levels of choline acetyltransferase (Luine et al., 1975, 1980; Singh et al., 1994), and in the performance of memory-related behavioral tasks (Singh et al., 1994) after estrogen treatment of ovariectomized rats. Collectively, these results indicate that estrogens are important in the maintenance of normal neuronal function related to cognition, an observation consistent with the studies of Sherwin et al. (Sherwin, 1988; Phillips and Sherwin, 1992) showing steroid modulation of memory and cognition in women subjected to surgical menopause.

Estrogens seem to exert neurotrophic and neuroprotective effects on a variety of cell types. Toran-Allerand (1976) first reported growth stimulation by estrogens of explants of the hypothalamus and preoptic area of the basal diencephalon. More recently, we have observed that estrogens protect transformed neurons and glia from the cytotoxic effects of a variety of insults, including serum deprivation (Bishop and Simpkins, 1994) and hypoglycemia (Bishop et al., 1994). 17β-E2 was particularly effective in protecting SK-N-SH neuroblastoma cells from the neurotoxic effects of serum deprivation (Bishop and Simpkins, 1994). This cell line is human in origin, expresses a neuronal phenotype, is estrogen-responsive (Ratka et al., 1991), and expresses estrogen receptors mRNA (Ratka et al., 1995) and the protein and message for nerve growth factor (Azar et al., 1991).

The studies cited above have used the 17β-isomer of E2, which is known to interact potently with the estrogen receptor (Korenman, 1969; Lubahn et al., 1985) and the estrogen receptor (ER)–17β-E2 complex binds with a longer duration to the estrogen-responsive element (Clark et al., 1982). By contrast 17α-E2 binds weakly to the estrogen receptor; the 17α-E2–ER complex only transiently binds to the estrogen-responsive element (Korenman, 1969; Clark et al., 1982; Lubahn et al., 1985). When administered acutely, α-E2 has only weak (Korenman, 1969) or no (Huggins et al., 1954; Kneifel et al., 1982) activity in peripheral estrogen-responsive tissues but seems to exert uterotropic effects with administration chronically at high doses (Clark et al., 1982; Clark and Markaverich, 1983).

The present study was undertaken to compare the relative activities of the β- and α-isomers of E2 in a test of the neuroprotective effect of estrogens. Surprisingly, both isomers of E2 were equally effective in protecting SK-N-SH cells from the cytotoxic effects of serum deprivation.

MATERIALS AND METHODS

Cell cultures. SK-N-SH cells were obtained from American Type Tissue Collection (Rockville, MD). Cell cultures were grown to confluency in

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Table 1. Effects of serum deprivation and 17α-estradiol on SK-N-SH cell number after plating at high and low density

Treatment	Plating density	Culture duration (hr) 24 Cells/well ($\times 10^3$)	48
FBS	1000	405 ± 21	452 ± 51
SF	1000	140 ± 11*	87 ± 7*
FBS	1000	480 ± 50	530 ± 100
SF	1000	230 ± 20*	130 ± 20*
17α-E2 (2 nM)	1000	480 ± 50	300 ± 60
FBS	250	235 ± 12	232 ± 6
SF	250	42 ± 2*	26 ± 2*

*$p < 0.05$ versus FBS group.

RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin (all reagents from Sigma, St. Louis, MO) in monolayers in plastic Corning 150 cm^2 flasks (Sigma) at 37°C and under 5% CO_2/95% air. Medium was changed three times weekly. Cells were observed with a phase-contrast microscope (Nikon Diaphot-300).

SK-N-SH cells were back-cultured every 5–7 d to maintain the cell line, and cells used in the following experiments were located in passes 9–12. The growth media were initially decanted, and the cells were rinsed with 6 ml of 0.02% EDTA, which was discarded. Then another 6 ml of 0.02% EDTA was added. After a 30 min incubation at 37°C, the cells were counted on a Neubauer hemacytometer (Fisher Scientific, Orlando, FL) and resuspended in appropriate media. Cells were plated at a density of 1.0×10^6 cells/ml.

Experimental media. Experiments were initiated by the back-culturing of SK-N-SH cells. Cells were suspended and centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in the appropriate treatment medium at a concentration of 1.0×10^6 cells/ml. Cells were plated either at 0.25 ml/well (low density plating) or 1 ml/well (high density plating) in 24 well Falcon plates (Fisher Scientific). In all studies, cells were cultured in RPMI-1640 media (serum-free, SF group), RPMI-1640 media supplemented with 10% FBS (FBS group), or RPMI-1640 media supplemented with one of the following steroids at the dose(s) indicated: 17β-estradiol (17β-E2, 0.2 or 2 nM; Pharmos, Alachua, FL); 17α-estradiol (17α-E2, 0.2 or 2 nM; Sigma); cholesterol (CHOL, 2 nM; Steraloids, Wilton, NH); testosterone (TEST, 2 nM; Steraloids); dihydrotestosterone (DHT, 2 nM; Steraloids); or corticosterone (CORT, 2 nM; Steraloids). In one study, a 10-fold molar excess of tamoxifen (20 nM; Steraloids) was administered at the same time as 17β-E_2 (2 nM) or 17α-E_2 (2 nM). In another study, tamoxifen (0 to 200 nM) was administered in the presence or absence of 17β-E2 (2 nM). All steroids were dissolved initially at 1 mg/ml in absolute ethanol and diluted in RPMI-1640 to a final concentration of 2 nM. To control for possible ethanol effects in the steroid-treated wells, we supplemented both the serum-free media (control group) and FBS media (FBS group) with absolute ethanol at a concentration of 0.0001% (v/v).

Quantitation of cell viability. Cell viability was assessed at 24 and/or 48 hr of treatment using the trypan blue dye exclusion method (Black and Berenbaum, 1964; Tennant, 1964). At the appropriate time, cell suspensions were made by decanting the media, rinsing each well with 0.2 ml of 0.02% EDTA, and then incubating cells with 0.2 ml of 0.02% EDTA at 37°C for 30 min. Cells were suspended by repeated pipetting of the EDTA over the cells. Aliquots (100 μl) from each cell suspension were incubated with 100 μl of 0.4% trypan blue stain (Sigma) for 5 min at room temperature. All suspensions were counted on a Neubauer hemacytometer (Fisher Scientific) within 15 min of the addition of trypan blue. Two independent counts of live cells were made for each aliquot.

Statistical analysis. The significance of differences among groups was determined by one-way ANOVA. Planned comparisons between groups used was done by Scheffe's F-test. For all tests, $p < 0.05$ was considered significant.

RESULTS

Serum deprivation had a marked effect on the viability of SK-N-SH cells at both high and low plating density (Table 1). At high plating density (1×10^6 cells/well) and in the presence of FBS, 52–60% of cells died in the first 24 hr, and then live cell number increased slightly for the next 24 hr. By contrast, serum deprivation at high plating density resulted in a loss of 77–86% plated cells by 24 hr and 83–91% of cells by 48 hr. At low plating density (0.25×10^6 cells/well), the presence of FBS maintained live cell number constant through 48 hr of the studies. Serum deprivation at low plating density reduced live cells by 82% at 24 hr and by 91% at 48 hr. The effects of FBS and serum deprivation on SK-N-SH cells at a high plating density are similar to our previously reported observation using flasks, as opposed to wells, for culturing of this line of neuroblastoma cells (Bishop and Simpkins, 1994).

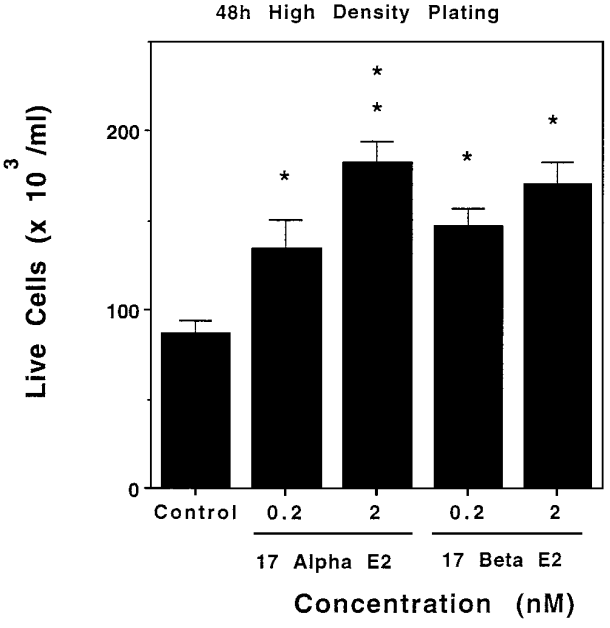

Figure 1. Effects of α- and β-estradiol on live SK-N-SH cell number after plating of cells at high density. Cells were plated at 1×10^6 cells per well, and cell number was determined 48 hr later. All wells were deprived of serum for the entire incubation period. Wells were treated either with no steroid (*Control*) or with α-estradiol or β-estradiol at the concentrations indicated. *$p < 0.05$ versus control; **$p < 0.05$ versus control and α-E2 groups at 2 nM. There were no differences between α- and β-estradiol groups at the same doses of the steroid. $n = 4$ wells per group.

We have observed previously a potent cytoprotective effect of 17β-E2 on SK-N-SH cultured at high density in SF media (Bishop and Simpkins, 1994). As a control, we tested the presumed weak or inactive optical isomer, 17α-E2, in an initial study (Table 1). Surprisingly, as we had reported previously for 17β-E2, 17α-E2 partially prevented the death of SK-N-SH cells in response to serum deprivation. When compared with the SF group, live cell numbers were 2.1- and 2.3-fold higher in culture grown for 24 and 48 hr, respectively, in the presence of 2 nM 17α-E2, indicating a neuroprotective effect of the α-isomer.

In a subsequent study, we compared the effectiveness of 17α-E2 effect with the naturally occurring 17β-E2 (Fig. 1). With initial plating at high density (1×10^6 cells/well), both the α- and β-isomers caused a dose-dependent increase in live cell number at 48 hr in culture (Fig. 1). For both isomers, SK-N-SH cells were protected by 65% at the 0.2 nM dose and by 88% at the 2 nM dose versus the nonsteroid-treated cells.

The time course of the effect of 17α-E2 and 17β-E2 was evaluated in SK-N-SH cultures plated at a low density (Fig. 2). At 24 hr of treatment (Fig. 2*A*), the low dose of both E2 isomers was

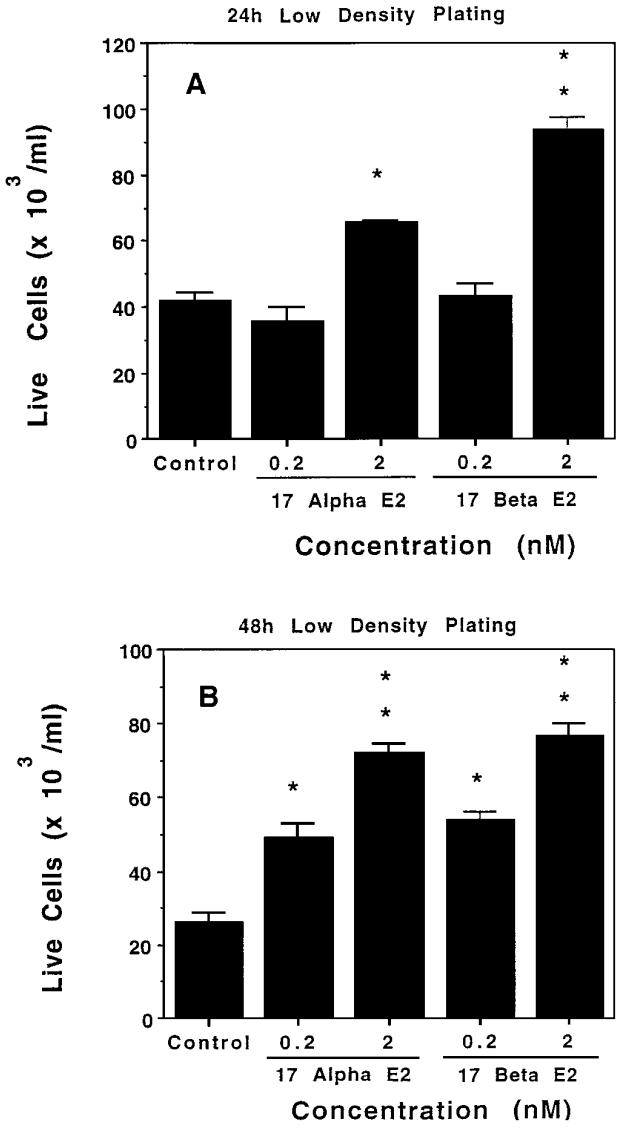

Figure 2. Effects of α- and β-estradiol on live SK-N-SH cell number after plating of cells at low density. Cells were plated at 0.25×10^6 cells per well, and cell number was determined at 24 (*A*) or 48 hr (*B*) later. All wells were deprived of serum for the entire incubation period. Wells were treated either with no steroid (*Control*) or with α-estradiol or β-estradiol at the concentrations indicated. *A*, $^*p < 0.05$ versus control; $^{**}p < 0.05$ versus control and both isomers at the 0.2 nM concentration. *B*, $^*p < 0.05$ versus control and α-E2 groups at 2 nM. $^{**}p < 0.05$ versus all other groups. $n = 6$ wells per group for both studies.

ineffective in preventing the serum deprivation-induced cell loss. However, the 2 nM concentration produced a 54% protection for the α-isomer and a 116% protection for the β-isomers. At 48 hr of culture, both α- and β-isomers caused a 86–106% and 172–189% protection of cells at the 0.2 and the 2 nM concentrations, respectively (Fig. 2*B*).

Tamoxifen alone had no effect on live cell number in SF cultures (Fig. 3), even when administered at concentrations ranging from 2 to 200 nM (data not shown). Coadministration of a 10-fold molar excess of tamoxifen with 17β-E_2 or 17α-E_2 reduced the neuroprotective effect of the estrogen by 39 and 32%, respectively (Fig. 3). The tamoxifen effect on 17β-E2 neuroprotection was not evident at 2 nM, was 32% at 20 nM, and was not enhanced further at 200 nM (Fig. 4).

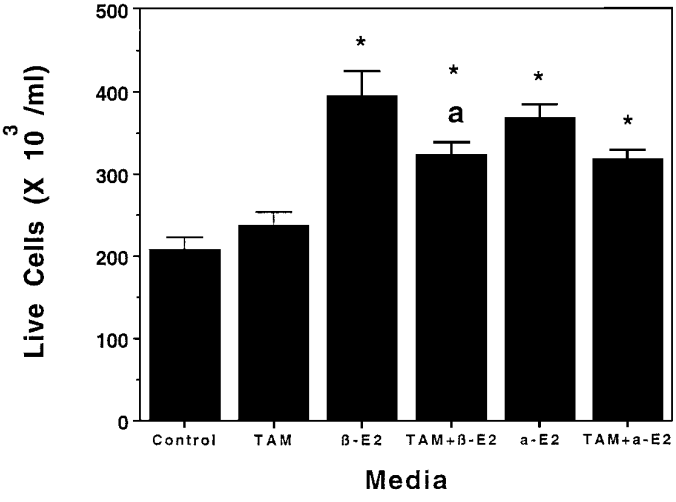

Figure 3. Effects of treatment with tamoxifen (*TAM*, 20 nM), 17β-estradiol (β-E_2, 2 nM), 17α-estradiol (*a-E2*, 2 nM), or their combination on live cell number. Cells were plated at 1×10^6 cell per well, and cell number was determined 48 hr later. Cells were deprived of serum during the entire incubation period. $^*p < 0.05$ versus *Control* (serum-free) and *TAM* groups. $a = p < 0.05$ versus the 17β-E_2 group. $n = 5$–6 wells per group.

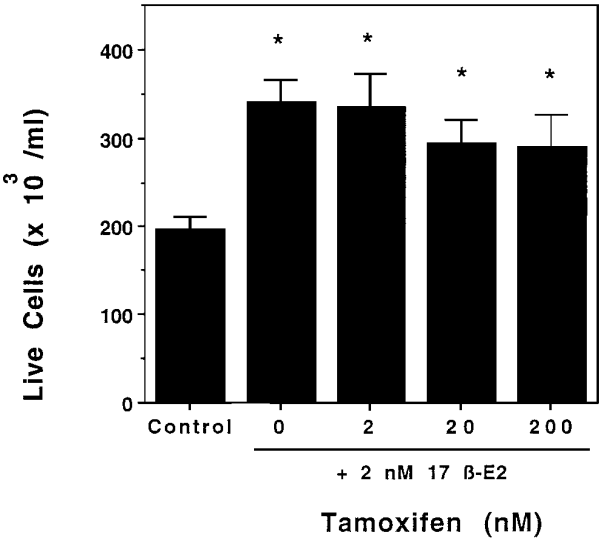

Figure 4. Effects of treatment with 17β-estradiol (2 nM) in the presence of tamoxifen (0–200 nM) on live SK-N-SH cell number. Cells were plated initially at 1×10^6 cells/ml, and cell number was determined 48 hr later. Cells were serum-deprived during the entire incubation period. $^*p < 0.05$ versus control (serum-free) cultures. $n = 4$–5 wells per group.

To assess the specificity of this estrogen effect on SK-N-SH cells, we tested in both high and low density cultures a variety of steroids, including cholesterol, progesterone, testosterone, dihydrotestosterone, and corticosterone. At 48 hr of culture, the time of the peak effect of both 17α- and 17β-E2, none of the substances tested protected cells from the cytotoxic effects of serum deprivation (Table 2).

DISCUSSION

The present study demonstrates for the first time that 17α-E2, a weak estrogen receptor agonist (Huggins et al., 1954; Korenman, 1969; Clark et al., 1982; Kneifel et al., 1982; Clark and Markaverich, 1983; Lubahn et al., 1985), is as effective as 17β-E2 in

Table 2. Effects of various steroids and plating density on the live SK-N-SH cell number at 48 hr

	Low plating density	High plating density
Treatment	Live cell number ($\times 10^3$)	Live cell number ($\times 10^3$)
Serum-free control	22.4 ± 1.4	94.4 ± 7.3
Cholesterol	18.3 ± 1.1	64.8 ± 3.9
Progesterone	21.3 ± 0.7	57.6 ± 6.2*
Testosterone	20.0 ± 4.2	87.2 ± 6.3
Dihydrotestosterone	20.7 ± 2.6	90.0 ± 7.7
Corticosterone	19.7 ± 1.3	68.8 ± 6.9

All cultures were deprived of serum and exposed to the respective steroid (2 nM) during the entire 48 hr incubation period. *$p < 0.05$ versus control group; $n = 5–8$ wells per group.

protecting SK-N-SH cells from the cytotoxic effects of serum deprivation. These results suggest that the estrogenic effect on neuroblastoma cell survival is mediated via a mechanism that does not require binding to the cytosolic estrogen receptor. This conclusion is consistent with the observation that the simultaneous addition of a 10-fold molar excess of the estrogen receptor antagonist, tamoxifen, reduced the neuroprotective effects of both estrogens by only one-third, and a 100-fold molar excess of tamoxifen had no additional effect on the 17β-E2 response. This effect is not attributable to a general steroid structure, because cholesterol, a progestin, androgens, and a glucocorticoid were ineffective in protecting SK-N-SH cells from the effects of serum deprivation. In fact, concentrations of progesterone and corticosterone as high as 200 nM were ineffective in protecting SK-N-SH cells from the cytotoxic effects of serum deprivation (J. W. Simpkins and P. S. Green, unpublished observations).

Serum deprivation is a well described insult to most neuronal cells in culture (Bishop and Simpkins, 1994). In the present study, we observed that at either low or high plating density, 90% of SK-N-SH cells failed to survive for 2 d in the absence of FBS. In the presence of FBS, plating conditions influenced the initial survival of cells, with higher plating density resulting in a lower percentage of cell survival. Presumably, with high plating density, competition for plating surface area resulted in lower cell survival. The exact mechanism by which serum deprivation kills neuronal cells in culture is not known. It is unlikely that the estrogen content of FBS is a factor. The fetal serum used has an E2 concentration of 0.06 nM (J. W. Simpkins, unpublished data) and is reported to contain estrone (0.1 nM) and estriol (0.04 nM) (Tissue Culture Technical Service, Sigma). With its dilution to 10% with RPMI medium, which contains no estrogens, the total estrogen content in the growth medium is 0.02 nM, which is too low to exert an estrogen effect on these cells *in vitro* (Simpkins, unpublished observations).

At both low and high density plating, both 17β-E2 and 17α-E2 protected SK-N-SH cells. The comparatively lower protective effects of the two isomers in the high density cultures is likely attributable to either (1) the fourfold lower steroid-to-cell ratio in the high density cultures or (2) the plating of cultures at near confluence in the high plating density cultures, a condition that results in the loss of cells because of competition for surface area of the plates. Nonetheless, the data indicate that at both high and low plating density, both estradiol isomers are neuroprotective.

We observed that both 17α- and 17β-E2 exerted effects on SK-N-SH cells at physiologically relevant concentrations. These data indicate that E2 concentrations observed in rodents and in women can serve a neuroprotective effect. In rats, neurotrophic (Toran-Allerand, 1976, 1980; Faivre-Bauman et al., 1981; Nishizuka and Arai, 1981; Toran-Allerand et al., 1983; Morse et al., 1986; Wooley et al., 1990; Wooley and McEwen, 1992) and neuroprotective (Bishop and Simpkins, 1994; Bishop et al., 1994; Singh et al., 1994; Behl et al., 1995; Goodman et al., 1996) effects of 17β-E2 have been described. However, to our knowledge, 17α-E2 has not been demonstrated previously to exert either neurotrophic or neuroprotective effects.

Because chronic exposure to high doses of 17α-E_2 has been reported to exert uterotropic effects in ovariectomized rats attributable to the chronic occupancy of the estrogen receptor with this weak agonist (Clark and Markaverich, 1993), we assessed the antagonism by tamoxifen of the neuroprotective effects of both 17β- and 17α-E_2. The absence of an effective antagonism by tamoxifen of this response to either isomer indicates that a cytosolic estrogen receptor mechanism is not the primary mechanism of the observed neuroprotection.

We considered the possibility that the observed effects of 17α-E2 may have been subsequent to its conversion by 17α-oxidoreductase to estrone and subsequent reduction of the 17-ketone to 17β-E2 (Breuer and Schott, 1966; Williams and Layne, 1967). However, this is an extremely unlikely possibility in that 17α-oxidoreductase activity is low in human tissue (Breuer and Schott, 1966; Williams and Layne, 1967), relative to that seen in other species (Mulay et al., 1968; Ivie et al., 1986). The conversion of 17α-E2 to estrone is reported to be <6%, both *in vivo* and in liver homogenates *in vitro* (Breuer and Schott, 1966; Williams and Layne, 1967). In our *in vitro* system, such a low conversion of 17α-E2 to estrone would express itself as a markedly lower potency for the α-isomer. Our observation of equal effectiveness of the two isomers indicates that metabolic activation of 17α-E2 is not involved in its cytoprotective effects.

Nongenomic actions of 17β-E2 are now well described. Direct effects of estrogens on neuronal membranes have been shown to involve specific membrane receptors (Pietras and Szego, 1979); 17β-E2 has been shown to increase hippocampal slice CA1 field potentials (Teyler et al., 1980) and to potentiate excitatory postsynaptic potentials of CA1 neurons within 2 min of its addition to slices (Wong and Moss, 1991, 1992), a time too short to involve a transcriptional mechanism. Local application of 17β-E2 also has been shown to alter Ca^{2+} fluxes in granulosa cells (Morley et al., 1992)and endometrial cells (Nemere and Norman, 1992) and to increase Ca^{2+} currents in GH_3 anterior pituitary cells (Richie, 1993). As such, we presume that both 17β- and 17α-E2 can exert their neuroprotective effects via a mechanism that does not require an interaction of the steroid with the estrogen receptor and the subsequent activation of genes. Indeed, recent evidence for the antioxidant activity of estradiol in a cell line that lacks the estrogen receptor (Behl et al., 1995) supports this contention.

The potential relevance of the observed neuroprotective effects of estrogen is demonstrated by the retrospective observation of a 40% reduction in the incidence of Alzheimer's disease in women who had used estrogens postmenopausally (Paganini-Hill and Henderson, 1994) and three reports of improvements in symptoms of Alzheimer's disease in some women with estrogen replacement therapy (Fillit et al., 1986; Honjo et al., 1989; Ohkura et al., 1994). The observed neuroprotective effects of the 17α-E2 isomer suggest that this compound may be particularly useful in achieving a selective neuroprotective action of estrogens without overstimulation of peripheral estrogen-responsive tissues.

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