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The synthetic estrogen 4-estren-3α,17β-diol (estren) induces estrogen-like neuroprotection

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Estrogen has demonstrated neuroprotective properties, which may underlie the observed preventive effect of estrogen-based hormone therapy (HT) against the development of neurodegenerative disorders such as Alzheimer's disease. Deleterious side effects of HT have increased efforts to develop safer compounds that selectively reproduce beneficial estrogen actions. Recently, 4-estren-3α,17β-diol (estren) was identified as having estrogen agonist properties in bone, without significantly stimulating growth of reproductive tissues. Here, we examined whether estren parallels the neuroprotective actions of estrogen against β-amyloid (Aβ) in cultured cerebrocortical neurons. Estren increased neuronal viability to a similar extent to that observed with 17β-estradiol (E_2) and 17α-estradiol. As we previously reported for E_2, estren rapidly increased PKC activity, and PKC inhibition prevented estren neuroprotection. In contrast, the estrogen receptor antagonist ICI 182,780 blocked E_2, but not estren neuroprotection. Our results indicate that estren-induced activation of rapid cell signaling pathways protects cultured neurons from Aβ toxicity.

Keywords: 4-Estren-3α, 17β-Diol; Estren; Estrogen; 17β-Estradiol; 17α-Estradiol; β-Amyloid; Neuroprotection; Protein kinase C; Alzheimer's disease

Introduction

The loss of estrogen that occurs with menopause places postmenopausal women at increased risk for certain age-related conditions, including osteoporosis (Riggs et al., 2002) and Alzheimer's disease (AD) (Launer et al., 1999). Restoring estrogen levels with hormone therapy (HT) at the time of menopause, but perhaps not later in life (Espeland et al., 2004; Shumaker et al., 2004), appears to reduce the risks of developing these conditions, supporting the position that estrogen has beneficial actions in specific tissues, including bone and brain (LeBlanc et al., 2001; Riggs et al., 2002). However, HT is also associated with detrimental effects, raising concern about its overall safety (Rossouw et al., 2002). Resolving this conundrum by selective activation of estrogen effects imparting health benefits in the absence of deleterious consequences would have important implications for treatment of several age-related disorders. One strategy to address this issue has been to develop synthetic compounds with selective agonist properties of estrogen. A promising recent advance was the identification of 4-estren-3α,17β-diol (estren), a compound that structurally differs from 17β-estradiol (E_2) only in its A ring, which contains one rather than three double bonds (Kousteni et al., 2002). Estren was demonstrated to bind both estrogen receptors (ER) α and β, although with less affinity than E_2 (Kousteni et al., 2002). Estren was found to be a potent estrogen agonist in bone but exerted no (Kousteni et al., 2002) or little (Moverare et al., 2003) proliferation of reproductive tissues. These differential actions of estren appear to result from its poor ability to induce classic ER transcriptional activity, which predominantly mediates the effects of estrogen in reproductive organs (Kousteni et al., 2002; Moverare et al., 2003). In contrast, estren mimics estrogen's rapid induction of cell signaling pathways in bone cells, an effect sufficient to maintain bone health in gonadectomized mice (Kousteni et al., 2003; Moverare et al., 2003).

Activation of rapid cell signaling pathways has also been shown to mediate at least some effects of estrogen in other non-reproductive targets, including the brain (Toran-Allerand et al., 1999). In particular, estrogen neuroprotection, which is thought to contribute to the neural benefits of HT (Lee and McEwen, 2001), depends in part upon activation of intracellular signaling pathways, including mitogen-activated protein kinase/extracellular-signal regulated kinase (MAPK/ERK) (Singer et al., 1999), phosphatidylinositol 3-kinase (Honda et al., 2000), and protein kinase C (PKC) (Cordey et al., 2003). Since estren was shown to parallel estrogen signaling in bone (Kousteni et al., 2002), we examined whether estren would mimic the protective actions of estrogen in neurons. We have previously shown that estrogen protects primary

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cultures of rat cerebrocortical neurons against β-amyloid (Aβ) toxicity, an AD-related insult. This beneficial effect was found to be dependent upon rapid and transient activation of PKC signaling pathways (Cordey et al., 2003). Here, we investigate whether estren treatment provides estrogen-like neuroprotection in an in vitro model of neurodegeneration.

Materials and methods

Materials

4-Estren-3α,17β-diol (estren) and 17α-estradiol (α-E_2) were obtained from Steraloids (Newport, RI). 17β-Estradiol (E_2), flutamide, and $A\beta_{25–35}$ were purchased from Sigma (St. Louis, MO), and $A\beta_{1–42}$ peptide was obtained from US Peptides (Rancho Cucamonga, CA). ICI 182,780 (ICI) was purchased from Tocris Cookson (Ellisville, MO). Gö 6983, bisindolylmaleimide V, myristoylated PKC peptide inhibitor (20–28), PKCζ pseudosubstrate peptide inhibitor, H-89, forskolin, U0126, and phorbol-12-myristate-13-acetate (PMA) were obtained from Calbiochem (La Jolla, CA). Phospho-ERK1/2 (1:4000) and ERK1/2 (1:5000) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Secondary HRP-conjugated antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA; anti-mouse 1:5000) and Pierce (Rockford, IL; anti-rabbit 1:5000). Cell culture reagents were acquired from Invitrogen (Buffalo, NY) and all other chemicals were purchased from Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Stock solutions of estren, E_2, and α-E_2 were prepared at 10 mM in 100% ethanol. Aβ peptides were solubilized in sterile H_2O under conditions that yield peptide aggregation, as previously described (Pike et al., 1993). PKC peptide inhibitors were solubilized in sterile H_2O. All other drugs were solubilized in dimethylsulfoxide (DMSO).

Cell culture

Experimental procedures involving animals were conducted in accordance with the University of Southern California guidelines based on National Institutes of Health standards. Primary rat cerebrocortical neuron cultures (~95% neuronal) were generated following a previously described protocol (Cordey et al., 2003). Briefly, dissected cerebral cortices of embryonic Sprague–Dawley rat pups day E17 were dissociated enzymatically with 0.125% trypsin and mechanically with a flame-polished glass pipette, then filtered through a 40-μm cell strainer (Falcon, Franklin Lakes, NJ). The resulting single cell suspension was diluted in Neurobasal medium supplemented with 410 nM biotin, 0.1 mg/mL bovine serum albumin, 10 μM carnitine-HCL, 10 μM ethanolamine-HCL, 85 μM galactose, 5 μg/mL insulin, 100 μM putrescine-2 HCL, 30 nM sodium selenite, 3 nM triiodo-L-thyronine, 5 μg/mL transferrin, 250 nM vitamin B12, 3.6 μM linoleic acid, 3.6 μM linolenic acid, 300 nM retinol-all trans, 350 nM retinyl acetate, 2.3 μM tocopherol, 2.1 μM tocopherol acetate, and 0.5 mM L-glutamine (modified from Brewer and Cotman, 1989). Cells were plated at a density of approximately 5×10^4 cells/cm^2 in 48-well plates for cell viability experiments, at 1×10^5 cells/cm^2 in 10-cm dishes for PKC activity assays, or at 1×10^5 cells/cm^2 in 12-well plates for Western blotting experiments. Cultures were maintained at 37°C in a humidified incubator with room air supplemented with 5% CO_2.

All experiments were started after 3 to 5 days in vitro. Estren, E_2, α-E_2, PMA, or forskolin were added to cultures beginning 1 h before administration of aggregated Aβ peptide (except for Fig. 3, see text). Inhibitors were added 1 h prior to exposure to estren, E_2, α-E_2, PMA, or forskolin. Final vehicle concentration did not exceed 0.04%, and control conditions were treated with the appropriate amount and type of vehicle.

Cell viability

Cell viability was estimated 20 to 24 h after addition of Aβ, as previously described (Cordey et al., 2003). In brief, cells labeled with the vital dye calcein-AM (Molecular Probes, Eugene, OR) and having normal morphology were scored as viable and counted in 4 fields per well, with 3 wells per condition in each culture preparation (each experiment was repeated in 3–4 independent culture preparations). The number of viable cells counted per well in vehicle-treated controls ranged from 150 to 250. Raw data from cell counts were analyzed with ANOVA followed by pairwise comparisons using the Fisher LSD test (significant effects indicated by $P < 0.05$). Cell viability is presented graphically as a percentage of the number of viable cells in the vehicle-treated control condition.

PKC activity assay

PKC activity was determined using a rapid filtration assay (Gopalakrishna et al., 1992), which specifically measures PKC activity, but does not discriminate between PKC isozymes. As previously described (Cordey et al., 2003), treated cells were collected in homogenization buffer [20 mM Tris–HCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 20 μM leupeptin, 0.15 μM pepstatin A, pH 7.5], and PKC was isolated from both soluble and 1% NP-40 detergent-solubilized membrane fractions by using a diethylaminoethyl (DEAE) cellulose column. The calcium- and phosholipid-stimulated PKC activity (proform) was eluted using 0.1 M NaCl. PKC reaction samples containing 20 mM Tris–HCl buffer, pH 7.4, 10 mM $MgCl_2$, 0.33 mM $CaCl_2$, 0.1 M ATP, 0.1 mg/mL histone H1, and 0.04 μM leupeptin were incubated in a 96-well filtration plate at 30°C for 5 min. Transfer of ^{32}P to histone H1 was determined after filtration of these samples. PKC activity was expressed as the difference in the activity that was observed in the presence of phosphatidylserine (20 μg/mL)/diolein (0.8 μg/mL) and the basal activity that was observed in the presence of EGTA (4 mM). For each condition, PKC activity was determined in duplicate samples from 4 independent cell culture preparations. Data from cytosolic and membrane fractions were adjusted for respective protein content, measured by the dye-binding method of Bradford (Bradford, 1976), and PKC activity was expressed as units/mg protein, where one unit of enzyme transfers 1 nmol of phosphate to histone H1 per minute at 30°C. Data from cytosolic and particulate fractions were combined to yield total PKC activity, which ranged from 0.42 to 1.58 units/mg protein in vehicle-treated control. Raw data from independent experiments were normalized to their respective vehicle-treated control condition and analyzed statistically with ANOVA followed by pairwise comparisons using the Fisher LSD test ($P < 0.05$).

Western blots

Cells were processed for Western blots using a standard protocol previously described (Cordey et al., 2003). Briefly, cell