Estrogen Receptor-Independent Neuroprotection via Protein Phosphatase Preservation and Attenuation of Persistent Extracellular Signal-Regulated Kinase 1/2 Activation

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ABSTRACT

The mechanism of estrogen-mediated neuroprotection is not yet clear. Estrogens have a variety of modes of action, including yet clear. Estrogens have a variety of modes of action, including transducing signaling events such as activation and/or suppression of the mitogen-activated protein kinase (MAPK) pathway. We have previously shown protein phosphatases to be involved in 17β-estradiol-mediated neuroprotection. In the present study, we assessed the role of estrogen receptors (ERs) in estrogen-mediated neuroprotection from oxidative/excitotoxic stress and the consequential effects on MAPK signaling. Okadaic acid and calyculin A, nonspecific serine/threonine phosphatase inhibitors, were exposed to cells at various concentrations in the presence or absence of 17α-estradiol, the enantiomer of 17β-estradiol, 2-(1-adamantyl)-3-hydroxyestra-1,3,5(10)-trien-17-one (ZYC3; non-ER-binding estrogen analog), and/or glutamate. All three compounds, which we have shown to have little or no binding to ERα and ERβ, were protective against glutamate toxicity but not against okadaic acid and calyculin A toxicity. In addition, in the presence of effective concentrations of these inhibitors, the protective effects of these estrogen analogs were lost. Glutamate treatment caused a 50% decrease in levels of protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), and protein phosphatase 2B (calcineurin) (PP2B). Coadministration of ZYC3 with glutamate prevented the decreases in PP1, PP2A, and PP2B levels. Furthermore, glutamate treatment caused a persistent increase in phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 that corresponds with the decrease protein levels of serine/threonine phosphatases. ZYC3 blocked this persistent increase in ERK phosphorylation. These results suggest that estrogens protect cells against glutamate-induced oxidative stress through an ER-independent mediated mechanism that serves to preserve phosphatase activity in the face of oxidative insults, resulting in attenuation of the persistent phosphorylation of ERK associated with neuronal death.

Estrogens have been shown to be potent neuroprotectants in a variety of systems and experimental models of neurodegeneration and cerebral ischemia (Gridley et al., 1997; Simpkins et al., 1997; Dubal et al., 1998; Green et al., 1998; Green and Simpkins, 2000; Yang et al., 2001). However, the mechanism of action remains elusive. Potential mechanisms of estrogens’ neuroprotective effects include the following: 1) classical estrogen receptor (ER)-dependent genomic actions; 2) ER-dependent, nongenomic actions; 3) ER-independent genomic actions; and 4) ER-independent nongenomic actions.

This laboratory and others have demonstrated that estrogens exert potent neuroprotective effects at physiologically relevant concentrations (for review, see Green and Simpkins, 2000). Both the naturally occurring feminizing estrogen, 17β-estradiol, and its inactive isomer, 17α-estradiol, can reduce toxicity caused by serum deprivation, β-amyloid treatment, and exposure to glutamate receptor agonists in cell model systems (Gridley et al., 1997, 1998; Green et al., 1998; Zauyámov et al., 1999). In addition, this laboratory has also demonstrated that treatment with either 17α-estradiol or enantiomer of 17β-estradiol (ENT E2) markedly reduces ischemic brain damage produced by middle cerebral artery occlusion in ovariectomized rats (Simpkins et al., 1997; Yang et al., 2000; Yang et al., 2001) and that estradiol administration reduces secondary ischemic damage and mortality after...
subarachnoid hemorrhage in vivo (Yang et al., 2001). ZYC3, a nonreceptor-binding estrogen analog, possesses both neuroprotective and vasoactive effects, which offers the possibility of clinical application for stroke without the side effects of estrogens (Liu et al., 2002). It also suggests that both the neuroprotective and vasoactive effects of estrogen are receptor independent because estrogen analogs with little or no binding to ERα and ERβ have been shown to be neuroprotective (Liu et al., 2002; Perez et al., 2005). In many studies, ER antagonists do not block or attenuate the protective actions of 17β-estradiol (Weaver et al., 1997; Moosmann and Behl, 1999), and estrogens exert their neuroprotective action even in the presence of transcription and translation inhibitors (Regan and Guo, 1997; Sawada et al., 1998). Furthermore, estrogens are known to activate ERK1/2 within 5 to 15 min, and this activation persists for at least 2 h (Singh et al., 2000). It has been speculated that the time course of ERK activation, among other factors, determines whether a cell commits to survival or death, which can explain the contradictory effects of MEK1/2 inhibition on neuronal cell survival.

Persistent activation of ERK has been shown to be involved in neuronal cell death (Alessandrini et al., 1999; Wang et al., 2003, 2004). Aberrant neuronal expression of phosphorylated ERK1/2 and other MAPKs are seen in brains with Alzheimer’s disease (Zhu et al., 2002b) and other neurodegenerative diseases characterized by δ deposits (Ferrer et al., 2003). In patients with Parkinson’s disease, increased phosphorylation of ERK is seen in the substantia nigra and midbrain (Zhu et al., 2002a). After acute ischemic stroke, there is an increased ERK phosphorylation in the penumbra (Slevin et al., 2000).

In the present study, we hypothesized that the neuroprotective actions of estrogens do not require receptor binding but the activation of phosphatases to mediate signaling through the MAPK pathway. We have previously shown that serine/threonine phosphatases are involved in neuroprotective mechanism of 17β-estradiol (Yi et al., 2005). We show that inhibition of serine/threonine protein phosphatases attenuates the neuroprotective effects of estrogen analogs that have little or no ERα- or ERβ-binding properties and involves the MAPK signaling pathway.

Materials and Methods

Chemicals. 17α-Estradiol was purchased from Steraloids, Inc. (Wilton, NH). ZYC3 and ENT E2 were prepared as described previously (Green and Simpkins, 2000; Liu et al., 2002). All steroids were dissolved in dimethyl sulfoxide at a concentration of 10 mM and diluted to appropriate concentrations in culture media. Calcein AM dissolved in dimethyl sulfoxide at a concentration of 10 mM and preincubated in seeding medium (DMEM; 4.5 g/l glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine) supplemented with B-27 with antioxidants in normal cell culture condition of 37°C in a humid atmosphere of 5% CO2. Primary cortical neurons were maintained in culture for 7 days before initiation of various experiments. Two hours before treatment with various inhibitors and/or 17α-estradiol, ENT E2, or ZYC3, the media were replaced with neurobasal medium supplemented with B-27 without antioxidants.

Calcine-AM Viability Assay. Primary cortical neurons were exposed to various treatments. After exposure to various treatment paradigms, cells were rinsed with phosphate-buffered saline, and cell viability was measured using the membrane-permeant calcine-AM dye (Molecular Probes). Calcine-AM is a fluorogenic esterase substrate that is hydrolyzed to a fluorescent product in cells having esterase activity and intact membranes. Cells were incubated in a solution of 2.5 μM calcine-AM in phosphate-buffered saline. Twenty minutes later, fluorescence was determined using a Bio-Tek FL6000 microplate reader (Bio-Tek Instruments, Winooski, VT) with an excitation/emission filter set of 485/530 nm, respectively. Cell culture wells treated with methanol served as blanks. The results, obtained in relative fluorescent units, are expressed as the percentage of untreated or vehicle-treated control values.

Immunoblot Analysis. Protein from whole cell lysates (25 μg) was separated by SDS-polyacrylamide gel electrophoresis and transferred to Immunobilon-P polyvinylidene difluoride (Millipore Corp., Bedford, MA) membrane. Membranes were rinsed in Tris-buffered saline (10 mM Tris-base, pH 8.0, 100 mM NaCl) containing 0.2% Tween 20, then blocked with 3% bovine serum albumin. Blots were then incubated with primary antibodies overnight at 4°C, rinsed, and incubated in the appropriate secondary antibody before detection using enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL). Enhanced chemiluminescence results were digitized and quantified using the UVP Bioimaging System. The immunoblots for protein phosphatases were normalized to β-actin and for pERK1/2 to ERK2 to ensure equal protein loading, and these normalized data were presented as percentage of control (100%).

Data Analysis. Statistical significance was determined by one-way analysis of variance followed by a Tukey’s multiple comparison test. p < 0.05 was considered significant for all experiments. The values are reported as the mean ± S.E.M.

Results

Effects of PP Inhibitors on Estrogen Analog-Mediated Neuroprotection against Glutamate-Induced Cytotoxicity. Estrogen analogs such as 17α-estradiol, ENT E2, and ZYC3 (structures in Fig. 1) have been shown to be potent neuroprotectants as 17β-estradiol (Bishop and Simpkins, 1994; Green et al., 2001; Perez et al., 2005). To determine the effectiveness of 17α-estradiol, ENT E2, and ZYC3 to protect neurons against oxidative stress or excitotoxicity induced by glutamate in the presence of a serine/threonine phosphatase inhibitor, we examined simultaneous treatment as well as 2- or 24-h pre-treatment of 17α-estradiol, ENT E2, or ZYC3 with glutamate and/or okadaic acid or calyculin A. In the absence of protein phosphatase inhibitors, the three estrogen analogs showed a dose-dependent neuroprotection against glutamate toxicity (Fig. 2). ZYC3 showed a greater potency than either ENT E2 or 17α-estradiol, consistent with our previous structure-activity relationship assessment (Perez et al., 2005). 17α-Estradiol, ENT E2, and ZYC3 were ineffective against okadaic acid or calyculin A toxicity when the serine/threonine inhibitor was
simultaneously administered with the steroids. In addition, nonlethal concentrations of okadaic acid or calyculin A attenuated the ZYC3-mediated neuroprotection against glutamate-induced toxicity (Fig. 3). Nearly complete abolishment of estrogen analog protection against glutamate toxicity was seen at 5 nM okadaic acid and 0.5 nM calyculin A (Fig. 3, A and B, respectively). Twenty-four-hour ZYC3 pretreatment did not prevent protein phosphatase inhibitor-induced cell death or the protein phosphatase inhibitor-induced abolishment of protection by ZYC3 of glutamate-induced neurotoxicity (Fig. 4). Data from 2-h pretreatment of ZYC3 or 24- and 2-h pretreatment with ENT E2 or 17α-estradiol are not shown, but the results were similar to those seen with the 24-h pretreatment with ZYC3.

**Time-Dependent Effects of ZYC3 and/or Glutamate on PP1 Protein Level.** We examined the time course of the re-

**Fig. 1.** Structures of 17β-estradiol, and three nonfeminizing estrogens, 17α-estradiol, ENT E2, and ZYC3.

**Fig. 2.** Effects of okadaic acid and calyculin A on 17α-estradiol-, ENT E2-, or ZYC3-mediated neuroprotection in primary rat cortical neurons. Primary cortical neurons were seeded into 96-well plates at a density of 25,000 cells/well. A, cells were treated simultaneously with 50 nM okadaic acid, 1 nM calyculin A, 50 μM glutamate, and/or varying concentrations of 17α-estradiol. B, cells were treated simultaneously with 50 nM okadaic acid, 1 nM calyculin A, 50 μM glutamate, and/or varying concentrations of ENT E2. C, cells were treated simultaneously with 50 nM okadaic acid, 1 nM calyculin A, 50 μM glutamate, and/or varying concentrations of ZYC3. Cell viability was determined by calcine AM assay (Molecular Probes) after 24 h of exposure to the various compounds. All data were normalized to a percentage of survival of nontreated control. Depicted are mean ± S.E.M. for 10 independent experiments with two replicates per experiment. *, p < 0.05 versus vehicle control; †, p < 0.05 versus glutamate-treated group.
MEK-Dependent Neuroprotection against Toxicity. Experimental data have shown that inhibition of ERK phosphorylation via MEK inhibition is neuroprotective (Chu et al., 2004). Hence, the neuroprotective effects of MEK inhibitors PD98059 (Fig. 7A) or U0126 (Fig. 7B) were evaluated against glutamate toxicity. One-hour pretreatment with PD98059 or U0126 had a neuroprotective effect against cytotoxicity caused by glutamate. This MEK-inhibitor-induced neuroprotection was blocked by increasing concentrations of okadaic acid (Fig. 7).

Effects of ZYC3 on ERK Phosphorylation during Oxidative Stress. Chronic phosphorylation of ERK1/2 is thought to send a neurotoxic or neurodegenerative signal (Stanciu et al., 2000; Kulich and Chu, 2001; Zhu et al., 2002a); thus, it has been speculated that estrogens attenuate the persistent ERK1/2 phosphorylation to mediate neuroprotection. Therefore, we examined the phosphorylation of ERK1/2 in glutamate toxicity in the presence of ZYC3 and/or okadaic acid. Twenty-four-hour glutamate treatment caused an ~2.5-fold increase in phosphorylation of ERK1/2 (Fig. 8). ZYC3 alone did not alter the phosphorylation state of ERK1/2 compared with vehicle control (Fig. 8). However, the presence of ZYC3 attenuated the elevated phosphorylation of ERK1/2 induced by glutamate. The presence of ZYC3 had no effect on okadaic acid-induced ERK1/2 phosphorylation, and the presence of okadaic acid abolished the ZYC3-mediated reduction in phosphorylation of ERK1/2 caused by glutamate (Fig. 8).

Discussion

The present study demonstrates that one of the mechanisms of estrogen-mediated neuroprotection is preservation of serine/threonine phosphatase protein expression via an ER-independent pathway that ultimately prevents persistent ERK1/2 phosphorylation. These observations are important in view of the Women’s Health Initiative Memory Study results that indicate chronic postmenopausal treatment of older women with estrogens (Rapp et al., 2003; Shumaker et al., 2003, 2004; Espeland et al., 2004) does not protect the brain from dementia but seems to cause an increase in strokes, heart attacks, deep venous thromboses, and sudden-onset dementia through a mechanism that likely involves induction of a hypercoagulative state through peripheral stimulation of prothrombotic and a decrease in antithrombotic factors (Hoibraaten et al., 2001; Scarabin et al., 2003; Canonico et al., 2007), some of which are known to be ER-mediated. Thus, alternatives to feminizing estrogens that protect the brain are needed.

We have previously reported that the nonfeminizing analogs of estrogens used in this study are protective against a variety of cytotoxic insults (Green et al., 1998) as well as in a
rat model for middle cerebral artery occlusion (Simpkins et al., 1997; Yang et al., 2000; Liu et al., 2002). The protective effects of these estrogen analogs against glutamate toxicity were antagonized by the protein phosphatase inhibitors, okadaic acid and calyculin A. In addition, we show that estrogen analogs rapidly and persistently antagonize the reduction in PP1, PP2A, and PP2B protein expression induced by oxidative and excitotoxic stresses, but not in the presence of okadaic acid. Phosphorylation of ERK1/2 in response to glutamate toxicity corresponds to decreases in serine/threonine phosphatase expression, and inhibitors of ERK1/2 phosphorylation attenuate the glutamate-induced cytotoxicity. Taken together, these data support the hypothesis that phosphatase regulation is a major component of estrogen-mediated neuroprotection that is not dependent upon the classical ER-mediated genomic effects.

Of the three estrogen analogs assessed, ZYC3 was more potent than either 17α-estradiol or ENT E2, consistent with our previous structure activity relationship studies (Green et al., 2001; Liu et al., 2002; Perez et al., 2005). Despite this, okadaic acid and calyculin A were effective in blocking the neuroprotective effects of all three compounds. This antagonism by protein phosphatase inhibition of estrogen neuroprotection occurred even at concentrations of okadaic acid and calyculin A that did not induce cell death. This latter observation indicates that abolition of estrogen neuroprotection by protein phosphatase inhibition is not due to an increase in the magnitude of the neurotoxic insult, which we have shown reduces the potency of estrogen neuroprotection (Perez et al., 2005).

The mechanism of this effect of estrogens on protein phosphatase levels and activity is not clear. There are no previous experimental data suggesting a direct interaction between estrogens and serine/threonine phosphatases. Although PP2A has been shown to regulate ERα by mRNA stabilization (Keen et al., 2005) as well as direct interaction with ERα in the absence of estrogen (Lu et al., 2003), our data indicate that effects of estrogens on protein phosphatases occur without ER interactions. An interesting but yet unexplained observation is that although 17β-estradiol (Yi et al., 2005) or ZYC3 (present study) alone have little effect on protein phosphatase levels, they cause a prompt increase in protein phosphatase concentrations and a persistent resistance to glutamate-induced decline in serine/threonine phosphatase levels. The absence of effects of estrogens alone and the rapidity of the response to estrogens in the face of insult suggest that estrogens either reduce or prevent the clearance of these serine/threonine phosphatases that are activated by oxida-

Fig. 4. Twenty-four-hour pretreatment of primary rat cortical neurons with ZYC3 does not attenuate glutamate neurotoxicity in the presence of okadaic acid or calyculin A. Primary cortical neurons were seeded into 96-well plates at a density of 25,000 cells/well. A, after 24-h pretreatment with varying concentrations of ZYC3 for 24 h, cells were treated with 50 nM okadaic acid and/or 50 μM glutamate. B, after 24-h pretreatment with varying concentrations of ZYC3 for 24 h, cells were treated with 1 nM calyculin A and/or 50 μM glutamate and/or varying concentrations of ZYC3. Cell viability was determined by calcein AM assay (Molecular Probes) after 24 h of exposure to the various compounds. All data were normalized to a percentage of survival of nontreated control. Depicted are mean ± S.E.M. for 10 independent experiments with two replicates per experiment. *, p < 0.05 versus vehicle control; †, p < 0.05 versus glutamate-treated group.
tive/excitotoxic insult, rather than causing expression of new protein. Therefore, it is likely that 17β-estradiol and its analogs are protecting cells by blocking the ubiquitination and/or degradation of protein phosphatases caused by oxidative or excitotoxic stresses.

In view of the relative lack of binding of these analogs to ERα or ERβ (Perez et al., 2005), intracellular signaling pathways may be involved in mediating their neuroprotective effects. Indeed, experimental studies have shown that the neuroprotective effects of estrogens are mediated at least in part through the rapid but acute phosphorylation of signaling proteins, such as adenylyl cyclase, protein kinase B, protein kinase A, protein kinase C, and MAPK (Migliaccio et al., 1996; Watters et al., 1997; Kelly et al., 1999; Zhang et al., 2001). Changes in the activity of these enzymes can regulate the phosphorylation of numerous intermediary signaling proteins such as protein of 90-kDa ribosomal S6 kinase, p38, c-Jun NH₂-terminal kinase, and the nuclear transcriptional factors cAMP-response element-binding protein and c-fos/c-Jun, which may ultimately mediate cell survival (for review, see Lee and McEwen, 2001). The present study shows that ERK1/2 is rapidly and persistently phosphorylated in response to oxidative and excitotoxic stresses caused by glutamate, and the presence of estrogens prevents this persis-

![Fig. 5](image1.png)  
**Fig. 5.** Time course of the effects of glutamate (A), ZYC3 (B), and their combination (C) on PP1 protein expression. Primary rat cortical neurons were treated with 50 μM glutamate and/or 10 nM ZYC3. Cells were harvested at the times indicated for Western blot analysis of PP1. The immunoblots for protein phosphatases were normalized to β-actin to ensure equal protein loading, and these normalized data were used to depict the data as percentage of control (100%). Depicted are mean ± S.E.M. for n = 5. *, p < 0.05 versus time 0 control.

![Fig. 6](image2.png)  
**Fig. 6.** PP1 (A), PP2A (B), and PP2B (C) protein levels in response to ZYC3 in the presence and absence of 50 μM glutamate and/or 50 nM okadaic acid in primary rat cortical neurons. Primary cortical neurons were treated with 50 nM okadaic acid (OA), 50 μM glutamate (Glut), and/or 10 nM ZYC3. Cells were harvested after 24 h of treatment for Western blot analysis of PP1, PP2A, and PP2B. The immunoblots for protein phosphatases were normalized to β-actin to ensure equal protein loading, and these normalized data were used to depict the data as percentage of control (100%). Depicted are mean ± S.E.M. for n = 5. *, p < 0.05 versus control; †, p < 0.05 versus glutamate-treated group.
Western blot analysis of pERK. The immunoblots for pERK1/2 were treated with 50 μM PD98059 for 1 h before treatment with varying concentrations of okadaic acid and 50 μM glutamate. Celis were harvested after 24 h of treatment for phospho-kinase inhibition protects against damage resulting from focal cerebral ischemia. Proc Natl Acad Sci USA 96:12866–12869.

B. Cells were pretreated with 10 nM U0126 for 1 h before treatment with varying concentrations of okadaic acid and 50 μM glutamate. Cell viability was determined by calcein AM assay (Molecular Probes) after 24 h of exposure to the various compounds. All data were normalized to a percentage of survival of nontreated control. Depicted are mean ± S.E.M. for 10 independent experiments with three replicates per experiment. *, p < 0.05 versus vehicle control; †, p < 0.05 versus glutamate-treated group.

Fig. 8. Phosphorylation of ERK after glutamate treatment in the presence and absence of ZYC3 and/or okadaic acid. Primary cortical neurons were treated with 50 nM okadaic acid (OA), 50 μM glutamate (Glut), and/or 10 nM ZYC3. Cells were harvested after 24 h of treatment for Western blot analysis of pERK. The immunoblots for pERK1/2 were normalized to ERK2 to ensure equal protein loading, and these normalized data were used to depict the data as percentage of control (100%). Depicted are mean ± S.E.M. for n = 5. *, p < 0.05 versus control.

The present study provides a potential ER-independent mechanism for the neuroprotective effects of estrogens. Estrogens seem to protect neurons through the prevention of insult-induced decrease in phosphatase activity and the resulting neurotoxicity, persistent hyperphosphorylation of proteins in multiple signaling pathways that are detrimental to cell survival. We have demonstrated that the neuroprotective effects of estrogen analogs against glutamate toxicity are opposed by phosphatase inhibition and that these analogs prevent insult-induced reduction in protein phosphatase levels as seen with 17β-estradiol (Yi et al., 2005). These data demonstrate that the protective effects of estrogens against neurotoxicity induced by oxidative stress and excitotoxicity are receptor-independent and involve protein phosphatase activation through a signal transduction pathway mediated by MAPK (ERK1/2).

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References


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