Neuroprotective Effects of Nonfeminizing Estrogens in Retinal Photoreceptor Neurons

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PURPOSE. Retinal diseases such as macular degeneration and glaucoma are disorders that target specific retinal neurons that can ultimately lead to vision loss. Under these conditions and pathologies, retinal neurons can die via apoptosis that may be due to increased oxidative stress. The neuroprotective effects of 17 β -estradiol (E2) and three synthetic nonfeminizing estrogen analogs (ZYC-26, ZYC-23, and ZYC-3) were investigated to examine their abilities to protect retinal neurons against glutamate toxicity.

METHODS. Using an in vitro model of glutamate-induced cell death in 661W cells, a mouse cone photoreceptor cell line, shown to express both estrogen receptors (ERs) via immunoblotting, was pretreated with E2 and its analogs and cell viability were assessed.

RESULTS. It was observed that E2 and estrogen analogs, ZYC-26 and ZYC-3, were protective against a 5 mM glutamate insult in 661W cells. The neuroprotective abilities of ZYC-26 and ZYC-3 were autonomous of estrogen receptor- α (ER α) and ER β demonstrated by their ability to protect in the presence of ICI 182780, a pan-ER antagonist with a high affinity for the estrogen receptor. Treatment with PPT and DPN, ER α - and ER β -specific agonists, respectively, did not protect the 661W cells from the glutamate insult. Studying the membrane ER (mER) or GPR30 did show that activation of the receptor by G1 protected the retinal neuron from insult, whereas G15, an antagonist of the mER was not able to antagonize the protection previously seen.

CONCLUSIONS. These data demonstrate that nonfeminizing estrogens may emerge as useful compounds for neuroprotection of retinal cells. (*Invest Ophthalmol Vis Sci.* 2012;53:4739–4747) DOI:10.1167/iovs.12-9517

Glutamate is the predominant excitatory neurotransmitter in the mammalian central nervous system (CNS) and the vertical pathway of the mammalian retina, but at high concentrations, it has been shown to be neurotoxic through inhibition of the glutamate/cystine antiporter. This inhibition disrupts glutathione synthesis, which increases reactive oxygen species (ROS).¹ The production of ROS is a normal

consequence of metabolism, but in the retina, elevated levels of ROS has been associated with diseases such as age-related macular degeneration (AMD)² and retinitis pigmentosa (RP).³ Although different enzyme systems within the cell can generate ROS, the mitochondrial electron transport chain is the predominant contributor.^{4–6}

In retinal diseases such as RP and AMD there is an eventual loss of photoreceptor cells that can lead to partial or complete blindness. AMD alone has been shown to be the leading cause of vision loss in the elderly population in the western hemisphere.^{7,8} It has been predicted that within the next decade the number of AMD cases is expected to reach 3 million.⁹ As with many of these retinal diseases and other neurodegenerative diseases, there are very few therapies to combat the neuronal cell loss due to a lack of understanding of the pathologies themselves.

With AMD, the positioning of the retinal pigment epithelium (RPE) and its role create an idyllic atmosphere for the elevation and accumulation of ROS,¹⁰ which in turn can disrupt mitochondrial function within these cells. The development of mitochondrial dysfunction can lead to the death of the RPE¹¹ via apoptosis, which as a consequence can result in death to the underlying photoreceptor cells.^{12,13} These factors can eventually lead to vision loss.

Epidemiologic studies have indicated a noticeable relationship between the development of AMD in women and the reduction of circulating estrogen as seen in menopause.^{14–17} These studies help to demonstrate the importance of estrogen. Estrogens have been shown to be neuroprotective in in vivo models, including transient and permanent middle cerebral artery occlusion,^{18–20} global forebrain ischemia,²¹ photothrombotic focal ischemia,²² and glutamate-induced focal cerebral ischemia,²³ and in in vitro systems including H₂O₂,^{24–29} serum deprivation,^{30–33} oxygen-glucose deprivation,^{34,35} and excitotoxicity.^{34,36–39} The ability of estrogen to protect the mitochondrial respiratory chain from oxidative damage has been demonstrated in a plethora of tissues, including the brain, breast, myocardium, and lens epithelium.⁴⁰ In particular, there has been over a decade of studies demonstrating convincingly the neuroprotective abilities of 17 β -estradiol (E2).^{18,24,30,36,41,42} The mechanism by which it protects, however, is still inadequately understood.^{42,43} Estrogens possess a phenolic ring that provides antioxidant/redox cycling activity in neurons and other tissues.^{44–47}

The discovery of a compound that can protect retinal neurons from insult or damage and reduce vision loss would be very beneficial. Although estrogens are viable candidates, their use as a neuroprotective agent is limited by their feminizing characteristics. We discovered nonfeminizing estrogens⁴⁸ that can provide potent neuroprotection, but lack binding to estrogen receptors (ERs). In the present study, we demonstrate the neuroprotective effects of E2 and estrogen analogs in 661W cells exposed to oxidative stress initiated by a glutamate insult. Here we assessed the neuroprotective effects of the

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FIGURE 1. Structures of compounds used to evaluate neuroprotection against glutamate toxicity in 661W cells.

derivatives of estrone, 2-adamantyl-estra-1,3,5(10)-trien-3-ol-17-one (ZYC-3), 2-adamantyl, 4-methyl estrone (ZYC-26), and 2-adamantyl, 3-O-methyl estradiol (ZYC-23) in a neuronal cell culture model. The mechanisms by which estrogens are capable of protecting against oxidative stress are thought to occur via an ER-mediated mechanism^{49,50} or their antioxidant activity.⁴⁶ Here we demonstrate that E2 and its analogs increase cell viability in 661W cells exposed to glutamate toxicity through a mechanism that is independent of the ER. The ability for estrogen and its analogs to protect within the 661W model was also dependent on the presence of a phenolic ring. These properties allow these compounds to function as neuroprotective agents that could provide protection from further damage to retinal neurons as well as provide prophylactic

protection from future damage due to eye disease or even eye trauma.

MATERIALS AND METHODS

Cell Culture

661W cells were generously donated by Muayyad Al-Ubaidi (University of Oklahoma Health Sciences Center, Oklahoma City, OK). The 661W cell line, a mouse retinal tumor cell line derived from cone photoreceptor cells, was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% dialyzed fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin. Cells were grown in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Only the first seven cell line passages were used for experiments.

FIGURE 2. ER α is expressed in the 661W cells.

Chemicals and Reagents

The following materials were used for experiments in our investigations: 17 β -estradiol (E2; Steraloids, Inc., Newport, RI); L-glutamic acid (Sigma-Aldrich, St. Louis, MO); G1 and G15, an agonist and antagonist, respectively, at the membrane ER G-protein-coupled receptor (GPR30) (Calbiochem, San Diego, CA); ICI 182780 (4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol [PPT]) and diarylpropionitrile (DPN; Tocris Bioscience, Ellisville, MO); and 2-adamantyl, 4 methyl estrone (ZYC-26), 2-adamantyl-estra-1,3,5(10)-trien-3-ol-17-one (ZYC-3), and 2-adamantyl, 3-0-methyl estradiol (ZYC-23) (synthesized in the laboratories of Covey and colleagues⁵¹).

Treatment Paradigm

661W cells in culture were removed using 0.25% trypsin-EDTA (Invitrogen Corp., Carlsbad, CA) and plated at a density of 3000 cells/well in 96-well plates. Cell media consisted of DMEM with 10% dialyzed FBS, and 1% penicillin-streptomycin solution. After 24 hours, cells were treated with either dimethyl sulfoxide vehicle control (DMSO; Sigma-Aldrich) or 5 mM glutamate in the presence of 1 nM to 10 μ M E2, DPN, PPT, ZYC-26, ZYC-3, ZYC-23, or G1. To study the specific involvement of the ER α and ER β , cells were also treated with the ER antagonist ICI 182780 in the presence of the different ZYC compounds. We also assessed the involvement of the membrane ER receptor (GPR30) by studying its antagonist G15 in the presence of concentrations of estrogens that provide neuroprotection in 661W cells. The structures of estrogen-related compounds used are depicted in Figure 1.

Calcein-AM Cell Imaging

Cell viability was assessed using the membrane-permeant calcein-AM dye (Molecular Probes, Eugene, OR), a fluorogenic esterase substrate that is capable of penetrating the membrane of live cells. Once inside the cell, calcein-AM undergoes hydrolysis by the intracellular esterases, causing the calcein to be released and to fluoresce strongly, thereby enabling the measurement of change in fluorescent units. Cells were plated at a density of 3000 cells/well in 96-well plates. After 24 hours of drug and glutamate treatment, the media was removed from each of the wells in the 96-well plates and the cells were rinsed once in phosphate buffer (pH 7.2) (PBS; Fisher Scientific, Pittsburgh, PA). Then to each well was added 100 μ L of 1 μ g/mL calcein-AM (Calbiochem) in PBS and the plate was then incubated at 37°C for 20 minutes. Fluorescence was determined using an excitation/emission filter set of 485/530 nm read with a commercial plate reader (Tecan Infinite M200; Tecan Systems, Inc., San Jose, CA).

Immunoblotting

For immunoblotting analysis, cells were harvested by scraping, washed in PBS, resuspended, then homogenized and sonicated in RIPA buffer (1 $\times$ PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 10 mg/mL aprotinin, and 100 mg/mL phenyl-methyl sulfonyl fluoride [PMSF]). Lysates were then centrifuged at 12,000g for 10 minutes at 4°C, and supernatants were collected for analysis. The protein concentrations were measured using a commercial protein assay (Bio-Rad Laboratories, Hercules, CA) before Western blot analysis. Protein from 661W cells and MCF7 cells was mixed with loading buffer, boiled for 5 minutes, separated by SDS-PAGE, and then transferred to polyvinylidene difluoride (PVDF) membranes (Immuno-

FIGURE 3. Effects of glutamate on cell viability of 661W cells. Depicted are mean $\pm$ SD ($n = 8$ per group). * $P < 0.05$ vs. vehicle control.

bilon-P; Millipore, Bedford, MA). Membranes were blocked with 5% dry milk in PBS. Proteins were probed with specific ER α or ER β antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 dilution and incubated overnight at 4°C. The blots were washed and applied with goat-anti-rabbit secondary antibodies (Santa Cruz Biotechnology) at 1:1000 dilution. After washing, the blots were developed with an enhanced chemiluminescent kit (Pierce, Rockford, IL).

Statistical Analysis

All data are presented as mean $\pm$ 1 SD. These data were analyzed using the ANOVA with Tukey's post hoc test for statistical evaluation against an α -level of 0.05. All bar graphs and EC50 (half-maximal effective concentration) calculations were made using a commercial statistics and data analysis software program (GraphPad Prism 5; GraphPad Software Inc., La Jolla, CA). For all groups, $n = 8$ wells and experiments were repeated three times to ensure consistency.

RESULTS

ER α Is Expressed in 661W Cells

To determine whether 661W cells expressed ER α or ER β , we performed Western blot analysis for the presence of these intracellular proteins. It has previously been shown that within the retina, ERs are distributed within the outer plexiform layer, the inner nuclear layer, the ganglion cell layer, and the nerve fiber.⁵² Our Western blot analysis confirmed the protein expression of ER α in 661W cells (Fig. 2). MCF7 cells were used as a positive control since it has previously been shown to express both ER α and ER β ,⁵³ and HEK 293T cells were used as a negative control since they do not express ER α or ER β .

Effects of Glutamate on Cell Viability in 661W Cells

To determine whether 661W cells were susceptible to glutamate toxicity, cells were cultured and incubated with concentrations of glutamate ranging from 1 to 10 mM for 24 hours. Exposure of 661W cells to increasing concentrations of glutamate resulted in a dose-dependent decrease in cell viability (Fig. 3). Viability was reduced from 100.0 $\pm$ 4.2% in the vehicle control to 97.9 $\pm$ 1.4, 77.2 $\pm$ 3.0, 60.0 $\pm$ 2.7, 42.9 $\pm$ 2.3, 34.3 $\pm$ 2.1, 21.0 $\pm$ 1.7, and 12.9 $\pm$ 1.4% in the presence of 1, 2, 3, 4, 5, 7.5, and 10 mM glutamate, respectively. At a concentration of 5 mM glutamate, cell viability was reduced by approximately 60–70%. We used the

FIGURE 4. Effects of E2 (A) and nonfeminizing estrogen analogs ZYC-26 (B), ZYC-3 (C), and ZYC-23 (D) on cell viability in glutamate-treated 661W cells. Depicted are mean $\pm$ SD ($n = 8$ per group). * $P < 0.05$ vs. glutamate alone-treated cells. ICI, ICI 182780.

concentration of 5 mM glutamate to evaluate the neuroprotective properties of each of the compounds of interest.

Effects of 17 β -Estradiol on Cell Viability in Glutamate-Treated 661W Cells

E2 has been well described as a neuroprotective agent in a variety of in vivo^{18–23} and in vitro models.^{24–39} Here we assessed its effects in 661W cells against oxidative damage following glutamate exposure. We tested E2 concentrations

ranging from 10 nM to 10 μ M in 661W cells treated with 5 mM glutamate (Fig. 4A). E2 modestly increased cell viability ($P < 0.05$) at the 10 μ M concentration only with an EC₅₀ of 4.4 μ M.

Effects of Nonfeminizing Estrogens on Cell Viability in Glutamate-Treated 661W Cells

Treatment of the 661W cells with phenolic compounds ZYC-26 and ZYC-3 provided protection, whereas treatment with the nonphenolic compound ZYC-23 did not. ZYC-26, one of the

FIGURE 5. Effects of ER α preferring agonist, PPT (A) and an ER β -preferring agonist DPN (B) on cell viability in glutamate-treated 661W cells. Depicted are mean $\pm$ SD ($n = 8$ per group). * $P < 0.05$ vs. glutamate alone-treated cells.

nonfeminizing estrogen compounds, protected 661W cells against 5 mM glutamate-induced oxidative damage. We saw statistically significant neuroprotection at concentrations ranging from 100 nM to 10 μ M (Fig. 4B). Viability increased from $79.2 \pm 2.1\%$ at 100 nM, to $85.0 \pm 1.2\%$ at 1 μ M to $94.6 \pm 1.5\%$ at 10 μ M, with an EC₅₀ of 65.4 nM. Preincubation with 500 nM ICI 182780 with 1 μ M ZYC-26 and 5 mM glutamate did not produce any significant changes in cell viability (Fig. 4B). Similarly, ZYC-3, also a nonfeminizing estrogen, was able to provide statistically significant protection in the 661W cells exposed to a 5 mM glutamate insult at concentrations ranging from 100 nM to 10 μ M (Fig. 4C). ZYC-3 increased cell viability at 100 nM from $32.3 \pm 0.9\%$ to $88.3 \pm 1.6\%$ at 10 μ M, with an EC₅₀ of 444 nM. In contrast, ZYC-23 was not able to provide any protection from a 5 mM glutamate insult at the concentration range of 10 nM to 10 μ M (Fig. 4D). ZYC-23 has an O-methyl group attached at the 3 position of the phenolic A ring (Fig. 1), abolishing the antioxidant characteristics of the compound. To determine whether the protection observed involved ER α or ER β , we used 500 nM ICI 182780, a concentration 1500-fold greater than the IC₅₀ (dose that causes half-maximal inhibition by an antagonist) of ICI 182780 in both ER α and ER β .⁵⁴ The addition of ICI 182780 at 500 nM during treatment with 1 μ M ZYC-3 produced no significant attenuation of cell viability. These data support the concept

FIGURE 6. Effects of G1 on cell viability in glutamate-treated 661W cells. Depicted are mean $\pm$ SD ($n = 8$ per group). * $P < 0.05$ vs. glutamate alone-treated cells.

that for E2 and nonfeminizing estrogens to be protective, it is necessary that they possess a phenol ring; this establishes the importance of the phenolic ring in the preservation of cell viability in 661W cells.

Effects of ER α - and ER β -Specific Agonists on Cell Viability in Glutamate-Treated 661W Cells

The inability of the ER antagonist ICI 182780 to block the neuroprotection by nonfeminizing estrogens suggested that the ERs are not involved in the action in 661W cells. We then assessed the effects of specific ER agonists to determine whether they were protective in 661W cells. Cells treated with PPT, an ER α -specific agonist, were not protected from a 5 mM glutamate insult at concentrations ranging from 10 nM to 10 μ M (Fig. 5A). Similarly, DPN, a relatively selective ER β agonist had no protective effect on 661W cells against a 5 mM glutamate insult at concentrations ranging from 10 nM to 10 μ M (Fig. 5B).

Effects of the GPR30 Agonist and Antagonist on Cell Viability in Glutamate-Treated 661W Cells

The membrane ER (mER) or GPR30 is a membrane-associated G_q-protein-coupled ER. Since our data did not implicate ER α and ER β in mediating the observed neuroprotection, we assessed another potential ER, the GPR30. To assess the role of this receptor, we utilized the GPR30-preferring ER agonist G1, which provided a dose-dependent, statistically significant neuroprotection against a 5 mM glutamate insult at concentrations 100 nM, 1 μ M, and 10 μ M (Fig. 6). Treatment with the GPR30 antagonist G15 did not markedly affect the neuroprotective activity of G1 (Fig. 7A). We assessed the ability of G15 to antagonize the neuroprotective effects of E2. G15 did not antagonize E2-induced protection from glutamate toxicity (Fig. 7B). G15 only modestly inhibited the protective effects of ZYC-26 and ZYC-3, again suggesting that their mechanism of protection was not via GPR30 (Figs. 7C, 7D).

FIGURE 7. Effects of the GPR30 antagonist, G15, on G1 (A), E2 (B), ZYC-26 (C), and ZYC-3 (D)-induced neuroprotection of cell viability in glutamate-treated 661W cells. Depicted are mean $\pm$ SD ($n = 8$ per group). * $P < 0.05$ vs. glutamate alone-treated cells.

DISCUSSION

Age-related macular degeneration (AMD) is the leading cause of visual loss in patients older than 60 years of age in the United States and developing countries. The etiology of AMD remains unknown, but researchers have shown that a combination of genetic factors⁵⁵⁻⁵⁷ and oxidative stress factors in the retina may influence the pathology of the disease.^{58,59} Oxidative stress was shown to contribute to the development of AMD by the Age-Related Eye Disease Study.⁶⁰ Other factors that have also been shown to influence AMD include environment, sex, smoking, diet, and nutrition. Several of these factors induce the

generation of elevated reactive oxygen species (ROS) in the retina, which can eventually lead to increased oxidative damage.^{61,62}

One of the earliest studies demonstrating the neuroprotective effects of estrogen focused on the ability of a synthetic estrogen analog to protect in an *in vivo* model of retinitis pigmentosa (RP). In that study, which used animals at postnatal day 9, the estrogen analog treatment resulted in an outer nuclear layer that was almost twice the thickness in untreated controls.⁶³ An *in vitro* model of N-methyl-D-aspartate-induced excitotoxic glaucomatous retinal ganglion cell (RGC) death also treated with the estrogen analog showed that the primary

TABLE. Potency of Estrogen-Related Compounds in Protecting 661W Cells from Glutamate-Induced Cell Death

Compounds	ED50 (μ M)
E2	4.4
ZYC-3	0.44
ZYC-26	0.065
G1	0.12
ZYC-23	>10
PPT	>10
DPN	>10

RGCs were protected from the insult when treated.⁶³ In another study, 17 β -estradiol protected RGCs from changes in the synaptic connectivity associated with ischemia prior to apoptosis and ischemia-induced apoptosis.⁶⁴ In a study that used RGCs, it was shown that RGC survival was greatly reduced in rats that had received oophorectomies, but in similar animals that received intravitreal 17 β -estradiol injections, RGC loss was reduced.⁶⁵

In our study, we demonstrated that the nonfeminizing estrogen analogs ZYC-26 and ZYC-3, as well as the GPR30 agonist G1, are able to protect 661W cells from glutamate-induced cell death. The neuroprotective abilities of these compounds were independent of ER α and ER β in view of the observations that PPT and DPN failed to offer any protection within this model and ICI 182780 did not antagonize the observed neuroprotection. Interestingly, GPR30 may mediate some level of protection, based on our observation that G1 was potentially neuroprotective. However, the putative membrane ER did not mediate the neuroprotection seen with the nonfeminizing estrogens, given that G15 was only minimally effective in antagonizing the neuroprotective effects of ZYC-26 and ZYC-3 (Figs. 7C, 7D).

The relative potency of the compounds tested for protection of 661W cells was ZYC-26 > G1 > ZYC-3 > E2 > > > ZYC-23, PPT, and DPN (the Table). The most potent of these compounds, ZYC-3, ZYC-26, and G1, were nonfeminizing^{51,66} compared with the inactive compounds: PPT, an ER α agonist, DPN, an ER β agonist, and ZYC-23. The inverse relationship between potency in this 661W model and ER binding argues that neuroprotection and ER binding are clearly distinguishable processes. We have made similar observations concerning separation of ER binding and neuroprotection in hippocampal cell types and in animal models of cerebral ischemia.⁴⁸

Inasmuch as PPT and DPN both possess phenol rings (Fig. 1) and exert antioxidant activity in other toxicity models,⁶⁷ we were surprised that neither compound provided protection of 661W cells against glutamate insult. Although these data clearly demonstrate the lack of ER mediation of the responses observed, they are at odds with the idea that phenolic rings are essential for neuroprotection of estrogen-like compounds. It is also worth noting that G1, which was potentially neuroprotective in this assay, is not an effective antioxidant^{66,68} and lacks a phenolic ring (Fig. 1). Collectively, the observed neuroprotection against a known prooxidant stressor, high concentrations of glutamate, is not likely due to the pure antioxidant activities of the compounds tested.

The present study demonstrates that E2, nonfeminizing estrogens, and G1 are neuroprotective through a mechanism that does not require classical ERs or GPR30. These data support the non-ER-mediated action of E2 and nonfeminizing estrogens that have been seen in a number of studies both *in vivo*^{18–23} and *in vitro*.^{24–39} There are multiple lines of evidence that the effects observed are not mediated by ERs. First, ZYC-3 and ZYC-26 do not bind to either ER α or ER β . Second, their

effects are not antagonized by the pan-ER antagonist, ICI 182780. Third, the ER-selective agonists PPT and DPN did not show any evidence of neuroprotection in this model. Although G1 was active in this model, its effects were not antagonized by G15. Similarly, the effects of E2, ZYC-26, and ZYC-3 were not antagonized either by ICI 182780 or G15. Finally, the effective doses of compounds were much higher than the low nM ED50 for estrogens at ER α or ER β . Collectively, these data provide strong support for the idea that neuroprotection in 661W cells is a non-ER-mediated mechanism.

In summary, the present observations suggest that the treatment of neurodegenerative eye diseases could be achieved with nonfeminizing estrogens and thereby could avoid the side effects of estrogens, even with chronic administration.

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