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Neuroprotection with Non-Feminizing Estrogen Analogues: An Overlooked Possible Therapeutic Strategy

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Abstract

Although many of the effects of estrogens on the brain are mediated through estrogen receptors (ERs), there is evidence that neuroprotective activity of estrogens can be mediated by non-ER mechanisms. Herein, we review the substantial evidence that estrogens neuroprotection is in large part non-ER mediated and describe in vitro and in vivo studies that support this conclusion. Also, we described our drug discovery strategy for capitalizing on enhancement in neuroprotection while at the same time, reducing ER binding of a group of synthetic non-feminizing estrogens. Finally, we offer evidence that part of the neuroprotection of these non-feminizing estrogens is due to enhancement in redox potential of the synthesized compounds.

Keywords

Estrogens; non-feminizing estrogens; neuroprotection; stroke; hormone therapy; estrogen receptors

1. Introduction

Increased risks for cardiovascular disease, stroke, blood clots, breast cancer, and dementia in women on estrogen therapy resulted in the early termination of the Women's Health Initiative (WHI). We have since struggled to reconcile these results with the numerous epidemiological, experimental, and clinical studies showing hormone therapy (HT) to be protective against a wide variety of pathological diseased states such as Alzheimer's disease (AD) and Parkinson's disease (PD), cerebrovascular stroke, and cardiovascular diseases (for review see Simpkins et al., 2009). Reevaluations of the WHI and other clinical trials and

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cohort studies, such as the Nurses' Health Study, suggest that effects of HT are dependent on post-menopausal stage and the extent of preexisting neurodegenerative or cardiovascular disease at the onset of HT (Dumas et al., 2008; Grodstein et al., 2006; Harman, 2004; Hodis et al., 2003; Sontag et al., 2004). These assessments indicate that younger women may derive neuro- and cardiovascular protection from HT, whereas the initiation of such therapy in older individuals is ineffective or even detrimental (Coker et al., 2009; Dubey et al., 2005; Maki, 2006; Manson et al., 2007; Resnick et al., 2009; Salpeter et al., 2006).

Sex differences in the incidence and outcome of stroke in human subjects suggest that hormonal factors may influence stroke occurrence and damage (Finucane et al., 1993; Paganini-Hill and Henderson, 1996). The severity of ischemic damage in spontaneously hypertensive rats is dependent upon estrogen status (Carswell et al., 2000a,b). While a few studies have shown neutral or negative effects of estrogens (Bingham et al., 2005; Harukuni et al., 2001; Santizo et al., 2002), exogenously administered 17 β -estradiol (β E2) is also able to reduce ischemic lesions in animals subjected to middle cerebral artery occlusion (MCAO) (Simpkins et al., 1997a; Dubal et al., 1998; Shi et al., 2001; Yang et al., 2000); estradiol-mediated protection against cerebral ischemia is seen in young (Liao et al., 2001; Simpkins et al., 1997a), middle-aged (Dubal and Wise, 2001), and diabetic rats (Toung et al., 2000), as well as in mice (Dubal et al., 2001) and gerbils (Shughrue and Merchenthaler, 2003). Injury due to MCAO is decreased in rats with either pretreatment with physiological levels of β E2 (Dubal et al., 1998) or with pharmacological post-treatment methods (McCullough et al., 2001; Yang et al., 2000).

The mechanisms of the neuroprotective actions of estrogen are characterized by classical estrogen receptor (ER)-dependent genomic and non-genomic actions. Estrogens have been shown to have an intrinsic antioxidant structure that lies in the phenolic ring of the compounds, which provide the antioxidant/ redox cycling activity in neurons (Prokai et al., 2003a,b). In addition, we have shown that both feminizing and non-feminizing estrogens potentiate L-type VGCC activity (Sarkar et al., 2008). As such, estrogens can have profound effects on neurons that are not dependent on ER interactions.

Many of chronic HT side-effects are most likely due to peripheral effects of orally administered estrogen preparations acting via known ERs (Coker et al., 2009; Dubey et al., 2005; Maki, 2006; Resnick et al., 2009; Salpeter et al., 2006). Several strategies have been undertaken to capitalize on the potent neuroprotective effects of estrogens, while avoiding the side effects of chronic peripheral ER activation. First, selective estrogen receptor modulators are compounds that act as agonists on one ER and antagonists at the other ER (Zandi et al., 2002). This strategy has attempted to enhance ER β agonist activity while antagonizing ER α (Brinton, 2004; Shelly et al. 2008). The rationale for this approach is that chronic activation of ER α , which is highly expressed in breast, uterus and liver, may account for the known side effects of chronic estrogen administration to post-menopausal women.

An alternative strategy that our laboratory is pursued for nearly two decades is the use of estrogen analogues that do not bind to ERs. This strategy is based on our seminal finding that the ER- α/β ligand β E2 and its presumed inactive diastomer, α E2, which binds to these

ER-isoforms with an approximately 40-fold lower affinity (Perez et al., 2005a,b), were equally potent in protecting neuronal cells and the effects were not blocked by an estrogen antagonist (Green et al., 1997a). This observation prompted a closer evaluation of the structural requirements for neuroprotection among steroids (Behl et al., 1997; Green et al., 1997b), which suggested that estrogen analogues with little ER binding affinity were just as protective as β E2; therefore, we reasoned that a substantial portion of the neuroprotective activity of estrogens was ER-independent.

2. Methods of assessing the estrogen receptor dependence of estrogen effects

There are at least 4 methods that can be used to assess the ER-dependence of effects of estrogens on neuroprotection.

- a. First, the use of a pan-ER antagonist, such as ICI182,780 (ICI), should inhibit compound effects if they are mediated through ERs. ICI has an IC_{50} of 0.29 nM, and as such is a potent ER antagonist. In many studies, it is used at concentrations 1000-times or greater than its IC_{50} . We observed that ICI 182,780 is extremely neurotoxic to primary cortical neurons, HT-22 cells and SK-N-SH with the lowest dose neurotoxicity seen at 0.3, 0.01 and 0.01 μ M in the three cell types, respectively (Perez, et al., Unpublished observations). In view of these data and the low nM IC_{50} of ICI for ERs, ICI should not be used to assess the ER-dependence of neuronal estrogen responses at concentrations greater than 100 nM, especially when neuroprotection is the measured estrogen response. In addition, ICI has been shown to be neuroprotective (Richardson and Simpkins, 2011; Zhao et al, 2006), complicating the interpretation of data suggesting effects of estrogens are antagonized by ICI.
- b. Second, given that the ED_{50} of estradiol for both $ER\alpha$ and $ER\beta$ is about 2-4 nM, ER-mediated neuroprotection should be seen at compound concentrations at or slightly above this concentration. In most studies, much higher concentrations of β E2 are required to achieve neuroprotection.
- c. For ER-mediated effects, structure-activity relationship studies should reveal that neuroprotection is correlated closely with ER binding for ER-mediated neuroprotection. This is not the case in multiple studies (See Behl et al.1997, Green et al. 1997b, Moosmann and Behl, 1999 and Perez et al, 2005b for example).
- d. Finally, ER-mediated neuroprotection should not be observed in cell types that lack ERs.

3. In vitro evidence for neuroprotection by non-feminizing estrogens

We (Green et al., 1997b) and others (Behl et al., 1997; Moosmann and Behl, 1999) have determined that the phenolic nature of the estradiol molecule is essential for neuroprotection. Therefore, we synthesized estrogen analogues to perform structure-activity relationship (SAR) studies using rationally designed compounds in β E2-neuroprotection assays. Over 70 compounds were tested in HT-22 (murine hippocampal) cells for their ability to inhibit

cytotoxicity against glutamate and iodoacetic acid. The EC₅₀ (or IC₅₀) values for neuroprotection, ER binding, and protection against lipid peroxidation were determined to ascertain potency comparisons with β E2 (Perez et al., 2005b). The structures of some of the compounds tested for neuroprotection are shown in Figure 1.

Neuroprotective estratrienes that have electron donating constituents increases the redox potential of the phenoxy radical, providing better neuroprotective properties (Perez, et al., 2005b). The donated hydrogen radical can quench free radicals formed in oxidative stress conditions. A-ring derivatives with electron donating constituents that stabilized the phenoxy radical were more potent than β E2 in protecting these cells from oxidative stress-induced toxicity. Our primary synthetic strategy was to replace the hydrogen with a bulky alkyl group at the 2- or 4-positions of the A-ring. For those compounds that included the addition of a single group to the 2-carbon position of the A-ring or 4-carbon position of the A-ring, there was an increase in potency by approximately ~2- to 170-fold as compared to the parent compounds, β E2 and estrone. When two groups flanked the 3-OH position, neuroprotection was enhanced, with approximately 9- and 4-fold decrease in EC₅₀ values for protection against neurotoxicity, respectively. However, C3 methoxy ether analogues of the addition of a single group to the 2-carbon position of the phenolic A-ring that lack an additional phenol substituent, failed to protect cells against toxicity induced cell death. Switching the hydroxyl group to the 2-position and adding bulky groups (1-adamantyl or tert-butyl) to the 3-position greatly improved neuroprotective potencies of the parent compound (Perez et al., 2005b).

Introducing conjugated double bonds into the B- or C-rings of estratrienes is another way to increase the stability of the phenoxy radical. Compounds with these modifications were approximately 180- and 490-fold more potent than β E2 against glutamate and IAA toxicity, respectively. Steroids having an aromatic B-ring, similar to conjugated equine estrogens, with intact phenolic hydroxyl groups were more potent against IAA toxicity, but performed equally as well against glutamate toxicity as compared to β E2 (Perez et al., 2005b). Polar groups attached to the B- and C-rings disrupted estrogen's ability to protect cells from oxidative stress. These hydroxyl groups, which impart hydrophilicity to the molecule, added to the middle of the structure influence the way these steroids fit into the center, hydrophobic lipid bilayer and thus its ability to react with lipid oxidation events. Opening of the B ring causes decreased rigidity of the molecule, but had no effect on the ability of β E2 to protect against IAA-induced cell death and decreased β E2's ability to protect against glutamate induced cell death by approximately 6- to 12-fold. Producing a nonplanar conformation, such that the A- and B rings lie perpendicular to the C- and D-rings, had no effects on neuroprotection.

D-ring substituents alter lipophilicity; however, the addition of norpregna, norcholesta, benzoate and methylethers to the 17-position did not result in better neuroprotection, instead decreased the potency of the parent compound. Introduction of the side chain found in 25-hydroxycholesterol, a 2-hydroxy-1-methylethyl side chain, spirocyclopentyl groups to the C-16 position did not enhance neuroprotection, possibly due to the fact that the spiro group lies orthogonal to the A-, B-, C-, D-rings and alters the position of the estratriene in the lipid

membrane. However, complete removal of a 17-substituent enhanced neuroprotection (Perez et al., 2005b).

These results indicate that protection against toxicity requires a phenolic hydroxyl group. The hydroxy-phenol group does not have to be part of the A-ring since the addition of a hydroxyphenol group to the 3-position was equally or more protective against glutamate and/or IAA-induced toxicities. This and the fact that the nonsteroidal compounds with phenolic groups are still protective demonstrate that the position of the hydroxyl phenol group is not restricted to the 3-position of the steroid backbone. In concert with glutathione, estrogens were also potent neuroprotectants (in the nanomolar range) against A β (25–35) toxicity in SK-N-SH (Gridley et al., 1998) and HT-22 cells (Green et al., 1998). All of these structure-activity studies found that the phenolic A-ring was an absolute requirement for neuroprotection, as compounds such as mestranol and quinestrol did not have protect effects. That EC₅₀ values seen with some of the more potent compounds were in the low nanomolar range is the key for therapeutic purposes.

Other estrogen-like substances, such as conjugated equine estrogens (Zhao et al., 2003), phytoestrogens (Gelinas and Martinoli, 2002; Roth et al., 1999; Wang et al., 2001), and estrogen metabolites (2-OHestradiol) (Teepker et al., 2003) were also found to be neuroprotective, although not as potent as the compounds screened in our studies.

In summary, a critical feature of the estradiol structure and the stabilization of the phenoxy radical state has been identified that increased the potency against oxidative stress damage; thus, advancing our understanding of estrogen-mediated neuroprotection.

4. Estrogen Protection in a Friedreich's Ataxia Model

Recently, we have shown that estrogen-like compounds, including a group of non-feminizing estrogens, can protect an *in vitro* Friedreich's Ataxia (FRDA) cell model against oxidative stress (Richardson et al., 2011). In this model, human FRDA skin fibroblasts are treated with L-buthionine (S,R)-sulfoximine (BSO) to inhibit *de novo* glutathione (GSH) synthesis, increasing the susceptibility of the cells to oxidative damage induced by naturally occurring reactive oxygen species (ROS). Previous studies have shown that FRDA fibroblasts are especially vulnerability to this treatment, while normal fibroblasts are not (Jauslin et al., 2002; Jauslin et al., 2003). Our results demonstrate that in this cell type, estrogens act in a manner independent of any known ER to produce their potent protective effects. Consistent with previous reports (Behl et al., 1997; Green et al., 1997b; Moosmann and Behl, 1999; Perez et al., 2005b; Prokai et al., 2003a), the potency and efficacy of these estrogen-like compounds was dependent on the presence of at least one phenol ring in its structure, and in this cell model the potency seems to be directly correlated to the number of phenol rings in the compound (Richardson et al., 2011).

β E2, which contains a single phenol ring (Fig. 1), provided protection across a wide range of concentrations (Fig. 2) with an EC₅₀ of 15.5 nM, and this effect was not significantly inhibited by the co-application of ICI. The 3 phenol ring containing ER α selective agonist 4,4',4'-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) and the 2 phenol ring containing ER β selective agonist diarylpropionitrile (DPN) similarly provided protection in

the very low nM range, with EC₅₀ values of 4.6 nM and 9.3 nM, respectively (Fig. 3). Again, these compounds were not antagonized by ICI. The non-feminizing estrogen 2-(1-adamantyl)-4-methylestrone (ZYC-26; Fig. 1), a compound with one phenol ring, but no ER binding ability due to the presence of an adamantyl group, was also potently cytoprotective in this system, with an EC₅₀ of 23.1 nM (Fig. 4A). In contrast, the non-feminizing estrogen 2-adamantyl, 3-O-methyl estradiol (ZYC-23; Fig. 1), which is very similar in structure to ZYC-26, with the phenol group replaced by an O-methyl group, had no significant protective effect (Fig. 4B). In addition, the membrane ER (mER) G protein-coupled receptor 30 (GPR30) agonist, G1, had no significant protective effect (Fig. 5A) and the GPR30 antagonist G15 over a concentration range of 1nM – 10 μ M was unable to inhibit the protective effect of 100 nM β E2 (Fig. 5B). These results are consistent with data showing that the level of ROS attenuation in this FRDA cell system is directly correlated to the presence and number of phenol rings present in the compound, substantiating the hypothesis that in the FRDA fibroblast model it is the phenol ring and its antioxidant potential that is the essential part of the estrogen molecule in terms of neuroprotection (Richardson et al., 2011).

These data clearly indicate that β E2 and estrogen-like drugs are capable of acting independently of ER α , ER β or GPR30, as long as at least one phenol ring is present in the molecular structure in this cell model. All tested phenolic estrogens were able to protect the FRDA fibroblasts from BSO-induced oxidative stress, ultimately preventing significant cell death, while the non-phenolic estrogens tested were not. This cell model provides evidence and support for the use of non-feminizing estrogens to potentially protect cells and tissues from stressors commonly associated with neurodegenerative diseases.

5. In vivo evidence for neuroprotection by non-feminizing estrogens

Both endogenous and exogenous β E2 administration attenuate infarct volume and are neuroprotective against the pathological events seen in experimental animal models of cerebral ischemia (Green et al., 1996, 1997a,b, 1998; Gridley et al., 1997; Alkayed et al., 1998; Dubal et al., 1998; Green and Simpkins, 2000; Green et al., 2001; Simpkins et al., 2005; Sudo et al., 1997; Yang et al., 2000). We selected several of our estratrienes for in vivo assessment against a routinely used model of cerebral ischemia, transient MCAO, in ovariectomized rats. To date, we have assessed eight compounds in this model, including β E2, estrone, α E2, ent-E2, enantiomer of 17-desoxyestradiol (ZYC-13), 2-(1-adamantyl) estrone (ZYC-3), 2-(1-adamantyl)-4-methylestrone (ZYC-26), and 3-O-methyl analog of (17 β)-2-(1-adamantyl)estradiol (ZYC-23). Consistent with our in vitro studies, β E2, estrone, α E2, ent-E2, enantiomer of 17-desoxyestradiol (ZYC-13), 2-(1-adamantyl) estrone (ZYC-3), and 2-(1-adamantyl)-4-methylestrone (ZYC-26) exert potent neuroprotective effects in focal cerebral ischemia/reperfusion injury (Fan et al., 2001; Green et al., 2001; Liu et al., 2002; Simpkins et al., 1997b, 2004). Subcutaneously implantation of α E2 24 hours before MCA occlusion showed neuroprotective effects (Simpkins et al., 1997b) even though α E2 binds to ER-isoforms with an approximately 40-fold lower affinity (Perez et al., 2005a). Two-hour pretreatment with E1 before the onset of transient MCA occlusion significantly reduced infarct volume compared with vehicle control following 24-hour reperfusion in ovariectomized rats. The quinol-conjugated E1 was equipotent with the parent

estrogen in reducing lesion. Ent-E2, which has shown to be less than one-eighth as active as β E2 in competitive bind assays for human recombinant ER α and ER β , is as effective as β E2 in reducing infarct size after MCA occlusions. There was no evidence of metabolic conversion of ent-E2 to β E2 in ovariectomized rats and no uterotrophic response to 3 days of administration (Green et al., 2001). These data suggests that non-feminizing enantiomers of estrogens can be potentially neuroprotective from human ischemic brain damage. ZYC-3 showed no binding to either ER α or ER β in competitive binding assays but was 6-times more potent than β E2 in a test for neuroprotection against glutamate toxicity. Further, in our in vivo model of transient MCA occlusion, ZYC-3 performed better than β E2 without affecting physiological parameters (Liu et al., 2002). The placement of a bulky adamantyl group in the 2-position of the A-ring of the estrone eliminates estrogenicity, enhances antioxidant potential (Perez et al., 2005b), and enhances the potent neuroprotective activity of estrogens against ischemic brain damage. ZYC-26 has an adamantyl moiety on the 2-carbon and a methyl group on the 4 carbon of estrone. ZYC-23 is similar, but the phenolic nature of the A-ring is eliminated through O-methylation of the 3-carbon, a chemical change that completely eliminate neuroprotection of estrogens (Simpkins et al., 2004). Neither of these compounds binds to either estrogen receptor. Similar to our in vitro study, ZYC-26 reduced lesion volume. As expected, no protective effects of ZYC-23 were seen in vitro or after transient MCA occlusion.

6. Future Directions

Having been extensively tested in in vitro and in vivo models for stroke, there is a need to conduct pharmacokinetic assessments of one or more of the most potent estrogen analogues and to begin to formulate these compounds for future clinical studies. Since these activities are not competitive for NIH funding, pharmaceutical support for a drug development program on non-feminizing estrogens for neuroprotection is needed.

7. Summary and conclusions

We are actively pursuing the optimization of estrogen analogues that do not bind ERs, but which are more potent neuroprotectants than β E2. This program has demonstrated that estrogen analogues with large bulky groups at the 2 and/ or 4 carbon of the phenolic A ring eliminate ER binding but enhance neuroprotective potency in cell culture screening systems. These A-ring derivatives work in part by increased electron donating capacity that stabilized the phenoxy radical. Our estrogen analogues are also potent neuroprotectants in an animal model of cerebral ischemia. Studies are in progress to understand the mechanism by which these analogues are neuroprotective. The discovery of these non-feminizing estrogens opens the possibility of making these compounds more “drug-able” and thereby producing nonfeminizing estrogens that can achieve the beneficial effects of estrogens without the unwanted side-effect related to interaction with ERs. Such an achievement could markedly change our approach to postmenopausal HT, as well as treatment of brain injuries.

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Highlights

- Estrogens have been shown to have an intrinsic antioxidant structure that lies in the phenolic ring of the compounds, which provide the antioxidant/ redox cycling activity in neurons.
- We and others have determined that the phenolic nature of the estradiol molecule is essential for neuroprotection.
- Synthetic neuroprotective estrogens that have electron donating constituents increases the redox potential of the phenoxy radical, providing better neuroprotective properties.
- We have shown that estrogen-like compounds, including a group of non-feminizing estrogens, can protect an *in vitro* Friedreich's Ataxia (FRDA) cell model against oxidative stress.
- Both endogenous and exogenous estradiol administration attenuates infarct volume and is neuroprotective against the pathological events seen in experimental animal models of cerebral ischemia.

Figure 1.
Structures of 17β-estradiol (E2) and several phenolic and non-phenolic estrogens assessed for neuroprotective effects.

Figure 2.

Effects of 17 β -estradiol (E2) on cell viability in BSO-treated FRDA fibroblasts. Depicted are mean $\pm$ 1 SD for n= 8 per group. * indicated p<0.05 versus BSO alone-treated cells. ICI indicated ICI 182,780. Republished from Richardson et al., 2011, with permission.

Figure 3. Effects of an ER α preferring agonist, PPT (A), and an ER β preferring agonist DPN, (B) on cell viability in BSO-treated FRDA fibroblasts. Depicted are mean $\pm$ 1 SD for n= 8 per group. * indicated $p < 0.05$ versus BSO alone-treated cells Republished from Richardson et al., 2011, with permission.

Figure 4.

Effects of non-feminizing estrogens on cell viability in BSO-treated FRDA fibroblasts. Depicted are mean $\pm$ 1 SD for $n=8$ per group. * indicated $p<0.05$ versus BSO alone-treated cells. ICI indicates ICI 182,780. ZYC-26 (A) indicates 2-adamantyl, 4 methyl estrone. ZYC-23 (B) indicates 2-adamantyl, 3-0-methyl estradiol. Republished from Richardson et al., 2011, with permission.

Figure 5.

Effects of the membrane ER-preferring agonist, G1 (A), on cell viability in BSO-treated FRDA fibroblasts, and effects of the membrane ER-preferring antagonist, G15 (B), on E2-induced enhancement of cell viability in BSO-treated FRDA fibroblasts. Depicted are mean ± 1 SD for n= 8 per group. * indicated $p < 0.05$ versus BSO alone-treated cells. Republished from Richardson et al., 2011, with permission.