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Estradiol Exerts Neuroprotective Actions Against Ischemic Brain Injury

Insights Derived from Animal Models

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Over the last 100 yr, the life-span of women has increased from 50 yr to over 80 yr, but the age of the menopause has remained unchanged, at 51 yr. Menopause is one of the most permanent physiologic changes that a woman will experience and is marked by a dramatic decrease in circulating levels of ovarian estrogens. Because the timing of menopause has remained fixed in the face of an increasing life-span, more women will live a greater proportion of their lives in a hypoestrogenic state. We appreciate more and more that the actions of ovarian steroid hormones are complex, and possibly exert opposing actions in different contexts. I review here the results of my laboratory's recent studies that clearly establish that low physiologic levels of estradiol replacement can exert profound neuroprotective actions when administered prior to an ischemic strokelike injury.

Key Words: Neuroprotection; estrogen receptors; apoptosis; menopause; ischemia; stroke.

Introduction

During most of the existence of the human species, the average life expectancy for women was between 20 and 40 yr. Today, the average life-span of women is 83 yr. In evolutionary terms, the rate of the increase in life expectancy that has occurred over the last century is nothing less than astounding. During this period, the age of the menopause has remained unchanged and continues today to occur at approx 50 yr of age. Hence, today most women will spend a third of their lives in the postmenopausal state, when estrogen secretion by the ovary is virtually nil. We once thought that ovarian steroids influenced predominantly reproductive targets, such as the hypothalamus, anterior pituitary, mammary glands, ovaries, and organs of the reproductive tract. More recently, we know that these hormones are pleiotropic and act beyond the scope of their reproductive functions to influence many nonreproductive organs, including regions of the brain such as the hippocampus, cerebral cortex, and striatum, areas that influence learning, memory, and balance; bone and mineral metabolism; the heart and vascular system; and the immune system. Because all of these physiologic systems depend upon estrogens to maintain normal function, it is not surprising that the absence of these hormones in postmenopausal women who do not take estrogen replacement therapy impacts the health of older women. Many clinical studies show that estradiol, the major estrogen synthesized by the ovary, protects against osteoporosis, cardiovascular and neurologic diseases, and brain injury sustained after a cerebrovascular stroke or other insults. Yet, other studies suggest otherwise: they report a lack of protection or increased health risks of hormone replacement therapy in the occurrence of cerebrovascular stroke *(1)*, cardiovascular disease *(2)*, Alzheimer disease *(3)*, and invasive breast cancer *(4)*. We will review on our work that clearly demonstrates that low physiological levels of estradiol replacement exert profound protective actions both in vivo and in vitro, and our work that establishes the multiple mechanisms that estradiol utilizes to achieve these neuroprotective effects. Other more extensive reviews (see refs. *5–9*) clearly show that several laboratories have contributed to our understanding of these novel nonreproductive actions of estrogens in maintaining normal brain function.

Estradiol Protects Against Permanent Middle Cerebral Artery Occlusion

Our initial work assessed whether physiologic levels of estradiol prevent brain injury in an in vivo model of permanent, focal ischemia *(10)*. Rats were ovariectomized (OVX) and implanted, immediately or at the onset of middle cerebral artery occlusion (MCAO), with Silastic capsules that produced physiologic low or physiologic high 17β-estradiol levels in serum (10 or 60 pg/mL, respectively). One week after ovariectomy, we occluded the middle cerebral artery using the methods of Longa et al. *(11)*. This method decreases

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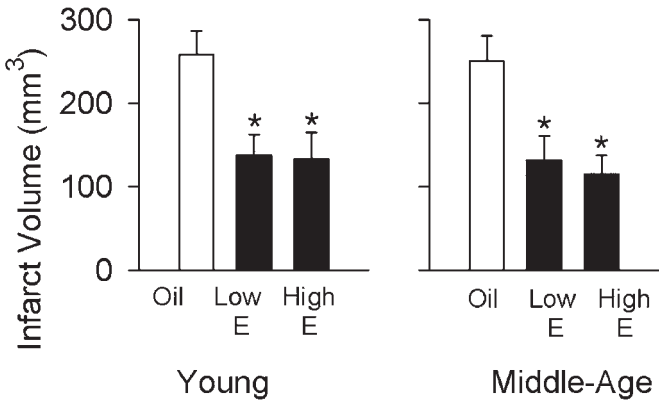

Fig. 1. Estradiol protects against MCAO in young and middle-aged rats. Low and high physiologic levels of estradiol decreased total injury ($p < 0.05$), as measured by staining of brain sections with 2% triphenyltetrazolium chloride and measurement of infarct size using a computer-assisted imaging system and NIH Image. (From ref. *12* with permission.)

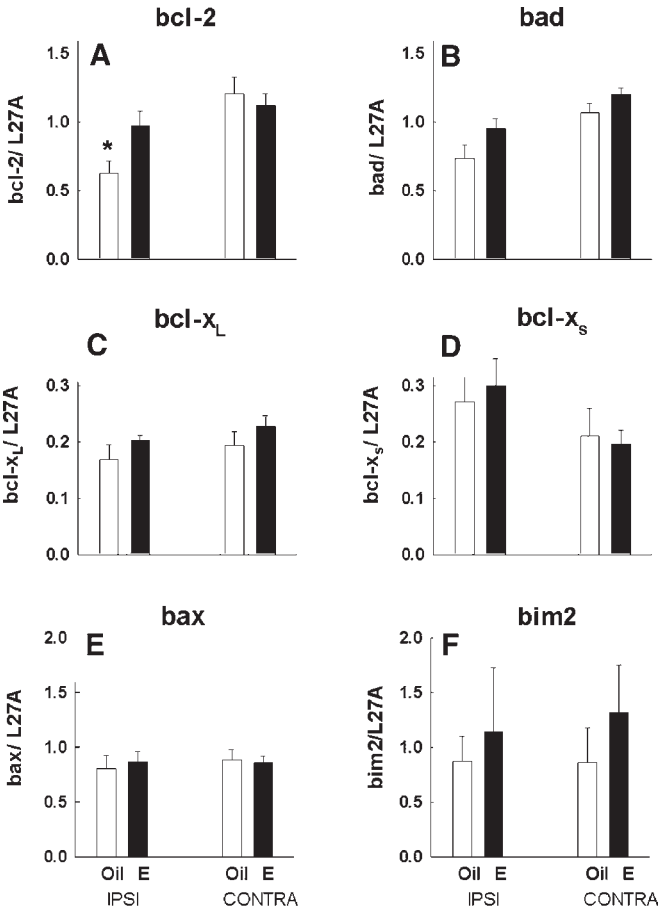

Fig. 2. MCAO injury and estradiol replacement interact and influence gene expression of the bcl family of genes in the cerebral cortex. Injury decreased the level of *bcl-2* gene expression on the ipsilateral side of injury ($p < 0.05$). Estradiol pretreatment prevented the injury-induced downregulation of *bcl-2* gene expression. No other member of this family of genes, which are critical to the evolution of apoptosis, was influenced by estradiol replacement.

cerebral blood flow to the cerebral cortex by approx 50% *(10)*. Estradiol pretreatment significantly reduced overall infarct volume compared to oil-pretreated controls in both young and middle-aged rats (Fig. 1). We were surprised to find that estradiol exerted equivalent protection in middle-aged rats *(12)*, since we have previously reported that responsiveness of the hypothalamus to estradiol is severely compromised, regardless of the end point measured, when animals reach this age *(13)*. The fact that estradiol continues to protect even as rats age suggests that estradiol's mechanisms of action in this brain region may require different factors than those in the hypothalamus, and that this constellation of factors may be preserved in a brain region–specific manner. This protective effect was regionally specific to the cortex, since no protection was observed in the striatum (data not shown). When estradiol replacement was delayed until the time of the MCAO, we could not detect any protection (data not shown). The lack of protection when estradiol is administered acutely contrasts with the findings of Green and Simpkins *(6)* and Hurn and Macrae *(7)*, who found that higher, more pharmacologic levels of estrogen replacement can effectively protect against brain injury even when administered at the time of ischemia or even after the injury is induced. Our findings that estradiol pretreatment reduces injury demonstrates that physiologic levels of estradiol can protect against neurodegeneration.

Estradiol Alters Expression of Multiple Genes That May Influence Ability of Cells to Survive Injury

Using our model of permanent cerebral artery occlusion and low physiologic levels of estradiol replacement, we began to elucidate potential mechanisms of estradiol action in injury. Bcl-2 is a protooncogene that promotes cell survival in a variety of tissues including the brain. Since it was known that estradiol promotes cell survival via Bcl-2 in non-neural tissues, we tested the hypothesis that estradiol decreases cell death by influencing *bcl-2* expression in ischemic brain injury. Furthermore, since estradiol may protect the brain through estrogen receptor (ER)–mediated mechanisms, we examined expression of both receptor subtypes, *ERα* and *ERβ* in the normal and injured brain. We analyzed gene expression by using reverse transcriptase polymerase chain reaction in microdissected regions of the cerebral cortex obtained from injured and sham female rats treated with estradiol or oil. Our data demonstrate that estradiol pretreatment prevents the injury-induced downregulation of *bcl-2* gene expression. This effect was specific to *bcl-2*; expression of other members of the Bcl-2 family (*bax, bcl-x$_L$, bcl-x$_S$, bad,* or *bim*) was unaffected by estradiol treatment (Fig. 2).

Using the same microdissected tissue punches, we found that ERs were differentially modulated in injury *(14)*. We discovered that the expression of *ERα* was dramatically

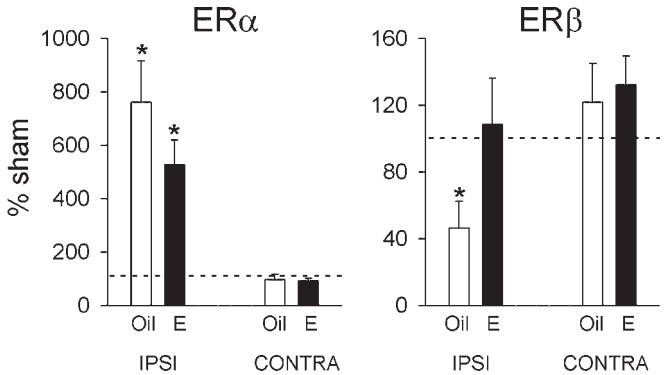

Fig. 3. ERα and ERβ gene expression are differentially modulated after MCAO. (**A**) ERα mRNA was dramatically upregulated in the ipsilateral cortex of oil (vehicle)- and estradiol-treated rats compared with the contralateral cortex ($p < 0.05$) and compared to constitutive levels in both oil- and estradiol-treated sham controls ($p < 0.05$). (**B**) Injury reduced ERβ gene expression on the ipsilateral side of injury ($p < 0.05$). Estradiol treatment prevented the injury-induced downregulation of ERβ mRNA in the ipsilateral cortex. (From ref. *14* with permission.)

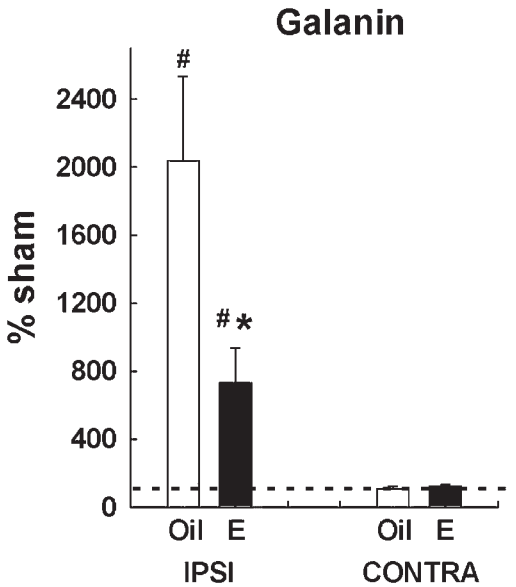

Fig. 4. Galanin gene expression is dramatically upregulated by MCAO and this effect is attenuated by estradiol pretreatment. Galanin gene expression was upregulated approx 20-fold within 24 h of MCAO ($^{\#}p < 0.05$). Estradiol pretreatment attenuated this increase ($^{*}p < 0.05$ compared to OVX oil-treated rats that underwent MCAO).

upregulated, following injury (Fig. 3). While estradiol did not influence the extent of this increase, the presence of elevated levels of ERα in the injured cortex may contribute to estradiol's ability to protect. The increase in *ERα* expression is reminiscent of its expression during early postnatal development, during the interval of sex-specific differentiation of the cortex and extensive neurogenesis and neuritogenesis *(15,16)*. It is possible that the injury-induced upregulation of *ERα* is a component of a dedifferentiation and/or recapitulation of this stage of development and an attempt to reenter the cell cycle, which is hypothesized to occur in response to injury *(17,18)*. Injury and estradiol influence the expression of the newly discovered ER, *ERβ*, and this effect was strikingly parallel to its effect on *bcl-2* gene expression; that is, estradiol prevented the injury-induced downregulation of *ERβ*. The function of this novel ER subtype is not clear. However, the discovery of ERβ in 1996 *(19)*, and the subsequent localization of its mRNA in the regions where ERα is sparse or absent, including the cerebral cortex and hippocampus *(20)*, suggested initially to us that estradiol may act through ERβ to protect against injury. Regardless of which receptor is involved, these initial findings suggested that ERs may be involved in hormone-mediated neuroprotection and that they may play novel and unique roles in the injured brain.

Another gene whose expression is regulated by both injury and estradiol is galanin. Galanin gene expression is upregulated in Alzheimer's disease and other circumstances of neurodegeneration. Therefore, we speculated that ischemic injury may alter its expression. To our great surprise, we found that galanin mRNA levels increase by approx 20-fold and that estradiol replacement therapy decreases its expression significantly (Fig. 4) *(21)*. At the present time, we are investigating whether these changes are functionally relevant and whether downregulation of this gene by estradiol may be a component of estradiol's ability to protect.

Ability of Physiologic Levels of Estradiol to Protect Require Presence of ERα

Our finding that injury affects the expression of both ERα and ERβ led us to investigate whether either or both of these receptors play pivotal roles in mediating neuroprotective actions of estradiol. To perform these studies, we utilized ERα-knockout (ERαKO) and ERβKO mice in an animal model of stroke. We performed our studies using the same controlled endocrine paradigm that we had previously used in our studies with rats. This was particularly important since endogenous levels of estradiol differ dramatically between ERαKO, ERβKO, and wild-type (WT) mice. Thus, we ovariectomized ERαKO, ERβKO, and respective WT mice and implanted them with capsules filled with either oil (vehicle) or a dose of estradiol that produces physiologic hormone levels in serum. One week later, mice underwent MCAO. Our results clearly demonstrate that deletion of ERα completely abolishes the protective actions of estradiol in all regions of the brain, whereas estradiol's ability to protect against brain injury is totally preserved in the absence of ERβ (Fig. 5) *(22)*. These results differ with those of Sampei et al. *(23)*, who reported that in gonadally intact WT and ERα-KO mice, protection does not depend on ERα. The compari-

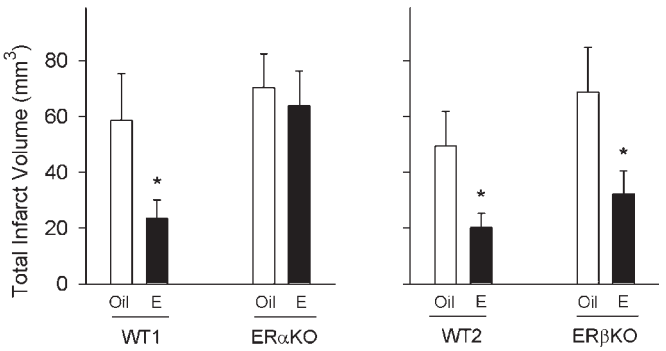

Fig. 5. Estradiol protects against MCAO in WT mice of both genetic backgrounds and in ERβKO mice, but not in ERαKO mice. (**A**) Estradiol significantly decreased infarct volume in WT compared with oil (vehicle)-treated controls ($p < 0.05$). By contrast, in ERαKO mice, estradiol did not exert any protective effect. (**B**) Estradiol significantly decreased infarct volume in WT and ERβKO mice ($p < 0.05$). Brain sections were stained with hematoxylin and eosin, and the volume of the infarct was quantified with a computer-assisted imaging system using NIH Image. (From ref. *22* with permission.)

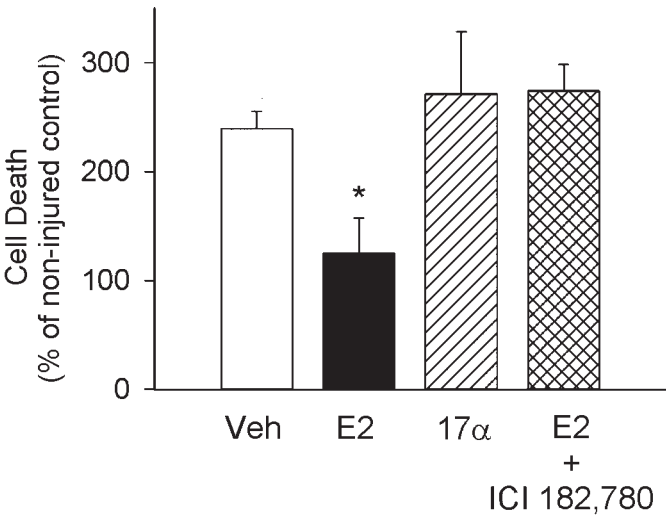

Fig. 6. Estradiol protects against ischemic injury–induced cell death in explant cultures of the cerebral cortex. Lactate dehydrogenase (LDH) release in controls and following ischemic injury reveals that 17β-estradiol protected against cell death ($p < 0.05$). However, 17α-estradiol failed to protect and ICI 182,780, an ER antagonist, blocked the protective actions of 17β-estradiol. (From ref. *24* with permission.)

son between WT and ERαKO mice that are gonadally intact is difficult to interpret because the estradiol concentrations in ERαKO mice are dramatically higher than in WT controls. This is important to consider because the mechanisms by which estradiol exerts protective actions are diverse and depend, in part, on the dose of the steroid. Thus, previous studies suggest that physiologic levels of estradiol may protect through receptor-dependent mechanisms, whereas higher concentrations of estradiol may act through mechanisms that do not require the presence of ERs (reviewed in ref. *5*). Hence, the work of Hurn and colleagues *(7,23)* further confirms that pharmacologic levels of estradiol protect through receptor-independent mechanisms, but it does not address the potential role of ERs in mediating the physiologic levels of estradiol to protect. Thus, our results clearly establish that the ER subtype ERα is a critical mechanistic link in mediating the protective actions of physiologic levels of estradiol in brain injury. We believe that these findings that ERα mediates protection of the brain carries far-reaching implications for the selective targeting of ERs in the treatment and prevention of neural dysfunction associated with normal aging or brain injury.

In Vitro Methods Complement Our Studies In Vivo

Methods to induce stroke-like injury in the adult and aging brain are extremely laborious. In addition, alone, results from in vivo studies may sometimes be difficult to interpret because it is not possible to differentiate the direct effects of estadiol or injury on the brain from the indirect effects on the vasculature, the endocrine system, or other possible targets of injury or estradiol. In vitro methods are invaluable tools that complement in vivo approaches. Multiple manipulations can be performed in vitro that are not technically or financially feasible using in vivo models. We have implemented organotypic explants because they provide a powerful way to manipulate cellular environments in vitro, while maintaining interneurons, spatial relationships, local synaptic connections, and interactions with the local glial environment. We established explant cultures of rat cerebral cortex to determine whether we can replicate estradiol's protective actions in an in vitro model of brain injury *(24)*. Using these organotypic cortical explant cultures, we began to decipher the mechanisms of estradiol's actions. Sections of the cerebral cortex were taken from rat pups on postnatal d 2 to 3 and cultured in roller tubes for 7 d in the presence or absence of estradiol. Injury was induced by exposure to kainic acid or potassium cyanide/2-deoxyglucose (KCN/2-DG) for varying lengths of time, and cell death was monitored by lactate dehydrogenase release at multiple times after injury. We found that exposure to this injury produced consistent delayed cell death. The presence of estradiol during the 7 d prior to injury significantly reduced the extent of cell death (Fig. 6). The protective effects of estradiol were dose dependent: low doses of E_2 (1, 10, and 30 n*M*) significantly reduced cell death; however, higher concentrations of E_2 (>60 n*M*) had no protective effect (data not shown). The observations that low levels of E_2 protect against cell death and that pretreatment is required suggested that the protective actions of estradiol may involve estradiol receptors. Therefore, we examined the ability of 17α-estradiol, which does not efficiently activate the estradiol recep-

tor, and the addition of the estradiol receptor antagonist, ICI 182,780, to influence the extent of cell death induced by KCN/2-DG and found that 17α-estradiol failed to protect and ICI 182,780 prevented 17β-estradiol from protecting against cell death (Fig. 6). Our results clearly show that in cortical explant cultures, estradiol protects cells against ischemic injury and strongly suggest that these protective actions involve estradiol receptors.

Conclusion

Our results firmly establish that low physiologic levels of estradiol replacement exert profound protective actions against brain injury induced by an MCAO, an experimental manipulation that mimics ischemic cerebrovascular stroke. We have further established that ERα plays a critical functional role in protecting against neuronal cell death. We suggest that estradiol, acting through its receptor, influences the expression of multiple genes to tip the balance toward cell survival and away from cell death. Our work using in vitro methods complements our in vivo studies and shows that injury can be attenuated when low concentrations of estradiol are included in the culture medium prior to injury and that similar mechanisms that depend on the presence of ERs are involved in neuroprotection.

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