

Are Estrogens Protective or Risk Factors in Brain Injury and Neurodegeneration? Reevaluation after the Women's Health Initiative

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Estrogens are essential for normal reproductive function. In addition, they exert important, complex, and diverse nonreproductive actions on multiple tissues. Although accumulating evidence from basic science studies using animal models suggests that estradiol plays a critical neuroprotective role against multiple types of neurodegenerative diseases and injuries, recent clinical studies have reported either inconclusive or untoward effects of hormone therapy on the brain. We focus herein on the work that we have done during the past 6 yr that strongly suggests that low levels of estradiol therapy exert dramatic protective actions in the adult injured brain.

Our results reveal that 17 β -estradiol slows the progression of this injury and diminishes the extent of cell death by suppressing apoptotic cell death pathways and enhancing expression of genes that optimize cell survival. Furthermore, we have found that estrogen receptors play a pivotal functional role in neuroprotection. Together, these results carry broad implications for the selective targeting of estrogen receptors in the treatment of neurodegenerative conditions resulting from disease or injury, particularly for aging, postmenopausal women. (*Endocrine Reviews* 26: 308–312, 2005)

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I. Introduction

NUMEROUS BASIC SCIENCE studies using animal models and *in vitro* cell and explant cultures, observational studies performed with human populations, and clinical trials led to the belief that ovarian hormones play an important role in providing women with protection against neurodegenerative diseases. However, whether hormone therapy (HT) in postmenopausal women is beneficial has become controversial because of the seemingly contradictory results when we compare these earlier studies with the recent results of the Women's Health Initiative (WHI) (1–4). The results that emerged from these clinical trials showed that use of conjugated equine estrogen (Premarin) with and without simultaneous medroxyprogesterone acetate (Prempro) lacked treatment benefit and increased risk of ischemic stroke. Before we accept these findings as the final word on the effects of HT on the adult brain, we should consider that this clinical trial used a specific HT regime on a group of older postmenopausal women, many of whom were obese. Whether or not different hormone preparations, initiated during the perimenopausal transition with little interruption with the normal menstrual cyclicity, would be efficacious remains unknown.

In our studies, we have used an animal model of cerebrovascular stroke to assess whether estradiol influences the extent of injury, under what circumstances it protects, and the mechanisms that underlie protective actions. We should emphasize that to date, most clinical studies have assessed whether estradiol therapy (ET)/HT alter the risk and mortality of stroke, but studies have not addressed whether estradiol decreases the extent of brain injury resulting from stroke or the cellular and molecular basis of decreased injury or improved repair. Most basic science studies have focused attention on whether ET/HT decrease the extent of injury and cell death after a stroke event has been experimentally induced. This is a very different question and must be appreciated when we compare data from clinical *vs.* basic science studies.

II. Definition and Incidence of Cerebrovascular Stroke

Cerebrovascular stroke has been classified into two broad categories: 1) ischemic stroke, in which there is an interruption of blood flow to the brain due to an embolism that becomes lodged in a cerebral vessel; or 2) hemorrhagic stroke, which involves a bursting of cerebral vessels or compression of brain tissue. According to the American Heart Association, ischemic stroke is over four times more common than hemorrhagic stroke (see Ref. 5 and <http://www.americanheart.org>). In stroke, decreased cerebral blood flow deprives neurons and surrounding cells of crucial substrates (*e.g.*, glucose and oxygen), and cells in the region will ultimately die. Neurons are particularly vulnerable to the ischemic insult. The pattern of cell death displayed depends upon the severity of the ischemic insult. Complete, permanent vessel occlusion leads to pan-necrosis, in which all cell types in the affected area die due

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Abbreviations: ER, Estrogen receptor; ET, estradiol therapy; HT, hormone therapy.

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to metabolic failure. In areas where collateral vessels lead to maintenance of some, albeit decreased, blood flow, neurons will undergo a delayed form of cell death, or apoptosis. Therefore, in a typical ischemic stroke, there is often a core area of necrotic damage in brain regions supplied solely by the blocked vessel. A border zone, known as the ischemic penumbra, surrounds this core area. This area is less severely affected due to the presence of some level of collateral circulation supplied by branches of other major vessels. It is salvageable if the environment is optimized through administration of growth factors (6–8) or hormones (9, 10) or suppression of the inflammatory response (11).

Ischemic stroke can involve a permanent blockage of a cerebral vessel by an embolism or a transient blockade of a cerebral vessel. Both the occlusion and the restoration of blood flow (reperfusion) to ischemic tissue cause cellular damage, possibly by different molecular mechanisms (12–14). Reperfusion injury may be mediated by various second messenger signaling pathways, particularly protein kinase C, unregulated neurotransmitter release, decrease in cellular energy stores, inhibition of electron transport, and inflammation (for review, see Ref. 15).

The risk of stroke is lower in premenopausal women than in age-matched men and increases in postmenopausal women to become equal to that of men by the time women are over 65 yr old (see Ref. 16 and <http://www.americanheart.org>). This observation led investigators to postulate that ET and HT may protect postmenopausal women. Indeed, several studies over the past 20 yr supported this contention; however, the clinical and epidemiological studies were not uniformly positive. Paganini-Hill (17) reviewed the studies that were performed before 1995 and concluded that the bulk of the evidence suggests that ET decreased the risk of stroke. In 2001, the Women's Estrogen for Stroke Trial indicated that estrogens do not protect against the rate of either nonfatal stroke or death in postmenopausal women with a history of stroke (18). The most recent findings of the WHI demonstrate that combined conjugated equine estrogens with and without concomitant medroxyprogesterone acetate exacerbates the risk of ischemic stroke in women who were postmenopausal for approximately 12 yr before the initiation of ET/HT (4, 19). The results of these studies are consistent with those that fail to detect estrogen-mediated protection of the heart in women who already suffer from cardiovascular disease (20) or of the brain in women who already exhibit signs of Alzheimer's disease (21). Together, these studies suggest that estrogen does not effectively protect against or reverse a disease process that has already been initiated. They suggest that this hormone may be able to protect against the initial stages of disease progression but is not an effective hormone of repair when damage from disease has progressed to a significant degree.

III. Experimental Models of Stroke and the Role of Estrogens

We have used an animal model in which the middle cerebral artery is permanently occluded to examine the effects

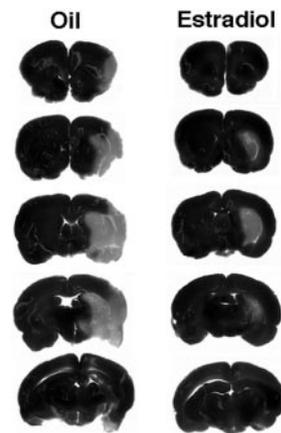


FIG. 1. Physiological levels of estradiol decrease ischemic brain damage after stroke injury. Representative coronal sections obtained from oil (*left*) and estradiol (*right*)-treated rat brains collected 24 h after the onset of ischemia and stained with hematoxylin and eosin. Ischemic injury, produced by permanent middle cerebral artery occlusion, appears unstained and is distributed across the cerebral cortex and striatum. Pretreatment with low levels of estradiol dramatically decreases the extent of stroke injury, compared with oil-treated controls.

of estradiol on neurodegeneration (22). In all of our studies that we describe below, we have used exclusively the 17 β form of estradiol. As discussed below, many studies, including our own, have investigated whether estradiol can attenuate cell death resulting from ischemic injury and whether the mechanisms of protection against cell death involve suppression of apoptotic signaling. We have found that low, physiological doses of ET are sufficient to exert dramatic protection against stroke injury in young female rats (Fig. 1) (23). Our endocrine paradigm produces fairly steady levels of approximately 15 pg/ml of estradiol in serum, which are strikingly similar to the levels we observe in rats during all days of the estrous cycle, except on proestrus, when the rise is in preparation for the ovulatory surge of gonadotropins. Using these concentrations of estradiol, we found that ET

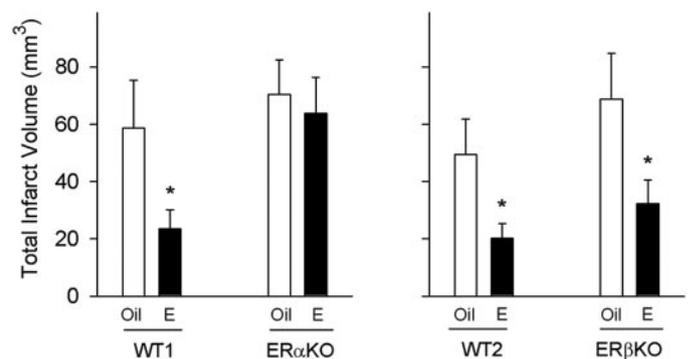


FIG. 2. ER α is critical in estradiol-mediated protection of the brain after stroke injury. Estradiol reduces ischemic infarct in both wild-type mice, WT1 and WT2, compared with respective oil-treated controls. Estradiol fails to protect in ER α knockout (ER α KO) mice, compared with oil-treated controls, but continues to protect in ER β KO mice, compared with oil-treated controls. Values represent mean \pm SE (n = 7–13 per experimental group). *, Estradiol-treated mice were statistically significantly different from oil-treated controls. [Adapted with permission from D.B. Dubal *et al.*, *Proc Natl Acad Sci USA* 98:1952–1957, 2001 (26), copyright by the National Academy of Sciences U.S.A.]

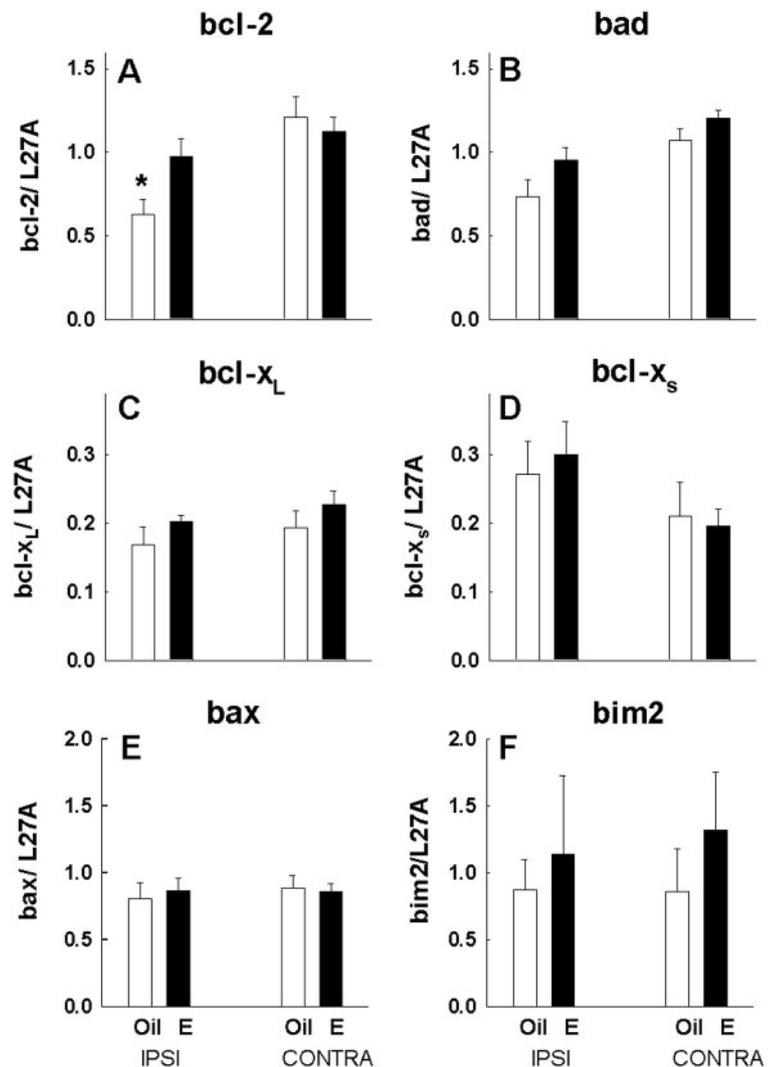


FIG. 3. Differential modulation of the bcl-2 family of genes in estradiol-treated rats that have undergone middle cerebral artery occlusion. Estradiol preserves expression of bcl-2 mRNA on the ipsilateral (IPSI) side of injury and has no effect on the contralateral (CONTRA) side of the brain. Estradiol did not influence the expression of any of the other members of the bcl-2 family that we investigated. Data represent mean \pm SE ($n = 7-11$ per experimental group). *, Oil-treated rats were statistically significantly different from estradiol-treated rats.

must be administered before middle cerebral artery occlusion and that treatment at the time of the injury does not decrease the extent of the infarct. We found that the protection does not involve estradiol-enhanced blood flow or increased blood glucose levels. In addition, we found that ET did not protect against the initial stages of injury, but instead protected against the later stages of injury. These initial results clearly established that very low levels of estradiol exert profound neuroprotective actions against stroke-like injury. They implied that ET protected predominantly by decreasing delayed cell death. Other researchers have used higher concentrations of estradiol, which reach 100 to 10,000 times the concentrations that we have used and found that animals can be treated up to 3 h after middle cerebral artery occlusion and still benefit from exposure to estradiol and estrogen-like compounds (24). Thus, these higher concentrations of estrogens appear to act through very different mechanisms: 1) estrogens affect cerebral blood flow and may prevent injury by causing vasodilation; 2) estrogens increase blood glucose concentrations and may allow neurons to be in a more protective metabolic environment; 3) estrogens act as antioxidants and may prevent free radical generation-induced cell

death; and 4) estrogens influence the production of nitric oxide and may influence vascular endothelial cell function to attenuate damage to the vasculature.

We investigated whether estradiol-induced neuroprotection requires estrogen receptor (ER) α and/or ER β and discovered that ER α plays an essential functional role in protecting against cell death. Our data reveal that physiological levels of estradiol induce the reappearance of ER α gene and protein expression on the ipsilateral side of injury (25). This gene is expressed at high levels during neonatal development when the cortex undergoes neurite outgrowth and synaptogenesis but decreases during later stages of development and in adulthood. Injury-induced reappearance of ER α gene and protein expression may indicate that the injured brain provides signals that communicate the need for expression of a receptor that is required for reinitiation of "developmental" events in the injured cortex. We found that the presence of this ER subtype is essential to protect against cerebral ischemia (26, 27). We used ER α or ER β knockout mice and found that when the ER α is absent, estradiol no longer protected against middle cerebral artery occlusion-induced cell death (Fig. 2). In marked contrast, in the absence

of ER β , estradiol continued to exert its protective effects. These studies clearly establish that the classic estrogen receptor, ER α , which was once thought to be more important in reproductive functions than in nonreproductive functions, is the critical mechanistic link in the ability of low levels of estradiol to protect against delayed cell death.

We have begun to assess the repertoire of downstream genomic targets of estradiol action through ERs. To date, we have reported that estradiol modulates the expression of multiple genes in ischemic brain injury, including ones that regulate the balance of cell death and cell survival factors (25) and immediate early genes, such as *c-fos* (28). Two examples of genes that respond to estradiol after middle cerebral artery occlusion illustrate the principle that multiple genes are either up-regulated or down-regulated.

As a first example, *Bcl-2* is a protooncogene that promotes cell survival in a variety of tissues including the brain. Because estradiol is known to promote cell survival via *Bcl-2* in nonneural tissues, we tested the hypothesis that estradiol decreases cell death by influencing *bcl-2* expression in ischemic brain injury. We analyzed gene expression in microdissected regions of the cerebral cortex adjacent to the infarct in ovariectomized rats and the equivalent regions of the brain in ET rats. Tissue samples were obtained from injured and sham female rats. We found that estradiol prevented the injury-induced down-regulation of *bcl-2* expression on the ipsilateral side of the brain. This effect was specific to *bcl-2*, because expression of other members of the *bcl-2* family (*bax*, *bcl-x_L*, *bcl-x_S*, *bad*, and *bim*) was unaffected by ET (Fig. 3).

In the second example, immediate early genes are induced by various forms of brain injury, and their induction is known to be a critical step in apoptotic cell death. We tested the hypothesis that the ability of estradiol to protect the brain against middle cerebral artery occlusion-induced cell death involves attenuation of the expression of one or more immediate early genes. We assessed immediate early gene mRNAs in regions ipsilateral and contralateral to the region of injury. Our results reveal that *c-fos*, *fosB*, *c-jun*, and *junB* mRNA levels were up-regulated 24 h after middle cerebral artery occlusion. Furthermore, among these, estradiol affected only the expression of *c-fos* mRNA and protein expression on both sides of the brain: the steroid treatment attenuated the injury-induced increase in a time-specific manner (Fig. 4). Our findings strongly suggest that the ability of estradiol to protect the brain against the late stages of cell death involve the attenuation of *c-fos* induction.

It is intriguing that injury influences some of these genes (*bcl-2*) only on the side of the infarct; whereas, in other cases, both sides of the brain are affected (*c-fos*). We interpret this to mean that even the uninjured side of the brain is involved in some aspects of protection and repair. Together, the up-regulation and down-regulation of gene expression enhance neuronal survival and suppress the expression of those that promote cell death.

It is important to remember that even in animal models, estrogens do not always exert beneficial effects. Under certain circumstances, 17 β -estradiol either fails to protect or even harms the brain. Although estradiol can decrease brain injury in the vast majority of studies, estradiol fails to attenuate cell death in some animal models (29–32). Several points should be emphasized. First, although a large body of literature suggests

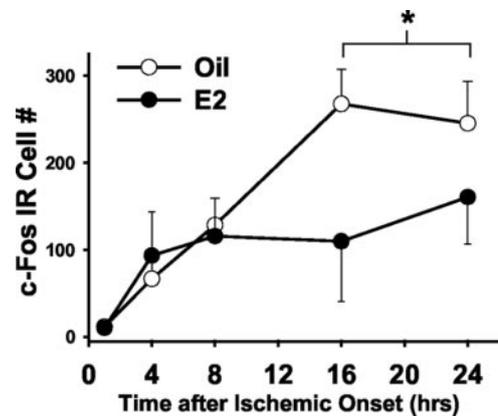


FIG. 4. Estradiol decreases the number of *c-fos* immunoreactive (IR) cells. *c-fos* IR cells were counted in ischemic cortex of coronal sections from animals euthanized at 1, 4, 8, 16, and 24 h after initiation of middle cerebral artery occlusion. Estradiol attenuates the number of *c-fos* IR cells during the late phase of ischemic injury (16–24 h). The number of *c-fos* IR cells increases between 1 and 4 h after the onset of injury in both oil- and estradiol-treated animals. In oil-treated animals, there is a secondary rise between 8 and 16 h. However, in estradiol-treated animals, there is an attenuation of this secondary rise in number of *c-fos* IR cells at 16–24 h in comparison to their oil-treated counterparts. Data are represented as mean \pm SEM ($n = 2-4$ per experimental group). *, Estradiol-treated mice were statistically significantly different from oil-treated controls. [Adapted with permission from D.B. Dubal et al., *J Neurosci* 19:6385–6393, 1999 (25) and S.W. Rau et al., *J Neurosci* 23:10487–10494, 2003 (28), copyright by The Society for Neuroscience.]

that estrogens are potent neuroprotective factors under numerous experimental circumstances *in vivo* and in many *in vitro* experimental conditions, in other experimental and disease/injury paradigms, estrogens either fail to protect or even exacerbate neural injury. It is possible that when the degree of injury is too severe, as may be the case in the hippocampus after prolonged global ischemia or when a disease state is already in progress, the actions of estradiol cannot overcome this degree of injury and are not sufficient to prevent cell death. Under other circumstances, estrogens can be deleterious to neural function. For example, in animal models of epilepsy, estradiol lowers the threshold for seizures and facilitates the induction and duration of excitatory neural firing. These data suggest that estrogen therapy may not always exert only beneficial actions in the brains of postmenopausal women, particularly in those with a medical history of epilepsy. As we continue to learn about the complexity of estrogen action with regard to dose, type of estrogen, and neurological condition, we may be better able to modify and transform ET into therapies that exert beneficial effects without the negative actions. Indeed, if we were able to develop estrogen-like compounds that did not have feminizing effects, these compounds could be used in both men and women.

IV. Summary

In summary, a large number of clinical and basic science studies have led to a new appreciation that estradiol acts far beyond the reproductive axis and exerts profound protective actions in the adult and aging brain. Although we have only begun to identify potential cellular and molecular mechanisms of this protection, our growing knowledge of estrogen

action in the injured brain will ultimately lead to a more complete understanding of the precise mechanisms underlying estradiol-mediated protection. This knowledge is crucial to developing both preventative and acute therapies for neurodegenerative conditions and carries great promise in improving the quality of life in our aging population.

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