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Mechanisms of Neuroprotection by Estrogen

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Over the past decade our recognition that estrogens function as important neurotrophic and neuroprotective factors has grown rapidly. Accumulating evidence from basic science studies demonstrates that estrogens exert profound protective actions against various forms of neurodegenerative diseases and injury. Although a thorough understanding of the mechanisms underlying the protective effect of estrogens is far from complete, significant progress has been achieved through the use of in vivo as well as in vitro models. Here we review the results from our laboratory demonstrating that low physiological levels of estradiol therapy exert powerful protection against ischemic stroke-like injury. Using an animal model of cerebrovascular stroke and in vitro explant cultures, we have begun to decipher under what circumstances 17β-estradiol protects against neuronal death and to uncover its mechanisms of action. In addition, we will review recent work demonstrating that estradiol may additionally enhance the ability of the adult brain to undergo repair by influencing the production of new neurons under neuropathological conditions, as well as by promoting an anti-inflammatory response. As we uncover the important protective roles of ovarian steroid hormones in brain disease and injury, we increasingly appreciate that the mechanisms by which estrogens achieve these effects are diverse and complex.

Key Words: Neuroprotection; estrogen; hormone replacement therapy.

Introduction

Menopause marks the end of ovarian hormonal production and the loss of reproductive function in women. While the age of menopause has remained fixed at age 51, the average life expectancy has increased to over 80 yr in most industrialized nations. Thus, many women will spend over one-third of their lives in a hypoestrogenic state. Hormone therapy (HT) has been touted as a palliative to replace estrogen and progesterone lost during this long period of hypoestrogenicity in postmenopausal women. Recently, HT and estrogen therapy (ET) have received a great deal of interest in the public media due to the premature termination of the Women's Health Initiative (WHI). The WHI consisted of two randomized, placebo-controlled clinical trials primarily designed to test whether HT and ET are protective against coronary heart disease in postmenopausal women. The HT (estrogen–progestin vs placebo) arm of the study was abruptly terminated in 2002 due to an increased risk of cardiovascular complications and breast cancer in women receiving HT *(1)*. Later, the ET (estrogen-only vs placebo) arm of the WHI was terminated in 2004 due to an increased risk of stroke in women receiving treatment *(2)*. As a result, many postmenopausal women have been advised to use HT/ET only for short-term management of postmenopausal symptoms. These outcomes of the WHI, however, must be interpreted with great caution based on the study design, hormone preparation, hormone dosage, the time when hormones were administered relative to the perimenopausal transition, and mean age of the study population at the initiation of HT/ET *(3,4)*. Previous clinical, observational, and epidemiological studies, as well as a large body of studies using animal models, demonstrate that HT and ET protect against various forms of neurodegenerative diseases and injury (for review, see refs. *5–7*). Further preclinical research that provides a more thorough understanding of the mechanisms underlying hormone's neuroprotective actions may bestow an insight into the disparity between the previous clinical/basic science studies and the WHI, and may lead to a safer and more effective design of future clinical studies.

Women are protected from stroke and other cardiovascular diseases until they reach the menopause. Stroke is the third leading cause of death in the United States, with ischemic strokes accounting for 88% of total strokes. Owing to the lack of ovarian hormone production, postmenopausal women are at high risk for stroke as their male counterparts and account for approx 61.0% of all stroke-related deaths (see ref. *8* and www.americanheart.org/statistics). To examine

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the mechanisms underlying the prevalence of stroke in postmenopausal women, our laboratory utilizes an animal model of stroke that mimics focal cerebral ischemia using a permanent middle cerebral artery occlusion (MCAO). The permanent MCAO model allows us to selectively study estradiol's neuroprotective effects on focal cerebral ischemia without the confounding effects of reperfusion injury.

Mechanisms of Neuroprotection by Estradiol

In addition to well-established roles in normal reproductive function, estradiol plays an essential role in the development of the central nervous system, as well as in maintaining normal brain function in adulthood. During fetal development, estradiol plays a critical role in sexual differentiation of the brain (for reviews, see ref. *9*). Estradiol exerts these actions by influencing numerous cellular functions in the developing brain, including neuronal and glial plasticity, dendritic growth, synaptogenesis, differentiation, neurogenesis, and cell migration *(10)*. The organizational effect of neonatal exposure to estradiol leads to sex-specific neuroanatomy, patterns of neurotransmitter/neuropeptide synthesis and release, and regulation of sex steroid–inducible gene expression, resulting in gender-specific sexual behaviors, secretion patterns of gonadotropin-releasing hormone and gonadotropins *(11,12)*.

Estradiol is not only essential to proper organization and sexual differentiation of the brain, but also plays a pivotal role in maintaining normal brain function and protecting the brain against various neurodegenerative diseases and injury. The neuroprotective actions of estradiol have been studied extensively using various animal models of neurological disease, including kainic acid–induced excitotoxicity *(13)*, cerebral contusion *(14)*, hypoxia *(15)*, amphetamine-substitute analog-induced toxicity *(16)*, and cerebral ischemia *(17–19)*. Taken together, these studies may provide insights into therapeutic as well as pharmacological approaches for the prevention and treatment of various neurological diseases and injury.

Our laboratory has used an animal model of stroke to decipher under what circumstances estradiol protects against neuronal death and to uncover its mechanisms of action. We have shown that low physiological levels of 17β-estradiol exert profound neuroprotective actions in a model of stroke injury in which the middle cerebral artery is permanently occluded *(17,20)*. This model reduces cerebral blood flow to the striatum and overlaying cortex by approx 50%, resulting in focal corticostriatal ischemic injury *(17)*. We have also demonstrated that estradiol effectively reduces the infarct volume in both young and middle-aged rats compared to oil-treated control animals, suggesting that a constellation of factors responsible for mediating estradiol's protective actions is preserved in aged animals *(21)*. In rats, estradiol protects the cortex against ischemic injury–induced neuronal death *(17)*, whereas in mice, it exerts neuroprotective actions in both the cortex and striatum *(20)*. Furthermore, we have found that at low physiological doses [approx 25 pg/mL serum *(20)*], estradiol must be administered prior to the onset of injury, because acute administration of estradiol at the time of ischemic injury does not decrease the extent of infarct *(17)*. This finding contrasts with the discoveries of Toung et al. *(19)* demonstrating that supraphysiological concentrations of estradiol administered immediately before the onset of ischemic injury exert neuroprotection. In addition, pharmacological levels of estradiol also effectively protected against ischemic brain injury when administered at as late as 6 h after the onset of injury *(22,23)*. The findings that acute or postischemic administrations of estradiol at supraphysiological doses exert protective actions against ischemic injury may have strong clinical implications, especially if non-reproductive estrogen-like compounds can be designed and used as the treatment as opposed to the preventive method. Moreover, a stereoisomer of 17β-estradiol, 17α-estradiol, has been shown to exhibit equivalent neuroprotection against ischemic damage, suggesting the existence of mechanisms independent of estrogen receptors (ERs) or those that depend on an alternative ER *(18,24)*. In summary, these findings clearly demonstrate that administration of even basal levels of estradiol profoundly protects the brain against stroke-like injury and that the mechanisms by which estrogens achieve their effects are diverse and complex.

Using our experimental paradigm of stroke injury and low physiological levels of estradiol replacement, we began to investigate potential mechanisms of neuroprotection. Using reverse transcription–polymerase chain reaction as well as *in situ* hybridization histochemistry, we found that ischemic injury upregulates ERα mRNA expression in the ipsilateral cortex of ovariectomized rats regardless of hormonal treatment *(25)*. Similarly, using immunocytochemistry, we discovered that stroke-like injury increases ERα protein levels on the injured side of the cortex of ovariectomized mice treated with either oil or estradiol. This upregulation of ERα protein expression was highest at 24 h after the onset of injury (Fig. 1), diminished by 48 h and subsequently became undetectable by 96 h (data not shown), suggesting that injury-induced increases in ERα protein expression are a relatively transient event. In contrast to ERα, ischemic injury did not change ERβ protein levels in ovariectomized mice (Fig. 1). The reappearance of ERα proteins in response to injury may represent a recapitulation of developmental stages of the brain, when ERα proteins show a transient increase in expression in specific brain regions including the cortex and hippocampus *(26,27)*. During this early postnatal interval, estradiol plays a pivotal role in the proper development and neuronal organization within these brain regions by influencing various neuronal functions such as dendritic outgrowth, synaptogenesis, and neurogenesis. Based on these findings, we speculate that the injury-induced upregulation of ERα mediates neuroprotective actions of estradiol in the adult brain. Using both ERα and ERβ knock-

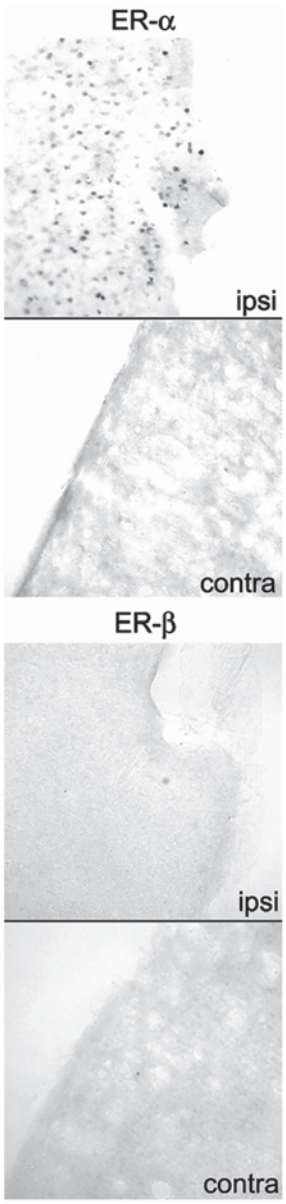

Fig. 1. ERα but not ERβ protein levels are upregulated in the ipsilateral cortex of ovariectomized mice at 24 h after the onset of ischemic injury. The number of cells that express ERα immunoreactivity is dramatically increased in the ipsilateral cerebral cortex of both oil- (not shown) and estradiol-treated mice, but not in the contralateral side of the cortex, or in sham-operated animals.

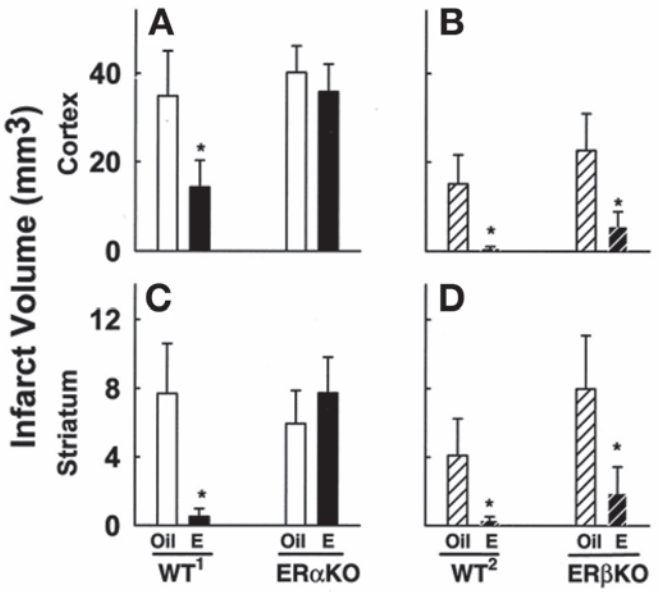

Fig. 2. ERα plays a critical role in neuroprotection mediated by estradiol. Estradiol significantly reduced the infarct volume both in the cortex ($*p < 0.02$) as well as in striatum ($*p < 0.05$) in wild-type mice (WT^1) compared with oil-treated controls. In contrast, estradiol did not protect in these brain regions of ERαKO mice compared with oil-treated controls. Estradiol significantly reduced cortical ($*p < 0.02$) and striatal ($*p < 0.04$) infarct volumes in wild-type mice (WT^2) compared with oil-treated controls, as well as in ERβKO mice. In all series, data represent the mean ± SEM. [Adapted with permission from D.B. Dubal et al. (2001). *Proc. Natl. Acad. Sci. USA* **98,** 1952–1957 *(20)*, copyright by the National Academy of Sciences USA.]

out mice, we found that the presence of ERα, but not ERβ, is a prerequisite for the ability of estradiol to exert protection against ischemic injury *(20)*; knocking out ERα blocks estradiol's ability to reduce the extent of infarct in both the cortex and striatum (Fig. 2). In marked contrast, estradiol continued to exert a powerful protection against injury-induced cell death in ERβ knockout mice (Fig. 2). Taken together, the injured brain seems to provide signals conveying the need for the reappearance of ERα, which may mediate the ability of estradiol to protect against neuronal death and possibly reinitiate differentiation of the injured brain.

To date, we have identified multiple downstream targets of estradiol action in neuroprotection. Estradiol modulates the expression of arrays of genes in ischemic brains, including those that influence the balance between cell death and cell survival. For instance, we reported that low physiological levels of estradiol prevents injury-induced downregulation of Bcl-2 without corresponding changes in the expression of other members of Bcl-2 family genes *(25)*. Because Bcl-2 is a protooncogene known to promote cell survival in a variety of tissues including the brain, we hypothesize that estradiol decreases the extent of neuronal death by preventing downregulation of Bcl-2 in response to ischemic injury. We also examined the effects of stroke injury and estradiol on immediate early genes (IEGs) because these are induced in response to various forms of brain injury, their expression is associated with injury-induced programmed cell death and steroids modulate their expression under a variety of experimental circumstances *(28,29)*. We have shown that the levels of IEGs including *c-fos*, *fosB*, *c-jun*, and *junB* increase following ischemic injury, and that estradiol specifically attenuates injury-induced upregulation of *c-fos* mRNA and protein *(30)*. These findings strongly suggest that the ability of estradiol to protect the brain against the late stages of neuronal death involves attenuation of *c-fos* upregulation. We then tested the hypothesis that estradiol

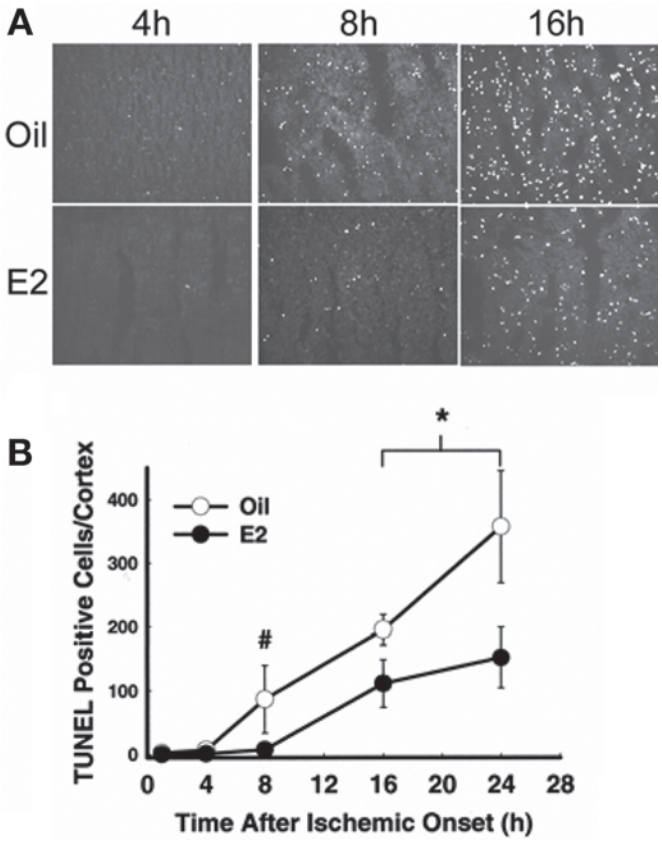

Fig. 3. (**A**) Composite of representative pictures from the ischemic cortex of ovariectomized female rats stained by the TUNEL technique. Representative 20× magnification fields taken from the parietal cortex of coronal sections showing cells that stained positive for TUNEL. These fields demonstrate TUNEL-positive cells from oil- and estradiol-treated rats at 4, 8, and 24 h after the onset of ischemic brain injury. (**B**) Estradiol delays and attenuates the number of TUNEL-positive cells in the ischemic cortex. The mean number of TUNEL-positive cells in the ischemic cortex rises dramatically at 4 h after ischemic injury in oil-treated animals and continues to rise through the remaining time points. In estradiol-treated animals, the number of TUNEL-positive cells rises dramatically after 8 h. ANOVAs were run for the early (1–8 h) and late (16–24 h) phases of injury. Estradiol significantly increased the number of TUNEL-positive cells during early ($^{\#}p < 0.05$) and late ($^{*}p < 0.05$) phases of ischemic injury compared to oil-treated animals. Data represent the mean ± SEM of 8–10 animals per group. [Adapted with permission from S.W. Rau et al. (2003). *J. Neurosci.* **23,** 11420–11426 *(31)*, copyright by the Society for Neuroscience.]

protects the brain against MCAO injury by attenuating neuronal apoptosis. Our data confirmed that estradiol attenuates markers of apoptosis including caspase-3 activation and injury-induced DNA fragmentation *(31)*. These events contribute to a reduction in apoptosis, as indicated by a decrease in the number of TUNEL-positive cells in the cerebral cortex of estradiol-treated ovariectomized rats compared to vehicle-treated rats (Fig. 3).

To further explore the molecular and cellular mechanisms mediating protective actions of estradiol, we have implemented organotypic cerebral cortical explant cultures *(32)*. Explants were exposed to potassium cyanide/2-deoxyglucose (KCN/2-DG) to model metabolic inhibition observed during ischemic injury. This in vitro model induced by metabolic inhibition exhibits considerable parallelisms with our in vivo model of brain ischemic injury. Using this method, we found that estradiol reduces cell death following injury in vitro, and that, as our in vivo model, this protection is likely to be mediated by estrogen receptor because (1) protection required pretreatment of hormone, (2) 17α-estradiol, the natural optical isomer of 17β-estradiol, which is generally considered to be less active due to its lower binding affinity to estrogen receptors, afforded no protection and (3) protective action of 17β-estradiol was blocked by the estrogen receptor antagonist ICI 182,780. We subsequently showed that protective action of estradiol in vitro also involves attenuation of apoptosis *(33)*. Estradiol pretreatment significantly reduced the numbers of cells undergoing apoptotic cell death as indicated by nuclear condensation and TUNEL staining. To begin to decipher the mechanism by which estradiol prevents apoptosis, we then analyzed the levels of Akt kinase activation and found that estradiol enhances activation of Akt kinase, an important mediator of cell survival signaling pathways. Taken together, our findings clearly demonstrate that low physiological levels of estradiol exhibit powerful neuroprotective actions both in vivo and in vitro, and that these protective actions are mediated by estrogen receptors and involve attenuation of neuronal apoptosis in response to ischemic brain injury.

Neurogenesis: Novel Roles for Estradiol in Brain Repair

During the past decade our laboratory and many others have begun to appreciate that estradiol plays important trophic as well as protective roles in the adult brain. In addition, recent work has shown that estradiol influences cell proliferation in the adult brain under normal physiological *(34)* and pathological conditions *(35)*. In adult mammals, the production of new neurons is highly restricted to two germinal niches in the brain, i.e., the hippocampal dentate gyrus and the forebrain subventricular zone (SVZ). In the adult female rats, the number of new granule neurons in the dentate gyrus of the hippocampus increases during proestrus when circulating estrogen levels are highest *(34)*. Consistently, removal of circulating estrogen by ovariectomy reduces the number of proliferating cells in the hippocampus, and subsequent administration of estradiol reverses this effect *(34)*. In contrast, estradiol does not seem to influence cell proliferation in the SVZ under normal physiological conditions in both rats *(34)* and mice *(36)*. However, Saravia et al. *(35)* have shown that the experimental diabetes induced by streptozotocin reduces the number of proliferating cells in the SVZ of mice, and that estradiol restores cell proliferation to the control level in diabetic mice. Thus, estradiol may have therapeutic potential under pathologi-

Table 1
Ischemic Injury[a]

	MCAO		
	Sham	Ipsilateral	Contralateral
Number of $BrdU^+$ cells	157 ± 9.95	111 ± 10.42*	121 ± 10.70*

[a]Ischemic injury induced by permanent occlusion of the middle cerebral artery (MCAO) reduced the number of BrdU-labeled cells on both the ipsilateral and contralateral sides of the subventricular zone of adult ovariectomized mice compared to sham-operated animals (*$p < 0.05$). Data represent the mean ± SEM of 6–7 animals per group.

cal conditions by influencing cell proliferation in the adult mammalian SVZ.

The forebrain SVZ lining the lateral ventricle, because it is a major repository of neural stem cells *(37,38)* and the majority of cells that are born in the rostral SVZ give rise to neuroblasts that migrate to the olfactory bulb and differentiate into the mature olfactory interneurons *(39)*. However, in response to brain insults such as ischemic stroke, a proportion of neuroblasts born in the SVZ instead migrate to the site of injury to potentially participate in self-repair and functional recovery of the nervous systems *(40,41)*. In rats, various forms of brain insults increase the number of newborn cells in the SVZ, including focal cortical ischemic injury *(42)*, cortical aspiration *(43)*, and corticostriatal ischemic injury *(44)*. However, in contrast to rats, the number of proliferating cells in the SVZ of mice decreases in response to neurodegenerative conditions including cortical aspiration *(45)* as well as experimental diabetes *(35)*. Consistent with these findings, we have found that experimental stroke decreases the number of BrdU-labeled newborn cells in the SVZ of ovariectomized mice (Table 1). Furthermore, pretreatment with physiological levels of estradiol in ovariectomized mice increased the number of newborn neurons (BrdU/double-cortin-dual-labeled cells; Fig. 4) in the SVZ following permanent MCAO *(46)*. Previous studies have shown that the forebrain SVZ is the birthplace of neurons capable of migrating to the cortex as well as striatum in the face of brain injury to potentially replace the damaged neurons *(40,41)*. Therefore, estradiol may enhance the ability of the brain to undergo repair by influencing the ability of the adult SVZ to mount a neurogenic response in an animal model of stroke. Whether immature neurons of the SVZ, after migrating to the sites of injury such as the cortex and striatum, are capable of forming appropriate synapses and become functionally active is yet to be determined.

Anti-inflammatory Properties of Estradiol

Many neurological disorders such as stroke, multiple sclerosis (MS), Alzheimer's disease (AD), and Parkinson's disease (PD) feature a common inflammatory component that

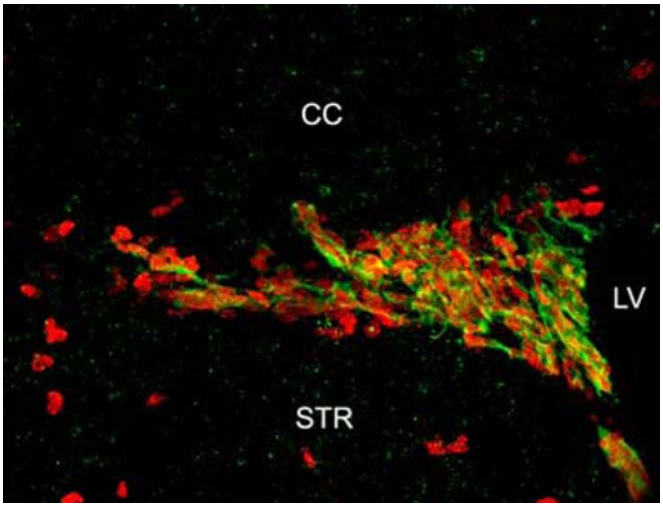

Fig. 4. Estradiol increases the number of BrdU/Dcx-dual-labeled cells in the subventricular zone (SVZ) after MCAO injury. Confocal photomicrograph (20× magnification field) of BrdU/double-cortin (Dcx)-dual-labeled newborn neurons in the ipsilateral SVZ of estradiol-treated ovariectomized adult mice. BrdU-labeled cells are shown in red, whereas Dcx-labeled cells are shown in green. CC, corpus callosum; LV, lateral ventricle; STR, striatum.

is manifest both inside and outside the nervous system. Inflammation is a complex process, comprising both innate and acquired immune responses, that share common pathways which converge to produce a delicate balance between neuroprotection and neurodegeneration *(47)*. A growing body of data suggests that estradiol-mediated molecular mechanisms interact with these inflammatory pathways to promote neuroprotection. Both in vivo and in vitro studies show that low, physiological concentrations of estradiol promote the brain anti-inflammatory response by suppressing the brain's innate immune response. In order to mediate this anti-inflammatory response, experimental evidence from several laboratories reveals that estradiol suppresses many pro-inflammatory factors such as free radicals, cytokines and chemokines, and the enzyme inducible nitric oxide synthase (iNOS), while enhancing the production of antiinflammatory cytokines, chemokines, and other growth factors *(6)*.

Microglia are the brain's macrophage and serve as the primary mediators of the brain's innate immune response. Microglial activation is a critical component of the inflammatory response during ischemic brain injury *(48)*. Studies using both microglial cultures *(49–51)* and an in vivo lipopolysaccharide (LPS) mouse model of neuroinflammation *(52)* reveal that physiological concentrations of estradiol suppress microglial production of free radicals (nitric oxide and superoxide), pro-inflammatory cytokines, and iNOS expression. Using a combination of both genetic and pharmacological approaches, these effects were shown to be mediated through ERα-mediated nonclassical, genomic mechanisms *(51,52)* and/or ERβ-mediated nongenomic signaling mechanisms *(49)*.

Table 2
Estradiol Suppresses Pro-inflammatory Cytokine Production Following MCAO[a]

	Cytokine Concentration (pg/mL)			
	TNFα (mean ± SEM)	*n*	IL-6 (mean ± SEM)	*n*
OVX + oil	4.498 ± 1.131	8	82.31 ± 14.89	8
OVX + estradiol	2.792 ± 0.2294*	9	33.25 ± 5.297*	10

[a]TNFα and IL-6 concentrations were measured using Luminex xMAP technology in plasma collected from 5-mo-old ovariectomized (OVX) mice 24 h after MCAO. Both TNFα and IL-6 concentrations were significantly lower (*$p < 0.05$, Student's *t*-test) in estradiol-treated mice compared with those that received an oil vehicle.

Astrocyte activation also plays a role in the inflammatory response during ischemic brain injury via astrogliosis, which promotes the formation of the glial scar present around the infarct. Astrocytes, like microglia, express both ERα and ERβ. During ischemia, the astrocyte marker glial fibrillary acidic protein (GFAP) is upregulated in cells surrounding the ischemic penumbra *(48)*. Estradiol suppresses astrocytic GFAP expression in in vivo *(53)* and in vitro *(54)* stab wound models of brain injury. However, whether estradiol-mediated suppression of GFAP is a neuroprotective mechanism in ischemic brain injury models is unclear and represents a fruitful area for further study.

Because inflammation plays a primary role in stroke pathophysiology, identifying molecules that preferentially suppress the chronic aspects of the inflammatory response will prove useful for developing stroke-related preventive therapies. Our laboratory has found that administration of low, physiological concentrations of estradiol to 5-mo-old, ovariectomized C57BL/6J mice prior to MCAO decreases plasma levels of the pro-inflammatory cytokines tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6) 24 h following MCAO compared to age-matched ovariectomized mice given an oil vehicle (Table 2). Brain microglia and peripheral macrophages serve as major sources of pro-inflammatory cytokines that affect infarct severity following cerebral ischemia *(48,55)*. Cytokine production is stimulated following stroke in both the CNS and peripheral tissues. Breakdown of the blood–brain barrier following stroke makes the brain more vulnerable to pro-inflammatory cytokine sources from peripheral tissues, resulting in increased cell death leading to a larger infarct. Both TNFα and IL-6 are commonly used as serum biomarkers in clinical stroke studies, and lower serum or plasma levels of both TNFα and IL-6 are correlated with smaller infarct volumes, a decrease in ischemic severity, and a better stroke outcome *(56,57)*. Our data are consistent with these clinical studies, suggesting that estradiol treatment suppresses the production of these proinflammatory cytokines, thereby suggesting another molecular mechanism through which estradiol exerts neuroprotection following stroke.

Conclusion

Estradiol clearly functions as more than a reproductive hormone by exhibiting a myriad of neuroprotective functions that are essential for neuronal survival in the adult and aging brain. Using rodent animal models, results from our laboratory and others clearly demonstrate that estradiol protects against cell death in the adult brain following cerebral ischemia. More recent studies reveal that neurogenic and anti-inflammatory mechanisms may provide additional modes of neuroprotection following ischemic insult. However, the disparate results between preclinical studies and clinical trials (e.g., WHI) emphasize the complexity of molecular mechanisms employed by estradiol as well as other neuroprotective hormones. A thorough understanding of estradiol action in the injured brain will ultimately lead to a clearer understanding of the complex mechanisms underlying its neuroprotective functions. It is essential that we continue to study the effects of estradiol and other hormones in the brain in order to explain the findings from the WHI and more importantly, to develop better preventative and/or therapeutic agents to protect the injured and aging brain.

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