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ESTROGEN THERAPY: DOES IT HELP OR HURT THE ADULT AND AGING BRAIN? INSIGHTS DERIVED FROM ANIMAL MODELS

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Abstract—Hormone therapy and estrogen therapy in postmenopausal women have been thought to ameliorate cognitive dysfunction and decrease the risk and/or progress of neurodegenerative conditions such as Alzheimer's disease and stroke. Furthermore, estrogens have been shown to exert neuroprotective actions in a variety of *in vitro* and *in vivo* models of brain injury. However, the findings of the Women's Health Initiative have made us re-evaluate these assumptions.

Our laboratory has shown that physiological levels of estradiol attenuate ischemic brain injury in young and middle-aged female rats. We have begun to probe the cellular and molecular mechanisms that underlie these novel non-reproductive actions of this steroid. Our findings demonstrate that in both young and aging rats, treatment with physiological concentrations of estradiol decreases ischemic injury by almost 50%, compared with oil-treated controls. Additionally, our data suggest that estradiol acts by altering the expression of genes that suppress apoptosis and enhance survival in the penumbral region of the infarct. These observations demonstrate that estrogen therapy protects against stroke-related injury in young and aging female rats and strongly suggest that middle-aged animals remain responsive to the protective actions of estradiol. Furthermore, they suggest that estrogen therapy protects against cell death by influencing the expression of genes that suppress apoptotic cell death pathways. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: ischemia, estrogen, menopause, neuroprotection, cell death, stroke.

It is well established that estradiol plays a critical reproductive role in the brain during fetal and neonatal development and during adulthood. More recently, it has become increasingly clear that estradiol can also play an important non-reproductive neurotrophic and neuroprotective role. During development estradiol stimulates neurite outgrowth and arborization of neuritic branches in organotypic cultures (Toran-Allerand, 1991) and in dispersed neuronal cell cultures (Murphy and Segal, 1996; Brinton et al., 1997). In the adult, estradiol stimulates the number of functional dendritic spines in the CA1 region of the hippocampus (Gould et al., 1990; Woolley and McEwen, 1993) and stimulates synaptogenesis (Woolley et al., 1996). It also protects against brain injury during adulthood in several experimental models. Estrogen therapy (ET) in ovariectomized (OVX) rats significantly decreases ischemic injury and injury induced by other neurotoxic stimuli (for reviews see McCullough and Hurn, 2003; Wise et al., 2001; Green and Simpkins, 2000; Garcia Segura et al., 2001). Moreover, in some strains of rats, ischemia-induced brain injury is less extensive on proestrus when estradiol is high, than on other days of the estrous cycle (Carswell et al., 2000) and females exhibit less cell death compared with males (Hall et al., 1991; Alkayed et al., 1998).

Neurodegenerative conditions such as Alzheimer's disease and cerebrovascular stroke occur more frequently in older postmenopausal than in equivalent-aged women who are premenopausal or in young women. Therefore, it is important to assess whether estradiol exerts protective actions in models of aging, such as surgically menopausal (e.g. OVX) animals and normally aging animals. We have assessed (1) the effects of physiological levels of estradiol on the extent of brain injury in young rats and (2) whether middle-aged rats remain responsive to these modest levels of estradiol. In addition, we have begun to decipher the cellular and molecular mechanisms that underlie estradiol's effects.

EXPERIMENTAL PROCEDURES

Detailed methods are described in our original peer-reviewed papers (Dubal et al., 1998, 1999) and are only described briefly below.

Animals and experimental treatments

Young (3–4 months, 250–300 g) and middle-aged (9–12 months, 350–400 g) female, Sprague–Dawley rats were ovariectomized (OVX) to eliminate endogenous ovarian estradiol and immediately implanted s.c. with a Silastic capsule, containing vehicle (sesame oil) or 17β-estradiol at two different doses (180 μg/ml or 1 mg/ml). To achieve identical serum concentrations of estradiol, young rats received a 30 mm capsule, whereas, middle-aged rats received a 40 mm capsule. The two doses of estradiol treatment (ET) produced serum levels of estradiol that are equivalent to basal or proestrous levels observed in the rat estrous cycle, respectively (Smith et al., 1975).

In vivo cerebral ischemia

One week after OVX and estradiol or vehicle treatment, permanent cerebral ischemia stroke was induced via insertion of a 4/0 (young rats) or a 3/0 (middle-aged rats) black monofilament suture to occlude the middle cerebral artery (MCA; $n=8$–14/experimental group) using methods described in detail in our previous publication (Dubal et al., 1998). Briefly, the right MCA was occluded via insertion of a poly-L-lysine-coated monofilament suture from the right external carotid artery, through the right internal carotid artery

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Abbreviations: ER, estrogen receptor; ET, estrogen therapy; MCA, middle cerebral artery; OVX, ovariectomized; TTC, triphenyltetrazolium chloride.

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to the base of the MCA. In a preliminary experiment, insertion of 4/0 and 3/0 sutures was tested to assess whether equivalent occlusion of the MCA in middle-aged rats necessitated the use of the larger diameter suture. Results showed that a 3/0 suture (larger diameter) was necessary for successful MCA occlusion (MCAo) in middle-aged rats (data not shown).

Histologic preparation

Brains were collected 24 h after the onset of ischemia and sectioned into 1 mm coronal slices using a brain matrix. Alternate slices were stained in 2% triphenyltetrazolium chloride (TTC) to visualize the injured part of the brain and analyze infarct volumes using coronal sections that span the brain via computer-assisted imaging (NIH v.1.60). The remaining alternate sections were frozen for mRNA analysis (see below).

RT-PCR studies

Alternate brain sections (1 mm thick) were frozen at −80 °C to monitor gene expression. The area of the cortex analyzed for gene expression was selected by first examining tissue from a 1 mm TTC-stained coronal section, corresponding to the middle of the infarct. Then, the adjacent fresh, frozen 1 mm section was used to analyze gene expression. A region adjacent to the infarct in OVX, oil- and estradiol-treated rats and the equivalent region on the contralateral side of the brain were microdissected. cDNA was produced by reverse transcribing total RNA and was amplified utilizing well-characterized RT-PCR methods to determine relative changes in gene expression at the mRNA level. After amplification, PCR products were resolved by polyacrylamide gel electrophoresis.

RESULTS

We found that estradiol decreased infarct volume in both young and middle-aged female rats, compared with respective vehicle-treated controls. Fig. 1 is a composite of representative coronal brain sections from oil- and estradiol-treated, young and middle-aged rats 24 h after MCAO. These data show that both doses of ET significantly reduced infarct volume as compared with oil-treated controls in both young (Fig. 2A) and middle-aged (Fig. 2B) rats. Estradiol pretreatment was equally effective in both age groups.

Fig. 3 shows *bcl-2* gene expression in microdissected punches taken from the the ipsilateral and contralateral

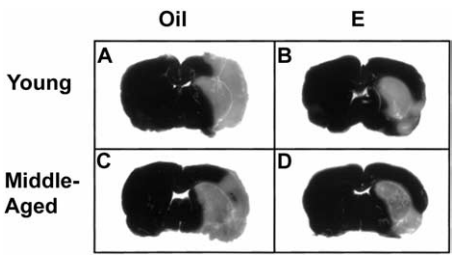

Fig. 1. Representative brain sections from an oil- and estradiol-treated, young and middle-aged rat after permanent cerebral ischemia. Infarcted tissue is white, while live tissue is darkly stained by TTC. In the absence of estradiol, brain injury was extensive in (A) young and (C) middle-aged rats. Physiological estradiol pretreatment (180 μg/ml) reduced the extent of infarct in both (B) young and (D) middle-aged rats. The volume of infarct included significant portions of the cerebral cortex and striatum.

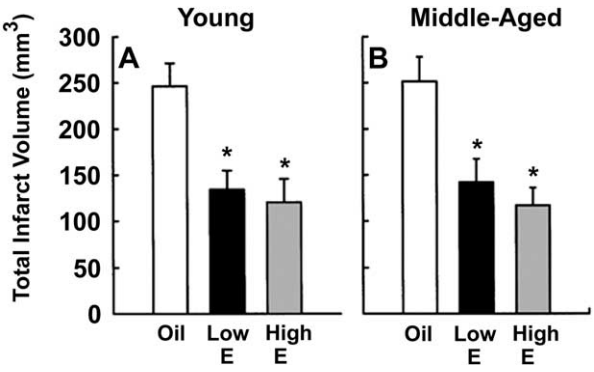

Fig. 2. Estradiol protects against total ischemic brain injury in young and middle-aged rats. Low and high physiological levels of estradiol decreased total injury in (A) young and (B) middle-aged rats. Infarct volume was the same in young and middle-aged rats which were OVX. Both low and high levels of estradiol treatment significantly decrease the infarct volume to the same extent in both age groups.

side of oil- and estradiol-treated rats that have undergone MCAO. To take equivalent anatomically placed tissues from both OVX and ET rats, the tissue was closer to the injury in OVX than in ET rats. Values are expressed as a percentage of sham to reflect changes in injury with respect to normal expression. On the ipsilateral side of the brain, ET prevented the injury-induced downregulation of *bcl-2*. In the absence of estradiol, *bcl-2* expression declined to approximately 60% of sham values on the injured hemisphere. Estradiol's action was specific to *bcl-2*, as expression of other members of the *bcl-2* family, *bax*, *bcl-x*$_L$, *bcl-x*$_S$, *bad*, and *bim* was not affected by ET (data not shown).

Fig. 4 shows modulation of *ER*α following MCAO. Twenty-four hours after MCAO, *ER*α was dramatically upregulated in the ipsilateral cortex of oil- and estradiol-treated brains, as compared with the contralateral cortex. Upregulation was similar in OVX and estradiol-treated rats,

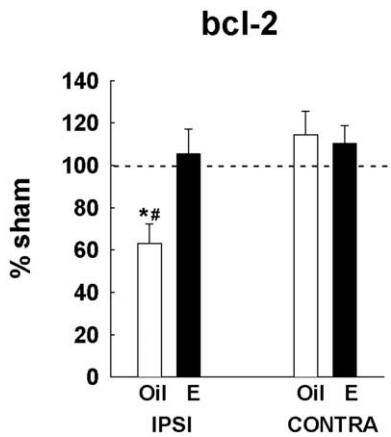

Fig. 3. Estradiol modulated *bcl-2* gene expression in cerebral ischemia. In injury, estradiol significantly prevented the injury-induced downregulation of *bcl-2* mRNA in the ipsilateral cortex, compared with oil-treated rats. In the absence of estradiol, *bcl-2* gene expression declined significantly below constitutive bcl-2 expression. Injury values are graphed as a percentage of sham values. These results were repeated four times, using two independent experimental groups.