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The loss of estrogen efficacy against cerebral ischemia in aged postmenopausal female mice☆

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HIGHLIGHTS

- Physiological doses of E2 show neuroprotective effects in adult female mice but not in aged female mice.
- ERα and ERβ expression in the cortex significantly decreased in the aged female mice.
- Estrogen replacement treatment could not recover ERα and ERβ expression in the aged female mice.

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ABSTRACT

Estrogen has been shown to have neuroprotective effects in numerous experimental studies involving young and adult animals. However, several clinical trials have found that in aged postmenopausal women who received estrogen replacement therapy, there did not appear to be a reduction in the incidence of stroke. The aim of this study was to investigate the effects of physiological dosages of estrogen on aged female mice subjected to ischemia-reperfusion injury. Adult ovariectomized (OVX) female mice and 22-month-old female mice received daily subcutaneous injections of 100 μg/kg or 300 μg/kg 17β-estradiol (E2) at the back of the neck for four weeks, and the expression levels of estrogen receptor (ER) α and β in the cerebral cortex were determined using real-time PCR and Western blotting analyses. To mimic ischemic stroke, the mice received middle cerebral artery occlusion (MCAO) treatment for 1 h followed by a 24-h reperfusion period. The mice were then subjected to neurological deficit testing and infarct volume evaluation. The aged mice showed higher neurological deficit scores and larger infarct volumes compared with the adult mice. Both the lower and higher physiological dosages of E2 significantly improved the neurological test scores and decreased the infarct volume in the adult mice; however, E2 showed no neuroprotective effects in the aged mice. Furthermore, the protein expression of ERα and ERβ in the cerebral cortex was significantly decreased in the aged mice compared with the adult mice, and this decrease was not rescued by E2 treatment. These results indicate that the down-regulation of ERα and ERβ in the cerebral cortex may contribute to the loss of estrogen efficacy against ischemic injury in aged females and may point to new therapies for ischemic stroke in aged postmenopausal women.

1. Introduction

Ischemic stroke is one of the leading causes of death worldwide [1]. Among the elderly, women are more likely than men to suffer from stroke [2], and the neurological outcomes for female patients are also worse compared with those for men [3]. Furthermore, compared with younger premenopausal women, older postmenopausal women are more likely to have stroke.

Numerous studies have shown that 17β-estradiol (E2) therapies are beneficial for ischemic stroke [4]. However, there has been significant controversy concerning the neuroprotective effects of E2. Two clinical studies from the Women's Estrogen for Stroke Trial

Abbreviations: CNS, central nervous system; CHIP, C terminus of heat shock cognate protein 70 (Hsc70)-interacting protein; E2, 17β-estradiol; ER, estrogen receptor; GCI, global cerebral ischemia; HRT, hormone replacement therapy; LTED, long-term 17β-estradiol deprivation; MCAO, transient middle cerebral artery occlusion; OVX, ovariectomized; rCBF, regional cerebral blood flow; TTC, 2,3,5-triphenyltetrazolium chloride.

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and the Women's Health Initiative found that E2 therapy failed to achieve protective effects and perhaps even increased the risk for dementia and stroke [5,6]. Other clinical trials have demonstrated that postmenopausal women who received E2 treatment were more likely to suffer from stroke [7] and had more severe outcomes than postmenopausal women who did not receive the therapy [8]. Furthermore, experimental studies in a long-term E2 deprivation (LTED) model revealed that the neurodegenerative effects of E2 are caused by multiple factors including decreased E2 sensitivity and decreased expression of ERα following ischemia [9].

In this study, we utilized 22-month-old female mice and a MCAO model to explore the effects of estrogen on ischemic injury in postmenopausal females. We also explored the mechanisms underlying the loss of estrogen efficacy against ischemic injury in aged females by examining the expression of estrogen receptors (ER) α and β in the cerebral cortex.

2. Materials and methods

2.1. Mice

Eighty 3-month-old (20–25 g) and 120 22-month-old (25–30 g) female C57BL/6 mice were obtained from the Experimental Animal Center of the Fourth Military Medical University. The mice were divided into a total of seven groups. The 80 adult mice were randomly divided into four groups ($n = 20$): (A) sham control; (B) OVX without E2; (C) OVX + low E2; and (D) OVX + high E2. The 120 aged mice were randomly divided into three groups ($n = 40$): (E) sham control; (F) aged + low E2; and (G) aged + high E2. In each group, five mice were used to detect the level of serum estrogen and the expression of ERα and ERβ, while others (15 or 35 mice) received MCAO treatment. The experiment protocol was approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University.

2.2. OVX and estrogen replacement

OVX was performed by dorsolateral incision, as previously described [10]. Briefly, after the animals were anesthetized, bilateral OVX surgery was carried out through two small lateral abdominal incisions, and both the right and left horns of the uterus were exposed. The ovaries were then carefully removed, leaving the uterus intact. Animals in the sham groups were subjected to the same operation, with the exception that the ovaries were left intact. Five days following OVX, the animals began receiving daily subcutaneous injections of 100 μg/kg or 300 μg/kg E2 (diluted in sesame oil solution) at the back of the neck for next four weeks.

2.3. Determination of serum estrogen levels

The levels of serum estrogen were measured to confirm HRT. Briefly, the animals were anesthetized with an overdose of pentobarbital sodium, and blood was collected from the ophthalmic artery. Serum estradiol levels were measured using an EIA kit (Cayman Chemical, Ann Arbor, MI).

2.4. Real-time PCR

To analyze the expression of the ERα and ERβ mRNAs in the cerebral cortex, real-time PCR was performed as previously described [11]. Briefly, total RNA was extracted from individual samples of cerebral cortex using TRIzol (Invitrogen, America) and reverse transcribed into cDNA using the RevertAidTM First-Strand cDNA synthesis kit (Fermentas, Lithuania). The mRNA transcript levels were determined by quantitative real-time PCR using cDNA as the template, appropriate specific primers and the SYBR Green RT-PCR kit (Takara, Japan). The following primer pairs (Takara, Japan) were used: ERα sense (5′-TCTGGGTATCATTACGGTGTCTGG-3′) and antisense (5′-GGTACACTGATTCGTGGCTGGA-3′); ERβ sense (5′-TCTGGGTATCATTACGGTGTCTGG-3′) and antisense (5′-GGTACACTGATT CGTGGCTGGA-3′); and GAPDH sense (5′-TCCTGTGGCATCCACGAAACT-3′) and antisense (5′-GGAGGGAGCAATGATCTTGATCTTC-3′). The expression levels of the target-gene mRNA transcripts were normalized relative to GAPDH controls.

2.5. Western blotting analyses

The expression of ERα and ERβ in the cortex was determined using Western blotting analysis as described previously [12]. Briefly, the cortex were rapidly harvested and were homogenized on ice in RIPA lysis buffer containing 1 mM PMSF. Equal amounts of protein (30 μg) were loaded into each lane of a 10% SDS-PAGE gel, subjected to electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked in 5% BSA for 1 h and were then incubated with 1:1000 dilutions of mouse anti-ERα (Abcam, USA), anti-ERβ (Abcam) or anti-β actin (Abcam) antibodies overnight at 4 °C. The membranes were then incubated with a HRP-conjugated secondary antibody for 2 h. Finally, the bands were visualized using Super Signal West PicoChemiluminescence Substrate.

2.6. MCAO

Four weeks after HRT, MCAO was conducted as previously described [12]. Cerebral blood flow was monitored using laser Doppler flowmetry (PeriFlux 5000; Perimed AB, Järfälla, Sweden). During surgery, an occlusion was considered to be successful when blood flow declined to less than 15% of the pre-ischemic baseline level; otherwise, the animals were discarded. Following 1 h of transient occlusion, cerebral blood flow was restored by removing the nylon suture for 24 h. Physiological parameters, blood pressure and blood gas, were monitored.

2.7. Neurobehavioral evaluation

Neurological behaviors in the mice were assessed 24 h after reperfusion using the Longa Score Scale [13]: (0) no deficit; (1) failure to extend left forepaw fully; (2) circling to the left; (3) falling to the left; (4) no spontaneous walking and decreased consciousness; and (5) dead. The neurological scores are expressed as median values with interquartile ranges.

2.8. Infarct volume assessment

The infarct volume was assessed by TTC staining as previously described [14]. Briefly, the mice were decapitated following blood collection. The brain was rapidly removed and coronal sections (1 mm) were prepared, immersed in 2% TTC at 37 °C for 30 min and then fixed with 4% paraformaldehyde for 24 h. The brain slices were photographed using a digital camera (Canon, Japan). Unstained areas were defined as infarcts and quantified using Photoshop CS3. The infarct volume was calculated by multiplying unstained areas and the slice thickness. The total infarct volume was calculated by summing the areas from all six slices.

2.9. Statistical analyses

The SPSS 13.0 software program for Windows was used for all statistical analyses. With the exception of the neurobehavioral scores, all values are expressed as means ± standard deviation (SD) and were analyzed using a one-way analysis of variance (ANOVA).