Estradiol Exerts Neuroprotective Effects When Administered After Ischemic Insult

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Background and Purpose—17β-Estradiol (E2) has been reported to exert neuroprotective effects when administered before an ischemic insult. This study was designed to determine whether E2 treatment after ischemia exerts the same effects and, if so, how long this therapeutic window remains open, and whether the effects are related to changes in cerebral blood flow (CBF).

Methods—Female Sprague-Dawley rats were subjected to permanent middle cerebral artery occlusion (MCAO). In protocol 1, E2 was administered (100 μg/kg IV followed immediately by subcutaneous implantation of crystalline E2 in a silicone elastomer tube) to ovariec-tomized females (OVX+E2) at 0.5 (n = 8), 1 (n = 6), 2 (n = 7), 3 (n = 6), or 4 (n = 9) hours after MCAO. Intact (INT; n = 6) and ovariec-tomized females (OVX; n = 12) were subjected to MCAO and received vehicle instead of E2. Two days after MCAO the animals were killed, and ischemic lesion volume was determined by 2,3,5-triphenyltetrazolium chloride staining. In protocol 2, CBF was monitored before and at 1, 24, and 48 hours in a group of animals receiving E2 or vehicle 0.5 hour after ischemia induction (INT, n = 6; OVX, n = 8; OVX+E2, n = 6).

Results—Lesion volume was 20.9 ± 2.2% and 21.8 ± 1.2% in the INT and OVX groups, respectively. E2 was found to decrease lesion volume significantly when administered within 3 hours after MCAO. The lesion volumes were 6.3 ± 0.5%, 10.3 ± 2.1%, 11.8 ± 1.8%, 13.5 ± 1.6%, and 17.9 ± 2.8% when E2 was administered at 0.5, 1, 2, 3, or 4 hours after MCAO, respectively. CBF decreased to 43.1 ± 2.2% and 25.4 ± 1.0% in the INT and OVX animals, respectively, at 5 minutes after MCAO. In comparison to OVX rats, CBF was not different at 1 hour after E2 administration but was increased significantly in the OVX+E2 group 1 and 2 days after E2 administration.

Conclusions—E2 exerts neuroprotective effects when administered after ischemia, with a therapeutic window in a permanent focal cerebral ischemia model of approximately 3 hours. This effect of estradiol was associated with no immediate change in blood flow but with a delayed increase in CBF. (Stroke. 2000;31:745-750.)

Key Words: cerebral blood flow • estrogens • ischemia • neuroprotection

Both retrospective and prospective epidemiological studies have demonstrated beneficial effects of estrogen replacement therapy in reducing stroke-related mortality that is associated with stroke in postmenopausal women.1,2 Recently, several laboratory studies have also emphasized the neuroprotective effects of estrogens.3–6 Both chronic and acute pretreatment can reduce ischemic damage in focal cerebral ischemia, indicating that estrogens may be a new therapeutic class of drugs to prevent neuronal damage associated with cerebral ischemia.

Presently, it is not known whether postischemic treatment with estrogen is beneficial. The purpose of this study was to determine (1) whether 17β-estradiol (E2) can protect against brain injury when administered after cerebral ischemia; (2) the duration of any therapeutic window offered by E2, and (3) whether any E2 neuroprotective effects are associated with changes in cerebral blood flow (CBF).

Materials and Methods

Female Charles River Sprague-Dawley rats (225 to 250 g; Wilmington, Mass) were maintained in laboratory acclimatization for 3 days before ovariectomy. Bilateral ovariectomy was performed 2 weeks before middle cerebral artery occlusion (MCAO) under methoxylurane inhalant anesthesia. All animal procedures were approved by the University of Florida Animal Care and Use Committee.

Middle Cerebral Artery Occlusion

Animals were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Rectal temperature was monitored and maintained between 36.5°C and 37.5°C during the procedure. MCAO was achieved according to the methods described by others, with the following modifications.7,8 With the aid of an operating microscope, the left common carotid artery, external carotid artery and internal carotid artery were exposed through a midline cervical skin incision. A 4-0 monofilament suture with its tip
rounded by heating was introduced into the internal carotid artery via the external carotid artery lumen and advanced until resistance was encountered. The distance between the common carotid artery bifurcation and the resistive point was approximately 1.9 cm. A 6-0 silk ligature was placed around the external carotid artery and tightened around the intraluminal monofilament suture to prevent bleeding and change of the suture position. The common carotid artery and pterygopalatine artery temporary ligatures were then released, and the skin incision was closed.

Measurement of Regional CBF
A laser-Doppler flowmeter was used for CBF measurements. The scalp was incised on the midline, and bilateral 2-mm burr holes were drilled 1.5 mm posterior and 4.0 mm lateral to the bregma. The dura was left intact to prevent cerebrospinal fluid leakage. Laser-Doppler flowmeter probes held in place by a micromanipulator were stereotaxically advanced to gently touch the intact dura mater. CBF was measured before and within 1.5 hours after MCAO. The incision was stapled, and the animals were then returned to their home cages. At 1 and 2 days after MCAO, the animals were reanesthetized with ketamine (60 mg/kg IP) and xylazine (10 mg/kg IP), and stable CBF recordings were obtained bilaterally at the same sites for at least 10 minutes. The CBF values were calculated and expressed as a percentage of the baseline values. CBF values reported represent the mean±SEM for the average of the CBF recordings obtained.

Measurement of Lesion Volume
Each group of animals was decapitated 2 days after MCAO, and the brain was removed and placed in a metallic brain matrix for tissue slicing (Harvard) immediately after decapitation. Five slices were made at 3, 5, 7, 9, and 11 mm posterior to the olfactory bulb. Each slice was incubated for 30 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride in physiological saline at 37°C and then fixed in 10% formalin. The stained slices were photographed by a digital camera (Sony MVC-FD5) and subsequently measured for the surface area of the slices and the ischemic lesion (Image-Pro Plus 3.0.1). Ischemic lesion volume was calculated as the sum of the areas of the ischemic lesion across the 5 slices divided by the total cross-sectional area of these 5 brain slices.

E2 Administration and Serum Concentration
To obtain a prompt and sustained elevation in serum E2 concentration, intravenous injection of an aqueous soluble E2 preparation combined with simultaneous implantation of a silicone elastomer pellet containing the steroid was used. To assess serum concentrations of E2 after this treatment regimen, 6 OVX animals were anesthetized with methoxyflurane inhalant, and a control blood sample was taken via the jugular vein. Then E2 (100 μg/kg body wt) complexed in hydroxypropyl-β-cyclodextrin (E2-HPCD, Sigma), which was dissolved in 0.9% normal saline, was administered via tail vein injection, and a 5-mm-long silicone elastomer tube (1.57 mm ID; 3.18 mm OD) containing crystalline E2 was immediately implanted subcutaneously. The animals were put back into their cages, and blood samples were then taken via the jugular vein at 5 minutes and 0.5, 1, 2, 4, 6, 12, 24, and 48 hours after steroid administration, under methoxyflurane anesthesia. Serum was separated from blood by centrifugation and stored frozen (−20°C). Serum E2 concentrations were determined with the use of duplicate serum aliquots in a radioimmunoassay (ultrasensitive estradiol kit, Diagnostic Laboratory).

Protocol 1
To determine whether E2 exerts any beneficial neuroprotective effect when administered after the ischemic insult and the duration of any therapeutic window, E2 was administered (100 μg/kg, by tail vein injection combined with subcutaneous implantation of an E2-containing silicone elastomer tube) in ovariectomized female rats (OVX+E2 group) at 0.5 (n=6), 1 (n=6), 2 (n=7), 3 (n=6), or 4 (n=9) hours after MCAO. As controls, ovariectomized females (OVX group; n=12) and intact females (INT group; n=6) were treated with equivalent volumes of saline and empty pellets at 0.5, 1, 2, 3, or 4 hours after MCAO.

Protocol 2
To determine whether any neuroprotective effects of E2 were associated with blood flow changes, a laser-Doppler flowmeter was used to monitor CBF. After a baseline CBF reading was obtained, CBF was continuously recorded for 1.5 hours after MCAO induction. E2 was administered (100 μg/kg tail vein injection and subcutaneous implantation of an E2 pellet) 0.5 hour after MCAO induction (OVX+E2 group; n=6), and CBF was obtained for 1 hour thereafter and at 24 and 48 hours after MCAO. Intact females (INT group; n=6) and ovariectomized females (OVX group; n=8) received equivalent volumes of saline and empty pellet as controls.

Statistical Analysis
Statistical analyses were performed with SigmaStat 2.0 Software (Jandel Scientific). All data were expressed as mean±SEM. The lesion volumes in each group comparison were analyzed with 1-way ANOVA. The CBF values in each group were analyzed among groups at each sampling time with 1-way ANOVA. The difference for each comparison was considered significant at the P<0.05 level.

Results
Effects of E2 Administration on Serum E2 Concentration
In young cycling female rats, serum levels of E2 vary between 11±1 pg/mL at diestrus and 41±5 pg/mL at proestrus. Serum E2 concentrations increased and peaked at 3487±110 pg/mL 5 minutes after E2 administration, then decreased to 76±16 pg/mL 24 hours after administration (Figure 1). With the slow release from the E2 pellet, serum E2 concentration remained high at 45±5 pg/mL 48 hours after administration, compared with 13±4 pg/mL in OVX animals.

Therapeutic Window of E2
E2 treatment after the ischemic insult exerted neuroprotective effects (Figures 2 and 3). The ischemic lesion volume was significantly reduced in the OVX+E2 group when E2 was administered at 0.5, 1, 2, or 3 hours after the ischemic insult, with lesion volumes of 6.3±0.5%, 10.3±2.1%, 11.8±1.8%,

![Figure 1. Effect of simultaneous intravenous injection of E2 (100 μg/kg complexed in hydroxypropyl-β-cyclodextrin) and subcutaneous implantation of an estradiol-filled silicone elastomer pellet on serum estradiol concentration in OVX rats. At the time indicated, 0.5-mL blood samples were obtained for subsequent E2 concentration analysis. n=6 rats per time point. Mean±SEM values are depicted. *P<0.05 vs all other sample times.](Image 314x571 to 530x718)
and 13.5±1.6%, respectively (P<0.05), indicating a therapeutic window of up to 3 hours in permanent focal cerebral ischemia. No significant difference of lesion volume was noted between OVX and INT groups (21.8±1.2% and 20.9±2.2%, respectively).

**Effect of E2 on CBF**

The ipsilateral CBF was higher immediately after MCAO in the INT group compared with the OVX and OVX+E2 groups: values for INT, OVX, and OVX+E2 groups were 43.1±2.2%, 26.2±1.5%, and 23.9±0.9%, respectively (P<0.01). After E2 administration, ipsilateral CBF increased at 1 and 2 days after E2 administration but not at 1 hour (Figure 4). The effects of MCAO on the contralateral CBF were similar in all groups and were independent of the estrogen status of the animal.

**Discussion**

This study demonstrates 3 potentially important clinical effects of E2. First, E2 exerts neuroprotective effects even when administered after the onset of an ischemic insult, with a therapeutic window up to 3 hours. Second, the neuroprotective effects of E2 are not associated with an immediate blood flow augmentation effect but with a later improvement in CBF. Third, at the dose used, neuroprotective effects of E2 are flow independent and in this permanent focal cerebral ischemia model are only observed with exogenous E2.

Several studies have demonstrated that E2 is a potent neuroprotective agent that decreases focal ischemia–induced lesion size by approximately 50% with E2 chronic pretreatment.3–6 E2 also exerts neuroprotective effects when administered immediately before occlusion.9 The present study, for the first time, systematically defines the therapeutic window of E2 in a model of permanent focal ischemia when the drug is administered after the ischemia has been induced.
The neuroprotective mechanisms of E2 are not yet elucidated, although both direct neuroprotective action on neurons and indirect effects on the cerebral vasculature are possible. Direct effects can include reduction in reactive oxygen species that accumulate during ischemia,10 blockade of excitatory amino acid toxicity,11,12 modulation of calcium homeostasis,13–15 induction of neurotrophins and their receptor and intracellular signaling pathway,16,17 induction of antiapoptotic protein,18,19 and/or enhancement of brain glucose uptake.20 E2 could also improve the outcome of cerebral ischemia through a protective effect on brain vascular endothelial cells,20 resulting in the presently observed delayed improvement in CBF in E2-treated rats.

E2 has been shown to act on both of the peripheral and intracranial vascular systems.21–24 In young cycling female rats, serum levels of E2 varied between 11±1 pg/mL on diestrus and 41±5 pg/mL on proestrus.23 In our study, deprivation of endogenous ovarian steroids resulted in low residual CBF ipsilateral to the MCAO.5,26 Acute administration of exogenous E2 (in which serum levels of E2 vary from 3487±110 to 45±5 pg/mL) increased ipsilateral CBF after stroke, but this effect was delayed until 1 to 2 days after occlusion. It appears that low levels of endogenous ovarian steroids resist the ipsilateral CBF effects after permanent MCAO. Acute treatment with high doses of E2 caused a delayed preserving effect on CBF, an effect that only occurred in the side ipsilateral to the MCAO.

The mechanism of any blood flow–preserving effects of E2 is still not well known, but 3 possibilities have been proposed. First, we have found that exposure of endothelial cells to E2 helps to maintain their viability during an ischemic episode.20 Findings in this experiment suggest that the delayed effect of E2 on CBF maybe be secondary to a vascular cytoprotective action of the hormone. Alternatively, estrogen could induce vasodilation in cerebral arteries.

Second, E2 has been found to modulate serum lipid levels, reducing aggregation of platelets and the thrombotic and vasoconstrictive effects of thromboxane.27,28 E2 withdrawal after ovariectomy increases the sensitivity of the rabbit basilar artery to serotonin.29 Using a mouse carotid model, Sullivan et al23 found that physiological levels of E2 replacement could significantly suppress the response of the carotid artery to injury. The endothelium produces a variety of vasoactive mediators such as prostacyclin and endothelium-derived nitric oxide, both of which have roles in regulating not only vascular tone but also smooth muscle cell proliferation.21 Goldman et al60 have also reported that within 10 minutes of injection of a supraphysiological dose of E2, CBF increases to most regions of the brain. In contrast, our study showed that the blood flow–preserving effects of E2 are not immediate but occur from 1 to 24 hours after E2 administration. These blood flow–preserving effects could be likely due to a slower genomic effect, since the cellular effects of E2 on gene expression occur hours to days after any insult.31

Finally, E2 could cause a delayed improvement in CBF through angiogenic mechanisms. Recently, Morales et al12 found that E2 exerted angiogenic effects in peripheral vessels. While angiogenic effects of E2 may play a potential role in protecting against cerebral ischemia, we are not aware of studies demonstrating that estrogens can induce angiogenesis within 2 days of steroid replacement. However, by promoting neovascularization and collateral formation, E2 could restore cerebral perfusion in ischemic areas and hence lessen the impact of occlusion.

Both low and high circulating concentrations of E2 have been reported to exert neuroprotective effects in the temporary cerebral ischemia model in E2 pretreatment studies.5,33 Both low and high physiological levels of E2 have exerted similar effects in a 1-day permanent cerebral ischemia study when administered before ischemia.6 The present study showed that E2 neuroprotective effects could be induced by high-level exogenous E2 in 2-day permanent cerebral ischemia when administered after ischemia. Subsequent assessment of the dose dependence of this neuroprotection is clearly needed.

Assessments of efficacy also need to be conducted in both male and female rats. E2 has been found to exert neuroprotective effects in males, although in males the effects are dependent in part on the suppression of testosterone secretion.34 Additionally, the neuroprotective effects of estrogens do not appear to be mediated by an estrogen receptor mechanism. 17α-Estradiol, a very weak estrogen, exerts neuroprotective effects equivalent to E2 both in vitro and in vivo.3,35 Additionally, we have recently reported that ent-estradiol, the enantiomer of E2 that lacks estrogenic activity, is as potent as E2 in protecting cerebral tissue from MCAO.36 These data indicate that several nonfeminizing estrogens that lack classic genomic-mediated estrogenic effects are potential clinical candidates for stroke neuroprotection.

In summary, our study demonstrates that E2 exerts neuroprotective effects when administered after an ischemic insult, with a therapeutic window of approximately 3 hours. The neuroprotective effect has a delayed CBF-preserving component and a blood flow–independent component. This study raises the possibility that estrogen compounds could be a useful therapy in preserving brain tissue, even if administered after the ischemic insult.

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References
Clinical studies have demonstrated that chronic estrogen use reduces stroke-related mortality.1,2 Along similar lines, animal models of cerebral ischemia have demonstrated that the presence of estrogen in physiological amounts is protective.3–8 Although much evidence exists that estrogen reduces stroke-related morbidity and mortality when present at the time of injury, it has been unclear whether estrogen is of therapeutic utility when administered after an ischemic event has occurred. To be of utility in the treatment, as opposed to the prevention, of stroke, estrogen must exert a protective effect when given within a reasonable time window after the ischemic event. The article by Yang et al demonstrates that postischemic administration of estrogen affords protection against ischemic damage similar to preischemic administra-

tion and that it acts within a clinically useful therapeutic window. However, this postischemic protection only occurs at supraphysiologic doses of estrogen. Another study9 has suggested that preischemic administration of supraphysiologic doses of estrogen lacks the neuroprotective activity exhibited by physiological doses of estrogen. Differences between the mechanisms of action of physiological and pharmacological amounts of estrogen must be determined to account for the differing actions when estrogen is administered before or after the ischemic event. It remains to be seen whether the mechanism by which supraphysiologic doses of estrogen exert a protective effect is a novel one or represents a nonspecific action of estrogen at a previously described neuroprotective site.
References


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