Estrogen Provides Neuroprotection in Transient Forebrain Ischemia Through Perfusion-Independent Mechanisms in Rats

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Background and Purpose—Estrogen-related neuroprotection in association with animal models of transient forebrain and focal ischemia has been documented in several recent reports. Some of those studies indicated that part of that benefit was a function of improved intraischemic vasodilating capacity. In the present study we examined whether chronic estrogen depletion and repletion affected ischemic neuropathology through perfusion-independent mechanisms.

Methods—Normal, ovariectomized (OVX), and OVX female rats treated with 17β-estradiol (E₂) were subjected to 30 minutes of transient forebrain ischemia (right common carotid occlusion plus hemorrhagic hypotension) and reperfusion. Neurological function and brain histopathology were assessed over the 72-hour recovery period. In all rats, preischemic and intraischemic cortical cerebral blood flow (CBF) levels were monitored with laser-Doppler flowmetry. In additional rats, CBF changes in the striatum and hippocampus were also monitored with laser-Doppler flowmetry probes and radiolabeled microspheres. In each experiment, the level of ischemia was targeted to a 75% to 80% reduction in cortical CBF.

Results—The similarity in ischemic severity among groups was supported by measurements of comparable patterns of electroencephalographic power changes during the ischemic period. Compared with normal females, OVX rats showed diminished neurological outcomes and more severe histopathology in the hippocampus and striatum. Two-week treatment of OVX rats with E₂ was accompanied by postischemic neuropathological changes similar to those seen in normal females. Intraischemic CBF reductions in the hippocampus and striatum were similar in all groups (to 35% to 50% of the preischemic value) but significantly less than the cortical CBF reductions.

Conclusions—These findings indicate that estrogen provides ischemic neuroprotection through mechanisms unrelated to improvement of intraischemic cerebral perfusion. (Stroke. 1999;30:630-637.)

Key Words: cerebral blood flow □ cerebral ischemia, global □ estradiol □ rats
ogy were examined. This was accomplished by monitoring cortical CBF, using laser-Doppler flowmetry (LDF), and controlling intraischemic CBF reductions. An intraischemic cortical CBF reduction target, to 20% to 25% of baseline, was used. In separate rats, we also measured and compared preischemic CBF levels and intraischemic CBF reductions in cortex, hippocampus, and striatum. This was done to establish that when cortical CBF is reduced to 20% to 25% in the 3 groups, the 2 most “vulnerable” regions (hippocampus and striatum) will display equivalent levels of hypoperfusion when groups are compared.

Materials and Methods

The study protocol was approved by the Institutional Animal Care and Use Committee. Female Sprague-Dawley rats (weight, 250 to 350 g) were used. Two experimental series were established. Both series consisted of 3 groups of rats: normal, OVX, and OVX+E2-treated females. In the first series (n = 30), the rats were subjected to 30 minutes of forebrain ischemia and 72 hours of reperfusion. In the second series (n = 18), the reperfusion phase was omitted. The rats from the first series were anesthetized with halothane, intubated endotracheally, and mechanically ventilated with 1% halothane in 70% N2O/30% O2. Catheters were inserted into a femoral artery and vein and the right subclavian vein. The catheters were used for pressure recording and sampling (for blood gas/pH and plasma glucose analysis), drug infusions, and intraischemic blood withdrawal, respectively. The right common carotid artery was isolated for later clamping. A muscle relaxant, vecuronium bromide, was given as an intravenous infusion to maintain paralysis. An area of the skull overlying the right parietal cortex was thinned to translucency with a drill, and an inverted 21-gauge needle was glued in place over the bone “window.” This was used as a guide for an 0.8-mm-diameter microsphere. According to procedures described previously,10 the microspheres were suspended in a 0.01% (in isotonic saline) Tween-80 solution. After thorough mixing, 0.2 mL of the solution (containing ~150,000 microspheres) was injected into the left ventricle and flushed with 0.2 mL of saline. Microsphere injections were made at 15 minutes before initiation of ischemia and at 30 minutes of ischemia. A reference arterial blood sample was withdrawn (at 0.4 mL/min), starting just before microsphere injection and continuing for 45 seconds after injection.11 Flows determined by LDF in the cortex, hippocampus, and striatum were simultaneously and continuously recorded throughout the experiments. During ischemia, blood withdrawal was adjusted to maintain cortical CBF at 20% to 25% of the preischemic value. At the end of the ischemic period, the rats were killed by halothane overdose, and tissue from 2 regions—the right cortex and the right subcortex (hippocampus + striatum)—was dissected out and weighed. Regional CBF values were calculated according to methods described previously.

Ovariectomies were performed by the supplier (Charles River, Wilmington, Mass) at 4 to 6 weeks before the study. The OVX+E2-treated group received daily intraperitoneal injections of 0.1 mg·kg−1·E2 (0.1 mL, prepared in dimethyl sulfoxide) for 2 weeks preceding the study, whereas untreated rats were given vehicle only. The E2 treatment regimen was designed to produce plasma E2 levels (at the time of ischemic onset) that were in the range of values seen in normal females. This dosing regimen was established in pilot studies in which plasma samples were obtained for E2 analysis (by radioimmunoassay14) in normal females and in OVX rats, either untreated or treated with E2 at 0.1 mg·kg−1·d−1. Blood was obtained, under light halothane anesthesia, by subclavian venipuncture. In the normal females (n = 5), blood was taken once per day (at noon) for 5 consecutive days to establish the range of E2 levels over the normal 4- to 5-day estrous cycle. In the E2-treated rats (n = 5), blood was withdrawn immediately before intraperitoneal E2 injection (at noon) and 2 hours after E2 (at 2 PM) for 5 consecutive days. In the untreated OVX rats (n = 5), only single samples were obtained in each animal.

For neurological function assessments, an 18-point scale was used. A blinded observer scored the rats each day for 3 days. There were 6 different categories: (1) consciousness (scores range from 0 [normal] to 4 [seizures]); (2) rope platform (scores range from 0 [climbs to platform] to 4 [no grasp reflex]); (3) limb tone (normal = 0, weak = 1); (4) walking (scores range from 0 [normal] to 4 [unable to stand]); (5) rotating screen (scores range from 0 [grasps to 80° to 25 movements] to 3 [falls from vertical screen]); and (6) pain reflex (normal = 0, hypotensive = 1). The summed daily scores could range from 0 (no dysfunction) to 54 (death on the first day after ischemia). For a rat to receive a score of 54, the animal had to regain consciousness after ischemia and had to experience at least 1 seizure before death. If both criteria were not satisfied, the rat was excluded. All rats surviving the full 3 days were anesthetized with isoflurane and subjected to perfusion fixation of the brain.15 The brains were subsequently removed and processed for histological examination (see above).

The E2 was obtained from Sigma. Arterial blood gasses and pH were measured with an Instrumentation Laboratories (model BGE) analyzer. Plasma glucose was measured with a Beckman Glucose Analyzer 2. Statistical comparisons of CBF results between the 3 groups were performed with a multivariate analysis (Systat). A nonparametric Kruskal-Wallis test was used for analysis of the histopathology and neurological outcome data. Preischemic versus intraischemic arterial data were analyzed with a paired t test. Statistical significance was taken at the P < 0.05 level.
**Results**

**Blood Variables**

In the rats subjected to ischemia and reperfusion, the arterial \( P_{O_2} \), \( P_{CO_2} \), pH, and MABP values, measured just before ischemia and at 30 minutes of ischemia, are summarized in the Table. The arterial \( P_{O_2} \), \( P_{CO_2} \), and pH values among groups were similar at comparable experimental time points, with no changes observed after imposition of ischemia. Similar reductions in MABP during ischemia were measured in the 3 ischemia/reperfusion groups. Similar 2- to 2.5-fold reductions in MABP during ischemia were measured in the 3 ischemia/reperfusion groups. Similar 2- to 2.5-fold increases in plasma glucose (from a preischemic value of \( \sim 160 \text{ mg} \cdot \text{dL}^{-1} \)) were observed at 30 minutes of ischemia in the normal, OVX, and OVX+E\(_2\)-treated females (data not shown). In the 3 groups in which regional CBF changes were monitored (data not shown), no significant changes in \( P_{O_2} \), \( P_{CO_2} \), and pH were observed when preischemic and intr/ischemic values were compared. When the 3 groups were compared, similar MABP values were measured before and during ischemia.

The results of the plasma \( E_2 \) evaluations are given in Figure 1. In the normal females (\( n=5 \)), mean peak plasma values were \( 62 \text{ pg} \cdot \text{mL}^{-1} \), while the nadir was \( 27 \text{ pg} \cdot \text{mL}^{-1} \). The lower values were obtained at 48 to 72 hours after the peak. In the \( E_2\)-treated rats (\( n=5 \)), the mean plasma \( E_2 \) values at 24 hours after injection (averaged over days 2 to 5) were \( 46 \text{ pg} \cdot \text{mL}^{-1} \) and were well within the normal range. Moreover, in \( E_2\)-treated rats subjected to TFI, that point would be equivalent to the onset of ischemia. At 2 hours after injection, the plasma \( E_2 \) levels (250 pg \( \cdot \text{mL}^{-1} \)) exceeded the normal range. The plasma \( E_2 \) concentration in untreated (\( n=5 \)) OVX rats (30 pg \( \cdot \text{mL}^{-1} \)) was equivalent to the lowest daily value seen in intact females.

**Ischemic Neuropathology**

Virtually identical intr/ischemic reductions in cortical CBF were achieved and maintained in the 3 groups subjected to ischemia and reperfusion. The average intr/ischemic cortical CBF values (expressed as a percentage of the preischemic perfusion unit level) in the normal (\( n=13 \)), OVX (\( n=10 \)), and OVX+E\(_2\)-treated (\( n=7 \)) groups were 23.6%, 23.8%, and 24.1%, respectively. Additional evidence that the levels of ischemia were comparable in the 3 groups was provided by measurements of intr/ischemic EEG power changes. The similarity in the pattern of the EEG power changes over the 30-minute ischemic period (Figure 2) is consistent with equivalent ischemic severities in the normal, OVX, and OVX+E\(_2\)-treated females. EEG power changes are a sensi

![Figure 1](https://via.placeholder.com/150)

**Figure 1.** Plasma \( E_2 \) levels in normal, OVX, and OVX+E\(_2\)-treated female rats (at 100 \( \mu \text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \) IP). In normal females (\( n=5 \)), venous samples were obtained once per day over 5 consecutive days. The nadir value represents the lowest level measured on any day over the 5-day sampling period; the peak represents the highest level obtained. In \( E_2\)-treated rats, samples were obtained twice daily (at the same times) over 5 consecutive days. The values given in the figure represent the means of those 5-day averages (\( n=5 \)). In OVX rats (\( n=5 \)), only a single sample was obtained. *\( P<0.05 \) vs nadir; †\( P<0.05 \) vs 24 hours after \( E_2 \). Values are mean±SE.

![Figure 2](https://via.placeholder.com/150)

**Figure 2.** Intr/ischemic EEG power reductions (in decibel units) in normal (\( n=13 \)), OVX (\( n=10 \)), and OVX+E\(_2\)-treated (\( n=7 \)) rats. The similarities in the patterns of EEG power reductions among groups are indicative of comparable ischemic severities (see Reference 1). Values are mean±SE.
ative indicator of ischemic magnitude, as described in a recent report in which we showed that decreases in CBF below current levels resulted in substantially greater intraischemic EEG power reductions. Thus, evidence from the present study, based on both perfusion and functional criteria, points to equivalent levels of ischemic severity being imposed in all groups.

Histopathologic assessments, obviously, can only be performed in surviving rats. In the present investigation we found that ovariectomy decreased and E2 treatment improved 72-hour survival rates. The percentage of animals surviving 3 days was 69% in the normal females (9 of 13), 40% in the untreated OVX females (4 of 10), and 71% (5 of 7) in the OVX+E2-treated rats. Using CA1 to CA4 pyramidal and dentate gyrus granule cell counts in the contralateral nonischemic hemisphere as an internal control (no signs of histopathology were observed in that hemisphere), we observed an ~45% loss of hippocampal neurons in normal females (Figure 3A). Almost all of that reduction was derived from loss of CA1 pyramidal cells (Figure 4). In OVX rats, the percent neuronal loss increased significantly, to almost 90%, and included extensive CA3, CA4, and dentate gyrus involvement (not shown). Two-week treatment of OVX rats with E2 (100 μg · kg⁻¹ · d⁻¹) was accompanied by a level of hippocampal cell loss (~60%) not significantly different from that observed in normal females, with some of that “recovery” seen in CA1 (Figure 4). Cell loss in CA3, CA4, and dentate gyrus was comparable to that seen in normal females (not shown). A similar pattern of histopathology was observed in the striatum when normal, OVX, and OVX+E2-treated females were compared (Figures 3B and 4). Again, with the histopathology-free contralateral hemisphere used as a point of reference, cell loss (Figure 3B) in normal females amounted to 33%, exceeded 50% in OVX rats (P<0.05 versus normal), and was reduced to <40% in E2-treated, OVX females (P>0.1 versus normal). The ischemic cerebral cortex was generally undamaged, except that in the untreated OVX group, but not in the remaining groups, a few isolated pyknotic neurons were observed in the intermediate layers (not shown).

Neurological outcome scores (which include both survivors and nonsurvivors) mirrored the histopathologic findings (Figure 5). Three-day outcome scores averaged 20 in normal females, 31 in the OVX-untreated group (P<0.05 versus normal), and 20 in the E2-treated rats (P>0.1 versus normal).

Regional CBF
Preischemic cortical and subcortical CBF values (by the microsphere technique) in normal females and OVX rats, with or without chronic E2 treatment, are summarized in Figure 6. Subcortical flows were obtained in samples that included tissue from striatum plus hippocampus. There were no significant differences in preischemic regional CBF levels when the 3 groups were compared.

The percent reductions in intraischemic blood flows, detected with the use of LDF probes positioned over the cortex...
and inserted into the striatum and hippocampus, are depicted in Figure 7. The CBF decreases in the striatum and hippocampus (35% to 50% of baseline for all groups) were significantly less than in the cortex (20% to 25% of baseline). The regional CBF relationships remained constant during the 30-minute ischemic period (Figure 7). The percent CBF reductions assessed through LDF were corroborated by microsphere flow data (Figure 8) for both the cortex and subcortex. The relationship among groups for absolute intraischemic regional CBF values reflected the relationship seen before ischemia. Thus, at 30 minutes of ischemia, the cortical CBF values in the normal, OVX, and OVX + E2 groups were 42 ± 6, 31 ± 9, and 38 ± 7 mL · 100 g⁻¹ · min⁻¹, respectively. The subcortical values were 43 ± 5, 37 ± 7, and 45 ± 3, respectively. The differences between groups (by ANOVA) were statistically insignificant. These findings established that, when the 3 study groups were compared, similar levels of ischemia were imposed in the cerebral cortex and in the more vulnerable hippocampus and striatum.

Discussion

The results of this study clearly show that under conditions of comparable ischemic severity, chronic estrogen depletion exacerbates and estrogen treatment lessens the brain damage associated with TFI. Recent results from our laboratory and others have also indicated the ability of estrogen to provide ischemic neuroprotection by virtue of improving brain perfusion. That particular benefit of estrogen was seen in association with global (forebrain) as well as focal ischemia (ie, transient middle cerebral artery occlusion) in rats1,3,4 and was linked, at least in part, to an E2-related enhancement of constitutive NOS expression and function.1,4,16 Thus, present and previous findings suggest that estrogen provides ischemic brain protection through perfusion-dependent as well as perfusion-independent processes.

In the present study we imposed the same level of ischemic severity in all experiments by targeting a specific percent reduction in cortical CBF. Additional evidence that all groups experienced similar ischemic severities was provided by our observation that all groups showed identical patterns of EEG power reductions during ischemia. We recently showed that EEG power analysis can be used as a sensitive indicator of ischemic severity.1 However, consistent with the classic pattern of ischemic vulnerability accompanying TFI,17 we found histopathology to be confined essentially to neurons of the hippocampus and striatum. Because of this, it was important to establish, in all experimental groups, that comparable levels of ischemic severity were achieved in the vulnerable regions, as well as in the cortex. The similarities in
regional intraischemic blood flow reductions among groups, coupled with the finding of no significant differences in preischemic regional CBF values (as others have reported\(^1\)) or absolute intraischemic regional CBF values, confirmed that comparable regional ischemic severities were imposed in all groups. Curiously, when cortical CBF was reduced by 75% to 80% of baseline, blood flow was diminished in the hippocampus and striatum by only 50% to 65%. Nevertheless, in relation to the cortex, these regions showed comparatively greater damage despite the fact that perfusion was reduced to a lesser extent. Although no explanation can be offered at this time, these findings suggest that, when different brain structures are compared, the degree of ischemia-induced neuronal loss is not solely related to the magnitude of the CBF decrease or the level to which CBF is reduced.

There are a variety of processes that may be affected by E\(_2\) and may promote ischemic brain protection through mechanisms unrelated to vasodilation. Brain damage after TFI has been linked to enhanced leukocyte adhesion/infiltration,\(^1\) free radical mechanisms,\(^2\) upregulation of the inducible NOS (iNOS) isoform,\(^3\) altered cerebral glucose transport,\(^4,5\) and apoptosis.\(^6\) Estrogens, E\(_2\) in particular, have been shown to possess anti-inflammatory functions,\(^7,8\) antioxidant actions,\(^9-11\) an ability to block the induction of iNOS,\(^12\) a capacity to improve glucose transport,\(^13,14\) and antiapoptotic effects.\(^15\) Because none of those possibilities were specifically addressed in this report, no further comment can be made at this time. Clearly, additional experiments are required.

In the present study only a single E\(_2\) replacement paradigm was used, ie, chronic treatment with a dose that was designed to produce circulating levels of E\(_2\), at the time of ischemic onset, that fell within the range of plasma E\(_2\) levels seen during the estrous cycle in normal females (Figure 1). The E\(_2\) administration protocol cannot be described as “physiological” because we elicited an E\(_2\) “surge” once each day, instead of once every 4 to 5 days (as occurs in intact females). Nevertheless, we did show that the 0.1 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) day\(^{-1}\) treatment protocol produced optimal neuroprotection in TFI,\(^1\) to the extent that with \(\geq 5\)-fold higher daily doses, ischemic neuroprotection was lost. This approach also did not permit us to establish whether the neuroprotection provided by estrogen replacement therapy in OVX rats is dependent on E\(_2\) interacting with “classic” or “nonclassic” estrogen receptors,\(^16\) is receptor independent, or involves genomic or non-genomic mechanisms. In fact, E\(_2\) replacement therapy in focal ischemia models has been shown to be efficacious with acute treatments.\(^1,3,3,3,3\) Moreover, protection has also been seen in animals given the purportedly receptor-inactive isoflur, 17 \(\alpha\)-estradiol.\(^1,3,3,3,3,3\) These findings would appear to be consistent with a nongenomic process and might be viewed as evidence of antioxidant actions of estrogens, as some investigators have suggested.\(^2,26,27,3,3,3,3,3,3\) On the other hand, there are indications that E\(_2\)-related neuroprotection, to some degree, may involve interactions with classic receptors (see, for example, Singer et al\(^3\)).

The present finding of detectable blood E\(_2\) levels in the OVX rats (30 pg \(\cdot\) mL\(^{-1}\)) is not unusual in that it falls within the range of values reported in the literature (from <5 pg \(\cdot\) mL\(^{-1}\) to >40 pg \(\cdot\) mL\(^{-1}\)).\(^3,3,3,3,3\) However, it is interesting to note that the intact females in the present study were neuroprotected compared with OVX rats, despite having circulating E\(_2\) levels, during most of their estrous cycles, similar to the levels seen in the OVX group. Whether the relatively brief E\(_2\) surge occurring on the day of proestrus or some other factor, such as progesterone, can account for the relative neuroprotection seen in the intact females remains to be established. Progesterone is a viable possibility in light of limited evidence showing it to be neuroprotective in focal and global ischemia models.\(^3,3,3,3,3\)

In conclusion, on the basis of the present and previously published results, using in vivo models, there is clear evidence that chronic estrogen depletion by ovariectomy (used as a model for menopause) is accompanied by an exacerbation of ischemic neuropathology. Studies to date have also indicated that both acute and chronic E\(_2\) replacement can diminish ischemic neuropathology and that the protection afforded by E\(_2\) repletion may involve both genomic and nongenomic (direct) actions and multiple sites of influence. The chronic E\(_2\) treatment protocol used in the present study was associated with a lessened neuropathology at 72 hours, although it remains to be established whether that neuroprotection can be maintained over longer periods of time. Finally, results from earlier studies implied that the palliative effects of estrogen were, at least in part, related to improving intraischemic cerebral perfusion.\(^1,3,3,3,3\) The present results strongly indicated that estrogen-induced neuroprotection, to a substantial degree, involves perfusion-independent mechanisms.

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References

Estrogen and Ischemic Brain Protection


Editorial Comment

Epidemiological and clinical data suggest that the risk of stroke is lower in premenopausal women, an observation that has been attributed to the beneficial effect of estrogen on vascular diseases. In support of this conclusion, experimental studies have provided evidence that estrogen is protective in different models of cerebral ischemia (eg, References 2 and 3). However, the mechanisms of the effect remain unclear. Evidence suggests that estrogen can ameliorate ischemic damage either by improving flow to the ischemic brain or by making the brain tissue intrinsically more resistant to the effects of cerebral ischemia. However, in vivo data supporting a neuroprotective role of estrogen independent of its vascular action are scarce.

In the accompanying study, Wang et al devised an experimental protocol that enabled them to minimize the vascular effects of estrogen. In a well-controlled model of transient forebrain ischemia in the rat, they reduced cerebral blood flow to a preset value in both intact and ovariec-tomized rats. They found that under these conditions of nearly identical ischemia, the ovariec-tomized rats had greater histological damage and neurological deficits than the intact rats. Importantly, estrogen replacement in ovariec-tomized rats re-established the protection to levels indistinguishable from those in intact females. The data provide convincing evidence that nonvascular factors play an important role in the protection exerted by estrogen in vivo. The nonvascular mechanisms by which estrogen exerts its protective effect following cerebral ischemia remain largely unknown, but they are likely to include both receptor-dependent and -independent effects on signaling pathways as well as direct effects on gene expression. Future studies addressing these issues are eagerly awaited.
The estrogen-induced protection observed in experimental studies raises the possibility that estrogen replacement in postmenopausal women could reduce the risk of stroke. However, recent well-controlled studies suggest that estrogen replacement does not influence the incidence of stroke in postmenopausal women. Therefore, despite its clear-cut beneficial effects in experimental models, the role of estrogen replacement in stroke prevention remains to be defined.

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