17β-Estradiol Reduces Stroke Injury in Estrogen-Deficient Female Animals

Renata Rusa, MD; Nabil J. Alkayed, MD, PhD; Barbara J. Crain, MD, PhD; Richard J. Traystman, PhD; Alane S. Kimes, PhD; Edythe D. London, PhD; Judy A. Klaus, RN; Patricia D. Hurn, PhD

**Background and Purpose**—The importance of postmenopausal estrogen replacement therapy for stroke in females remains controversial. We previously showed that female rats sustain less infarction in reversible middle cerebral artery occlusion (MCAO) than their ovariectomized counterparts and that vascular mechanisms are partly responsible for improved tissue outcomes. Furthermore, exogenous estrogen strongly protects the male brain, even when administered in a single injection before MCAO injection. The present study examined the hypothesis that replacement of 17β-estradiol to physiological levels improves stroke outcome in ovariectomized, estrogen-deficient female rats, acting through blood flow–mediated mechanisms.

**Methods**—Age-matched, adult female Wistar rats were ovariectomized and treated with 0, 25, or 100 µg of 17β-estradiol administered through a subcutaneous implant or with a single Premarin (USP) injection (1 mg/kg) given immediately before ischemia was induced (n=10 per group). Each animal subsequently underwent 2 hours of MCAO by the intraluminal filament technique, followed by 22 hours of reperfusion. Ipsilateral parietal cortex perfusion was monitored by laser-Doppler flowmetry throughout ischemia. Cortical and caudate-putamen infarction volumes were determined by 2,3,5-triphenyltetrazolium chloride staining and digital image analysis. End-ischemic regional cerebral blood flow was measured in ovariectomized females with 0- or 25-µg implants (n=4 per group) by 14C-iodoantipyrine quantitative autoradiography.

**Results**—Plasma estradiol levels were 3.0±0.6, 20±8, and 46±10 pg/mL in the 0-, 25-, and 100-µg groups, respectively. Caudate-putamen infarction (% of ipsilateral caudate-putamen) was reduced by long-term, 25-µg estrogen treatment (13±4% versus 31±6% in the 0-µg group, P<0.05, and 22±3% in the 100-µg group). Similarly, cortical infarction (% of ipsilateral cortex) was reduced only in the 25-µg group (3±2% versus 12±3% in the 0-µg group, P<0.05, and 6±3% in the 100-µg group). End-ischemic striatal or cortical blood flow was not altered by estrogen treatment at the neuroprotective dose. Infarction volume was unchanged by acute treatment before MCAO when estrogen-treated animals were compared with saline vehicle–treated animals.

**Conclusions**—Long-term estradiol replacement within a low physiological range ameliorates ischemic brain injury in previously ovariectomized female rats. The neuroprotective mechanism is flow-independent, not through preservation of residual ischemic regional cerebral blood flow. Furthermore, the therapeutic range is narrow, because the benefit of estrogen in transient vascular occlusion is diminished at larger doses, which yield high, but still physiologically relevant, plasma 17β-estradiol levels. Lastly, unlike in the male brain, single-injection estrogen exposure does not salvage ischemic tissue in the female brain. Therefore, although exogenous steroid therapy protects both male and female estrogen-deficient brain, the mechanism may not be identical and depends on long-term hormone augmentation in the female. (Stroke. 1999;30:1665-1670.)

**Key Words:** cerebral blood flow ■ cerebral ischemia ■ 17β-estradiol ■ estrogen replacement therapy ■ stroke ■ rats

The risk of cerebrovascular events in women rises after menopause,1 but the benefit of postmenopausal estrogen replacement therapy (ERT) for stroke is not clear. Although ERT reduces risk of cardiovascular disease,2 increased,3 decreased,4,5 and unchanged risks6 of stroke have been reported in postmenopausal hormone users. Furthermore, the effect of ongoing ERT on clinical outcome after stroke has not been well studied. Estrogen exhibits both vascular7–9 and nonvascular effects10–12 through genomic and nongenomic mechanisms, many of which could confer neuroprotection in the hormone-treated brain. We previously showed that female animals sustain less brain tissue injury from middle cerebral artery occlusion (MCAO) than do age-matched males and that this advantage is abolished by ovariectomy.13 In females with native estrogens, intrainschemic cerebral blood flow (CBF) is partially preserved in striatal regions, and these animals have smaller infarcts.13 Furthermore, exogenous estradiol, when administered by implant or as a single intravenous injection at the

---

**See Editorial Comment, page 1670**

Received January 7, 1999; final revision received May 12, 1999; accepted May 12, 1999.

From the Department of Anesthesiology and Critical Care Medicine (R.R., N.J.A., R.J.T., J.A.K., P.D.H.) and Department of Pathology (B.J.C.), Johns Hopkins University School of Medicine, and National Institute on Drug Abuse (A.S.K., E.D.L.), Baltimore, Md.

Correspondence to Dr Patricia D. Hurn, Department of Anesthesiology and Critical Care Medicine, Johns Hopkins Medical Institutions, 600 N Wolfe St/Blalock 1404, Baltimore, MD 21287. E-mail: phurn@jhmi.edu

© 1999 American Heart Association, Inc.

*Stroke* is available at [http://www.strokeaha.org](http://www.strokeaha.org)
onset of MCAO, protects the male brain during experimental stroke. The purpose of the present study was to determine whether replacement of estrogen to physiological plasma levels also protects the female brain deprived of ovarian estrogen and whether protection through the exogenous steroid, like the native hormone species, occurs through vascular mechanisms.

Materials and Methods

This study was conducted in accordance with the National Institutes of Health guidelines for the care and use of animals in research, and protocols were approved by the Animal Care and Use Committee of Johns Hopkins University. All methods were as previously described. In brief, age-matched, sexually mature female Wistar rats (200 to 250 g, Harlan) were ovariectomized, and 17β-estradiol pellets (0-, 25-, or 100-μg; 21-day controlled time release) were implanted in the subcutaneous tissue of the dorsal neck (n = 10 per group). All rats received estrogen implants exactly 1 week after ovariectomy to ensure a uniform time of estrogen depletion before replacement. Time from implant to MCAO ranged from 7 to 16 days. In additional cohorts, ovariectomized females were injected, through a femoral venous catheter, with Premarin (1 mg/kg, USP) or an equivalent volume of saline 30 minutes before occlusion. For MCAO, each animal was anesthetized with halothane (1% to 1.5% delivered by mask in O₂-enriched air), and a femoral artery catheter was placed for continuous monitoring of mean arterial pressure and measurement of arterial blood gases. Rectal temperature was controlled by a heating lamp. Cortical perfusion was measured by laser-Doppler flowmetry (LDF), with the probe placed 6 mm lateral and 2 mm posterior to the bregma. Focal cerebral ischemia was induced by using a modified intraluminal filament technique as previously described. In brief, the common and external carotid arteries were exposed and ligated. The occipital artery was cauterized, and the pterygopalatine artery was ligated. After baseline LDF was determined, a 4.0 nylon monofilament surgical suture with a heat-blunted tip was introduced through the common and internal carotid arteries until the LDF signal showed an abrupt and significant reduction, confirming ongoing ischemia. Then, the suture was secured in place. LDF was measured during ischemia over 15-minute intervals and for the first 15 minutes of reperfusion, which was achieved by withdrawing the suture. Five-minute recording averages were analyzed. The brain was harvested for staining with 2,3,5-triphenyltetrazolium chloride and subsequently fixed in 10% neutral buffered formalin. Infarction volumes in cerebral cortex and caudate-putamen complex were determined by digital image analysis and expressed as a percentage of the ipsilateral structure. Blood was obtained for steroid analysis during MCAO in the single-injection animals or at 22 hours of reperfusion in animals with long-term implants. Plasma estradiol and progesterone levels were measured in duplicate by radioimmunoassay as previously described. End-ischemic regional CBF (rCBF) was measured in an additional cohort of ovariectomized Wistar rats (n = 4 per group), with or without 25-μg 17β-estradiol implants, using quantitative autoradiography with 14C-iodoantipyrine. Femoral arterial and venous catheters were inserted, and the middle cerebral artery was occluded as in the previous cohorts. At 2 hours of MCAO, arterial blood pressure and blood gases were measured, and then 40 μCi of 14C-iodoantipyrine in 1 mL of isotonic saline were infused intravenously for 45 seconds. During infusion, fifteen 20-μL samples of free-flowing arterial blood from the femoral artery catheter were collected in heparin-coated sample tubes. With the filament still in place and with a constant LDF signal, the rat was decapitated 45 seconds after the start of infusion. The brain was subsequently frozen and sectioned. Autoradiographic images were obtained at 3 different coronal levels (+2.7, +0.2, and −1.8 mm from the bregma, 6 to 9 images each). Each section was digitized, and rCBF was determined by image analysis. Rates of rCBF were calculated as previously described.

All values reported are mean ± SE. All physiological variables were analyzed by 2-way ANOVA and a post hoc Newman-Keuls test. Infarction volumes were analyzed by 1-way ANOVA, and post hoc comparisons were made by using the Newman-Keuls test. The criterion for statistical significance was set at P = 0.05.

Results

Physiological parameters during MCAO and reperfusion for the long-term treatment groups are summarized in Table 1. Mean arterial pressure, blood gases, and hemoglobin and glucose concentrations were also equivalent in the single-injection groups. Plasma estradiol levels were 3.0 ± 0.6, 20 ± 8, and 46 ± 10 pg/mL in the 0-, 25-, and 100-μg implant groups, respectively (for the 25- and 100-μg groups, P ≤ 0.05 versus 0-μg group; for the 100-μg group, P ≤ 0.05 versus 25-μg group). Plasma progesterone levels were not different among groups.

<table>
<thead>
<tr>
<th>Group*</th>
<th>Time Interval</th>
<th>pH</th>
<th>PaCO₂, mm Hg</th>
<th>PaO₂, mm Hg</th>
<th>Temperature, °C</th>
<th>MAP, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μg (n=10)</td>
<td>Preischemia 7.3±0.02</td>
<td>45±3</td>
<td>149±12</td>
<td>37.4±0.3</td>
<td>93±7</td>
<td></td>
</tr>
<tr>
<td>1 h after MCAO 7.4±0.02</td>
<td>45±3</td>
<td>127±15</td>
<td>38.1±0.2</td>
<td>96±7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reperfusion 7.4±0.02</td>
<td>45±2</td>
<td>121±16</td>
<td>37.8±0.3</td>
<td>95±7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 μg (n=10)</td>
<td>Preischemia 7.4±0.02</td>
<td>43±4</td>
<td>122±10</td>
<td>37.7±0.2</td>
<td>94±4</td>
<td></td>
</tr>
<tr>
<td>1 h after MCAO 7.4±0.02</td>
<td>48±3</td>
<td>107±7</td>
<td>37.9±0.2</td>
<td>97±10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reperfusion 7.4±0.01</td>
<td>46±3</td>
<td>123±6</td>
<td>37.5±0.3</td>
<td>90±4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μg (n=10)</td>
<td>Preischemia 7.3±0.01</td>
<td>44±2</td>
<td>128±7</td>
<td>37.0±0.3</td>
<td>94±4</td>
<td></td>
</tr>
<tr>
<td>1 h after MCAO 7.3±0.01</td>
<td>48±4</td>
<td>115±12</td>
<td>37.3±0.2†</td>
<td>94±3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reperfusion 7.3±0.01†</td>
<td>52±3†</td>
<td>110±9</td>
<td>37.0±0.3†</td>
<td>85±4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SE. PaCO₂ indicates arterial carbon dioxide tension; PaO₂, arterial oxygen tension; and MAP, mean arterial blood pressure.

*Groups are based on dose of 17β-estradiol delivered by subcutaneous implant.

†P < 0.05 from 0-μg group.
occlusion. Figure 2 summarizes residual LDF (expressed as % of baseline signal) and emphasizes the lack of difference in reduction of LDF signal among treatment groups. End-ischemic LDF in ovariectomized rats that received estradiol replacement (37±8% in 25-μg group; 31±7% in 100-μg group) was not different from that observed in ovariectomized females that did not receive estradiol replacement (31±11%). Acute estradiol administration did not alter tissue outcomes in treated rats as compared with ovariectomized control animals treated with equivalent volumes of intravenous saline (Figure 3). Ischemic reduction in LDF signal was not different between the short-term intervention groups.

We next measured end-ischemic rCBF autoradiographically in cohorts of ovariectomized animals treated with either 0 μg or the neuroprotective 25-μg estradiol dose (Figure 4). Contralateral, nonischemic rCBF was not different between hormone-deficient and hormone-repleted groups (=200 mL/min per 10 g). Similarly, striatal and cortical rCBF during MCAO was not altered by estradiol replacement. To further examine differences in end-ischemic CBF distribution in these animals, we quantified brain tissue volume that experienced near-zero CBF (potentially ischemic core) as well as tissue volumes experiencing less severely reduced CBF (likely penumbra). Figure 5 shows the results of this partitioning of brain volumes into incremental levels of absolute rCBF in estrogen-deficient versus estrogen-repleted animals. There are no differences between groups at any flow increment, suggesting that estrogen did not recruit tissue from a low “flow state” to a partially preserved flow state.

Discussion

This study demonstrates 3 important findings. First, exogenous estrogen reduces infarction volume in cortex and striatum after reversible vascular occlusion of the middle cerebral artery in ovariectomized female rats. Second, the observed neuroprotection is dose-dependent, requiring low physiological plasma estrogen levels and long-term exposure to the hormone. Third, unlike native estrogens, the exogenous steroid does not act through blood flow-mediated mechanisms to protect the brain during ischemic insult. These findings suggest a direct neuroprotective effect of exogenous estrogen in the ischemic female brain.

The importance of postmenopausal ERT for stroke risk in women remains controversial. Furthermore, it is not known whether estrogen availability alters the pathophysiology of cerebral ischemia in female patients. Estrogen treatment has been shown to augment cerebral perfusion in some experimental ischemic models. However, estrogen reduces hippocampal and striatal injury after global forebrain ischemia in ovariectomized rats by blood flow–independent mechanisms. Our present animal studies, and others, demonstrate that the female brain deprived of natural ovarian sources of estrogen can benefit from hormone repletion before the onset of MCAO and focal injury. These findings are not explained by differences in physiological parameters among treatment groups or by elevations in intraischemic CBF. We previously showed that normal Wistar female rats sustain less infarction than their male or ovariectomized counterparts using the same ischemic model, in part because of partial preservation of blood flow during vascular occlusion. Furthermore, exogenous estrogen strongly protects the male brain in a wider dose range, including both the 25- and 100-μg long-term implants used in the present study. We also observed that short-term Premarin treatment at 1 mg/kg, the dose used in the present study, provides neuroprotection equivalent to that provided by long-term hormone therapy in male animals that undergo MCAO. The present results indicate that although estrogen replacement also improves tissue outcome in hormone-deficient female rats, the steroid has a surprisingly more narrow therapeutic range and is not protective when used to treat an acute condition as it is in the male.

Although variations in plasma estradiol level reflected individual differences in subcutaneous absorption and metabolism of the implanted drug, the 100-μg group had an average estradiol level of 46 pg/mL, compared with 20 pg/mL in the low-dose group. Both levels are physiologically relevant; ie,
the baseline plasma 17β-estradiol level reported in the rat is 17±2 to 21±2 pg/mL, with spikes to 80 to 140 pg/mL during various stages of the menstrual cycle. However, only the 20-pg/mL level results in significant neuroprotective capability. It may be that the constant imposition of higher plasma levels provided by implants in a fixed “steady state” can result in adverse effects not ordinarily observed as estradiol periodically spikes to high physiological levels. The latter condition occurs during the transient stages of the normal menstrual cycle, diestrus and proestrus. For example, our data differ somewhat from those of Simpkins et al because large doses of 17β- or 17α-estradiol delivered only over 24 hours before MCAO reduced infarction volume. Long-term steroid delivery may be required to best assess the precise therapeutic window for estrogen, including potential adverse effects that modulate neuroprotective potential. Early clinical studies reported numerous adverse cardiovascular sequelae when supraphysiological estrogen doses were used as an oral contraceptive measure.

Additional studies are needed to determine whether there is a clear threshold above which neuroprotection is lost as well as whether the dose-response relationship of estrogen is altered by tonic versus cyclic administration or by concomitant progesterone replacement.

The beneficial effect of physiological doses of estrogen does not appear to be related to preservation of blood flow during MCAO. The LDF signal was reduced by a similar percentage from the baseline level in all groups, suggesting that the ischemic insult was equivalent among groups. However, because LDF measures only relative changes in cortical perfusion, rather than absolute CBF, we also quantified end-ischemic CBF in a separate cohort of animals treated with the protective dose. Using 14C-iodoantipyrine autoradiography, we found no difference in ischemic CBF within the territory of the occluded middle cerebral artery. Similarly, end-ischemic tissue volumes at near-zero, low, and relatively preserved flow were not altered by exogenous estrogen. There also were no baseline blood flow differences in the contralateral, nonischemic hemisphere in estrogen- versus non–estrogen-treated animals. The latter observation is consistent with our previous work, in which intact and ovariec-tomized, anesthetized females were found to have equivalent baseline CBF. Thus, although these data suggest that both endogenous and exogenous estrogen reduce stroke injury in the female brain, the mechanisms of protection may differ. In particular, it appears that the protection provided by ERT in ovariec-tomized females may be due to a direct neuroprotective action on vulnerable neurons or glia.

Estradiol interacts with and alters function in diverse neuronal target sites in different parts of the brain, including areas not associated with reproductive function, via estrogen receptor-dependent and –independent actions. Estrogen receptor mRNA has a widespread but varying distribution in different parts of the brain, but whether classic estrogen receptors, either subtype α or the more recently identified subtype β, are important in stroke is not clear. Infarction volume is exacerbated by anties-
trogen treatment in female mice; however, damage is paradoxically less extensive in female mice deficient in estrogen receptor α than in their wild-type counterparts. Nonclassic, membrane “receptor” mechanisms may also be relevant. For example, estradiol reduces calcium currents in rat neostriatal neurons via a membrane receptor. Alternatively, several members of the estrogen family are potent antioxidants and inhibit iron-catalyzed lipid peroxidation. Estradiol can also regulate expression of the antioxidant, antipapoptotic protein bcl-2 in steroid-sensitive tissue. Estradiol-mediated neuroprotection in our MCAO model is associated with higher postischemic expression of bcl-2 mRNA in cortex and striatum. Furthermore, estradiol induces a receptor-mediated antioxidant effect in culture, inhibiting superoxide anion production and enhancing the biological activity of nitric oxide. This observation could be relevant in vivo, because estrogen-treated animals have been reported to show limited 3-nitrotyrosine immunoreactivity after forebrain ischemia. Finally, although no difference in plasma progesterone levels was apparent between ovariectomized and estradiol-treated female rats, progesterone receptor pathway in modulating ischemic injury cannot be excluded.

In conclusion, we demonstrated that long-term 17β-estradiol replacement therapy within the physiological range ameliorates ischemic brain injury in ovariectomized female rats. The neuroprotective mechanism is not through preservation of ischemic CBF and is likely due to a direct parenchymal effect of estradiol.

Acknowledgments

This study was supported by grants NS3368, NR03521, and NS20020 from the National Institutes of Health.

References


The study of Rusa et al investigated further the mechanisms of the protective effect exerted by estrogen in a model of the focal cerebral ischemia produced by transient occlusion of the rat middle cerebral artery. It was found that estrogen replacement in ovariectomized female rats reduces the volume of brain injury. The effect was observed only with long-term treatment at low (25-μg) but not high (100-μg) doses of estrogen replacement. To determine whether the reduction in ischemic injury was related to the cerebral hemodynamic effects of estrogen, cerebral blood flow was measured after induction of ischemia. It was found that estrogen replacement did not affect the dynamics of flow reduction, which was assessed qualitatively by laser-Doppler flowmetry. Furthermore, the authors measured cerebral blood flow quantitatively using 14 C-iodoantipyrine as a tracer, a technique with a high degree of spatial resolution. It was found that estrogen replacement does not affect the reduction in blood flow throughout the ischemic territory. These data provide the most convincing evidence to date that, in focal ischemia, the protective effect of estrogen replacement is independent of cerebral hemodynamic effects.

Previous epidemiological and experimental studies have suggested sex differences in the incidence and outcome of cerebral ischemia. However, the mechanisms of such sex differences have not been elucidated. In female rats, depletion of endogenous estrogen by ovariectomy worsens ischemic injury, an effect related to a more profound reduction in cerebral blood flow in the ischemic territory. On the other hand, the observations of Rusa et al and Wang et al indicate that the effect of exogenous estrogen on ischemic damage is not related to improved cerebral perfusion. The collective evidence suggests that the mechanisms by which estrogen modulates ischemic injury vary depending on the source of estrogen and include both flow-dependent and flow-independent actions. The flow-independent mechanisms of estrogen are likely to be complex and include effects on gene expression, reactive oxygen species, as well as membrane function (see Rusa et al article for references).

This elegant study opens the way to additional investigations of the mechanisms by which estrogen replacement influences ischemic brain injury. These studies would not only help clarify the mechanistic basis of the effects of estrogen replacement in postmenopausal females but also suggest new therapeutic approaches based on modulation of estrogen levels.

Costantino Iadecola, MD, Guest Editor
Laboratory of Cerebrovascular Biology and Stroke
Department of Neurology
University of Minnesota
Minneapolis, Minnesota

References
17β-Estradiol Reduces Stroke Injury in Estrogen-Deficient Female Animals

Stroke. 1999;30:1665-1670
doi: 10.1161/01.STR.30.8.1665
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/30/8/1665

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/