Stroke in Estrogen Receptor-α–Deficient Mice

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Background and Purpose—Recent evidence suggests that endogenous estrogens or hormone replacement therapy can ameliorate brain damage from experimental stroke. Protective mechanisms involve enhanced cerebral vasodilation during ischemic stress as well as direct preservation of neuronal viability. We hypothesized that if the intracellular estrogen receptor subtype-α (ERα) is important to estrogen’s signaling in the ischemic brain, then ERα-deficient (knockout) (ERαKO) female mice would sustain exaggerated cerebral infarction damage after middle cerebral artery occlusion.

Methods—The histopathology of cresyl violet–stained tissues was evaluated after reversible middle cerebral artery occlusion (2 hours, followed by 22 hours of reperfusion) in ERαKO transgenic and wild-type (WT) mice (C57BL/6J background strain). End-ischemic cerebral blood flow mapping was obtained from additional female murine cohorts by using [14C]iodoantipyrine autoradiography.

Results—Total hemispheric tissue damage was not altered by ERα deficiency in female mice: 51.9±10.6 mm3 in ERαKO versus 60.5±5.0 mm3 in WT. Striatal infarction was equivalent, 12.2±1.7 mm3 in ERαKO and 13.4±1.0 mm3 in WT mice, but cortical infarction was paradoxically smaller relative to that of the WT (20.7±4.5 mm3 in ERαKO versus 30.6±4.1 mm3 in WT). Intraocclusion blood flow to the parietal cortex was higher in ERαKO than in WT mice, likely accounting for the reduced infarction in this anatomic area. There were no differences in stroke outcomes by region or genotype in male animals.

Conclusions—Loss of ERα does not enhance tissue damage in the female animal, suggesting that estrogen inhibits brain injury by mechanisms that do not depend on activation of this receptor subtype. (Stroke. 2000;31:738-744.)

Key Words: estrogen ■ cerebral ischemia ■ gender ■ menopause ■ stroke

Estrogen is a natural neuroprotectant and a potential therapeutic agent in many forms of cardiovascular and cerebrovascular disease. Although women are at lower risk for stroke than men, this native protection is lost in the postmenopausal years. Consequently, there has been much interest in determining whether hormone replacement therapy improves cerebrovascular disease or alters stroke pathophysiology. Estrogen has been the best studied of the sex steroids and blocks estrogen-induced neuroprotection in cultured neurons. Nevertheless, administration of 17β-estradiol salvages the brain from injury after cerebrovascular occlusion in ovariecotomized or estrogen-deficient female6–14 and male15,16 animals, as well as in aged, reproductively senescent rodents.17 The likely mechanisms by which the native steroid acts to protect the brain involve both enhanced vasodilation and recruitment of collateral circulation during cerebral artery occlusion and direct, perfusion-independent neuronal rescue.

As an initial step in understanding how estrogen signaling alters cerebral ischemic injury, the contribution of the steroid’s classic intracellular receptors has come under investigation.18–20 Two subtypes of the estrogen receptor (ER) are present and biologically active in the brain21,22 and act as ligand-activated transcription factors that alter gene expression in target cells: ERα and the recently identified ERβ.23,24 Generalized pharmacological ER blockade with pure antiestrogens exacerbates ischemic injury in wild-type (WT) mice19 and blocks estrogen-induced neuroprotection in cultured neu-
rons. However, studies with presently available ER antagonists can be criticized on the grounds of the lack of subtype specificity and poor bioavailability to the brain in vivo. In the current study, we examined histopathological outcomes after middle cerebral artery (MCA) occlusion and the regulation of cerebral blood flow in ischemic and nonischemic brain in a transgenic mouse strain deficient in ERα, known as ER knockouts (ERαKO). As previously reported, the start codon and amino-terminal domain of the gene are disrupted in these mice, yielding a small expression of incomplete ERα transcripts but no functional α-subtype receptors. ERαKO homozygotes of both sexes are healthy but have abnormal reproductive function and sex behavior. We demonstrate herein that loss of ERα does not enhance perfusion defects after vascular occlusion or increase tissue damage after ischemic stroke in female ERαKO mice, suggesting that this ER subtype does not mediate estrogen’s neuroprotective activity.

Materials and Methods

The study was conducted in accordance with National Institutes of Health guidelines for the use of experimental animals, and the protocols were approved by the institutional Animal Care and Use Committee. Somatosensory and motor behavior was evaluated in male and female ERαKO mice and compared with that of WT controls (C57BL/6J background strain; Harlan, Indianapolis, Ind) 1 week before MCA occlusion. These tests assessed balance (time to fall from a narrow pole up to a maximum of 120 seconds), agility (turning in a blind alley or on an inclined screen), forelimb strength (hanging from suspended wire), and autogrooming time.

Cerebral ischemia was then induced by reversible MCA occlusion in these animals, as previously published. In brief, mice were anesthetized with 1% to 1.2% halothane in O2-enriched air by face mask, and rectal and temporalis muscle temperatures were controlled at 37±0.5°C throughout the experiment with heating lamps and water pads. Unilateral MCA occlusion was performed by inserting a 6-0 nylon monofilament into the internal carotid artery via an external carotid artery stump and then positioning the filament tip for occlusion at a distance of 6 mm beyond the internal carotid/pterygopalatine artery bifurcation. After securing the filament in place, the surgical site was sutured closed and infiltrated with 0.5% bupivacaine as needed for postoperative analgesia. The animal was then awakened and grossly assessed for neurological damage as follows: 0=no deficit, 1=failure to extend forelimb, 2=circling, 3=unilateral weakness, 4= no spontaneous motor activity. Mice with clear neurological deficits were reassanesthetized with halothane for suture removal at 2 hours of occlusion. At 22 hours of reperfusion, each animal was again reassanesthetized for transectional perfusion with normal saline followed by neutral buffered 10% formalin. The brain was then postfixed in formalin and 30% sucrose in phosphate buffer, cut as serial coronal sections (40 μm) on a freezing microtome, and stained with cresyl violet. A set of 12 evenly spaced sections through the forebrain was mounted for determination of infarction volume by image analysis (Inquiry, Loats Inc.). Numeric evaluations in ERαKO animals of both sexes demonstrated no abnormalities. There were no differences among groups in gross neurological score as assessed during MCA occlusion (2.4±0.2 and 2.6±0.2 in WT and ERαKO female mice; 2.1±0.2 and 2.2±0.2 in WT and ERαKO male mice). Total tissue damage within the ischemic hemisphere was unchanged by ERα deficiency in females: hemispheric infarction volume was 51.9±10.6 mm3 in ERαKO females versus 60.5±5.0 mm3 in WT females. Similarly, striatal injury was equivalent: 12.2±1.7 mm3 in ERαKO and 13.4±1.0 mm3 in
WT mice. Only cortical infarction was altered in ERαKO females: it appeared paradoxically less (20.7 ± 4.5 mm³) than would be anticipated from corresponding measurements in WT mice (30.6 ± 4.1 mm³). Figure 1 depicts these values normalized as a percentage of total ipsilateral structure. In agreement with histopathological outcome, neurological function scores at 22 hours of recovery were also unchanged (1.6 ± 0.2 in ERαKO versus 1.9 ± 0.1 in WT). There were no differences in stroke outcomes by region or genotype in male ERαKO versus WT animals. Arterial blood pressure and respiratory gas composition were monitored before and during MCA occlusion and were comparable among groups (the Table).

To map cortical and subcortical perfusion deficits, intracerebral blood flow ipsilateral and contralateral to the occlusion was quantified throughout the brain at 2 hours of MCA occlusion (Figure 2). The distribution of tissue volume recruited into near-zero and low-flow zones within the ischemic hemisphere was not different in ERαKO and WT females (Figure 3), suggesting a similarity of ischemic insult. Absolute blood flow in all regions evaluated within the nonischemic hemisphere was equivalent in ERαKO and WT mice, indicating that the loss of ERα does not alter baseline cerebral blood flow in the female. Furthermore, intracerebral blood flow was not different between groups in all brain regions examined, with the exception of the parietal cortex (Figure 4). Flow to this area during occlusion was elevated in ERαKO relative to WT females, likely accounting for our observation of reduced infarction in this anatomic area. In addition, LDF data obtained over the parietal cortex suggested that localized perfusion was less severely reduced throughout occlusion in ERαKO females (Figure 5).

**Discussion**

We hypothesized that if ERα was important to estrogen’s signaling in the ischemic brain, then ERαKO mice would sustain an exaggerated cerebral infarction after MCA occlusion. The main finding of the study is that loss of ERα neither enhances tissue damage in the female animal nor exacerbates intracerebral tissue perfusion defects. Total hemispheric infarction was unchanged in ERαKO relative to age-matched WT mice of the same background strain. These data suggest that estrogen inhibits brain injury by mechanisms that do not depend on activation of the ERα subtype. Alternative signaling pathways include activation of the intracellular ERβ subtype or non–receptor-initiated mechanisms.

Clinical ischemic stroke is frequently the sequela of atherothrombotic vascular occlusion, with varying degrees of persistent tissue perfusion from collateral and anastomotic microvessels. Endogenous brain protectants may therefore act by 1 or both of 2 distinct pathophysiological mechanisms: by maximally dilating collateral circulation and partially ameliorating intraocclusion loss of blood flow or by direct cell preservation of parenchymal neurons and glia. Previous work emphasized that endogenous estrogen utilizes both approaches to salvage brain tissue in the female after experimental ischemic stroke. We used a novel transgenic strain to dissect the role of 1 ER subtype in cerebrovascular pathophysiology. Currently available pharmacological antiestrogens do not provide receptor subtype–selective antagonism; therefore, ERαKO animals have provided many new insights into estrogen’s signaling mechanisms in a variety of tissue and cell types (for a review, see Reference 27). The present results suggest that ERα-mediated mechanisms are not important to tissue outcome in experimental stroke.

Estrogens clearly have direct and rapid effects on nonreproductive neuronal tissue and on the cerebral vasculature. For example, synaptic architecture within areas such as the hippocampus changes with the estrous cycle and can be altered in <24 hours by exogenous estradiol. Furthermore, complementary fluctuations in the volume of astrocytic processes and synaptic numbers occur in response to ovarian steroids. The steroid may utilize diverse signaling pathways to produce biological effects. These include (1) nuclear ER-linked modulation of target gene transcription efficiency; (2) nonnuclear effects involving estrogen receptor signaling and protein synthesis; and (3) modulation of receptor expression.

**Figure 1.** Loss of ERα does not increase stroke damage in female mice. Cortical tissue was paradoxically spared in ERαKO females; however, the effect was restricted specifically to the cortex. Overall damage was unaffected by ERα deletion. Values are mean ± SEM, expressed as a percentage of contralateral cortex or hemisphere in female C57BL/6J WT and ERαKO mice, n = 9 per group. Ipsilat indicates ipsilateral. *P < 0.05 vs WT group.

### Intraischemic Physiological Measurements

<table>
<thead>
<tr>
<th>Group</th>
<th>WT, g</th>
<th>pH</th>
<th>PaO₂, mm Hg</th>
<th>PaCO₂, mm Hg</th>
<th>Rectal Temperature, °C</th>
<th>MAP, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>♂ WT</td>
<td>22±0.6</td>
<td>7.35±0.01</td>
<td>122±1</td>
<td>34±1</td>
<td>36.4±0.1</td>
<td>81±2</td>
</tr>
<tr>
<td>♂ ERαKO</td>
<td>21±0.9</td>
<td>7.33±0.01</td>
<td>120±7</td>
<td>33±2</td>
<td>36.6±0.4</td>
<td>89±1</td>
</tr>
<tr>
<td>♀ WT</td>
<td>27±0.3</td>
<td>7.32±0.01</td>
<td>132±13</td>
<td>33±2</td>
<td>37.1±0.1</td>
<td>105±3</td>
</tr>
<tr>
<td>♀ ERαKO</td>
<td>26±0.6</td>
<td>7.35±0.01</td>
<td>138±3</td>
<td>35±1</td>
<td>36.7±0.2</td>
<td>95±5</td>
</tr>
</tbody>
</table>

For ♂ WT and ♀ ERαKO, n = 4 per group; pH is arterial pH; PaO₂ is arterial oxygen tension; PaCO₂ is arterial carbon dioxide tension; and MAP is mean arterial blood pressure. No differences were observed in WT vs ERαKO mice for either sex. Data expressed as mean ± SE.

*ERKO relative to age-matched female mice. Cortical tissue was paradoxically spared in ERαKO females, likely accounting for our observation of reduced infarction in this anatomic area. In addition, LDF data obtained over the parietal cortex suggested that localized perfusion was less severely reduced throughout occlusion in ERαKO females (Figure 5).**
(2) ER-dependent but nontranscriptional mechanisms; (3) non–ER-linked transcriptional mechanisms that utilize generalized signaling molecules; and (4) cell membrane–associated activity that is far too rapid to involve mRNA transcription and protein synthesis (for recent reviews, see References 33 and 34). In addition, cross-talk between membrane-mediated events and nuclear receptor activation has also been described, particularly within the vasculature.34 There are few data that distinguish which of these signaling modalities are used by estrogen to initiate (or integrate) its many putative anti-ischemic mechanisms. Such mechanisms...
include induction of neuroprotective gene products bcl2,35,36 and neurotrophic growth factors,37,38 nontranscriptional modulation of excitatory neurotransmission and glutamate toxicity,26,40 and antioxidant activity.41,42 The present data allow the exclusion of 1 signaling pathway by which estrogen acts in ischemic brain: nuclear ERα activation. Although deficiency in this subtype does not exacerbate histological damage in females, we have recently observed increased damage after MCA occlusion in WT female mice chronically treated with ICI 182,780, an inhibitor of both known ER subtypes.19 Therefore, it is likely that loss of functional ERβ, rather than ERα, is responsible for amplifying stroke damage in these mice.

We and others have also observed that endogenous estrogen amplifies residual cerebral blood flow in female animals during vascular insult or occlusion.5,8,11 Such promotion of blood flow during ischemic stress is lost in estrogen-deficient animals and is absent in the male.5,8,11 Potential mechanisms include estrogen-induced increases in vascular diameter; enhanced vasodilatory capacity through increased elaboration of nitric oxide, prostacyclin, or other endothelium-derived mediators; and reduced sensitivity to selected vasoconstrictor stimuli. Such observations are not surprising, since estrogen has well-known vasoactive properties in the cerebral circulation. Gray-matter blood flow is higher in women versus men,43–45 but sex differences disappear by 50 to 60 years of age.43,44 Premenopausal women demonstrate greater cerebral vasodilatory capacity to stimuli such as increased systemic hypercapnia when compared with men of the same age.46 Exogenous estrogen replacement also increases blood flow throughout brain regions, including the cortex, cerebellum, basal ganglia, and hippocampus and produces cerebral vasodilation in animals47,48 and humans with49 and without50 significant cerebrovascular disease. However, the current findings indicate that cerebral blood flow is not depressed in the healthy brain or within the ischemic lesion in ERαKO females relative to WT C57BL/6J mice, suggesting that estrogen’s basal or stress-evoked vasodilator properties are not likely dependent on ERα.

An unanticipated finding was the selective reduction of cortical injury observed in ERαKO females, potentially explained by a relative preservation of intraocclusion blood flow to the parietal cortex. Whether this result represents a unique response to loss of the receptor subtype is unclear; however, the anatomic limitation (parietal cortex only) and sex bias (females only) would argue against a nonspecific compensatory physiology within the transgenic strain. A plausible explanation for this finding is related to the chronically elevated plasma estrogen levels sustained in the ERαKO female, consistent with their hormone insensitivity (84 pg/mL, ~3 times that of the WT female mouse).27 Because estradiol is vasoactive, high endogenous levels may have improved outcome by flow-dependent, ERα-independent means in steroid-sensitive cortical regions. We have previously observed intraocclusion preservation of cerebral blood flow in WT female rodents and rabbits.5,8 If so, such protective effects could be mediated by the recently identified ERβ and/or by nonreceptor, membrane-associated binding to target cells. Although expression of ERβ in cerebral vessels has not yet been shown, ERβ mRNA is present in the ERαKO aorta41 and is induced by vascular injury in both endothelial and vascular smooth muscle cells.52 Furthermore, ERβ mRNA is present in the cortex of ERαKO mice,21 and there is evidence of translation into a 17β-estradiol-binding, biologically active protein.52

In conclusion, ERα deficiency does not enhance tissue damage in female animals, indicating that estrogen inhibits brain injury by mechanisms that do not depend on activation of this receptor subtype. Our findings may have clinical relevance to the current search for selective estrogen receptor agonists that are useful hormone replacement agents from the perspective of bone and heart but that have adjunctive neuroprotective properties. Such agents could be helpful to women who elect estrogen therapy in their middle years but who also carry the risk for or a history of ischemic stroke and cerebrovascular disease. This animal study would argue against targeting ER agonists with selective ERα activity in the brain.

Acknowledgments
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References


20. Gibbs RL. Levels of txa2 and BDFN mRNA, but not NGF mRNA, fluctuate across the estrous cycle and increase in response to acute hormone replacement. Brain Res. 1998;787:259–268.


The recent discovery of two distinct estrogen receptors, α and β, expands possibilities for development of more selective therapies. Transgenic mice deficient for the α- or β-estrogen receptor have already been used to demonstrate differential actions of estrogen mediated by each receptor type.

Protective effects of estrogen have been well documented in animal models of stroke. The present study by Sampei et al clearly demonstrates that these actions of estrogen are not solely dependent on the α-estrogen receptor. Whether protective effects of estrogen are mediated by the β-receptor or by another as yet undescribed mechanism remains to be determined. Further delineation of the nature of the estrogen receptor involved will contribute to better understanding of the mechanism of estrogen’s protective effect and, perhaps, improved prevention and/or treatment of stroke.

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