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 [^{14}C]iodoantipyrine autoradiography.

Results—Total hemispheric tissue damage was not altered by ERα deficiency in female mice: 51.9$\pm$10.6 mm^3 in ERαKO versus 60.5$\pm$5.0 mm^3 in WT. Striatal infarction was equivalent, 12.2$\pm$1.7 mm^3 in ERαKO and 13.4$\pm$1.0 mm^3 in WT mice, but cortical infarction was paradoxically smaller relative to that of the WT (20.7$\pm$4.5 mm^3 in ERαKO versus 30.6$\pm$4.1 mm^3 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 in both clinical and laboratory settings. Although it is still unclear whether estrogen replacement therapy reduces stroke risk,[1,2] available data agree that chronic estrogen use reduces stroke-related mortality.[3,4] Our laboratory and others have shown that female animals sustain less brain damage after stroke compared with their male counterparts and that this benefit disappears with reproductive senescence or on removal of endogenous ovarian steroids.[5–7] Furthermore, administration of 17β-estradiol salvages the brain from injury after cerebrovascular occlusion in ovariectomized or estrogen-deficient female[8–14] and male[15,16] 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.

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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 brain[21,22] 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) mice[19] and blocks estrogen-induced neuroprotection in cultured neu-

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rons.[20] 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,[25,26] 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.[27] 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.[28,29]

Cerebral ischemia was then induced by reversible MCA occlusion in these animals, as previously published.[19,30] In brief, mice were anesthetized with 1% to 1.2% halothane in O_2-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% buvivacaine 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 reanesthetized with halothane for suture removal at 2 hours of occlusion. At 22 hours of reperfusion, each animal was again reanesthetized for transcardial 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). The following areas were measured in each section: cortical infarct, total ipsilateral cortex, total contralateral cortex, striatal infarct, total ipsilateral striatum, and total contralateral striatum. Because larger infarcts were associated with significant edema, areas in each section were corrected for edema as follows. The relative size of the cortical infarct was expressed as a percentage: 100%×[contralateral cortex−(total ipsilateral cortex−cortical infarct)]/ipsilateral cortex. The relative size of each striatal infarct was similarly corrected. Corresponding volumes were then calculated for the total set of slices. All measurements were carried out by an investigator blinded to treatment assignment.

Physiological measurements were carried out in separate, age-matched ERαKO and WT animal cohorts. Femoral arterial blood pressure and cortical laser-Doppler flowmetry ([LDF] Moor Instruments Ltd) were determined during occlusion and the first 30 minutes of reperfusion. A shallow indentation was made in the parietal skull (2 mm posterior, 3 mm lateral to the bregma) with a low-speed drill for placement of the LDF probe (DP3 optical, 1-mm diameter). A thin oil interface and the probe were applied with a hood to block ambient light. The LDF signal was recorded semicontinuously and averaged over 15-minute intervals for comparison among treatment groups. Arterial blood samples via femoral catheter (100-μL sample volume) were analyzed for pH, P_{O_2}, P_{CO_2}, and standard base excess at baseline and at end-ischemia.

In an additional set of female animals, regional cerebral blood flow was measured by [^{14}C]iodoantipyrine autoradiography, as previously described[5,14] and modified for the mouse. Mice with clear neurological deficits during MCA occlusion were reanesthetized, and arterial (Clay Adams PE 10; 0.28-mm ID, 0.61-mm OD, 15 cm long) and venous (PE 10; 10 cm long) femoral catheters were inserted. At 120 minutes of MCA occlusion, arterial blood pressure, pH, P_{CO_2}, and P_{O_2} were measured, and intravenous infusion and arterial sampling were started. A total of 4 μCi of [^{14}C]iodoantipyrine in 81 μL of isotonic saline was infused intravenously over 45 seconds at a constant infusion rate of 6.48 mL/h. Simultaneously, the arterial catheter was opened, and blood was allowed to flow freely into heparinized saline drops of known volume placed in paraffin wells. Nine blood samples were collected at 5-second intervals; the mouse was decapitated at 45 seconds; and the brain was quickly removed (<60 seconds), frozen in 2-methylbutane on dry ice, and stored at −80°C. Each brain was sectioned on a cryostat (20-μm-thick coronal sections at −18°C) and thaw-mounted onto glass coverslips. Sections were apposed for 1 week to film (Kodak, SB-5) with ^{14}C standards. Sample volume was measured by using a pipette and calculated by subtracting the volume of a saline drop from the total volume of blood sample plus saline. Parallel time-control saline drops were used to account for changes in volume due to evaporation. The concentration of [^{14}C]iodoantipyrine was determined by liquid scintillation spectroscopy after decolorization with 0.2 mL of tissue solubilizer (Soluene-350, Packard Instruments Co). Autoradiographic images representing 7 coronal levels (+4, +3, +2, +1, 0, −1, and −2 mm from the bregma; 3 images each) were digitized, and regional cerebral blood flow was determined by image analysis software (Inquiry, Loats Associates). Rates of regional cerebral blood flow were calculated at discrete 0.1-mm^2 regions within cortical and subcortical regions within the MCA distribution and averaged over 3 to 7 consecutive coronal slices; the images of each coronal slice were scanned and pixels were stratified according to corresponding blood flow rates. Pixels with flow rates falling within a range of blood flow were summed and converted to volume units.[14]

All data are expressed as mean±SEM. Statistical evaluation was performed by Student's *t* test to compare infarction volumes and regional cerebral blood flow between animal groups. Physiological and behavioral variables were analyzed by 2-way ANOVA and a post hoc Newman-Keuls test to determine differences between groups. Postischemic neurological scores were analyzed by the Mann-Whitney *U* test. The criterion for statistical significance was set at $P \leq 0.05$.

Results

Baseline gross neuroanatomic and sensorimotor behavioral 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 mm^3 in ERαKO females versus 60.5±5.0 mm^3 in WT females. Similarly, striatal injury was equivalent: 12.2±1.7 mm^3 in ERαKO and 13.4±1.0 mm^3 in

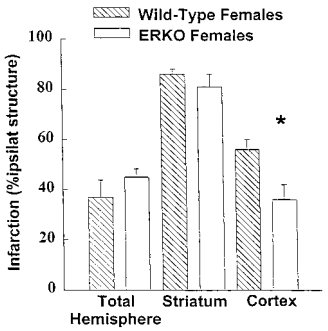

Figure 1. Loss of ERα does not increase stroke damage in female mice. Cortical tissue was paradoxically spared in ERαKO females; however, the effect was restricted specifically to the cortex. Overall damage was unaffected by ERα deletion. Values are mean±SEM, expressed as a percentage of contralateral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

WT mice. Only cortical infarction was altered in ERαKO females: it appeared paradoxically less (20.7±4.5 mm^3) than would be anticipated from corresponding measurements in WT mice (30.6±4.1 mm^3). 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 by ERα deficiency (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, intraischemic 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, intraischemic 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 intraischemic 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[5,8,11] 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.[31] Furthermore, complementary fluctuations in the volume of astrocytic processes and synaptic numbers occur in response to ovarian steroids.[32] The steroid may utilize diverse signaling pathways to produce biological effects. These include (1) nuclear ER-linked modulation of target gene transcription efficiency;

Intraischemic Physiological Measurements

Group	Wt, g	pH	Pa_{O_2}, mm Hg	Pa_{CO_2}, mm Hg	Rectal Temperature, °C	MAP, mm Hg
♀ WT	22±0.6	7.35±0.01	122±1	34±1	36.4±0.1	81±2
♀ ERαKO	21±0.9	7.33±0.01	120±7	33±2	36.6±0.4	89±1
♂ WT	27±0.3	7.32±0.01	132±13	33±2	37.1±0.1	105±3
♂ ERαKO	26±0.6	7.35±0.01	138±3	35±1	36.7±0.2	95±5

For ♂ WT and ♂ ERαKO, n=4 per group; pH is arterial pH; Pa_{O_2} is arterial oxygen tension; Pa_{CO_2} 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.

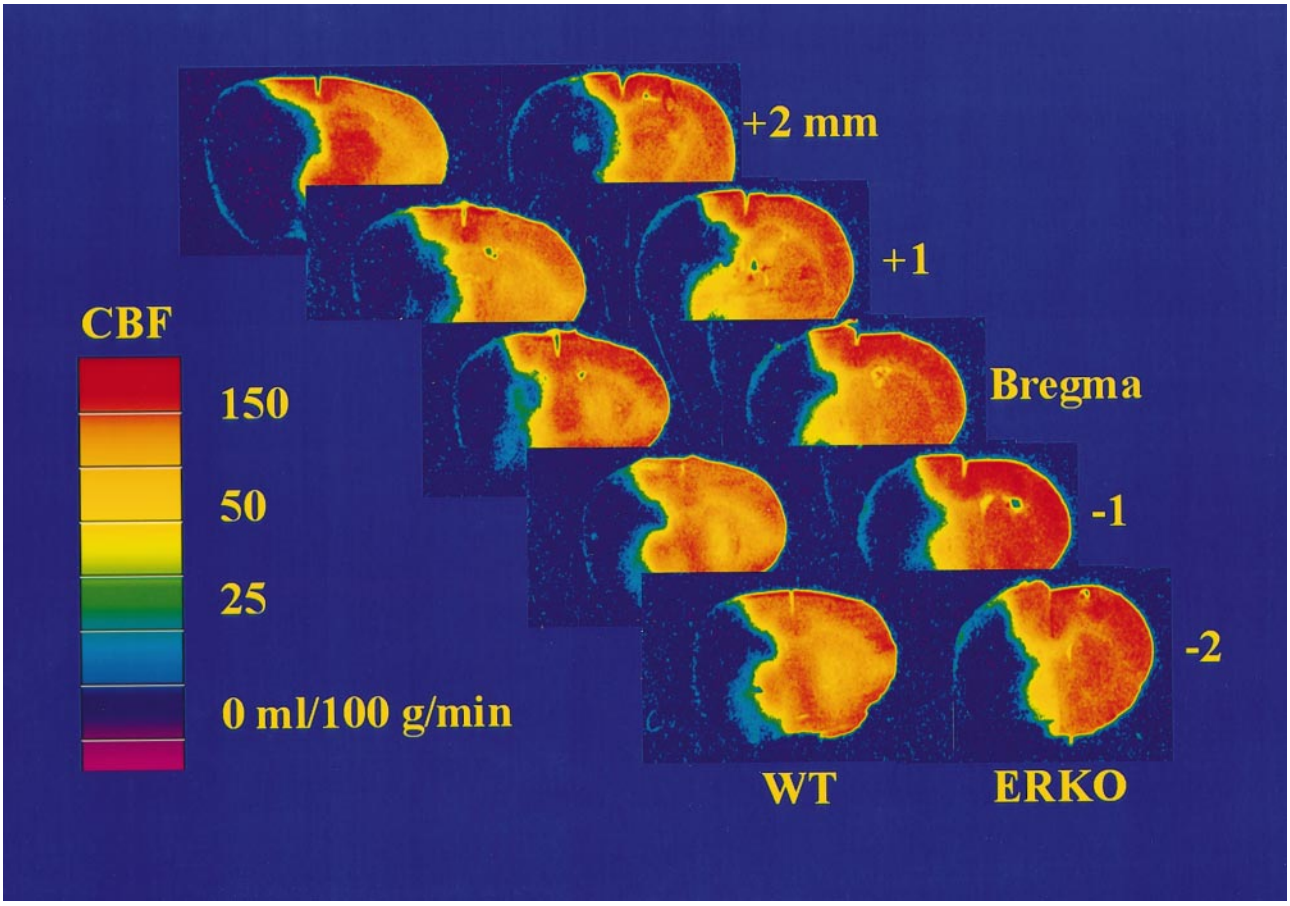

Figure 2. End-occlusion regional cerebral blood flow (CBF) as measured by [^{14}C]iodoantipyrine autoradiography in brain slices −2 to +2 mm from the bregma. Large areas of near-zero CBF are visible across the cortex and caudate-putamen complex (striatum); this area typically evolves into the core of the infarct. Reduction of CBF by the end of 2 hours of MCA occlusion appeared similar in most areas in both WT (n=5) and ERαKO (n=6) females.

(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

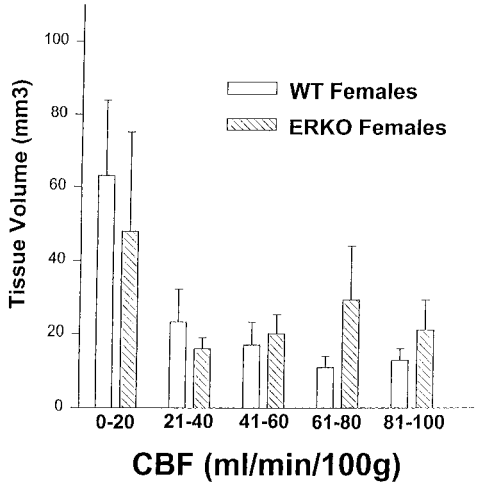

Figure 3. Tissue volume throughout the brain during near-zero flow is equivalent in WT (n=6) and ERαKO (n=5) mice, as are flow distribution patterns across the brain. Data are hemispheric tissue volume (mm^3) partitioned at incremental levels of cerebral blood flow (CBF) as measured at end-occlusion.

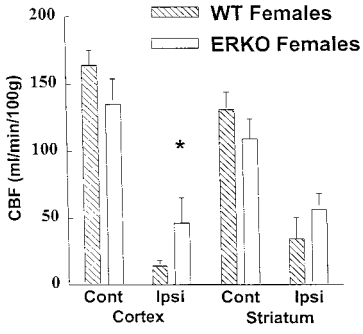

Figure 4. Regional cerebral blood flow (CBF) at 2 hours of MCA occlusion as measured by [^{14}C]iodoantipyrine autoradiography in WT (n=5) and ERαKO (n=6) female mice. Regions shown are representative of CBF within the ischemic MCA territory; contralateral (cont) and ipsilateral (ipsi) parietal cortex and striatum. Cortical blood flow during occlusion was higher in ERαKO mice, likely explaining the observation of smaller infarction volumes in the cortex. *$P \leq 0.05$ vs WT.

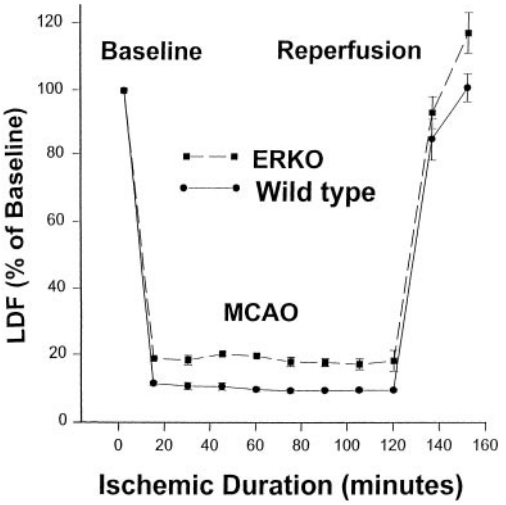

Figure 5. LDF signal from the parietal cortex partially preserved during MCA occlusion (MCAO) in ERαKO compared with WT female mice, n=3 per group.

include induction of neuroprotective gene products bcl2[18,35,36] and neurotrophic growth factors,[37,38] nontranscriptional modulation of excitatory neurotransmission and glutamate toxicity,[20,39,40] 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 animals[47,48] and humans with[49] and without[50] 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, $\approx$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 aorta[51] 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.[22]

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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Editorial Comment

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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