Estrogens Decrease Reperfusion-Associated Cortical Ischemic Damage

An MRI Analysis in a Transient Focal Ischemia Model

Jiong Shi, MD, PhD; Jonathan D. Bui, BS; Shao-Hua Yang, MD; Zhen He, MD, PhD; Timothy H. Lucas, BS; David L. Buckley, PhD; Stephen J. Blackband, PhD; Michael A. King, PhD; Arthur L. Day, MD; James W. Simpkins, PhD

Background and Purpose—Early identification of irreversible cerebral ischemia is critical in defining strategies that influence neuronal survival after stroke. We used MRI to investigate the effects of 17β-estradiol (E2) on the temporal evolution of focal ischemia.

Methods—Female rats were ovariectomized and divided into 1 of 2 groups: ovariectomy alone (OVX; n=4) or ovariectomy with estrogen replacement (OVX+E2; n=3). Both groups were then subjected to 1-hour middle cerebral artery occlusion (MCAO), with the use of a standardized endovascular monofilament model, followed by reperfusion. Sequential diffusion-weighted (DWI) and T2-weighted (T2WI) MRI were obtained during and after the MCAO. In separate groups of animals (n=5 for OVX and OVX+E2), cerebral blood flow (CBF) was measured by laser-Doppler methods before, during, and after occlusion.

Results—DWI detected similar lesion characteristics during MCAO in both groups. In the OVX group, lesion size did not change during reperfusion, but the signal intensity ratio increased early and stabilized during the latter stages. In contrast, DWI lesion size decreased during reperfusion in OVX+E2 rats by 50% to 60% (P<0.05), a size reduction almost exclusively limited to cortical regions. During MCAO, the signal intensity ratio in OVX+E2 rats was reduced compared with OVX rats. Reperfusion further attenuated the signal intensity ratio in cortical but not subcortical regions (P<0.05 versus OVX). T2WI revealed no lesions in either group during MCAO, but it detected lesion sizes similar to that of DWI during reperfusion. Furthermore, similar patterns and magnitudes of estrogen treatment–related decrease in lesion size were noted after reperfusion. T2WI demonstrated less intense signal intensity ratio changes in both groups compared with DWI. There were no differences in CBF between groups either during occlusion, early reperfusion, or 1 day after reperfusion.

Conclusions—This study strongly suggests that estrogens selectively protect cortical tissue from ischemic damage during MCAO and that this protection is exerted during both the occlusion and reperfusion phases of ischemia and does not involve an estrogen-related change in CBF. (Stroke. 2001;32:987-992.)

Key Words: cerebral ischemia, focal ■ estrogens ■ magnetic resonance imaging ■ neuroprotection ■ reperfusion injury

Stroke ranks as the third leading cause of death and the leading cause of disability in the United States.1 Stroke patients must not only survive the acute stages of infarction but must then cope with significant mental, physical, and economic stresses associated with neurological impairment. When one considers the cost in both loss of life and loss of self-esteem and productivity, the need for effective therapeutic interventions is obvious. Most strokes occur when perfusion to the middle cerebral artery (MCA) is reduced by a clot within the major cerebral arteries, producing a region of focal cerebral ischemia and a subsequent cascade of neuronal and microvascular changes ultimately leading to infarction.2 Experimental ultrastructural evidence suggests that some of the damage occurs in the interval of reduced or absent perfusion (the occlusion phase), but most arises during the reperfusion stage, after flow has been restored by clot lysis or opening of collateral channels.3 Since new thrombolytic therapy can now dissolve clots and restore arterial patency, the search for neuroprotective agents that can blunt the reperfusion-
associated injury and elongate the tissue interval for safe intervention assumes critical strategic importance.4

Observations from our and other laboratories indicate that estrogens are potent neuroprotective agents and decrease focal and global ischemia-induced lesion size by as much as 50%.5–15 An understanding of the events affected by estrogen during occlusion and reperfusion will allow us to define the therapeutic window for application of estrogens in stroke. The histological methods used in these previous studies limited our ability to dynamically assess the protective effects of estrogen during occlusion and reperfusion.

MRI can provide a wealth of critical information about the initiation, progression, and localization of cerebral ischemic events during their occurrence. Diffusion-weighted MRI (DWI), a sensitive indicator of random movement of water molecules, is thought to reveal the early changes associated with stroke-induced cytotoxic edema.16 On the other hand, conventional T2-weighted MRI (T2WI), a sensitive indicator of vasogenic edema that occurs later in the pathophysiology of stroke, can detect subacute ischemic damages, even though it fails to show acute ischemic changes.16 As such, MRI can quantify the progression of 2 major pathological consequences of cerebral infarction. In the present study we applied MRI techniques to noninvasively analyze, for the first time, the temporal and spatial effects of 17β-estradiol (E2) in focal cerebral ischemic events.

Materials and Methods

Animals
Sprague-Dawley female rats (250 g body wt) purchased from Charles Rivers Laboratories, Inc (Wilmington, Mass), were housed in pairs in hanging, stainless steel cages in a temperature-controlled room (25±1°C) with daily light cycle (light on 7 AM to 7 PM daily) for a minimum of 3 days before surgery. All rats had free access to Purina Rat Chow and tap water. All procedures performed on animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida before initiation of the study. Two weeks before the focal ischemia was induced, all rats were ovariec-tomized to eliminate endogenous estrogens. Rats in the ovariec-tomy with estrogen replacement (OVX+E2) group were administrated a single dose of E2 (100 μg/kg) 2 hours before focal ischemia surgery, while those in the ovariec-tomy alone (OVX) group received an oil vehicle injection. The sample size needed for the MRI study was calculated on the basis of published observations by us and others that E2 treatment caused a 50% reduction in MCA occlusion (MCAO)–induced lesion size.5–15 A small number of animals sufficient to show statistical significance were assigned to each group.

Focal Ischemic Model
MCAO was achieved according to the methods described previously.5 Briefly, after administration of anesthetics, the left common carotid artery, external carotid artery, and internal carotid artery on the left side were exposed and dissected through a midline cervical incision. A 3-0 monofilament suture was introduced into the left internal carotid artery lumen and gently advanced until resistance was felt, indicating MCAO and compromised blood flow. The suture

Table 1. Physiological Parameters in Rats Subjected to Transient MCAO (n=4 for Each Group)

<table>
<thead>
<tr>
<th>Group</th>
<th>MABP, mm Hg</th>
<th>pH</th>
<th>PO₂</th>
<th>PO₂</th>
<th>Hematocrit, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before MCAO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td>101±8</td>
<td>7.29±0.03</td>
<td>55±3</td>
<td>67±4</td>
<td>42±1</td>
</tr>
<tr>
<td>OVX+E2</td>
<td>90±3</td>
<td>7.27±0.02</td>
<td>61±2</td>
<td>69±4</td>
<td>43±2</td>
</tr>
<tr>
<td>Ischemia 30 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td>124±4</td>
<td>7.33±0.02</td>
<td>50±4</td>
<td>70±13</td>
<td>41±1</td>
</tr>
<tr>
<td>OVX+E2</td>
<td>116±7</td>
<td>7.31±0.05</td>
<td>51±5</td>
<td>70±8</td>
<td>43±3</td>
</tr>
<tr>
<td>Reperfusion 30 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td>99±11</td>
<td>7.35±0.01</td>
<td>45±1</td>
<td>74±5</td>
<td>44±1</td>
</tr>
<tr>
<td>OVX+E2</td>
<td>92±10</td>
<td>7.28±0.06</td>
<td>56±7</td>
<td>76±9</td>
<td>43±3</td>
</tr>
</tbody>
</table>

Figure 1. Sequential DWI from representative OVX and OVX+E2 rats during MCAO and after reperfusion. Two weeks after ovariec-tomy, female rats were divided into OVX (n=4) and OVX+E2 (n=3) groups. Both groups were then subjected to 1-hour MCAO. Sequential DWI were then obtained for each animal beginning at 30 minutes of the MCAO and at 2, 4, and 6 hours after monofilament removal (reperfusion interval). The imaging sections shown were captured at 9 mm caudal to the end of olfactory bulb.
was kept in place for 60 minutes and then withdrawn to allow MCA reperfusion. The operating procedure was performed within 20 minutes with little bleeding. Rectal temperature was monitored and maintained between 36.5°C and 37.0°C during the entire stroke procedure.

**Magnetic Resonance Imaging**
Imaging was performed in a 4.7-T, 33-cm magnet with a Brucke console with an actively shielded gradient set capable of 220 mT/m. The animals were supported on a cradle, and their heads were placed in a home-built birdcage coil with a 5-cm outer diameter (operating in quadrature transmit/receive mode). After the acquisition of scout images, 6 coronal plane images were prescribed beginning 3 mm behind the olfactory bulb. The slices were each 1.5 mm thick and were separated by 2 mm. All images were acquired over a 5-cm field of view with a 128×128 matrix (0.39×0.39-mm in-plane resolution), with a repetition time of 1.75 seconds and 2 signals averaged. Each set of 6 images was acquired in 7.5 minutes. DWI was performed with a standard pulsed gradient, spin-echo technique with an echo time of 33 ms. The gradient pulses were each applied for 9 ms and were separated by 13 ms around the 180° refocusing pulse. The gradient amplitude used was 152 mT/m, resulting in a b value of 1400 s/mm². T2WI was performed with a standard spin-echo technique with an echo time of 75 ms. Both DWI and T2WI were captured sequentially for each animal (n=4 for OVX and n=3 for OVX+E2 group) at 30 minutes during MCAO (the occlusion interval) and 2, 4, and 6 hours after withdrawal of the monofilament (the reperfusion period).

**Quantification of Ischemic Lesion Sizes and Intensity**
The ischemic lesion sizes and lesion intensity of the MR images were anatomically measured with Image-Pro Plus software (Media Cybernetics). The lesion area was subdivided into cortical and subcortical areas according to neuroanatomic landmarks. The percentage of the lesion size over the whole brain coronal section was calculated. The lesion intensity ratio was calculated, with the intensity of the nonlesioned hemisphere assigned a value of 1.

**Cerebral Blood Flow Measurement**
Cerebral blood flow (CBF) was measured in a separate group of rats (n=5 in each group for OVX and OVX+E2) that underwent focal ischemia surgery by methods that we have previously described in detail. A middle line section exposed the small area around bregma. Two symmetrical holes were drilled through the skull and adjacent to the dura. These 2 holes were located at 1.5 mm posterior to and 3.5 mm left/right of bregma. Two probes of a digital laser perfusion monitor (MICROFLO DSP, Oxford Optronix Ltd) were placed on the dura to record the second-to-second change in CBF before, during, and after MCAO. Recordings were made from each animal for at least 10 minutes before occlusion, during the entire hour of occlusion, and beginning at 10 minutes of reperfusion and again at 24 hours after the occlusion. For each recording period, data representing a 10-minute period of stable CBF measurements were used to determine CBF for that sample period for each animal.

**Physiological Parameters**
Physiological parameters were monitored in a separate group. The left femoral artery was catheterized for mean arterial blood pressure monitoring and arterial blood sampling in the OVX and OVX+E2 animals (n=4 in each group). Physiology parameters were measured with a portable clinical analyzer (i-STAT).

**Statistical Analysis**
The Mann-Whitney U test was applied to determine the significance of the difference between OVX and OVX+E2 groups. R² was calculated to analyze the coherence of the 2 measurements. A value of P<0.05 was considered significant.

**Results**
The mean arterial blood pressure was kept in the normal range during the experiment. The relatively lower P O₂ and relatively higher P CO₂ were due to the anesthesia. There were no significant differences in the determined parameters between OVX and OVX+E2 groups (Table 1).

DWI detected early changes in lesion sizes at 30 minutes into the MCAO. The total MCAO-induced lesion size was similar in both groups (33.7% and 33.5% of the whole hemisphere in the OVX and OVX+E2 groups, respectively) (Figures 1 and 2A) but was larger in cortical regions in the OVX group (26.5% versus 17.1% of OVX+E2) (Figures 1 and 2B). During reper-
fusion, the lesion size remained constant in the OVX group but decreased in the OVX+E2 group by 50% to 60% (P<0.05) (Figures 1, 2A, and 2B). The size reduction was primarily located in cortical regions (Figures 1, 2A, and 2B).

The intensity of the ischemic lesion, as measured by the signal intensity ratio (SIR), increased at 30 minutes during MCAO in the OVX group (lesion side versus nonlesion side, 2.35 and 2.20 in cortical and subcortical regions, respectively) and reached a plateau during the latter stages of reperfusion until 6 hours of reperfusion, when SIR dropped to 1.81 and 1.87 in cortical and subcortical regions, respectively (Table 2). By comparison, the SIR during MCAO in OVX+E2 animals was reduced (1.63 and 1.51 in cortical and subcortical regions, respectively; P<0.05).

Reperfusion further attenuated the SIR in cortical but not subcortical regions to 1.28 (P<0.05 versus OVX) in the OVX+E2 group (Table 2).

T2WI failed to detect the early vasogenic edema induced by MCAO in either group. During reperfusion, the OVX group demonstrated a continuous increase in lesion sizes in cortical (19.7%, 22.1%, and 24.7% at 2, 4, and 6 hours during reperfusion, respectively) and subcortical (8.3%, 10.1%, and 12.5% at 2, 4, and 6 hours during reperfusion, respectively) regions (Figures 3, 4A, and 4B). In contrast, the OVX+E2 group showed a 70% to 80% decrease in lesion size in cortical regions (P<0.05) (Figures 3 and 4B) and a 10% to 25% decrease in subcortical regions (Figures 3 and 4C). T2WI showed less attenuation of SIR by E2 treatment compared with DWI. E2 treatment did not significantly decrease SIR in subcortical regions compared with OVX groups (Table 2) but did cause a 20% decrease (P<0.05) in SIR in cortical regions during later reperfusion.

In separate groups of animals, CBF was measured before and during MCAO and 2 times during reperfusion. In both groups, CBF was reduced to approximately 20% of baseline during MCAO. Consistent with our previous observations, CBF gradually recovered during the reperfusion phase, reaching 81% and 97% of baseline in OVX and OVX+E2 groups, respectively, by 24 hours of reperfusion. There were no significant differences between groups at any of the sampling times assessed (Figure 5).

**Discussion**

Early detection and localization of potentially reversible ischemic damage are crucial for designing and investigating clinical therapeutic interventions against stroke. The present study demonstrates that MRI can provide a wealth of critical information about the initiation, progression, and localization of cerebral ischemic events and herein is used to define the location and the component of the developing ischemic lesion as affected by estrogens. DWI, which is sensitive to the random movement of water molecules, is thought to reveal the early changes associated with stroke-induced cytotoxic edema. On the other hand, conventional T2WI is sensitive to vasogenic edema that occurs later in the pathophysiology of

| Table 2. Effects of Estrogen in SIR of Ischemic Lesions Assessed by MRI |
|-------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                        | SIR             |                 |                 |                 |                 |                 |                 |
|                        | DWI 2 h         | 4 h             | 6 h             | T2WI 2 h        | 4 h             | 6 h             |                 |
|                        | Cortex          |                 |                 | Subcortex       |                 |                 |                 |
| OVX                    | 2.35±0.15       | 2.43±0.25       | 2.34±0.27       | 1.81±0.09       | 0.14±0.05       | 1.67±0.06       | 1.84±0.05       |
| OVX+E2                 | 1.63±0.12*      | 1.15±0.38*      | 1.23±0.40*      | 1.28±0.26       | 0.12±0.08       | 1.36±0.08*      | 1.40±0.11*      |
|                        | 2.20±0.21       | 2.38±0.15       | 2.27±0.22       | 1.87±0.06       | 1.40±0.04       | 1.59±0.06       | 1.76±0.06       |
|                        | 1.51±0.32       | 1.86±0.05*      | 2.11±0.11       | 1.94±0.09       | 1.28±0.01       | 1.35±0.04       | 1.52±0.06       |

Values are mean±SEM. Changes in ischemic lesion intensity were measured by SIR. The Mann-Whitney U test was applied to determine the significance of the difference between OVX (n=4) and OVX+E2 (n=3) groups.

*P<0.05 vs OVX group.
It can detect subacute ischemic damages, although it fails to show acute ischemic changes.

The early detection of ischemic lesion volumes by DWI is predictive of the clinical severity and outcome of stroke patients. In our study DWI provided the earliest detected evidence of cerebral ischemia and was the parameter most affected by E2 treatment. This reduction in DWI changes by E2 treatment can account for most of the observed beneficial effects of estrogen pretreatment.

The preferential protection provided by estrogen to cortical versus subcortical tissue could reflect the differential severity of the ischemic damage of these 2 brain regions. The differential severity could result from their different blood supplies. The penumbra of the cortical ischemic region receives collaterals from leptomeningeal anastomosis, as well as from the watersheds between the anterior cerebral artery and the MCA and between the posterior cerebral artery and the MCA, while the cores of the cortical ischemic region and the subcortical region are supplied by terminal arteries of the MCA only. During MCAO, the penumbra may continue to receive limited blood flow from the anterior cerebral artery, while the core and basal ganglion are believed to be more severely occluded. Alternatively, subcortical white matter is more vulnerable to the effects of focal ischemia than cortex. These differences in the vulnerability and blood supply to the core and penumbra area could lead to the difference in neuroprotective effects of estrogen between these 2 areas.

The protective effects of E2 treatment appear to CBF independent. We have previously reported the neuroprotective effects of estradiol in the absence of changes in CBF. In addition, others have reported a blood flow-independent neuroprotective effect of estrogens in both transient and permanent occlusion models. Consistently in this study, we saw no estrogen-related changes in the extent of decline in CBF during the occlusion and no significant difference in CBF during reperfusion.

E2 treatment appears to exert part of its protective effects by preventing permanent damage associated with reperfusion. Reperfusion causes structural alteration of the Golgi apparatus and compromises the energy supply to brain cells. Hoehn-Berlage et al applied bioluminescence and fluorescence techniques to correlate DWI and energy disturbance during MCAO and found a depletion of ATP in the ischemic core, while the area of tissue acidosis spread beyond the ATP-depleted core region. These findings are consistent with our triphenyltetrazolium chloride staining observation (data not shown) that the white core infarct region is surrounded by the pink ischemic penumbra. Additionally, since part of the E2-related improvement in DWI outcome occurs during the reperfusion phase, this delayed effect of the steroid may help to explain the observation that E2 treatment can be delayed until up to 3 hours after the onset of ischemia and lesion size reductions are still observed.

Figure 4. Effects of estrogen on MCAO-induced lesion sizes in female rats assessed by T2WI. Serial T2WI was applied to assess the total (A), cortical (B), and subcortical (C) lesion sizes in both OVX (n=4) and OVX+E2 (n=3) groups. Average measurements of lesion sizes at 7 and 9 mm were used for statistical analysis because they represented the widest extent of the MCAO lesions. The Mann-Whitney U test was applied to determine the significance of the difference between OVX and OVX+E2 groups. Mean±SEM values are depicted. When SEM is not shown, it is too small to be depicted. *P<0.05 vs OVX group.

Figure 5. Effects of estrogen on CBF during MCAO and reperfusion in OVX and OVX+E2 rats. Mean±SEM values for OVX (n=5) and OVX+E2 (n=4) rats are shown. There were no differences between the groups at any of the sampling times.
Reperfusion of ischemic tissue can produce an influx of oxygen followed by an accumulation of oxygen-derived free radicals. The oxidative stress may damage unsaturated fatty acids in the plasma membrane, which in turn could increase calcium influx into the cell and worsen ischemia-initiated neuronal injuries. We and others have shown that estrogens can attenuate free radical-induced peroxidative damage, modulate calcium homeostasis in neurons, and interact with neurotrophins, their receptors, and signaling pathways. All of these effects of estrogen may contribute to its protective effects during reperfusion.

The suggestion that estrogens may have significant protective properties during reperfusion could have profound impact in stroke therapies. Many centers in the United States are now treating stroke acutely, using thrombolytic agents to dissolve the offending clot. Clinical trials have demonstrated a significant clinical improvement in such patients, especially when the treatment is delivered within 3 hours of stroke onset. Reopening an occluded intracranial vessel, however, is not without serious risks, which may include acute or delayed intracerebral hemorrhage, reperfusion hyperemia, and progression to infarction despite a patent lumen. The identification of an agent that can protect against such mechanisms, if delivered before or early after the vessel is reopened, could minimize an otherwise preprogrammed infarction and perhaps also positively influence hemorrhagic risks by stabilizing energy metabolism in vascular endothelium.

The present study suggests that estrogens are good candidates for producing such effects. During reperfusion, E2 treatment dramatically decreases ischemic lesion sizes and intensities, as demonstrated by both DWI and T2WI, and these decreases are almost exclusively located in cortical regions. In our previous study we found that a single dose (100 g/kg) of E2 could increase the serum E2 concentration to physiological level in rats, producing levels sufficient for neuroprotection. These findings are also consistent with a study showing that OVX can increase the ischemic lesion volume in a focal stroke model. In summary, we applied MRI techniques to demonstrate the temporal and spatial ischemic changes in a focal ischemic animal model. We have demonstrated that estrogens selectively protect cortical tissue from ischemic damage and that this protection is exerted during both the occlusion and reperfusion phases of ischemia. This study suggests that estrogen could have direct clinical applications by protecting against thrombolytic-induced reperfusion injury.

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References

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