Effects of Estrogen on Leukocyte Adhesion After Transient Forebrain Ischemia

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Background and Purpose—Recent findings indicate that estrogen (ie, 17β-estradiol [E2]) provides neuroprotection in models of transient global and focal ischemia. Enhanced postischemic leukocyte adhesion and infiltration have been linked to neuropathology in the brain as well as other tissues. We recently showed that estrogen reduces leukocyte adhesion in the cerebral circulation of female rats during resting conditions.

Methods—We compared leukocyte adhesion in pial venules in vivo in intact, ovariectomized (OVX), and E2-treated OVX female rats subjected to transient forebrain ischemia (30-minute right common carotid artery occlusion and hemorrhagic hypotension) and reperfusion. Adherent rhodamine-6G–labeled leukocytes were viewed through a closed cranial window with the use of intravital microscopy. Leukocyte adhesion was measured before ischemia and at different times after reperfusion.

Results—Before ischemia, leukocyte adhesion (measured as a percentage of venular area occupied by adherent leukocytes) was 2 to 3 times greater in OVX versus intact or E2-treated OVX rats (7.0%, 3.4%, and 2.2%, respectively). This difference disappeared at 120 minutes of reperfusion, when comparable levels of enhanced leukocyte adhesion were observed in all groups. In OVX rats, leukocyte adhesion remained elevated after 4 and 6 hours of reperfusion (11.6% and 12.9%, respectively), while the other 2 groups showed significantly lower levels (5.0% and 5.8% for intact rats and 7.0% and 7.2% for E2-treated OVX rats).

Conclusions—Present results demonstrate that estrogen modulates leukocyte adhesion in the cerebral circulation after transient forebrain ischemia. This effect suggests that decreased leukocyte adhesion may be an important mechanism in estrogen-mediated neuroprotection. (Stroke. 2000;31:2231-2235.)

Key Words: adhesion molecules ■ cerebral ischemia ■ estradiol ■ neuroprotection ■ nitric oxide ■ rats

We recently showed that ovariectomy-induced chronic estrogen depletion was associated with an enhanced leukocyte adhesion in the cerebral circulation of female rats during resting conditions and in the presence of protein kinase C activation.1 There is abundant evidence that the increased leukocyte adhesion and infiltration that accompany cerebral ischemia exacerbate ischemic neuropathology.2–4 In previous studies from our laboratory, we demonstrated that estrogen, particularly 17β-estradiol (E2), provides neuroprotection in transient forebrain ischemia.5,6 The possibility therefore exists that E2-related neuroprotection may, at least in part, be attributable to a repression of postischemic leukocyte adhesion. In the present study we sought to evaluate whether the increased leukocyte adhesion seen in estrogen-depleted rats during nonischemic conditions would translate into higher levels of leukocyte adhesion after transient forebrain ischemia and perhaps even increased leukocyte transmigration during the initial hours of reperfusion. We used a rat transient forebrain ischemia model that combined right common carotid artery occlusion and hemorrhagic hypotension. Adherent leukocytes in pial venules were visualized with the use of a closed cranial window model and intravital microscopy.

Materials and Methods

The Institutional Animal Care and Use Committee approved the study protocol. Thirty-three adult female Sprague-Dawley rats (Charles River, Wilmington, Mass) weighing 300 to 400 g were used. Rats were divided in 3 groups: intact rats (n=10), ovariectomized (OVX) females (n=13), and E2-treated OVX females (n=10). The supplier performed ovariectomies 4 to 6 weeks before the study. The E2 was prepared in dimethyl sulfoxide (DMSO), and 100 μg/kg per day was given intraperitoneally for 1 week before the study. This dose provides average daily plasma levels of E2 within physiological limits (~50 pg/mL).8 On the day of the study, rats were anesthetized with halothane, tracheostomized, paralyzed with curare, and ventilated with 0.8% halothane in 70% N2O/30% O2. Femoral arterial and venous catheters were placed for monitoring of mean arterial blood pressure (MABP) and arterial blood gases and for drug infusion, respectively. A midline incision in
the neck was made, and the right common carotid artery was located and isolated, followed by placement of a right subclavian venous catheter. Rats were secured in a head holder in prone position to facilitate placement of a closed cranial window. Details of this procedure are provided in previous publications. Briefly, a 10-mm-diameter craniotomy was performed over the skull midline, the underlying dura was carefully removed, and an 11-mm-diameter acrylic window, outfitted with 3 ports for inflow, outflow, and intracranial pressure monitoring, was placed and fixed to the skull with cyanoacrylate gel. After window placement, halothane was discontinued, and a bolus of intravenous fentanyl was given (10 µg/kg), followed by a maintenance dose of intravenous fentanyl of 25 µg/kg per hour and ventilation with 70% N₂O/30% O₂. These conditions were maintained throughout the study. Cannulas were inserted in the ports, and the space under the window was filled with artificial cerebrospinal fluid, which was suffused at a rate of 1 mL/min and maintained at a temperature of 37°C. Pco₂ of 40 to 45 mm Hg, Po₂ of 50 to 60 mm Hg, and pH = 7.35. Intracranial pressure was maintained at 5 to 10 mm Hg by adjusting the height of the outflow cannula. MABP was continuously monitored, and rectal temperature was servo-controlled at 37°C. Arterial blood samples were taken for measurement of pH, Pco₂, and Po₂. Those analyses were performed on an ABL 520 Blood Gas System (Radiometer).

Pial venules were viewed through a microscope (Nikon) equipped with a color video camera (Sony, Fryer Co Inc). An epi-illumination system with a mercury lamp was used. Magnifications of ×800 were displayed on a video monitor. Pial venules (35 to 70 µm in diameter) were localized. Leukocytes were labeled with rhodamine-6G (200 µg/mL in 0.9% saline) given initially as an intravenous bolus (1 mL) and followed by continuous infusion at a rate of 1 mL/h. An appropriate rhodamine filter set was inserted into the light path, and baseline leukocyte dynamics were recorded. Illumination was limited to 60 seconds to avoid photobleaching.

An 0.8-mm-diameter laser-Doppler flow probe (Perimed) was secured to the cranial window above the right parietal cortex, and baseline measurements, in perfusion units, were recorded. Right forebrain ischemia was produced by clamping the right common carotid artery and by blood withdrawal from the subclavian vein to decrease cortical cerebral blood flow to 20% of baseline, as measured by laser-Doppler flowmetry. Reperfusion was established after 30 minutes. Leukocyte dynamics were again monitored after 30, 60, and 120 minutes of reperfusion. A videotape record of each experiment was made for subsequent analysis of leukocyte adhesion. This analysis was performed by capturing multiple frames of taped images in a computer with the use of the Image Pro Plus analysis system (Media Cybernetics). Leukocyte adhesion was measured and calculated in all experiments as the percentage of leukocytes (stickers and rollers) occupying the venular area as captured in each frame.

In an attempt to study leukocyte adhesion at longer reperfusion times, each of the 3 experimental groups was further divided into 2 subgroups: in the first one (group A), leukocyte adhesion was measured at 4, 5, and 6 hours of reperfusion. To assess the effect of the vehicle used to administer E₂ (DMSO), another group of OVX rats was studied in which DMSO was given intraperitoneally for 1 week and leukocyte adhesion was measured after 4, 5, and 6 hours of reperfusion. The results of this group were compared with those of the untreated OVX group.

At the end of the experiments (group B), we sought to evaluate whether there were any differences in leukocyte infiltration into the brain tissue in the 3 groups studied (intact, OVX, and E₂-treated OVX). For this, we employed immunofluorescence analyses using an antibody against MPO, a marker for leukocytes. The brains were perfusion-fixed with 4% paraformaldehyde in PBS, pH 7.0, and paraffin-embedded with techniques previously described. Coronal sections (7 µm) were prepared at the level of the striatum and hippocampus. Slides were pretreated in 10% poly-L-lysine solutions for 5 minutes and cleaned with 1% HCl in 70% ethanol. Slides were drained and dried in a 60°C oven for 1 hour. The brain tissue sections were adhered to the slides and were deparaffinized in xylene with 3 changes of 15, 5, and 5 minutes each. Sections were gradually hydrated through graded alcohols, beginning with 100% ethanol twice for 5 minutes each, followed by 95% ethanol twice for 5 minutes each, then 80% ethanol once for 2 minutes, and finally with distilled water for 1 minute. Slides were incubated for 10 minutes with 30% H₂O₂ in methanol (1:9) to block endogenous peroxidase activity. Slides were rinsed in distilled water twice for 2 minutes and once with PBS for 2 minutes and immersed in a beaker containing 1 L of 10 mmol/L citrate buffer (pH 6.0) and heated for 20 minutes.

The solution was allowed to cool to 42°C, and the slides were rinsed with PBS. To decrease background, 100 µL of 3% goat serum with 0.3% Triton-X blocking solution was added to each slide and incubated at 37°C for 30 minutes. The blocking solution was drained, and any excess blocking serum was wiped from the section. This was followed by the addition of 100 µL of 1:100 rabbit antihuman myeloperoxidase immunoglobulin fraction of rabbit antisemur primary antibody (Dako) and incubated overnight. On the next day, the slides were washed with PBS 3 times for 3 minutes each, followed by incubation with 100 µL of biotin-SP-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch). The slides were washed in PBS again 3 times for 3 minutes, and 100 µL of 1:1000 FITC-conjugated streptavidin (Jackson ImmunoResearch) was added for 30 minutes in a dark room. The sections were washed extensively with PBS, and coverslips were mounted with the use of VECTashield mounting medium H-100. Finally, the sections were examined under fluorescence microscopy with a 530-nm filter in a dark location at room temperature.

Statistical analyses were performed with Kruskal-Wallis 1-way ANOVA on ranks with multiple comparison procedure (Dunnett’s method) for comparisons of leukocyte adhesion between groups; 1-way ANOVA for comparisons of physiological variables between groups; repeated-measures ANOVA with a post hoc Tukey test for comparisons of leukocyte adhesion and physiological variables within a given experiment; and paired t test for comparisons of leukocyte adhesion between untreated OVX and DMSO-treated

**TABLE 1. Physiological Variables for Group A**

<table>
<thead>
<tr>
<th>Group</th>
<th>Preischemia</th>
<th>Intraischemia</th>
<th>30-min Reperfusion</th>
<th>120-min Reperfusion</th>
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<tbody>
<tr>
<td></td>
<td>pH</td>
<td>Pco₂</td>
<td>MABP</td>
<td>pH</td>
</tr>
<tr>
<td>Intact</td>
<td>7.42±0.01</td>
<td>38.7±1.5</td>
<td>123±6</td>
<td>7.40±0.03</td>
</tr>
<tr>
<td>OVX</td>
<td>7.41±0.02</td>
<td>40.6±1.5</td>
<td>122±4</td>
<td>7.43±0.02</td>
</tr>
<tr>
<td>OVX+E₂</td>
<td>7.40±0.02</td>
<td>38.6±0.7</td>
<td>125±5</td>
<td>7.39±0.03</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n=5 per group). Pco₂ and MABP are expressed in millimeters of mercury.

*P<0.05 vs preischemia; †P<0.05 vs OVX; ‡P<0.05 vs intraischemia.
B, C) and E2-treated OVX rats (D, E, F) are shown in Figure 1. Representative images of pial venules with adherent rhodamine-labeled leukocytes at different times are shown. The top panels (A, B, C) correspond to OVX rats, and the bottom panels (D, E, F) to E2-treated OVX rats, at different times during the experiments: before ischemia (A, D), after 120 minutes of reperfusion (B, E), and after 6 hours of reperfusion (C, F). Before ischemia, leukocyte adhesion was higher in OVX rats than in E2-treated OVX rats. This difference disappears after 120 minutes of reperfusion, when both groups have similar levels of enhanced leukocyte adhesion. However, after 6 hours of reperfusion, leukocyte adhesion in E2-treated OVX animals is significantly lower than that in OVX animals, in which levels of adhesion remain elevated.

### Table 2. Physiological Variables for Group B

<table>
<thead>
<tr>
<th>Group</th>
<th>Preischemia</th>
<th>Intraischemia</th>
<th>4-h Reperfusion</th>
<th>6-h Reperfusion</th>
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<tr>
<td></td>
<td>pH</td>
<td>Pco₂</td>
<td>MABP</td>
<td>pH</td>
</tr>
<tr>
<td>Intact</td>
<td>7.46±0.01</td>
<td>38.2±1.0</td>
<td>138±3</td>
<td>7.41±0.04</td>
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<tr>
<td>OVX</td>
<td>7.46±0.02</td>
<td>39.8±1.4</td>
<td>132±6</td>
<td>7.43±0.02</td>
</tr>
<tr>
<td>OVX+E₂</td>
<td>7.44±0.01</td>
<td>36.7±1.3</td>
<td>133±7</td>
<td>7.41±0.02</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n=5 per group). Pco₂ and MABP are expressed in millimeters of mercury.

*P<0.05 vs preischemia.

OVX animals. All values are reported as mean±SEM. Statistical significance was considered at the P<0.05 level.

### Results

The arterial blood variables measured before ischemia, during ischemia, and at different times after reperfusion are summarized in Tables 1 and 2. There were no significant differences in pH, Pco₂, and MABP among the 3 study groups or between subgroups. A few significant differences in Pco₂ and MABP were seen over the course of the experiments in all 3 groups (Tables 1 and 2). However, all values remained within normal limits. Pco₂ (data not shown) was maintained above 100 mm Hg in all experiments. Table 1 shows the results of group A, in which the cranial window was placed before ischemia, and Table 2 represents group B, in which ischemia was produced before placement of the cranial window. The severity of the ischemia produced in intact, OVX, and E2-treated OVX rats was the same in both subgroups. In all animals studied, intraschismic cortical CBF was controlled at 20% of baseline, as measured by laser-Doppler flowmetry (data not shown). Representative images of pial venules with rhodamine-labeled leukocytes at different times in OVX (A, B, C) and E2-treated OVX rats (D, E, F) are shown in Figure 1. Leukocyte adhesion was calculated at different times, and the results are shown in Figure 2. Before ischemia, the percentage of adherent leukocytes in OVX rats was significantly higher than that in the intact and E2-treated OVX groups (7.0%, 3.4%, and 2.2%, respectively). However, after 30 minutes of reperfusion, this difference was lost, mainly between the intact and OVX groups (14.1% versus 12.7%, respectively). The percent leukocyte adhesion in the E2-treated group was lower (6.0%) but not significantly different compared with the values for the other 2 groups. After 120 minutes of reperfusion, adherent leukocytes increased compared with preischemic levels (P<0.05), to 18.6%, 14.4%, and 14.6%, in intact, OVX, and E2-treated OVX, respectively, demonstrating no differences among them. After 4 hours of reperfusion, OVX rats had a significantly higher percentage of adherent leukocytes of 11.6%, compared with 5.0% and 7.0% in intact and E2-treated OVX rats, respectively. A similar relationship was found after 5 and 6 hours of reperfusion; in the latter, OVX rats had significantly higher levels of adherent leukocytes (12.9%) than intact and E2-treated OVX (5.8% and 7.2%, respectively). Thus, increased leukocyte adhesion appears to persist in estrogen-depleted animals after longer reperfusion times. Leukocyte adhesion was also measured in DMSO-treated OVX rats, finding that at 4 and 6 hours of reperfusion this group also showed increased leukocyte adhesion (14.9% and 15.8%, respectively). These levels are similar to the levels found in untreated OVX animals at the same times, suggesting that DMSO has no effect in leukocyte adhesion. The results are summarized in Figure 3.

In group B animals, at the end of each experiment, brains were fixed, sectioned, and stained with an antibody against MPO protein to assess leukocyte infiltration into the brain tissue (cortex, striatum, and hippocampus). No evidence of leukocytes in the brain parenchyma was observed in any of the 3 groups studied, although a few leukocytes trapped in pial venules of OVX rats could be seen after 6 hours of reperfusion, as shown in Figure 4.

### Discussion

In confirmation of recently published findings from our laboratory, preischemic leukocyte adhesion was 2 to 3 times
higher in OVX rats than in intact rats and E₂-treated OVX rats. After 30 minutes of reperfusion, some increased leukocyte adhesion was seen in all groups. At 120 minutes of reperfusion, comparable levels of enhanced leukocyte adhesion were observed in all groups. At longer reperfusion times (4 and 6 hours), the percentage of adherent leukocytes remained elevated in the OVX group, while intact and E₂-treated groups showed significantly lower levels. There was no evidence of leukocyte infiltration into the brain tissue in any of the 3 groups studied after 6 hours of reperfusion.

Our results indicate that estrogen modulates leukocyte adhesion in the cerebral circulation of female rats during resting conditions and after ischemia. Recent publications point to an estrogen-related limitation of inflammatory responses in peripheral tissues due to a decreased expression of adhesion molecules, including vascular cell adhesion molecule-1, E-selectin, and intercellular adhesion molecule-1. There is not, however, a clear consensus on whether estrogen acts directly or indirectly to decrease leukocyte adhesion. E₂ treatment has been shown to upregulate the expression of endothelial nitric oxide synthase (NOS) and neuronal NOS. NO derived from endothelial NOS is well known for its antiadhesive properties in the microvasculature of postischemic tissue. Recently, Garcia-Duran et al. showed that estrogen stimulates neuronal NOS protein expression in human neutrophils. This was associated with a reduction in the expression of the CD18 antigen on the surface of E₂-incubated neutrophils, producing a decrease in adhesive capacity. In further support of an antiadhesive action of NO, Gauthier et al. found that expression of P-selectin and intercellular adhesion molecule-1 was attenuated in hypercholesterolemic rats treated with the NO donor CAS1609. This treatment produced a decrease in rolling and adherence of leukocytes in the mesenteric circulation. Hypercholesterolemia was also accompanied by a repression of endothelial NOS-mediated vasodilating function (impaired relaxation to acetylcholine) that was also prevented by infusion of the NO donor. These studies would therefore suggest that estrogen acts through NO, at least in part, in reducing leukocyte adhesion.

An unexpected finding in the present study was that the level of leukocyte adhesion at 4 and 6 hours of reperfusion in intact and E₂-treated OVX rats, but not untreated OVX rats, was much lower than the levels of adhesion seen at 120 minutes of reperfusion. That reduction in the number of adherent leukocytes could not be attributed to de-adhesion and subsequent tissue infiltration. That is, no MPO expression within the brain parenchyma, irrespective of estrogen status, was observed in brain sections obtained from rats exposed to 6 hours of reperfusion. These results would appear to suggest a process whereby estrogen promotes de-adhesion, allowing leukocytes to return to the circulation. The specific mechanisms involved in that suspected estrogen action must await identification in future experiments.

Figure 2. Percent venular area occupied by adherent leukocytes in intact (n=5), OVX (n=5), and E₂-treated OVX (n=5) rats in group A (preischemia, 30 and 120 minutes of reperfusion) and group B (4 and 6 hours of reperfusion). Values are mean ± SEM. *P<0.05 vs OVX; #P<0.05 vs 120 minutes of reperfusion.

Figure 3. Percent venular area occupied by adherent leukocytes in untreated OVX (n=5) and vehicle-treated OVX (n=3) rats after 4 and 6 hours of reperfusion.

Figure 4. Photomicrograph of 2 MPO-positive leukocytes trapped in a pial venule of an OVX rat after 6 hours of reperfusion. There was no evidence of leukocyte infiltration into the brain tissue in any of the 3 groups studied. Bar ~20 μm.
(MPO immunoreactivity) in the cortex, striatum, and hippocampus of OVX rats, but not intact and E2-treated OVX rats, after 48 and 72 hours of reperfusion.

In conclusion, we have shown that chronic E2 treatment attenuates leukocyte adhesion in the rat cerebral circulation during resting conditions and after transient forebrain ischemia. This anti-inflammatory effect of estrogen may account, at least in part, for the ischemic neuroprotection afforded by estrogen treatment, as reported in earlier studies.

Acknowledgments

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References


Editorial Comment

It is well established that estrogen has protective effects against stroke. In the preceding article, Santizo and his colleagues provide additional evidence, which clarifies further this neuroprotective effect of estrogen. They found that estrogen reduced leukocyte adhesion following transient forebrain ischemia in rats. Work by others suggests that at least one of the mechanisms through which this is accomplished is upregulation of nitric oxide synthase. Nitric oxide reduces the expression of adhesion molecules and thereby induces reduced leukocyte adhesion. This is additional evidence of the role of inflammation in stroke.

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