Estrogen Replacement Treatment in Diabetic Ovariectomized Female Rats Potentiates Postischemic Leukocyte Adhesion in Cerebral Venules

Hao-Liang Xu, MD, PhD; Verna L. Baughman, MD; Dale A. Pelligrino, PhD

Background and Purpose—Chronic 17β-estradiol (E$_2$) replacement therapy in ovariectomized (OVX) female rats reduces leukocyte adhesion and brain damage after transient forebrain ischemia. Recently, we found that E$_2$ treatment in diabetic OVX females was associated with enhanced postischemic neuropathology. We tested the hypothesis that in chronically hyperglycemic diabetic OVX females, chronic E$_2$ replacement potentiates post-transient forebrain ischemia leukocyte adhesion.

Methods—Pial venules were observed through closed cranial windows. Adherence of rhodamine 6G–tagged leukocytes was monitored before and 10 hours after transient forebrain ischemia (20 minutes right common carotid artery occlusion plus hemorrhagic hypotension) in intact, untreated OVX and E$_2$-treated OVX females rendered diabetic via streptozotocin. Leukocyte adhesion was quantitated as the percentage venular area occupied by adherent leukocytes.

Results—At 2 hours after transient forebrain ischemia, a similar low level of leukocyte adhesion was seen in the 3 groups (<3% of the venular area). Starting at ~4 hours after ischemia, leukocyte adhesion in the E$_2$-treated OVX females rose to significantly higher levels compared with the other groups. Relative to the 2-hour value, the level of adhesion at 10 hours was 12.5-fold, 4-fold, and 5-fold greater in the E$_2$-treated OVX, OVX, and intact groups, respectively. Leukocyte extravasation (beginning after 6 hours of reperfusion) was observed in a majority (64%) of the E$_2$-treated animals, with limited or no extravasation seen in the intact or OVX groups.

Conclusions—These results suggest that factors associated with diabetes and chronic hyperglycemia convert E$_2$ from a counterinflammatory to a proinflammatory substance in an ischemic setting. (Stroke. 2004;35:1974-1978.)

Key Words: hyperglycemia ■ ischemia ■ inflammation ■ estrogen

Laboratory evidence supports the neuroprotective and vasculoprotective actions of 17β-estradiol (E$_2$). Despite this, the results of recently published large-scale prospective clinical studies have indicated that hormone replacement therapy not only fails to provide any benefits with respect to stroke and cardiovascular disease but may actually be detrimental. In line with this, findings from our laboratory, and more recently from Zhang et al, led to the identification of a clinically relevant circumstance (diabetes, chronic hyperglycemia) in which E$_2$ replacement therapy in ovariectomized (OVX) animals was ineffectual in providing ischemic neuroprotection. In fact, in our study, E$_2$ treatment actually exacerbated neuropathology.

Postischemic leukocyte adhesion and infiltration have been linked to tissue damage. E$_2$ has been reported to reduce leukocyte accumulation in cardiac tissue after ischemia. In the brain, our findings support a lesser-magnitude postischemic leukocyte adhesion in nondiabetic, intact, and E$_2$-treated OVX female rats compared with untreated OVX females. This may relate to a repression of adhesion molecule “availability” in the presence of E$_2$ caused by blocking nuclear factor κB activation.

In nonbrain models of ischemia/reperfusion, diabetic (chronically hyperglycemic) animals versus nondiabetics show a more intense postischemic inflammatory response. There is currently no information regarding chronic hyperglycemia and postischemic inflammatory activity in cerebral ischemia/reperfusion models. In acutely hyperglycemic rats subjected to forebrain ischemia/reperfusion, Lin et al reported a substantial increase (at 24 to 72 hours) in the numbers of adherent and infiltrated leukocytes in and around pial vessels, as well as parenchymal vessels in vulnerable brain regions. Normoglycemic animals were virtually devoid of adherent/infiltrated leukocytes and compared with the hyperglycemic rats, displayed less brain damage 72 hours after ischemia. However, leukocyte behavior was not monitored before 24 hours after ischemia. Thus, it is uncertain whether the increased leukocyte “presence” actually contributed to that damage or occurred in response to cellular injury.
In fact, conditions may exist in which postischemic leukocyte adhesion contributes to brain damage. When combining published findings from our laboratory obtained in nondiabetic females, one finds that the severity of neuropathology 3 days after ischemia parallels the levels of leukocyte adhesion measured during the initial 6-hour period after restitution of blood flow. On the basis of this, we hypothesized that the relative levels of postischemic leukocyte adhesion in streptozotocin-induced diabetic, OVX, and E2-treated OVX female rats will follow the same general pattern as seen for postischemic neuropathology (ie, postischemic leukocyte adhesion during the initial hours of reperfusion will be highest in E2-treated OVX females).

**Methods**

The study protocol was approved by the Institutional Animal Care and Use Committee. Three groups of female Sprague–Dawley rats (200 to 250 g at arrival) were used: intact female, OVX, and 17β-estradiol–treated (E2; 0.1 mg · kg⁻¹ · day⁻¹ · IP for 7 to 8 days before ischemia) OVX rats (OVX+E2). Ovariectomies were performed by the vendor (Charles River) 1 week before shipment. As shown in a previous report from our laboratory, the 0.1 mg · kg⁻¹ · day⁻¹ dose results in an average daily plasma E2 concentration that falls between the peak and nadir levels observed during the normal rat estrous cycle. In the present study, blood samples were obtained in 4 rats from each group via percutaneous venipuncture for analysis of serum E2 levels before ischemia induction. Diabetes was induced via streptozotocin (60 mg/kg IV) at ~1 week after arrival, and the rats were studied ~6 to 8 weeks later. All displayed plasma glucose levels were in the range of 25 to 30 mmol/L at the time transient forebrain ischemia was imposed. Anesthesia was induced with halothane. The rats were then paralyzed (curare), tracheotomized, and mechanically ventilated. For surgery, anesthesia was maintained with 0.8% halothane/70% N2O/30% O2. Catheters were placed in the femoral artery to measure systemic arterial pressure and to obtain arterial blood samples, and in the femoral vein for infusion of drugs. Rectal temperature was maintained at 37°C with a servocontrolled heating pad. A midline incision in the neck was made, and the right common carotid artery was isolated, followed by insertion of a right subclavian venous catheter. Rats were secured in a head holder to facilitate placement of a closed cranial window. After surgery, the halothane was discontinued, and the rat was maintained on 70% N2O/30% O2-fentanyl (25 μg · kg⁻¹ · h⁻¹ · IV) thereafter. The space under the window was filled with artificial cerebrospinal fluid (aCSF) that was equilibrated with gas consisting of 10% O2/5%CO2 with balance of N2. The 37°C aCSF was suffused at 0.5 mL/min.

Pial venules were viewed through a microscope (Nikon) equipped with a color video camera (Sony) and displayed on a video monitor.

**Arterial Blood Variables and Body Weights**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight (g)</th>
<th>Serum E2 (pg/ml)</th>
<th>Paco2 (mm Hg)</th>
<th>pHa</th>
<th>Mean Arterial Blood Pressure (mm Hg)</th>
<th>Plasma Glucose (mmol/L)</th>
<th>Paco2 (mm Hg)</th>
<th>pHa</th>
<th>Mean Arterial Blood Pressure (mm Hg)</th>
<th>Plasma Glucose (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact (n = 11)</td>
<td>260 ± 28†</td>
<td>57.6 ± 7.8†</td>
<td>31.8 ± 5.4</td>
<td>7.43 ± 0.07§</td>
<td>115 ± 11</td>
<td>28.2 ± 4.2</td>
<td>30.4 ± 3.2</td>
<td>7.27 ± 0.13</td>
<td>100 ± 14*</td>
<td>26.4 ± 4.1</td>
</tr>
<tr>
<td>OVX (n = 10)</td>
<td>320 ± 25</td>
<td>17.1 ± 9.2</td>
<td>30.8 ± 3.9</td>
<td>7.44 ± 0.05</td>
<td>120 ± 17</td>
<td>28.9 ± 1.9</td>
<td>31.0 ± 6.5</td>
<td>7.29 ± 0.13*</td>
<td>109 ± 14*</td>
<td>25.1 ± 5.9</td>
</tr>
<tr>
<td>OVX+E2 (n = 11)</td>
<td>278 ± 27†</td>
<td>41.6 ± 6.6†</td>
<td>31.0 ± 4.1</td>
<td>7.43 ± 0.04</td>
<td>120 ± 15</td>
<td>29.3 ± 1.8</td>
<td>31.3 ± 6.0</td>
<td>7.29 ± 0.13*</td>
<td>103 ± 18*</td>
<td>25.8 ± 5.5</td>
</tr>
</tbody>
</table>

Values are means ± SD.

Sample in OVX+E2 group was obtained 12 hours after most recent E2 injection. Intact group samples were obtained without consideration of stage of estrous cycle.

$t^P < 0.05$ vs preischemia value.

$†P < 0.05$ vs OVX.

$§P < 0.05$ vs OVX+E2.

Autologous leukocytes were labeled in vivo with rhodamine 6G. A baseline record of leukocyte dynamics was obtained before ischemia. Illumination was limited to 60 seconds at a time to avoid photobleaching. A 0.8-mm-diameter laser-Doppler flow probe (Perimed) was secured to the cranial window above the right parietal cortex, and baseline measurements were recorded. Right forebrain ischemia was produced by clamping the right common carotid artery, combined with 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 20 minutes. The volume of blood withdrawn (into syringes containing ~25 U heparin) and subsequently infused was in the range of 7 to 10 mL. Skull temperature during ischemia remained >36.5°C. Leukocyte dynamics were again monitored after 2, 4, 6, 8, and 10 hours of reperfusion. A videotape record of each experiment was made for subsequent analysis of leukocyte adhesion using the Image Pro Plus analysis system (Media Cybernetics). Leukocyte adhesion was measured and calculated in all experiments as the percentage of adherent leukocytes occupying the venular area, as captured in each video frame. In some instances (but only at time points >6 hour), leukocyte infiltration was observed. In those cases, extravasation was quantitated and expressed as the percentage of the total leukocyte population found outside of blood vessels. It should be noted that when extravasation was observed, the measurement of “leukocyte adhesion” included only rhodamine-positive cells in contact with the vessels.

Statistical analyses were performed using an unpaired t test (comparisons between groups). For time-related comparisons within groups, a repeated-measures 1-way ANOVA with a post hoc Tukey test for multiple comparison procedures was used. A level of $P < 0.05$ was considered significant in all statistical tests. Values are presented as mean ± SD. All drugs/chemicals were obtained from Sigma unless otherwise stated.

**Results**

The arterial PCO2, pH, and mean pressure data obtained before ischemia and at 10 hours after reperfusion are summarized in the table. Arterial PO2 remained >100 mm Hg during the monitoring period. When comparing initial and end-reperfusion values, modest reductions in mean arterial blood pressure were seen in the 3 groups, although the change in the OVX group was not statistically significant. Preischemic pH was significantly lower in the intact versus the other 2 groups. The end-reperfusion arterial pH value was significantly lower than the preischemic value in all experimental groups. This progressive acidosis was quantitatively similar in all groups, with the arterial pH value falling from 0.09 to 0.15 U during the course of the study. No significant changes in PCO2 and plasma glucose were observed during the experiments, and
the values among groups were statistically similar at equivalent time points. The body weight in the OVX group was significantly higher compared with the other 2 groups. Serum E2 levels were similar in the intact and OVX/E2 groups and significantly higher than the value in the OVX group.

Representative frames captured from videotaped recordings obtained during the reperfusion period are provided in Figure 1. Figure 2 summarizes the levels of leukocyte adhesion (percentage of the viewed venular area occupied by adherent leukocytes) observed in the 3 groups before ischemia and 2, 4, 6, 8, and 10 hours after reperfusion. The level of preischemic leukocyte adhesion was relatively low (≈2% to 3%) in the untreated and E2-treated OVX groups; but in the intact group, preischemic adhesion was significantly higher (≈6%) compared with the 2 OVX groups. At 2 hours after reperfusion, leukocyte adhesion was relatively low (2% to 3%) in all 3 groups. In fact, the level of adhesion in the intact females was significantly lower than the level measured before ischemia. In the OVX/E2 group, significant increases in leukocyte adhesion relative to the 2-hour value were seen already at 4 hours (2.6-fold rise), with further significant elevations at each subsequent time point represented in both figures, eventually achieving a level at 10 hours after reperfusion, 12.5× higher than the level measured at 2 hours. In the OVX females not treated with E2, a significant increase over the 2-hour value (2-fold rise) was not seen until 6 hours after reperfusion, with modest additional increases observed up to 10 hours after reperfusion, achieving a level 3.9× greater than the 2-hour value. The pattern of postischemic leukocyte adhesion in the intact female group generally mirrored the pattern seen in the OVX (untreated) group up to 6 hours. However, significant differences, relative to the 2-hour value, were only seen at 8 and 10 hours after reperfusion. Also, compared with the OVX group, the intact females showed a steeper rise in adhesion during the 6- to 10-hour interval (1.7-fold [6 hours] to 4.8-fold [10 hours] rise, relative to the 2-hour value). Furthermore, at 10 hours after reperfusion, leukocyte adherence in intact females was significantly greater than in untreated OVX females.

Leukocyte infiltration, if it occurred at all, began at ≧6 hours after reperfusion. The OVX+E2–treated group displayed the greatest incidence of infiltration, with 7 of 11 (64%) of these animals demonstrating an extravascular leukocyte presence by 10 hours after reperfusion (Figure 1). Fewer intact females displayed infiltration at 10 hours (3 of 11% to 27%); whereas no infiltration was detected in untreated OVX females. We estimated infiltration by dividing the area occupied by extravascular leukocytes by the sum of the areas occupied by extravascular and intravascular leukocytes and then multiplying by 100%. In the 7 OVX+E2 females showing extravasation at 10 hours, an “infiltration index” of 44±8% was calculated; whereas in the 3 intact females, the 10-hour value was 28±5%. It should be noted that extravascular cells were excluded from the data summarized in Figure 2. Thus, limiting the measurement to cells within (or in contact with) venules is likely to cause one to underestimate “true” leukocyte activity. As such, the differences in relative inflammatory behavior among the 3 study groups at 10 hours after ischemia are probably greater than depicted by the data presented in Figure 2.

Discussion

One of the key findings in this study was that chronic E2 replacement therapy in diabetic, chronically hyperglycemic OVX female rats exacerbates postischemic inflammation, as reflected in the heightened adherence of leukocytes to pial venules, and in many instances, leukocyte extravasation. In addition, untreated OVX females displayed a somewhat lower level of postischemic leukocyte adhesion compared
with intact females. These results are in contrast to published findings from our laboratory in nondiabetic female rats, in which ovariectomy was accompanied by an enhanced level of postischemic leukocyte adhesion compared with intact females. Moreover, that exacerbation of inflammatory activity in nondiabetic OVX females could be prevented by chronic E2 replacement.4 Thus, present findings suggest that factors associated with diabetes and chronic hyperglycemia convert E2 from a counterinflammatory to a proinflammatory substance.

Generally speaking, the far more robust leukocyte response in the diabetic E2-treated OVX females compared with the other diabetic groups appears to correlate with the level of postischemic neuropathology observed in this group.2 However, the magnitude of the neuropathology and postischemic leukocyte adhesion were not strictly correlated in the 3 female groups. That is, in diabetic females, although the order of neuropathologic severity was OVX+E2>OVX>intact,2 the order for postischemic leukocyte activity was OVX+E2>OVX>intact (present study). On the other hand, in nondiabetic females, neuropathology and adhesion followed the same order (ie, OVX>OVX+E2= intact).5,10 Additional differences emerge when comparing earlier results on nondiabetics5 with findings in diabetics in the current study. One relates to the temporal patterns of postischemic leukocyte adhesion, which is particularly evident in intact and E2-treated OVX females (and males),18 in which nondiabetics exhibited a transient increase in adhesion during the initial 6 hours of reperfusion, with a return toward baseline levels by 10 hours. In nondiabetics, only the OVX group showed the progressive rise that was seen in all groups of the present study. Furthermore, there are even differences to be considered when comparing the diabetic rats of our recent article2 with those of the present study. The degree of stress imposed on the rats in the current investigation may have been greater because of a more extensive surgical preparation (ie, cranial window) and the fact that the rats were maintained under anesthesia and mechanical ventilation for >10 hours. One manifestation of this may be the postischemic arterial acidosis observed in all rats. However, although acidosis can contribute to leukocyte adhesion,19 the fact that all groups displayed similar arterial pH levels during reperfusion would seem to limit the possibility of any “differential” pH effect on postischemic leukocyte adhesion in the 3 groups of the present study; although the lower preischemic arterial pH in the intact group could have contributed to the higher preischemic adhesion level of this group.

At any rate, the above implies that higher-magnitude leukocyte activity during reperfusion does not always translate into an exacerbation of neuronal damage. Indeed, as covered in a recent review,11 there are examples in experimental cerebral ischemia studies of a lack of correlation between postischemic leukocyte behavior and neuropathology. Certainly, in experiments in which assessments of leukocyte presence were performed at ≥24 hour, one cannot disregard a scenario in which meaningful neuronal damage precedes the appearance of significant leukocyte adhesion and infiltration. Thus, such leukocytes may only play a “bystander” role, heralding the presence of damage rather than causing the damage itself. Conversely, the results from a number of animal studies provide evidence favoring leukocyte contributions to ischemic neuropathology.12 Another possibility to consider is that leukocyte activity in pial venules may not be representative of leukocyte behavior in venules of vulnerable brain structures. However, in forebrain ischemia/reperfusion, Lin et al9,10 showed that leukocyte behavior in pial vessels does track that seen in parenchymal vessels.

The possibility remains that circumstances may exist in which postischemic leukocyte adhesion contributes to brain damage. Furthermore, evidence implies that the initial 6- to 10-hour reperfusion period may be the critical time frame. That is, in diabetic E2-treated OVX females, as well as untreated nondiabetic OVX females, the level of venular leukocyte adhesion does not diminish between 4 and 8 hours and actually expands, at least in diabetics, with signs of infiltration beginning at ≥8 hours after ischemia. Perhaps not coincidentally, the same groups showing the most robust progressive rise in postischemic adhesion also exhibited greater neuropathology. However, the presence of “other factors” could modulate the pathologic effects linked to increased leukocyte presence. This could account for the finding that intact diabetic females, despite exhibiting a level of leukocyte adhesion, during 6 to 10 hours of reperfusion exceeding that seen in their OVX counterparts, displayed a comparatively moderate degree of subsequent brain damage.2 Indeed, there is support for other factors essentially overriding the potentially deleterious actions associated with an increased presence of leukocytes during the initial hours of reperfusion. Thus, Ahmed et al20 reported that in rats pretreated with lipopolysaccharide (LPS) 24 hours before temporary MCA occlusion, leukocyte activity was enhanced substantially after 6 hours of reperfusion. Despite this, infarct volumes were actually lower in the LPS group. Because it has been established that LPS pretreatment (8 to 24 hours before ischemia) leads to ischemic tolerance,21 it is likely that “preconditioning factors” helped to overcome any damaging effects associated with an increased presence of leukocytes.

In diabetes, we can only speculate why, relative to untreated OVX females, intact females seemed to tolerate a greater level of postischemic leukocyte activity, whereas E2-treated OVX females clearly did not. One intriguing possibility relates to progesterone, which represents the most obvious element differentiating intact females from both E2-treated and untreated OVX rats. To address this, future studies should include groups of OVX females receiving combined E2+progesterone replacement.

In conclusion, results from our laboratory support the possibility that chronic hyperglycemia not only prevents the benefits of E2 but actually provides an environment in which E2 replacement therapy potentiates postischemic inflammatory activity and exacerbates ischemic neuropathology. Thus, rather than reducing postischemic leukocyte activity and neuropathology (as seen in nondiabetic rats), E2 supplementation in diabetic OVX rats increased both. However, that loss of E2-related neuroprotection was not evident in gonadally intact diabetic females, despite the presence of enhanced postischemic inflammation. We suspect that the latter may
relate to the presence of progesterone, although this remains to be tested experimentally.

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References
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