Estrogen restores postischemic pial microvascular dilation

Y. WATANABE, M. T. LITTLETON-KEARNEY, R. J. TRAYSTMAN, AND P. D. HURN
Department of Anesthesiology and Critical Care Medicine, Johns Hopkins Medical Institutions,
Baltimore, Maryland 21287

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Watanabe, Y., M. T. Littleton-Kearney, R. J. Traystman, and P. D. Hurn. Estrogen restores postischemic pial microvascular dilation. Am J Physiol Heart Circ Physiol 281: H155–H160, 2001.—Estrogen protects the brain from experimental cerebral ischemia, likely through both vascular and neuronal cellular mechanisms. The purpose of this study was to determine whether chronic estrogen treatment in males and repletion in ovariectomized (Ovx) females reverses abnormalities in pial arteriolar reactivity during reperfusion from global forebrain ischemia (4-vessel occlusion, 15 min) and whether the site of protection is vascular endothelium. Male and Ovx female rats were implanted with either placebo or a 25-μg 17β-estradiol pellet 10 days before ischemia. With the use of intravital microscopy, pial vessel dilation to ACh (10 μM) and S-nitroso-N-acetyl-penicillamine (SNAP; 1 μM) and vasoconstriction to serotonin (10 μM) was examined in situ at 30–60 min of reperfusion. Postischemic changes in vessel diameter were compared with preischemic values for each agent. Postischemic response to both ACh and SNAP was lost in males and Ovx females, but not in estrogen pellet-implanted males and estrogen-implanted Ovx females, suggesting that estrogen protects both endothelial and smooth muscle-mediated vasodilation. Ischemia blunted vessel constriction to serotonin regardless of treatment. These data demonstrate that estrogen acts as a vasoprotective agent within the cerebral circulation and can improve microvascular function under conditions of an acutely evolving ischemic pathology.

cerebral ischemia; microvasculature; pial circulation

ESTROGEN HAS BEEN WELL ESTABLISHED as a neuroprotectant in cerebral ischemic injury. The ability of the steroid to salvage brain has been linked to multiple cellular mechanisms, and its protective targets potentially include neurons and cerebral blood vessels (for reviews, see Refs. 24 and 44). Chronic estrogen treatment in animals can improve cerebral blood flow (CBF) during global (21, 39) and focal cerebral ischemia (2). Recent findings (31) also indicate that acute estrogen infusion during postischemic reperfusion acts as a potent cerebral vasodilator in the pathological, but not in the un injured, brain. The ability of estrogen to augment vascular signaling has been well described in normal cerebral vessels (16, 17, 41), particularly with regard to enhancing endothelium-derived nitric oxide (NO) and cyclooxygenase signaling (18). It has not been reported whether estrogen improves cerebral vessel function during and after neuroinjury.

Cerebral ischemia is well known to produce early vascular abnormalities during reperfusion: hyperemia, delayed hyperperfusion, and a markedly depressed responsiveness to both endothelium-mediated vasodilators, e.g., acetylcholine (ACh) (8, 30, 35) and vasoconstrictors, e.g., serotonin (5-HT) (7, 32, 42). Alternatively, increased sensitivity to vasoconstrictors is present in some types of cerebral vascular injury (30, 32). Because estrogen improves intra- and postischemic CBF, we hypothesized that the steroid normalizes impaired vasodilation after ischemic stress. However, estrogen also modulates selected vasoconstrictor stimuli, which could shift the net balance of vascular function toward dilation under some conditions. For example, steroid withdrawal increases basilar artery sensitivity to the neurotransmitter and platelet product 5-HT (11, 15). Endothelium-dependent vasoconstriction to 5-HT is putatively mediated through tyrosine kinase activation in large cerebral arteries (27), but through non-NO mechanisms in pial vessels (13, 45).

This previous work suggests that estrogen amplifies vascular endothelial signaling under physiological conditions. However, it is not known whether the steroid can normalize vascular behavior, which is ordinarily dysfunctional after cerebral ischemia. As a first step in understanding the functional actions of estrogen within the postischemic circulation in vivo, we sought to broadly characterize pial vascular responsivity to both dilator and constrictor agonists. The purpose of this study was to determine the following: 1) whether chronic estrogen treatment in males and repletion in ovariectomized (Ovx) females protects pial vasodilation versus constriction during early reperfusion after four-vessel occlusion (4-VO), and 2) whether this protection is restricted to endothelium-dependent signaling.

METHODS

Animals. All protocols for this study were approved by the Johns Hopkins Medical Institutional Animal Care and Use Committee. Three-month-old, sexually mature Wistar rats (204–252 g body wt) were randomized into four groups (n =...
7 rats per group): placebo-implanted males, placebo-implanted Ovx females, estrogen pellet-implanted males (EMale) and estrogen-implanted Ovx females (EOvx). One investigator (Y. Watanabe), who was blinded to animal treatment status, conducted the experiments. Rats were fitted with a closed cranial window over the pia mater cortex and baseline pial arteriolar responses to ACh, the endothelium-independent NO donor S-nitroso-N-acetyl-penicillamine (SNAP), and 5-HT were determined by using video microscopy under fentanyl (25 μg·kg⁻¹·h⁻¹, intravenous infusion) and 70% NO₂-30% O₂ anesthesia. Reversable global forebrain ischemia was induced by 4-VO for 15 min. Reperfusion was initiated, and changes in vessel diameter in response to ACh, SNAP, and 5-HT were reevaluated between 30 and 60 min postischemia. All methods are as previously published or as detailed below (21, 25, 28).

Animal preparation. Female rats were ovariolectomized under halothane anesthesia, as previously described (2). All rats received subcutaneous implants of either 17β-estradiol (25 μg, 3 wk-timed release pellet; Innovative Research) or placebo pellets (in close carriers) and were randomized to treatment group at not <7 days and not >14 days of steroid administration. The implants delivered a consistent dose over 21 days (1.2 pg/day for 21 days) producing stable, reproducible plasma estrogen levels, as previously published (2, 23, 38, 48), by 7 days of implantation. The dose and duration of estradiol treatment was selected on the basis of our previous findings of efficacy in neuroprotection for male, Ovx females, and reproductively senescent Wistar rats at this same dose and duration of implantation (2, 48, 49). Treatment of males with this dose and duration of estradiol does not alter plasma testosterone level, as previously reported (49). The implant results in plasma steroid levels that are physiologically for the reproducibly active female rat, i.e., achieved 10 ± 3 pg/ml in males and 20 ± 8 pg/ml in Ovx females (2, 48, 49). Implants were inserted aseptically under 1–2% halothane anesthesia in the dorsal neck.

Global cerebral ischemia was produced by the 4-VO technique, as previously described (42, 43). On the day before ischemia, both vertebral arteries were permanently occluded and bilateral vessel occluders placed on the carotid arteries. Briefly, 1–1.5% halothane was delivered via snout mask, and the carotid arteries were then isolated via a midline neck incision. Silastic vessel ties were placed loosely around each carotid vessel, and a silk suture (1-0) was threaded through a trochar placed posterior to the trachea but above the large cervical muscles and other vessels (43). After the trochar was withdrawn, the suture ends were taped to the neck, and the carotid vessel occluders were secured. The rat was placed in the prone position, and an incision was made over the first cervical vertebrae. Both vertebral arteries at the level of the alar foramina were permanently occluded with the use of electrocautery. All wound edges were closed with wound clips, and were infiltrated with 1% bupivicaine.

Animals were allowed to recover for 24 h, and anesthesia was again induced with 1% halothane and NO₂ in enriched O₂ (70–30% mixture) via snout mask. A tracheostomy was performed, and the animal was mechanically ventilated throughout the remaining protocol. A femorotomy was cannulated for arterial blood pressure and blood gas monitoring during ischemia-reperfusion. Arterial blood gases were determined before each set of pial arteriolar diameter measurements, and mechanical ventilation adjusted as needed to assure physiological stability.

A closed cranial window was inserted over the pia mater cortex, as previously described (21, 25, 28). Briefly, the animal was placed in a stereotactic head holder and a craniotomy was performed over the parietal cortex with a cooled high-speed drill. Bone bleeding was controlled, and the site was sealed with dental acrylic, which served as a “well” for superfusion of artificial cerebrospinal fluid. The dura was excised for exposure of the pial vessels. The 70% NO₂-30% O₂ mixture was administered for the remainder of the experiment, but halothane was discontinued and intravenous fentanyl was begun (10 μg/kg loading dose, followed by a continuous infusion of 25 μg·kg⁻¹·h⁻¹). The cranial window was suffused with artificial cerebrospinal fluid at a constant physiological temperature (37°C), pH, PCO₂, and PO₂ and was sealed with dental acrylic. Inlet and outlet tubing allowed superfusion of experimental agents and control of intra-window pressure at 5 mmHg. Pial artery and arteriolar diameters were measured with the use of a Zeiss compound microscope with fiber-optic epic-illumination interfaced to a charge-coupled device camera and high-resolution monitor, a Super VHS video recorder, and a graphic printer. Arteriolar diameter measurements were obtained in each animal from a single main pial vessel with 3–5 daughter branches, which served as benchmarks within the vessel; these individual measurements were averaged and analyzed per animal as a single case. Inner diameters of vessels within individual arteriolar networks were measured with a resolution of ~2–3 μm. Absolute vessel diameter was measured off-line and expressed as percentage of the baseline diameter established before each drug superfusion.

Experimental protocol. After a 30-min recovery period, preischemic pial vessel responses were evaluated to a single concentration of three agents administered in randomized order: ACh (10 μM), SNAP (1 μM), and 5-HT (10 μM). Because the objective of the study was to test multiple aspects of vascular behavior within the same postischemic animal, full dose-response curves could not be constructed for each agent. Therefore, the optimal single infusion concentration to be used in the study was determined in preliminary experiments in which each agent was evaluated (10⁻⁴ to 10⁻¹ M) in nons ischemic animals equipped with cranial windows. Dilatation produced by ACh was 16.2 ± 1.5, 12.6 ± 0.6, and 8.3 ± 2.8% of baseline was at 10⁻⁵, 10⁻⁶, and 10⁻⁷ M, respectively. For SNAP, dilation was 18.0 ± 1.2 and 15.2 ± 1.2% of baseline at 10⁻⁶ and 10⁻⁷ M, respectively. For 5-HT, constriction was −11 ± 0.9, −9.6 ± 1.4, and −8.4 ± 1.3% of baseline at 10⁻⁵, 10⁻⁶, and 10⁻⁷ M, respectively. These doses are consistent with those employed in previous reports from our laboratory (8, 25, 28) and others (13, 14, 30, 41). The optimal infusion concentration was selected for its ability to produce maximum changes in vessel diameter. To further confirm that the selected doses for each agent produced significant responses in postischemic vessels under halothane/NO₂ anesthesia, additional preliminary experiments were conducted in a separate cohort of control animals not treated with either placebo or estrogen implants (n = 7). We observed that ACh, SNAP, and 5-HT at the selected doses produced robust responses in the preischemic period and diminished but clearly measurable changes in vessel diameter when infused post-4-VO (data not shown).

4-VO was initiated by tightening both carotid artery ties, as well as tightening of external suture ties as a means of reducing collateral blood flow from extracranial muscle. Loss of cortical blood flow was visualized through the cranial window. After 15 min of occlusion, all ties were released and CBF was reestablished. The animal were allowed to recover for 15 min, postischemic superfusions were then repeated in randomized order, and diameters were measured at 30–60 min of reperfusion.
Table 1. Physiological data pre- and postischemia

<table>
<thead>
<tr>
<th></th>
<th>Male Placebo</th>
<th>Female Placebo</th>
<th>Male Estrogen</th>
<th>Female Estrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mmHg</td>
<td>105 ± 3.5</td>
<td>104 ± 4.0</td>
<td>109 ± 3.5</td>
<td>109 ± 3.5</td>
</tr>
<tr>
<td>Postischemia</td>
<td>104 ± 3.6</td>
<td>100 ± 4.0</td>
<td>114 ± 3.2</td>
<td>104 ± 3.6</td>
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<tr>
<td>pH</td>
<td>7.47 ± 0.01</td>
<td>7.46 ± 0.01</td>
<td>7.46 ± 0.01</td>
<td>7.45 ± 0.02</td>
</tr>
<tr>
<td>Postischemia</td>
<td>7.49 ± 0.01</td>
<td>7.45 ± 0.02</td>
<td>7.48 ± 0.01</td>
<td>7.44 ± 0.03</td>
</tr>
<tr>
<td>PaCO2, mmHg</td>
<td>36 ± 1.1</td>
<td>36 ± 0.7</td>
<td>38 ± 0.8</td>
<td>36 ± 1.1</td>
</tr>
<tr>
<td>Preischemia</td>
<td>36 ± 1.4</td>
<td>36 ± 2.0</td>
<td>33 ± 0.9</td>
<td>36 ± 1.2</td>
</tr>
<tr>
<td>Postischemia</td>
<td>143 ± 8.9</td>
<td>142 ± 11.4</td>
<td>145 ± 14.8</td>
<td>148 ± 16.7</td>
</tr>
<tr>
<td>Postischemia</td>
<td>143 ± 17.2</td>
<td>146 ± 14.4</td>
<td>152 ± 16.3</td>
<td>147 ± 15.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, 7 rats per group. MAP, mean arterial pressure; PaCO2, arterial PCO2; PaO2, arterial PO2.

**Statistical analysis.** All values are reported as means ± SE unless otherwise indicated. Physiological parameters and vessel diameters were evaluated by using a two-way analysis of variance (ANOVA) or Kruskal-Wallace ANOVA on ranks in cases when the data were not normally distributed (vessels tested with SNAP and 5-HT). Post hoc comparisons were made with a Newman-Keuls test. Significance was set at $P < 0.05$.

**RESULTS**

Arterial blood gases and blood pressure and intrawindow temperature and pressure were constant throughout the study (Table 1). Intrawindow temperature was held constant at 37.8 ± 0.03°C, and intrawindow pressure was maintained at 5 mmHg. Baseline, preischemic pial vessel diameter among groups ranged from 31.8 ± 0.48 to 69.9 ± 1.8 μm. Absolute preischemic and postischemic vessel diameters are summarized in Table 2. There were no differences in the mean vessel diameters between treatment groups either before or after 4-VO. Furthermore, postischemic vessel diameters were allowed to rapidly return to preischemic values before the randomized set of agonist infusions was initiated (see Table 2).

Before ischemia, pial vessels of all treatment groups reacted normally to stimulation with ACh, SNAP, or 5-HT (Figs. 1–3). Both ACh and SNAP superfusions produced brisk vasodilation, increasing by ~18–20% of presuperfusion diameter in all animals. Superfusion of 10 μM 5-HT resulted in a small but consistent vasoconstriction, decreasing by ~8–10% of presuperfusion diameter. However, postischemic dilation to 10 μM ACh in Males (2.6 ± 1.6% of presuperfusion diameter) and Ovx females (6.3 ± 2.0% presuperfusion diameter) was strikingly depressed compared with preischemic dilation (see Fig. 1). In contrast, estrogen treatment preserved vasodilatory responses to SNAP in postischemic pial vessels. In EMales and EOvx, postischemic vessel diameter increased with SNAP (16.4 ± 1.0 and 15.8 ± 1.7% of presuperfusion values, respectively), which did not differ from preischemic vasodilation (Fig. 2).

Table 2. Baseline pial arteriolar diameter before and after ischemia

<table>
<thead>
<tr>
<th></th>
<th>Preischemic</th>
<th>Postischemic</th>
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</thead>
<tbody>
<tr>
<td>Male</td>
<td>48.0 ± 5.1</td>
<td>51.1 ± 5.9</td>
</tr>
<tr>
<td>EMale</td>
<td>42.6 ± 2.1</td>
<td>45.4 ± 3.2</td>
</tr>
<tr>
<td>Ovx</td>
<td>48.2 ± 3.6</td>
<td>50.0 ± 4.3</td>
</tr>
<tr>
<td>EOvx</td>
<td>44.3 ± 2.4</td>
<td>48.0 ± 3.3</td>
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</tbody>
</table>

All values are means ± SE; n, 7 rats per group. EMale, 17β-estradiol implanted male; Ovx, ovariectomized female; EOvx, 17β-estradiol implanted ovariectomized female. Vessel diameters among groups were measured in micrometers.
In contrast, ischemia blunted normal constriction to 10 μM 5-HT in all groups regardless of treatment. Figure 3 illustrates that unlike the beneficial effect of estrogen on arteriolar dilation to ACh and SNAP, hormone pretreatment failed to restore posts ischemic vasoconstriction to 5-HT in the male or female rat.

**DISCUSSION**

This study demonstrates two novel findings. First, chronic estrogen treatment reverses the loss of response to endothelium-mediated vasodilator, but not vasoconstrictor, agents that is ordinarily observed after ischemia in untreated animals. This indicates that physiologically relevant plasma estrogen levels in animals of either gender could enhance blood flow by restoring dilation rather than by mitigating excessive vasoconstriction. Although male sex steroids were not measured in this study, Sampei et al. (49) observed that plasma testosterone is unaffected by estrogen treatment in male rats. Thus the preservation of posts ischemic vasoreactivity by chronic estrogen availability is unlikely to have been influenced by testosterone. Second, the action of estrogen is not limited to endothelium-dependent agonists because dilation to ACh and SNAP is almost completely restored by steroid treatment. These data clearly demonstrate that estrogen is "vasoprotective" in the ischemic cerebral circulation and preserves function in vessels at high risk of reperfusion injury. This preservation of vasodilatory capacity may account for our recent observation that estrogen rapidly restores CBF after vascular occlusion to near preischemic levels, whereas hypoperfusion persists in untreated male animals (31).

Abnormal vasomotor activity has been documented in numerous models of global forebrain ischemia (5, 8, 10, 30, 47), and, to our knowledge, no agents have been shown to reverse defective posts ischemic vasodilation in vivo. Chronic estrogen exposure can achieve this end and could be therapeutically useful in restoring sensitivity to physiological dilator stimuli within recovering brain. The present study focused on specific vasomotor pathways that are known to be dysfunctional after ischemic stress and that have been shown to be potential targets for the activity of estrogen under physiological conditions. There is extensive evidence (12, 24, 34, 44) in coronary, uterine, and peripheral vessels that estrogen modulates multiple aspects of normal and atherosclerotic vessel function by targeting vascular smooth muscle cells, endothelium, and platelets. Our findings extend this concept by demonstrating that estrogen preserves NO-mediated vasodilation, but not NO-independent vasoconstriction, after a global ischemic insult. Whether the gain in posts ischemic functionality is limited to NO-guanylate cyclase signaling remains to be shown, and other vasodilatory mechanisms not tested here may be spared in the estrogen-treated vasculature. Furthermore, the protection of estrogen cannot be necessarily assured for all endothelium-dependent function, because posts ischemic vasoconstriction to a constrictive platelet product, 5-HT, was unaffected by estrogen treatment. Serotonin is a known constrictor of cerebral blood vessels, with complex mechanisms that are vessel size and site dependent, likely through endothelial generation of prostanoioids (45, 46). Preischemic pial vasoconstriction to 5-HT was modest in our preparation, as was expected from previous findings (13, 14, 19, 44, 45) in small versus large cerebral arterioles. However, a significant loss of response to 5-HT was readily demonstrated during reperfusion, and this was not reversible by estrogen treatment.

The present data do not show the specific mechanisms by which estrogen preserves posts ischemic NO vasodilation after cerebral ischemia. Estrogen receptors (ER) have been identified in endothelium and vascular smooth muscle cells of many species (for a review, see Ref. 34), including both known ER-α and ER-β subtypes (26). Furthermore, endothelial injury and denudation result in a threefold upregulation of ER-β and stable, low-level ER-α expression in vascular tunica media (29). Therefore, ER-dependent and -independent mechanisms of vascular protection may be important. Although testing the importance of ER-dependent mechanisms in vivo is challenged by the lack of ER subtype-specific pharmacological antagonists, ER-α-deficient transgenic mice demonstrate abnormal CBF responses during middle cerebral artery occlusion (49). Reports from some laboratories (18, 33, 41) suggest that estrogen enhances endothelial NO synthase expression and microvascular cGMP content (38), amplifies cGMP protein kinase signaling (50, 51), and increases enzyme activity and NO production (3, 4, 16, 17, 22, 33, 37). Estradiol also induces phosphorylation and rapid activation of endothelial NO synthase via receptor-dependent, nongenomic means that are mediated by phosphatidylinositol 3-kinase-Akt pathways (20). Given these extensive studies, one hypothesis would be that chronic estrogen treatment in the present study enhanced basal NO availability, resulting in a relative preservation of NO vasodilatory sig-

![Fig. 3. Presischemic pial artery response to stimulation with serotonin (5-HT) compared with postischemic response in rats treated with either placebo or 17β-estradiol (n = 7 per group). Data are means ± SE. *P < 0.05.](image)
naling after ischemia. However, this mechanism seems unlikely because preischemic dilation to either ACh or SNAP was not different between estrogen-deficient males and EMales or Ovx females. Although loss of vasodilation to ACh has been reported (41) in estrogen-deficient female rats, we did not observe abnormal baseline pial vasodilation to ACh (10 μM) or SNAP (1 μM) in any animal regardless of treatment status. Nevertheless, estrogen treatment resulted in preserved sensitivity to ACh and SNAP during reperfusion. This observation is consistent with recent observations that 17β-estradiol has very modest effects on basal CBF but strongly increases CBF after ischemic stress. The efficacy of chronic estrogen treatment in restoring responsiveness to SNAP during reperfusion could be through preservation of cGMP activity, cGMP phosphorylation of cGMP-dependent protein kinases (50, 51), large conductance Ca2+-dependent K⁺ (BKCa) channel function (9). For example, estrogen mimics the action of SNAP on coronary artery smooth muscle, increasing cGMP and stimulating BKCa channel opening (9).

The present model of global cerebral ischemia produced clear vascular abnormalities during early reperfusion in pial vessels, which were largely reversed by preischemic hormone implantation. The estrogen dose was selected for its previous efficacy in reducing neuronal damage after focal cerebral ischemia (2, 48, 49). Although the present findings emphasize early function recovery in estrogen-treated vessels, further studies are needed to determine whether morphological vascular damage is also averted. It is unlikely that the vasoprotection of estrogen can be explained by differences in baseline arterial tone, because preischemic vessel diameters were not different between treatment groups. Furthermore, vessel diameters returned to preischemic values within minutes of restoring CBF in all animal cohorts (see Table 2). Finally, systemic variables, which affect vascular tone such as arterial blood pressure, were quite comparable between estrogen- and placebo-treated animals.

To our knowledge, these data are the first to demonstrate that estrogen provides a gender-independent restoration of agonist-induced pial vasodilation that is ordinarily dysfunctional during early reperfusion. Estrogen-induced preservation of small vessel responsiveness to endothelium and nonendothelium-dependent dilators could have significant effects on postischemic hemodynamics and tissue recovery. Taken together, our results suggest that chronic estrogen replacement mitigates early and evolving endothelial and vascular smooth muscle vascular dysfunction associated with global cerebral ischemic injury.

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