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Effects of estrogen on postischemic pial artery reactivity to ADP

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Abstract

Objective—To determine if (1) ischemia alters pial artery responsiveness to the partially nitric oxide (NO)–dependent dilator ADP, (2) the alteration depends on 17 β -estradiol (E2), and (3) NO contributes to E2 protective effects.

Methods—Response to ADP and the non-NO-dependent dilator PGE₂ were examined through closed cranial windows. Ovariectomized (OVX) and E2-replaced (E25, 0.025 mg; or E50, 0.05 mg) rats were subjected to 15-minute forebrain ischemia and 1-hour reperfusion. Endothelial NO synthase (eNOS) expression was determined in pre- and post-ischemic isolated cortical microvessels.

Results—In OVX rats, ischemia depressed pial responses to ADP but not to PGE₂. Both doses of E2 maintained responses to ADP and had no effect on the response to PGE₂. eNOS inhibition decreased the ADP response by 60% in the E25 rats and 50% in the E50 rats but had no effect in the OVX rats. Compared to the OVX group, microvessel expression of eNOS was increased by E2, but postischemic eNOS was unchanged in both groups.

Conclusions—The nearly complete loss of postischemic dilation to ADP suggests that normal non-NO-mediated dilatory mechanisms may be acutely impaired after ischemic injury. Estrogen's protective action on ADP dilation may involve both NO- and non-NO-mediated mechanisms.

Keywords

estrogen; ischemia; reperfusion; pial artery dilation; nitric oxide synthase

Introduction

Abnormally low cerebral blood flow (CBF) accompanies stroke and cardiac arrest, with subsequent functional damage of the neurovascular unit. It is conceivable that persistently depressed cerebrovascular responsiveness might compound tissue injury. Few data exist to indicate if estrogen has a significant effect than on the microvasculature or on other structures of the neurovascular unit after cerebral ischemic injury. Cerebral vasomotor dysfunction has been associated with worsening of stroke [17], but it remains unclear if estrogen's vascular effects protect or modulate postischemic CBF, evoked release of vasoactive substances, and autoregulation capacity. Maintenance of postischemic vasomotor

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Disclosure/Conflict of Interest

The authors have no conflict of interest to declare concerning this research.

reactivity requires the integrity of vascular endothelium. Endothelial cells are essential in that they can regulate vascular tone via release of vasodilators such as nitric oxide (NO), prostacyclin (PGI₂), and endothelium-derived hyperpolarizing factors (EDHF) and vasoconstrictors such as endothelin-1 and thromboxane A₂ [9]. Estrogen may protect cerebrovascular reactivity after transient ischemia by affecting vascular endothelial and/or smooth muscle function. Earlier studies showed that estrogen's neuroprotective actions may be dose dependent, with lower doses producing a more beneficial effect following focal stroke [32]. However, few studies have investigated if estrogen's beneficial effects on the postischemic pial dilatory response are dose dependent.

In the healthy pial vasculature, topical estrogen causes estrogen receptor-dependent dilation, but only at supraphysiologic concentrations [15]. However, physiologic concentrations of estrogen can modify endothelial-dependent dilatory mechanisms that involve NO, PGI₂, and EDHF [10,22,34]. How these mechanisms are altered in the postischemic cerebrovascular bed and how estrogen modulates these mechanisms postischemia have not been well studied. Ischemia triggers vascular dysfunction and endothelial cell injury [49] and, not surprisingly, impairs responses to endothelium-dependent vasodilators [3]. Such loss of vasodilatory capacity may reflect impaired pial arteriolar ability to adjust nutrient blood flow to penetrating arterioles resulting in inability to meet local cortical neuron demands [43]. Very little is understood regarding the impact of pial artery vasomotor dysregulation after ischemic brain injury. Postischemic loss of the pial dilatory capacity may intensify neuronal injury by disruption of neurovascular coupling. There is a paucity of data regarding the effects of chronic estrogen replacement on pial arteriole reactivity after ischemic injury and whether these effects might be detrimental or protective. Our previous work demonstrates that estrogen affords some protection of postischemic vascular responsiveness [27], including endothelium, NO-dependent vasodilation to acetylcholine (ACh) [39]. However, it is unclear if chronic estrogen replacement can also partially preserve postischemic pial artery responsiveness to other dilators such as ADP.

In the healthy female rat brain the mechanisms by which ADP elicits pial artery dilation is more complex than that of ACh [41]. Recent data indicates that approximately half of the vasorelaxant properties of ADP are endothelium-independent regardless of hormone status [46]. These endothelium-independent vasodilatory effects have been attributed to ADP interaction with purinergic receptors (likely to be the P₂Y₁ receptor sub-type) located in astrocyte end feet comprising the glia limitans (GL) [46]. Furthermore, neither pial vascular smooth muscle nor neuronal nitric oxide synthase (NOS) appear to contribute to the vasorelaxant effects of ADP [46]. Recent data indicate that mechanisms of endothelium-dependent ADP-stimulated pial artery dilation differ between estrogen competent (naïve) and estrogen deficient females. [42]. In naïve rats ADP-evoked dilation entails NO production via endothelial NOS (eNOS) and possibly interaction with an endothelial P₂Y₁ receptor [46]. However, when estrogen deficiency exists ADP elicits dilation via an alternative pathway using a gap-junctional dependent process involving an endothelium-dependent hyperpolarizing factor (EDHF) [41,44,46].

The first goal of the present study was to determine if forebrain ischemia and reperfusion impair pial arteriolar dilation to the partially endothelial-dependent dilator ADP to the same extent as that seen with ACh in ovariectomized rats [39]. Responses were compared to the endothelial-independent dilator prostaglandin E₂ (PGE₂). The second aim was to determine if chronic estrogen treatment dose-dependently protects postischemic reactivity to ADP. Estrogen is known to increase expression of eNOS in cerebral microvessel [18], and our previous work suggests that estrogen protects postischemic vasodilatory responses. Therefore, the third aim was to determine if chronic estrogen replacement enhanced postischemic microvascular eNOS expression.

Materials and Methods

Animal Preparation

The Johns Hopkins Medical Institutional Animal Care and Use Committee approved all animal protocols used for this study. Sexually mature female Wistar rats (Harlan, Indianapolis, IN; body weight: 240 ± 25 g) were used in all studies. For all studies to determine the effects of estrogen on postischemic pial artery response to ADP, rats were randomly divided into three groups: ovariectomized (OVX) rats and OVX rats that received a low dose (0.025 mg—E25) or a higher dose (0.05 mg—E50) of 7 β -estradiol (E2). We used OVX and E50 rats to determine if the effects of ADP on postischemic pial arteriolar dilation were similar to the responses Ach that we previously observed. In our earlier studies we only examined the effects of E50 on postischemic pial artery dilation. However, recent literature suggests that there may be dose-dependent effect of estrogen. Therefore in these studies we added a group of animals treated with the lower E25 estrogen dose to determine if lower plasma estrogen concentrations alter the protective effect on postischemic pial artery dilation to ADP. To determine if estrogen's protective effect on postischemic pial artery response to ADP involves eNOS or cyclooxygenase metabolites, a separate cohort of E50 rats was used. In these groups we tested if eNOS and COX metabolite inhibition reverses the beneficial effects of estrogen on postischemic dilatory capacity. We selected the E50 treatment because we have previously observed a robust postischemic response to other dilators. The estradiol was administered via subcutaneously implanted 21-day, slow-release pellets (Innovative Research of America, Sarasota, FL). After ovariectomy or E2 treatment, rats were caged for a period of at least 7 days until the day of the experiment. These estrogen doses were chosen based on previous work in our laboratory showing that the slow-release pellets produce a stable range of physiologically relevant estrogen levels between 7 and 21 days after implantation [27]. Terminal blood samples were collected at the end of the experimental protocols, and plasma estrogen was measured by radioimmunoassay using commercially available kits (Coat-a-Count; DPC, Los Angeles CA).

Transient Forebrain Ischemia

The four-vessel occlusion (4-VO) model was used to induce transient global ischemia, as previously described [16,26,39]. Briefly, the day before experiment, the rat was anesthetized with halothane (4% induction; 1% maintenance). Both vertebral arteries were permanently occluded using electrocautery, and silastic vessel ties were loosely placed around the carotid arteries bilaterally. A silk suture (2-0) was threaded under the trachea, carotid arteries, and vagus nerve, but above the large cervical muscles and loosely secured at the posterior neck with an elastic bandage. The animal was allowed to recover overnight. On the following day, the rat was anesthetized with halothane, and the femoral vein and artery were cannulated with PE50 catheters for drug infusion and measurements of blood gases and mean arterial blood pressure (MABP). All rats were mechanically ventilated via a tracheostomy to maintain oxygen and carbon dioxide concentrations within normal limits. Rectal temperature was maintained at between 36.5°C and 37.5°C using warming mats and heat lamps.

Transient, incomplete cerebral ischemia was induced for 15 minutes by occluding the carotid arteries and tightening the cervical ties (to reduce collateral blood flow from extracranial sources). Reduction of CBF was confirmed by dilation of pupils and by visualization of blood flow stasis in the cranial microvessels. At end-ischemia, the ties were released and CBF was reestablished.

Measurement of Cerebral Artery Diameter

Using the cranial window technique, pial artery responses were determined via measurement of the inner diameter, as previously described [15,39]. Under halothane anesthesia, the scalp was reflected back and a craniotomy was performed over the right parietal cortex using a cooled high-speed drill. After removing the bone flap, a polypropylene ring (7-mm outer diameter) was cemented to the skull. The ring was equipped with inflow and outflow cannulae, a cannula for intrawindow pressure measurement, and a thermistor for intrawindow fluid temperature monitoring. The well formed by the ring was filled with aCSF which was pH, PCO₂, PO₂, and temperature-controlled artificial cerebrospinal fluid (aCSF). Once the dura was carefully removed to expose superficial pial vessels, the window was sealed using a glass cover slip. The intrawindow pressure was maintained at 5–8 mmHg, and temperature was controlled with a warming lamp. Upon completion of the window, halothane was discontinued, and an N₂O/O₂ mixture (70%/30%) via endotracheal tube was started. In addition, a loading dose of fentanyl (10 µg/kg) was administered intravenously followed by a continuous intravenous infusion of 25 µg/kg/hr.

Pial arteriolar responses were visualized through the cranial window using a microscope coupled to a computer video recording system. MetaMorph software (Molecular Devices Corporation, Sunnyvale, CA) was used to measure the changes in arterial diameters. For each rat, pial arteriolar responses were measured on 1–2 main pial vessels (mean-47±15 µm) and 1–3 daughter branches (mean-33±8 µm). Vessel diameters (resolution, ~2–3 µm) were expressed as the percentage of the baseline diameter prior to infusion of each drug. Because ADP-evoked vasodilatory response was similar between these parent and daughter vessels the responses were averaged for analysis.

Isolation of Cerebral Microvessel

Cerebral microvessels were isolated according to the method of Silbergeld and Ali-Osman [35]. Briefly, the animals were sacrificed with an overdose of halothane, the thoracic cavity was opened, and the heart was perfused with ice-cold saline to remove red blood cells. The brain was harvested, and the cortex was separated from the rest of the brain. Cortices from two animals were pooled, minced with a scalpel, suspended in 12 mL of ice-cold minimal essential medium (MEM, Gibco Laboratories, Grand Island, NY), and then homogenized at low speed for 40 seconds. The homogenate was centrifuged at 250 g for 10 minutes at 4°C. The supernatant was discarded; the pellet was resuspended in 25% dextran (molecular weight, 100,000–200,000 daltons; Sigma-Aldrich Company, St. Louis, MO) in MEM, and centrifuged at 2000 g for 20 minutes at 4°C. After discarding the supernatant, the pellet was resuspended in MEM and run three times through a nylon mesh sieve (60 µm). The microvessel fraction, which contained small arterioles, venules, and capillaries trapped on the mesh, was collected and stored at –80°C. A small aliquot of the microvessels was evaluated using light microscopy to confirm the purity of the preparation.

Western Blots

To determine the eNOS protein expression, the microvessel samples were thawed and suspended in an ice-cold RIPA buffer (Sigma-Aldrich) and one mini-pellet of protease inhibitors (Roche Applied Science, Germany) for every 10 mL of buffer. After homogenization, the sample was centrifuged (12,000 g; 20 minutes; 4°C) and the supernatant was collected for determination of total protein concentration using the Bradford method. For each sample, 2 µg of microvessel protein was loaded and separated on NuPAGE Novex 4%–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA). After electrophoresis separation, proteins were transferred to a nitrocellulose membrane (Invitrogen) and the membrane was incubated (20°C) with blocking buffer (5% nonfat dry milk; 0.1% Tween; 1% BSA). The membrane was then incubated overnight (4°C) with either monoclonal

mouse anti-eNOS (BD Transduction Laboratories, Franklin Lakes, NJ; 1:200 dilution in blocking buffer) or monoclonal mouse anti-actin antibodies that recognize the C-terminal of all isoforms (Sigma; 1:10,000 dilution in blocking buffer). Following overnight incubation, the membrane was rinsed with Tween-PBS for 45 minutes and incubated (20°C) for 90 minutes with anti-mouse IgG antibody conjugated to horseradish peroxidase (Amersham Biosciences, Piscataway, NJ). The membrane was rinsed with Tween-PBS for 45 minutes, incubated with electrochemiluminescence reagent (Amersham) for 60 seconds, and exposed to hyperfilm (Amersham) to visualize eNOS expression. Human endothelial cell lysate was used as positive control. Initially, we tested the purity of our microvessel preparation by comparing the expression of von Willebrand factor (an endothelial marker) in the cortical microvessel fraction to expression in the remaining cortical tissue. Expression in aorta was used as a positive control. A polyclonal rabbit anti-human Von Willebrand Factor (1:200), (Dako, Carpinteria, CA 93013, USA) was used as the primary antibody and goat anti-rabbit IgG (H+L) - HRP Conjugate (1:10,000; Bio-Rad Laboratories, Hercules, CA 94547, USA) as the secondary antibody. Actin was used as a protein loading control.

Experimental Protocols

After construction of the cranial window, the animals were allowed to recover for 30 minutes. Baseline pial artery diameters were measured as described above; either ADP (10 μ M) or PGE₂ (500 ng/mL) was slowly then infused into the cranial window and allowed to dwell for 5 minutes. We also tested responses to ACh (10 μ M) to confirm findings from our earlier work. After the 5-minute dwell time, vessel diameters were remeasured, and percent change in vessel diameter was calculated. The doses of ADP and PGE₂ were chosen based on dose-response curves (data not shown) and previous studies in our laboratory. After determination of the preischemic response, the window was rinsed for 5 minutes with aCSF, and 15-minute 4-VO ischemia was induced. The animals were allowed to reperfuse for 60 minutes, basal diameters were remeasured. After obtaining the postischemic baseline measurements, ADP or PGE₂ was infused into the window and pial artery diameters were retested in the presence of the drugs. The drugs were then washed out of the window by infusing warmed aCSF. After being assessed for postischemic response to ADP and the vessels had returned to baseline, the cranial windows in the E2-treated rats were superfused with the NOS inhibitor N^ω-nitro-L-arginine (L-NNA; 10 μ M; Sigma-Aldrich). Superfusion of this concentration of L-NNA in a cranial window inhibits NOS activity in underlying cortex and blocks pial artery dilation to ACh [29]. In preliminary studies (data not shown), we confirmed that 10 μ M L-NNA inhibited postischemic dilation to the endothelium-dependent vasodilator ACh in both OVX and estrogen-treated rats. L-NNA was infused for 5 minutes and allowed to dwell in the window for an additional 20 minutes prior to measurement of vessel diameters. Responses to ADP were repeated in the presence of L-NNA. To determine if estrogen's beneficial effect on ADP-evoked postischemic pial arteriole dilation involves cyclooxygenase metabolites, an additional cohort of E50 rats were treated with indomethacin (10 mg/kg i.v; Sigma-Aldrich) after reperfusion. As detailed previously, preischemic pial arteriole dilation to ADP was determined, ischemia was induced and rats were allowed to reperfuse. After 60 minutes reperfusion postischemic dilation to ADP was retested and the window was rinsed with aCSF. Indomethacin was infused for 20 minutes and ADP-evoked pial artery dilation retested. The window was again rinsed with aCSF and L-NNA (10 μ M) was superfused into the window as previously described. Arteriolar response to ADP was then evaluated. Others have shown this indomethacin dose effectively inhibits COX synthesis [45].

Data Analysis

All data are reported as mean $\pm$ SD. Physiologic parameters and vessel diameters were evaluated using two-way ANOVA or Kruskal-Wallis ANOVA on ranks in cases where data

were not normally distributed. All eNOS proteins were expressed as the ratio of eNOS to actin. Differences in eNOS expression between microvessel preparations from preischemic and postischemic cortex from E50-treated and OVX rats were evaluated by one-way ANOVA. A p value ≤ 0.05 was considered significant. Statistical analyses were performed using SigmaStat Statistical Software (version 3.0, SPSS, Chicago, IL).

Results

Physiologic Parameters

As expected, plasma estrogen levels were low in the OVX group (6.2 ± 1.7 pg/mL), and both E25 (19.6 ± 6.6 pg/mL) and E50 (64.7 ± 31.7 pg/mL) estradiol significantly increased plasma estrogen levels ($p < 0.003$). Rat estrogen levels have been reported to range from 5 to 20 pg/mL [36,40] and up to 57 pg/mL during pregnancy [40]; therefore, the plasma values that we achieved were within physiologically relevant ranges in rat. All physiologic variables were maintained within normal range throughout experiments (representative values in Table 1). Body temperature was kept at $37 \pm 0.2^\circ\text{C}$, intrawindow pressure was held at between 5 and 8 mmHg, and intrawindow temperature was maintained at $36 \pm 0.6^\circ\text{C}$.

Effects of Ischemia on Pial Artery Vasodilatory Response

Preischemic pial arteriole diameters ranged in size from 25 μm to 64 μm . Both preischemic and postischemic baseline diameters were similar in OVX and both the E25 and E50-treated groups (Table 2). Confirming previous work [39], ischemia was found to cause a profound depression of vasodilatory response to 10 μM of the NO and endothelium-dependent dilator ACh in OVX rats (preischemia = $27 \pm 7\%$; postischemia = $2 \pm 4\%$), whereas chronic E50 treatment partially preserved the response (preischemia = $22 \pm 6\%$; postischemia = $17 \pm 5\%$). Without ischemia, the dilator response to 10 μM ACh remained stable over a 3-hour period in a time-controlled group (1 hr = $24 \pm 1\%$; 2 hr = $25 \pm 1\%$; 3 hr = $25 \pm 1\%$).

We have tested the preischemic and postischemic ADP response in naïve rats (data not shown) and observed that the responses are essentially the same as those previously reported in our earlier studies using Ach [39]. Similar to effects of Ach, loss of postischemic ADP-evoked vascular dilatory response was also observed in the OVX rats (Figure 1). Before ischemia, dilation to ADP was similar in OVX and both estrogen-treated groups. However, postischemic pial arteriolar dilation to ADP was lost in the OVX group ($3 \pm 2\%$), compared to preischemic measurements ($26 \pm 7\%$; $p < 0.001$). Compared to preischemic ADP-evoked pial dilation, the postischemic response was diminished in both the E25 ($p = 0.08$) and the E50 group ($p = 0.003$). However, compared to the OVX group, chronic estrogen replacement with either E25 or E50 significantly improved postischemic ADP responses (3 ± 2 vs. 18 ± 5 and $21 \pm 1\%$, respectively) (Figure 1). Subsequent superfusion of L-NNA during reperfusion had no effect on basal pial arteriole diameters (data not shown). However, L-NNA attenuated the postischemic ADP response by $\sim 60\%$ in the E25 group and by 50% in the E50 group (Figure 2). Although others have shown that COX metabolites do not contribute to ADP-evoked pial artery dilation [45], but it is unclear if residual dilation after NOS inhibition may be due to postischemic synthesis of a COX metabolite. Infusion of indomethacin during reperfusion had no effect on ADP-evoked pial artery dilation in E50-treated rats, whereas superfusion of L-NNA retained its ability to reduce the response to ADP (Figure 3).

In contrast to the postischemic vasodilatory depression observed in response to ACh and ADP, postischemic pial artery responses to PGE_2 were not significantly changed from preischemic values in either the OVX or the estrogen-treated group (Figure 4). In addition, the response to PGE_2 was unaffected by topical application of L-NNA.

Effects of Estrogen on Isolated Cerebral Microvessels

It is well established that chronic estrogen treatment increases eNOS in cerebral microvessels [18], but the effect of ischemia remains unclear. Therefore, we examined the effects of ischemia alone and in combination with chronic estrogen (0.05 mg) treatment on cortical cerebral microvascular eNOS expression. We confirmed the purity of our microvessel preparation by demonstrating that Von Willebrand factor was enriched nearly sixfold in the isolated microvessel fraction compared to the whole tissue. As expected, eNOS expression in cortical microvessels was greater in E50-treated rats than in OVX rats that were not subjected to ischemia (Figure 5). Ischemia had no effect on eNOS immunoreactivity at 2 hours of reperfusion after ischemia in either the OVX or E50-treated groups. However, eNOS expression in E50-treated rats continued to remain greater than in OVX rats after ischemia.

Discussion

This study resulted in three major findings. First, transient global forebrain ischemia causes loss of pial artery vasodilation to stimulation with the partially endothelial-dependent dilator ADP during the early reperfusion period, whereas dilation to the endothelial-independent dilator PGE₂ remains unaffected in estrogen-depleted female rats. Estrogen's protective effect does not appear to be dose dependent, as treatment with E25 or with E50 improved postischemic pial sensitivity to ADP. Second, in the estrogen-treated rats, postischemic dilation to ADP, but not to PGE₂, was depressed by 50%–60% in the presence of L-NNA. A COX metabolite was not responsible for the residual ADP response with estrogen repletion. Third, prolonged estrogen treatment increases cerebral microvessel eNOS expression, and this increase is maintained for at least 2 hours of reperfusion. Our data imply that lower doses of estrogen may be equally as effective as higher estrogen doses to sustain postischemic pial artery vasodilatory capacity. In addition, ischemia may affect both the NO-dependent and the NO-independent components of ADP-induced dilation, and estrogen can moderate these deleterious effects on the cerebral microvasculature.

Postischemic vasomotor dysfunction [1,5,27,39] and loss of myogenic tone [11] in the cerebral vasculature have been previously reported and may reflect Ischemia-induced endothelial and/or vascular smooth muscle (VSM) injury. In the present study, we used two different agents—ADP (a partially endothelium/NO-dependent vasodilator) and PGE₂ (an endothelium/NO-independent vasodilator) to evaluate pial arterial vascular function after a transient global ischemic insult. In the nonischemic cerebral vasculature, pial arterial response to the NO donor S-nitrosoacetylpenicillamine (SNAP) was unaffected by estrogen, thereby suggesting no direct effect of estrogen on healthy VSM reactivity to NO [24]. In contrast, we previously showed that postischemic pial artery dilatory response to SNAP was markedly attenuated and that estrogen pretreatment partially preserved sensitivity to SNAP [39]. Others have suggested that estrogen may reduce cerebral microvascular dysfunction by preserving basal levels of cGMP [23] and by diminishing NADPH oxidase production of superoxide [20], which is known to scavenge NO. In the present study, PGE₂ was used to determine if loss of postischemic pial vasodilatory response involves VSM dysfunction not associated with a defect in NO signaling and if estrogen treatment alters the response. Our current data show that postischemic pial artery response to topical PGE₂ was unaffected by ischemia, by estrogen, or by eNOS blockade. Our observations are consistent with reports that newborn piglets subjected to transient global ischemia retain the ability to dilate pial arteries in response to topically applied PGE₂ [2]. Thus, the impaired postischemic reactivity seen with agonists targeting VSM such as SNAP, serotonin, and a thromboxane analog [28,39] cannot be generalized to all agonists acting on VSM.

ADP-induced pial artery dilation in estrogen depleted rats is insensitive to NOS inhibition and likely involves an EDHF, gap junctional conduction and astrocytic elements in the GL [46]. In OVX rats, we found nearly complete loss of postischemic dilation to ADP, suggesting that these normal non-NO-mediated dilatory mechanisms may be acutely impaired after ischemic injury. Further investigation is warranted to elucidate how ischemia produces injury to pial dilatory mechanisms.

Chronic estrogen treatment increases cerebrovascular endothelium-derived vasorelaxant mediators produced by eNOS, cyclooxygenase, and prostaglandin synthase [8,18,21,22]. The current study reveals an acute fall in postischemic vasodilatory response to the partially endothelium-dependent vasodilator ADP that is largely reversed by estrogen replacement. Loss of responsiveness to ADP following cerebral endothelial denudation or injury has been previously described [30,47]. Taken together, these earlier studies and our present data confirm that ischemic injury alters normal cerebral vascular dilatory sensitivity to ADP. To our knowledge, the present study is the first to demonstrate that postischemic ADP-induced pial artery dilation is improved in the presence of estrogen and that lower estrogen doses produce essentially the same effect as higher doses. The fact that chronic estrogen repletion clearly protects pial responsiveness to the partially NO-dependent agonist ADP and that this effect of estrogen is unaffected by COX metabolite inhibition by indomethacin, but markedly reduced in the presence of L-NNA suggests that protective actions of estrogen partially involve NO during early reperfusion. Our current studies do not fully elucidate how estrogen may affect postischemic pial artery NO production, but activation of both genomic and non-genomic pathways is possible, as both are recognized targets of estrogen [14].

In healthy pial arteries of estrogen-treated rats L-NNA depresses ADP-stimulated pial arteriolar as much as 81% [42]. In the current studies we examined only postischemic responses to ADP in the presence of L-NNA to determine the effects of estrogen status on pial dilatory capacity after ischemic brain injury. Inhibition of NOS had virtually no effect on the OVX group, presumably because eNOS expression was low and the residual postischemic dilatory response was already largely depressed. However, in estrogen-treated rats postischemic reactivity to ADP was reduced by ~50% in the presence of L-NNA. Although we cannot exclude the possibility that L-NNA was insufficient to completely block NO synthesis or that NO synthesis increases after ischemia, our data support the concept that estrogen's beneficial effect on pial artery dilatory capacity may partly involve preservation of the non-NO-dependent component of ADP-evoked dilation.

Estrogen augments NO production in nonischemic cerebral vessels [7] and increases eNOS protein expression both *in vivo* and *in vitro* [19]. However, the effect of cerebral ischemia on eNOS expression is less clear. As early as 1 hour after permanent focal ischemia, eNOS immunostaining of cerebral vessels was observed to increase in ischemic brain regions [48]. In contrast, others failed to see early eNOS upregulation but did observe heightened eNOS protein expression at 24 hours after reversible focal ischemia [38]. With global cerebral ischemia, we failed to find a significant increase in microvessel eNOS during early reperfusion. Moreover, the increased eNOS expression seen with chronic estrogen treatment was preserved during this early reperfusion period. In healthy cerebral vessels, estrogen increases COX-1, prostacyclin synthase, and PGI₂ [21], but it is unclear if this is true in the postischemic microvasculature. Our current data show that COX inhibition with indomethacin has little effect on postischemic pial dilation to ADP in estrogen-treated rats alone or in the presence of L-NNA. Therefore, it is unlikely that estrogen-mediated increase in COX metabolites is responsible for the hormone's protective effect on dilatory responsiveness to ADP. We cannot rule out the possibility that estrogen increases other arachidonic acid derivatives such as epoxyeicosatrienoic acids (EETs) after global ischemic injury. Another consideration is that our microvessel preparation contained variable amounts

of glial elements, and this effect of diluting endothelial proteins may have been different in postischemic preparations. However, our samples were routinely examined under light microscopy to confirm the purity of microvessels and we used Western blots for the vascular endothelial marker von Willebrand Factor to verify microvessel enrichment of our samples.

In the present study, we establish that estrogen amplifies eNOS expression on microvessels and this may partially explain why the vasodilatory response to ADP is normalized in estrogen-treated rats. Our data do not show the specific mechanism by which estrogen enhances microvascular eNOS. Estrogen receptors have been detected on endothelium and VSM cells of cerebral blood vessels [13,25,31,37] and may contribute to the preischemic increases in eNOS expression with E2 treatment. Moreover, estrogen can exert rapid non-genomic effects that modulate cerebral vasodilation via eNOS [14]. It is possible that estrogen acts non-transcriptionally via the phosphoinositide-3 kinase/Akt/eNOS signaling pathway to increase NOS sensitivity to calcium [6,12,37] or to inhibit caveolin-1 expression thereby increasing NO synthesis [4,33,42].

CONCLUSIONS

In conclusion, the present study showed that transient forebrain ischemia impairs postischemic vasodilatory sensitivity to ADP, possibly involving both the NO-dependent and the NO-independent components of the response. It is unlikely that loss of vasodilatory capacity resulted from nonspecific VSM injury because normal postischemic responsiveness to PGE₂ was intact. Estrogen repletion partially protects the pial arterial responsiveness to ADP and augments basal and postischemic eNOS expression in cerebral microvessels. Lower estrogen doses are as effective as higher doses in protecting pial dilatory responses to ADP. Estrogen's protective effect on ADP-evoked postischemic pial artery dilation may partly depend on NOS activity. Clear effects of estrogen on vascular recovery after ischemic brain injury have yet to be established. Results of the present study extend our earlier work showing that chronic estrogen replacement mitigates evolving postischemic vascular dysfunction to a variety of stimuli. Reestablishment of near normal dilatory capacity by estrogen may help to preserve the ability of pial arteries to supply downstream penetrating vessels and to attenuate tissue injury after ischemic brain injury.

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Figure 1.

Preischemic and postischemic pial artery response in ovariectomized (OVX; n = 7), OVX 0.025 mg estrogen-treated (E25; n = 5), and OVX 0.05 mg estrogen-treated (E50; n = 6) rats to stimulation with 10 μ M ADP, reported as percentage of baseline diameter. Data are mean $\pm$ SD; *p $\leq$ 0.001 from all preischemic responses; †p $\leq$ 0.001 from postischemic E25 and E50.

Figure 2.

Postischemic pial artery response in ovariectomized (OVX; $n = 6$), OVX estrogen-treated with 0.025 mg (E25; $n = 5$), and OVX estrogen-treated with 0.05 mg (E50; $n = 6$) rats to stimulation with 10 μ M ADP alone and in the presence of 10 μ M L-NNA, reported as percentage of baseline diameter. Data are mean $\pm$ SD; * $p \leq 0.05$ postischemic E25 vs. E25 + L-NNA; † $p \leq 0.05$ postischemic E50 vs. E50 + L-NNA.

Figure 3.

Preischemic and postischemic pial artery response to ADP in OVX estrogen-treated (0.05 mg) rats (n=5). Postischemic dilation to ADP alone (ADP), after COX inhibition with indomethacin (INDO; 10 mg/kg i.v. 20 minutes prior to testing) and in the presence of indomethacin and 10 μ M L-NNA (IND-LNNA). Data are mean $\pm$ SD; * $p \leq 0.001$ from postischemia; ** $p \leq 0.001$ from ADP and IND.

Figure 4. Preischemic and postischemic pial artery response in ovariectomized (OVX; $n = 5$) and OVX estrogen-treated (E2; $n = 5$) rats to stimulation with 500 ng/mL PGE₂ alone and in the presence of L-NNA, reported as percentage of baseline diameter. Data are mean $\pm$ SD.

Figure 5.

Western blot of eNOS protein relative to actin in isolated cerebral microvessels from preischemic and postischemic OVX and E2-treated rats. Data are mean ± SD (n = 3); *p ≤ 0.03 OVX vs. E2 preischemia; †p ≤ 0.03 OVX vs. E2 postischemia.

Table 1

Representative physiologic parameters before ischemia and at 60 minutes of reperfusion.

	Preischemia	60 min of Reperfusion
MABP (mmHg)	92 ± 7	91 ± 6
pH	7.39 ± 0.03	7.39 ± 0.03
PaO ₂ (mmHg)	127 ± 21	123 ± 15
PCO ₂ (mmHg)	38.8 ± 2.9	40.0 ± 2.7
Hemoglobin (g/dL)	10.3 ± 1.1	10.9 ± 1.3 ^a

^a p ≤ 0.05. MABP, mean arterial blood pressure; PaO₂, arterial oxygen tension; PCO₂, arterial carbon dioxide tension.

Table 2

Pial vessel diameter before ischemia and at 60 minutes of reperfusion.

Diameter (μm)	OVX (n = 8)	E25 (n = 6)	E50 (n = 6)
Preischemia	41 $\pm$ 7	39 $\pm$ 8	41 $\pm$ 4
60 min of reperfusion	47 $\pm$ 12	39 $\pm$ 8	47 $\pm$ 12

OVX, ovariectomized; E25, 0.025 mg estrogen; E50, 0.05 mg estrogen.