Postischemic Estrogen Reduces Hypoperfusion and Secondary Ischemia After Experimental Stroke

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Background and Purpose—Estrogen is a known neuroprotective and vasoprotective agent in experimental cerebral ischemia. Preischemic steroid treatment protects animals of both sexes from focal cerebral ischemia. This study determined whether intravenous estrogen acts as a vasodilator when administered on reperfusion and whether the resulting increase in cerebral blood flow (CBF) provides tissue protection from middle cerebral artery occlusion.

Methods—Adult male Wistar rats were treated with reversible middle cerebral artery occlusion (2 hours), then infused with intravenous estrogen (Premarin; 1 mg/kg) or vehicle during the first minutes of reperfusion (n=15 per group). Cortical laser-Doppler flowmetry was used to assess adequacy of occlusion. Ischemic lesion volume was determined at 22 hours after occlusion by 2,3,5-triphenyltetrazolium chloride staining and image analysis. Cortical and striatal CBF was measured by ^{14}[C]iodoantipyrine autoradiography at 10 (n=10) or 90 (n=11) minutes of reperfusion.

Results—As expected, supraphysiological plasma estrogen levels were achieved during reperfusion (estrogen, 198±45 pg/mL; vehicle, 6±5; P=0.001). Physiological variables were controlled and not different between groups. Total hemispheric infarction was reduced in estrogen-treated rats (estrogen, 49±4% of ipsilateral structure; vehicle, 33±5%; P=0.02), which was most pronounced in striatum (estrogen, 40±6% of ipsilateral striatum; vehicle, 60±3%; P=0.01). CBF recovery was strikingly increased by estrogen infusion at 10 minutes in frontal (estrogen, 102±12 mL/100 g per minute; vehicle, 45±15; P=0.01) and parietal cortex (estrogen, 74±15 mL/100 g per minute; vehicle, 22±13; P=0.028) and throughout striatum (estrogen, 87±13 mL/100 g per minute; vehicle, 25±20; P=0.02). Hemispheric volume with low CBF recovery (eg, <20 mL/100 g per minute) was smaller in estrogen-treated animals (estrogen, 73±18 mm³; vehicle, 257±46; P=0.002). However, differences in CBF recovery could not be appreciated between groups by 90 minutes of reperfusion.

Conclusions—Acute estrogen therapy during reperfusion improves tissue outcome from experimental stroke. The steroid rapidly promotes CBF recovery and reduces hemispheric no-reflow zones. This beneficial effect appears only during early reperfusion and likely complements other known mechanisms by which estrogen salvages brain from focal necrosis. (*Stroke*. 2001;32:796-802.)

Key Words: cerebral ischemia, focal ■ estrogens ■ gender ■ middle cerebral artery occlusion ■ reperfusion ■ stroke, acute ■ women

Estrogen has been widely shown to protect brain in numerous models of experimental brain injury. Preischemic steroid treatment has been well studied as a neuroprotective agent in adult animals of both sexes and in reproductively senescent, middle-aged female rats (for reviews, see 1, 2). From the perspective of cerebrovascular disease and stroke, estrogen has been primarily of interest as a postmenopausal hormone therapy that could reduce stroke incidence. The therapeutic utility of the steroid in postischemic treatment paradigms has been understudied. 17β-Estradiol administered up to 3 hours after permanent middle cerebral artery (MCA) occlusion reduces infarction in ovariectomized female rats.[3] Whether estrogen is efficacious when adminis-

tered during postischemic reperfusion and whether such protection can be observed in the male animal have not previously been determined.

Furthermore, estrogen has known vasodilatory and dose-restricted, antithrombotic properties that could be exploited to promote cerebral blood flow (CBF) recovery after transient vascular occlusion. Specifically, acute 17β-estradiol administration transiently increases CBF in rat[4] and dilates pial vessels in situ at high concentrations,[5] while chronic exposure increases endothelial nitric oxide synthase (eNOS)[6] and cGMP activity in brain microvessels.[7] While estrogen can improve intraischemic CBF in some animal models,[8–10] its potential for enhancing reperfusion CBF is unclear. Pro-

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longed cerebral ischemia produces profound vascular abnormalities during recirculation: hyperemia, delayed hypoperfusion, and markedly depressed responsiveness to endothelium-mediated vasodilators such as acetylcholine.[11,12] Chronic estrogen treatment can restore responsivity to eNOS-dependent vasodilators.[13] Furthermore, we have observed recently that estrogen therapy in male or ovariectomized rats normalizes postischemic pial vessel sensitivity to acetylcholine.[14] Therefore, we hypothesized that the postocclusion cerebral circulation could be responsive to estrogen and reduce postischemic perfusion defects in penumbral brain regions. This study determined whether intravenous estrogen acts as a cerebral vasodilator when administered on reperfusion and whether the resulting increase in CBF accounts in part for tissue protection from MCA occlusion.

Materials and Methods

This study was conducted in accordance with the National Institutes of Health guidelines for the care and use of animals in research, and protocols were approved by the Animal Care and Use Committee of Johns Hopkins University. All methods were as previously described.[9,15–17] In brief, age-matched male Wistar rats (weight, 220 to 280 g; Harlan) were subjected to 2 hours of reversible MCA occlusion followed by 22 hours of reperfusion. Each animal was anesthetized with halothane (1% to 1.3% delivered by face mask in O_2-enriched air). Rectal and temporalis muscle temperatures were monitored with a MONO-THERM system and maintained at approximately 36.5°C with a warming blanket and heat lamp during surgery and ischemia. The femoral artery was cannulated for measurement of arterial blood gases, glucose, and blood pressure before, during, and immediately after ischemia. A femoral venous catheter was placed for the injection of intravenous estrogen (Premarin; 1 mg/kg in 1 mL saline) or an equivalent volume of isotonic saline during reperfusion (n=15 per group). The rationale for treatment selection was that our previous study in male rats showed Premarin (USP) 1 mg/kg to reduce infarction when administered before MCA occlusion.[15] Premarin (USP) is a widely used compound for estrogen replacement therapy in a variety of clinical disease processes and in postmenopausal women. The compound is a pH-balanced mixture of conjugated estrogens occurring as sodium salts of water-soluble estrogen sulfates (including estrone, equilin, 17α-dihydroequilin, 17α-estradiol, equilenin, 17α-dihydroequilenin) with lactose, sodium citrate, and simethicone binders. Confirmation that a single injection resulted in prolonged elevation of plasma 17β-estradiol level was determined in a separate animal cohort (n=6), with sampling at 20 minutes, 40 minutes, and 1, 2, 4, 6, and 8 hours after injection. Values at these time points were 13.1±2.9, 6.4±0.9, 4.8±1.2, 2.4±1.4, 1.1±0.7, 0.9±4.0, and 0.26±0.2 ng/mL, respectively. Plasma 17β-estradiol was measured by radioimmunoassay as previously described.[8]

Unilateral MCA occlusion was induced by the intraluminal filament technique, as previously described.[9,15–17] Cortical laser-Doppler flowmetry (LDF) was used to confirm the adequacy of vascular occlusion in each animal. The right common carotid artery was exposed via a lateral neck incision, separated from the vagus nerve, and ligated. The external carotid artery was ligated, the occipital branch was cauterized, and the pterygopalatine artery was exposed and ligated. After baseline LDF was determined, a 4.0 nylon monofilament surgical suture with a heat-blunted tip was advanced through the common carotid artery and into the internal carotid artery until an abrupt reduction in LDF signal was observed. The filament was secured in place; LDF was recorded at 15-minute intervals throughout the 120 minutes of occlusion, during hormone infusion, and during 30 minutes of reperfusion. Removal of the intraluminal suture at the end of the ischemic period was associated with a rapid restoration of LDF signal. Estrogen or saline (n=15 per group) was given as a bolus on removal of the occluding filament. The animal recovered, and the brain was harvested after 22 hours of reperfusion.

A terminal blood sample was obtained for measurement of plasma estrogen. The brain was removed and sliced into seven 2-mm coronal sections, and infarction volume was quantified with the use of 2,3,5-triphenyltetrazolium chloride (TTC) staining, digital photography, and image analysis software, as previously described.[9,15–17]

Regional CBF (rCBF) was measured in additional nonsurvival cohorts of male Wistar rats with the use of quantitative [14C]iodoantipyrine (IAP) autoradiography under halothane anesthesia, as described previously.[9] Femoral vascular catheters and LDF monitoring were used in each animal, and the MCA was occluded as described above. Premarin (n=11) or saline (n=10) was administered on removal of the filament, and arterial blood gases were measured 5 minutes after injection. At either 10 or 90 minutes of reperfusion, 40 μCi of [14C]IAP (New England Nuclear) in 0.8 mL of isotonic saline was infused intravenously for 45 seconds. During infusion, fifteen 10-μL samples of free-flowing arterial blood from the femoral artery catheter were collected in heparin-coated sample tubes. The rat was decapitated 45 to 50 seconds after the start of infusion, and the brain was quickly removed and frozen at −50°C in 2-methylbutane on dry ice. Each brain was sectioned by cryostat into 20-μm-thick coronal sections at −20°C and thaw-mounted onto cover glasses. Sections were apposed for 1 week to film (Kodak, Bio-Max MR) with 14C standards. The concentration of [14C]IAP in blood samples was determined by liquid scintillation spectroscopy (Beckman, model 3801) after decolorization with 0.2 mL of tissue solubilizer (Soluene-350, Packard Instruments Co). Autoradiographic images representing 3 different coronal levels (+2.2, +0.2, and −1.8 mm from the bregma, 6 to 9 images each) were digitized, and rCBF was determined with the use of image analysis software (Inquiry, Loats). Rates of rCBF were calculated as previously described.[9,17] Two methods of analysis were used to determine rCBF. First, CBF was measured by sampling 0.1-mm² squares within gray matter of regions most vulnerable to MCA occlusion, the parietal and frontal cerebral cortex and lateral and medial striatum. Flow rates were averaged within 6 to 9 consecutive brain slices from each of 3 coronal levels. In the second method, areas perfused with predetermined intervals of CBF were isolated by digital image scanning and summed to construct a histogram distribution of brain tissue over incremental ranges of blood flow rates. Areas were averaged among 3 images from each of 3 coronal levels (+2.2, +0.2, −1.8 mm from bregma) and then were numerically integrated to obtain an estimate of tissue volume for each CBF interval.

All values reported are mean±SEM. All repeated-measures data, including physiological variables, LDF during MCA occlusion and early reperfusion, and rCBF measurements, were analyzed by 2-way ANOVA. If significant differences were found, then a post hoc Newman-Keuls test was used to detect the source of the difference. Infarction volume was analyzed by unpaired t test, comparing estrogen- and saline-treated groups. The criterion for statistical significance was set at P<0.05.

Results

Baseline arterial blood pressure, blood gases, hemoglobin, and glucose concentrations were equivalent between both groups and were maintained at near-baseline values throughout the experimental protocol (Table). Infusion of estrogen did not alter arterial blood pressure. Plasma estrogen as measured at 22 hours was higher in estrogen- versus vehicle-treated males (198±45 versus 6±5 pg/mL; P=0.001). Estrogen treatment reduced total infarction (49±4% of ipsilateral hemisphere versus 33±5%; P=0.015) and striatal infarction (40±6% of ipsilateral striatum versus 60±3%; P=0.010) relative to the control cohort (Figure 1). Cortical infarction was not statistically different in vehicle- (38±7% of ipsilateral cortex) versus estrogen-treated animals (25±7%; P=0.22). Intraischemic LDF was sharply reduced in all animals regardless of treatment group during ischemia, sug-

Physiological Variables During MCA Occlusion

Group	pH	Paco$_2$, mm Hg	Pao$_2$, mm Hg	Hg, g/dL	Glu, mg/dL	MAP, mm Hg	Temperature, °C	End-Occlusion LDF, % of Baseline Signal	Rep LDF, % of Baseline Signal
Saline	7.39±0.04	42±2	154±6	12.1±1.3	109±8	92±5	36.4±0.1	28±0.1	43±5
Estrogen	7.40±0.05	42±3	151±6	12.2±1.4	103±6	92±5	36.5±0.2	28±0.1	66±7

Values are mean±SEM. Groups are as follows: estrogen (Premarin 1 mg/kg; n=15) and equivolumetric saline (n=15). Hg indicates arterial hemoglobin concentration; Glu, arterial plasma glucose concentration; MAP, mean arterial blood pressure; and Rep LDF, LDF at 5 minutes of reperfusion with estrogen infusion in progress.

gesting adequacy of occlusion (Table). Early recovery of LDF signal also occurred in all animals by minutes of occlusion release. There were no statistical differences between treatment groups in LDF at any time point during ischemia or at 5, 15, and 30 minutes of reperfusion.

Early recovery of CBF at 10 minutes of reperfusion was evaluated in separate cohorts of saline- (n=5) or estrogen-treated (n=5) animals. Contralateral, nonischemic CBF was not different between estrogen and vehicle groups in any area of the MCA territory: frontal cortex (150±6 versus 151±11 mL/100 g per minute); parietal cortex (145±7 versus 139±7 mL/100 g per minute); lateral striatum (150±7 versus 146±9 mL/100 g per minute); or medial striatum (140±6 versus 136±11 mL/100 g per minute). However, recovery of rCBF in ipsilateral cortical and striatal regions was strikingly improved at 10 minutes of reperfusion with estrogen treatment (Figure 2). Figure 3 shows absolute rCBF in these brain regions, as well as average CBF within the contralateral hemisphere. To further examine differences in CBF recovery in these animals, we quantified total hemispheric brain tissue volume with near-zero CBF (likely areas of continued ischemia or no-reflow) and tissue volumes experiencing some degree of blood flow restoration (Figure 4). In estrogen-treated brain, low-flow regions were small (P=0.002 versus vehicle), and volumes with high CBF recovery were substantial (P=0.010 versus vehicle). We monitored LDF during occlusion and reperfusion up to within 5 minutes of IAP injection and decapitation to

ensure uniformity of insult. Although cortical CBF at 10 minutes was clearly higher as measured by IAP in estrogen-treated animals (Figure 3), we did not observe differences in either intraischemic LDF (26±4% of baseline signal in vehicle versus 26±3% in estrogen group). LDF before IAP injection was not different between groups (78±7% of baseline signal in vehicle versus 69±4% in estrogen group).

To determine whether the enhancement of CBF seen in estrogen-treated animals persisted over time, recovery of CBF was measured at 90 minutes of reperfusion in another animal cohort. There was no further enhancement of CBF in any brain area by estrogen relative to saline treatment at 90 minutes (Figure 3). Furthermore, volumetric analysis indicated that brain volume with extremely low blood flow (0 to 20 mL/100 g per minute) was resolving in both treatment groups (71±34 mm^3 in vehicle versus 66±29 mm^3 in estrogen group). The tissue volume distribution over blood flow increments was not different between estrogen and vehicle groups (Figure 4). Again, there were no differences between groups in intraischemic LDF (21±4% of baseline signal in vehicle versus 27±2% in estrogen group) or LDF assessed approximately 5 minutes before IAP injection/decapitation (82±30% of baseline signal in vehicle versus 64±19% in estrogen group).

Discussion

There are 3 major findings of this study. First, acute estrogen is a potent cerebral vasodilator of the postischemic cerebral circulation, reducing tissue volumes of early no-reflow and increasing absolute CBF in both cortex and striatum. The treatment does not increase blood flow to contralateral, nonischemic brain, suggesting that its vasodilatory properties are specific to areas of potentially compromised perfusion. Second, the blood flow–promoting effect of estrogen appears to be effective only during early reperfusion, since saline-treated animals sustain equivalent CBF recovery by 90 minutes. Therefore, the utility of estrogen as a vasodilator may be time limited. Third, estrogen treatment to supraphysiological plasma levels reduces total infarction size when given during reperfusion. With this estrogen preparation and animal model, regional infarction reduction was most robust in striatum. These findings provide evidence for the efficacy of estrogen in the male brain when administered during reperfusion after reversible focal ischemia. Taken together, the data suggest that acute estrogen strongly improves early CBF recovery and may have an impact on early reperfusion injury. While this mechanism occurs early in injury evolution and is unlikely to fully account for the neuroprotection of the
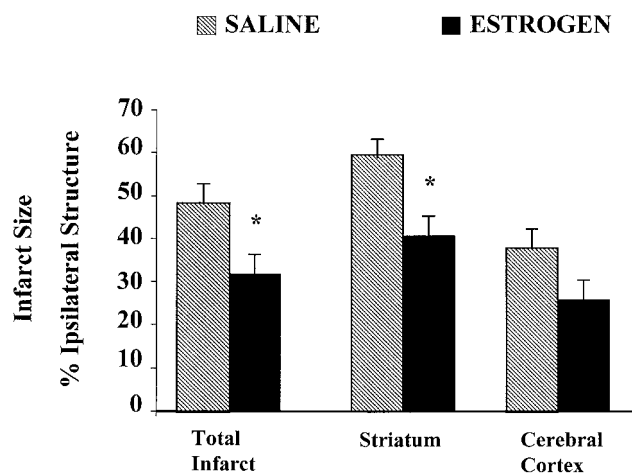
Figure 1. Effect of estrogen (1 mg/kg Premarin; n=15) vs equivolumetric saline (n=15) given at reperfusion on total, striatal, and cortical infarct size as measured by TTC histology at 22 hours. *P<0.05.
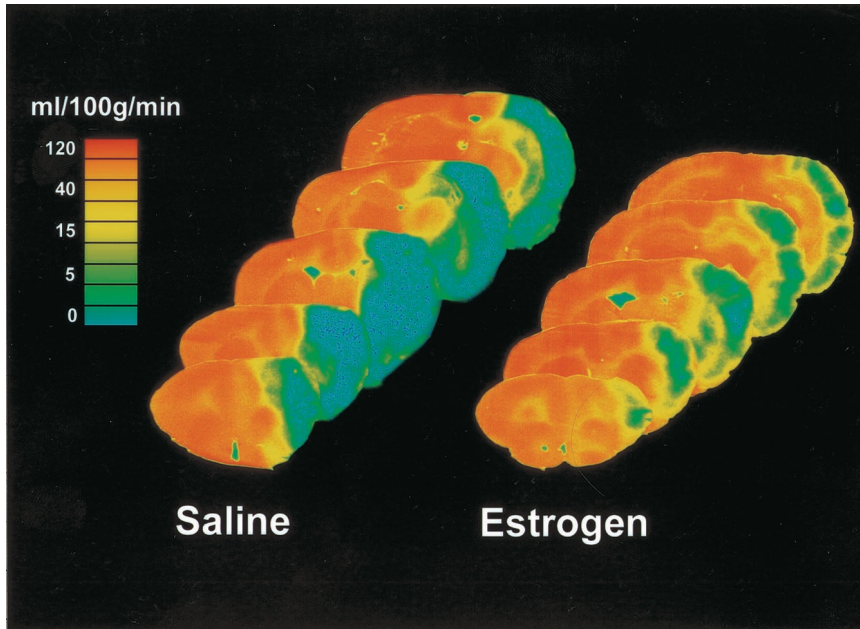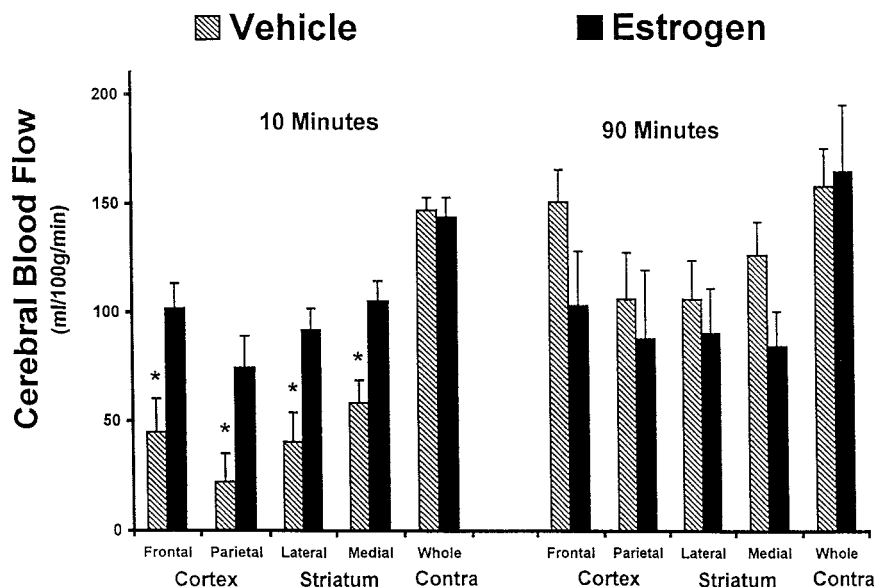
Figure 2. Effect of estrogen (1 mg/kg IV Premarin) vs equivolumetric saline on recovery of CBF as measured by [^{14}C]IAP autoradiography at 10 minutes of reperfusion after MCA occlusion.

steroid, estrogen may be useful in clinical therapy as a vasodilator that restores immediate postocclusion microcirculatory flow.

The present histological findings are consistent with numerous studies that demonstrate that brain injury after experimental stroke is sex-specific and linked to reproductive hormone status.[1,2] Female animals with endogenous sex steroids enjoy substantial neuroprotection when confronted with an ischemic episode, and this protection is lost during reproductive senescence.[17] Estrogen treatment at physiologically relevant concentrations clearly reduces infarction size after global or focal cerebral ischemia in rodents of both sexes and in reproductively senescent female rats (for reviews, see 1, 2). The therapeutic window for the protection of estrogen in irreversible MCA occlusion has recently been shown to be approximately 3 hours in the young adult, ovariectomized

female rat.[3] Our study extends these observations and is the first to show that estrogen is effective (1) in reducing total infarction when administered during reperfusion after reversible vascular occlusion and (2) in the adult male rat with intact male sex steroids. The latter point is consistent with our previous finding that preischemic estrogen therapy is equally efficacious in intact or gonadectomized male animals.[15] Further studies are needed to demonstrate the utility of estrogen in aged males or to determine whether there are interactions between native testosterone and estrogen therapy delivered during reperfusion.

The neuroprotection of estrogen is likely the result of several complementary mechanisms called into play throughout brain injury evolution. We hypothesized that the vascular properties of estrogen could provide a first temporal line of defense by enhancing CBF recovery or accelerating the rate

Figure 3. Effect of estrogen (1 mg/kg IV Premarin) vs equivolumetric saline on CBF in 4 brain regions within the ischemic MCA territory at 10 (n=5 for each treatment group) or 90 (n=5, vehicle group; n=6, estrogen-treated group) minutes of reperfusion. Contralateral tissue CBF is shown as averaged hemispheric blood flow. *$P<0.05$.
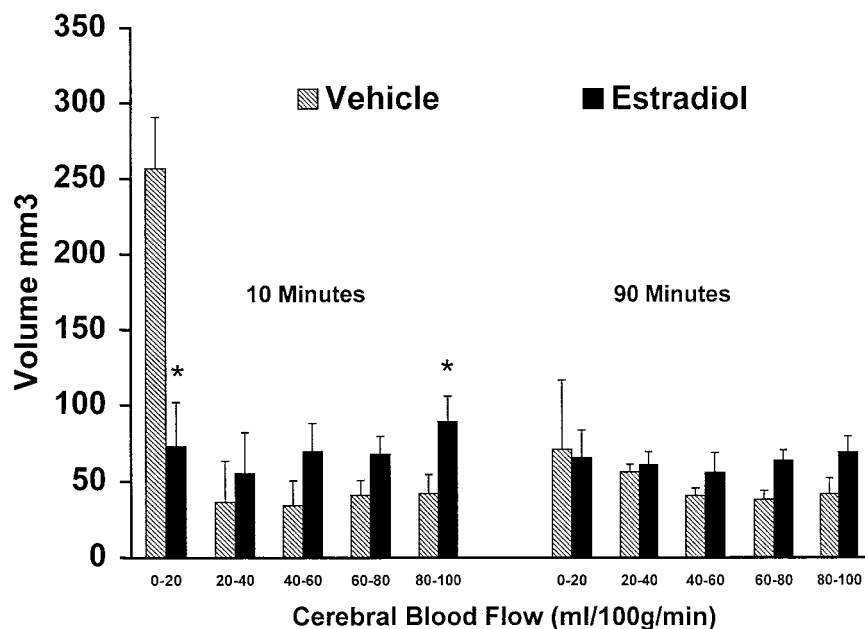
Figure 4. Effect of estrogen (1 mg/kg IV Premarin) vs equivolumetric saline on tissue volume distribution when partitioned into CBF increments at both 10 (n=5, vehicle group; n=6, estrogen-treated group) and 90 (n=5, vehicle group; n=6, estrogen-treated group) minutes of reperfusion. *P<0.05.

of CBF restoration after vascular occlusion. Although estrogen is a known vasodilator of peripheral blood vessels,[18–21] it appears to be a modest dilator of the cerebral vasculature under normal conditions. For example, ovariectomy does not alter baseline CBF in young female rats,[22] and acute estrogen infusion either transiently increases (ie, 10-minute duration) or fails to increase CBF in rat,[4] rabbit,[5] or sheep.[23] High concentrations of 17β-estradiol are required to dilate pial vessels in situ.[5] Therefore, it appears that estrogen is at best a modest cerebral vasodilator in the intact, nonischemic cerebral vasculature. Our observation in the present study that postischemic estrogen did not increase CBF in the contralateral, nonischemic hemisphere is consistent with these previous reports.

In contrast, estrogen is thought to act as a significant cerebral vasodilator and to protect vascular integrity under pathological conditions such as atherosclerosis, cerebral ischemia, and head injury. The present study findings support this hypothesis. Several studies show that endogenous and exogenous estrogens preserve intraischemic CBF in global[8,10] or focal cerebral ischemia,[9] protect cerebral vasodilatory capacity,[10] and reduce postischemic hyperemia.[8] Although CBF was not quantified, 2 studies report that estrogen improves cortical LDF when measured 1 hour after reversible MCA occlusion[24] or when measured daily at 1 and 2 days after permanent MCA occlusion.[3] However, CBF-preserving effects during vascular occlusion have not been universally reported with exogenous steroid treatment,[16] and few studies have quantified absolute CBF during ischemia. In spontaneously hypertensive, stroke-prone rats, Carswell et al[25] demonstrated smaller infarcts in proestrus (high estrogen state) than in metestrus (low estrogen state). However, there were no associated differences in intraischemic CBF, as measured by [^{14}C]IAP.[25] In other ischemic models such as global forebrain ischemia, vigorous neuroprotection has been demonstrated in estrogen-treated versus ovariectomized rats, even

when intraischemic CBF reduction was controlled to allow comparable blood flow between treatment groups.[26]

Data addressing estrogen during reperfusion are limited. Improved perfusion has been measured by LDF in female versus male animals after closed-head injury, an effect that is partially eliminated by ovariectomy.[2] We now show that intravenous estrogen results in significant vasodilation in all postischemic brain areas examined at 10 minutes of reperfusion. We compared tissue volume with rCBF <20 mL/100 g per minute between treatment groups, with the rationale that this volume of tissue experiences continued ischemia and/or no-reflow over 10 minutes and could therefore influence eventual infarction size. Estrogen clearly reduced this component of injury and accordingly increased the amount of tissue with restored CBF. This outcome could be mediated through any of several recently described interactions between estrogen and vasodilatory effectors, eg, eNOS and microvascular cGMP,[6,7] prostacyclin,[27] and large-conductance, calcium- and voltage-activated vascular smooth muscle K$^+$ channels.[28–30]

By 90 minutes of reperfusion, CBF recovery was essentially static in estrogen-treated males but had greatly progressed in saline vehicle–treated animals so that cortical and striatal blood flow levels were no longer different between groups. Comparison of absolute CBF in the MCA territory between groups (Figure 3) suggests that the pattern of CBF restoration was different in estrogen- versus vehicle-treated animals. With estrogen, CBF responded promptly at 10 minutes and with little further change by 90 minutes. With saline, there was little recovery at 10 minutes, with the bulk of CBF restoration evident by 90 minutes. Although the initial rate of CBF recovery may have been different, blood flow recovery then equalized among animals within the second window of our discrete observations. Whether the level of CBF recovery observed at 90 minutes is maximal for this model of occlusion and reperfusion remains to be shown. Furthermore, the results cannot exclude that estrogen may

again exert vasodilatory action later in reperfusion, should secondary hypoperfusion occur.[31] However, this small [^{14}C]IAP time course emphasizes that the beneficial effect of estrogen on CBF is nearly immediately operant during early reperfusion and likely complements other antioxidant[24,32–37] and anti-inflammatory mechanisms[38,39] by which estrogen salvages brain from focal necrosis. Other possible nonvascular effects of estrogen include enhancement of the antiapoptotic protein bcl-2,[40–42] reduced glutamate toxicity,[43–45] increased mitogen-activated protein kinase activation,[46] and increased growth factor signaling.[47,48]

Steroid-induced CBF recovery throughout the ischemic hemisphere did not translate into smaller infarction volumes in all brain regions within the MCA territory. While total hemispheric infarction was reduced by estrogen treatment, the bulk of the protection occurred in striatum rather than cortex. We have previously observed that striatum, rather than cortex, is particularly responsive to estrogen-induced flow preservation. This may be due to the architecture of striatal blood supply, which is predominately end-arterial rather than rich with collateral vessels, as is present in cortex.[49] If estrogen shortens the period of postischemic hypoperfusion, then striatal tissue may benefit most greatly. Lastly, only a single estrogen dose and pharmaceutical preparation was tested in these experiments. Our rationale for dose selection was based on previous efficacy with the same treatment when given to male rats immediately before MCA occlusion.[15] In the present study the 1-mg/kg dose resulted in large plasma 17β-estradiol concentrations that persisted throughout the 22-hour reperfusion period, and it is therefore unlikely that insufficient steroid was available to promote CBF over time. However, the lack of full dose-response testing clearly limits our understanding of the most efficacious hormone therapy for poststroke estrogen treatment, and further studies will be needed to optimize both dose and pharmaceutical composition.

In conclusion, estrogen is neuroprotective in male animals even when administered after prolonged ischemia. The steroid promotes CBF recovery almost immediately on administration and reduces hemispheric no-reflow zones throughout the ischemic hemisphere. This beneficial effect appears only during early reperfusion and may be primarily targeted toward brain regions with restricted vascular collateralization such as the striatum. While the blood flow–enhancing, or flow-preserving, mechanism is unlikely to fully account for the neuroprotection of the steroid, estrogen may be useful in clinical stroke therapy as a vasodilator that restores postocclusion microcirculatory flow.

Acknowledgments

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