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Effects of Estrogen on Platelet Reactivity After Transient Forebrain Ischemia in Rats

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Abstract

Estrogen's prothrombotic effects are of increasing concern, particularly in stroke risk and recovery. Using an ischemic rodent model, the authors sought to determine (a) if estrogen replacement increases post-ischemic platelet reactivity, (b) if changes in estrogen status alter intraplatelet endothelial nitric oxide synthase (eNOS) synthesis, and (c) if estrogen-mediated effects on platelets alter cerebral blood flow during reperfusion. Intact (I), ovariectomized (OVX), and OVX + 17 β -estradiol (E50) rats were subjected to 30 min of forebrain ischemia and 60 min of reperfusion. Using the platelet activation marker P-selectin, postischemic platelet reactivity was quantified by flow cytometry. In a separate cohort (I, OVX, E50), the authors quantified platelet eNOS by Western blot. Another cohort (OVX, E50) was subjected to ischemia/reperfusion, and cerebral blood flow was determined using the iodoantipyrine technique. Collagen-stimulated platelet P-selectin expression was increased in the OVX rats at 60 min of reperfusion, and this effect was reversed by estrogen treatment. No differences in platelet eNOS expression were detected among groups. Cerebral blood flow at 60 min reperfusion was comparable between the OVX and the E50 rats. The authors conclude that during reperfusion, estrogen deficiency increases postischemic platelet sensitivity to stimuli in estrogen-deficient rats. Estrogen treatment mitigates effects of estrogen loss on platelets, but this early effect is apparently not caused by intraplatelet eNOS depression. Neither estrogen deficiency nor estrogen treatment changes early postischemic regional brain blood flow. In this rodent global cerebral ischemic model, physiologic doses of estrogen are not deleterious to platelet reactivity and may initially reduce postischemic platelet reactivity.

Keywords

estrogen; hormones; platelets; P-selectin; forebrain ischemia; eNOS

Platelet accumulation in brain vasculature following ischemic cerebral injury may contribute to lingering perfusion abnormalities and impaired vascular reactivity once circulation is reestablished (Abumiya et al., 2000; Grau et al., 1998; Littleton-Kearney, Hurn, Kickler, & Traystman, 1998). Physiologic stimulants, such as altered local production of endothelial antiaggregants, expression of endothelial adhesion molecules (Rosenblum, 1997), and shear

stress can contribute to adhesion/activation of platelets to precipitate shape change and surface expression of the adhesive glycoprotein P-selectin. In patients, circulating platelet aggregates (Dougherty, Levy, & Weksler, 1977) and platelet leukocyte complexes (McCabe et al., 2004) increase after transient ischemic attacks or stroke, as does the platelet activation marker P-selectin (Grau et al., 1998; Love & Barber, 2001; Marquardt et al., 2002). The effects of estrogen on platelet biology are understudied, but some investigators suggest that estrogen replacement therapy may augment platelet sensitivity to endogenous aggregants following stroke or ischemic injury. Although estrogen preserves cerebral blood flow (CBF) during global (Pelligrino, Santizo, Baughman, & Wang, 1998) and focal ischemia (Alkayed, Murphy, Traystman, & Hurn, 2000) in rodent models, recent data from the Women's Health Initiative (WHI) study suggest that estrogen repletion after the onset of menopause heightens the risk for thrombosis and stroke (Anderson et al., 2004; Rossouw et al., 2002; Wassertheil-Smoller et al., 2003). Consequently, the prothrombotic effects of estrogen are of increasing concern, particularly in stroke risk and recovery.

Conflicting data exist regarding the effect of estrogen replacement therapy on the platelet biology of postmenopausal women. Hormone replacement therapy (HRT) reportedly depresses thrombin-stimulated aggregation (Aune, Oian, Omsjo, & Osterud, 1995; Nakano, Oshima, Matsuura, Kajiyama, & Kambe, 1998), platelet P-selectin expression (Nakano et al., 1998), and calcium influx (Nakano et al., 1998; Raman, Standley, Rajkumar, Ram, & Sowers, 1995). In contrast, others observe no effect of estrogen on platelet aggregation (Teede, McGrath, Turner, & Majewski, 2001), whereas still another study reveals a 22% increase in platelet P-selectin expression 12 weeks after initiation of HRT (Thijs et al., 2002). Furthermore, some data suggest the existence of an inverse relationship between plasma estrogen levels and P-selectin expression (Jilma et al., 1996; Lip, Blann, Jones, & Beevers, 1997). Platelet-derived nitric oxide (NO) suppresses aggregation (Radomski, Palmer, & Moncada, 1987), and estrogen may alter platelet NO synthase (isoform of endothelial NOS; eNOS) expression in a manner detrimental to recovery from cerebral ischemic injury.

The effect of estrogen on platelets is likely multifactorial, and it is unclear if estrogen status modulates platelet sensitivity during postischemic reperfusion or if the mechanism involves estrogen-induced modification of platelet eNOS. As a first step in clarifying the effects of estrogen on platelet function following cerebral ischemia, we sought to determine (a) if estrogen deficiency or estrogen replacement alters postischemic platelet biology, (b) if estrogen deficiency over time alters platelet eNOS expression, and (c) if estrogen replacement has an effect on regional CBF during early reperfusion. We hypothesized that (a) estrogen deficiency heightens postischemic platelet reactivity following transient global ischemia, and estrogen replacement decreases platelet reactivity during postischemic resuscitation; (b) estrogen deficiency results in depression of platelet eNOS expression; and (c) estrogen replacement improves regional CBF during early postischemic resuscitation.

Methods

Animals

All protocols for this study were approved by the Johns Hopkins Medical Institutions Institutional Animal Care and Use Committee. A total of 49 sexually mature (2–3 months old, 225–270 g) female Wistar rats (Harlan, Indianapolis, IN) were randomized into the following groups: intact (I), ovariectomized (OVX), and estrogen-treated (E50). All animals were maintained on a 12-hr day/night cycle in the university vivarium with free access to standard rat chow and water. Group size was set to obtain a power of at least 0.50.

Animal Preparation

Fourteen to 16 days prior to the study, all rats were subjected to either sham surgery (I) or ovariectomy (OVX, E50) under halothane anesthesia as previously described (Alkayed et al., 2000). The rodent/platelet turnover rate is approximately 3 to 5 days (Bannerman, 1983). Therefore, to ensure adequate time for new platelet formation under estrogen-deficient conditions, a 50- μ g 17 β -estradiol pellet (21-day, timed-release pellet; Innovative Research Labs, Seattle, WA) was surgically implanted in the dorsal neck of all estrogen-treated groups 7 days after ovariectomy and 7 to 8 days prior to study. All studies were conducted at least 2 weeks after ovariectomy. This method produces consistent, reproducible plasma estrogen levels over 21 days (1.2 μ g/day) and, in our hands, has been shown to reduce cerebral infarction size (Alkayed et al., 2000; Hurn, Littleton-Kearney, Kirsch, Dharmarajan, & Traystman, 1995; Palmon et al., 1998). Like humans, rats have cyclical elevation in plasma estrogen over a 5-day cycle, giving rise to wide variation in circulating hormone levels. In the E50 group, our goal was to raise plasma estrogen to levels within the normal range for sexually mature rats. Plasma estrogen levels achieved with the implant are within physiologically relevant ranges for intact, sexually mature rats (3.0 ± 0.6 to 88 ± 2 pg/mL; Butcher, Collins, & Fugo, 1974; Hurn & Macrae, 2000; Rusa et al., 1999). Rats in the I and OVX groups were implanted with a placebo nitrocellulose pellet (Innovative Research Labs) 7 to 8 days prior to the study.

Ischemia Protocol

A total of 33 rats was used for the ischemic protocol studies—platelet P-selectin and [14 C]IAP ([14 C] iodoantipyrine) autoradiography. Carotid arteries were isolated and vertebral arteries were coagulated under light anesthesia, and the animals were recovered overnight. The next day, anesthesia was induced using a mixture of halothane (3%) enriched with oxygen. The trachea was intubated (16-gauge intercath), and animals were mechanically ventilated (halothane 1%–1.5%) throughout the protocol. A femoral arterial catheter was inserted to monitor blood pressure and to evaluate blood gases during ischemia/reperfusion. Over the right parietal skull, the bone was exposed and thinned (6 mm from sagittal suture and 2 mm distal from bregma) for laser-Doppler flow probe placement. Finally, the right external jugular vein was cannulated with the catheter oriented cephalically to obtain samples for platelet counts and flow cytometry. Cerebral ischemia was induced by 4-vessel occlusion for 30 min, as previously described (Harukuni, Hurn, & Crain, 2001; Pulsinelli & Brierley, 1979; Watanabe, Littleton-Kearney, Traystman, & Hurn, 2001). Reduction in cerebral perfusion was ensured by reduction in cortical laser-Doppler flow to 45% or less of the preischemic values. At the end of 30 min, animals were allowed to reperfuse spontaneously for 60 min. Whole-blood samples for platelet counts and flow cytometry were collected from the jugular vein immediately prior to ischemia (baseline) and at 10, 30, and 60 min of reperfusion.

Flow Cytometry Studies

For these studies, 24 rats ($n = 8$ /group) were randomly assigned to the I, OVX, or E50 groups. Samples for analysis of platelet surface expression of platelet P-selectin (CD 62P) were obtained from free-flowing blood from the jugular catheter, collected into ethylenediaminetetraacetic acid (EDTA) tubes, and immediately diluted with ice-cold phosphate-buffered saline. CD 61, a platelet membrane specific marker for the GP IIIa subunit located only on platelet surfaces, was used to separate out platelets from other cells in whole blood, and CD 62P was used as a marker of platelet activation. A 5- μ L sample was incubated with the strong platelet aggregant collagen (15 min; 20 $^{\circ}$ C; final concentration, 2.5 μ g) to evaluate platelet sensitivity to stimulation. To determine unstimulated platelet P-selectin, 50 μ L of blood was immediately fixed with cold paraformaldehyde (1.0%; final concentration) in EDTA. Both stimulated and unstimulated cells were added to HEPES-buffered saline (1:50, final dilution), containing fluorescein isothiocyanate (FITC)–conjugated mouse antirat

monoclonal CD 61 (IgG1κ; final concentration, 0.05 μg; Research Diagnostics, Inc., Flanders, NJ) and R-phycoerythrin-conjugated rabbit antihuman polyclonal CD 62P (final concentration, 0.1 μg; BD PharMingen, San Jose, CA), and they were incubated in the dark for 20 min. Analysis gates were set based on the fluorescence intensity of the CD 61-FITC-positive platelets (gated dot plot of CD61 vs. forward scatter) and the percentage of CD62P-positive cells (gated histogram of CD62 vs. forward scatter). The mean fluorescence intensity of platelets positive for CD 62P was evaluated using flow cytometry (FACScan, Becton, Dickinson, and Co., Franklin Lakes, NJ) within 4 hr of the completion of the experiment. Increasing fluorescence intensity was used as an indicator of platelet activation, as only activated platelets express P-selectin on the platelet surface. Platelet fluorescence was quantified relative to molecules of equivalent soluble fluorochrome (MESF) using commercially supplied calibration standards (Bangs Laboratories, Fishers, IN). The calibration standard values were used to generate a standard curve. Fluorescence of platelets positive for CD 62P at each time point (baseline, 20, 30, and 60 min reperfusion) was then interpolated from this standard curve. Data were analyzed using CELLQuest software (Becton, Dickinson). Because significant variation exists among individual rats, data were normalized to baseline values. Therefore, platelet surface P-selectin was expressed as a ratio of the preischemic MESF to MESF at 10, 30, and 60 min of reperfusion. Platelet counts were determined using a Coulter automated cell counter (Beckman Coulter, Irvine, CA) and were normalized to baseline.

Quantitative [¹⁴C] Iodoantipyrine ([¹⁴C]IAP) Autoradiography

A total of 16 rats were used for these studies. CBF was examined in the OVX ($n = 4$) and the E50 ($n = 5$) groups. Regional CBF was determined as previously described (Alkayed et al., 1998; Murphy, Littleton-Kearney, & Hurn, 2002). Briefly, at 60 min of reperfusion, 40 μCi of [¹⁴C]IAP (New England Nuclear, Boston, MA) in 0.8 mL of isotonic saline was infused intravenously over 45 s. Free-flowing blood samples (20 μL) were collected into EDTA tubes at 3-s intervals, and the rats were decapitated 48 to 52 min after the start of infusion. Brains were quickly removed and frozen in 2 methylbutane on dry ice at -50°C . Each brain was cryostat-sectioned in 20-μm-thick coronal sections at -20°C and thaw-mounted onto cover glasses. Sections were opposed to film (Bio-Mace MR, Eastman Kodak, Rochester, NY) for 1 week with [¹⁴C]IAP. Decolorized blood samples were subjected to liquid scintillation spectroscopy to determine the concentration of [¹⁴C]IAP. Autoradiographic images representing different coronal levels were digitized, and regional CBF was determined using image analysis software (Inquiry, Loats Associates, Westminster, MD). The Kety-Schmidt modification of the Fick principle was used to calculate CBF, as previously described (Alkayed et al., 1998). Regional CBF was averaged within three consecutive brain slices at two coronal levels, +0.2 and -1.8 mm from bregma, to give an estimate of CBF in the cortex, striatum, and hippocampus.

Effect of Estrogen Status on Platelet eNOS Protein Expression

In a separate set of experiments, 16 rats were randomized into I ($n = 5$), OVX ($n = 6$), and E50 ($n = 5$) groups. Animals were anesthetized with 3% halothane, and blood for platelet isolation was collected via open-chest cardiac puncture. Blood was collected in 3.8% trisodium citrate (w/v), centrifuged at 1800 RPM (3 min, 20°C), and the top layer of platelet-rich plasma was removed. The platelet purity of the samples was examined using an automated cell counter (Beckman Coulter) to ensure less than 0.1% contamination with leukocytes and red blood cells. A platelet pellet was formed by centrifugation ($3000\text{ RPM} \times 5\text{ min}$; 20°C), the supernatant was discarded, and the pellet was gently washed twice with calcium-free Tyrodes washing buffer (163.8 mM NaCl; 2.7 mM KCl; 11.9 mM NaHCO_3 ; 0.4 mM NaH_2PO_4 ; 1 mM MgCl₂; 5.6 mM glucose; BSA 0.35%; pH 6.3; Rucinski & Niewiarowski, 1989). Platelets were solubilized in lysis buffer (50 mM Tris-HCl/0.1M NaCl/1% TritonX-100; pH 6.0) containing a mixture of protease inhibitors and centrifuged ($10,000\text{ RPM} \times 10\text{ min}$) to pellet the cells. The

pellet was resuspended in lysis buffer, run through a 25-gauge needle, centrifuged ($10,000 \times g$; 20 min) to remove the insoluble fraction, and frozen at -80°C for quantification of platelet eNOS protein by Western blot.

Samples were thawed immediately prior to running Western blots. Total protein concentrations of solubilized platelet samples were determined using the Bradford technique. Proteins were separated using polyacrylamide gel electrophoresis (7.5% SDS-PAGE gel; Bio-Rad minigel, Hercules, CA). For each sample from the cytosolic fraction, 20 μg of total protein was heated at 100°C for 5 min with an equivalent volume of $1\times$ sample buffer (4% SDS, 10% β -mercaptoethanol). Gels were electrophoresed at room temperature for 90 min at 140 mV and transferred to a nitrocellulose membrane (Polyscreen PVDF, New England Nuclear) in tris-glycine-methanol buffer for 1 hr at 100 V at 4°C . The membrane was blocked with 5% nonfat dry milk in Tween Tris-buffered saline (TTBS), pH 7.5, for 1 hr and incubated with the primary antibody (polyclonal anti-eNOS, 1:500, Chemicon, Temecula, CA) overnight at 4°C . The membrane was washed 3 times with TTBS for 15 min, and the blot was then incubated with horseradish peroxidase-conjugated goat antirabbit IgG (1:5,000; Zymed, San Francisco, CA) for 60 min and rinsed again as described. All samples were incubated overnight with Actin (1:10,000, Sigma, St. Louis, MO) to control for protein transfer. Samples were run with positive controls for eNOS (Transduction Laboratories, Lexington, KY). Enhanced ChemiLuminescence Assay (ECL, Amersham, Chicago, IL) was used to view the protein bands. The eNOS signal appeared as a band at ≈ 140 kDa, and the optic density was quantified (Inquiry, Loats Associates) and expressed as a ratio of eNOS to β -actin protein.

Measurement of Physiologic Variables and Plasma Estrogen

All animals were weighed on an electronic scale prior to the study (Scout Pro, Pinebrook, NJ). Arterial blood pressure was measured with Statham 23Db pressure transducers (Gulton-Statham Transducers Inc., Costa Mesa, CA) and continuously recorded on a Gould-Brush polygraph (Modul Company, Danbury, CT). Arterial blood samples were analyzed with a Radiometer electrode system for pH, PO_2 , and hemoglobin (Chiron Diagnostics Corporation, Emeryville, CA). Plasma 17β -estradiol was measured in triplicate using a commercially available clinical radioimmunoassay kit (Coat-a-Count, Diagnostic Products Corp., Los Angeles, CA) with a range of 0 to 3600 pg/mL and a linear sensitivity of 8 pg/mL or greater.

Data Analysis

All data are reported as mean $\pm$ SEM unless otherwise indicated. Physiologic variables were analyzed using a two-way analysis of variance (ANOVA). Plasma estrogen was analyzed using a one-way ANOVA. The ratio of eNOS protein to β -actin protein was analyzed using a one-way ANOVA. The postischemic changes in P-selectin expression over time and between groups were analyzed using one-way and two-way repeated measures ANOVA, respectively. Post hoc comparisons were made using a Newman-Keuls test. If data did not meet the assumptions of the ANOVA, they were analyzed using a Kruskal-Wallis test on ranks. Differences in regional CBF between OVX and E50 groups were determined using t tests for independent samples. Significance was set at $p \leq .05$ for all tests.

Results

Physiologic Parameters

No differences in weights were noted among the I (242.4 ± 6.0 g), OVX (248.6 ± 5.0 g), or E50 (245.5 ± 4.7 g) groups ($p = .71$). Physiologic parameters for baseline and 60-min reperfusion are depicted in Table 1. In all groups, preischemic mean arterial blood pressure exceeded the postischemic values. The OVX rats required higher oxygen supplements to maintain physiologic support, likely due to lower hemoglobin values (Table 1). Cortical laser-

Doppler flowmetry was used to validate ischemia and was not different between groups (data not shown). We were unable to obtain enough blood to evaluate plasma estrogen levels in 1 OVX and 1 E50 rat, so plasma estrogen levels were obtained in a total of 22 rats. Plasma estrogen levels in the E50 group (43.14 ± 12.06 pg/mL) were higher than the I (3.84 ± 1.07 pg/mL) and the OVX (1.18 ± 0.59 pg/mL) groups. Normally, there is a wide variation in rat platelet count (500,000–1,000,000 μ L). Therefore, differences in platelet count among the groups were evaluated as the ratio of preischemic counts to 60-min reperfusion counts. No significant differences between the groups were noted (I: 0.99 ± 0.14 ; OVX: 0.97 ± 0.22 ; E50: 0.79 ± 0.09 ; $p = .622$).

Effect of Estrogen Deficiency on Platelet P-Selectin Expression

All P-selectin data are normalized to baseline preischemic platelet P-selectin values. Data are expressed as the ratio of baseline MESF to MESF at 10, 30, and 60 min of reperfusion. Compared to the OVX group, estrogen treatment transiently lowered P-selectin expression at 10 min of reperfusion (OVX: 1.107 ± 0.05 vs. E50: 0.91 ± 0.04) in resting (unstimulated) platelets. However, no differences were observed in the I and OVX groups throughout the 60-min reperfusion (Fig. 1A). Because we observed modest changes in P-selectin expression in platelets over the 60-min observation window, we sought to determine if platelet reactivity to stimulation is altered by estrogen insufficiency. By 60 min of reperfusion, we observed that, compared to the intact rats (1.02 ± 0.10), estrogen depletion increased platelet reactivity to collagen (1.62 ± 0.2 ; $p = .016$), and estrogen repletion abolished the hypersensitivity to stimulation created by ovariectomy (1.12 ± 0.10 ; Fig. 1B).

Platelet eNOS Protein

In some conditions, platelets have been reported to make inducible NOS (iNOS). We therefore tested for the presence of iNOS in our samples but found no protein for iNOS. Estrogen deficiency did not alter platelet eNOS protein expression. Furthermore, estrogen treatment following ovariectomy had minimal effect on platelet eNOS production (Fig. 2; $p = .407$).

Iodoantipyrine Studies

Because differences in platelet responsiveness were noted between the OVX and the E50 group, we measured regional CBF to ascertain if differences in blood flow could be detected. Regional CBF at 60 min of reperfusion was comparable in the ovariectomized and the estrogen-treated rats. Likewise, we found no differences in CBF between the groups in the cortex ($p = 1.0$), striatum ($p = .948$), and hippocampus ($p = .902$; Fig. 3).

Discussion

The results of this study demonstrate several major findings. First, estrogen deficiency increases postischemic platelet reactivity during early reperfusion after global cerebral ischemia. This study also shows that estrogen replacement in the face of estrogen deficiency is not detrimental to platelet reactivity and can reverse postischemic increases in platelet sensitivity to aggregants. Furthermore, the mechanism by which estrogen reduces platelet reactivity during estrogen deficiency does not involve depression of platelet eNOS expression. Finally, the heightened platelet sensitivity to stimulation that we observed in the OVX group does not seem to reduce regional CBF, at least during early reperfusion.

We and others have demonstrated that estrogen replacement at doses similar to those in the present study moderates focal ischemic brain injury in rodents (Alkayed et al., 1998; Wise, 2003). However, the importance of estrogen deficiency and replacement to postischemic platelet function and the effects on microvascular perfusion remain unclear. In the present study, we uncovered no detrimental effect of estrogen on platelet reactivity in the face of

estrogen deficiency. Surprisingly, platelets harvested from estrogen-treated rats were transiently more quiescent and expressed less P-selectin compared to their ovariectomized, untreated counterparts, but the effect was lost by 30 min of reperfusion. A possible explanation for this result may be that we observed minimal P-selectin expression in the other groups and, by 30 min, what little P-selectin was located on the surfaces of platelets in the OVX and I groups had been shed. The fact that P-selectin is rapidly shed from platelet surfaces unless ongoing activation persists (Michelson et al., 1996) may partially explain our inability to detect a significant increase in P-selectin expression on platelets from the ovariectomized and the intact group.

In the present study, we sought to determine if platelet activation begins early after restoration of CBF. If platelets are activated and become adherent in small vessels, this may lead to regional hypoperfusion and intensification of injury. Early in reperfusion, the bulk of platelets expressing P-selectin may be tethered to other cells, such as monocytes or leukocytes, and would be undetectable in our studies, as we analyzed only platelet-bound P-selectin. It is also possible that endothelial injury may not be fully established by 60 min of reperfusion, so that the stimulus for platelet activation is insufficient to trigger the activation of large numbers of platelets. Acute cerebral ischemia is associated with increased expression of platelet P-selectin in humans and in rats (Grau et al., 1998; Marquardt et al., 2002), and measurable platelet aggregates appear in the cerebral microvasculature as late as 48 hr after middle cerebral occlusion in rodents (Garcia et al., 1994). After experimental cardiac arrest (an injury similar to that incurred with the four-vessel occlusion model), a time-dependent increase in platelet aggregates occurs along bifurcations of cerebral microvessels during postischemic reperfusion (Okada et al., 1994), as does a delayed appearance of adherent P-selectin-positive platelets (Love & Barber, 2001) along vascular endothelium.

A major finding of this study is that once reperfusion is established, circulating platelets from estrogen-deficient rats are hyperresponsive to stimulation with collagen and subsequently express more platelet P-selectin. To our knowledge, this is the first study to demonstrate that after a cerebral ischemic insult, estrogen deficiency augments platelet sensitivity to aggregants during the early reperfusion period. However, our results do agree with other studies showing that in healthy swine and rats, estrogen deficiency can increase platelet aggregation and secretion (Jayachandran, Owen, & Miller, 2003), as well as P-selectin expression (Littleton-Kearney, Rebel, Jones, & Hurn, 2001). Ischemia triggers vascular endothelial cell injury with subsequent exposure of the underlying vascular collagen matrix (Zhang et al., 2001). Amplified postischemic platelet responsiveness to collagen may reflect increased platelet activation, as circulating platelets are exposed to areas of damaged endothelium. Our observation that estrogen deficiency heightens platelet sensitivity during reperfusion may reflect the intensification of evolving subendothelial lesions that results in partial activation of platelets during transient exposure to the injured microvasculature.

We found that compared to the OVX rats, estrogen replacement in the E50 rats blocked postischemic hypersensitivity to collagen and reduced platelet P-selectin expression. However, the mechanism by which estrogen modulated this response is unclear. It is possible that the reversal of hypersensitivity to collagen that we observed in the E50 group is related to preservation of vascular endothelial responses. Severity of vascular injury modulates the degree of platelet activation (Pluta, Lossinsky, Walski, Wisniewski, & Mossakowski, 1994). Estrogen replacement may reduce subendothelial ischemic injury and preserve endothelial synthesis of antiaggregant products, such as NO. Alternatively, estrogen replacement normalizes postischemic pial arteriole response (Watanabe et al., 2001) and may reduce platelet shear stress during early reperfusion. The degree of shear stress can modulate the release of vonWillebrand factor from endothelial cell storage sites, and platelets can adhere to

vonWillebrand factor at the GP 1b complex on the platelet surface (Kunicki & Ruggeri, 2001).

The relative instability of blood pressure that we noted during reperfusion is likely a result of vasomotor dysregulation associated with severe brain injury or falling hemoglobin levels. However, these changes in blood pressure had no effect on CBF. Both the OVX and the E50 group demonstrated lower hemoglobin levels relative to the I group. This is likely due to some degree of hemodilution, as both the OVX and E50 groups required intravenous fluid to maintain blood pressure during reperfusion. Our data show that neither the reduction in blood pressure nor the differences in hemoglobin concentrations among groups altered circulating platelet counts. Therefore, it is doubtful that the differences noted in platelet P-selectin expression were due to changes in hemoglobin or blood pressure. We found significant variation between the plasma estrogen levels of the I and the OVX groups. This finding was not surprising given that sexually mature rats demonstrate significant variation in plasma estrogen levels during the estrous cycle. Although we did not control for normal plasma estrogen variability in the intact group, the plasma estrogen levels in the current study were virtually identical to those we have measured in other studies (Rusa et al., 1999; Toung, Traystman, & Hurn, 1998). In those studies, we demonstrated that estrogen replacement reduces infarction volume but the neuroprotective dose has a narrow therapeutic range (Rusa et al., 1999). In pilot studies, we found that an estrogen implant dose of 50 μ g (slow-release pellet) would consistently raise plasma estrogen to this therapeutic range (46 ± 10 pg/mL).

We had hypothesized that estrogen replacement restores intraplatelet eNOS expression and, in doing so, depresses platelet sensitivity to aggregatory stimulus, which is evidenced by a subsequent fall in platelet surface P-selectin. However, our data do not support this hypothesis, as we detected no change in platelet eNOS expression in the estrogen-deficient or the estrogen-treated rats. Others have noted that estrogen deficiency increases platelet eNOS expression in platelets of sexually mature pigs subjected to acute loss of ovarian sex steroids (Jayachandran & Miller, 2002), but we did not observe this effect in the current study. The differences between our studies and that of Jayachandran and Miller (2002) are likely related to species variation, technical differences, handling of platelets, or the duration of estrogen deficit. We studied our rats at 2 weeks postovariectomy, as opposed to at 1 month. Therefore, it is possible that up-regulation of eNOS occurs gradually and requires a longer period of estrogen deficiency. In addition, the considerable variability in platelet eNOS expression that we noted in the OVX group may reflect incomplete platelet turnover under estrogen-deficient conditions. Although rodent platelets turn over in 3 to 5 days (Bannerman, 1983), it is possible that 2 weeks is not long enough for total replacement of circulating platelets and may explain why we could not detect significant increases in platelet eNOS. Further investigation is needed to ascertain if, indeed, this is the case.

Estrogen reportedly depresses platelet aggregation (Bar et al., 1993; Nakano et al., 1998; Selles, Polini, Alvarez, & Massheimer, 2001) and reduces the size of platelet aggregates (Haque et al., 2001). Estrogen depletion may promote formation of platelet aggregates and impede regional CBF during postischemic reperfusion. In the present study, we found no differences in cortical or striatal blood flow between the ovariectomized and the estrogen-replaced rats, despite differences in stimulated platelet P-selectin expression. However, regional blood flow measures may not be sensitive enough to detect smaller variation in blood flow—associated occlusion of small vessels by platelet aggregates. Platelet and leukocyte accumulation have been documented in evolving brain infarcts in the rat as early as 4 to 6 hr and as late as 48 hr of reperfusion (Garcia et al., 1994). Taken together, our data suggest that platelets are activated by exposure to microvasculature injury, but not enough platelets accumulate in the microvasculature to alter total CBF during the early stages of reperfusion. Treatment with estrogen does not seem to change postischemic regional blood flow. Although statistically we

did have a 50% power, the interpretation of our data may be limited by our small sample size. [¹⁴C]IAP autoradiography is technically very difficult in healthy rats, as a successful study depends on free-flowing arterial blood collection at 3-s intervals. Because of the severity of the ischemic injury, several rats died during the blood collection phase as a result of cardiovascular instability. We had significant attrition in both groups; 1 of the E50 and 3 of the OVX rats died before the brain was harvested. It is possible that more OVX rats died compared to the E50 rats because estrogen treatment had a beneficial effect on the cardiovascular status.

Conclusion

Miller, Shuster, and Hayes (2003) suggested that preclinical studies such as ours are essential to determine links between estrogen, platelet function, and thrombosis and to generate safe, effective hormone treatment regimens. The issues surrounding hormone replacement therapy (HRT) are of growing concern to women, particularly in light of the controversy generated by the WHI study, which suggested that estrogen therapy might actually worsen the risk for thrombosis and stroke for women. The results of the WHI study are limited by the type of estrogen and the dosage employed, as well as the average age of the women sampled. Furthermore, it is unclear if estrogen treatment influences recovery after a cerebrovascular ischemic event. It must be emphasized that the aim of WHI was to determine if postmenopausal estrogen treatment affects risk for stroke in women, whereas the present study addresses the consequences of estrogen treatment after a global ischemic cerebral injury in a rodent model. Although the results of preclinical studies such as ours cannot be generalized to humans, this study is a first step in beginning to understand the effects of estrogen on postischemic platelet function after brain injury. Our data show that during early reperfusion, acute estrogen deficiency increases postischemic platelet reactivity to aggregatory stimuli and that treatment with estrogen doses that produce physiologically relevant levels mitigates the hypersensitivity. The effect of estrogen deficiency and replacement on platelets does not seem to be modulated by depression of intraplatelet eNOS. Furthermore, neither estrogen deficiency nor estrogen replacement changes early postischemic regional brain blood flow. Taken together, our results indicate that in this rodent model of global cerebral ischemia, physiologic doses of estrogen are not deleterious to platelet reactivity during early reperfusion and may initially reduce platelet sensitivity to proaggregatory stimulants.

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Figure 1.

P-selectin positive platelets expressed as a ratio of baseline (preischemic) mean estimated soluble fluorescence (MESF) to MESF at 10, 30, and 60 min of postischemic reperfusion. (A) Ratio in resting (unstimulated) platelets positive for P-selectin (* $p \leq .05$, E50 vs. OVX; $n = 8$ /group). (B) Ratio in platelets stimulated with collagen positive for P-selectin († $p \leq .02$, OVX vs. E50 and N; $n = 8$ /group).

Figure 2. Platelet lysate expression of endothelial nitric oxide synthase (eNOS) protein normalized to β -actin in intact (I; $n = 5$), ovariectomized (OVX; $n = 6$), and OVX + 17 β -estradiol (E50; $n = 5$) groups. (A) Representative Western blot from platelet lysates from I, OVX, and E50 rats. (B) Densitometric measurement of eNOS protein from platelet lysates.

Figure 3. Cerebral blood flow (CBF) measured by iodoantipyrine and expressed as mL/100 g/min in the cortex, striatum, and hippocampus of ovariectomized (OVX; $n = 4$) and OVX + 17β -estradiol (E50; $n = 5$) rats at 60 min postischemic reperfusion.

Table 1
 Physiologic Data Preischemia and at 60 Min of Reperfusion

	Intact	Ovariectomized	Ovariectomized Plus Estrogen
Mean arterial blood pressure (mm Hg)			
Preischemia	78.7 ± 4.5	81.0 ± 4.3	94.5 ± 5.7
Reperfusion	60.7 ± 3.6 *	73.8 ± 2.4 *	57.2 ± 7.6 *
pH			
Preischemia	7.38 ± 0.10	7.41 ± 0.06	7.47 ± 0.06
Reperfusion	7.35 ± 0.03	7.31 ± 0.06	7.38 ± 0.03
Arterial oxygen tension (PaO ₂ ; Torr)			
Preischemia	165.6 ± 21.3	214.8 ± 51.8	224.4 ± 25.54
Reperfusion	151.6 ± 30.0	332.4 ± 51.8 **	192.4 ± 17.30
Hemoglobin (mg/dL)			
Preischemia	10.8 ± 0.6	9.0 ± 0.4	9.2 ± 0.4
Reperfusion	9.4 ± 0.3	8.1 ± 0.7 **	7.1 ± 0.2 **

NOTE: Data are presented as mean ± SEM.

* $p \leq .05$ from preischemic values.

** $p \leq .05$ from I.