Estrogen Increases Endothelial Nitric Oxide Synthase via Estrogen Receptors in Rat Cerebral Blood Vessels
Effect Preserved After Concurrent Treatment With Medroxyprogesterone Acetate or Progesterone

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Background and Purpose—In vivo and in vitro rat models of hormone therapy were used to test the following hypotheses: (1) estrogen acts directly on cerebrovascular estrogen receptors to increase endothelial nitric oxide synthase (eNOS); (2) increased protein correlates with higher NOS activity; and (3) effects of estrogen on eNOS are altered by concurrent treatment with either medroxyprogesterone acetate (MPA) or progesterone.

Methods—Blood vessels were isolated from brains of ovariectomized female rats; some were treated for 1 month with estrogen, estrogen and progesterone, or estrogen and MPA. Isolated cerebral vessels were also treated in vitro with estrogen in the absence and presence of progesterone, MPA, tamoxifen, and the estrogen receptor antagonist ICI 182 780. Levels of eNOS were measured by Western blot, and NOS activity was measured by [14C]arginine-[14C]citrulline conversion.

Results—Chronic hormone treatment in vivo resulted in plasma levels of 17β-estradiol, progesterone, and MPA in the range of values found in humans. Estrogen treatment resulted in higher levels of cerebrovascular NOS activity that paralleled increases in eNOS protein. In vitro estrogen treatment for 18 hours also resulted in a concentration-dependent increase in eNOS protein (EC50 ≈300 pmol/L) that was completely prevented by estrogen receptor antagonists tamoxifen or ICI 182 780. However, cotreatment with progesterone or MPA, either in vivo or in vitro, did not alter the effect of estrogen on eNOS protein.

Conclusions—Estrogen receptor activation in cerebrovascular tissue results in increased eNOS activity and protein levels. The latter effect persists in the presence of either progesterone or MPA. Thus, increased NO production by eNOS may contribute to the neuroprotective effects of estrogen. (Stroke. 2002;33:1685-1691.)

Key Words: cerebral vessels ■ estrogens ■ nitric oxide synthase ■ rats

Sex and age differences in stroke incidence suggest that female gonadal hormones provide neuroprotection for premenopausal women.1 Although clinical use of hormone replacement therapy (HRT) for stroke remains controversial, estrogen is clearly neuroprotective in experimental models of cerebral ischemia.1–5 For example, after middle cerebral artery occlusion, intact female rats sustain a smaller infarct volume than ovariectomized females or males, and this is associated with greater cerebrovascular blood flow during ischemia.2 Administration of estrogen to ovariectomized female rats immediately after middle cerebral artery occlusion also decreases infarct volume and increases cerebral blood flow after 1 to 2 days.3 While a number of mechanisms likely contribute to neuroprotection, one important action appears to be the ability of estrogen to increase the vasodilatory capacity of the cerebral vasculature during cerebral ischemia.4,5

Recent evidence suggests that estrogen acts, in part, by increasing nitric oxide (NO) in the cerebrovascular endothelium.6–9 NO is produced by the enzyme endothelial nitric oxide synthase (eNOS) to cause vasodilation and inhibit platelet aggregation, both of which may be important in protection against stroke. Indeed, the effects of experimental stroke are worsened by pharmacological eNOS inhibition10 or genetic eNOS knockout,11 suggesting that eNOS is important for protection against brain injury. We have previously demonstrated that chronic, in vivo estrogen treatment of gonadectomized male and female rats increases levels of cerebrovascular eNOS protein6 with a parallel increase in NOS-mediated modulation of cerebrovascular contractility.7,8 However, cerebrovascular NOS activity after estrogen treatment has not been measured. Therefore, we investigated the hypothesis that in vivo treatment with estrogen increases NOS activity in cerebral blood vessels.

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Mechanisms underlying in vivo effects of estrogen on cerebrovascular eNOS are not known. Estrogen could act indirectly by altering one of a number of physiological factors known to influence eNOS, such as blood flow or plasma lipids. Conversely, endothelial cells in culture exhibit increased eNOS mRNA and protein expression in direct response to estrogen. Therefore, we investigated the hypothesis that estrogen acts directly on receptors in cerebral blood vessels to increase eNOS protein. Isolated vessels were incubated in vitro with estrogen and several estrogen receptor antagonists.

Possible clinical application of these findings to human therapy may be limited, in part, by the fact that, in women with an intact uterus, estrogen is usually prescribed in combination with a progestin to decrease risk of endometrial carcinoma. One of the most commonly used progestins, medroxyprogesterone acetate (MPA), may have adverse effects on vascular protective effects of estrogen. Despite the clinical relevance, there are few animal studies on effects of MPA on vascular function. In monkeys, MPA was found to decreased eNOS mRNA and protein expression in direct response to estrogen.

In ovariectomized rats, however, progesterone was found to increase levels of subcortical infarction after middle cerebral artery occlusion. To model postmenopausal HRT, we chronically administered hormones via subcutaneous silicone elastomer (Silastic, Dow Corning) capsules. Ovariectomized female rats were compared with ovariectomized females treated with estrogen alone, both estrogen and progesterone, or estrogen together with MPA. Serum hormone levels were measured, and capsule sizes were selected to give serum levels within the clinically relevant range. This model was then used to investigate whether estrogen-induced increases in eNOS would be blocked by concurrent treatment with MPA or progesterone in ovariectomized female rats.

Materials and Methods

Hormone Treatment In Vivo
All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of California at Irvine. Ovariectomized female rats received 1 of 4 possible hormone treatments: no treatment (control), estrogen, estrogen and progesterone, or estrogen and MPA. Three-month-old female Fischer 344 rats (Harlan Sprague-Dawley) were gonadectomized while anesthetized with ketamine 21.1 mg/kg and xylazine 4.2 mg/kg IP. Hormone-filled Silastic tubing (1.57 mm inner diameter /H1103/ 3.18 mm outer diameter) implanted subcutaneously. Tubing lengths from 1 to 150 mm were used for administration of estrogen and progesterone, and MPA. Stock solutions of cation exchange resin (Bio-Rad AG50W, Na+ form) and counted. NOS inhibitor-sensitive NOS activity was calculated as the difference between activity measured in the absence and presence of L-NAME/L-NA. Protein content was determined by modified Lowry assay. NOS activity values were expressed as picomoles per minute per milligram protein.

In Vitro Hormone Treatment
Intact cerebral vessels from ovariectomized female rats were aliquoted into mini petri dishes containing 1.5 mL Dulbecco’s modified Eagle’s medium (without phenol red or sodium pyruvate) plus penicillin (10 IU/mL) and streptomycin (10 µg/mL). Intact vessels were maintained at 37°C in 95% O2/5% CO2 for 18 hours with drug(s) as indicated: cycloheximide-encapsulated 17β-estradiol, ICI 182 780, tamoxifen, cycloheximide-progesterone, or MPA. Stock solutions (0.1 mM/L) of ICI 182 780 and tamoxifen were prepared in 95% ethanol and diluted so that the final concentration of ethanol in the medium was approximately 0.001%. Vehicle controls contained either ethanol or 2-hydroxypropyl-β-cyclodextrin.

Western Blot
Cerebral vessels were homogenized in 50 mM/L β-glycerophosphate, 100 µM/L sodium orthovanadate, 2 mM/L magnesium chloride, 1 mM/L EGTA, 0.5% Triton X-100, 1 mM/L β-dithiothreitol, 20 µM/L pepstatin, 20 µM/L leupeptin, 0.1 U/mL aprotinin, and 1 mM/L phenylmethylsulfonyl fluoride and then incubated on ice for 20 minutes. Samples were centrifuged at 180g for 25 minutes at 4°C, and the supernatant was collected. After protein determination, samples were diluted in Tris-glycine SDS Sample Buffer (NoveX), boiled for 4 minutes, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). For each vessel preparation, 15 µg of protein was loaded in duplicate onto 8% Tris-glycine gels and separated by SDS-PAGE.
The positive control for eNOS (endothelial cells provided by Transduction Laboratories) and biotinylated broad-range molecular weight markers (Bio-Rad) also were loaded. After separation, proteins were transferred to a nitrocellulose membrane by electrophoretic blotting, and membranes were incubated overnight at 4°C in blocking buffer. Incubation with a monoclonal mouse anti-eNOS antibody (Transduction Laboratories) or mouse anti-α-smooth muscle actin antibody (Sigma) was followed by incubation with anti-mouse IgG antibody conjugated to horseradish peroxidase (Transduction Laboratories). Positive bands were detected by electrochemiluminescence reagent and Hyperfilm (Amersham) and quantified with a computer-based image analysis system (MCID).

To compare in vivo treatments, 1 vessel preparation from each hormonal group was run together on a single gel. Experiments were repeated a number of times (n), with preparations from different sets of animals. For each in vitro experiment, vessels were isolated and treated, and conditions were analyzed in parallel.

### Data Analysis and Statistics

Data are expressed as mean ± SE. Statistical significances of Western blot and radioimmunoassay data were determined by ANOVA with Prism software, with the use of repeated measures for Western blots. Post hoc analysis was done with the Bonferroni multiple comparison test. For NO synthase studies, measurements were made in triplicate in each of 4 separate experiments, and data were analyzed by Student’s t test. The acceptable level of significance was defined as P < 0.05.

### Results

In vivo estrogen treatment of ovariectomized females for 1 month significantly increased serum levels of 17β-estradiol (Table 1). Additional treatment with progesterone implants increased serum progesterone levels without affecting levels of 17β-estradiol. To develop a model for MPA treatment, rats had been removed (data not shown).

Cerebral blood vessels isolated from the 4 hormonal treatment groups were analyzed for eNOS protein (Figure 1). In all vessel preparations, immunoreactivity for eNOS was detected as a 140-kDa band. Only 1 band per lane was observed with anti-eNOS antibody, and this corresponded to that of the positive control. Chronic in vivo estrogen treatment of ovariectomized females resulted in a 12-fold increase in eNOS protein in cerebral vessels compared with ovariectomized females (Figure 1). In contrast, estrogen treatment did not affect levels of α-smooth muscle actin (data not shown). Concurrent in vivo treatment with estrogen and either progesterone or MPA also increased cerebrovascular eNOS, and these effects were not significantly different from those seen with estrogen alone.

Specific NOS activity, ie, activity sensitive to inhibition by L-NAME and L-NA, was measured in freshly isolated cerebral vessels. NOS activity was greater in vessels from ovariectomized females treated in vivo with estrogen compared with vessels from ovariectomized females (P < 0.05; Figure 2). Similar results, ie, increased NOS activity after estrogen treatment, were obtained when intraparenchymal vessels were isolated from brains in which the pial vessels had been removed (data not shown).

For in vitro experiments with isolated cerebral vessels, cyclodextrin was used as vehicle for 17β-estradiol and progesterone. Although cyclodextrin in millimolar concentrations can affect caveolae and eNOS, preliminary experiments indicated that none of the concentrations of cyclodextrin used in the present study (10⁻¹² to 10⁻⁷ mol/L) had any significant effect on eNOS activity or protein levels.

### Table 1. Effect of Hormone Treatment on Serum Concentrations of 17β-Estradiol, Progesterone, and MPA, Body Weight, and Uterine Weight

<table>
<thead>
<tr>
<th></th>
<th>17β-Estradiol, ng/mL</th>
<th>Progesterone, ng/mL</th>
<th>MPA, ng/mL</th>
<th>Body Weight, g</th>
<th>Uterine Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovariectomized</td>
<td>15 ± 4</td>
<td>5 ± 2</td>
<td>&lt;0.20</td>
<td>175 ± 6</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>Ovariectomized+estrone</td>
<td>65 ± 10*</td>
<td>7 ± 2</td>
<td>&lt;0.20</td>
<td>152 ± 4*</td>
<td>0.90 ± 0.04*</td>
</tr>
<tr>
<td>Ovariectomized+estrone and progesterone</td>
<td>49 ± 10*</td>
<td>24 ± 3*</td>
<td>&lt;0.20</td>
<td>169 ± 4</td>
<td>0.55 ± 0.05†</td>
</tr>
<tr>
<td>Ovariectomized+estrone and MPA (3 mm)</td>
<td>50 ± 5*</td>
<td>7 ± 3</td>
<td>1.0 ± 0.1*</td>
<td>165 ± 4</td>
<td>0.49 ± 0.10†</td>
</tr>
</tbody>
</table>

Values are mean ± SE; n = 3–7.

*Significantly different from ovariectomized.

†Significantly different from ovariectomized with estrogen.

In vivo estrogen treatment of ovariectomized females increased uterine weight and decreased body weight compared with ovariectomized females (Table 1). Concurrent treatment with either MPA or progesterone significantly attenuated the effects of estrogen on uterine weight and body weight.
effect on eNOS protein levels. Therefore, only the highest concentration of cyclodextrin vehicle used (10^{-8} to 10^{-7} mol/L) was assayed as the control.

In vitro incubation with 17β-estradiol (0.3 to 10 nmol/L) for 90 minutes had no effect on eNOS protein levels in cerebral blood vessels isolated from ovariectomized females (data not shown). Treatment for 18 hours in vitro, however, resulted in significant, concentration-dependent increases in eNOS protein compared with vehicle (P<0.001; Figure 3). As indicated in Figure 3, the concentrations of 17β-estradiol that increased eNOS protein levels in vitro correlated with serum levels measured in animals treated in vivo with estrogen. In contrast, the concentration of 17β-estradiol found in sera of ovariectomized rats had no significant effect on eNOS protein levels in vitro.

![Figure 1](image1.png) **Figure 1.** Effect of in vivo hormone treatment on eNOS levels in cerebral blood vessels. Representative immunoblot shows eNOS (140 kDa) in vessels from ovariectomized females (O), ovariectomized females with estrogen treatment (OE), ovariectomized females treated with estrogen plus MPA (OE/MPA), and ovariectomized females treated with estrogen plus progesterone (O/EP). eNOS band optical density was expressed as percentage of O value obtained in the same experiment. Mean±SEM values for 4 different vessel preparations of each animal group are shown. *Significantly different from O, P<0.01.

![Figure 2](image2.png) **Figure 2.** Effect of in vivo estrogen treatment on NOS activity in cerebral blood vessels. NOS activity was expressed as picomoles of citrulline produced per minute per milligram tissue protein. NOS inhibitor–sensitive NOS activity in vessels from ovariectomized females (O) and ovariectomized females with estrogen replacement (OE) is shown. *Significantly different from O, P<0.05; n=6.

![Figure 3](image3.png) **Figure 3.** Effect of in vitro estrogen treatment on eNOS protein in cerebral vessels from ovariectomized female rats. Vessels were incubated for 18 hours with either vehicle or estrogen (10^{-12} to 10^{-8} mol/L) and analyzed by Western blot. Optical density was quantified and plotted relative to vehicle-treated tissues from the same experiment. Serum estrogen levels of ovariectomized females (O) and ovariectomized females with estrogen replacement (OE) are indicated. *Significantly different from vehicle, P<0.05; n=5 to 8 experiments.
In cerebral vessels isolated from ovariectomized females, in vitro coincubation for 18 hours with 17β-estradiol (1 nmol/L) and either the estrogen receptor partial agonist tamoxifen (10 nmol/L) or the antagonist ICI 182 780 (1 nmol/L) completely prevented estrogen-induced increases in eNOS protein (P<0.01; Figure 4A and 4B). The vehicle for the estrogen receptor antagonists (ethanol) had no significant effect on eNOS protein levels (data not shown).

Coincubation of MPA with 17β-estradiol in vitro did not prevent the estrogen-induced increase in eNOS (5.0-fold increase in eNOS protein with 17β-estradiol alone compared with 5.4-, 5.0-, and 5.9-fold increases after coincubation with 1, 10, and 100 ng/mL MPA, respectively; Figure 5A). Similarly, in vitro coincubation of 17β-estradiol with progesterone did not prevent the estrogen-induced increase in eNOS (5.2-, 5.4-, and 5.6-fold increases after coincubation with 1, 10, and 100 nmol/L progesterone, respectively; Figure 5B).

**Discussion**

With the use of in vivo and in vitro rat models of HRT, this study demonstrates several important effects of estrogen on cerebral blood vessels. First, in vivo treatment of ovariectomized females with estrogen increases levels of both eNOS protein and NOS activity in cerebral blood vessels. Second, in vitro treatment of the vessels with estrogen also increases levels of eNOS protein, demonstrating that estrogen acts directly on cerebrovascular tissue. Concentrations of estrogen effective in vitro correlate with serum concentrations of estrogen that effectively increase eNOS protein in vivo, validating the relevance of the in vitro results. Third, the effect of estrogen to increase eNOS protein in vitro is completely inhibited by estrogen receptor antagonists, indicating that this action of estrogen is receptor mediated. Finally, effects of estrogen on eNOS protein are not affected by concomitant in vivo or in vitro treatment with either progesterone or MPA. These findings suggest that the various forms of HRT should all provide potentially beneficial enhancement of cerebrovascular eNOS.

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**Figure 4.** Effect of estrogen receptor blockade on estrogen-induced increase in eNOS protein in cerebral blood vessels from ovariectomized female rats. Tissues were incubated in vitro for 18 hours with either vehicle, estrogen alone, or estrogen plus estrogen receptor blockers. Protein was analyzed by Western blot. Optical density was quantified relative to vehicle-treated tissues from the same experiment. A, Immunoblot showing eNOS protein in cerebral vessels incubated in duplicate with vehicle (V), 1 nmol/L estrogen alone, or 1 nmol/L estrogen in combination with either 10 nmol/L tamoxifen (Tam) or 1 nmol/L ICI 182 780 (ICI). B, Mean levels of eNOS protein in experiments illustrated in A. E indicates estrogen. *Significantly different from vehicle, P<0.01; n=4.

**Figure 5.** Effect of in vitro hormone treatment on eNOS in cerebral blood vessels from ovariectomized female rats. Vessels were incubated for 18 hours with either vehicle (V), estrogen alone (10⁻⁹ mol/L), or estrogen plus MPA or progesterone (P) and analyzed by Western blot. Each blot represents 2 experiments, each showing similar results. A, eNOS in vessels incubated in duplicate with vehicle, 1 nmol/L estrogen alone, or 1 nmol/L estrogen plus 1, 10, or 100 ng/mL MPA. B, eNOS in vessels incubated in duplicate with vehicle, 1 nmol/L estrogen alone, or 1 nmol/L estrogen plus 1, 10, or 100 nmol/L progesterone.
Serum concentrations of estrogen after in vivo treatment of ovariectomized rats correlate with levels of estrogen seen physiologically in rats and measured in premenopausal females and in postmenopausal women taking estrogen replacement therapy. The effectiveness of the in vivo estrogen treatment was confirmed by measurement of the expected increase in uterine weight.

In vivo estrogen treatment of ovariectomized rats increased levels of cerebrovascular eNOS protein, as we and others have shown previously. In the present study, we also found a parallel increase in NOS activity in cerebral vessels isolated from estrogen-treated animals. Intraparenchymal vessels contain only eNOS, but pial arteries can also contain perivascular neuronal NOS (nNOS)–positive nerves; therefore, we confirmed our NOS activity results using vessels isolated from brains in which the pial vessels had been removed. Furthermore, preliminary studies in our laboratory indicate that estrogen has no effect on nNOS protein (K.J. Pak, BS, et al, unpublished data, 2002). Thus, the increase in vascular NOS activity after estrogen treatment is likely due to eNOS, which is consistent with the hypothesis that estrogen increases cerebrovascular eNOS protein, resulting in increased NOS activity. These findings correlate with previous studies showing that estrogen treatment modulates vascular contractility of rat cerebral arteries by increasing NOS-dependent vasodilation.

In vitro treatment of isolated cerebral vessels with physiological concentrations of 17β-estradiol also increased eNOS protein. These data indicate that estrogen acts directly on cerebral vessels to influence NO production. Estrogen is likely acting via vascular estrogen receptors, because low concentrations of the estrogen receptor antagonist ICI 182 780 or the partial agonist tamoxifen completely prevented estrogen-induced increases in eNOS protein, resulting in increased NOS activity. These findings correlate with previous studies showing that estrogen treatment induces vascular contractility of rat cerebral arteries by increasing NOS-dependent vasodilation.

In summary, we have found that in vivo estrogen treatment increases eNOS protein and NOS activity in cerebral vessels. In vitro treatment of intact cerebral vessels with estrogen also causes a concentration-dependent increase in eNOS protein that is completely inhibited by estrogen receptor blockade. These data are consistent with the hypothesis that estrogen acts on estrogen receptors in cerebrovascular tissue, resulting in increased eNOS protein and NOS activity. Furthermore, using a rodent model for 3 different modalities of postmenopausal hormone therapy, we found that estrogen-induced increases in eNOS are preserved when estrogen is combined with either progesterone or MPA. It is not known, however, whether other possible neuroprotective actions of estrogen are influenced by MPA. This question can now be addressed with the use of the rat model developed in the present study.

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References

19. McNeill et al. Estrogen Increases Endothelial eNOS in Cerebral Vessels
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