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. Although clinical use of hormone replacement therapy (HRT) for stroke remains controversial, estrogen is clearly neuroprotective in experimental models of cerebral ischemia. 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 cerebral blood flow during ischemia. 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. 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.

Recent evidence suggests that estrogen acts, in part, by increasing nitric oxide (NO) in the cerebrovascular endothelium. 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 inhibition or genetic eNOS knockout, 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 protein with a parallel increase in NOS-mediated modulation of cerebrovascular contractility. 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.
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 negate the protective effects of estrogen against coronary artery hyperreactivity. MPA also attenuated the beneficial effects of estrogen on coronary atherosclerosis in monkeys, but progesterone did not. 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 and 3.18 mm outer diameter) implanted subcutaneously. Tubing lengths from 1 to 150 mm were used for administration of 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.

#### 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 mmol/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-cycloheximide.

#### Western Blot
Cerebral vessels were homogenized in 50 mmol/L β-glycerophosphate, 100 μmol/L sodium orthovanadate, 2 mmol/L magnesium chloride, 1 mmol/L EGTA, 0.5% Triton X-100, 1 mmol/L dithiothreitol, 20 μmol/L pepstatin, 20 μmol/L leupeptin. 0.1 U/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride and then incubated on ice for 20 minutes. Samples were centrifuged at 180 g for 25 minutes at 4°C, and the supernatant was collected. After protein determination (modified Lowry assay), 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 electroblotting, 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) or mouse anti-β-estradiol. To develop a model for MPA treatment, rats were subcutaneously implanted for 1 month with various lengths of Silastic tubing (2 to 120 mm) packed with MPA. 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.

Data Analysis and Statistics

Data are expressed as mean±SE. Statistical significances of Western blot and radioimmunnoassay 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 NOS activity 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 were subcutaneously implanted for 1 month with various lengths of Silastic tubing (2 to 120 mm) packed with MPA. Serum MPA levels increased in proportion with tubing length (Table 2). The 3-mm length achieved serum levels (1 ng/mL) similar to those found in humans treated therapeutically with MPA (0.8 to 1.6 ng/mL), and this size was used in subsequent studies of MPA. MPA treatment had no significant effect on serum levels of either 17β-estradiol or progesterone (Table 1).

As expected, 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.** 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.** 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.** 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.

**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 the cerebrovasculature 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 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 the cerebrovasculature 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 modulates vascular contractility of rat cerebral arteries by increasing NOS-dependent vasodilation.7,8

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Although not specifically addressed in this study, it is likely that estrogen affects transcription of eNOS in cerebral vessels. Increases in eNOS protein were evident after 18 hours but not 90 minutes of estrogen treatment in vitro, consistent with a genomic effect. Estrogen increases eNOS mRNA in cultured endothelial cells. Similarly, 18-hour in vitro incubation with estrogen increases NO release in gracilis muscle arterioles of hypertensive rats. This effect appears dependent on transcription and estrogen receptor activation; however, eNOS protein or mRNA was not measured in the latter study.

Women taking HRT are often given a combination of estrogen and a progestin such as MPA. Of concern is the possibility that MPA may negate some of the “vascular protective” effects of estrogen. Few studies have used animal models to examine interactions between estrogen and MPA; however, several reports suggest detrimental effects of MPA. In monkeys, MPA, at clinically relevant serum levels, blocked the effect of estrogen to decrease vascular smooth muscle hyperreactivity.13 MPA also prevented estrogen-induced reduction in coronary atherosclerosis in monkeys.17 In rats, estrogen-mediated inhibition of neointima formation in the carotid artery after balloon injury was attenuated by MPA, although serum levels were not measured.14

In our study, serum concentrations of 17β-estradiol, progesterone, and MPA achieved by in vivo treatment correlate with human serum levels, both physiological (for progesterone, 1 to 25 ng/mL) and therapeutic (for estrogen, 15 to 250 pg/mL; for MPA, 0.8 to 1.6 ng/mL). This treatment with progesterone and MPA effectively prevented estrogen-induced increases in uterine weight, which in normal female rats averages 0.40 ± 0.04 g. In contrast, clinically relevant doses of progesterone and MPA had no effect on the ability of estrogen to increase eNOS levels in cerebral vessels. Similarly, in vitro exposure to progesterone and MPA did not alter estrogen-induced increases in eNOS at concentrations corresponding to in vivo serum levels (1 nmol/L and 1 ng/mL, respectively). Higher concentrations of progesterone and MPA were also without effect. Therefore, estrogen-mediated increases in eNOS protein in the cerebral vasculature appear unaffected by concurrent administration of either progesterone or MPA.

Our findings are in agreement with those of several human studies showing that estrogen alone, or in combination with MPA, improves vascular function. For example, transdermal estrogen reduces internal carotid and middle cerebral artery pulsatility indexes in postmenopausal females, and MPA supplementation does not modify this effect. Postmenopausal women receiving estrogen alone or with MPA show improved ischemic vasodilator responses measured by brachial artery reactivity and increased plasma levels of total oxidized products of NO. Therefore, in humans, estrogen in combination with MPA appears to increase vasodilation, with parallel increases in NO production. However, it remains to be determined whether these effects are mediated by increases in eNOS.

In summary, we have found that in vivo estrogen treatment increases eNOS protein and NOS activity in cerebral blood 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

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