Chronic Estrogen Treatment Increases Levels of Endothelial Nitric Oxide Synthase Protein in Rat Cerebral Microvessels

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Background and Purpose—A number of studies indicate that the female gonadal hormone, estrogen, confers protection against cerebrovascular disorders such as stroke. One postulated mechanism for these effects of estrogen is an action on the enzyme endothelial nitric oxide synthase (eNOS), which produces the vasodilatory molecule NO. We have investigated the hypothesis that estrogen increases expression of eNOS in cerebral microvessels of male and female rats.

Methods—We measured levels of eNOS protein by Western blot in cerebral microvessels isolated from 7 groups of animals: females, ovariectomized females, ovariectomized females treated with estrogen, males, castrated males, castrated males treated with estrogen, and castrated males treated with testosterone.

Results—Ovariectomized female rats treated with estrogen had 17.4-fold greater levels of eNOS protein in cerebral microvessels than ovariectomized females, and intact females had 16.6-fold greater levels than ovariectomized females ($P<0.01$). In intact females, cerebral microvessel eNOS protein levels were 9.2-fold higher than those of intact males ($P<0.05$). Levels of eNOS protein in castrated males, castrated males treated with testosterone, and males were not different from each other. Estrogen treatment of castrated animals resulted in an 18.8-fold increase in cerebral microvessel eNOS protein ($P<0.05$).

Conclusions—Chronic estrogen treatment increases levels of eNOS protein in cerebral microvessels of male and female rats. This increase in eNOS protein correlates with our previous functional findings indicating that estrogen exposure increases NO modulation of cerebrovascular reactivity in both male and female animals. Upregulation of eNOS expression may contribute to the neuroprotective effect of estrogen. (Stroke. 1999;30:2186-2190.)

Key Words: cerebral vessels • estrogens • gender • nitric oxide synthase • rats

A number of studies indicate that the female gonadal hormone, estrogen, confers protection against stroke. For example, during middle cerebral artery occlusion (MCAO), a model for stroke, untreated female rats sustain approximately one third of the total tissue infarction observed in males or ovariectomized females. Treatment of male rats with estrogen protects male brains during MCAO, reducing infarct volume by similar amounts. Structural and functional consequences of common carotid artery thrombosis are worsened by endothelial nitric oxide synthase (eNOS) inhibition, indicating that eNOS protects against brain injury. Action of estrogen on the enzyme eNOS may contribute to the protective effects of this hormone. We recently demonstrated that estrogen treatment increases nitric oxide synthase (NOS)–mediated modulation of cerebrovascular reactivity in gonadectomized female and male rats. Cerebral microvessels contribute a considerable portion of the total resistance to blood flow and are important in the coupling of brain metabolism to regional blood flow. Therefore, we investigated the hypothesis that estrogen upregulates expression of eNOS in cerebral microvessels by measuring levels of eNOS protein in animals of differing hormonal status.

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Materials and Methods

Animal Treatment

Seven groups of rats were compared: females, ovariectomized females, ovariectomized females treated with estrogen, males, castrated males, castrated males treated with estrogen, and castrated males treated with testosterone. Gonadectomies were performed on 3-month-old Fischer 344 rats (Harlan Sprague-Dawley) anesthetized with ketamine 90 mg/kg and xylazine 10 mg/kg IP. In some animals, hormone treatment (estrogen or testosterone) was started at the time of gonadectomy by implanting hormone-filled silicone elastomer capsules subcutaneously. The capsules remained in place until the animal was killed. Animals were killed by decapitation 4 weeks after gonadectomy, and brains were immediately frozen at $-20^\circ$C. Serum levels of estradiol were determined with the use of a double-antibody $^{125}$I radioimmunoassay kit (Diagnostic Products) as previously described. Serum levels of testosterone were determined with the use of a double-antibody $^{125}$I radioimmunoassay kit (ICN Biochemicals).
Microvessel Isolation
Cerebral microvessels were isolated from rat brain parenchyma by methods described previously. Briefly, 4 brains were pooled, homogenized gently with a Dounce tissue grinder in ice-cold PBS (0.01 mol/L, pH 7.4), and centrifuged (with the use of a Beckman GS15R swinging bucket rotor) at 2000 g for 10 minutes at 4°C. The supernatant was then discarded, and the pellet was washed by resuspension in PBS and recentrifuged at 4000 g for 10 minutes. The supernatant was discarded; the pellet was resuspended in PBS, gently rinsed with a strong stream of cold PBS, and centrifuged at 2000 g for 10 minutes. The final pellet was washed by resuspension in PBS, layered over dextran, and centrifuged at 2000 g for 10 minutes. The supernatant was then discarded; the pellet was resuspended in PBS, gently layered on top of a dextran solution (15%; molecular weight, 2000,000) and centrifuged at 3500 g for 55 minutes. The pellet was then collected, resuspended in PBS, layered over dextran, and centrifuged at 4000 g for 20 minutes. The final pellet was poured over a nylon mesh screen (50 μm) and washed extensively with a strong stream of cold PBS. The microvessel fraction, containing small arterial stromal, venules, and capillaries, was collected from the top of the screen and stored at −20°C.

Lysis and Protein Content Determination
Microvessel samples were glass homogenized in a lysis buffer consisting of 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 N,N-dithiothreitol, 20 μmol/L pepstatin, 20 μmol/L leupeptin, 0.1 U/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride, then incubated on ice for 20 minutes. Next, samples were centrifuged at 180 g for 25 minutes at 4°C, and the supernatant was collected and stored on ice for protein determination (by a modified Lowry assay) and analysis by SDS-PAGE/Western blot. As a positive control for eNOS, cultured human umbilical vein endothelial cells (generously provided by Christopher Hughes, University of California at Irvine) were lysed in the same manner. Lysed macrophages and brain homogenate were supplied by Transduction Laboratories as a positive control for inducible NOS (iNOS) and neuronal NOS (nNOS), respectively.

SDS-PAGE/Western Blot
For each animal group, 20 μg of microvessel protein was loaded onto 8% Tris-glycine gels and separated by SDS-PAGE. Endothelial cell lysate and biotinylated broad-range molecular weight markers (Bio-Rad) were loaded onto the gels as well. After electrophoretic separation, proteins were transferred to a nitrocellulose membrane by electroblotting, and membranes were incubated overnight at 4°C in blocking buffer (PBS containing 1% Tween-20 [T-PBS] and 6.5% nonfat dry milk). Membranes were then incubated for 4 hours at room temperature with either a monoclonal mouse anti-eNOS, anti-iNOS, or anti-nNOS antibody (Transduction Laboratories, 1:10 000 dilution in blocking buffer). Membranes were rinsed with T-PBS for 30 minutes, incubated at room temperature with either a monoclonal mouse anti-eNOS, anti-iNOS, or anti-nNOS antibody (Transduction Laboratories, 1:500 dilution in blocking buffer), rinsed with T-PBS for 30 minutes, and incubated with anti-mouse IgG horseradish peroxidase (Transduction Laboratories, 1:10 000 dilution in blocking buffer). Membranes were rinsed with T-PBS for 30 minutes, incubated with electrochemiluminescence reagent (Amersham) for 2 minutes, and apposed to hyperfilm (Amershams).

Data Analysis and Statistics
Densitometric analyses were performed with a computer-based image analysis system (MCID). In each of 4 separate experiments, microvessels from all 7 treatment groups were isolated and analyzed in parallel. In addition, measurements were made in duplicate in 3 of the 4 experiments. Data are expressed as mean±SE. Statistical significance of Western blot and radioimmunoassay data was determined by ANOVA with the Prism software package, with repeated measures used for the Western blot data. Post hoc analysis was performed with Bonferroni's multiple comparison test (Prism software package). With comparisons made as follows: females versus males; ovariectomized females treated with estrogen and females versus ovariectomized females; and males, castrated males treated with estrogen, and castrated males treated with testosterone versus castrated males. Acceptable level of significance was defined as P<0.05.

Results
As indicated in the Table, serum estrogen levels were greater in females and ovariectomized females treated with estrogen compared with ovariectomized females (both P<0.001), in females compared with males (P<0.01), and in castrated males treated with estrogen compared with castrated males (P<0.05). Testosterone treatment of castrated males in-
creased serum testosterone levels \( P<0.001 \) but not serum estrogen levels. Females had significantly lower body weights than males \( P<0.001 \), and surgery and/or hormone treatment had no significant effect on body weight in males or females. Uterine weight was significantly higher in females and ovariectomized females treated with estrogen compared with ovariectomized females (both \( P<0.001 \)).

Immunoreactivity for eNOS was detected as a 140-kDa band in all microvessel preparations studied. Only 1 band was observed, and it corresponded to that of the positive control (cultured endothelial cells). Omission of the eNOS primary antibody or use of a negative control tissue (macrophages) resulted in omission of the band of interest (data not shown). No iNOS or nNOS immunoreactivity was detected in the isolated microvessels, although bands were detected for each of the positive controls (macrophages or brain homogenate, respectively; data not shown).

In females, estrogen treatment of ovariectomized animals resulted in a 17.4-fold increase in eNOS protein in cerebral microvessels, while intact females had 16.6-fold higher protein levels than ovariectomized females (both \( P<0.01 \); Figures 1A and 2A). In intact females, eNOS protein levels in cerebral microvessels were 9.2-fold higher than those of intact males \( P<0.05 \); Figure 1A).

In males, neither castration nor testosterone treatment of castrated males altered levels of cerebral microvessel eNOS protein. Estrogen treatment of castrated animals resulted in an 18.8-fold increase in eNOS protein in cerebral microvessels \( P<0.05 \); Figures 1B and 2B).

**Discussion**

This study demonstrates 4 important findings. First, estrogen treatment increases the amount of eNOS protein in cerebral microvessels of both male and female gonadectomized rats. Second, neither castration of intact males nor testosterone treatment in castrated male rats alters the amount of eNOS protein. Third, ovariectomized female rats have less eNOS protein than intact females. Finally, intact female rats have greater levels of eNOS protein than intact males. These findings clearly demonstrate that exogenous or endogenous estrogen, but not testosterone, increases levels of eNOS protein in cerebral microvessels.

While exogenous or endogenous estrogen may have many sites of action in the animal, a direct effect on microvessel eNOS expression is likely. Genomic effects of chronic estrogen treatment on eNOS have been shown in other systems. Ovine pulmonary artery endothelial cells incubated with estrogen in culture show an increase in NOS activity as well as increases in eNOS mRNA and protein levels, and these effects are inhibited by treatment with ICI 182780, an estrogen receptor antagonist. In cultured human endothelial cells, estrogen increases eNOS mRNA and protein via a receptor-mediated system, without affecting eNOS mRNA stability, and enhances binding of transcription factor Sp1, which is essential for the activity of the human eNOS promoter. In the rat aorta, eNOS mRNA increases with pregnancy or estrogen treatment but not with progesterone or testosterone treatment. Therefore, it is likely that chronic estrogen treatment specifically increases both transcription and translation of eNOS, resulting in higher levels of eNOS protein throughout the vascular endothelium.

**Figure 1.** Effects of chronic estrogen treatment on eNOS protein in cerebral microvessels from male and female rats. eNOS protein was detected by SDS-PAGE/Western blot analysis with the use of an anti-eNOS antibody. Equivalent amounts of protein extracted from cerebral microvessels from various groups of animals were run on the lanes as marked. The Western blots shown are representative of 4 experiments, 3 of which were done in duplicate. Brains from 4 animals were pooled for each group in an experiment. Protein molecular weight markers are indicated in kilodaltons. A, Immunoblot showing eNOS protein in cerebral microvessels isolated from ovariectomized females (O), untreated females (F), and untreated males (M). B, Immunoblot showing eNOS protein in cerebral microvessels isolated from untreated males (M), castrated males (C), castrated males with estrogen treatment (CE), and castrated males with testosterone treatment (CT).

**Figure 2.** Effect of chronic estrogen treatment on levels of eNOS protein in cerebral microvessels from male and female rats. Optical density of eNOS-positive bands was quantified by scanning densitometry and plotted relative to either ovariectomized females (A) or castrated males (B) from the same experiment. Values are mean \( \pm \) SE; \( n=4 \). A, Levels of eNOS protein in cerebral microvessels from ovariectomized females with estrogen replacement (OE), ovariectomized females (O), untreated females (F), and untreated males (M) are shown. *Significantly different from ovariectomized females, \( P<0.01 \); #Significantly different from untreated males, \( P<0.05 \). B, Levels of eNOS protein in cerebral microvessels from untreated males (M), castrated males (C), castrated males with estrogen treatment (CE), and castrated males with testosterone treatment (CT) are shown. *Significantly different from castrated males, \( P<0.05 \).
How these findings might relate to protection from stroke is not completely clear. Chronic estrogen treatment results in improved stroke outcome after vascular occlusion in both male and female rats.1,2 Some of the actions of estrogen that might contribute include the following: increased availability of vasodilatory molecules (such as NO), increased angiogenesis, functional differences in vascular reactivity, or antioxidant activity.3 After MCAO, female rats have a smaller infarct size in both cerebral cortex and caudoputamen compared with males and ovariectomized females. Furthermore, during MCAO, female rats have higher laser-Doppler flow in the cerebral cortex and caudoputamen compared with male and ovariectomized female rats, suggesting that a flow-preserving effect of estrogen mediates neuroprotection in these areas. However, end-ischemic cerebral blood flow is greater in females than in males and ovariectomized females only in the caudoputamen (not in the cerebral cortex). Furthermore, when areas with similar levels of very low cerebral blood flow are compared, intact females sustain smaller infarction volumes than do ovariectomized females.1 This implies that estrogen may improve stroke outcome by more than 1 mechanism, exerting both flow-preserving as well as other neuroprotective effects.

Whether the flow-preserving effect of estrogen during ischemia is mediated via increased eNOS activity is not known. In nonischemic conditions, there are no differences in laser-Doppler flow rates in the caudoputamen or cerebral cortex when female, male, and ovariectomized female rats are compared.1 However, in parallel to this study, our laboratory has shown that estrogen reduces myogenic tone in rat middle cerebral arteries through an \( N^2 \)-nitro-L-arginine methyl ester (L-NAME)–sensitive mechanism, suggesting that NO production and/or sensitivity is greater when estrogen is present.4,5 Because the results from the present study suggest that more eNOS protein is present in the cerebral circulation of estrogen-treated animals, it is likely that this functional effect of estrogen treatment is mediated by greater NO production in response to myogenic tone. While increased levels of eNOS may not affect resting blood flow, the capacity of females to respond to conditions of altered flow or perfusion pressure may be enhanced by estrogen treatment.

A number of studies in both male and female humans suggest that chronic estrogen exposure increases the capacity of the endothelium to produce NO in response to changes in flow. For example, estrogen replacement in postmenopausal women both decreases blood pressure14 and increases the flow. For example, estrogen replacement in postmenopausal women decreases blood pressure and increases the flow. For example, estrogen replacement in postmenopausal women decreases blood pressure and increases the flow. For example, estrogen replacement in postmenopausal women decreases blood pressure and increases the flow. For example, estrogen replacement in postmenopausal women decreases blood pressure and increases the flow. For example, estrogen replacement in postmenopausal women decreases blood pressure and increases the flow.

In summary, many studies suggest that estrogen acts in males and premenopausal and postmenopausal females to increase levels of NO, which then mediate vasodilation. The present study indicates that this increase in NO production may be secondary to an effect of estrogen to increase levels of eNOS protein in both males and females. Together with our previous studies showing an NO-dependent effect of estrogen to modulate cerebral vascular reactivity, these studies support the hypothesis that an increase in eNOS protein due to estrogen treatment may contribute to the flow-preserving neuroprotective effects of estrogen.

Acknowledgments

This work was supported in part by National Institutes of Health grant R01HL07775 and an Alpha Omega Alpha Student Research Fellowship. The authors thank Jonnie Sephus for the radioimmunoassay and animal surgeries. Greg Geary, PhD, for animal surgeries, and Susan Javaheri for the iNOS experiments.

References

It is well established that estrogen has a protective effect against stroke. The accompanying article by McNeill and colleagues sheds light on one of the mechanisms by which this effect is mediated. The authors showed that estrogen treatment caused increases in the amount of eNOS protein in cerebral microvessels in both male and female gonadectomized rats. They also found that ovariectomy in female rats caused a decrease in eNOS protein. In addition, female rats had greater amounts of eNOS protein than male rats. Testosterone did not appear to have any effect on cerebral eNOS concentration. It therefore appears likely that the protective effect of estrogen against stroke may be mediated for the most part through increased generation of eNOS, which results in increased production of NO and in vasodilation.

There is some evidence in the literature, as the authors point out, that suggests that this effect of estrogen may be also present in humans. Studies of this type may ultimately explain more fully the sex-related differences in the incidence of stroke and sex-related differences in the consequences of ischemia that have long puzzled physicians and scientists.

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Stroke. 1999;30:2186-2190
doi: 10.1161/01.STR.30.10.2186
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/30/10/2186

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