Effect of estrogen on cerebrovascular prostaglandins is amplified in mice with dysfunctional NOS

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Li, Xiangduan, Greg G. Geary, Rayna J. Gonzales, Diana N. Krause, and Sue P. Duckles. Effect of estrogen on cerebrovascular prostaglandins is amplified in mice with dysfunctional NOS. *Am J Physiol Heart Circ Physiol* 287: H588–H594, 2004. 10.1152/ajpheart.01176.2003.—Chronic estrogen treatment increases endothelial vasodilator function in cerebral arteries. Endothelial nitric oxide (NO) synthase (eNOS) is a primary target of the hormone, but other endothelial factors may be modulated as well. In light of possible interactions between NO and prostaglandins, we tested the hypothesis that estrogen treatment increases prostanoid-mediated dilation using NOS-deficient female mouse models, i.e., mice treated with a NOS inhibitor [N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME)] for 21 days or transgenic mice with the eNOS gene disrupted (eNOS<sup>−/−</sup>). All mice were ovariectomized; some in each group were treated chronically with estrogen. Cerebral blood vessels were isolated for biochemical and functional analyses. In vessels from control mice, estrogen increased protein levels of eNOS but had no significant effect on prostacyclin production. However, when NO production was dysfunctional, there is often compensatory upregulation of COX-1 levels, constriction to indomethacin, and functional analyses. In vessels from eNOS<sup>−/−</sup> mice, estrogen treatment increased levels of COX-1 protein and constriction to indomethacin. In L-NAME-treated mice, however, cerebrovascular NO effects were mediated via estrogen receptor (ER)-α (11), which is present on cerebral arterial endothelium (40).

Evidence suggests estrogen may also upregulate endothelial production of the dilator prostaglandin PGI<sub>2</sub> (17, 28, 32, 33, 35, 42). However, data from cerebral blood vessels are not straightforward. In the rat, estrogen treatment enhances cerebrovascular PGI<sub>2</sub> synthesis by elevating levels of cyclooxygenase (COX)-1 and PGI<sub>2</sub> synthase proteins (32, 33). However, there is no functional consequence of this effect when vascular reactivity of first-order branches of the female rat MCA are studied (9). In this artery, there appears to be an inverse relationship between the role of PGI<sub>2</sub> and vessel diameter (32), in contrast to NO, which plays a more prominent role in the larger diameter branches (9). When smaller branches of the rat MCA are studied, estrogen does indeed increase COX-dependent dilation as well as endothelial PGI<sub>2</sub> production (32). In mouse cerebral arteries, the study of estrogen modulation is complicated by apparent interactions among endothelial factors. COX inhibition has no effect on vascular tone in vitro unless NOS is inhibited (10). However, after estrogen treatment of ovariectomized (OVX) mice, NOS inhibition has a greater effect on isolated cerebral artery diameter but only when a COX inhibitor is present (10). These data suggest that estrogen increases NO production as well as compensation by a prostanoid dilator in mouse cerebral arteries.

Because of possible interactions between the NOS and COX dilator pathways, it is difficult to determine whether estrogen directly influences PGI<sub>2</sub> production or whether the effect of estrogen is dependent on NO, because it has been shown to increase PGI<sub>2</sub> in some studies (6, 13, 45). In the former case, PGI<sub>2</sub> could compensate for the loss of NO, but this would not be true in the latter situation. It is also possible that when NO is present, it suppresses PGI<sub>2</sub> production (1, 8, 14, 19, 20). If...
so, estrogen-induced increases in NO production may mask the functional effects of concomitant increases in the COX-1-PGI2 pathway. The goal of the present study was to address these questions by examining effects of estrogen on COX-dependent dilation in the absence of NO. To accomplish this, we used two animal models of NOS dysfunction: transgenic mice with the eNOS gene disrupted [eNOS−/− (37)] and wild-type mice treated for 21 days with an inhibitor of NOS in the drinking water (18). For each condition, female mice were divided into two groups: OVX and OVX with estrogen replacement (OVX + E). Cerebral blood vessels were isolated, and levels of COX-1 protein as well as basal production of PGI2 were measured. To assess functional effects of endothelium-derived factors, the diameters of isolated, pressurized MCAs were recorded in the presence and absence of NOS and COX inhibitors.

MATERIALS AND METHODS

Animals. Animal procedures were approved by the Animal Care and Use Committee of the University of California-Irvine. Female C57/B6 and eNOS−/− mice were supplied from Jackson Laboratories (Bar Harbor, ME). The latter animals were obtained by targeted disruption of the eNOS gene in C57B/6 mice (37). Mice were housed under a 12:12-h light-dark cycle with food and water available ad libitum. All mice were OVX, and some were also treated with estrogen (OVX + E). Ovariectomy and estrogen replacement were performed while the animals were under anesthesia (90 mg/kg ketamine and 10 mg/kg xylazine). Estrogen was replaced at the time of ovariectomy by subcutaneous insertion of a 1-mm silicone elastomer capsule made from Dow Corning Silastic medical grade tubing (1.57 mm inner diameter × 3.18 mm outer diameter), sealed with silicone elastomer adhesive type A (Dow Corning) and packed with 17β-estradiol. We have previously shown that this method of estrogen replacement results in serum estrogen levels within the physiological range (10). All animals were euthanized 3–4 wk after surgery.

OVX and OVX + E animals were compared in three different groups of mice: wild-type C57/B6 OVX (C57/B6 and C57/B6 + E), eNOS−/− OVX (eNOS−/− and eNOS−/− + E), and wild-type C57/B6 OVX mice treated chronically with an inhibitor of NOS, l-NAME (l-NAME and l-NAME + E). In the latter group, 1.5 mg/ml l-NAME was added to the drinking water of C57/B6 and C57/B6 + E mice for 21 days before euthanasia (18).

Tissue preparation. Mice were euthanized in the middle of the day by exposure to CO2. The uterus was removed from each animal, dried, and weighed. Brains were rapidly removed from the cranial cavity and placed in cold physiological salt solution (PSS) containing (in mM) 118 NaCl, 4.8 KCl, 1.6 CaCl2, 1.2 KH2PO4, 25 NaHCO3, 1.2 MgSO4, and 11.5 glucose, equilibrated with 95% O2-5% CO2. For the measurement of contractile responses, 1- to 2-mm segments of the MCA were carefully dissected and studied on the same day. Alternatively, 4-mm segments from both sides of the MCA were carefully dissected for prostanooid assay. The brains were then frozen at −80°C until use for whole brain blood vessel isolation and Western blot analysis.

Prostanoid assay. MCA segments were incubated in HEPES buffer containing (in mM) 10 HEPES, 130 NaCl, 4 KCl, 4 MgSO4, 4 NaHCO3, 1.8 CaCl2, 1.18 KH2PO4, 6 dextrose, and 0.025 EDTA and maintained in a tissue culture incubator with 95% O2-5% CO2 for 6 h. Samples of the media were then collected and stored at −80°C for subsequent prostanooid assay. PGI2 was determined as the stable metabolite, 6-keto-PGF1α, using the enzyme-linked immunosorbent assay kit (Amersham) according to the protocol provided by the manufacturer.

Cerebral vessel isolation. One to two brains from each treatment group were pooled, homogenized with a loosely fitting Dounce tissue grinder in ice-cold 0.01 M (pH 7.4) PBS, and then centrifuged at 3,500 rpm for 5 min at 4°C. The supernatant was discarded, and the pellet was washed several times by resuspension in PBS followed by centrifugation at 3,500 rpm for 5 min. The pellet was then resuspended in PBS, gently layered over 15% dextran (mol. mass, 43 kDa), and finally centrifuged at 5,000 rpm for 30 min at 4°C. Pellets containing blood vessels were collected over a 50-μm nylon mesh and washed for several minutes with cold PBS. Isolated vessels, inspected by light microscopy, were a mixture of arteries, arterioles, veins, venules, and capillaries.

Protein blot analysis. Isolated vessels were incubated in lysis buffer (containing 50 mM β-glycerophosphate, 100 μM NaVO3, 2 mM MgCl2, 1 mM EGTA, 0.5% Triton X-100, 1 mM dl-dithiothreitol, 20 μM pepstatin, 0.1 U/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) on ice for 20 min. After homogenization, samples were centrifuged at 5,000 rpm for 10 min at 4°C. Supernatants were collected, and protein content was determined by a modified Lowry assay. Equal amounts of protein isolated from OVX and OVX + E animals (20 μg) were loaded onto 8% Tris-glycine gels and separated by SDS-PAGE. Biotinylated molecular mass markers (Bio-Rad) were loaded at the same time. After electrophoretic separation, protein was transferred to nitrocellulose membranes (Amershams), which were then incubated overnight at 4°C in blocking buffer (0.01 M PBS, 0.1% Tween 20, and 6.5% nonfat dry milk). All primary antibodies were mouse anti-eNOS (Transduction Laboratories). Human endothelial cell lysate was used as a positive control. COX-1 was detected with mouse anti-COX-1 polyclonal antibody (Cayman Chemical), and the positive control was a lysate of RAW 264.7 macrophages. Anti-mouse IgG antibody conjugated to horseradish peroxidase (Transduction Laboratories) was used as the secondary antibody, and electrochemiluminescence reagent and Hyperfilm (both from Amershams) were used to image protein levels. The computer-based electrophoresis analysis program UN-SCAN-IT (Silk Scientific) was used for densitometric quantification of the films. For each animal model, band densities from OVX + E were expressed as the fold increase over OVX samples run on the same gel.

Contractile studies. A 1- to 2-mm segment of the MCA, taken ~1 mm from the circle of Willis, was cannulated and mounted in an arteriograph (Living Systems; Burlington, VT) as described previously (10). All experiments were conducted under no-flow conditions. A constant-flow peristaltic pump continuously superfused (30 ml/min) the artery with PSS. During the 60-min equilibration period, a pressure servo system maintained transmural pressure at 40 mmHg. The artery was viewed with an inverted microscope equipped with a video camera and monitor. A video-electronic dimension analyzer was used to measure luminal diameter and wall thickness defined as distance from the inside to the outside arterial edge. Changes in transmural pressure and lumen diameter were digitized by a MacLab analog-to-digital converter and recorded on a Macintosh computer.

Changes in artery diameter at two transmural pressures (40 and 80 mmHg) were measured under the following conditions: 1) PSS, 2) in the presence of l-NAME (100 μM), 3) in the presence of l-NAME plus indomethacin (10 μM), and 4) in the presence of 0 mM Ca2+ -EDTA (3 mM) plus sodium nitroprusside (100 μM). The last condition defined the passive response of the vessel. Maximum passive diameters of the isolated arteries (80 mmHg) were similar for all of the animal groups studied and were not affected by chronic estrogen treatment (C57/B6: 155 ± 2 μm vs. estrogen treated: 154 ± 2 μm; l-NAME treated: 154 ± 2 μm vs. estrogen-treated: 155 ± 3 μm; eNOS−/−: 148 ± 1 μm vs. estrogen-treated: 152 ± 1.5 μm; P > 0.05, ANOVA). In the case of cerebral arteries from l-NAME-treated mice, the first condition (PSS) was omitted, so all studies of vessels from these animals were done in the presence of l-NAME. All drugs were perfused for 20 min before the first pressure step, and each pressure step (10 mmHg) was maintained for 5–10 min to allow the vessel to reach a stable condition before diameter was measured. Control arteries showed consistent responses to four sequential series of

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pressure steps. Indomethacin-induced constriction was determined by subtracting the steady-state diameter in L-NAME alone from the steady-state diameter with indomethacin plus L-NAME. All drugs were purchased from Sigma Chemical (St. Louis, MO).

Data analysis. Data are expressed as mean ± SE, and data from OVX and OVX + E animals were compared within each treatment group (C57/B6, eNOS−/−, and t-NAME treated). For measurements of 6-keto-PGF₁₀, body weight, and uterine weight, statistical significance was compared between OVX and OVX + E mice by unpaired Student’s t-test for each treatment group. For measurements of Western blot band densities, a paired analysis was used to compare optical density compared with C57B6 mass and positive control for eNOS. Representative bands migrating at 140 kDa correspond to the molecular mass of eNOS.

Table 1. Effect of estrogen treatment on body and uterine weights and middle cerebral artery wall thickness

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Body Weight, g</th>
<th>Uterine weight, mg</th>
<th>Wall thickness, μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57B/6</td>
<td>24.5±0.5</td>
<td>2.9±0.3</td>
<td>6.7±0.7</td>
</tr>
<tr>
<td>C57B/6 + E</td>
<td>24.1±0.3</td>
<td>30.2±3*</td>
<td>7.0±0.5</td>
</tr>
<tr>
<td>t-NAME treated</td>
<td>22.0±0.4</td>
<td>5.7±0.8</td>
<td>7.1±0.5</td>
</tr>
<tr>
<td>t-NAME treated + E</td>
<td>23.6±0.5*</td>
<td>25.2±2*</td>
<td>7.7±0.9</td>
</tr>
<tr>
<td>eNOS−/−</td>
<td>21.6±0.4</td>
<td>3.2±0.1</td>
<td>7.0±0.4</td>
</tr>
<tr>
<td>eNOS−/− + E</td>
<td>20.7±0.5</td>
<td>29.2±3*</td>
<td>7.3±0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE; N, number. t-NAME, Nω-nitro-l-arginine methyl ester; E, estrogen; eNOS, endothelial nitric oxide synthase. All mice were ovariectomized (OVX). *Significantly different from respective OVX group without estrogen (P < 0.05).

RESULTS

Body and uterine weights. Body and uterine weights for the various animal groups studied are shown in Table 1. As expected, estrogen treatment significantly increased the dry uterine weight of all groups of OVX mice: C57/B6, eNOS−/−, and l-NAME treated. The uterine weights of estrogen-treated mice were similar to what was found in ovary-intact female mice (10); thus it appears that appropriate levels of hormone were achieved with the estrogen implants. With regard to body weight, estrogen treatment had no effect in C57/B6 control and eNOS−/− mouse groups. However, in t-NAME-treated mice, there was a statistically significant increase in body weight with estrogen treatment. There were no significant effects of estrogen treatment on arterial wall thickness (Table 1).

C57/B6 control mice. As we have demonstrated previously in both rats and mice (11, 25, 40), chronic estrogen treatment results in a significant increase in the levels of cerebrovascular eNOS (Fig. 1A). In contrast, the levels of COX-1 protein were not significantly different between vessels from estrogen-treated and control C57/B6 mice (Fig. 1B). Furthermore, there was no effect of estrogen treatment on prostacyclin production in cerebral vessels from wild-type C57/B6 mice (Fig. 1C).

The contribution of prostaglandins to vascular tone was assessed in isolated, pressurized arteries using the nonselective COX inhibitor indomethacin. As shown in Fig. 1D, arteries from C57/B6 mice constricted to indomethacin (10 μM) in the presence of l-NAME. These findings suggest that production of a dilator prostaglandin, most likely prostacyclin, modifies vascular diameter in these isolated, pressurized cerebral arteries. Estrogen treatment, however, did not significantly affect constriction to indomethacin in cerebral arteries from C57/B6 mice.

Thus, in wild-type C57/B6 mice, estrogen caused an increase in eNOS levels, but no apparent effect on the COX-1 pathway, as assessed by measuring COX-1 levels, PGI₂ production and constriction after COX blockade. This result was...
unexpected based on the clear effects of estrogen that we previously demonstrated on the COX pathway and COX-dependent dilation in rat cerebral vessels (32, 33). Because of potential interactions between NO- and COX-dependent pathways (1, 23, 34) and effects of estrogen on eNOS, we hypothesized that NO may confound our ability to assess effects of estrogen on COX-dependent dilation. Therefore, we studied two models of NOS dysfunction: l-NAME-treated and eNOS−/− mice.

**l-NAME-treated mice.** After treatment of C57/B6 mice for 21 days with l-NAME, effects of estrogen observed in cerebral blood vessels were altered compared with mice that were not treated with l-NAME. As shown in Fig. 2, chronic estrogen implants in l-NAME-treated OVX mice significantly increased levels of eNOS. However, in contrast to mice not exposed to l-NAME, there was also a significant increase in COX-1 in cerebral vessels from l-NAME + E mice. Furthermore, in contrast to C57B6 mice that were not exposed to l-NAME, estrogen exposure of l-NAME-treated mice resulted in significant increases in prostacyclin production (Fig. 2C). In addition, as shown in Fig. 2D, arteries from l-NAME-treated mice exposed to estrogen showed significantly greater constriction to indomethacin compared with arteries from l-NAME OVX mice.

**eNOS−/− mice.** Levels of COX-1 were also significantly increased after estrogen treatment in cerebral arteries from eNOS−/− mice (Fig. 3A). However, no significant increase in prostacyclin production was measured in arteries isolated from eNOS−/− mice. Interestingly, constriction to indomethacin (in the presence of l-NAME in the bath) was significantly increased by estrogen treatment in arteries isolated from eNOS−/− mice (Fig. 3C). This effect, however, was not as great as the effect of estrogen on constriction to indomethacin seen in arteries from l-NAME-treated mice (compare Figs. 2D and 3C). As expected, eNOS protein was not detected in cerebral blood vessels from eNOS−/− mice (data not shown).

**DISCUSSION**

The key finding of this study of mouse cerebral arteries is that, when NOS function is chronically disrupted, the impact of estrogen on COX-dependent vasodilation is revealed. Two complementary approaches were used to prevent NOS function in vivo: transgenic disruption of the eNOS gene and chronic treatment with an inhibitor of NOS, l-NAME. In contrast to findings in wild-type mice, in both l-NAME-treated and eNOS−/− mice, estrogen treatment resulted in significant increases in levels of COX-1 and constriction to indomethacin. In l-NAME-treated mice, estrogen treatment also caused a significant increase in cerebrovascular prostacyclin production, but this was not detected in vessels from eNOS−/− mice. These findings indicate that important interactions among endothelial factors impact the net effect of estrogen. Because estrogen enhances production of more than one endothelium-dependent dilator, hormone-mediated decreases in cerebral vascular tone may be maintained if one factor, e.g., NO, is compromised.

In OVX control mice, estrogen treatment increased levels of cerebrovascular eNOS protein, as shown previously (11, 39). This finding correlates well with the effect of estrogen to increase eNOS mRNA (39) and l-NAME-sensitive dilation in mouse cerebral vessels (10, 11). In animals with disrupted ERα, these effects do not occur (11), suggesting that this receptor, which is present in cerebral vessels (40), mediates the increase in eNOS protein and function. In the present study, the ability of estrogen to elevate eNOS levels persisted during chronic exposure to l-NAME, indicating that NO, per se, is not responsible for this effect. Cerebral vessels from eNOS−/− mice did not express eNOS protein, as expected (30, 37).
The diameter difference between exposure to L-NAME (100 mM) alone and in the presence of Indo. Drug solutions were superfused for 20 min before constriction was determined at 40 or 80 mmHg (N = 6).

To understand the complex results of this study, several key interrelated questions will be addressed. First, through what routes could estrogen influence the prostaglandin pathway, and does estrogen increase PGI2 production indirectly by altering NO production? Another important question is: How could removal of eNOS or blockade of NOS function unmask an action of estrogen on the prostaglandin pathway?

Through what routes could estrogen influence the prostaglandin pathway? In several studies of cultured endothelial cells, NO was found to increase PGI2 production (6, 13, 45); thus one hypothesis is that estrogen indirectly increases cerebrovascular PGI2 by increasing NO. However, in the present study, the action of estrogen on prostaglandin-mediated dilation is actually enhanced after chronic suppression of NO. This supports the proposition that estrogen directly modulates the PGI2 synthetic pathway (4, 35) by, for example, genomic upregulation of COX-1 expression (17). In rat cerebral blood vessels, estrogen treatment enhances production of endothelial-derived PGI2 and increases levels of the synthesizing enzymes, COX-1 and PGI2 synthase (32, 33). In the present study, estrogen treatment also elevated COX-1 protein in cerebral vessels from L-NAME-treated and eNOS+/− mice, consistent with the hypothesis that estrogen directly upregulates expression of the synthetic pathway for PGI2 in the cerebral circulation.

In the mouse MCA, both NO and prostanoïds appear to contribute to endothelium-dependent dilation (10). Thus we were surprised that, in the present study, estrogen treatment had no effect on the indomethacin-sensitive contribution to vascular tone. However, when NO production was chronically inhibited in mice, either through pharmacological or genetic manipulation, the effect of estrogen on the COX pathway was manifest. This leads us to the second important question. Because, in the mouse cerebrovascular, estrogen can up-regulate COX-1 protein and prostacyclin production, apparently by an action independent of NO, why was this effect only seen when there was chronic NOS dysfunction? There appears to be at least two possibilities: NO inhibits the production of prostacyclin, or NO prevents the action of estrogen to upregulate COX-1. Of course these possibilities are not mutually exclusive.

It is clear from the work of others that chronic NOS inhibition does indeed increase prostaglandin-mediated vasodilation. For example, dilation to acetylcholine becomes substantially indomethacin sensitive in coronary arteries from eNOS−/− compared with wild-type mice (21). In skeletal muscle arterioles from eNOS−/− mice, flow-dependent vasodilation is entirely dependent on an indomethacin-sensitive mechanism, in contrast to the critical role of both NOS and COX in wild-type mice (41). NO was shown to inhibit prostacyclin production in a number of studies in which exogenous NO or NO donors were applied directly to vascular tissue (8, 19, 22, 23, 44). NO or its metabolite peroxynitrite may directly alter enzymatic activity of the hemeproteins COX (5, 36, 43) and PGI2 synthase (14, 44), but these data are not conclusive. Other evidence suggests NO may indirectly affect COX activity by increasing cGMP levels and PKG activity (8, 22, 23) or by modulating K+ channels (13). One or more of these mechanisms may contribute to the apparent suppression of PGI2-mediated dilation by NO in mouse cerebral arteries.

Inhibition of NO can also increase release of constrictor prostanoïds, as shown in cremaster muscle arterioles (20). Endothelial production of constrictor prostanoïds, such as PGH2, predominates in rat cerebral arteries after ovariectomy, whereas estrogen treatment causes a shift to dilator prostanoïds, primarily PGI2 (32). If similar phenomena were present in mice with NOS dysfunction, they would contribute to the differences observed in cerebral artery responses to indomethacin.

Our study suggests that NO may suppress the ability of estrogen to increase protein levels of COX-1 in cerebral ves-

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**Fig. 3.** Cerebrovascular effects of in vivo estrogen treatment of OVX eNOS−/− mice. Results from eNOS−/− mice are shown by open bars; those from eNOS+/− + E by hatched bars. *Significantly different (P < 0.05) compared with eNOS−/− mice. A: Western blot analysis of COX-1 protein. Protein bands migrating at 70 kDa correspond to the molecular mass and positive control for COX-1 (N = 4). B: prostacyclin production was measured as the stable metabolite 6-keto-PGF1α in media incubated with isolated middle cerebral arteries (N = 7). C: effect of estrogen treatment on constriction to Indo. Endothelium-intact pressurized cerebral arteries were studied. Constriction to Indo (expressed as the change in μm) was calculated as the diameter difference between exposure to L-NAME (100 mM) alone and in the presence of Indo. Drug solutions were superfused for 20 min before constriction was determined at 40 or 80 mmHg (N = 6).
Estrogen may not enhance EDHF compensation in cerebral arteries. In our study, but a recent report suggests that estrogen modulation of endothelium-dependent dilation may differ among vascular beds depending on the relative contributions and interactions of the various endothelial factors. In the cerebral circulation, estrogen clearly upregulated production of NO by eNOS and PG12 by COX-1. The latter effect can be suppressed by NO; thus it is most evident in smaller vessels with a minor NO component (32) or under conditions of NO dysfunction.

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