Estrogen regulates myogenic tone in pressurized cerebral arteries by enhanced basal release of nitric oxide

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Estrogen regulates myogenic tone in pressurized cerebral arteries by enhanced basal release of nitric oxide. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2248–H2256, 1997.—Second-order middle cerebral arteries (135.0 ± 4.6 µm ID) from male, female, ovariectomized female (no endogenous estrogen), and estrogen-treated ovariectomized female Sprague-Dawley rats were harvested and mounted in a pressure myograph. Myogenic response was recorded over a range of 10–100 mmHg and was repeated in the pressure myograph. Myogenic response was recorded over a range of 10–100 mmHg and was repeated in the pressure myograph.

METHODS

Animals, Preparation of Vessels, and Instrumentation

Age-matched Sprague-Dawley rats from four groups were used: male, female, ovariectomized female (no endogenous estrogen), and ovariectomized female with estrogen replacement. Ovariectomized animals were obtained from Charles River Canada (Montreal, Quebec, Canada). For estrogen replacement, animals (n = 14) were anesthetized by halothane inhalation, and then 17β-estradiol pellets (3-wk sustained release, 0.5 mg 17β-estradiol) were placed in dorsal subcutaneous pockets. Incisions were closed with a single suture of 3–0 prolene. There were no operative or perioperative deaths. Estrogen-treated animals were killed 3 wk after operation.

Second-order middle cerebral arteries (135.0 ± 4.6 µm ID, range 100–202 µm) were obtained from the animals and used for all experiments. After pentobarbital sodium (Somnotol, 30 mg/kg) and heparin sodium (Hepalean, 500 U/kg) were injected intraperitoneally, the anesthetized animals underwent a midline laparotomy, and blood for serum estrogen measurement was drawn from the inferior vena cava. The animals were then killed by decapitation, and the brain was removed and immersed in cold oxygenated physiological saline solution (PSS). A second-order middle cerebral artery (0.6–1.0 mm long) was carefully dissected from surrounding connective tissues and transferred to the experimental chamber of a pressure myograph filled with oxygenated PSS at 37°C. The proximal aspect of the artery was fed onto a glass microcannula (tip diameter 70–90 µm) and tied with a single strand (20 µm) of braided 4–0 nylon suture. After the artery was flushed with PSS to remove intraluminal blood, the distal aspect of the vessel was similarly cannulated and tied. With the use of no-flow conditions, the intraluminal pressure was set to 60 mmHg by using an electronic pressure servo system (9), and the vessel was equilibrated for 60 min, during which time the vessels spontaneously and reliably developed myogenic tone, with significantly reduced luminal diameters. Once attained, myogenic tone and vessel diameter remain stable unless perturbed by changes in transmural pressure or the addition of vasoactive compounds (9, 18, 21).

The PSS in the experimental chamber was continuously superfused around the pressurized artery at a flow rate of 20–25 ml/min, passing through an external reservoir that was bubbled with a 95% O2-5% CO2. A heating pump connected to a heat exchanger maintained the PSS at 37°C. Buffer pH, monitored by a micro pH probe in the tissue bath, was maintained at 7.40 ± 0.04 by adjustment of the gassing rate.

The arteriograph containing a pressurized cerebral artery was placed on the stage of an inverted microscope with a monochrome video camera attached to a viewing tube. Arterial dimensions were measured using a video system that provides automatic continuous read-out measurements of arteries and test our hypothesis that estrogen increases basal production of NO, and in so doing, regulates myogenic tone.

GENDER-BASED DIFFERENCES in vascular disease have been known since the 1930s (7), and although much effort has been directed toward the study of estrogen and coronary artery disease, the smaller but significant correlation between estrogen exposure and cerebrovascular disease has received less attention. Zhang et al. (35) recently published male-to-female stroke mortality ratios of 1.33–1.50 for North America, with a mean for industrialized countries of 1.68. These sex ratios have been increasing globally over the last 35 years. Supporting a role for estrogen in this cerebrovascular protection, Finucane et al. (4) showed a 31% reduction in stroke incidence and a 63% reduction in stroke mortality; vasoprotection.

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luminal diameter and wall thickness (9). The information is updated every 17 ms, and the precision of the diameter measurements is within 1%.

**Endothelium Removal**

Endothelium removal was achieved by rubbing the luminal surface of the vessel with the cannula tip and was confirmed by loss of dilatation to A-23187 (10⁻⁶ M). Because A-23187 is irreversible, confirmation of endothelium removal was done at the end of the experiment.

**Solutions and Drugs**

The ionic composition of the PSS was (in mM) 118 NaCl, 24.9 NaHCO₃, 4.7 KCl, 1.18 KH₂PO₄, 1.17 MgSO₄, 1.6 CaCl₂, 11.1 glucose, and 0.026 EDTA. Calcium-free solution contained 2.0 mM ethylene glycol-bis(β-aminoethoxy ether)-N,N,N',N'-tetracetic acid (EGTA) and no CaCl₂. N-nitro-L-arginine methyl ester (L-NAME), A-23187, and sodium nitroprusside were obtained from Sigma Chemical (St. Louis, MO). 17β-Estradiol pellets were purchased from Innovative Research of America (Toledo, OH).

**Experimental Protocols**

Effect of subcutaneous estrogen pellet implantation on serum estrogen level. Blood samples (3 ml) were collected into a syringe and then transferred to Eppendorf ultracentrifuge containers. After centrifugation for 8 min at 14,000 revolutions/min, plasma was collected and kept frozen at −20°C. Plasma 17β-estradiol concentration was measured using a 125I radioimmunassay kit (ICN Biomedical, Carson, CA). Briefly, 1 ml of 125I-labeled estradiol was added to assay tubes containing 100 µl plasma or standard solution. After incubation for 90 min at 37°C to allow binding of estrogen (plasma or labeled), the liquid content of the tubes was aspirated and discarded. 125I activity of the empty tubes in a gamma counter then reflects the content of estrogen in each plasma sample when referenced to a standard curve.

Effect of chronic estrogen exposure on myogenic tone. After the vessel equilibrated at 60 mmHg for 1 h, transmural pressure was decreased to 10 mmHg. The vessel (4 groups, each n = 5) was subjected to stepwise increase in transmural pressure, from 10 to 100 mmHg, to determine the degree of myogenic tone at each pressure. Each pressure was maintained until a stable diameter reading was attained (5–6 min). The protocol was then repeated, and the results were averaged. To examine the contribution of NO to net myogenic tone, l-NAME (2 × 10⁻⁴ M), a competitive inhibitor of NO synthase (NOS) (constitutive and inducible isofoms), was added to the superfusing buffer and allowed to circulate for 30 min, during which time the vessels (pressurized at 60 mmHg) all decreased in diameter to varying degrees. As above, the vessels were subjected to stepwise increases in transmural pressure, their steady-state diameters reflecting the underlying myogenic tone without the influence of basal NO production. As above, this was repeated, and the results were averaged. Finally, calcium-free EGTA buffer was substituted and circulated for 20 min. The vessel was cycled through the same pressure steps to determine the “passive” diameters at each pressure to calculate the percentage of myogenic constriction in the presence and absence of l-NAME. Myogenic tone at each pressure was expressed as percent decrease in diameter from the passive diameter or

\[ \% \text{Constriction} = 100\% \times \frac{(D_{\text{Ca-free}} - D_{\text{PSS,l-NAME}})}{(D_{\text{Ca-free}})} \]

where D is the arterial diameter in calcium-free, PSS, or l-NAME-containing buffer. Percent constriction in endothelium-denuded vessels was calculated similarly.

NO donor sensitivity and stimulated NO release by A-23187. Myogenically active vessels from the ovariectomized and estrogen-treated groups were used to examine the effect of estrogen exposure on the sensitivity to NO and on stimulated NO release. Vessels (n = 5 in each group) were equilibrated at 60 mmHg as above, during which time they all spontaneously constricted between 20 and 25%. Vessels were then exposed to increasing concentrations of sodium nitroprusside (10⁻⁸ to 10⁻³ M), and the resulting luminal diameter at each concentration was measured. For stimulated NO release, vessels (n = 5 in each group) were similarly equilibrated and then exposed to increasing concentrations of the calcium ionophore A-23187 (10⁻⁶ to 10⁻³ M). After this, the vessels were bathed in calcium-free buffer to determine the passive diameter. Vasodilation responses were expressed as percent increase in diameter from the initial diameter (due to myogenic tone) or

\[ \% \text{Dilation} = 100\% \times \frac{(D_{\text{SNP,A-23187}} - D_{\text{MT}})}{(D_{\text{Ca-free}} - D_{\text{MT}})} \]

where D is the measured arterial diameter, subscripts SNP and A-23187 denote concentrations of vasodilators, and subscripts Ca-free and MT denote passive and initial myogenic tone diameters, respectively.

Effect of genetic sex and estrogen exposure on mechanical characteristics. Cerebral artery mechanical characteristics are expressed as passive distensibility, which is the relative incremental change in internal diameter per unit change in pressure in the absence of smooth muscle activation. This was determined for each vessel using a variation of the relationship described by Baumbach et al. (2).

Passive distensibility = 100% × \[ \frac{(D_{P_2} - D_{P_1})}{(D_{P_1} \times (P_2 - P_1))} \]

where \( P_1 \) is the transmural pressure (2 > 1), and \( D_2 \) is the vessel diameter at the corresponding pressure. To prevent myogenic constriction at each pressure, vessels were bathed in calcium-free EGTA buffer, such that the resultant diameter reflects the cumulative stretch properties of the connective tissue, smooth muscle, and endothelial cells of each vessel.

Effect of estrogen exposure on wall tension. Wall tension was calculated using the Laplace relation (wall tension = transmural pressure × vessel radius), with 1 mmHg = 1.33 × 10⁻⁴ N/m².

**Statistical Analysis**

Results are presented as means ± SE, and n represents the number of vessels in each group. One vessel was taken from each animal. Differences between groups were compared using analysis of variance, with multiple comparisons by Newman-Keuls test for significant differences. A value of P < 0.05 was considered significant.

**RESULTS**

**Animal Characteristics**

All animals had comparable weights at the time of delivery, and male, female, and ovariectomized animals gained weight during the 3-wk treatment period. However, estrogen-treated animals had lower weight than the other groups at day 21 and, in fact, had significantly lower weight from their own initial measurement (Table 1).
Effects of Estrogen Exposure on Extent of Myogenic Tone

Although male arteries had slightly less passive distensibility than female arteries, this was statistically significant for only one data point. There were no differences in distensibility between ovariectomized and estrogen-treated arteries (Fig. 7, A and B).

DISCUSSION

There are four main findings in this study. 1) Pharmacological inhibition of NOS and endothelium removal causes a greater potentiation of pressure-induced myogenic tone in cerebral arteries chronically exposed to estrogen, suggesting a greater basal release of endothelium-derived NO. 2) Chronic estrogen exposure does not alter artery sensitivity to an NO donor, nor does it alter stimulated release of NO by a calcium ionophore, arguing against increased expression of NOS as the mechanism of increased NO release. 3) There are no differences in passive distensibility of cerebral arteries between genetically male arteries and genetically female arteries, with or without chronic estrogen exposure. 4) Despite the increase in basal release of NO, chronic estrogen exposure does not alter net arterial tone, suggesting the presence of compensatory myogenic mechanisms that may function to maintain consistent arterial diameter, at each pressure, even in the face of a chronic and sustained vasodilatory stimulus.

Our data are consistent with an increase in the basal production of NO due to chronic exposure to physiological levels of 17β-estradiol. Several lines of evidence lend support to this finding. Using rabbit aortic rings precontracted with phenylephrine, Hayashi et al. (11) found greater potentiation of contractile tension by NOS inhibition with L-NAME in female vessels as compared with male. Furthermore, this gender-based
difference was abolished by ovariectomy, suggesting that physiological levels of female sex hormones (of which estrogen is one) stimulate basal NO release. Enhanced unstimulated production of NO from rat aortas from females as compared with males using a bioassay technique has been reported by Kauser and Rubanyi (16). Furthermore, incubation of cultured endothelial cells (human umbilical vein and bovine aortic) with physiological levels of 17β-estradiol increases NOS activity as well as NO and NO metabolite release (12).

We found no difference in sensitivity of ovariectomized female arteries to NO, regardless of estrogen exposure, and this is in agreement with published data (11, 17). Also, we have shown no effect of estrogen on nonreceptor-mediated NO release by A-23187. Similarly, Gisclard et al. (6), using rabbit femoral artery rings, found that chronic estrogen treatment did not increase relaxations to A-23187, whereas receptor-mediated relaxations to acetylcholine were increased, suggesting an effect of estrogen on the number or sensitivity of muscarinic receptors. In a model of guinea pig pregnancy (when circulating estrogens are increased), Weiner et al. (32) showed no change in A-23187-stimulated relaxation, whereas acetylcholine-stimulated relaxation was increased in preconstricted uterine and carotid arteries. However, Miller and Vanhoutte (19) observed increased relaxations to A-23187 in rabbit aortic rings, indicating that nonreceptor-mediated mechanisms of increased NO release by estrogen may be species and vessel dependent. Our findings support an increase in NOS activity only at the basal state, and because stimulated NO release was unaffected, argue against increased NOS expression as the underlying mechanism.

An increase in basal but not stimulated NO release could be due to alterations in endothelial calcium signaling. For example, an increase in basal endothelial free calcium would increase basal NO release, but stimulated NO release (by further elevation of intracellular calcium) would not necessarily be altered. In estrogen-exposed aortic rings, greater endothelium-dependent dilations due to the endoplasmic reticulum Ca2+-ATPase inhibitor cyclopiazonic acid implicate intracellular free calcium in this process, possibly through increased plasmalemmal calcium leak (23).

It is important to note that our experiments examined the vascular effects of chronic estrogen exposure, and not acute estrogen exposure, which has been described in other preparations (5, 10, 14, 20, 22, 30). In such experiments, 17β-estradiol (10 µM) is proposed to enhance fura 2-recorded free calcium levels in freshly isolated endothelial cells by increasing the driving force for calcium entry. This calcium entry is initiated by estrogen-induced hyperpolarization due to activation of tetraethylammonium-sensitive potassium channel openings (24).

Interestingly, in the face of greater NO production, estrogen-exposed cerebral resistance arteries develop
opened myogenic constrictions and arterial diameters at each pressure that were equal to male and ovariectomized vessels, suggesting the existence of an adaptive constriction mechanism that can be recruited to maintain a consistent arterial tone despite significant vasodilatory stimuli. Because arterial diameter profoundly affects blood flow (by the Poiseuille relationship), sustained cerebral dilations would be expected to increase cerebral blood flow significantly, given a consistent perfusion pressure. Lending clinical support for an adaptive constriction mechanism, several reports have failed to demonstrate significant gender-based differences in cerebral blood flow (26, 28, 31).

Removal of the endothelium did not significantly alter this adaptive constriction, indicating that endothelium-borne constrictors or dilators are not involved and instead suggesting a myogenic basis. A rationale for myogenic adaptation is provided by Johnson's hypothesis (15), which proposed that it is wall tension rather than transmural pressure that acts as the stimulus for a myogenic constriction. By this hypothesis, any sustained vasodilation would then act as a stimulus for a myogenic constriction by increasing the arterial wall tension according to the Laplace equation (wall tension = transmural pressure x radius). This view is supported by the observation of Burrows and Johnson (3) that a pressure increase in cat mesenteric arterioles is followed by a constriction just sufficient to keep the arterial wall tension constant. In rat cremasteric arterioles, levels of free intracellular calcium and myosin light-chain phosphorylation correlate with calculated wall tension rather than transmural pressure or diameter (36). In very elegant experiments, Van-Bavel and Mulvany (29) showed that in pressurized cat mesenteric small arteries, graded agonist responses under isobaric conditions were converted to all-or-none

![Fig. 3. Myogenic constriction of male and female arteries (A) and ovariectomized and estrogen-treated arteries (B) with functionally intact endothelium. There was no difference in degree of myogenic tone (P > 0.05).](http://ajpheart.physiology.org/)
responses under isometric conditions, suggesting that agonist sensitivity is wall tension dependent, such that sensitivity increases with increasing wall tension. If agonist response is analogous to the myogenic response (and indeed some agonists induce myogenic activity, Ref. 29), then increasing wall tension would be expected to increase myogenic sensitivity, with subsequent contraction, until the force of contraction by enhanced sensitization becomes equal to the force of contraction.

Fig. 6. Dilation of myogenically active cerebral arteries to a nitric oxide donor, SNP (A), and a calcium ionophore, A-23187 (B), showing no difference between ovariectomized and estrogen-treated vessels (P > 0.05).

Fig. 5. Effect of NOS inhibition on myogenic tone. A: myogenic constriction of male and female arteries in presence of L-NAME (2 × 10^-4 M). Although myogenic tone increased in both groups, there was greater potentiation of myogenic tone in female vessels at 100 mmHg. *P < 0.05. B: myogenic constriction of ovariectomized and estrogen-treated animals in presence of L-NAME, showing greater potentiation of tone in estrogen-exposed vessels at 60–100 mmHg. *P < 0.05. C: myogenic constriction of male and female arteries after endothelium removal, showing greater potentiation in female arteries. *P < 0.05.
An adaptive mechanism to maintain a consistent degree of myogenic constriction despite long-term exposure to vasodilatory stimuli might exist to safeguard the delicate balance of cerebral hydrostatic and oncotic pressures and protect the physiologically important blood-brain barrier. With equal cerebral perfusion pressure, enhanced cerebral vasodilation would increase blood flow, possibly to levels that become injurious. For example, it has been shown that vasodilator stimuli such as tissue acidosis, hypercapnea, and papaverine enhance blood-brain barrier damage and cerebral edema formation caused by severe hypertension. In acute brain injury, where regions of loss of autoregulation and high cerebral blood flow exist, further increase in flow by systemic hypertension can lead to enhanced blood-brain barrier damage, cerebral edema, and rising intracranial pressure (27).

Adaptive constriction appears reversible; the adaptation seen in cycling females (Fig. 5A) is not seen after ovariectomy (Fig. 5B), and the fact that adaptation is observed after NOS inhibition or endothelium removal indicates that adaptation is long term in nature. We observed persistence of potentiation by L-NAME for the duration of each experiment, so adaptation must be regulated over a period of at least several hours. Pathologically, this would have to be the case: instantaneous adaptation of resistance vessels would abolish physiological vasomotion entirely.

Fig. 7. A: distensibility of male and female arteries. Although a slight trend toward greater distensibility in female vessels is evident, this is significant only for one data point. *P < 0.05. B: distensibility of ovariectomized and estrogen-treated arteries, showing no differences.

Fig. 8. Wall tension (with intact endothelium) of male and female (A) and ovariectomized and estrogen-treated (B) arteries. No differences are evident in wall tension between groups (P > 0.05). Passive wall tensions (without SE) for male, female, ovariectomized, and estrogen-treated arteries (pass m, pass f, pass o, pass e, respectively) are provided for reference.
In myogenically active rat coronary septal arteries, estrogen exposure was found to increase the basal release of endothelium-derived NO (32). In this set of experiments using larger vessels (~200 µm diameter), no myogenic adaptation was observed; rather, estrogen exposure resulted in decreased net myogenic tone, whereas the degree of underlying myogenic tone (with NOS inhibition) was unaltered. This indicates that in addition to organ-specific differences, there likely are size-related differences in resistance artery physiology.

The vascular protective effect of estrogen is partially mediated by improvement in plasma lipid profile (1), and enhanced NO production by the cerebral circulation may provide vascular protection through other cellular effects mediated by NO. Endothelial adhesion and recruitment of monocytes into the subendothelial space is one of the earliest events in atherogenesis and results in lipid-laden foam cell deposition, the hallmark lesion of atherosclerosis (25). NO inhibits the expression of monocyte chemoattractant protein 1, a chemotactic factor which functions to direct monocyte migration across the endothelium (34). In addition, NO has inhibitory effects on lipoprotein oxidation (13), a fundamental step in the lipid oxidation theory of atherogenesis. Furthermore, in established atherosclerosis, the inhibition of platelet aggregation by physiological levels of NO (8) may function to prevent platelet plug formation at sites of atherosclerotic narrowing, thus preventing this early step in acute arterial occlusion.

In summary, we have shown a greater potentiation of myogenic tone in cerebral arteries exposed to estrogen by inhibition of NOS, suggesting increased basal release of NO. Estrogen exposure did not alter sensitivity of the arteries to NO, or stimulate release of NO through a non-receptor-mediated mechanism. Together, these findings support a greater activity of endothelial NOS at the basal state, but argue against increased expression of NOS (which would be expected to increase stimulated NO release as well). We have also shown that these estrogen-exposed arteries mount a greater underlying myogenic tone so that with functional endothelium there is no difference in net arterial tone or diameter despite greater vasodilatory stimulus from NO.

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