Evidence That Estrogen Suppresses Rho-Kinase Function in the Cerebral Circulation In Vivo

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Background and Purpose—Premenopausal women are less susceptible to cardiovascular diseases than men or postmenopausal women. Such disease states are often associated with increased vascular RhoA/Rho-kinase activity and decreased activity of nitric oxide (NO). This study tested whether female gender is associated with lower Rho-kinase activity or higher NO activity in cerebral arteries in vivo and whether estrogen contributes to any such gender differences.

Methods—Changes in basilar artery diameter were measured with the use of a cranial window preparation in anesthetized Sprague-Dawley rats. Some female rats were ovariectomized (OVX) and treated subcutaneously daily for 14 days with vehicle (dimethyl sulfoxide) or 17β-estradiol. Vascular expression of RhoA or Rho-kinase was assessed by Western blotting.

Results—The Rho-kinase inhibitor Y-27632 was selectively 3-fold more potent as a cerebral vasodilator in males versus females. Expression of total RhoA or Rho-kinase did not differ between males and females. In OVX rats, vasodilator responses to Y-27632 resembled responses in males. Treatment of OVX rats with 17β-estradiol normalized the vasodilator effects of Y-27632 to be equivalent to responses in intact female controls. The NO synthase inhibitor N-nitro-L-arginine methyl ester caused 50% greater constriction of the basilar artery in females versus males, but responses in OVX rats treated with either vehicle or 17β-estradiol did not differ from those recorded in intact females.

Conclusions—These data indicate that vascular Rho-kinase function is suppressed in females because of the effects of estrogen, whereas the higher NO activity in females is estrogen independent. (Stroke. 2004;35:2200-2205.)

Key Words: cerebral arteries ■ estrogens ■ gender ■ nitric oxide

The incidence of cardiovascular disease, including stroke and cerebrovascular disease, is lower among premenopausal women than in age-matched men or in postmenopausal women.1,2 Although the specific mechanisms by which female gender is vasoprotective are not fully elucidated, these effects are believed to be due in part to higher endogenous levels of estrogen in premenopausal women contributing to increased bioavailability of nitric oxide (NO).2,3

Activation of the G protein RhoA and its downstream effector, Rho-kinase, results in decreased myosin light chain phosphatase activity.4 This leads to increased phosphorylation of myosin light chain and thus vascular smooth muscle cell contraction.5 Evidence is accumulating to suggest that diseases such as hypertension and coronary and cerebral vasospasm are associated with increased vascular Rho-kinase activity,5 but no study has tested whether Rho-kinase function or expression might be influenced by gender.

There is increasing functional evidence that vascular NO signaling may interact with and inhibit activity of the RhoA/Rho-kinase pathway. For example, one of the mechanisms of NO-induced aortic relaxation appears to be via inhibition of RhoA/Rho-kinase–mediated contraction.6 Furthermore, Rho-kinase–mediated vascular tone of the basilar artery is augmented by chronic NO synthase (NOS) inhibition,7 consistent with a normal modulatory effect of NO on Rho-kinase function. In this study we first tested whether Rho-kinase function or expression in the cerebral circulation is gender dependent. Second, given the potential for NO to inhibit vascular Rho-kinase function, we tested whether basal NOS activity in cerebral arteries in vivo is influenced by gender. Third, we tested which gender differences in vascular function can be attributed to the effects of estrogen.

Materials and Methods

All procedures were approved by the institutional animal experimentation ethics committee. Experiments were performed in Sprague-Dawley rats of either gender (male: n=37; mean±SE weight, 400±18 g; female: n=79; mean±SE weight, 269±7 g). Rats were bred in the Department of Pharmacology at The University of Melbourne, Australia.
In Vivo Experimental Protocol

The surgical procedure for animal preparation to measure basilar artery diameter via a cranial window has been described. Arterial blood gases and pH were maintained at normal levels for the duration of the experiment (pH = 7.37 ± 0.01; P CO 2 = 37 ± 1 mm Hg; P O 2 = 188 ± 5 mm Hg). When sampled from the cranial window, mock cerebrospinal fluid pH and gases were as follows: pH = 7.38 ± 0.01; P CO 2 = 35 ± 1 mm Hg; P O 2 = 129 ± 1 mm Hg. The basilar artery was allowed to stabilize for 30 minutes after surgical preparation before responses were obtained to topical application of vasoactive agents. The following drugs were tested: Y-27632 (1 to 100 μmol/L), a Rho-kinase inhibitor; acetylcholine (1 to 100 μmol/L), an endothelium-dependent vasodilator; sodium nitroprusside (0.01 to 1 μmol/L), a NO donor; aprikalim (0.3 to 3 μmol/L), an ATP-sensitive K\(^{+}\) (K\(_{ATP}\)) channel opener; N-nitro-l-arginine methyl ester (L-NAME) (1 mmol/L), a NOS inhibitor; and serotonin (0.1 to 100 μmol/L), a 5-HT\(_{1}\) receptor agonist. The following drugs were diluted in mock cerebrospinal fluid, then were superfused over the cranial window in cumulatively increasing concentrations. Diameter of the basilar artery was recorded under basal conditions and when stable during application of each agonist. After vessel diameter had returned to the control level, an additional 15- to 30-minute recovery period was allowed before application of another drug. The sequence of application of drugs was randomized. Up to 4 vasoactive agents were tested in each animal.

Ovariectomized Rats

Twenty-five female rats (weight, 240 ± 8 g) were anesthetized (methohexitone 65 mg/kg IP) and administered buprenorphine (0.01 mg/kg SC) for analgesia. A bilateral ovariectomy was then performed through a small dorsal incision. The skin was sutured closed, and the rat was allowed to recover. After 4 weeks, the ovariectomized (OVX) rats had gained +100 g body weight (341 ± 8 g). They were then injected subcutaneously daily for an additional 14 days with either vehicle (10% dimethyl sulfoxide; n = 12) or 17β-estradiol (0.1 mg/kg; n = 13). Animals were anesthetized, the cranial window was prepared, and concentration-response data for all vasoactive agents were obtained. At the conclusion of each of these experiments, the uterus was harvested and weighed.

Expression of RhoA and Rho-Kinase in Rat Basilar Artery

Expression of RhoA and Rho-kinase in rat basilar artery was measured by Western blotting. Basilar arteries were harvested after euthanasia, immediately frozen in liquid nitrogen, and stored at −80°C. Each basilar artery was homogenized in ice-cold lysis buffer with the following composition (in mmol/L): NaCl, 100; Tris-HCl, 10; EDTA, 2; NaF, 10; β-glycerophosphate, 10; benzamidine, 1.27; 1% Triton X-100, pH 7.4; and containing protease inhibitors (Complete Mini, Roche), with the use of a hand-held glass homogenizer, on ice. Homogenates were cleared by centrifugation, and protein concentration was determined with the use of the Bradford assay (BioRad). For blotting, gel sample buffer was added to tissue homogenates and boiled. Equal amounts of protein were loaded onto 12% (RhoA) or 7.5% (Rho-kinase) polyacrylamide gels and transferred to polyvinylidene difluoride or nitrocellulose membranes, respectively. Membranes were blocked for 1 hour at room temperature in TBS containing 0.1% Tween 20 and 5% skim milk powder. Membranes were incubated overnight at 4°C with primary antibody for RhoA (1:1000; sc-179, Santa Cruz Biotechnology, Santa Cruz, Calif) or antiserum raised against a GST fusion protein of the Rho-kinase (ROKα) coiled-coil domain, residues 429 to 968 expressed in Escherichia coli from a pGEX plasmid provided by T. Leung (1:2000; DMVS, Adelaide, Australia). This was followed by anti-rabbit (1:1000) secondary antibody linked to horseradish peroxidase (Chemicon International, Temecula, Calif). Immunoreactive bands were detected by ECL (Amersham) and quantitated densitometrically with the use of Kodak Image Station 440CF (Perkin-Elmer Life Sciences, Boston, Mass).

Drugs

Nimodipine was obtained from Calbiochem. Aprikalim was obtained from Rhone-Poulenc Rorer. Y-27632 [R-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide] was obtained from Welfide Corporation. All other drugs were obtained from Sigma Chemical Co. Stock solutions of aprikalim (1 mmol/L) and nimodipine (10 mmol/L) were prepared by dissolving in 50% dimethyl sulfoxide and 50% saline. Subsequent dilutions were made in saline. All other drugs were dissolved and diluted in saline. At the final concentration used, dimethyl sulfoxide alone (<0.15%) had no effect on basilar artery diameter.

Statistical Analysis

Vasodilator-induced increases in basilar artery diameter from baseline are expressed as percentage of the maximum dilator response achievable by 100 μmol/L sodium nitroprusside plus 10 μmol/L nimo- dipine. Vasoconstrictor responses to L-NAME and serotonin are expressed as percent change in diameter over baseline. All data are expressed as mean ± SE. Comparisons were made with the use of Student paired or unpaired t tests or ANOVA, as appropriate. Concentration-response data were fitted to the following 4-parameter Hill equation with the use of Prism 4 (GraphPad):

\[
\text{Response} = \frac{E_{\text{max}}}{1 + 10^{\left(\log EC_{50} - \text{X}\right)/\text{nh}}}
\]

where E\(_{\text{max}}\) is maximal asymptotic response, Basal is minimal asymptotic response, LogEC\(_{50}\) is logarithm of the calculated concentration that would elicit 50% of the maximum response, X is logarithm of the agonist concentration, and nh is the Hill slope.

For comparison between pairs of treatment groups, concentration-response data were fitted to the Hill equation either individually (ie, groups significantly different) or pooled (ie, no difference between groups), and the better model was determined by F test (extra sum of squares test) with the use of GraphPad Prism 4. An advantage of this approach is that the entire data set is utilized in the determination of statistical significance rather than any 1 model parameter. A probability value <0.05 was considered significant.

Results

Basilar artery diameter was 248 ± 6 μm in males (n = 25) and 250 ± 5 μm (n = 45) in females under control conditions. Maximum artery diameter was 400 ± 9 μm in males (n = 25) and 368 ± 6 μm (n = 45) in females (P < 0.05). Arterial blood pressure averaged 97 ± 3 mm Hg (n = 25) in males and 77 ± 2 mm Hg (n = 45) in females (P < 0.05). Arterial pressure was not affected by application of any vasoactive substance to the cranial window (data not shown).

Effect of Gender on Basilar Artery Responses to Vasodilators

Application of Y-27632 (1 to 100 μmol/L) to the cranial window caused marked concentration-dependent increases in basilar artery diameter of male and female rats (Figure 1a). Y-27632 concentration-response curves were selectively 3-fold leftward in males versus females (P < 0.05; Figure 1a). By contrast, vasodilator responses to acetylcholine and aprikalim were each similar in males versus females (Figure 1b and 1c, respectively).

Effect of Gender on Responses to Vasoconstrictors

L-NAME (1 mmol/L) caused profound constriction of the basilar artery in both male and female rats, but responses were 47% greater in females (P < 0.05; Figure 2a). By contrast, serotonin (0.1 μmol/L) caused equivalent constritor responses in males and females (Figure 2b).
Effects of Ovariectomy and Estradiol Therapy

OVX rats treated with vehicle for 14 days gained weight (353 ± 9 to 364 ± 10 g; n = 12), whereas OVX rats treated with 17β-estradiol lost weight (330 ± 11 to 314 ± 10 g; n = 13). Arterial blood pressure did not differ between the 2 OVX treatment groups (83 ± 5 mm Hg [n = 12] in vehicle-treated OVX rats and 90 ± 5 mm Hg [n = 10] in 17β-estradiol–treated OVX rats). Ovariectomy reduced uterine weight (control females = 579 ± 38 mg; n = 18; OVX + vehicle = 197 ± 22 mg; n = 8; P < 0.05 versus control females), and treatment with 17β-estradiol restored uterine weight to normal (580 ± 107 mg; n = 9; P < 0.05 versus OVX + vehicle). Basilar artery diameter was 266 ± 11 μm in OVX rats treated with vehicle (n = 12) and 242 ± 8 μm in OVX rats treated with 17β-estradiol (n = 10). Maximum artery diameter was 392 ± 15 μm in OVX + vehicle rats (n = 11) and 401 ± 9 μm (n = 10) in OVX + 17β-estradiol rats.

In OVX + vehicle rats, vasodilator responses to Y-27632 were left-shifted relative to intact females and were identical to responses observed in males (Figure 3a). By contrast, 17β-estradiol treatment in OVX rats caused a rightward shift in the concentration-response curve to Y-27632 in association with similar levels of arterial pressure (see above), restoring responses to resemble effects observed in control females (Figure 3a). Vasodilator responses to acetylcholine and aprikalim did not differ between vehicle- or 17β-estradiol–treated OVX groups (Figure 3b and 3c, respectively). Constrictor responses of the basilar artery to L-NAME (Figure 4a) and serotonin (Figure 4b) were each similar in OVX rats treated with vehicle or 17β-estradiol.

Effect of Gender on RhoA and Rho-Kinase Expression in the Rat Basilar Artery

Western blot analysis of RhoA (Figure 5a) and Rho-kinase (Figure 5b) expression revealed no significant gender difference in levels of either protein in the basilar artery of male versus female rats.
Discussion

Our present findings provide the first evidence that a gender difference exists in the vascular activity of Rho-kinase in any vascular bed. This protein is now known to have increased function in several major cardiovascular diseases, and its functional inhibition is thought to be a major mechanism of the cholesterol-independent protection afforded by statins. The present study shows that estrogen suppresses Rho-kinase function in the cerebral circulation of female rats in vivo. Specifically, the major findings are as follows: (1) Y-27632, a pharmacological inhibitor of Rho-kinase, produced a stronger dilator response of the basilar artery in males than in females, consistent with a higher basal Rho-kinase function in males; (2) the NOS inhibitor L-NAME caused greater cerebral vasoconstriction in females than in males; (3) in OVX rats, vasodilator responses to Y-27632 closely resembled responses recorded in males. However, chronic treatment of OVX rats with 17β-estradiol normalized the effects of Y-27632 to be similar to those observed in intact females, indicating that estrogen normally suppresses vascular Rho-kinase function in females. These findings are novel and important because (1) they are obtained in vivo; (2) they report direct cerebral vessel effects; (3) they indicate that a gender difference exists, with Rho-kinase function lower in arteries of females; (4) they show that endogenous estrogen fully accounts for the gender difference in Rho-kinase function; and (5) they show that differences within physiological levels of blood pressure and basal NO activity do not mediate the gender difference in Rho-kinase function. Gender-dependent differences in the level of vascular Rho-kinase function may therefore contribute to the differing susceptibility to cardiovascular diseases between males and females.
Rho-Kinase Function Is Lower in Female Cerebral Arteries

Rho-kinase is now believed to play an important role in maintaining vascular tone and mediating contractile responses to vasoconstrictor agonists. In the present study we have shown for the first time that pharmacological inhibition of Rho-kinase results in weaker dilatation of the basilar artery in females than in males, suggesting that Rho-kinase function may contribute less to basal vascular tone in female rats. The gender-related difference in the vasodilator effect of Y-27632 was selective because responses to acetylcholine and aprikalim were similar in males and females. This indicates the weaker dilator response to Y-27632 in females was not simply due to a greater general functional antagonism opposing vasodilators in females. Greater levels of basal NO release have been reported in several vessels from females than from males, as observed here in the rat basilar artery. In these studies we used a relatively large concentration of L-NAME (1 mmol/L) to ensure that endothelial NOS (eNOS) was fully blocked in both genders, and hence the resulting constrictor effect could be reliably regarded as an index of total basal NO activity. Thus, our data do not necessarily disagree with a recent study that found no gender difference in much smaller (<10%) basilar artery constrictor responses to 1 to 10 μmol/L N0-monomethyl-L-arginine (L-NMMA), a less potent eNOS inhibitor, because those effects are likely to reflect submaximal inhibition of eNOS. Despite the gender difference in basal NO activity in the present study, equivalent responses to acetylcholine in both genders indicate that there was no gender difference in stimulated, endothelium-dependent, NO-mediated dilatation of the rat basilar artery, as previously reported in this vessel and in the rabbit basilar artery. Our data also show that there is no gender difference in responses mediated by KATP channels. Lower Rho-kinase function in the female basilar artery is not due to reduced expression of RhoA or Rho-kinase but could instead be related to lower activity of either protein.

Basal NOS Function Is Higher in Female Cerebral Arteries

The NOS inhibitor L-NAME induced a relatively strong constriction of the basilar artery in males and females, consistent with previous reports in studies of males in which related NOS inhibitors were used. Thus, it appears that a substantial basal release of NO occurs in the cerebral vessels of both genders in vivo. Interestingly, L-NAME caused selectively greater vasoconstriction in females, indicating that total basal NOS activity is greater in the basilar artery of females versus males. This is analogous to findings reported in rabbit aorta and rat middle cerebral artery, in which vasoconstriction to NOS inhibitors under basal conditions was higher in females than in males. On the basis of our data with OVX animals (see below), the enhanced basal NO generation in cerebral arteries of intact females cannot account for the weaker contribution of Rho-kinase to vasoconstriction in female vessels.

Effects of Estrogen

The increased risk of cardiovascular disease in women after menopause may be attributed in part to reduced estrogen levels. We found that after ovariectomy in female rats, vasodilator responses to Y-27632 were substantially enhanced and became identical to those observed in male rats. Furthermore, treatment of a separate group of OVX rats with 17β-estradiol resulted in vasodilator responses to Y-27632 identical to those observed in control females. This effect of 17β-estradiol on Rho-kinase activity occurred without any change in blood pressure. Thus, one way in which chronic exposure to estrogen may be vasoprotective is via suppression of vascular Rho-kinase function. Notably, there was no effect of ovariectomy or 17β-estradiol treatment on vasodilator responses to acetylcholine and aprikalim, suggesting again that these effects were selective for Rho-kinase function.

Surprisingly, L-NAME–induced vasoconstrictions were similar in OVX rats treated with vehicle or 17β-estradiol, suggesting that ovariectomy does not reduce basal NOS function and that chronic estrogen treatment after ovariectomy does not enhance basal NOS function in the basilar artery. This contrasts with findings reported in rat middle cerebral artery and rat gracilis muscle arterioles, in which NOS inhibitors caused greater constrictions in intact or OVX females treated with 17β-estradiol compared with effects in males or control OVX females. Our treatment regimen was
effective in replacing estrogen given that treatment with 17β-estradiol restored OVX uterine weight to that of normal females. Furthermore, our data indicate that higher NOS function is not the cause of estrogen-induced suppression of Rho-kinase activity. A potential but as yet untested explanation for our findings may be that estrogen-induced suppression of Rho-kinase activity is due to greater expression of Rnd1, a member of the Rho family that is constitutively bound to GTP and able to block Ca2+ sensitization by RhoA.22

**Gender Differences in Myogenic Tone**

Dilatation of the basilar artery in vivo by Y-27632 is consistent with Rho-kinase activity making an important contribution to myogenic tone in this artery.7 Augmented Rho-kinase–mediated myogenic tone appears to occur during chronic hypertension in rats7,23 and in humans.24 In the present study we observed a stronger vasodilator response to Y-27632 in males, suggesting that Rho-kinase function is more prominent in the male cerebral circulation of rats. Furthermore, enhanced vasoconstriction in response to L-NNAME in females suggests greater basal NOS activity. Previous studies have similarly reported greater myogenic tone in cerebral and noncerebral arteries of males than in females.11,14,21

In summary, the present findings show that Rho-kinase function is suppressed in the cerebral circulation of female rats and that this effect is dependent on endogenous estrogen. Such an effect may contribute to the lower incidence of stroke in premenopausal women.

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**References**

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