Altered Calcium Dynamics Do Not Account for Attenuation of Endothelium-Derived Hyperpolarizing Factor–Mediated Dilations in the Female Middle Cerebral Artery

Elke M. Golding, PhD; Dorota M. Ferens, BSc(Hons); Sean P. Marrelli, PhD

Background and Purpose—The contribution of endothelium-derived hyperpolarizing factor (EDHF) to ATP-mediated dilations is significantly attenuated in the rat middle cerebral artery of intact and estrogen-treated ovariectomized (OVX) females compared with males and vehicle-treated OVX females. Since an increase in endothelial calcium appears to be a critical prerequisite in the EDHF response, we tested the hypothesis that endothelial cell intracellular calcium ([Ca\(^{2+}\)]) fails to reach sufficient levels to elicit robust EDHF-mediated dilations in females and that this effect is mediated by estrogen.

Methods—Vascular diameter and [Ca\(^{2+}\)], were measured concomitantly in perfused middle cerebral artery segments with the use of videomicroscopy and fura 2 fluorescence, respectively.

Results—In the presence of N\(^{\text{G}}\)-nitro-L-arginine methyl ester and indomethacin, the dilation to 10\(^{-5}\) mol/L ATP was significantly reduced (P<0.05) in intact females (42±8%; n=6) and estrogen-treated OVX females (25±6%; n=9) compared with intact males (89±5%; n=6) and vehicle-treated OVX females (92±2%; n=7). Contrary to our initial hypothesis, endothelial cell [Ca\(^{2+}\)], increased to comparable levels in intact females (461±116 nmol/L), estrogen-treated OVX females (417±50 nmol/L), intact males (421±77 nmol/L), and vehicle-treated OVX females (530±92 nmol/L). In response to luminal ATP (10\(^{-5}\) mol/L), smooth muscle cell [Ca\(^{2+}\)], decreased to a greater degree in males (37±4%; n=8) compared with females (21±5%; n=7) and in vehicle-treated OVX females (18±7%; n=7) compared with estrogen-treated OVX females (3±5%; n=9).

Conclusions—Our data suggest that loss of a factor coupling EDHF to reduction of ionized smooth muscle cell [Ca\(^{2+}\)], accounts for the attenuated EDHF-mediated dilations in the female middle cerebral artery. (Stroke. 2002;33:2972-2977.)

Key Words: brain ● endothelium ● endothelium-derived factors ● gender ● rats

An emerging concept is being developed in the cerebral microcirculation that exposes a central role for endothelium-derived hyperpolarizing factor (EDHF) in the modulation of vascular tone. While many laboratories are currently working toward pinpointing the actual identity of EDHF, we know that the initial stimulus for EDHF-mediated dilations involves calcium influx into the endothelium either directly by virtue of calcium ionophores or indirectly via stimulation of endothelial receptors. Such endothelial calcium mobilization culminates in hyperpolarization of the vascular smooth muscle with ensuing relaxation of the artery.

Sex-specific differences in vascular reactivity have recently been described both in the periphery and in the cerebral circulation. In particular, nitric oxide (NO)\(^{6,7}\) and prostacyclin\(^{8}\) appear to be upregulated in females. We have recently shown that in the female middle cerebral artery (MCA), EDHF-mediated dilations are significantly attenuated compared with their male counterparts.\(^{9}\) These dilations in females were enhanced to levels similar to that of an intact male after ovariectomy and subsequently were lost after chronic estrogen replacement, suggesting that this effect is mediated by estrogen. In contrast to our findings in cerebral vessels, estrogen appears to potentiate EDHF-mediated dilations in peripheral vessels.\(^{10,11}\) This adds to the accumulating data supporting the notion that the mechanism for the EDHF response is distinct in the periphery and the cerebrovasculature.\(^{12}\)

The specific mechanisms through which estrogen acts to reduce EDHF-mediated dilations have not yet been identified. This task is confounded by the fact that the identity of EDHF in the brain still remains unclear.\(^{12}\) Nevertheless, it is well established that the primary stimulus for the production and/or release of EDHF is a rise in endothelial cell (EC) intracellular calcium ([Ca\(^{2+}\)].\(^{13,14}\) Moreover, the magnitude of the [Ca\(^{2+}\)]\(^{15}\) increase appears to distinguish an NO-mediated dilation from an EDHF-mediated dilation, with the latter requiring a slightly higher increase in [Ca\(^{2+}\)].\(^{3}\) Given the
critical role of EC [Ca\(^{2+}\)], in the EDHF pathway, it is reasonable to speculate that alterations in endothelial calcium regulation may account for the attenuated EDHF-mediated dilations in the female MCA.

The present study has tested the hypothesis that in the female MCA, EC [Ca\(^{2+}\)], fails to reach critical threshold levels to elicit a robust EDHF response and that this effect is mediated by estrogen. We demonstrate herein that EDHF-mediated increases in EC [Ca\(^{2+}\)], were sufficiently elevated to potentially elicit an EDHF-mediated dilation in intact females and estrogen-treated ovariectomized (OVX) females. However, the EDHF-associated reduction in smooth muscle cell (SMC) [Ca\(^{2+}\)], was markedly attenuated. Our results suggest that estrogen acts to uncouple the EDHF response at a point beyond the change in EC [Ca\(^{2+}\)]. This uncoupling prevents the reduction of SMC [Ca\(^{2+}\)], and resultant dilatation.

**Materials and Methods**

Experiments were performed in strict accordance with National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Animal Protocol Review Committee at Baylor College of Medicine. Rats were housed under a 12-hour/12-hour light/dark cycle with unrestricted access to food and water. Experiments were performed on age-matched males and females (2% isoflurane). Rectal temperature was maintained spontaneously (2% isoflurane). All surgical procedures were undertaken under aseptic conditions.

Experiments were performed in strict accordance with National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Animal Protocol Review Committee at Baylor College of Medicine. Rats were housed under a 12-hour/12-hour light/dark cycle with unrestricted access to food and water. Experiments were performed on age-matched (aged 70 to 90 days) male (weight, 275 to 324 g) and female (weight, 200 to 224 g) Long-Evans rats. Four groups of rats were used in the present study: (1) intact males (n = 14); (2) intact females (n = 13); (3) vehicle-treated OVX females (n = 14); and (4) estrogen-treated OVX females (n = 18).

**Estrogen Depletion and Repletion**

All surgical procedures were undertaken under anesthetic conditions. Animals were secured to a nose cone and allowed to breathe spontaneously (2% isoflurane). Rectal temperature was maintained at 37°C at room temperature followed by a washout period with fresh PSS at 37°C. The vessel was allowed 30 minutes for complete de-esterification of fura 2-AM before SMC [Ca\(^{2+}\)].

**EDHF-Mediated Dilations**

EDHF-mediated dilations were assessed by luminal application of ATP, a P2Y, purinoceptor agonist, in the presence of N\(^{6}\)-nitro-L-arginine methyl ester (L-NAME) and indomethacin. After the development of spontaneous tone, L-NAME (3 × 10\(^{-5}\) mol/L) and indomethacin (10\(^{-5}\) mol/L) were added to the luminal and abluminal baths to remove the NO synthase and cyclooxygenase contributions, respectively. A concentration-response curve to luminal application of ATP (10\(^{-9}\) to 10\(^{-5}\) mol/L) was determined in all 4 groups. Vascular diameter and either EC or SMC [Ca\(^{2+}\)], were assessed in parallel (see Measurement of Vascular Diameter and Calcium). In some experiments, a concentration-response curve to luminal application of the calcium ionophore A23187 (10\(^{-7}\) to 10\(^{-4}\) mol/L) was determined. To assess whether female MCA's were responsive to SMC hyperpolarization, 15 mmol/L KCl was added to the abluminal bath. This concentration of KCl has been demonstrated to produce inwardly rectifying potassium channel–dependent dilations in cerebral arteries. Experiments were terminated by replacing PSS with calcium-free PSS containing 1 mmol/L EGTA to obtain the maximum dilation of the vessel.

**Reagents and Buffers**

All chemicals were purchased from Sigma with the exception of fura 2-AM and Phloronic F-127 (Tet Labs). The ionic composition of PSS contained the following (mmol/L): NaCl 119, NaHCO\(_3\) 21, KCl 4.7, KH\(_2\)PO\(_4\) 1.18, MgSO\(_4\) 1.17, CaCl\(_2\) 1.6, glucose 5.5, and EDTA 0.026. MOPS buffer consisted of the following (mmol/L): NaCl 145, NaH\(_2\)PO\(_4\) 1.2, KCl 4.7, MgSO\(_4\) 1.17, CaCl\(_2\) 1.6, glucose 5, pyruvate 2, EDTA 0.02, and MOPS 3. The MOPS buffer was adjusted to pH 7.4 at room temperature. Solutions of ATP (10\(^{-7}\) mol/L) and L-NAME (3 × 10\(^{-5}\) mol/L) were prepared in distilled water, aliquoted, and then frozen. A stock solution of indomethacin (10\(^{-5}\) mol/L) was prepared in a solution of NaCO\(_3\) and distilled water. Fura 2-AM was mixed with 50 μL dimethyl sulfoxide and 25 μL Phloronic F-127 in dimethyl sulfoxide.

**Data Analysis and Calculations**

All data are presented as mean ± SEM. Both diameter and [Ca\(^{2+}\)], measurements were averaged over a 2-minute period immediately after luminal exposure to ATP. Changes in vascular diameter are
Results

Ovariectomized Rats

At 2 weeks after OVX and pump insertion, plasma estradiol levels were significantly elevated in estrogen-treated OVX females (11 ± 2 pg/mL; P < 0.05, 1-way ANOVA). Estrogen-treated OVX females also gained significantly less weight compared with vehicle-treated OVX females (1.9 ± 5% versus 47 ± 3%; P < 0.05, 1-way ANOVA).

Endothelial Calcium Changes to EDHF-Mediated Dilations

Previous studies in our laboratory have shown that EDHF-mediated dilations are attenuated in the intact female and estrogen-treated OVX rat MCA. To determine whether this could be attributed to an insufficient elevation in EC [Ca2+], changes in vascular diameter and EC [Ca2+] were measured simultaneously.

After the development of tone, resting MCA diameters were similar between groups: 241 ± 9 μm (intact males),
The EDHF-mediated dilation was significantly reduced in intact females and estrogen-treated OVX females (Figure 3). ATP-induced dilations were significantly reduced in intact females and estrogen-treated OVX females (174±13 nmol/L) compared with intact males (126±10 nmol/L) and in estrogen-treated OVX females (113±15 nmol/L) compared with vehicle-treated OVX females (62±5 nmol/L) (P<0.05, 1-way ANOVA). However, in response to 10⁻⁵ mol/L ATP, EC [Ca²⁺], increased to similar levels in intact males (421±77 nmol/L), intact females (461±116 nmol/L), vehicle-treated OVX females (530±92 nmol/L), and estrogen-treated OVX females (417±50 nmol/L) (P=NS, 1-way ANOVA) (Figures 1B and 3B, respectively).

To rule out the possibility that there is modulation of calcium sensitivity at the level of the endothelium, MCAs were luminally exposed to the calcium ionophore A23187 in the presence of L-NAME and indomethacin. A23187 increases [Ca²⁺], by directly facilitating Ca²⁺ influx into the cell. As shown in Figure 4, A23187-induced dilations were significantly attenuated in the intact female MCA compared with the intact male MCA.

**Smooth Muscle Calcium Changes to EDHF-Mediated Dilations**

To further dissect out the mechanisms associated with the effect of estrogen on EDHF-mediated dilations, changes in SMC [Ca²⁺], in response to luminal application of ATP were assessed. The diameter of the pressurized MCAs was not affected by loading the SMCs with fura 2-AM. In the presence of L-NAME and indomethacin, resting SMC [Ca²⁺] was comparable between estrogen-treated OVX females (237±12 nmol/L), vehicle-treated OVX females (221±10 nmol/L), and estrogen-treated OVX females (237±11 nmol/L) (Figures 5 and 6). In response to luminal application of 10⁻⁵ mol/L ATP, SMC [Ca²⁺] decreased in both intact males (170±9 nmol/L) and vehicle-treated OVX females (176±17 nmol/L), while it increased in intact females (244±15 nmol/L) and estrogen-treated OVX females (264±17 nmol/L). The increase in SMC [Ca²⁺] reflects the fact that these vessels dilated transiently, followed by constriction. We therefore calculated the minimum value that SMC [Ca²⁺] reached in response to 10⁻⁵ mol/L ATP and verified that SMC [Ca²⁺] decreased to a greater degree in males (37±4%) compared with females (21±5%) and in vehicle-treated OVX females (18±7%) compared with estrogen-treated OVX females (3±5%).

SMC hyperpolarization induced by 15 mmol/L KCl caused a comparable dilation in intact males (71±9%) and intact females (64±4%), with a similar corresponding decrease in SMC [Ca²⁺], by 22±6% (intact males) and 24±3% (intact females). Comparable dilations were also observed in estrogen-treated (69±5%) and vehicle-treated OVX females (73±6%). The concomitant decrease in SMC [Ca²⁺], was also comparable between estrogen-treated OVX females (29±2%) and vehicle-treated OVX females (30±4%).
The results of the present study suggest that a factor or mechanism coupling EDHF to reduction of ionized SMC $\text{Ca}^{2+}$ accounts for the attenuated EDHF-mediated dilations in the female MCA. This conclusion is supported by 3 lines of evidence. First, EC $\text{Ca}^{2+}$ reached sufficient levels to potentially elicit an EDHF response in all experimental groups. However, this was not accompanied by a robust dilation in intact females and estrogen-treated OVX females. Second, imposed increases in EC $\text{Ca}^{2+}$ using the calcium ionophore A23187 failed to elicit a substantial EDHF-mediated dilation. Third, SMC $\text{Ca}^{2+}$ decreased to a greater degree in intact male MCAs compared with intact females, ruling out the possibility of an alteration of the $\text{Ca}^{2+}$ sensitivity of the vascular smooth muscle contractile apparatus in females ($P<0.05$ compared with males, 2-way repeated-measures ANOVA).

**Discussion**

The results of the present study suggest that a factor or mechanism coupling EDHF to reduction of ionized SMC $\text{Ca}^{2+}$, accounts for the attenuated EDHF-mediated dilations in the female MCA. This conclusion is supported by 3 lines of evidence. First, EC $\text{Ca}^{2+}$, reached sufficient levels to potentially elicit an EDHF response in all experimental groups. However, this was not accompanied by a robust dilation in intact females and estrogen-treated OVX females. Second, imposed increases in EC $\text{Ca}^{2+}$, using the calcium ionophore A23187 failed to elicit a substantial EDHF-mediated dilation. Third, SMC $\text{Ca}^{2+}$, decreased to a greater degree in intact male MCAs compared with intact females, ruling out the possibility of an alteration of the $\text{Ca}^{2+}$ sensitivity of the vascular smooth muscle contractile apparatus in females ($P<0.05$ compared with males, 2-way repeated-measures ANOVA).

**Endothelial Calcium Changes to EDHF-Mediated Dilations**

In the presence of L-NAME and indomethacin, resting but not ATP-stimulated EC $\text{Ca}^{2+}$, was significantly elevated in MCAs isolated from intact females and estrogen-treated OVX females compared with intact males and vehicle-treated OVX females. Our findings agree with those of Knot and colleagues, who found that in coronary arteries, basal EC $\text{Ca}^{2+}$ but not acetylcholine-stimulated $\text{Ca}^{2+}$, was significantly elevated in females compared with their male counterparts. Although they agree, the latter studies do not reflect an EDHF-mediated dilation. To substantiate this, we have previously shown that the ATP-induced dilation resistant to L-NAME and indomethacin in intact males and OVX females can be abolished either by denudation or inhibition of $\text{Ca}^{2+}$-sensitive potassium channels (charybdotoxin).

Our finding that basal EC $\text{Ca}^{2+}$, is elevated in the MCA isolated from intact females and estrogen-treated OVX females deserves some attention. An elevated EC $\text{Ca}^{2+}$ could suggest a greater driving force for calcium, perhaps instigated by greater hyperpolarization. Furthermore, the notion that female ECs are more hyperpolarized may offer an explanation for the attenuated EDHF-mediated dilations. The amplitude of the ATP-induced hyperpolarization depends on the resting membrane potential, as shown in cells with a more negative membrane potential, where the hyperpolarization in response to endothelium-dependent vasodilators was much smaller in magnitude. In other words, the magnitude of the agonist-induced hyperpolarization may be a result of the difference in resting membrane potential between male and female MCAs.

While EDHF-mediated increases in EC $\text{Ca}^{2+}$, were sufficiently elevated to potentially elicit an EDHF-mediated dilation, a robust dilation was not observed in intact females and estrogen-treated OVX females. One could reason that a reduced calcium sensitivity at the level of the endothelium may account for this phenomenon. To address this possibility, the calcium ionophore A23187 was delivered to the endothelium in the presence of L-NAME and indomethacin. If there was a $\text{Ca}^{2+}$ sensitivity issue at the level of the endothelium,
one would expect that imposed increases in EC Ca\(^{2+}\) would elicit robust EDHF-mediated dilations. However, A23187 was also ineffective in eliciting a robust EDHF relaxation in intact females compared with intact males (Figure 4). Taken together, our data suggest that at the level of the endothelium, neither insufficient agonist-induced increases in [Ca\(^{2+}\)], nor calcium sensitivity can account for the attenuated EDHF-mediated dilations in the female rat MCA.

**Smooth Muscle Calcium Changes to EDHF-Mediated Dilations**

Resting SMC [Ca\(^{2+}\)], was comparable in MCAs isolated from intact males, intact females, vehicle-treated OVX females, and estrogen-treated OVX females (Figures 5 and 6). However, in response to luminal ATP (10^{-5} mol/L), SMC [Ca\(^{2+}\)], decreased to a greater degree in males (37\(\pm\)4\%) and vehicle-treated OVX females (18\(\pm\)7\%) compared with females (21\(\pm\)5\%) and estrogen-treated OVX females (3\(\pm\)5\%). Previous studies have reported that basal levels of [Ca\(^{2+}\)], are reduced in aortic SMCs isolated from intact females and estrogen-treated OVX females.\(^{24}\) The disparity in the 2 studies is most likely a reflection of differences in vascular bed (MCA versus aorta), strain (Long-Evans versus Wistar-Kyoto rats), and experimental paradigm (pressurized versus nonpressurized vessels).

Since dilation to EDHF in the male MCA is mediated by hyperpolarization of the SMC,\(^{1,25}\) we investigated the possibility that the attenuation of the EDHF-mediated dilation in females is attributed to lack of hyperpolarization of the SMC. However, exposure to 15 mmol/L KCl elicited decreases in SMC [Ca\(^{2+}\)], and a concomitant dilation, suggesting that females can respond appropriately to hyperpolarization of the SMC. Although this is indirect evidence, it suggests that hyperpolarization of the SMC is inadequate in the EDHF-mediated pathway to produce a dilation in intact female and estrogen-treated OVX female MCAs.

In conclusion, the results of the present study suggest that a factor or mechanism coupling EDHF to reduction of ionized SMC [Ca\(^{2+}\)], accounts for the attenuated EDHF-mediated dilations in the female MCA. Our results support the idea that there is inadequate hyperpolarization of the SMC in the female MCA to elicit a robust EDHF-mediated dilation. This study underscores the fact that sex-related differences in vascular reactivity exist and promotes new perspectives for clinical research.

**Acknowledgment**

This work was supported by American Heart Association National Scientist Development grant 0130250N.

**References**


Altered Calcium Dynamics Do Not Account for Attenuation of Endothelium-Derived Hyperpolarizing Factor–Mediated Dilations in the Female Middle Cerebral Artery
Elke M. Golding, Dorota M. Ferens and Sean P. Marrelli

Stroke. 2002;33:2972-2977; originally published online October 31, 2002;
doi: 10.1161/01.STR.0000035907.82204.39
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 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/33/12/2972

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/