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 \(N\)-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
critical role of EC [Ca\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{2+}], was markedly attenuated. Our results suggest that estrogen acts to uncouple the EDHF response at a point beyond the change in EC [Ca\textsuperscript{2+}]. This uncoupling prevents the reduction of SMC [Ca\textsuperscript{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 (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 aseptic conditions. Animals were secured to a nose cone and allowed to breathe spontaneously (2% isoflurane). Rectal temperature was maintained at 37°C with the use of a heating pad and a temperature controller (Harvard Apparatus Inc). A bilateral ovariectomy was then performed by accessing the ovaries through 2 lateral abdominal incisions. A dorsal incision was made, and an osmotic minipump (model 2004, Alza Corporation) was subcutaneously implanted to deliver either 17β-estradiol or 50% dimethyl sulfoxide/0.045% NaCl (vehicle). 17β-Estradiol was administered at a physiological dose of 4 μg/kg per day. Validation of estrogen depletion and repletion comprised monitoring body weight and measuring plasma estradiol levels to elicit a robust EDHF response and that this effect is mediated by estrogen.

**Measurement of Vascular Diameter and Calcium**

Vascular diameter and intracellular calcium were measured simultaneously as recently described in detail\textsuperscript{16} with the use of a charge-coupled device video system and photomultiplier tube, respectively (Intracellular Imaging). Frequency of acquisition was 8 Hz for vascular diameter and 4 and 5 Hz for EC and SMC [Ca\textsuperscript{2+}], respectively. For the measurement of EC or SMC [Ca\textsuperscript{2+}], the Ca\textsuperscript{2+}-sensitive indicator fura 2 was selectively loaded into either the EC or SMC, respectively (see below). Fura 2 was excited at 340 and 380 nm, and the emission light was sampled at 510 nm. The fluorescence ratio (340/380 nm) was calculated after subtraction of the background fluorescence.

Selective loading of the endothelium was achieved by perfusing fura 2-AM (0.67 μmol/L) through the lumen for a period of 4 minutes. The fura 2-AM containing buffer was subsequently washed out, and an additional period of 15 minutes was allowed for complete de-esterification of the dye. Smooth muscle loading was achieved from the adventitial side by replacing the abluminal PSS with MOPS buffer containing fura 2-AM (1 μmol/L). Loading was continued for 5 minutes 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\textsuperscript{2+}] measurements were acquired. Previous studies have confirmed the selectivity of loading the ECs and SMCs with the use of this paradigm.\textsuperscript{16}

**EDHF-Mediated Dilations**

EDHF-mediated dilations were assessed by luminal application of ATP, a P2Y, purinoreceptor agonist, in the presence of N\textsuperscript{o}-nitro-l-arginine methyl ester (L-NAME) and indomethacin.\textsuperscript{17} After the development of spontaneous tone, L-NAME (3×10\textsuperscript{−5} mol/L) and indomethacin (10\textsuperscript{−6} 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\textsuperscript{−7} to 10\textsuperscript{−3} mol/L) was determined in all 4 groups. Vascular diameter and either EC or SMC [Ca\textsuperscript{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\textsuperscript{−7} to 10\textsuperscript{−4} mol/L) was determined. To assess whether female MCA 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.\textsuperscript{18} 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 Pluronic F-127 (TefLabs). The ionic composition of PSS contained the following (mmol/L): NaCl 119, NaHCO\textsubscript{3} 21, KC\textsubscript{l} 4.7, KH\textsubscript{2}PO\textsubscript{4} 1.18, MgSO\textsubscript{4} 1.17, CaCl\textsubscript{2} 1.6, glucose 5.5, and EDTA 0.026. MOPS buffer consisted of the following (mmol/L): NaCl 145, NaH\textsubscript{2}PO\textsubscript{4} 1.2, KCl 4.7, MgSO\textsubscript{4} 1.17, CaCl\textsubscript{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\textsuperscript{−7} mol/L) and L-NAME (3×10\textsuperscript{−2} mol/L) were prepared in distilled water, aliquoted, and then frozen. A stock solution of indomethacin (10\textsuperscript{−6} mol/L) was prepared in a solution of NaCO\textsubscript{3} and distilled water. Fura 2-AM was mixed with 50 μL dimethyl sulfoxide and 25 μL Pluronic F-127 in dimethyl sulfoxide.

**Data Analysis and Calculations**

All data are presented as mean±SEM. Both diameter and [Ca\textsuperscript{2+}], measurements were averaged over a 2-minute period immediately after luminal exposure to ATP. Changes in vascular diameter are...
presented as a percentage of the maximum diameter of the MCAs, calculated as follows

\[
\% \text{ Maximum Diameter} = \frac{(D_{\text{ATP}} - D_{\text{Base}})}{(D_{\text{Max}} - D_{\text{Base}})} \times 100,
\]

where \( D_{\text{ATP}} \) is the diameter of the MCA after luminal administration of ATP, \( D_{\text{Base}} \) is the baseline diameter of the MCA before addition of ATP, and \( D_{\text{Max}} \) is the maximal diameter of the MCA in the presence of calcium-free conditions. In situ calibration \(^{16} \) was performed on both intact male MCAs (n=8) and intact female MCAs (n=5). The calibration curves did not differ statistically and were therefore averaged to yield the following values: 2.017 (\( R_{\text{max}} \)), 0.1365 (\( R_{\text{min}} \)), and 5.218 (\( \beta \)). The in situ \( K_d \) was previously determined by Knot and Nelson \(^{19} \) to be 282 nmol/L.

Statistical comparisons of body weight changes and 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 had also gained significantly less weight compared with vehicle-treated OVX females (1.9 ± 5% versus 47 ± 3%; \( P < 0.05 \), 1-way ANOVA).

**Results**

Ovariectomized Rats

At 2 weeks after OVX and pump insertion, plasma estradiol levels were significantly elevated in estrogen-treated OVX females (36 ± 7 pg/mL; \( P < 0.05 \), 1-way ANOVA). Estrogen-treated OVX females had also gained significantly less weight compared with vehicle-treated OVX females (1.9 ± 5% versus 47 ± 3%; \( P < 0.05 \), 1-way ANOVA).

**Figure 1.** Percent diameter change (A) and endothelial \([\text{Ca}^{2+}]_i\) (B) of male (n=6) and female (n=6) MCAs in response to luminal delivery of ATP (L-NAME and indomethacin present). The EDHF-mediated dilation was significantly reduced in female MCAs compared with male MCAs. However, the concomitant increase in endothelial \([\text{Ca}^{2+}]_i\) was comparable in both groups. (#\( P < 0.05 \) compared with males, 2-way repeated-measures ANOVA). Resting endothelial \([\text{Ca}^{2+}]_i\) was significantly greater in intact females compared with intact males (#\( P < 0.05 \) compared with males, 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. \(^9 \) To determine whether this could be attributed to an insufficient elevation in EC \([\text{Ca}^{2+}]_i\), changes in vascular diameter and EC \([\text{Ca}^{2+}]_i\) were measured simultaneously.

After the development of tone, resting MCA diameters were similar between groups: 241 ± 9 \( \mu \)m (intact males),
Exposure to L-NAME and indomethacin resulted in significantly reduced in intact females and estrogen-treated OVX females (Figure 3). ATP-induced dilations were significantly reduced in intact females (Figure 1), and vehicle-treated and estrogen-treated females (Figure 3). Percent diameter change of intact male (n=7) and estrogen-treated OVX female (OVX/E2) (n=9) MCAs in response to luminal delivery of ATP (L-NAME and indomethacin present). The EDHF-mediated dilation was significantly reduced in OVX/E2 MCAs compared with OVX/Vehicle MCAs. However, the concomitant increase in endothelial [Ca2+]i was significantly greater in OVX/E2 compared with OVX/Vehicle (n=7) and estrogen-treated OVX female (OVX/E2) (n=3% (intact females), 24% (estrogen-treated OVX females), and 19% (vehicle-treated OVX females), and 19% (estrogen-treated OVX females)).

Changes in diameter and EC [Ca2+]i in response to luminal delivery of ATP were then assessed in intact males, intact females (Figure 1), and vehicle-treated and estrogen-treated females (Figure 3). ATP-induced dilations were significantly reduced in intact females and estrogen-treated OVX females compared with intact males and vehicle-treated OVX females (P<0.05, repeated-measures ANOVA) (Figures 1A and 3A, respectively). Representative dynamic responses of MCAs isolated from intact males and females are shown in Figure 2A and 2B, respectively. The diameter of the pressurized MCAs was not affected by loading the endothelium with fura 2-AM. In the presence of L-NAME and indomethacin, resting EC [Ca2+]i was significantly greater in intact 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, l-way ANOVA). However, in response to 10-5 mol/L ATP, EC [Ca2+]i 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+]i 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+]i, 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+]i was comparable between estrogen-treated OVX females (69±6% (vehicle-treated OVX females), and 6% (intact males) and 23% (estrogen-treated OVX females)). The increase in SMC [Ca2+]i by directly facilitating Ca2+ influx into the cell. We therefore calculated the minimum value that SMC [Ca2+]i, reached in response to 10-5 mol/L ATP and verified that SMC [Ca2+]i, decreased to a greater degree in 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+]i, reflects the fact that these vessels dilated transiently, followed by constriction. We therefore calculated the minimum value that SMC [Ca2+]i, reached in response to 10-5 mol/L ATP and verified that SMC [Ca2+]i, decreased to a greater degree in males (37±4% (vehicle-treated OVX females (18%±7%) compared with and 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+]i, 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+]i, was also comparable between estrogen-treated OVX females (29±2%) and vehicle-treated OVX females (30±4%).

**Figure 3.** Percent diameter change (A) and endothelial [Ca2+]i (B) of vehicle-treated OVX female (OVX/Vehicle) (n=7) and estrogen-treated OVX female (OVX/E2) (n=9) MCAs in response to luminal delivery of ATP (L-NAME and indomethacin present). (P<0.05 compared with males, 2-way repeated-measures ANOVA). The diameter of the pressurized MCAs was not affected by loading the endothelium with fura 2-AM. In the presence of L-NAME and indomethacin, resting EC [Ca2+]i was significantly greater in intact 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, l-way ANOVA). However, in response to 10-5 mol/L ATP, EC [Ca2+]i 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).

**Figure 4.** Percent diameter change of intact male (n=4) and intact female (n=4) MCAs in response to luminal delivery of the calcium ionophore A23187 (L-NAME and indomethacin present). The EDHF-mediated dilation was significantly reduced in female MCAs compared with male MCAs (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 $[\text{Ca}^{2+}]_i$ accounts for the attenuated EDHF-mediated dilations in the female MCA. This conclusion is supported by 3 lines of evidence. First, EC $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ using the calcium ionophore A23187 failed to elicit a substantial EDHF-mediated dilation. Third, SMC $[\text{Ca}^{2+}]_i$ decreased to a greater degree in intact male MCAs compared with intact females, ruling out the possibility of an alteration of the $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$, was significantly elevated in MCAs isolated from intact females and estrogen-treated OVX females. Our findings agree with those of Knot and colleagues, who found that in coronary arteries, basal EC $[\text{Ca}^{2+}]_i$, but not acetylcholine-stimulated $[\text{Ca}^{2+}]_i$, 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 $[\text{Ca}^{2+}]_i$ is elevated in the MCA isolated from intact females and estrogen-treated OVX females deserves some attention. An elevated EC $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$, 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 $[\text{Ca}^{2+}]_i$ 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\(^{2+}\)]\(_i\) 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\(^{2+}\)]\(_i\) decreased to a greater degree in males (37±4%) and vehicle-treated OVX females (18±7%) compared with females (21±5%) and estrogen-treated OVX females (3±5%). Previous studies have reported that basal levels of [Ca\(^{2+}\)]\(_i\) 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,\(^{1,2}\) 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\(^{2+}\)]\(_i\) 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\(^{2+}\)]\(_i\) 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.

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**References**

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