CHRONIC ESTROGEN DEPLETION ALTERS ADENOSINE DIPHOSPHATE-INDUCED PIAL ARTERIOULAR DILATION IN FEMALE RATS

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We recently reported (21) that estrogen depletion via ovariectomy in rats was accompanied by a nearly complete loss of pial arteriolar responses to the endothelial nitric oxide (NO) synthase (eNOS)-dependent vasodilator acetylcholine (ACh). Those responses were restored in ovariectomized (Ovx) rats given chronic estrogen replacement. The apparent estrogen-associated changes in eNOS function probably can be attributed, at least in part, to the reported ability of estrogen in cerebral vessels to positively influence eNOS expression (15, 21), while negatively affecting the expression of the endogenous eNOS inhibitor caveolin-1 (21). In the present study, using a closed cranial window system and intravital microscopy, we sought to corroborate the aforementioned estrogen influence on cerebrovascular eNOS function by monitoring pial arteriolar reactivity to another purported eNOS-dependent vasodilator ADP (14, 22). Intact females, Ovx females, and 17β-estradiol (E2)-treated Ovx females were studied. The eNOS-dependent portion of the ADP response was ascertained by comparing ADP-induced pial arteriolar dilations before and after suffusion of a NOS inhibitor, Nω-nitro-L-arginine (L-NNA; 1 mM) in intact, ovariectomized (Ovx), and 17β-estradiol (E2)-treated Ovx females. We also examined whether ovariectomy altered the participation of other factors in the ADP response. Those factors were the following: 1) the prostaglandin indomethacin (Indo); 2) the Ca2+-dependent K+ (KCa) channel, ibeiriotoxin (IbTX); 3) the ATP-regulated K+ (KATP) channel glibenclamide (Glib); 4) the KCa-regulating epoxygenase pathway miconazole (Mic); and 5) the adenosine receptor 8-sulfophenyltheophylline (8-SPT)

In intact females, the eNOS-dependent (i.e., L-NNA sensitive) portion of the ADP response was ascertained by comparing ADP-induced pial arteriolar dilations before and after suffusion of a NOS inhibitor, Nω-nitro-L-arginine (L-NNA). The ADP response was first established in intact, ovariectomized (Ovx), and 17β-estradiol (E2)-treated Ovx females. We also examined whether ovariectomy altered the participation of other factors in the ADP response. Those factors were the following: 1) the prostaglandin indomethacin (Indo); 2) the Ca2+-dependent K+ (KCa) channel, ibeiriotoxin (IbTX); 3) the ATP-regulated K+ (KATP) channel glibenclamide (Glib); 4) the KCa-regulating epoxygenase pathway miconazole (Mic); and 5) the adenosine receptor 8-sulfophenyltheophylline (8-SPT).

METHODS

The study protocol was approved by the Institutional Animal Care and Use Committee. In initial experiments, three groups of Sprague-Dawley rats (250–350 g) were used: intact female, ovariectomized (Ovx), and E2-treated (0.1 mg·kg−1·day−1 ip for 1–2 wk) Ovx rats (Ovx + E2). The vendor (Charles River) performed the ovariectomies 1 wk before the rats were shipped. The Ovx rats were then used at 4–6 wk postovariectomy. As shown in a previous report from our laboratory (21), the 0.1 mg·kg−1·day−1 dose results in an average daily plasma E2 concentration that falls between the peak and nadir levels observed over the normal rat estrous cycle. Further details regarding animal treatments are provided in earlier publications from our laboratory (21, 23, 26). Anesthesia was induced with halothane. The rats were then paralyzed with curare, tracheotomized, and mechanically ventilated. For surgery, anesthesia was maintained with 0.8% halothane and 70% N2O:30% O2. Catheters were placed in the femoral artery to measure systemic arterial pressure and to obtain arterial blood samples, and in the femoral vein for infusion of drugs. Rectal temperature was maintained at 37°C with a servo-controlled heating pad. The rats were then

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prepared for placement of a closed cranial window, according to established procedures (21, 23). Briefly, the surface of the skull was exposed after a midline scalp incision. A craniotomy (10-mm diameter) was performed over the midline of the skull, and the dura was carefully removed, keeping the sagittal sinus intact. An acrylic cranial window equipped with three ports (inflow, outflow, and intracranial pressure monitoring) was fixed to the skull with cyanoacrylate gel. After the surgery, the halothane was discontinued and a loading dose of intravenous fentanyl (10 μg/kg) was given. The rat was maintained on 70% N2O-30% O2 and fentanyl (25 μg·kg⁻¹·h⁻¹ iv) thereafter. The space under the window was filled with artificial cerebrospinal fluid (aCSF) that was equilibrated with 10% O2-5% CO2, with a balance of N2 (9). The aCSF was suffused at 0.5 ml/min and was maintained at a temperature of 37°C. Cerebral arterioles were observed using a microscope equipped with a video camera. Magnifications of ×800 were displayed on a video monitor. Measurements of arteriole diameters were made using a calibrated video microscler.

In all experiments, initial diameter measurements were made after a 40-min period of cortical suffusion with drug-free aCSF (at 1 h after halothane). Hypercapnia (Paco2, 0–65 mmHg) was then imposed for 3 min, and the arterioles displaying an adequate response to CO2 (pial arteriolar “reactivity” >1.0% diameter increase/mmHg CO2 change) were used in each experiment. In the first series of experiments, ADP was suffused at concentrations of 1, 10, and 100 μM (10 min at each level). Baseline diameters were then restored via 10- to 15-min suffusion of drug-free aCSF. At this time, the nonselective NOS inhibitor, Nω-nitro-l-arginine (L-NNA, 1 mM), was introduced into the suffusate and applied for 60 min. The ADP suffusion sequence was then repeated.

In the second series of experiments, only intact and Ovx females were evaluated. Series 2 rats were further divided into five subgroups, according to drug treatment: 1) cyclooxygenase inhibitor, 2) KCa, channel blocker, 3) KATP channel blocker, 4) cytochrome P-450 epoxygenase inhibitor, and 5) adenosine receptor antagonist. After suffusions of ADP (1, 10, and 100 μM), baseline diameters were restored with suffusion of drug-free aCSF for 10 min. At this point, experimental protocols in the different groups diverged. In group 2.1, the effect of indomethacin on the pial arteriolar response to ADP was examined before and 20 min after an intravenous injection of indomethacin (10 mg/kg). After return to baseline, L-NNA (1 mM) was added to the suffusate, and the ADP response was evaluated after 45–60 min. In group 2.2, before ADP infusion, IbTX (0.1 μM) was suffused for 30 min. After the return to IbTX suffusion alone, L-NNA (1 mM) was added, and the ADP response was evaluated 45–60 min later. In one-half of the animals, the pial arteriolar responses to suffusions of the NO donor S-nitroso-N-acetylpenicillamine (SNAP; 0.1 and 1.0 μM) were measured. SNAP was administered before ADP both in the absence and in the presence of IbTX. In group 2.3, glibenclamide (5 μM) was suffused for 10 min before cosuffusion with ADP. In group 2.4, miconazole (10 μM) was added to the suffusate, and ADP cosuffusion was initiated 20 min later. In group 2.5, a slight modification in the initial vasodilator application sequence was introduced. Thus, adenosine (100 μM) was suffused for 5 min, and then after 10 min of drug-free aCSF, the ADP application was initiated. The general adenosine receptor antagonist 8-SPT (10 μM) was then suffused for 15 min before repeating the additions of adenosine and ADP. Several intact and Ovx females were utilized as time controls, where ADP and SNAP responses were evaluated in the absence of any inhibitor additions. No changes from the initial ADP or SNAP response were observed (data not shown). Mean arterial blood pressure (MABP) was continuously monitored and arterial blood samples were taken at 30-min intervals for measurement of PO2, PCO2, and pH by using a blood gas/pH analyzer (model ABL 520, Radiometer; Copenhagen, Denmark). The rats were euthanized with a halothane overdose at the termination of the experiment.

Statistical comparisons of pial arterioles diameter values within groups were made using one-way analysis of variance combined with a post hoc Tukey’s test. A level of P < 0.05 was considered significant in all statistical tests. Values are presented as means ± SE.

ADP, L-NNA, Indo, IbTX, miconazole, and 8-SPT were purchased from Sigma (St. Louis, MO), glibenclamide was obtained from Research Biochemicals (Natick, MA). ADP, L-NNA, and miconazole were prepared in aCSF. Indo was dissolved by sonication in a Na2CO3 solution. IbTX was dissolved in ethanol and diluted in aCSF before suffusion. The stock glibenclamide was made by initially dissolving this agent in a small amount of dimethyl sulfoxide and the balance in ethanol. This vehicle was then diluted 1:1,000 in aCSF to make the working solution. All concentrations were expressed as the final molar concentration suffused under the cranial window.

RESULTS

In animals from both experimental series, the arteriolar PCO2, pH, and MABP did not show any significant variations over the length of the study. Thus, initial and final pH, PCO2, and MABP values were (in mmHg) 7.38 ± 0.01 and 7.37 ± 0.01, 38.8 ± 1.2 and 34.6 ± 1.7, and 125 ± 3 and 132 ± 2, respectively. PO2 was maintained above 100 mmHg during all experiments.

No significant changes in pial arteriolar diameters were observed in the presence of 1 μM ADP. Thus, in the results described below, only the findings obtained during suffusions of 10 and 100 μM ADP are reported. The results of series 1 experiments are shown in Fig. 1. The initial ADP response evaluations revealed similar dose-dependent vasodilations (at 10 and 100 μM ADP) in the intact (17 ± 2 and 28 ± 2%, respectively), Ovx (14 ± 1 and 22 ± 2%, respectively), and Ovx + E2 (15 ± 2 and 28 ± 3%, respectively) groups. In all groups, pial arteriolar diameters reverted to pre-ADP levels within 10 min after return to control aCSF. In intact females, topical application of the NOS inhibitor L-NNA (1 mM) was accompanied by significant 60 and 41% reductions in pial arteriolar dilations at 10 and 100 μM ADP, respectively. No reductions in the ADP response at any dose were observed in the Ovx group. However, in the E2-treated Ovx females, the L-NNA sensitivity was restored, to the extent that the response was reduced by 81 and 50% at 10 and 100 μM ADP, respectively. Before ADP suffusion, application of L-NNA for 60 min was associated with virtually no change in pial arteriolar diameters (data not shown).

The results of series 2 experiments are summarized in Figs. 2–7. In the cyclooxygenase inhibition experiments (Fig. 2), before administration of Indo, ADP elicited statistically similar dose-dependent responses in intact and Ovx females (18 ± 1 vs. 14 ± 2%, at 10
Treatment with Indo had no effect on baseline diameters (data not shown), and did not alter responses of pial arterioles to ADP in either group. When L-NNA was subsequently added to the suffusate, baseline diameters were not altered but the ADP response in the intact females was reduced to an extent (68 and 51% at 10 and 100 μM, respectively). IbTX alone did not significantly alter baseline diameters, although modest (2–6%) reductions were observed (not shown). However, IbTX was found to have a significant effect on ADP reactivities in both groups. Thus, at 10 μM ADP, the diameter increases were reduced by 76% and 97% in the intact and Ovx females, respectively. At 100 μM ADP, the diameter increases were reduced by 45% and 59%, respectively. After ADP withdrawal, and in the presence of continued IbTX suffusion, baseline diameters were restored. At this point, a cosuffusion of IbTX and L-NNA was initiated and ADP reactivities were subsequently retested. No further changes in ADP reactivities were observed, compared with those measured in the presence of IbTX alone. The most important information revealed by this experimental manipulation (coupled to the results

![Graph A](image)

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Fig. 1. Pial arteriolar diameter increases (expressed as a percentage of the baseline diameters) during suffusions of increasing concentrations of ADP, both in the absence (initial) and in the presence of topically applied Nω-nitro-arginine (L-NNA; 1 mM). Results for intact (A), ovariectomized (B), and 17β-estradiol (E2)-treated ovariectomized (C) females are presented. Baseline diameters were 36.7 ± 5.9, 37.5 ± 1.9, and 37.9 ± 2.1 μm for the intact, ovariectomized, and E2-treated ovariectomized rats, respectively. Values are means ± SE. *P < 0.05 vs. initial; n = 4–5 rats per group.

μM; and 37 ± 3 vs. 24 ± 1% at 100 μM, respectively). Treatment with Indo had no effect on baseline diameters (data not shown), and did not alter responses of pial arterioles to ADP in either group. When L-NNA was subsequently added to the suffusate, baseline diameters were not altered but the ADP response in the intact females was reduced to an extent (68 and 51% at 10 and 100 μM, respectively) similar to that seen in the data summarized in Fig. 1. The addition of L-NNA had no effect on the ADP response in Ovx females. In all cases, pre-ADP diameters were rapidly restored on cessation of ADP suffusions.

In experiments examining the roles of KCa and KATP channels in ADP-induced vasodilation in intact and Ovx rats, contrasting findings were obtained. The results of the experiments examining KCa channel contributions are given in Fig. 3. Consistent with the results described above, no differences in the initial reactivities to 10 and 100 μM ADP were seen in the intact versus Ovx females (17 ± 2 vs. 18 ± 2% at 10 μM, and 32 ± 3 vs. 35 ± 5% at 100 μM, respectively). IbTX alone did not significantly alter baseline diameters, although modest (2–6%) reductions were observed (not shown). However, IbTX was found to have a significant effect on ADP reactivities in both groups. Thus, at 10 μM ADP, the diameter increases were reduced by 76% and 97% in the intact and Ovx females, respectively. At 100 μM ADP, the diameter increases were reduced by 45% and 59%, respectively. After ADP withdrawal, and in the presence of continued IbTX suffusion, baseline diameters were restored. At this point, a cosuffusion of IbTX and L-NNA was initiated and ADP reactivities were subsequently retested. No further changes in ADP reactivities were observed, compared with those measured in the presence of IbTX alone. The most important information revealed by this experimental manipulation (coupled to the results

![Graph D](image)

Fig. 2. ADP-induced pial arteriolar diameter increases in intact (A) and ovariectomized (B) females before (initial) and after indomethacin (Indo; 10 mg/kg iv), and then Indo plus topically applied L-NNA (1 mM). Baseline diameters were 38.8 ± 4.4 and 37.5 ± 7.5 μm for the intact and ovariectomized rats, respectively. Values are means ± SE. *P < 0.05 vs. initial and Indo; n = 4 rats per group.
provided in Fig. 1) is that the NO- and KCa-dependent portions of the ADP response in intact females completely overlap. Ostensibly, these findings might be explained by a mechanism whereby NO elicits pial arteriolar dilations in rats via a KCa-dependent process (19). However, recent findings (25) from our laboratory indicated that SNAP-induced pial arteriolar relaxation in male rats was unaffected by IbTX. Therefore, we examined the effects of IbTX on SNAP-induced pial arteriolar dilations in intact and Ovx females. Sufussions of SNAP at 0.1 and 1.0 μM produced identical pial arteriolar responses in the intact and Ovx females (Fig. 4). Furthermore, those initial responses were significantly reduced (by ~50% at 0.1 μM SNAP and by 30–40% at 1.0 μM SNAP) in both groups in the presence of IbTX. Miconazole had no significant effect on baseline diameters (not shown) and did not alter ADP-induced vasodilations in either intact or Ovx female rats (Fig. 6), suggesting that the KCa dependency revealed by the results summarized in Fig. 3 was not related to generation of P-450 epoxygenase products.

Finally, to establish whether ADP-induced pial arteriolar dilations were in any way related to nucleotidase-influenced adenosine production, experiments were conducted in the absence and presence of sufussions of the adenosine receptor antagonist 8-SPT (10 μM). As in all of the other experiments, ADP reactivities before inhibitor addition (Fig. 7) were statistically similar in intact and Ovx females (at 10 and 100 μM ADP, intact vs. Ovx females, respectively). Also, similar responses to topically applied adenosine were observed in the two rat groups. In the

Fig. 4. Pial arteriolar diameter increases elicited by increasing sufussion concentrations of the nitric oxide donor, S-nitroso-N-acetyl penicillamine (SNAP), in intact (A) and ovariectomized (B) rats, in the absence (initial) and in the presence of topically applied ibotin toxin (IbTX; 0.1 μM), followed by combined suffusion of IbTX and L-NNA (1 mM). Baseline diameters were 38.9 ± 2.1 and 36.0 ± 1.1 μm for the intact and ovariectomized rats, respectively. Values are means ± SE. *P < 0.05 vs. initial; n = 8 rats per group.

Fig. 3. Pial arteriolar diameter increases elicited by increasing sufussion concentrations of ADP, in intact (A) and ovariectomized (B) rats, in the absence (initial) and in the presence of topically applied ibotin toxin (IbTX; 0.1 μM), followed by combined suffusion of IbTX and L-NNA (1 mM). Baseline diameters were 40.4 ± 2.0 and 33.8 ± 0.6 μm for intact and ovariectomized rats, respectively. Values are means ± SE. *P < 0.05 vs. initial; n = 4 rats per group.

linked to KCa channel activation (7), we tested whether the epoxygenase inhibitor miconazole (10 μM) had any effect on ADP reactivities. At 10 μM, miconazole has been shown to be a selective epoxygenase inhibitor, with no effects on NOS activity (1). Before miconazole was given, and identical to the findings given in Figs. 1–5, statistically similar ADP responses were seen in the intact and Ovx females (17 ± 2 vs. 13 ± 3% at 10 μM and 27 ± 2 vs. 25 ± 3% at 100 μM, respectively). Miconazole had no significant effect on baseline diameters (not shown) and did not alter ADP-induced vasodilations in either intact or Ovx female rats (Fig. 6), suggesting that the KCa dependency revealed by the results summarized in Fig. 3 was not related to generation of P-450 epoxygenase products.
presence of 8-SPT, no changes in baseline diameters were observed (not shown). However, in both groups, the adenosine response was completely inhibited, indicating the effectiveness of the adenosine receptor blockade. Despite this, ADP-induced dilations were not reduced, demonstrating that ADP does not act via adenosine receptors.

**DISCUSSION**

The principal findings in this investigation can be summarized as follows. First, although the magnitude of ADP-induced pial arteriolar dilation is essentially unaffected by chronic estrogen depletion and subsequent repletion, the NO dependency of the ADP response is lost in ovariectomized females, but restored to the levels observed in intact females on chronic estrogen replacement. Second, in both intact and Ovx female rats, the ADP responses were substantially reduced in the presence of the large-conductance K<sub>Ca</sub> channel (big K<sub>Ca</sub>) channel blocker, IbTX. Moreover, in the intact females, the effects of the NOS and big K<sub>Ca</sub> channel inhibitors completely overlapped. However, the implication of these findings, that the NO- and K<sub>Ca</sub>-dependent portions of the ADP response involve a common pathway, was only partly supported on further study. Third, cytochrome P-450 epoxygenase products are not involved in the K<sub>Ca</sub> channel-dependency of the ADP response. Fourth, irrespective of chronic estrogen status, vasodilator prostanoids, K<sub>ATP</sub> channels, and adenosine receptors, do not participate in ADP-induced pial arteriolar dilations.

The present findings would indicate that the mechanism or mechanisms through which ADP promotes cerebral vasodilation can be substantially altered by estrogen. Thus in the face of chronic estrogen depletion, the K<sub>Ca</sub> channel and NO-dependent portion of the ADP-induced vasodilating process is essentially replaced by a mechanism that continues to rely on K<sub>Ca</sub> channel activation, but not NO.

Evidence obtained in studies (10, 14, 20, 22) on rodent pial arterioles in vivo clearly supports at least a partial NO contribution to the ADP-induced vasodilating response; although some disagreement exists as to the precise percentage of the ADP response affected by NOS inhibition. That is, the ~50% reduction in ADP reactivity seen in the intact females of the present study is less than the 65–90% reductions observed in our previous studies (10, 20). Because the experiments were performed on Sprague-Dawley rats of the same age, exposed to similar surgical and anesthetic conditions and NOS inhibitor doses, gender represents the only major model difference, when comparing the present (female) and previous (male) studies from our...
Thus the possibility exists that the NO-dependent portion of the ADP response is greater in male versus female rats. Results to date also suggest that the source of that NO is eNOS. The latter can be inferred from studies in isolated rat cerebral arteries, or murine pial arterioles in vivo, where ADP-induced relaxation was repressed on endothelial removal (28) or endothelial injury (22), respectively; and from preliminary data obtained in our laboratory (unpublished communications) showing that ADP-induced pial arteriolar dilation in vivo was not altered in the presence of a neuronal NOS-selective inhibitor.

The current findings would appear to be in line with the concept that ADP-induced cerebral arteriolar dilation can occur via more than one pathway. Thus, if one pathway is blocked, a “compensatory switch” takes place, thereby maintaining ADP reactivity, but via an alternative route. The pathway utilized, at least in female animals, is determined by chronic estrogen status. Our present results bear some resemblance to recent findings by Golding and Kepler (6), which showed that ATP-induced dilations in cerebral arteries isolated from intact and estrogen-treated Ovx female rats were much more sensitive to NO inhibition than vessels obtained from untreated Ovx females (6). Moreover, like the present study, KCa channel (but not cyclooxygenase) blockade substantially reduced the response in the estrogen-depleted group. One plausible explanation for the retention of agonist-induced vasodilating function relates to the production of another endothelium-derived relaxing factor (EDRF); the endothelium-derived hyperpolarizing factor (EDHF). Previous studies involving both cerebral (2) and peripheral (13, 18) vessels have suggested that NO may prevent agonist-induced increases in EDHF generation. Thus, only when NO synthesis is blocked, will an increase in EDHF be revealed. However, the results from other laboratories do not support this view (4). Nevertheless, in the absence of any measure of vascular smooth muscle membrane potential, the contribution of an EDHF to agonist-induced vasodilation is often identified by the sensitivity of the vessel to KCa channel blockade combined with its insensitivity to NOS and cyclooxygenase blockade. Those criteria were met in the Ovx rats of the present study, as well as in the study by Golding and Kepler (6).

Of some relevance to the present model of chronic reduction in eNOS function (i.e., estrogen depletion) are the findings of Meng et al. (16), who reported a fairly normal pial arteriolar ACh reactivity in eNOS knockout mice. The data obtained in that study indicated that the retention of ACh reactivity was partly attributable to a compensatory increase in the role of nNOS, although the authors did suggest the possibility of an increase in the contribution from an EDHF.

Recent findings from our laboratory showed that eNOS-dependent vasodilating function (ACh reactivity) in pial arterioles in vivo is almost completely lost in Ovx females but restored with chronic estrogen replacement (21). Those findings, in principal, agree with the present results showing a complete loss of the NO-sensitive component of ADP-induced dilations in estrogen-depleted females. On the other hand, in Ovx rats, the absence of any ACh reactivity, as opposed to a “normal” ADP-induced vasodilating response would seem to indicate that the signal transduction pathways activated by endothelial muscarinic and ADP-sensitive P2Y1 purinoceptor stimulation are different. Although specific differences have not as yet been identified, one possible (but perhaps oversimplified) speculation would be that in rat pial arterioles, P2Y1 receptor activation is capable of activating both eNOS and an “EDHF generator,” whereas muscarinic agonists stimulate only eNOS. In the former case, the NO produced would prevent EDHF release.

As appealing as the above model is in explaining the data obtained in the present study, it remains only speculative, both because of published evidence that appears to oppose features of this model and because of limitations in our experimental approach. Regarding the former, relaxation of mesenteric arteries harvested from female versus male rats (27), or aortas from female rats with pregnancy-induced chronic elevations in circulating estrogen (3), displayed a greater dependence on a non-NO/nonprostanoid hyperpolarizing factor when acetylcholine was applied. These findings, suggesting a direct relationship between chronic estrogen status and the relative EDHF contribution to an endothelium-mediated vasodilating stimulus, would seem to contradict the results obtained for cerebral vessels (Ref. 6 and present results). However, the possibility that estrogen negatively influences EDHF activity only in cerebral vessels is unlikely: in another study (8) that compared mesenteric arteries from male and female rats, the apparent EDHF component was greater in the “estrogen-deprived” males. Although methodological factors (i.e., the use of different rat...
strains and mesenteric artery preparations) or endocrine factors (differences in the levels of circulating testosterone, progesterone, or estrogen before the time of vessel harvest) may have played a role in establishing these differences, a definitive explanation for this apparent contradiction cannot be offered at this time.

Unlike the results obtained in untreated Ovx females, the portion of the ADP response in intact females that was unaffected by NOS (or combined NOS and cyclooxygenase) inhibition was apparently not associated with K\textsubscript{Ca} channel activation because combining NOS and K\textsubscript{Ca} channel blockers produced no further attenuation of the ADP response than that seen in the presence of either inhibitor by itself. Put another way, big K\textsubscript{Ca} channel blockade appeared to affect only the NO-dependent portion of the ADP response in the “estrogen normal” female. At first glance, one might conclude that this reflects a NO (therefore, not EDHF)-induced K\textsubscript{Ca} channel activation (and smooth muscle hyperpolarization?). However, in 1998, we reported (25) that in male rats, SNAP-induced pial arteriolar relaxation was insensitive to IbTX suflusion. The absence of any IbTX effect in our earlier study, versus the 30–55% reductions found in the current investigation, could be taken to indicate that NO-induced pial arteriolar dilation is much more dependent on vascular (smooth muscle?) K\textsubscript{Ca} channel activation in females than it is in males. Moreover, that K\textsubscript{Ca} dependency does not appear to be influenced by chronic estrogen status, because both intact and Ovx females showed the same sensitivity to IbTX. Not consistent with a gender-based explanation are the findings of Paterno et al. (19), who found that the pial arteriolar dilations elicited by the NO donor sodium nitroprusside in male rats were reduced by 40–50% in the presence of IbTX. Nevertheless, present and previous experiments from our laboratory were conducted under nearly identical conditions of anesthesia, surgery, and NO donor administration, leaving only gender as the principal difference. This affords us a less complicated evaluation of the data obtained, as opposed to the study of Paterno et al. (19), where a different anesthetic, cranial window preparation, and NO donor were used. However, despite the presence of some K\textsubscript{Ca} channel dependency in the NO-induced dilation of pial arterioles, more than one-half of the NO donor response in the present study was unaffected by IbTX. Thus one cannot readily conclude that the overlapping and nonadditive effect of l-NNA and IbTX on the ADP response in intact females was entirely due to NO-associated K\textsubscript{Ca} channel activation. Indeed, one cannot ignore the prospect that these results were influenced by IbTX acting on the K\textsubscript{Ca} channels shown to exist on cerebral vascular endothelial cells (12). One manifestation of this arrangement might be endothelial K\textsubscript{Ca} channel potentiation of ADP-induced, eNOS-derived NO release (12).

The specific identity of the EDRF remains elusive. To date, several candidate EDRFs have been proposed. These include an epoxide derived from the cytochrome P-450 epoxygenase pathway (5, 7), a cannabinoid receptor agonist (anandamide) (17), and carbon monoxide (11). The first has received the greatest attention. However, because miconazole did not affect ADP-induced relaxation in the current study, it is unlikely that an epoxide played any role in the responses observed in the Ovx rats. Whether anandamide, carbon monoxide, or some other EDHF is involved will require additional experimentation.

Studies to date consistently indicate that, irrespective of the factor involved, ADP-induced cerebrovascular dilation is strongly endothelium dependent (see METHODS). This also includes pial arterioles studied in vivo, at least in the mouse (22). Nevertheless, because the endothelium dependence of the ADP response under current experimental conditions was not evaluated, we cannot eliminate the prospect that this non-NO, nonprostanoid but K\textsubscript{Ca}-dependent mechanism, which is switched on in the face of estrogen depletion, may indeed involve a paracrine factor that is nonendothelial in origin. Moreover, lack of information as to the extent of endothelial participation also does not permit us to ignore the possibility that ADP-induced pial arteriolar relaxation may occur via direct interactions with P2Y\textsubscript{1} receptors on vascular smooth muscle. Such a process merits consideration in light of recent findings (24) showing a K\textsubscript{Ca} channel-dependent hyperpolarization in isolated pig aortic smooth muscle cells in the presence of ADP and other P2Y\textsubscript{1} agonists.

In conclusion, present findings suggest that chronic estrogen depletion, while not influencing the magnitude of ADP-induced dilations, is associated with a change in the mechanism mediating the vasorelaxant response. That is, the partial NO dependence of the ADP action is completely lost in ovarioectomized females but is restored on chronic estrogen replacement. The NO-independent component was strongly influenced by K\textsubscript{Ca} channel blockade only in the estrogen-depleted group. Also, the ADP response was unaffected by blockade of prostanoid synthesis, K\textsubscript{ATP} channels, or adenosine receptors. Taken together, these results suggest the appearance of an EDHF-like factor as an important participant in the pial arteriolar response to ADP when estrogen is chronically depleted.

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