Nascent EDHF-mediated cerebral vasodilation in ovariectomized rats is not induced by eNOS dysfunction

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Xu, H. L., R. A. Santizo, V. L. Baughman, and D. A. Pelligrino. Nascent EDHF-mediated cerebral vasodilation in ovariectomized rats is not induced by eNOS dysfunction. Am J Physiol Heart Circ Physiol 285: H2045–H2053, 2003.—In estrogen-depleted [i.e., ovariectomized (Ovx)] animals, an endothelium-derived hyperpolarizing factor (EDHF)-like mechanism may arise to, at least partially, replace endothelial nitric oxide (NO) synthesize (eNOS)-derived NO in modulating cerebral arteriolar tone. Additional findings show that eNOS expression and function is restored in estrogen-treated Ovx female rats, while the nascent EDHF-like activity disappears. Because NO has been linked to repression of EDHF activity in the periphery, the current study was undertaken to examine whether the nascent EDHF role in cerebral vessels of Ovx females relates to a chronically repressed eNOS-derived NO-generating function. We compared the effects of chronic NO inhibition with Nω-nitro-L-arginine-methyl ester (L-NAME; 100 mg·kg⁻¹·day⁻¹ for 3 wk) on EDHF-mediated pial arteriolar vasodilation in anesthetized intact, Ovx, and 17β-estradiol-treated (0.1 mg·kg⁻¹·day⁻¹ ip, 1 wk) Ovx (OVE) female rats as well as in male rats that were prepared with closed cranial windows. In the chronic NOS inhibition groups, pial arteriolar responses were monitored in the absence (all groups) and presence (females only) of indomethacin (Indo; 10 mg/kg iv). Finally, the gap junction inhibitory peptide Gap 27 (300 μM) was applied to block EDHF-related vasodilation. NO donor (S-nitroso-N-acetyl-penicillamine) responses were similar in all rats studied. Acetylcholine (ACh) reactivity was virtually absent in control Ovx rats and chronically NOS-inhibited intact female, OVE, and male rats. However, a partial recovery of ACh reactivity was seen in L-NAME-treated Ovx females. In addition, in the presence of L-NAME, a normal CO₂ reactivity was observed in all females, whereas a 50% reduction in CO₂ reactivity was seen in males. In intact and OVE rats, both chronic and acute (Nω-nitro-L-arginine suffusion) NOS inhibition, combined with Indo, depressed ADP-induced dilation by ≥50%, and subsequent application of Gap 27 had no further effect on ADP-induced vasodilation. ADP reactivity was retained in Ovx rats after combined chronic NOS inhibition and acute Indo, but was attenuated significantly by Gap 27. In males, Gap 27 had no effect on arteriolar reactivity. Taken together, our data demonstrate that in the cerebral microcirculation, NO does not have an inhibitory effect on EDHF production or action. The increased EDHF-like function in chronic estrogen-depleted animals is not due to eNOS deficiency, suggesting a more direct effect of estrogen in modulating EDHF-mediated cerebral vasodilation.

VASCULAR ENDOTHELIAL CELLS play a vital role in cerebral vasodilation. These cells synthesize and release a variety of vasorelaxant substances, including prostacyclin, nitric oxide (NO), and an additional vasodilating entity, collectively termed endothelium-derived hyperpolarizing factor (EDHF). Although the chemical identity of EDHF is controversial, a consensual functional definition of EDHF has emerged, identifying EDHF as an agonist-induced, endothelium-dependent vasodilating entity (or process) that elicits hyperpolarization of smooth muscle via opening Ca²⁺-dependent K⁺ channels and persists after effective inhibition of NO synthase (NOS) and cyclooxygenase (COX). Indeed, the tissue- and species-related heterogeneity of EDHF-like mechanisms implies that multiple EDHFs might exist. Candidates include products of arachidonate metabolism (epoxides, anandamide), potassium ions, cAMP, reactive oxygen species, or a transfer of hyperpolarizing current via intercellular gap junctions (2, 20).

In the periphery, there is evidence to suggest that EDHF-like behavior may appear only when the NO-generating function is repressed. One explanation for this phenomenon is that NO exerts a tonic inhibitory effect on the production and/or action of EDHF. Studies (1, 8, 13) have shown that EDHF influence is revealed after acute or chronic reductions in NO, implying that it may involve multiple sites. However, in cerebral vessels, acute manipulation of NO levels was not found to alter EDHF-like behavior (17). On the other hand, the effects of chronic NO depletion on EDHF-related mechanisms in cerebral vessels has not been specifically examined.

Golding and Kepler (5) reported that EDHF-mediated cerebral vasodilations, which are negligible in intact female rats, are significantly enhanced after ovariectomy. Recent findings also indicated a substantially diminished expression of cerebral eNOS (11, 16)

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and repression of eNOS-dependent cerebral vasodilatating function (16) in chronically ovariec-tomized (Ovx) rats that was reversed by chronic estrogen replacement. Taken together, these findings imply that chronic loss of endothelial NO generating function may result in a compensatory upregulation of EDHF-like vasodilating influences in cerebral blood vessels. Accordingly, we hypothesized that in animals with chronic estrogen depletion, the enhancement of EDHF-like behavior is due to chronic suppression of the negative influence of NO. To that end, we compared intact, Ovx, and Ovx + 17β-estradiol (E2)-treated (OVE) female rats in the absence or presence of chronic treat-
ment with a NOS inhibitor.

MATERIALS AND METHODS

The study was approved by the Institutional Animal Care and Use Committee. Sprague-Dawley rats (280–340 g body wt) were used throughout the experiment. The animals were housed in standard cages, with a 12:12-h light-dark cycle. Most rats were randomly assigned to three different groups: intact female, Ovx female, and OVE female (0.1 mg/kg body wt) for 3 wk). Average daily intake of L-NAME was 39.2 ± 36.7 mg/kg body wt. This L-NAME treatment protocol has previously been shown to elicit a >95% reduction in Ca2+-dependent NOS activity in the brain (18).

The technique for cranial window preparation was previously described in detail (29). Briefly, the rats were anesthe-
tized with halothane, paralyzed with curare, and mechan-
ically ventilated with 0.8% halothane-70% N2O-30% O2 after tracheotomy. After insertion of bilateral femoral arterial and venous catheters, the rat was secured in a head holder to facilitate placement of a closed cranial window. A 10-mm-
diameter craniotomy was performed over the skull midline and the dura was removed. The window (11 mm in diameter), equipped with outflow, inflow, and intracranial pressure monitoring ports, was fixed to the skull with the use of cyanoacrylate gel. After window placement, halothane was discontinued, and anesthesia was maintained with a bolus of fentanyl (10 μg/kg iv), followed by a maintenance dose of fentanyl (25 μg·kg−1·h−1) and ventilation with 70% N2O-30% O2. The space under the window was filled with artificial cerebrospinal fluid (aCSF, PCO2 = 45 mmHg; 37°C; pH = 7.35). Intracranial window pressure was maintained at 5–10 mmHg during the experiment. Mean arterial blood pressure (MABP) was continuously monitored, and a servo-controlled heating pad was employed to keep the body temperature at 37°C. Arterial blood samples were taken for blood gas anal-
yses every hour (ABL 520 Blood Gas System, Radiometer). Arterioles with diameters between 25 and 50 μm were used for the experiments. The in vivo diameter measurement system consisted of a microscope (Nikon), video camera (Sony), and calibrated video microscaler (Optech).

In all experiments, initial arteriolar diameters were mea-
sured after a 40-min period of cortical suffusion with drug-
free aCSF (1 h after halothane). Hypercapnia [arterial PaCO2 (PaCO2) ~65 mmHg] was then imposed for 3 min, and CO2 reactivity was calculated as the percent diameter increase per mmHg of PaCO2 change. At 10 min after return to base-
line, a suffusion of the partially eNOS-dependent vasodilator ADP was initiated at a concentration of 10 and then 100 μM (5 min at each level). This was followed by suffusion with the almost entirely eNOS-dependent vasodilator acetylcholine (ACH; 10 and 100 μM, 10 min for each level). After return to baseline, a suffusion with the NO donor S-nitroso-N-acetyl-
penicillamine (SNAP; 0.1 and 1.0 μM, 3 min for each level) was initiated. In the chronically NOS-inhibited female (but not male) groups, 20 min after return to baseline, the COX inhibi-
ator indomethacin (Indo; 10 mg/kg body wt iv) was administered and the above sequence of vasodilator applica-
tions was repeated. For the female groups not exposed to chronic L-NAME treatments, the pial arterioles were subjected to acute NOS inhibition via suffusion of Nω-nitro-l-
arginine (l-NNA; 1 mM) for 1 h before Indo treatment. Finally, in both control and chronically L-NAME-treated fe-
male rats, the gap junction blocking peptide Gap 27 (300 μM), which interferes with connexin43 and -37-related gap junctional communication (3), was suffused for 1 h before reevaluation of ADP, ACh, CO2, and SNAP responses. In male rats, Gap 27 treatments were only given to controls. L-NAME, l-NNA, Indo, ADP, ACh, and SNAP were pur-
chased from Sigma (St. Louis, MO). Gap 27 peptide (SRPT-
EKTIFII) was synthesized by the Protein Research Lab at the University of Illinois (Chicago, IL). The purity was >95%. Statistical comparisons of pial arteriole diameter changes between groups in the NOS inhibitor treatment experiments were made using two-way ANOVA combined with a post hoc Tukey analysis. For comparisons of diameter values within a given experiment, repeated-measurements, two-
way ANOVA design and post hoc Tukey analysis were used. A level of P < 0.05 was considered significant in all statistical tests. Values are presented as means ± SE.

RESULTS

The MABP levels, measured at the start of the ex-
periments (Table 1), were significantly higher in the rats chronically treated with L-NAME compared with controls. The MABP did not change significantly during any of the interventions except after intravenous Indo in the vehicle control group, where the MABP increased slightly (not shown). The arterial pH and P CO2, with the exception of periods of imposed hyper-

<table>
<thead>
<tr>
<th>Group</th>
<th>Pial Arteriolar Diameter, μm</th>
<th>PaCO2, mmHg</th>
<th>pHa</th>
<th>MABP, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>36.8 ± 3.3</td>
<td>41.8 ± 1.9</td>
<td>7.37 ± 0.03</td>
<td>131 ± 4</td>
</tr>
<tr>
<td>L-NAME</td>
<td>36.1 ± 0.5</td>
<td>34.2 ± 0.9*</td>
<td>7.42 ± 0.01*</td>
<td>155 ± 7*</td>
</tr>
<tr>
<td>Male</td>
<td>36.9 ± 1.6</td>
<td>36.7 ± 4.4</td>
<td>7.41 ± 0.03</td>
<td>130 ± 4</td>
</tr>
<tr>
<td>L-NAME</td>
<td>36.4 ± 2.1</td>
<td>33.5 ± 2.8</td>
<td>7.44 ± 0.02</td>
<td>151 ± 4*</td>
</tr>
<tr>
<td>Vehicle</td>
<td>35.4 ± 1.8</td>
<td>36.4 ± 2.7</td>
<td>7.41 ± 0.02</td>
<td>125 ± 4</td>
</tr>
<tr>
<td>L-NAME</td>
<td>36.1 ± 1.2</td>
<td>36.9 ± 3.9</td>
<td>7.41 ± 0.04</td>
<td>158 ± 3*</td>
</tr>
<tr>
<td>Male</td>
<td>36.1 ± 2.0</td>
<td>33.3 ± 1.9</td>
<td>7.43 ± 0.01</td>
<td>122 ± 6</td>
</tr>
<tr>
<td>L-NAME</td>
<td>39.2 ± 1.8</td>
<td>36.7 ± 1.3</td>
<td>7.42 ± 0.02</td>
<td>147 ± 2*</td>
</tr>
</tbody>
</table>

Values are means ± SE. PaCO2, arterial partial pressure of CO2; MABP, mean arterial blood pressure; pHa, arterial pH; L-NAME, Nω-nitro-l-arginine methyl ester; Ovx, ovariec-tomized; OVE, 17β-
estradiol-treated Ovx rats. *P < 0.05 vs. vehicle.
capnia, did not show significant variations over the course of the studies. Arterial $P_O_2$ was maintained >100 mmHg during all experiments (not shown).

**Effect of chronic NOS inhibition on ADP-induced vasodilation.** In confirmation of earlier results published from our laboratory (24, 25), initial suffusions with ADP, at 10 and 100 $\mu$M, elicited similar dose-dependent vasodilations in intact (13.4 ± 1.9% and 29.8 ± 5.8%, respectively), Ovx (14.1 ± 2.4% and 35.3 ± 8.4%, respectively), and OVE (12.3 ± 3.0% and 28.4 ± 4.9%, respectively) females (Fig. 1). All the arteriolar diameters returned to baseline after 10-min suffusion with drug-free aCSF. In intact and OVE groups, topical applications of L-NNA for 60 min combined with 10 mg/kg iv Indo resulted in 70% and 50% reductions in the vasodilations produced by 10 and 100 $\mu$M ADP, respectively. These changes can be ascribed entirely to l-NNA, because it was previously shown that Indo alone has no effect on ADP reactivity in the same female groups (25). Chronic l-NAME treatment affected ADP responses in a manner similar to that observed in vehicle control rats after acute topical application of a NOS inhibitor (l-NNA). Thus the intact female group exhibited 78% and 35% reductions in the response to 10 and 100 $\mu$M ADP, respectively, whereas the OVE group displayed 70% and 35% reductions, respectively (Fig. 1). Those results were not altered on addition of Indo. However, in agreement with the results of our earlier study (22), in the Ovx group, ADP reactivity was not diminished by combined acute NOS and COX inhibition. Furthermore, that response was also not altered by chronic NOS inhibition or subsequent administration of Indo (Fig. 1). In males, ADP suffusions elicited pial arteriolar dilations similar in magnitude to those observed in the female groups (Fig. 2). Like in estrogen-normal females, chronic NOS inhibition in male rats was accompanied by significant reductions in ADP reactivity compared with vehicle-treated controls (62% and 49% at 10 and 100 $\mu$M ADP, respectively).

The addition of a synthetic gap junction inhibitory peptide Gap 27 after the NOS-COX combined inhibition in intact and OVE groups elicited no further reductions in ADP reactivity, irrespective of whether the NOS inhibition was acute or chronic. On the other hand, Gap 27 administration was accompanied by significantly reduced ADP-induced dilations in both the vehicle control (75% and 65% reductions at 10 and 100 $\mu$M ADP, respectively) and chronic L-NAME treatment (60% and 36%, respectively) Ovx groups (Fig. 1). Administration of Gap 27 in male rats had no effect on ADP reactivity (Fig. 2). This was identical to published findings from our laboratory (24) showing an absence of any influence of Gap 27, by itself, in intact and $E_2$-treated Ovx female rats.

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**Fig. 1.** Effect of acute [topical application of 1 mM $N^O$-nitro-L-arginine (l-NNA)] or chronic [3 wk of $N^O$-nitro-L-arginine methyl ester (l-NAME) in drinking water] nitric oxide (NO) synthase (NOS) inhibition in the absence and presence of cyclooxygenase inhibition with indomethacin (Indo; 10 mg/kg iv) and gap junctional blockade (topical Gap 27; 300 $\mu$M) on ADP (10 and 100 $\mu$M)-induced pial arteriolar dilation in intact (A), ovariectomized (Ovx) (B), and 17$\beta$-estradiol-treated Ovx (OVE) (C) rats. Results are expressed as the percentage of pial arteriolar diameter increase from baseline value. Values are means ± SE; n = 4 in each group; *P < 0.05 vs. initial; †P < 0.05 vs. vehicle; §P < 0.05 vs. Indo + l-NNA; and ¶P < 0.05 vs. Indo alone.
Effect of chronic NOS inhibition on ACh-induced vasodilation. To confirm that chronic L-NAME was effective at blocking cerebral vascular eNOS activity, the arteriolar responses to the established eNOS-dependent vasodilator ACh were evaluated (Fig. 3). In the vehicle control group, ACh (10 and 100 μM) induced pial arteriolar dilations of 9.0 ± 0.7% and 19.4 ± 1.2%, respectively, in intact females. Consistent with
results published from our laboratory (16), ACh reactivity was completely lost in Ovx rats but recovered after chronic estrogen replacement (10.7 ± 0.7% and 21.4 ± 1.1% in OVE rats). In vehicle controls, after acute suffusion of the nonselective NOS inhibitor L-NNA (1 mM), ACh reactivity was eliminated completely in all the groups (data not shown). Indo was found to have no effect on ACh-induced pial arteriolar relaxation (not shown). The finding that ACh-induced pial arteriolar dilations in estrogen-normal females are essentially entirely dependent on NO confirms previous results from our laboratory (16). Chronic NOS inhibition was also accompanied by a substantial loss of ACh reactivity. In the intact and OVE groups, no significant ACh-induced diameter increases were observed. Curiously, in L-NAME-treated Ovx rats not given E2 replacement, there was a statistically significant partial restoration of the ACh response, to the extent that a >10% increase in diameter was observed at 100 μM ACh, compared with a completely repressed ACh reactivity in the absence of L-NAME treatment (i.e., control Ovx). However, in the chronically L-NAME-treated Ovx females, the subsequent addition of Indo was accompanied by a significant reduction in and complete loss of ACh reactivity, suggesting the appearance of a prostaglandin-dependent component in the ACh response. No statistically significant effects of Indo were seen in the chronically L-NAME-treated intact and OVE female rats. Chronic L-NAME treatment in male rats produced an effect similar to that observed in intact and OVE females, i.e., a virtually complete loss of ACh reactivity. It is of some relevance to note that we previously reported (14, 21) that acute NOS inhibition in male rats also is accompanied by nearly complete suppression of ACh-induced dilations of pial arterioles.

Effect of chronic NOS inhibition on hypercapnia-induced vasodilation. Among all the female vehicle control groups, no significant differences were observed in the initial responses to hypercapnia (Fig. 4). In the three vehicle-treated female groups, after suffusion with 1 mM L-NNA for 60 min, CO2 reactivity was significantly reduced by 60%. Subsequent treatment with Indo resulted in a further significant reduction in

![Fig. 4. Pial arteriolar responses to hypercapnia in vehicle and chronically L-NAME-treated intact (A), Ovx (B), and OVE females (C) and in male rats (D) in the absence (all groups) or presence (L-NAME-treated females) of intravenous Indo. CO2 reactivity is expressed as the percent pial arteriolar diameter increase per mmHg arterial PCO2 (Paco2) increase. The Paco2 values (in mmHg) measured during hypercapnia in the intact females were 68.5 ± 2.0 (vehicle control), 67.8 ± 3.9 (chronic L-NAME), and 69.1 ± 3.5 (chronic L-NAME + acute Indo). In the Ovx group, the respective values were 68.0 ± 1.6, 66.5 ± 4.5, and 65.2 ± 4.0. The Paco2 values in the OVE females were 78.1 ± 6.2, 70.5 ± 3.4, and 64.6 ± 4.7, respectively. In the males, hypercapnic Paco2 values were 68.4 ± 2.1 (vehicle control) and 71.3 ± 3.1 (chronic l-NAME). Values are means ± SE; n = 4 in each group. *P < 0.05 vs. vehicle; †P < 0.05 vs. chronic l-NAME.](http://ajpheart.physiology.org/)
CO$_2$ reactivity (~75%). Because the results in the intact, Ovx, and OVE females were virtually the same, the data are represented as a composite of the three groups (see Fig. 5). This result indicates that CO$_2$ reactivity and its dependence on NO and prostanoids is not affected by variations in estrogen status. Interestingly, no significant changes in CO$_2$ responses were found in any female groups when NOS was chronically inhibited (Fig. 4), suggesting that a compensatory mechanism replaces NO when NO production is chronically blocked. The absence of any apparent reduction in CO$_2$ reactivity did not appear to relate to incomplete NOS inhibition because subsequent topical application of L-NNA (1 mM) did not affect CO$_2$ reactivity (data not shown). Because Indo reduced the CO$_2$ response by ~80% from baseline in the chronic L-NAME group (Fig. 4), it would appear that prostanoids are capable of replacing NO in mediating hypercapnia-induced pial arteriolar dilation when NO-generating function is chronically repressed. In contrast, CO$_2$ reactivity in male rats was significantly affected by chronic NOS inhibition, falling to ~50% of the value observed in control animals (Fig. 4).

**Effect of chronic NOS inhibition on SNAP-induced vasodilation.** Suffusion of the NO donor SNAP at 0.1 and 1.0 μM elicited identical dose-dependent vasodilating responses in intact, Ovx, and OVE rats. Those responses were not altered by acute or chronic NOS inhibition or by treatment with Indo (Fig. 6).

**DISCUSSION**

The key findings of this study can be summarized as follows. First, chronic loss of NO-generating function has the same effect on ADP-induced pial arteriolar dilation as acute blockade of NOS activity. That is, ADP reactivity was suppressed in intact and OVE females, but unaltered in Ovx females, in the presence of acute (see Refs. 24 and 25) or chronic NOS inhibition. Second, irrespective of whether a NOS inhibitor was given, gap junctional blockade did not alter ADP responses in estrogen-normal (i.e., intact and OVE) females but was associated with a significantly reduced ADP reactivity in Ovx females. These results strongly indicated that the repression of eNOS function that occurs in chronically estrogen-depleted states (16) does not account for the appearance of an EDHF-like component in the pial arteriolar response to ADP in Ovx females. Third, in accord with previously published findings (16), the eNOS-dependent, prostanoid-independent pial arteriolar response to ACh was completely repressed in Ovx and acutely and chronically NOS-inhibited, estrogen-normal females. Surprisingly, a significant partial restitution of ACh-induced pial arteriolar relaxation was observed in chronically NOS-inhibited, estrogen-depleted Ovx females. That recovery appeared to be related to the nascent appearance of a prostanoid influence. Fourth, some gender-related differences in the effects of chronic NOS inhibition on pial arteriolar vasodilating function were observed. This was particularly evident in the responses to hypercapnia. That is, in both males (22) and females, acute NOS inhibition elicited a substantial attenuation in the CO$_2$ response, whereas after chronic L-NAME treatment, a complete recovery of CO$_2$ reactivity was only seen in females. Like the ACh response in chronically L-NAME-treated Ovx females, the recovery of CO$_2$-induced vasodilating function appeared to be related to an increase in prostanoid influence.

The results to date in peripheral vascular tissue indicate that EDHF-like activity may appear under circumstances where NO-generating function is repressed. Furthermore, it has been proposed that this relates to NO acting to inhibit EDHF production or function (1, 13). Because cerebrovascular eNOS expression and activity are diminished in Ovx females (16, 25), it is tempting to ascribe the nascent EDHF dependency in the pial arteriolar response to ADP in these animals to the loss of a NO-associated inhibitory influence. However, in cerebral arteries, present and previously published results do not support such a mechanism, irrespective of whether the changes in NO are imposed chronically or acutely (17). Therefore, earlier findings indicating the appearance, after ovarectomy, of EDHF-like behavior in cerebral vessels, both in vivo (24) and ex vivo (5), suggests that sex hormone depletion per se, rather than NO, may be directly involved.

There is evidence from the literature that supports a mechanism involving a direct estrogen action. Thus Herve et al. (7) reported that E$_2$ can directly interfere
with gap junctional communication. Therefore, in the presence of estrogen, one might envisage a basal repression of gap junctional activity. On the other hand, in estrogen-depleted states, a disinhibition of gap junctional function may occur. Such a phenomenon could explain our recent findings (24) showing the advent of an EDHF-like, gap junction-dependent component of ADP-induced pial arteriolar dilations in chronically ovariectomized females. Nevertheless, that postulate is not entirely consistent with findings in male rats. Thus, in contrast to results in Ovx females, findings from our laboratory indicate a substantial NO dependency in the pial arteriolar response to ADP in male rats (14), a response that is insensitive to gap junctional blockade (Fig. 2). On the other hand, in middle cerebral arteries harvested from male versus Ovx female rats (5), an equivalent EDHF-like dilating response (to ATP) was observed, as opposed to the substantially lower ATP reactivity in arteries from estrogen-normal females. These seemingly contradictory findings may simply be a reflection of dissimilarities in the experimental models. For example, in addition to differences inherent in in vivo versus in vitro preparations, one might also consider differences related to the vessel type studied (i.e., large artery vs. arteriole), regional factors, and the vasodilating agonist employed. Indeed, the apparent disagreement in results from cerebral vessels may be yet another example of the multiple endothelium-dependent vascular smooth muscle hyperpolarizing mechanisms described in a number of published reports (for a review, see Ref. 2).

As mentioned above, we recently reported the appearance of a nascent EDHF dependency in the pial arteriolar dilations elicited by ADP in Ovx females (25) and that this EDHF-like behavior involved gap junctional communication (24). The identification of an EDHF-like mechanism was based on experimental evidence that satisfied three of the four widely accepted major criteria used in establishing the presence of an EDHF. That is, the ADP response did not involve NO or a prostanoid, it was attenuated by a Ca^{2+}-dependent K^{+} channel blocker, and it required an intact endothelium. The only criterion that could not be established was the presence of a pial vascular smooth muscle hyperpolarization. Accurate in vivo measurements of membrane potentials in pial vessels are beyond currently available technology. It is important to note that, in our earlier studies, in estrogen-normal rats, application of endothelial injury after NO (and prostanoid) synthesis inhibition elicited no further reductions in ADP reactivity (24). Moreover, unlike Ovx females, the ADP response in the intact and E_{2}-supplemented rats was completely insensitive to gap junctional block-
ade. Thus, although ~50% of the ADP response remained, that NO, prostanoid, and endothelium-independent component could not have been an EDHF, because endothelium did not contribute, and it certainly did not involve gap junctions. Although the precise nature of that endothelium-independent and inhibitor-resistant component of ADP-induced dilation of pial arterioles remains elusive, recent preliminary findings from our laboratory have suggested that it may involve astrocytic influences (15).

There were additional findings in the present study that were not directly related to the initial objectives of this investigation, but were, nevertheless, of some interest. In particular, we observed some hormone- and gender-based differences with respect to the manner in which chronic NOS inhibition influenced vasodilating function. The most striking example was in the pial arteriolar response to hypercapnia. Thus, all female groups, whether hormone depleted or not, displayed a normal CO₂ reactivity, whereas the male rats exhibited a substantially attenuated response. The normalization of the hypercapnic response in the females appeared to result from a compensatory increase in prostanoid influence. Furthermore, because this compensation occurred in females irrespective of hormone status, it would seem that the mechanisms involved somehow relate to estrogen/progesterone-independent, but nevertheless gender-associated, factors. However, one cannot discount the possibility that testosterone could have played a role in preventing compensation in males.

There was one other unexpected finding. Thus, in Ovx females not provided with E₂ replacement, chronic NOS inhibition was associated with a significant partial recovery of the pial arteriolar response to ACh. In the absence of NOS inhibitor treatments, present and earlier (16) findings indicate a complete loss of ACh reactivity in Ovx rats. In contrast, in males and estrogen-normal females, current and previous (14, 16, 18, 22) results indicate that both acute and 2- to 3-wk NOS inhibition are associated with a statistical absence of ACh reactivity in cerebral arteries and arterioles. In other words, no “recovery” of the ACh response occurred over that time frame. However, Sercombe et al. (18) did report some restitution of ACh-induced relaxation in cerebral arteries of male rats on extending the period of NOS inhibition from 2 to 6 wk. The partial recovery of ACh reactivity seen in the Ovx females of the present study was prevented by Indo, thus suggesting the appearance of a prostanoid involvement in the ACh response. Evidence of a compensatory increase in prostanoid contributions to the vasodilating function of mesenteric arteries in rats subjected to 3 wk of L-NAME treatment has been published (6). In that case, the mechanism appeared to involve an upregulation in the expression of COX-2. A similar increase in prostanoid influence was reported for skeletal muscle arterioles in eNOS knockout mice (19). In the brain, the possibility of a compensatory increase in prostanoid influence in ACh-induced vasodilation in eNOS-knockout mice has not been examined. However, in such animals, an increase in a NO-independent component in the response of pial arterioles to ACh was observed (12), leaving room for a possible role for prostanoids.

Finally, some consideration should be given to the implications associated with the presence of chronic hypertension in the L-NAME-treated rats. In fact, the chronic hypertension that develops is not simply a function of eNOS blockade but is also likely to include a sympathetic activation component arising from loss of neuronal NOS activity (4). When combining hypertension and sympathoadrenal activation-related changes with the differences in hormone status among the various groups of the present study, one might envisage a variety of interactions occurring that could result in different mechanisms governing cerebral vascular reactivities among these groups. One possible example may very well be the partial recovery of ACh reactivity (and the advent of a prostanoid dependency) seen only in the Ovx group.

In conclusion, our data demonstrate that, in the in vivo cerebral microcirculation, NO does not have an inhibitory effect on EDHF production and/or function. The appearance of EDHF-like behavior in chronically estrogen-depleted females, therefore, is not due to eNOS deficiency, suggesting a direct effect of estrogen in modulating EDHF-mediated cerebral vasodilation.

**DISCLOSURES**

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