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Nitric-Oxide-Dependent Pial Arteriolar Dilation in the Female Rat: Effects of Chronic Estrogen Depletion and Repletion

Dale A. Pelligrino, Shuhua Ye, Fulong Tan, Roberto A. Santizo, Douglas L. Feinstein, and Qiong Wang
Department of Anesthesiology, University of Illinois at Chicago, Chicago, Illinois 60616

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In this study, we compared endothelial nitric oxide synthase (eNOS)-mediated cerebral vasodilating responses in intact female rats, chronically ovariectomized (OVX) rats, and OVX rats treated for 2 weeks with 17β-estradiol (E_2). Under anesthesia, using intravital microscopy and a closed cranial window system, pial arteriolar diameter changes were monitored during sequential cortical suffusions of an eNOS-dependent dilator [acetylcholine (ACh)] and a direct NO donor [*S*-nitrosoacetylpenicillamine (SNAP)]. In separate rats from the same groups, we compared eNOS and caveolin-1 (CAV-1) protein abundance in pial arterioles (via immunofluorescence analyses). In untreated and low-dose E_2-treated (1.0 $\mu g \cdot kg^{-1} \cdot day^{-1}$) OVX rats, ACh-induced vasodilations were virtually absent. High-dose E_2 treatment (100 $\mu g \cdot kg^{-1} \cdot day^{-1}$) restored ACh-induced pial arteriolar dilations to levels seen in intact females. The vasodilations elicited by SNAP and ADO were unaffected by chronic estrogen changes, indicating no direct estrogen influence on vascular smooth muscle (VSM) reactivity. Pial arteriolar eNOS protein abundance was diminished by ovariectomy and restored by high-dose E_2 treatment. Pial arteriolar CAV-1 expression was higher in OVX versus intact and E_2-treated OVX females. These results suggest that long-term changes in estrogen directly influence brain eNOS functional activity. The estrogen-related changes in eNOS-dependent vasodilating function appear to be related, in part, to a capacity for E_2 to increase eNOS protein expression and, in part, to an E_2-associated diminution in endothelial CAV-1 expression. © 2000 Academic Press

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Premenopausal females, compared to males, are less susceptible to stroke and stroke-related brain damage (1). Following onset of menopause, that gender-based advantage may disappear altogether. Differences in levels of circulating estrogens, to a large extent, could account for those observations. Estrogen-associated protection may be a function of greater levels and activity of a Ca^{2+}-dependent nitric oxide synthase, in particular, the endothelial form (eNOS). The increased capacity for NO generation that would accompany eNOS upregulation could, for example, act to resist reductions in cerebral blood flow (CBF) under conditions that may lead to ischemic events, thereby providing neuroprotection (2). In the periphery, correlations exist between levels of circulating estrogen, Ca^{2+}-dependent NOS expression or activity, and vasodilating capacity (3–10). A positive correlation between circulating levels of E_2 and eNOS protein expression in whole brain microvessel fractions was recently reported (11). Nevertheless, such microvascular preparations are composed primarily of capillary tissue (12), and, thus, do not permit any firm conclusions regarding vasodilating function. Limited findings suggest a correlation between chronic estrogen status and basal NO release in cerebral vessels (13, 14). However, it is not known whether *stimulated* NO release in cerebral vessels is influenced by estrogen.

The principal aim of this investigation was to determine whether chronic reductions in circulating estrogen levels result in concomitant reductions in brain eNOS activity to the extent that cerebral (pial) arteriolar vasodilating capacity is diminished. Within that context, we endeavored to establish whether the suppressed vascular reactivity, if present, could be "corrected" by E_2 treatment. Three groups were evaluated: intact female, ovariectomized (OVX), and E_2-treated OVX rats. The major experimental approach involved the use of intravital microscopy and a closed cranial window system. We monitored pial arteriolar diameter changes during cortical suffusions of an eNOS-dependent dilator, acetylcholine (ACh) and a direct NO

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donor, *S*-nitrosoacetylpenicillamine (SNAP). To confirm NO dependency in these experiments, arteriolar responses were measured prior to and after initiating suffusion of the nonspecific NOS inhibitor, nitro-L-arginine (L-NNA). Because initial findings indicated that eNOS-dependent vasodilating function was affected, we sought to examine whether the eNOS functional sensitivity to variations in chronic E_2 status related to effects on eNOS protein abundance or "post-translational" influences. Thus, we also compared, in separate rats from the same groups, using immunofluorescent techniques, pial arteriolar endothelial expression of eNOS and the endogenous inhibitor of eNOS activity, caveolin-1 (CAV-1) (15).

METHODS

The study protocol was approved by the Institutional Animal Care and Use Committee. Female Sprague-Dawley rats, 250–350 g, were used. The pial arteriolar cNOS functional assessments employed a well-established closed cranial window system. The cranial window design and surgical implantation were described in detail in previous publications (16, 17). Anesthesia was induced with halothane and the rat was paralyzed (curare), tracheotomized, and mechanically ventilated. Surgical anesthesia for insertion of the cranial window and bilateral femoral arterial and venous catheters consisted of 0.8% halothane/70% N_2O/30% O_2. Following catheterization, the animal was placed in a head-holder and the cranial window was exposed. The halothane was discontinued and a loading dose of intravenous (iv) fentanyl was given (10 $\mu g \cdot kg^{-1}$). Anesthesia during the study was iv fentanyl (25 $\mu g \cdot kg^{-1} \cdot h^{-1}$) plus ventilation with 70% N_2O/30% O_2. Cannulae were secured into the inflow, outflow and intracranial pressure (ICP)-monitoring ports of the cranial window and the space under the window was filled with artificial cerebrospinal fluid (aCSF). The composition of the aCSF was provided elsewhere (16). The aCSF was suffused at 1.0 $ml \cdot min^{-1}$ and was maintained at a temperature of 37°C, a PCO_2 = 40–45 mmHg, PO_2 = 50–60 mmHg and pH ≈7.35. The ICP was controlled at 5–10 mmHg by adjustments of the height of the outflow cannula. The reactivity of 25–45 μm pial arterioles on the exposed cortical surface was assessed via measurement of diameter changes. A microscope (Nikon) and color video camera (Sony) arrangement was equipped with an epi-illumination, darkfield system (Fryer Co. Inc., Carpentersville, IL). Magnifications of ≥800× were displayed on a video monitor. Measurements of vessel diameters were made using a calibrated video microscaler (Optech).

Three groups of female rats were used in the vascular reactivity experiments: intact (n = 5); OVX and E_2-treated (n = 10); and OVX-untreated (n = 5). Ovariectomies were performed by the supplier (Charles River, Wilmington, MA) at 4–6 weeks prior to study. The ovariectomized-E_2 treated group was further divided into 2 subgroups—rats receiving daily ip injections of either 1.0 or 100 $\mu g \cdot kg^{-1}$ E_2 for ~2 weeks preceding study. As reported in an earlier study from our laboratory (18), the higher dose produced an average daily plasma levels of E_2 (assessed via radioimmunoassay) approximating the levels seen in intact females during proestrus (~60 $pg \cdot ml^{-1}$). The lower dose produced average daily plasma E_2 levels of ~30 $pg \cdot ml^{-1}$—a value only slightly greater than the level seen during the "quiescent" phases (diestrus/metestrus) of the estrus cycle (18). In all experiments, initial diameter measurements were made at ≥1 h post halothane and following a 30 min period of cortical suffusion with drug-free aCSF. Next, topical applications of aCSF containing SNAP (0.1 μM) then ACh (100 μM) were sequentially applied (3–5 min each). L-NNA (1 mM) was then introduced, and after 1 h, the above sequence was repeated (with L-NNA in the aCSF). The L-NNA step was omitted in the low-dose E_2 subgroup. The doses of vasodilators and L-NNA used in the present study were taken from preliminary dose-response evaluations. For the vasodilators, we used doses that were greater than the ED_{50} but less than the doses producing maximal responses for each agent. The L-NNA dose we administered (1 mM) is commonly used in *rat* studies employing topical application of L-NNA or nitro-L-arginine methylester (reviewed in refs. 2, 17).

Mean arterial pressure (MABP) was continuously monitored, and rectal temperature was servo-controlled at 37°C. Arterial blood samples were taken at ~30 min intervals for measurement of PO_2, PCO_2, and pH. Those analyses were performed on an Instrumentation Laboratories (model BGE) blood gas/pH analyzer. At the termination of the experiments, the rats were euthanized with a halothane overdose.

One additional series of intact female, ovariectomized, and E_2-treated (100 $\mu g \cdot kg^{-1} \cdot day^{-1}$) ovariectomized rats was studied. These rats were used to gather corroborative evidence for the functional data obtained in the intravital microscopy experiments, with respect to estrogen-related expression of eNOS. Using immunofluorescence analyses, we compared eNOS and caveolin-1 (CAV-1) immunoreactivity in pial vessels using double-labeling techniques. The CAV-1 analysis was included because CAV-1 has been reported to be a potent endogenous negative regulator of eNOS activity in vascular endothelial cells (15). For these evaluations, the brains were perfusion-fixed (4% paraformaldehyde in phosphate-buffered saline, pH 7.0) and paraffin-embedded according to procedures described in earlier reports from our laboratory (18, 19). For each brain studied, 7 μm coronal sections were prepared at the level of the striatum and hippocampus. The sections were deparaffinized (ethanol/xylene) and endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol. The slides were rehydrated in phosphate-buffered 0.3% Triton X-100. To block nonspecific binding, the sections were incubated in 2% horse serum for 20 min. Each section was then exposed to the first primary antibody (CAV-1, polyclonal, Transduction Laboratories, Lexington, KY; 1:200 dilution), incubated at 37°C for 1 h and then kept overnight at 4°C. The sections were subsequently exposed to the second antibody (donkey anti-rabbit conjugated to FITC, from Jackson Immuno Research, 1:400 dilution) and maintained at 37°C for 1 h. The slides were then washed in PBS and exposed to 2% horse serum. The second primary antibody, eNOS (monoclonal from Transduction Laboratories; 1:200 dilution), was then introduced, and the slides were kept at 37°C for 1.5 h. The second antibody (goat anti-mouse IgG, Cy™3-conjugated, from Jackson Immuno Research, 1:1000 dilution) was then added and the slides were maintained at 37°C for one hour. Measurements of the relative area of pial vessels expressing immunoreactive eNOS and CAV-1 were made using the Image Pro Plus system (Media Cybernetics, Silver Spring, MD). We examined 25–50 μm pial arterioles lying from 0–4 mm laterally on each side of the dorsal midline. The images were acquired and digitized using a fluorescent microscope (Nikon Eclipse E400) equipped with a Spot2 cooled digital camera (Diagnostic Instruments, Inc.). In each rat, 2 slices at the level of the striatum and 2 at the level of the hippocampus were viewed. One additional slice was used as a control (i.e., primary antibody omitted). Endothelial NOS and CAV-1 abundance for each pial vessel viewed was expressed as the area of immunopostivity divided by the cross-sectional area of the vessel (times 100%). The results for all vessels analyzed (typically 1–2 vessels per slice) were averaged for each rat. Care was taken to select vessels that appeared to be cut at right angles to the slice plane. Such arterioles could be identified by the uniformity in vessel wall thickness throughout the entire vessel circumference. Background corrections were made from a vessel-free area immediately adjacent to the pial vessel. Endothelial immunoreactivity was found to be absent in control slices where the primary antibody was omitted.

Statistical comparisons of pial diameter values within groups were made using a 2-way analysis of variance (ANOVA), with a post-hoc C matrix test for multiple comparisons (Systat, Evanston, IL). For

TABLE 1

Arterial Blood Variables

Group	Initial				60 min L-NNA				120–150 min L-NNA			
	PO_2 (mmHg)	PCO_2 (mmHg)	pH	MABP (mmHg)	PO_2 (mmHg)	PCO_2 (mmHg)	pH	MABP (mmHg)	PO_2 (mmHg)	PCO_2 (mmHg)	pH	MABP (mmHg)
Intact female	155 ± 15	36.5 ± 1.4	7.39 ± 0.02	138 ± 4	141 ± 19	36.5 ± 3.6	7.37 ± 0.01	140 ± 10	165 ± 18	31.2 ± 1.7	7.39 ± 0.04	144 ± 4
OVX + E_2*	131 ± 9	34.9 ± 1.6	7.42 ± 0.02	129 ± 7	131 ± 8	32.8 ± 0.7	7.41 ± 0.01	131 ± 6	144 ± 12	31.2 ± 1.0	7.39 ± 0.01	130 ± 9
OVX	105 ± 13	41.6 ± 3.7	7.37 ± 0.01	136 ± 5	112 ± 13	35.4 ± 3.2	7.34 ± 0.01	141 ± 5	119 ± 9	35.6 ± 1.8	7.27 ± 0.06	138 ± 4

Note. Values are means ± SE. * E_2 dose = 100 μg/kg/day. n = 5 in each group.

comparisons of arteriolar diameter changes, we employed a multiway ANOVA (Systat). For analysis of Ca^{2+}-dependent NOS activity, or immunoreactivity, between groups, we employed a one-way ANOVA, with a post-hoc Tukey analysis. Statistical significance was taken at the $P < 0.05$ level.

RESULTS

Arterial blood variables. The key arterial blood variables measured immediately prior to the initial vasodilator suffusion, following the initiation of L-NNA suffusion, and at the end of the experiment (120–150 min after the onset of L-NNA administration) are summarized in Table 1. No significant variations in PO_2, PCO_2, pH, or MABP were observed in any of the groups over the course of each experiment. It should be noted that MABP was maintained, via blood withdrawal, at control levels following L-NNA administration. Under normal circumstances, MABP would be expected to rise in the presence of L-NNA. Data from the OVX group treated with E_2 at 1.0 $\mu g \cdot kg^{-1} \cdot day^{-1}$ are not included in Table 1, since the L-NNA step was omitted in that group. Nevertheless, the initial values for PO_2, PCO_2, pH, or MABP in that group were similar to those in the other 3 groups represented in Table 1.

Pial arteriolar diameter changes. The initial pial arteriolar diameters measured in the intact female, OVX + E_2, and OVX groups were 31.2 ± 4.6 μm, 32.7 ± 2.6 μm, and 33.0 ± 4.5 μm, respectively. Baseline diameters measured between exposures to the various vasodilating stimuli showed only minor variations (<10%, $P > 0.1$) from the initial values. The pial arteriolar responses are presented in Fig. 1. Untreated OVX rats exhibited little response to ACh (Fig. 1, upper). On the other hand, intact females displayed the expected ACh-induced vasodilating response. In OVX rats treated with 100 $\mu g \cdot kg^{-1} \cdot day^{-1}$ E_2, the ACh-induced diameter increase was equivalent to that seen in intact females. In rats treated with 1.0 $\mu g \cdot kg^{-1} \cdot day^{-1}$, the ACh response remained repressed. The NO-dependence of the vasodilations during ACh (high-dose E_2-treated and intact females) suffusions was confirmed by the finding that those responses were completely blocked after L-NNA (Fig. 1, upper), but were repeatable in time control rats where L-NNA was omitted (data not shown). The absence of any differences in the responses to SNAP (Fig. 1, lower) indicates that neither chronic E_2 changes nor L-NNA suffusions had any direct influence on vascular smooth muscle function in general and NO reactivity in particular.

eNOS/CAV-1 immunoreactivity. Pial arteriolar immunoreactive eNOS, as one might expect, was concentrated in the endothelial layer (Fig. 2). A similar distribution was also observed for CAV-1 (Fig. 2). Subsequent image analysis of 20–50 μm pial vessels overlying the dorsal cortex (in the area exposed in

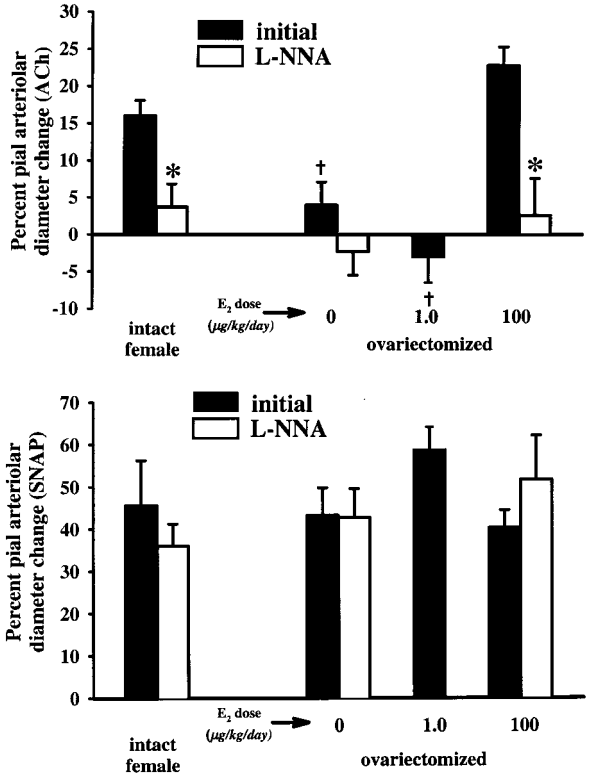

FIG. 1. Pial arteriolar diameter changes during suffusions of the eNOS-dependent vasodilator, ACh (100 μM, upper) and the NO donor, SNAP (0.1 μM, lower) prior to and following topical application of the NOS inhibitor, nitro-L-arginine (L-NNA, 1.0 mM). Values are means ± SEM (n = 5 in each group). *$P < 0.05$ versus pre-L-NNA; †$P < 0.05$ versus intact female and OVX + 100 μg/kg/day E_2.

cranial window experiments) revealed some striking differences in the extent of eNOS and CAV-1 expression in the 3 main study groups. To facilitate comparisons, the area of immunoreactivity for each protein within a given vessel was expressed relative to the cross-sectional area of that vessel. The results of those analyses are summarized in Fig. 3. The relative area of eNOS immunoreactivity was found to be ~15% of the total cross-sectional area of the pial arterioles analyzed in intact and OVX plus E_2-treated (@100 $\mu g \cdot kg^{-1} \cdot day^{-1}$), but only 8% of the cross-sectional area in untreated OVX rats ($P < 0.05$ vs the other 2 groups, Fig. 3A). On the other hand, when comparing CAV-1 expression in the same 3 groups (Fig. 3B), the pattern was reversed, with CAV-1 expression amounting to ~15% of the total cross-sectional area in the untreated OVX group, but only 3–5% of the cross-sectional area in the intact and E_2-treated groups ($P < 0.05$). Those differences were even more pronounced when evaluating the relative eNOS/CAV-1 expression ratios (Fig. 3C). Thus, the relative expression of eNOS exceeded that of CAV-1 by 3.5-fold in the intact and treated rats; whereas in the untreated OVX rats, the relative eNOS expression was found to be one-half that measured for CAV-1.

DISCUSSION

The major findings of the present study were: (1) In pial arterioles, following chronic estrogen depletion (ovariectomy), eNOS-dependent vasodilation was substantially attenuated. (2) Chronic 17β-estradiol treatment of OVX rats was accompanied by a dose-dependent restoration of normal reactivity to the eNOS-dependent vasodilator, ACh. (3) Expression of pial arteriolar eNOS protein was reduced by ovariectomy and normalized upon E_2 treatment. (4) The pial arteriolar endothelial expression of the endogenous regulator of eNOS activity, CAV-1, was increased by ovariectomy and reverted to normal levels of expression with chronic E_2 replacement.

It is well-established that the vasodilation of pial arterioles elicited by topical applications of ACh in rats and other adult species is largely dependent upon eNOS-derived NO (20). That fact was supported in the present study by the findings that the non-selective nNOS inhibitor, L-NNA, completely blocked Ach-induced dilations. Thus, with the present experimental model, evaluations of pial arteriolar reactivities to ACh can be used as a valid index of changes in eNOS functional expression. The findings of this study, indicating an E_2-related enhancement of eNOS-dependent cerebral vasorelaxation, is consistent with recent findings published by Geary *et al.* (14). These authors showed that myogenic tone was lower in pressurized rat middle cerebral artery segments harvested from intact and E_2-treated OVX females compared to untreated OVX females. Those differences appeared to be related to a greater NO production in the former groups.

A plausible explanation for the marked E_2-associated influence on pial arteriolar reactivity to ACh would be a direct E_2-related effect on eNOS protein expression (11). The conclusion that the changes in ACh reactivity were indeed a reflection of E_2 effects on eNOS expression was strengthened by the additional findings that estrogen depletion and repletion: (a) did *not* affect vascular smooth muscle *reactivity* to NO, as indicated by our finding of similar vasodilating responses to the NO donor, SNAP, in all the groups studied; and (b) *did* correlate positively with eNOS protein expression in pial arterioles.

One possible mechanism of estrogen action is via binding to specific soluble receptor proteins in the cytoplasm, forming mobile complexes that translocate to the nucleus, bind to estrogen response elements in the promoter region of target genes [e.g., eNOS (21)], leading to activation of gene transcription (22, 23). However, there is also evidence that genes, including eNOS, need not possess any conventional estrogen response element sequences for estrogen-induced transcriptional activation to occur (23, 24). Estrogens have been associated with an increased immediate early gene expression and enhancement of transcription factor activity (25, 26). Thus, the complex formed by estrogen binding to its receptor may facilitate immediate early gene binding to specific motifs (e.g., AP-1) within the promoter regions of target genes (27). That type of interaction could ultimately lead to eNOS enzyme upregulation. With regard to estrogen, not unlike other transcriptional promoters, such a process may involve a time frame of many hours (28). However, for the present, the estrogen receptor (ER) dependence of the E_2 effect on cerebrovascular eNOS expression remains unconfirmed. Future studies should be designed to establish the presence of ERs in cerebral endothelium and to examine whether E_2 effects on eNOS expression and function can be blocked by ER antagonists.

There are other possibilities that merit consideration before one can conclude that the E_2-associated effects on ACh responsivity in pial arterioles relates exclusively to changes in eNOS protein expression. These include: (1) E_2-associated alterations in muscarinic receptor density or in the signal transduction pathway linking muscarinic receptors to eNOS activation; and (2) possible posttranslational influences of E_2 on eNOS function.

It is possible that the profound differences in arteriolar reactivity to ACh among the groups may be related to E_2-associated changes in pial arteriolar muscarinic receptor density (or agonist binding) or a change in the ability of the receptor, when activated, to enhance eNOS activity. To date, these issues have not been addressed in the cerebral vasculature. However, in other structures, evidence does not seem to favor an

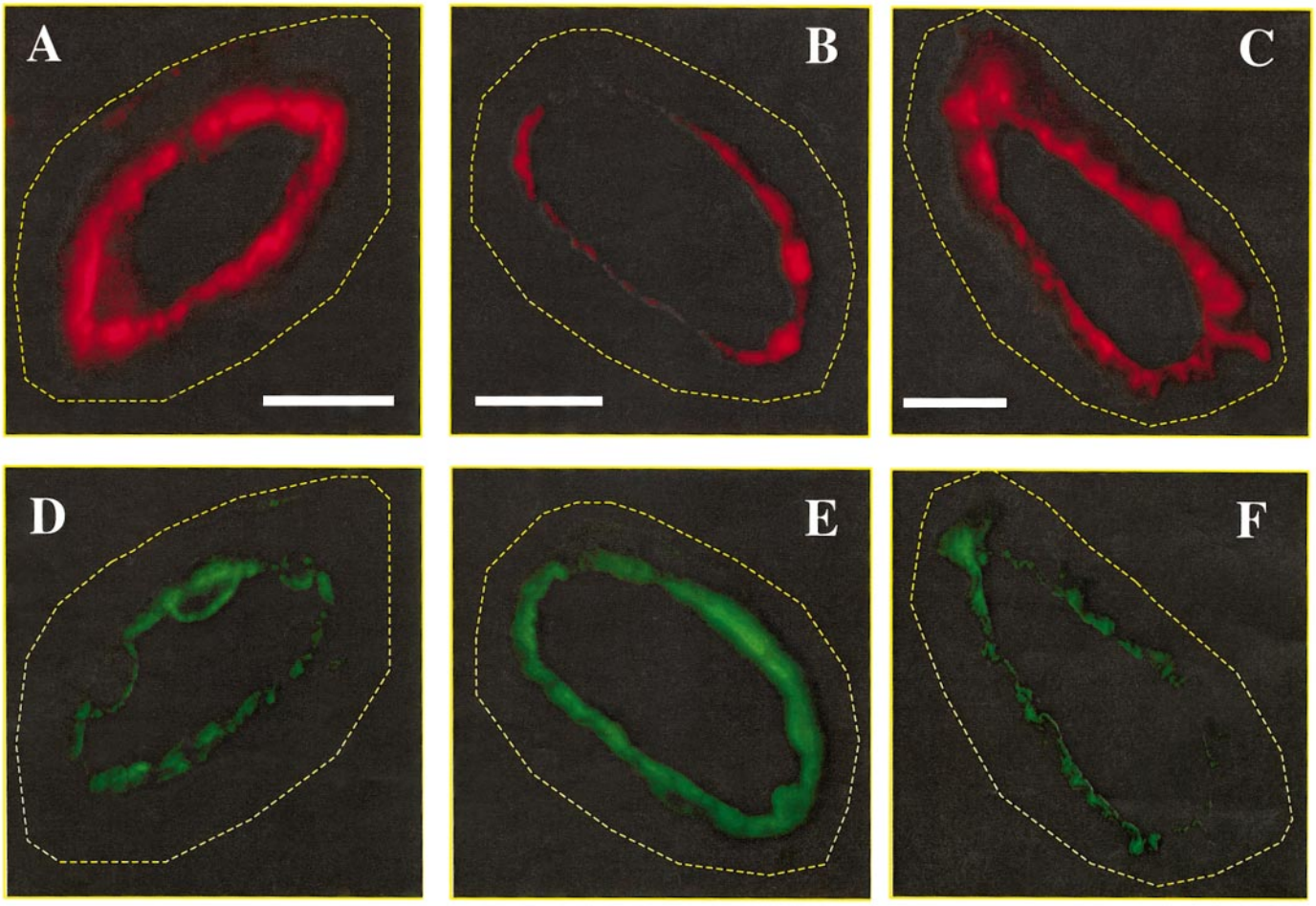

FIG. 2. Representative photomicrographs depicting eNOS (red, A–C) and CAV-1 (green, D–F) pial arteriolar immunoreactivities in intact (A, D), OVX (B, E), and 100 μg/kg/day E_2-treated OVX (C, F) females. For each rat group, both eNOS and CAV-1 expression were evaluated (see Fig. 3) in the same vessel using a dual immunofluorescent staining technique. The hatched line approximates the outer wall of the arteriole. Note that the two proteins were concentrated around the vessel lumen (presumably endothelium). Note also the similarities in the wall-to-lumen dimension ratios of the vessels shown. This is typical of the arterioles selected for analysis, the results of which are summarized in Fig. 3. The horizontal bar (A–C) represents 10 μm. See text for further details.

E_2-associated enhancement of receptor density or agonist affinity. In brain *tissue*, receptor binding capacity has been reported to be diminished by E_2 (29). In the periphery, E_2 treatment of OVX animals was associated with increases (30), decreases (31), or no change (32) in muscarinic receptor density in a variety of structures. It was recently shown that treatment of cultured human umbilical vein endothelial cells with E_2 for 1 day enhanced the intracellular Ca^{2+} ($[Ca^{2+}]_i$) increases accompanying muscarinic receptor activation (33). Because eNOS is activated by increased $[Ca^{2+}]_I$, this raises the possibility that estrogen may indeed augment muscarinic receptor to eNOS signal transduction. However, the concentration of E_2 applied to these cells (100 nM) was excessive and considerably higher than the circulating levels of E_2 seen in intact females and in our high-dose E_2-treated group (0.1–1.0 nM, see ref. 18). Thus, the physiologic relevance of those *in vitro* findings remains open to question.

The endothelial NOS isoform is subject to a considerable degree of post-translational control. A number of recent reports [reviewed by Michel and Feron (34)] have shown that optimal activity of eNOS requires localization of eNOS to plasmalemmal structures called caveolae. The importance of caveolar localization for optimal eNOS regulation may relate to the observation that most of the elements needed for activation eNOS are concentrated in the microdomains of these structures. These include cofactors; L-arginine transporters; (35); proteins regulating Ca^{2+} influx, extrusion, and intracellular release (36); and even muscarinic receptors (37). A recently characterized mechanism for regulating eNOS activity is its binding to the caveolar protein, CAV-1. That association represses

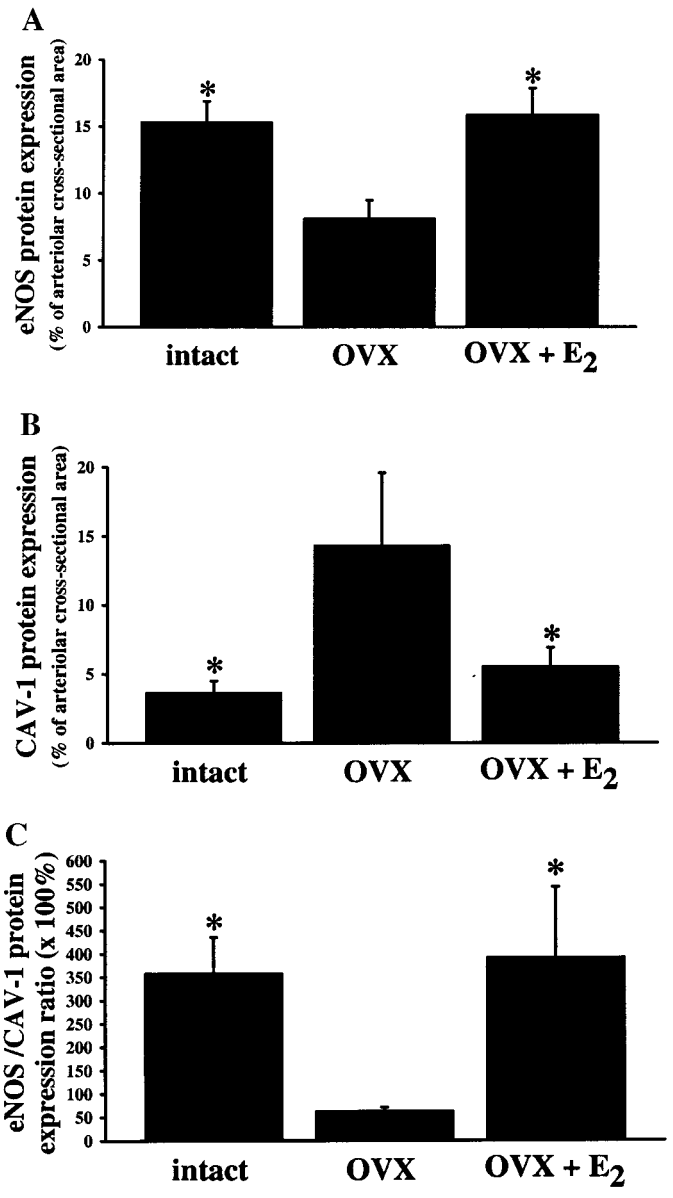

FIG. 3. Pial arteriolar relative expression of eNOS (A) and CAV-1 (B), together with the ratios of eNOS/CAV-1 expression (C) within vessels (ratios calculated for each arteriole analyzed). The relative abundance of eNOS and CAV-1 protein in each vessel was expressed as the area of eNOS or CAV-1 immunoreactivity divided by the total cross-sectional area of that vessel (times 100%). Values are means ± SE. *$P < 0.05$ vs OVX.

eNOS activity (34). Stimulation of eNOS occurs when Ca^{2+}-activated calmodulin displaces CAV-1 from its binding site on the eNOS molecule. In the present study, we found that E_2, in addition to its association with higher pial arteriolar eNOS expression, was also associated with lower endothelial CAV-1 expression. Those effects of E_2 were much more clearly revealed when relating eNOS and CAV-1 endothelial expression within the same vessel. Thus, the eNOS/CAV-1 expression ratios were substantially higher in the intact and E_2-treated OVX rats compared to the untreated OVX rats. These findings, therefore, point to the possibility that the E_2-related potentiation of eNOS-dependent cerebral arteriolar vasodilating function is due not only to an upregulation of eNOS protein expression, but also to a combination of increased eNOS and diminished CAV-1 endothelial expression. As a cautionary note, immunofluorescence evaluations do not reveal whether the proteins are interacting in any way. Nevertheless, a higher endothelial expression of eNOS relative to CAV-1 can be viewed as suggestive of a lesser eNOS/CAV-1 interaction and a greater functional eNOS activity. Furthermore, in preliminary evaluations of brain surface tissue (unpublished), we found that immunoprecipitated CAV-1 displayed substantial eNOS immunoreactivity upon subsequent Western blot analysis. This suggests a strong potential for interaction between these two proteins. The ability of E_2 to affect eNOS function via modulating CAV-1 represents one of variety of potential mechanisms for E_2 influence on eNOS. The list of endogenous "eNOS regulators" continues to grow. One recent example is the phosphoinositol-3-kinase-activated Akt protein (or protein kinase B), which has been reported to phosphorylate a specific serine residue on the eNOS molecule, thereby increasing eNOS activity (38). Moreover, in breast cells, E_2 has been linked to increased Akt levels and activity (39). However, whether E_2 has any influence over the cerebrovascular endothelial activity of that protein or other cellular targets must await future investigation.

In conclusion, these results suggest that long-term changes in circulating estrogen levels can have a profound influence on pial arteriolar eNOS expression and functional activity. We obtained additional evidence that estrogen not only increases eNOS protein expression, but also may potentiate eNOS activity through downregulation of endothelial CAV-1.

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REFERENCES

1. Wren, B. (1992) *Med. J. Austral.* **157,** 204–208.
2. Iadecola, C., Pelligrino, D. A., Moskowitz, M. A., and Lassen, N. A. (1994) *J. Cereb. Blood Flow Metab.* **14,** 175–192.
3. Bell, D. R., Rensberger, H. J., Koritnik, D. R., and Koshy, A. (1995) *Am. J. Physiol.* **268,** H377–H383.
4. Goetz, R. M., Morano, I., Calovini, T., Studer, R., and Holtz, J. (1994) *Biochem. Biophys. Res. Commun.* **205,** 905–910.
5. Hayashi, T., Yamada, K., Esaki, T., Mutoh, E., and Iguchi, A. (1997) *Gerontology* **43,** 24–34.
6. Hishikawa, K., Nakaki, T., Marumo, T., Suzuki, H., Kato, R., and Saruta, T. (1995) *FEBS Lett.* **360,** 291–293.

7. MacRitchie, A. N., Jun, S. S., Chen, Z., German, Z., Yuhanna, I. S., Sherman, T. S., and Shaul, P. W. (1997) *Circ. Res.* **81,** 355–362.
8. Miller, V. M., and Vanhoutte, P. M. (1991) *Am. J. Physiol.* **261,** R1022–R1027.
9. Rahimian, R., Laher, I., Dube, G., and Vanbreemen, C. (1997) *J. Pharmacol. Exp. Ther.* **283,** 116–122.
10. Weiner, C. P., Lizasoain, I., Baylis, S. A., Knowles, R. G., Charles, I. G., and Moncada, S. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5212–5216.
11. Mcneill, A. M., Kim, N., Duckles, S. P., Krause, D. N., and Kontos, H. A. (1999) *Stroke* **30,** 2186–2190.
12. Galea, E., Glickstein, S. B., Feinstein, D. L., Golanov, E. V., and Reis, D. J. (1998) *Am. J. Physiol.* **276,** H2053–H2063.
13. Skarsgard, P., Vanbreemen, C., and Laher, I. (1997) *Am. J. Physiol.* **273,** H2248–H2256.
14. Geary, G. G., Krause, D. N., and Duckles, S. P. (1998) *Am. J. Physiol.* **275,** H292–H300.
15. Feron, O., Saldana, F., Michel, J. B., and Michel, T. (1998) *J. Biol. Chem.* **273,** 3125–3128.
16. Koenig, H. M., Pelligrino, D. A., and Albrecht, R. F. (1993) *J. Neurosurg. Anesth.* **5,** 264–271.
17. Wang, Q., Pelligrino, D. A., Koenig, H. M., and Albrecht, R. F. (1994) *J. Cereb. Blood Flow Metab.* **14,** 944–951.
18. Wang, Q., Santizo, R., Baughman, V. L., and Pelligrino, D. A. (1999) *Stroke* **30,** 630–637.
19. Pelligrino, D. A., Santizo, R., Baughman, V. L., and Wang, Q. (1998) *NeuroReport* **9,** 3285–3291.
20. Pelligrino, D. A., Wang, Q., Koenig, H. M., and Albrecht, R. F. (1995) *Brain Res.* **704,** 61–70.
21. Venema, R. C., Nishida, K., Alexander, R. W., Harrison, D. G., and Murphy, T. J. (1994) *Biochim. Biophys. Acta 1* **218,** 413–420.
22. Smith, S. S. (1994) *Prog. Neurobiol.* **44,** 55–86.
23. Sukovich, D. A., Mukherjee, R., and Benfield, P. A. (1994) *Mol. Cell. Biol.* **14,** 7134–7143.
24. Marsden, P. A., Heng, H. H., Scherer, S. W., Stewart, R. J., Hall, A. V., Shi, X. M., Tsui, L. C., and Schappert, K. T. (1993) *J. Biol. Chem.* **268,** 17478–17488.
25. Hyder, S. M., Nawaz, Z., Chiappetta, C., Yokoyama, K., and Stancel, G. M. (1995) *J. Biol. Chem.* **270,** 8506–8513.
26. Zhou, Y., and Dorsa, D. M. (1994) *Horm. Behav.* **28,** 376–382.
27. Umayahara, Y., Kawamori, R., Watada, H., Imano, E., Iwama, N., Morishima, T., Yamasaki, Y., Kajimoto, Y., and Kamada, T. (1994) *J. Biol. Chem.* **269,** 16433–16442.
28. Clark, J. H., Schrader, W. T., and O'Malley, M. (1992) *in* William's Textbook of Endocrinology, pp. 63–65, Saunders, Philadelphia.
29. Al-Dahan, M. I., and Thomas, P. J. (1987) *Pharmacology* **34,** 250–258.
30. Batra, S. (1990) *J. Endocrinol.* **125,** 185–189.
31. Batra, S., and Andersson, K. E. (1989) *Acta Physiol. Scand.* **137,** 135–141.
32. Klangkalya, B., and Chan, A. (1988) *Life Sci.* **42,** 2307–2314.
33. Moini, H., Bilsel, S., Bekdemir, T., and Emerk, K. (1997) *Endothelium* **5,** 11–19.
34. Michel, T., and Feron, O. (1997) *J. Clin. Invest.* **100,** 2146–2152.
35. Mcdonald, K. K., Zharikov, S., Block, E. R., and Kilberg, M. S. (1997) *J. Biol. Chem.* **272,** 31213–31216.
36. Fujimoto, T., Miyawaki, A., and Mikoshiba, K. (1995) *J. Cell Sci.* **108,** 7–15.
37. Feron, O., Smith, T. W., Michel, T., and Kelly, R. A. (1997) *J. Biol. Chem.* **272,** 17744–17748.
38. Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999) *Nature* **399,** 597–601.
39. Ahmad, S., Singh, N., and Glazer, R. I. (1999) *Biochem. Pharmacol.* **58,** 425–430.