Vascular Biology, Atherosclerosis and Endothelium Biology

The Estrogen Effects on Endothelial Repair and Mitogen-Activated Protein Kinase Activation Are Abolished in Endothelial Nitric-Oxide (NO) Synthase Knockout Mice, but Not by NO Synthase Inhibition by *N*-Nitro-L-arginine Methyl Ester

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We have previously shown that estrogen exerts a vasoprotective effect by accelerating reendothelialization after perivascular artery injury through activation of the estrogen receptor α . Because 17 β -estradiol (E_2) is known to increase the bioavailability of nitric oxide, in this study, we used the same perivascular model to characterize the role of the endothelial nitric oxide synthase (eNOS) pathway in reendothelialization. Surprisingly, we found that the stimulatory effect of E_2 on reendothelialization was not altered following pharmacological inhibition of nitric-oxide synthase enzymatic activity by *N*-nitro-L-arginine methyl ester, whereas it was abolished in eNOS-deficient ($eNOS^{-/-}$) mice. This discrepancy between eNOS gene inactivation and the pharmacological inhibition of eNOS was confirmed in a classical model of endovascular injury. When assessing the involvement of eNOS in short-term membrane-associated signaling events induced by E_2 , we found that E_2 stimulated phosphorylation of extracellular signal-regulated kinase 1/2 in isolated perfused carotid arteries from wild-type mice in the absence or presence of *N*-nitro-L-arginine methyl ester, whereas this stimulation was abolished in carotid arteries from $eNOS^{-/-}$ mice.

Similar results were obtained in primary cultures of mouse aortic endothelial cells. These data reveal an original and unexpected role of eNOS, in which its presence but not its enzymatic activity appears to be a determinant for estrogen signaling in the endothelium. The consequences of this novel function of eNOS with respect to vascular diseases should be explored. (*Am J Pathol* 2008, 172:830–838; DOI: 10.2353/ajpath.2008.070439)

The endothelium is a major regulator of vascular homeostasis, influencing vascular tone and exerting anticoagulant, antiplatelet adhesion/aggregation, and fibrinolytic activities. Loss of endothelial monolayer integrity represents an important early step in atherosclerotic plaque development, and likewise, endothelial destruction provoked by endoluminal angioplasty is a major effector of the subsequent restenosis.¹

17 β -Estradiol (E_2) exerts several vasoprotective effects, such as vascular healing. We previously demonstrated that E_2 accelerates reendothelialization² and that E_2 was also shown to prevent neointimal hyperplasia in rats³ and medial hyperplasia in mice.⁴ In addition, E_2 promotes vasorelaxation and inhibits platelet aggregation through potentiation of endothelial NO and prostacyclin production. Indeed, E_2 stimulates endothelial NO

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synthase (eNOS) activity acutely through a membrane-derived nongenomic effect.^{5,6} E₂ also increases NO bioactivity chronically through a decreased breakdown of NO, as a consequence of a reduced production of reactive oxygen species.^{7,8}

The effects of E₂ can be mediated by estrogen receptor α (ER α) or β (ER β), two members of the nuclear receptor superfamily that are encoded by two distinct genes.⁹ We and others previously showed that ER α , but not ER β , is responsible for important vascular effects of E₂ such as NO production^{10–12} and arterial healing.^{2,13} Although ERs are classically defined as ligand-activated transcription factors,¹⁴ it has become clear that short-term “extragenomic” responses play an important role in cultured endothelial cells, leading to phosphatidylinositol 3-kinase (PI3K)-AKT and eNOS activation.^{6,10,15} Both eNOS and a fraction of ER α are present in caveolae. Even though, to date, no direct interaction between the two proteins have been reported, they appear to be organized into a functional signaling complex in caveolae.¹⁶

Losordo and colleagues¹⁷ have previously shown that the accelerating effect of E₂ on vascular reendothelialization is abolished in eNOS^{−/−} mice, demonstrating the implication of eNOS. Concomitantly, we observed a persistence of the E₂ effect on reendothelialization in a perivascular injury model using a pharmacological approach where eNOS was inhibited by *N*-nitro-L-arginine methyl ester (L-NAME). We have pursued this initial and contradictory observation through more thorough studies using both pharmacological inhibition of nitric-oxide synthase (NOS) activity by L-NAME and mice deficient in eNOS in both endovascular and perivascular injury models. In both models, we confirmed that the accelerating effect of E₂ was abolished in eNOS^{−/−} mice but also unexpectedly that the effect of E₂ was unaltered after pharmacological inhibition of NOS activity. A similar discrepancy was also observed on the phosphorylation status of mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 1 (ERK1) and ERK2, after E₂ treatment in an *ex vivo* model of isolated perfused carotid artery and *in vitro* in primary culture of mouse aortic endothelial cells. Thus, these data argue for a crucial role of eNOS, which is independent of its nitric-oxide synthase activity in the mediation of the estrogen effect in mouse carotid artery.

Materials and Methods

Animals

The investigation was in agreement with the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institutes of Health (Bethesda, MD). The mice were housed in stainless steel cages in groups of five and kept in a specific pathogen-free facility. C57Bl/6J wild-type mice were purchased from Charles River (L'Arbresle, France). eNOS^{−/−} mice¹⁸ and ER α ^{−/−} mice¹⁹ were maintained in our animal facility.

For all surgical procedures, female mice were anesthetized by intraperitoneal injection of a mixture of ket-

amine and xylazine and allowed to recover on a 37°C heat pack. They were ovariectomized at 4 weeks of age and given or not given 60-day time release E₂ pellets (0.1 mg of E₂, releasing 80 μ g/kg/day) (Innovative Research of America, Sarasota, FL) implanted subcutaneously into the backs of the animals with a sterile trochar. The NOS inhibitor, L-NAME (Sigma, Saint Quentin Fallavier, France) (50 mg/kg/day), was given in the drinking water after ovariectomy. Electric carotid artery injury was performed 2 weeks later in 6-week-old mice.

cGMP Measurements

cGMP (the second messenger of NO) content in the thoracic aorta, the lung, and the cerebellum was measured to assess the level of inhibition of NO synthase activity. Mice given L-NAME (0 or 50 mg/kg/day) (eight mice in each group) were sacrificed with an overdose of ketalar and used for determination of cGMP (the second messenger of NO) content in the thoracic aorta, in the lung, and in the cerebellum. Thoracic aortae, lungs, and cerebella were removed and frozen in liquid nitrogen, and the tissues were stored frozen (−80°C) until measurement of the cGMP level. Frozen samples were thawed in glass potter in the presence of 3-isobutyl-1-methylxanthine (10^{−4} M) and sonicated 10 times using ten 20-seconds bursts. The homogenates were centrifuged (4000 rpm at 4°C during 15 minutes), and the supernatant fraction was taken for radioimmunoassay of cGMP using a commercial kit (Amersham, Piscataway, NJ). The samples were acetylated to increase the sensitivity of the assay. Data are the average of values from triplicate incubations. cGMP content was expressed as picomole per milligram of protein.

Perivascular Injury Model

The perivascular injury model was performed as described previously.² In brief, the left common carotid artery was exposed via an anterior incision of the neck. The electric injury was applied to the distal part of the common carotid artery. The optimal conditions were determined as follows: electric power of 2 W applied for 2 seconds to each millimeter of carotid artery over a total length of 4 mm, with the help of a size marker placed parallel to the long axis of the carotid.

Endovascular Injury Model

The endovascular injury model was adapted from Lindner et al.²⁰ The right common carotid artery was exposed from the bifurcation via an anterior incision of the neck. The external carotid was ligated distally and looped proximally with 8-0 silk suture. Silk loops were placed around the internal and common carotids to temporally restrict blood flow in the area of surgical manipulation. The ligature on the common carotid was precisely positioned against the sternocleidomastoid muscle 4 mm from the carotid bifurcation. This distance was very uniform from one animal to another. The external carotid

artery was incised. The injury was performed using a 0.3-mm-diameter swab built by loosely winding and sticking an 8-0 silk suture around a 0.16-mm-diameter blunted guide wire. The swab was introduced, advanced through the common carotid artery, and withdrawn three times. A 4-mm-length denudation from the bifurcation of the common carotid artery was performed using the silk ligature on the common carotid as injury limit. The device was removed, and the external carotid was ligated proximally. Blood flow was then restored by loosening the loops around the internal and common carotids. The skin incision was closed, and animals were allowed to wake up under warm conditions.

Quantification of Endothelial Regeneration

Three or 5 days after injury, the endothelial regeneration process was evaluated by staining the denuded areas with Evans blue dye (Sigma) as described previously.² In brief, the common carotid artery was dissected with an adjacent portion of the aortic arch and carotid bifurcation. The artery was then opened longitudinally and placed between slides with Fluoprep (BioMérieux, Marcy l'Etoile, France). The ratio between the area stained in blue and the total carotid artery area was calculated. The surface of the area that remained deendothelialized was indexed to the total carotid artery area to take into account the changes in vessel area due to both elasticity of the carotid artery and the flattening of the vessel between slides.

Organ Culture

Female mice between 8 and 10 weeks of age were sacrificed by a lethal injection of sodium pentobarbital (50 mg/kg i.p.). Carotid arteries were immersed in an organ culture bath filled with Dulbecco's modified Eagle's medium containing antibiotics (100 IU/mL penicillin, 100 mg/L streptomycin, and 10 μ g/L fungizone) and supplemented with 5% steroid-free fetal calf serum. Each arterial segment was maintained for 72 hours in a closed perfusion circuit described previously,²¹ and exposed to an intraluminal hydrostatic pressure of 80 mmHg with continuous renewal of medium within the intraluminal space at minimal shear stress (0.5 dynes/cm²). Organ culture of the carotid segments was performed under sterile conditions in an incubator containing 5% CO₂ at 37°C. At the end of the organ culture period, 10 nmol/L E₂ (Sigma) or vehicle (dimethyl sulfoxide) was injected in the intraluminal compartment for 15 to 60 minutes in the presence (100 μ mol/L) or absence of L-NAME. Thereafter, vessels were quickly removed and processed as described below.

Cultured Endothelial Cells

Endothelial cells were obtained from mouse aortae according to the primary explant procedure adapted from a previously described protocol,²² allowing the isolation of limited but pure cell preparations. Serum-starved endothelial cells were exposed for the indicated periods of

time to E₂ (10 nmol/L) in the presence or absence of L-NAME (100 μ mol/L) or N^G-monomethyl-L-arginine acetate (100 and 300 μ mol/L; Sigma).

Western Blot

Cultured vessels were ground in ice-cold lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 5 mmol/L EGTA, 150 mmol/L NaCl, 20 mmol/L sodium glycerophosphate, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1% Triton X-100, 0.1% Tween 20, and protease inhibitors (Roche, Neuilly-sur-Seine, France). Detergent-soluble fractions were retained, and protein concentrations in samples were measured using a Bradford protein assay (Bio-Rad, Marnes-la-Coquette, France). For Western blot analysis, lysates containing 30 μ g of protein were electrophoresed on polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Amersham). Membranes were incubated with anti-phospho-ERK1/2, anti-ERK1/2 (Cell Signaling Technology, Beverly, MA) or anti-ERK2 antibodies (Santa Cruz Biotechnology, Inc., Heidelberg, Germany). An enhanced chemiluminescence system was used as the detection method (ECL Plus; Amersham).

Small Interference (siRNA) Transfection

siRNA duplex oligonucleotides (ON-TARGET Plus pre-designed siRNA) were from Dharmacon, Inc. (Lafayette, CO). EA.hy926 cells (courtesy of C. J. Edgell, Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, NC) were cultured as described previously²³ and transfected with siRNA (100 nmol/L) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the instruction provided by the supplier. Forty-eight hours later, cells were harvested and subjected to Western blot analysis as described above. The antibodies used were from Santa Cruz Biotechnology, Inc. (anti-eNOS) and Cell Signaling Technology (anti-phospho-ERK1/2 and anti-ERK1/2).

Immunohistochemical Analysis

Arteries were embedded in paraffin, and sections perpendicular to the long axis of the carotid were cut at the level of the reendothelialized area of the injured carotid artery. Sections were subjected to standard hematoxylin/eosin staining and eNOS immunodetection. Detection of eNOS expression was determined with rabbit polyclonal anti-eNOS (1:200, catalog number sc-654; Santa Cruz Biotechnology, Inc.). The specificity of eNOS antibody was tested on carotid artery cross-sections from eNOS^{-/-} mice.

Statistics

Data are expressed as mean $\pm$ SEM. For cultured vessels, Western blot band density was quantified using Image Gauge software (Fuji, Saint Quentin en Yvelines,

Figure 1. Perivascular injury model: the effect of E_2 on the reendothelialization process in ovariectomized wild-type (A) and $eNOS^{-/-}$ (B) mice treated or not treated (placebo) with E_2 (80 μ g/kg/day) and receiving or not receiving L-NAME (50 mg/kg/day) in the drinking water. Results show percentage of de-endothelialization calculated as ratio of remaining de-endothelialized area to total carotid artery area at day 5. * $P < 0.05$ vs respective placebo group.

France). One-way analysis of variance (or two-way) was used to compare data for different genotypes or inhibitors. When analyses of variance yielded significant results, comparisons were done using Bonferroni's *t*-test.

Results

eNOS, but Not NO Production, is Required for the Effect of E_2 on Reendothelialization

To define the role of NO production in the stimulating effect of E_2 on reendothelialization in the perivascular injury model, we first used a pharmacological approach; ovariectomized wild-type C57Bl/6J mice were treated chronically with L-NAME (50 mg/kg/day). As shown in Figure 1A, pharmacological inhibition of eNOS activity did not alter the accelerating effect of E_2 on reendothelialization 5 days postinjury. Similar results were obtained at day 3 postinjury (data not shown). Similar results were also obtained at day 5 postinjury in C57Bl/6J mice treated chronically with a twofold higher dose of L-NAME (100 mg/kg/day; data not shown).

Given that these results were inconsistent with a previous study by Losordo and colleagues,¹⁷ implicating eNOS in the E_2 effect on reendothelialization, we decided to repeat the study in the endovascular injury model. As observed in the perivascular injury model, treatment with L-NAME did not influence the capacity of E_2 to speed up reendothelialization in the endovascular injury model (Figure 2A). To verify that the dose of L-NAME used (50 mg/kg/day) efficiently blocked NO production in our experiments, the cGMP content in the thoracic aorta of C57Bl/6J mice treated with L-NAME (0 or 50 mg/kg/day) for 8 days were 1.06 ± 0.05 and 0.35 ± 0.03 pmol/mg protein, respectively ($P < 0.0001$), demonstrating a threefold drop in the second messenger of NO in L-NAME-treated mice.

Figure 2. Endovascular injury model: the effect of E_2 on the reendothelialization process in ovariectomized wild-type (A) and $eNOS^{-/-}$ (B) mice treated or not treated (placebo) with E_2 (80 μ g/kg/day) and receiving or not receiving L-NAME (50 mg/kg/day) in the drinking water. Results show percentage of de-endothelialization calculated as ratio of remaining de-endothelialized area to total carotid artery area at day 5. * $P < 0.05$ vs respective placebo group.

The cGMP content in the lung of C57Bl/6J mice given placebo or L-NAME 50 mg/kg/day were 0.22 ± 0.04 and 0.11 ± 0.02 pmol/mg protein, respectively ($P < 0.001$). These decreased levels of vascular cGMP were not further altered by doubling the dose of L-NAME (ie, 100 mg/kg/day for 8 days), strongly suggesting that the dose of 50 mg/kg/day used in this study as well as in previous works^{24,25} maximally blocks the eNOS activity. Moreover, we observed a 4.5-fold drop in cGMP in cerebellum preparations from L-NAME-treated mice, demonstrating an inhibition of neuronal NOS (data not shown). Taken together, our data show that inhibiting NO production with L-NAME did not influence the effect of E_2 on endothelial regrowth *in vivo*, irrespective of the arterial injury model.

The divergence between our findings and those previously published¹⁷ prompted us to reassess the role of eNOS five days after injury using a transgenic approach. Indeed, in agreement with the reported data, the accelerating effect of E_2 on reendothelialization was abolished in $eNOS^{-/-}$ mice, both in the perivascular injury model (Figure 1B) and in the endovascular injury model (Figure 2B). Again, similar results were obtained at day 3 postinjury (data not shown).

As shown in Figures 1B and 2B, the absence of E_2 effect on reendothelialization was confirmed in these animals, and no "additional" effect of L-NAME was observed in either vascular injury model. Altogether, these data showed that *in vivo*, eNOS, but not NO production, is required for the accelerating effect of E_2 on reendothelialization.

eNOS Expression is Enhanced in the Reendothelialized Intimal Area

Given the importance of the eNOS protein in the reendothelialization process, we next performed immunohisto-

Figure 3. Immunostaining of eNOS in noninjured (A, C) and reendothelialized (B, D) carotid arteries of wild-type (A, B) and *eNOS*^{-/-} mice (C, D) at day 5 after perivascular injury.

chemical analyses to compare its expression levels in injured and uninjured carotid arteries. Compared with the endothelium of an intact (uninjured) artery (Figure 3A), eNOS expression was clearly enhanced in the regenerated endothelium of healing carotid artery, day 5 postinjury in wild-type mice (Figure 3B), in agreement with previous observations made in regenerated endothelium in rat²⁶ and in proliferating cultured endothelial cells.²⁷ In this electric injury model, the regenerated endothelial area can be easily recognized by the characteristic absence of underlying smooth muscle cells. The expression of eNOS was restricted to the intima in both intact and injured vessels, suggesting that it was mainly expressed at the level of the endothelium even after injury. As anticipated, no eNOS-specific staining was detected in arteries of *eNOS*^{-/-} mice (Figure 3, C and D).

eNOS, but Not NO Production, is Required for E₂-induced ERK1/2 Phosphorylation in Isolated Perfused Carotid Artery and Primary Culture of Mouse Aortic Endothelial Cells

We then sought to explore the respective roles of eNOS deficiency and eNOS enzyme inhibition in E₂-induced ERK1/2 activation using an *ex vivo* model. As shown in Figure 4, ERK1/2 phosphorylation was enhanced in wild-type carotid arteries exposed to E₂, reaching an activa-

tion peak at 30 minutes and declining at 60 minutes. A delayed ERK1/2 activation has previously been reported in cultured endothelial cells exposed to E₂.²⁸ We also found that ERK1/2 phosphorylation by E₂ was preserved

Figure 4. Acute phosphorylation of ERK1/2 induced by E₂ in carotid arteries from wild-type mice is insensitive to L-NAME but is abolished in *eNOS*^{-/-} carotid arteries. **A:** Representative Western blot showing short-term phosphorylation kinetics of ERK1/2 induced by E₂ (10 nmol/L), administered in the intraluminal compartment of carotid arteries exposed 3 days in organ culture. ERK1/2 phosphorylation is equivalent in untreated and L-NAME-treated (100 μmol/L) carotid arteries from wild-type mice but abolished in carotid arteries from *eNOS*^{-/-} and *ERα*^{-/-} mice. **B:** Bar graph represents the quantification of the phospho-ERK1/2/ERK2 ratio, normalized to the control within each group. **P* < 0.05 and ****P* < 0.001 vs respective control group.

Figure 5. eNOS deficiency prevents E₂-induced ERK1/2 activation in MAECs. Isolated mouse endothelial cells were treated with E₂ (10 nmol/L) for different times. **A:** Representative immunoblotting experiments show that phosphorylation of ERK1/2 is enhanced by E₂ in endothelial cells from wild-type but not eNOS^{-/-} or ERα^{-/-} mice. **B:** Bar graph represents the quantification of the P-ERK/ERK ratio, normalized to the control within each group (*n* = 2 experiments).

in carotid arteries from wild-type mice treated with 100 μmol/L L-NAME, demonstrating that E₂ acted independently of NO production. In contrast, E₂-induced ERK1/2 activation was abolished in carotid arteries from eNOS^{-/-} mice. Similarly, E₂ failed to induce ERK1/2 phosphorylation in carotid arteries from ERα^{-/-} mice.

We then reassessed the respective roles of eNOS deficiency and eNOS enzyme inhibition in cultured endothelial cells from mouse aortae. As shown in Figure 5, we found that E₂ stimulated the phosphorylation of ERK1/2 equally in untreated and in L-NAME-treated cells from wild-type mice. N^G-Monomethyl-L-arginine acetate, another NOS inhibitor, did not alter the effect of E₂-stimulated phosphorylation of ERK1/2 in endothelial cells from wild-type mice (data not shown). Moreover, pretreatment with 0.5 mmol/L dipropyleneetriamine NONOate, a NO donor, did not restore the effect of E₂ in endothelial cells from eNOS^{-/-} mice (data not shown). On the other hand, activation of ERK1/2 by E₂ was abolished in eNOS^{-/-} and ERα^{-/-} cells. A deficiency in intracellular BH4 levels does not appear to explain these differences, because neither the supplementation of BH4 (3 μmol/L) nor that of the BH4 synthesis substrate sepiapterin (50 μmol/L) to cultured aortic endothelial cells from C57BL/6 wild-type mice altered the phosphorylation of ERK1/2 in response to E₂ (data not shown).

Given that gene inactivation is known to favor gene expression remodeling, particularly "gene compensa-

tions", we tried to restore eNOS expression in eNOS-deficient endothelial cells through transfection. However, we did not succeed with this approach due to the difficulty of transfecting these fragile cells, which underwent apoptosis soon after transfection.

Therefore, we sought to abolish eNOS expression in eNOS-expressing endothelial cells using siRNA against eNOS. We first performed this experiment in endothelial cells from eNOS^{+/+} mice, but again these cells did not tolerate transfection. We next tried a more classical cell model of endothelial cells: human umbilical vein endothelial cells. Unfortunately, we were unable to elicit ERK1/2 activation in response to E₂ in human umbilical vein endothelial cells, even in freshly harvested cells at very early passages. This was probably due to the lack of ER gene expression (assessed by reverse transcription-polymerase chain reaction and Western blotting) at variance with previous work,²⁹ but in agreement with other.³⁰

Finally, we tried a human umbilical vein endothelial cell-derived cell line (EA.hy926) that has been shown to express ERα.³¹ In this model, we were able to elicit MAPK activation in response to E₂ and a clear decrease of this signal after eNOS siRNA treatment (Supplemental Figure S1, available at <http://ajp.amjpathol.org>). These data indicate that the level of eNOS protein expression influences the capacity of E₂ to activate ERK1/2 *in vitro*. However, we were struck by the poor reproducibility of this model, because in two of four experiments, we did not observe an increase in ERK1/2 phosphorylation in response to E₂ in control cells.

Altogether, these data suggest that the E₂ effect leading to ERK1/2 phosphorylation is mediated by ERα and appears to be strictly dependent on the presence of eNOS protein, but not its activity, in both *ex vivo* and *in vitro* models. It is noteworthy that the reproducibility of the effects observed *in vivo* or *ex vivo* was much more robust than the ones observed in cultured endothelial cells.

Discussion

Altogether, our results reveal an original and unexpected role for eNOS in endothelial regeneration and in plasma membrane E₂ signaling, acting as a signaling protein independently of its NO-producing function. Indeed, our finding that accelerated reendothelialization induced by E₂ was abolished in eNOS^{-/-} mice agreed with a previous report;¹⁷ however, we were surprised to observe that the pharmacological inhibition of NOS with L-NAME had no influence on the beneficial effect of E₂ in two models of vascular injury.

Our finding that the *in vivo* effects of E₂ differ between L-NAME-treated and eNOS^{-/-} mice appears robust for the following reasons. 1) It was established in two different experimental models, endovascular and electric perivascular carotid artery injury. 2) Pharmacological inhibition of eNOS using L-NAME was previously shown to maximally block NO synthase activity,^{24,25,32} and its efficacy was verified in the present work. 3) Abrogation of the effect of E₂ by eNOS gene inactivation has now been demonstrated in two different eNOS gene inactivation

models. Indeed, the model used by Losordo and colleagues¹⁷ targets exon 12 in the eNOS gene,³³ whereas the one used in the present study targets exons 24 to 25.

To better understand the interaction between E_2 and eNOS, we explored the respective influences of eNOS gene inactivation and eNOS activity inhibition on the short-term effects of E_2 in an *ex vivo* artery model. This model was used to specifically study endothelium in its native matrix and mechanical (submitted to physiological pressure) environment, in the absence of confounding hormonal or circulating factors encountered *in vivo*.³⁴ As previously described in cultured human, bovine, and porcine endothelial cells,^{28,35,36} we found that E_2 induced ERK1/2 activation in isolated perfused carotid arteries. Interestingly, and in agreement with our reendothelialization data, the plasma membrane signaling of E_2 was strictly dependent on the presence of eNOS protein but not eNOS activity in isolated perfused carotid arteries. The limitation of this model in interpreting ERK1/2 activation is that both endothelial and smooth muscle cells express ERK1/2. However, the exclusive expression of eNOS in the endothelium (Figure 3) on the one hand and the similarity in results between *in vivo* and *ex vivo* models on the other hand reinforce the concept that plasma membrane signaling of E_2 is strictly dependent on the presence of eNOS protein but not eNOS activity in the endothelial cells of carotid arteries.

eNOS gene disruption and NOS pharmacological inhibition are often viewed as comparable strategies, as they in some aspects generate similar results, such as the induction of hypertension and endothelial dysfunction.^{18,24,25,32,33} However, discrepancies between these two models have already been suggested, but never directly demonstrated. For instance, *eNOS*^{-/-}*apoE*^{-/-} mice develop twofold more fatty streak lesions than *apoE*^{-/-} mice,³⁷ whereas inhibition of NO production by L-NAME does not influence fatty streak deposit in *apoE*^{-/-} mice.^{25,37} Similarly, de Mey and colleagues³⁸ have previously shown an abrogation of shear stress induced vascular remodeling in *eNOS*^{-/-} mice, but not in L-NAME-treated rats,³⁹ suggesting that eNOS, but not eNOS activity, could play a role in vascular remodeling. This kind of dissociation between protein expression and enzymatic activity was nicely demonstrated for PI3K γ ,⁴⁰ which participates in two distinct signaling cascades: a kinase-dependent pathway that controls PKB/AKT activity and a kinase-independent pathway that relies on protein interactions with a phosphodiesterase to negatively modulate cardiac contractility.

Given that eNOS is largely present in caveolae and a fraction of ER α is also localized in this membrane compartment, these two molecules could associate in a functional molecular complex.^{16,31} Moreover, both ER α and eNOS were shown to interact with heat shock protein 90⁴¹ and caveolin 1,^{42,43} which reinforces the probability of a caveolar interaction of these proteins and supports of the hypothesis that eNOS acts in a multiprotein assembly, allowing ERK1/2 activation in response to E_2 .

Even though L-NAME is not a specific inhibitor of eNOS but inhibits other NOS isozymes, such as neuronal NOS and to some extent inducible NOS, the lack of

specificity could hardly be influencing our conclusions concerning the effects on the carotid artery. Indeed, the inhibition of inducible NOS by L-NAME is only partial *in vivo* at the dose used in the present study.^{44,45} However, L-NAME did not alter the observed loss of E_2 effect in *eNOS*^{-/-} mice, ruling out an inference of the other NOS isoforms in the abrogation of the E_2 effects in *eNOS*^{-/-} mice. Finally, a significant contribution of superoxide production by eNOS uncoupling is unlikely because 1) BH4 supplementation did not influence the result observed in wild-type cultured cells; 2) L-NAME inhibits superoxide production, as demonstrated in previous *in vivo*⁴⁶ and *ex vivo*⁴⁷ studies; and 3) L-NAME inhibits superoxide production of purified neuronal NOS.^{48,49}

The *in vivo* effects of E_2 probably involve both genomic and nongenomic mechanisms. Although ERs are classically defined as ligand-activated transcription factors,¹⁴ it has become clear that short-term "extragenomic-to-genomic" responses play an important role in cultured endothelial cells,⁶ but also in osteoblasts and in cortical neurons (as for instance the activation of PI3K-AKT pathways as well as MAPK pathways).^{50,51} The characterization of the respective roles of these "membrane" and "classical" effects represents an important but difficult challenge for the next few years.^{15,52,53}

Beyond this unexpected role of eNOS in E_2 signaling, the clinical implications of our observations could be significant. First, the strong correlation between our *in vivo* and *ex vivo* findings support an implication of plasma membrane estrogen signaling in its protective effects. Second, given that deficiency in E_2 has been recognized to modulate endothelial function,⁵⁴ altered eNOS expression or trafficking in the context of endothelial dysfunction could hinder the beneficial effects of E_2 , thus sustaining a vicious circle. This could for instance help to explain the highly negative relationship reported between smoking and estrogen atheroprotection.⁵⁵ Third, the proposed role of eNOS, acting within a molecular complex independently of its NO producing activity, may not be restricted to E_2 signaling but may also influence endothelial response to other vasoactive compounds. These specific roles of eNOS should be more precisely explored in future work.

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