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Estrogen Modulation of Endothelial Nitric Oxide Synthase

KEN L. CHAMBLISS AND PHILIP W. SHAUL

Department of Pediatrics, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390

Over the past decade, clinical and basic research has demonstrated that estrogen has a dramatic impact on the response to vascular injury and the development of atherosclerosis. Further work has indicated that this is at least partially mediated by an enhancement in nitric oxide (NO) production by the endothelial isoform of NO synthase (eNOS) due to increases in both eNOS expression and level of activation. The effects on eNOS abundance are primarily mediated at the level of gene transcription, and they are dependent on estrogen receptors (ERs), which classically serve as transcription factors, but they are independent of estrogen response element action. Estrogen also has potent nongenomic effects on eNOS activity mediated by a subpopulation of ERα localized to caveolae in endothelial cells, where they are coupled to eNOS in a functional signaling module. These observations, which emphasize dependence on cell surface-associated receptors, provide evidence for the existence of a steroid receptor fast-action complex, or SRFC, in caveolae. Estrogen binding to ERα on the SRFC in caveolae leads to $G_{\alpha i}$ activation, which mediates downstream events. The downstream signaling includes activation of tyrosine kinase-MAPK and Akt/protein kinase B signaling, stimulation of heat shock protein 90 binding to eNOS, and perturbation of the local calcium environment, leading to eNOS phosphorylation and calmodulin-mediated eNOS stimulation. These unique genomic and nongenomic processes are critical to the vasoprotective and atheroprotective characteristics of estrogen. In addition, they serve as excellent paradigms for further elucidation of novel mechanisms of steroid hormone action. (*Endocrine Reviews* 23: 665–686, 2002)

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I. Introduction

ESTROGEN IS AN important atheroprotective molecule with marked effects on the vasculature that are mediated, at least in part, by increased availability of the signaling molecule nitric oxide (NO) (1, 2). The endothelial isoform of NO synthase (eNOS) is the principal source of NO in the vascular wall (3). This article will review our current knowledge of estrogen modulation of eNOS expression and activity in vascular endothelium. The role of estrogen and NO in vascular function and disease in humans will first be summarized. Important insights obtained in animal models will also be addressed. Recent investigations in cell culture revealing that the actions of estrogen on eNOS are both genomic and nongenomic in nature will be discussed. In addition, the roles of the two known estrogen receptor (ER) isoforms, ERα and ERβ, will be described. Finally, the cellular and molecular mechanisms underlying the genomic and nongenomic regulation of eNOS by estrogen will be considered in depth. Further knowledge about the complex impact of estrogen on endothelial cell biology is needed to understand the normal actions of the hormone in the cardiovascular system, and to guide future considerations of

Abbreviations: Ach, Acetylcholine; apoE, apolipoprotein E; E_2, 17β-estradiol; eNOS, endothelial isoform of NOS; ER, estrogen receptor; ERE, estrogen response element; GDPβS, guanosine 5′-O-(2-thiodiphosphate); GPCR, G protein-coupled receptor; GTN, glyceryl trinitrate; HRT, hormone replacement therapy; HSP90, heat shock protein 90; HUVEC, human umbilical vein endothelial cells; L-NAME, nitro-L-arginine methyl ester; L-NMMA, L-N^G-monomethyl arginine; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOS, NO synthase; PI3-kinase, phosphatidylinositol 3-kinase; RGS, regulator of G protein signaling; SERM, selective ER modulator; SOD, superoxide dismutase; SRFC, steroid receptor fast-action complex; THC, tetrahydrochrysene; VSM, vascular smooth muscle.

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hormone therapy to prevent or treat cardiovascular disease (4).

II. Estrogen, Nitric Oxide, and Vascular Function and Disease in Humans

A. Estrogen and human vascular disease

The dramatic impact of estrogen on cardiovascular health is apparent from population-based studies, which indicate that premenopausal women have very little coronary artery disease compared with men, that the incidence of the disease rises markedly after menopause, and that hormone replacement therapy (HRT) under certain conditions reduces the risk to premenopausal levels (5–8). In addition, women who undergo surgical menopause without estrogen replacement have two times the risk of coronary artery disease than other premenopausal women (9). Whereas the mechanisms underlying these findings are not completely understood, considerable emphasis has been placed previously on the capacity of the hormone to modify circulating lipid levels (10). However, only approximately one third of the clinical advantages of estrogen can be attributed to effects on lipid balance (6, 11–13). Instead, recent evidence indicates that estrogen acts directly on the blood vessel wall to provide a major atheroprotective effect (14).

B. Sources of estrogens in humans

The estrogen compounds to which the vascular endothelium may be exposed in women are multiple, and they arise from both endogenous and exogenous sources. Before menopause, the major endogenously derived circulating estrogen compound is 17β-estradiol (E_2) produced by the ovary. Other endogenous estrogens and estrogen metabolites have also been described. After menses, when circulating E_2 levels increase and begin to cycle, levels range from less than 0.36 nM during the follicular phase to 2.8 nM during midcycle. During pregnancy, estrogen levels rise to 70 nM due to placental production, which increases near term and further with the onset of parturition. After menopause, E_2 concentrations fall to levels that are equivalent to those in males (0.04–0.21 nM). During menopause, the primary form of endogenous circulating estrogen is estrone derived peripherally from androstenedione by conversion in adipose tissue (15). Endothelial cells in both women and men may also be exposed to estrogens derived from the local conversion of testosterone or Δ^4-testosterone to E_2 by aromatase (16). Aromatase, E_2-hydroxysteroid dehydrogenase, and 17-ketoreductase enzyme activities were first demonstrated in rat arterial vascular smooth muscle (VSM) cells in cell culture (17), and more recently aromatase expression was noted in human VSM by *in situ* hybridization (18). Only trace levels were detected in samples from infants, and greater aromatase mRNA and activity were found in adult samples. In addition, studies in cultured human VSM demonstrated upregulation of aromatase mRNA in response to cAMP, phorbol ester, or dexamethasone (18). Thus, there is evidence that aromatase expression in VSM is developmentally regulated and also modulated by multiple signal transduction pathways and by glucocorticoids. However, the relative impacts of circulating *vs.* locally derived estrogens on endothelial cell function have not yet been clearly elucidated.

In addition to endogenously derived estrogens, there are often important exogenous sources in humans. Contraception is frequently accomplished using a combination of ethinyl E_2 and levonorgostrel or norethindrone. HRT is also often provided to postmenopausal women. This is frequently in the form of conjugated equine estrogens, and other oral and transdermal forms are also often considered. HRT frequently entails conjugated equine estrogens in combination with medroxyprogesterone acetate. In addition, selective estrogen receptor modulators, or SERMs, such as raloxifene are used for the treatment of osteoporosis, and it is likely that vascular-specific SERMS will also soon be available. Furthermore, phytoestrogens are a class of compounds found in certain plant-derived beverages and foods, and they have both estrogenic and antiestrogenic effects (14, 19, 20). As such, the endothelium may be exposed to a variety of forms and levels of estrogen compounds depending on the sex and age of the individual, his/her diet, and therapies that he/she may be receiving.

C. Long-term effects of estrogen on eNOS function

The onset of menopause provides a natural model of estrogen deprivation in which the effects of endogenous levels of the hormone on vascular function can be revealed. In studies of changes in brachial artery diameter after reactive hyperemia, which provides an assessment of flow-mediated, endothelium-dependent, and NO-dependent vasodilation, responses were greater in premenpausal *vs.* postmenopausal women (21). Similarly, determinations of forearm blood flow constrictor responses to NOS antagonism with L-N^G-monomethyl arginine (L-NMMA), which also evaluate vascular NO activity, revealed greater constriction in premenopausal *vs.* postmenopausal women. Importantly, blood flow responses to the NO donor glyceryl trinitrate (GTN) were similar in the two study groups, indicating comparable VSM responses to NO. As might be predicted, the responses in postmenopausal women were comparable to those observed in men (Fig. 1 and Ref. 22).

The menstrual cycle certainly provides another natural model of varying estrogen status. In studies of healthy young women, the capacity for endothelium-dependent vasodilation in the brachial artery paralleled serum E_2 levels, and, additionally, there was evidence of progesterone antagonism of this effect (23, 24). More recently, endothelium-dependent responses to bradykinin in resistance vessels have also been observed to be enhanced at midcycle during the period of greatest serum E_2 levels, whereas responses to GTN are unchanged (Fig. 2 and Ref. 25). In agreement with these findings, sex hormone deprivation after ovariectomy for uterine leiomyoma is associated with a decline in endothelium-dependent vasodilation, whereas the response to the NO donor sodium nitroprusside is unaltered (26). Although the exact sources of NO are undetermined, the enhanced endothelium-dependent vasodilation observed during periods of greater estrogen abundance during the menstrual cycle correlates with higher serum levels of the NO metabolites nitrate

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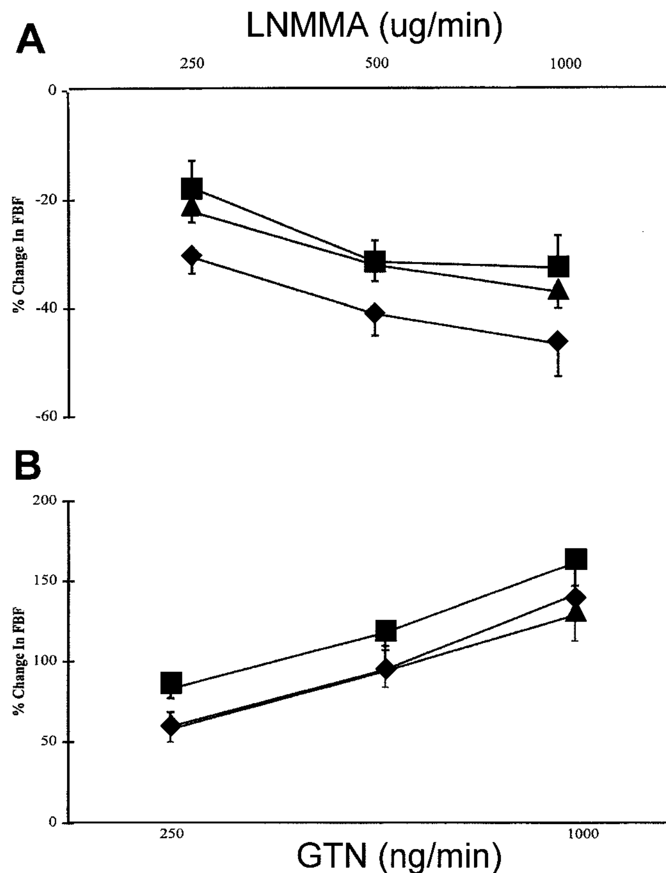

FIG. 1. Menopause is associated with a decline in endothelium-dependent, NO-dependent vasodilation. Constrictor responses to brachial artery infusion of L-NMMA (A) and vasodilator responses to brachial artery infusion of GTN (B) were assessed by measurements of forearm blood flow (FBF) in 15 premenopausal women (*diamonds*), 12 postmenopausal women (*squares*), and 14 men (*triangles*). L-NMMA responses were increased in premenopausal women *vs.* postmenopausal women and men, $P < 0.05$. In contrast, vasodilator responses to GTN were similar between groups. Values are mean ± SEM. [Reprinted with permission from: N. G. Majmudar *et al.*: *J Clin Endocrinol Metab* 85:1577–1583, 2000 (22). © The Endocrine Society.]

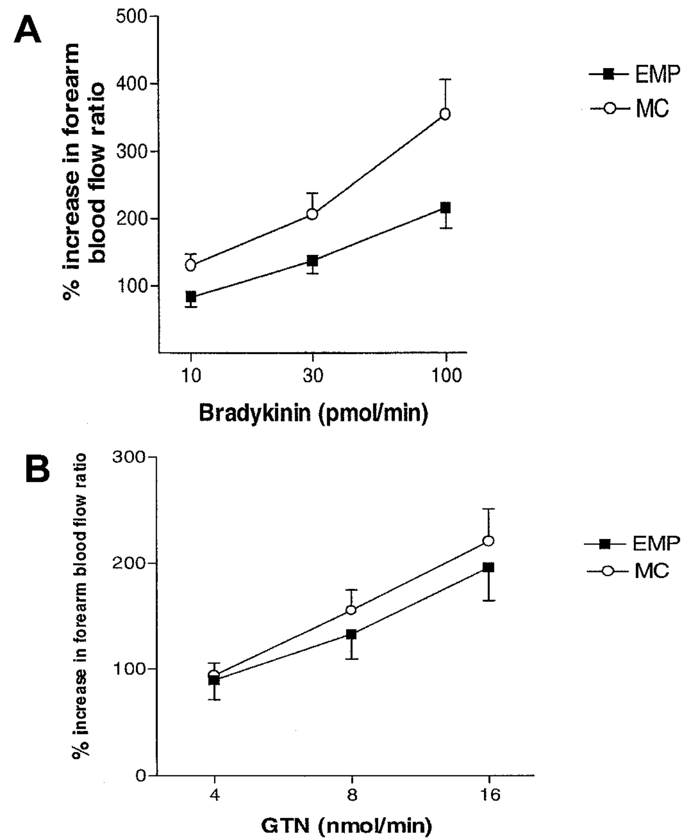

FIG. 2. The capacity for endothelium-dependent vasodilation varies during the menstrual cycle. Forearm blood flow response to bradykinin (A) and to GTN (B) at midcycle (MC) and during the early menstrual phase (EMP) were evaluated in healthy women. The response to bradykinin was greater during MC *vs.* EMP, whereas the response to GTN was similar, $P < 0.05$. Data are mean ± SEM, n = 15 subjects, and statistical analysis was performed on area under the curve values. [Reprinted with permission from N. N. Chan *et al.*: *J Clin Endocrinol Metab* 86:2499–2504, 2001 (25). © The Endocrine Society.]

and nitrite and greater exhaled NO (23, 27, 28). Thus, although many other parameters may vary in parallel with the changes in estrogen status that accompany menopause or surgical ovariectomy or the menstrual cycle, there is a strong correlation between endogenous estrogen levels and the capacity for endothelial NO production in women.

The effects of HRT also provide insights into NOS regulation by estrogen. In landmark studies reported in 1994 it was demonstrated that endothelium-dependent vasodilation of the brachial and coronary arteries is enhanced after estrogen replacement therapy in postmenopausal women (29, 30). Estrogen replacement therapy also causes elevations in plasma NO and NO metabolites (31, 32). In addition, there is evidence that forearm blood flow constrictor responses to NOS antagonism are greater after estrogen replacement therapy, whereas responses to glyceryl trinitrate are unchanged, indicating augmented basal NO availability (22). Furthermore, flow-mediated, endothelium-dependent vasodilation of the brachial artery is enhanced similarly by HRT with estrogen and by raloxifene therapy, whereas endothelium-independent responses are not altered (Fig. 3). In addition, the interventions yield comparable increases in plasma NO levels (33). Interestingly, the inclusion of progestin in postmenopausal HRT appears to blunt the effects of estrogen on endothelial NO production (21), perhaps paralleling the findings noted above for variations in endothelial function during the menstrual cycle (24). Premenopausal women receiving estrogen replacement after ovariectomy also have enhanced endothelial function after therapy, and similar effects have been documented in young women receiving oral contraceptives (34). Long-term estrogen administration also improves vascular function in males, and this is at least partially mediated through endothelium-derived NO (35, 36). As such, prolonged exposure to exogenously derived estrogen has positive effects on endothelium-derived NO availability, which parallel those noted with altered endogenous levels and provides further evidence of potential long-term modulation of eNOS function by the hormone in humans.

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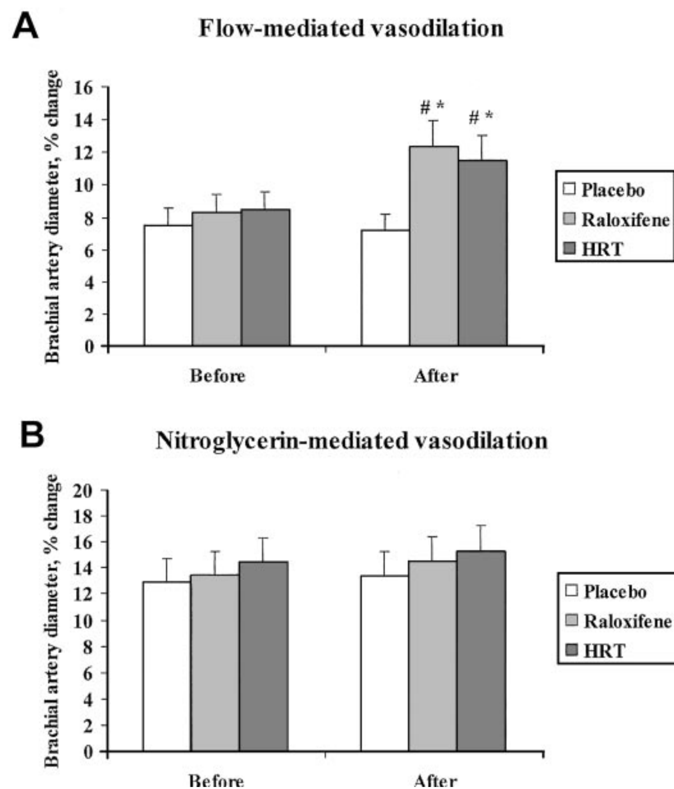

FIG. 3. Flow-mediated vasodilation is increased after chronic HRT or raloxifene treatment in humans. Flow-mediated endothelium-dependent vasodilation (A) and nitroglycerin-mediated endothelium-independent vasodilation (B) were examined in postmenopausal women treated for 6 months with placebo, raloxifene, or HRT. #, $P < 0.05$ *vs.* placebo; *, $P < 0.05$ *vs.* before treatment. Values are mean ± SD, n = 30 per group. [Reprinted with permission from: A. Saitta *et al.*: *Arterioscler Thromb Vasc Biol* 21:1512–1519, 2001 (33).]

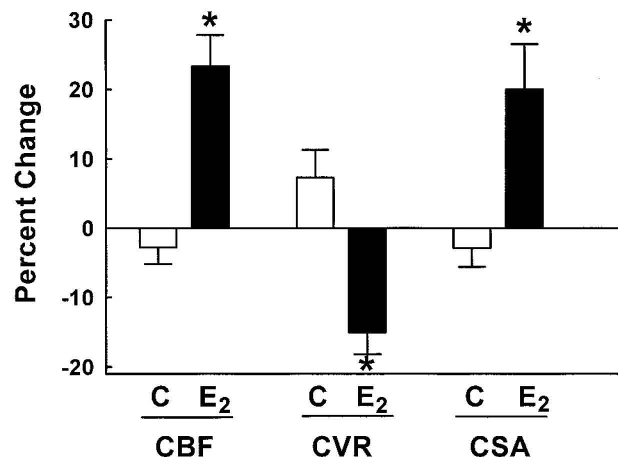

FIG. 4. Ethinyl E_2 causes rapid coronary vasodilation in humans. Changes in coronary blood flow (CBF), coronary vascular resistance (CVR), and epicardial coronary cross-sectional area (CSA) were determined within 15 min of iv ethinyl E_2 (E_2) administration in postmenopausal women. Values are mean ± SEM, n = 11 and 22 subjects for placebo control (C) and ethinyl E_2, respectively, *, $P < 0.05$ *vs.* control. [Reprinted with permission from: S. E. Reis *et al.*: *Circulation* 89:52–60, 1994 (37).]

D. Short-term effects of estrogen on eNOS function

There is also evidence of short-term effects of estrogen on eNOS function in humans. In key early studies reported in 1994, iv ethinyl E_2 was found to cause a direct decrease in coronary vasomotor tone within 15 min in postmenopausal women (Fig. 4). In addition, ethinyl E_2 caused attenuation of vasoconstrictor responses to acetylcholine (Ach) when given 15 min before Ach (37). Experiments with intracoronary E_2 administered in a manner yielding premenopausal levels did not demonstrate changes in basal coronary vasomotor tone, but there was greater vasodilatory response to Ach when E_2 and Ach were given simultaneously. In these studies, E_2 had no effect on vasodilation with sodium nitroprusside, suggesting that the hormone rapidly modifies the availability of endothelium-derived NO (38). Findings were similar when E_2 effects on forearm vascular responses were assessed in postmenopausal women, with the exception that endothelium-independent vasodilation was also enhanced (39). In further studies in the coronary circulation of older women, the capacity of E_2 to cause augmented Ach-induced responses was prevented by NOS antagonism, providing additional evidence that the underlying mechanism involves enhanced bioavailability of NO (40). In contrast to the observations made in postmenopausal women, intracoronary E_2 administration to similarly aged men caused neither a change in basal vasomotor tone nor greater vasodilation with Ach tested 20 min later (41). However, the provision of iv conjugated estrogens 15 min earlier caused greater Ach-induced coronary blood flow and less cold-induced coronary vasoconstriction in men (42, 43). In a similar manner, phytoestrogens cause direct, rapid, NO-dependent dilation of the forearm vasculature of both men and women, which is comparable in degree to that observed with E_2 (Fig. 5). Lower concentrations of phytoestrogen also potentiate the vasodilatory response to Ach. It is important to note that the threshold concentration of phytoestrogen yielding these effects does not greatly exceed those found in East Asian subjects in whom dietary phytoestrogen intake has been suggested to contribute to an extremely low incidence of atherosclerosis and coronary artery disease (44). These cumulative observations indicate that, in addition to the long-term effects of estrogen and estrogen-like compounds on eNOS action, there are short-term effects on the bioavailability of NO. Importantly, these effects may generally be comparable in men and women, and the estrogen formulation is a critical variable.

E. Role of estrogen receptors

Little experimental data are available in humans to reveal the role of ER in vascular function and health in the intact state. However, a young adult male with a disruptive mutation in the ERα gene was found to have premature coronary artery disease at age 31 yr (45, 46). Flow-mediated vasodilation of the patient's brachial artery was also attenuated, whereas vasodilation with sublingual nitroglycerin was intact, suggesting a diminished capacity for endothelial NO production. Interestingly, rapid vasodilation in response to sublingual E_2 was normal (46). These observations may be explained by recent studies demonstrating that either ERα or

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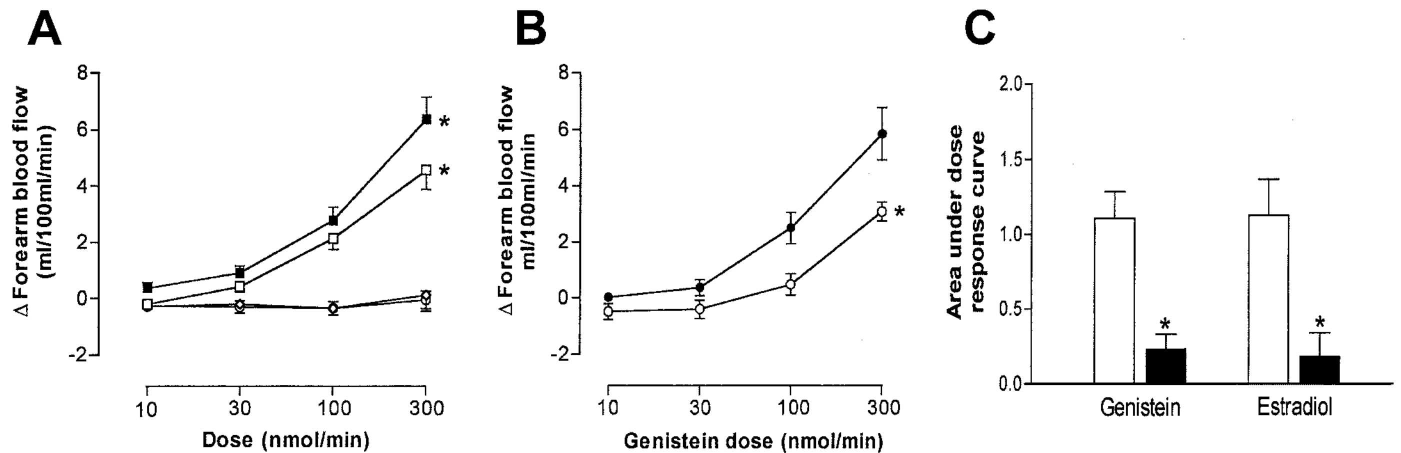

FIG. 5. Both estrogen and the phytoestrogen genistein cause rapid, NO-dependent vasodilation in humans. A, Increase above baseline in forearm blood flow (Δforearm blood flow) after brachial artery infusion of genistein/daidzein vehicle (*open circles*, n = 6), daidzein (*open diamonds*, n = 6), and genistein in men (*closed squares*, n = 9) and premenopausal women (*open squares*, n = 6). *, $P < 0.0001$ compared with vehicle by ANOVA for all doses. B, Increase above baseline in forearm blood flow (Δforearm blood flow) during coinfusion of genistein with saline (*closed circles*) and then after coinfusion with L-NMMA (8 μmol/min, *open circles*) on the same occasion (n = 6). *, $P < 0.002$ for comparison of genistein plus saline *vs.* genistein plus L-NMMA by ANOVA for all doses. C, Vasodilator effects of genistein and 17β-estradiol (AUC) during coinfusion of saline (*open bar*) and L-NMMA (*closed bar*, 8 μmol/min, n = 5). *, $P < 0.01$ for L-NMMA *vs.* saline. [Reprinted with permission from: H.A. Walker *et al.*: *Circulation* 103:258–262, 2001 (44).]

ERβ can mediate rapid eNOS activation in cell culture. These findings are presented below in *Section V.D*.

More direct studies of ER involvement in endothelial NO production have been performed in isolated human mammary artery segments. In precontracted rings obtained from men at the time of coronary artery bypass surgery, E_2 at concentrations of 100 nM or greater caused relaxation that was prevented by either endothelium removal or NOS antagonism. The response to E_2 was also attenuated 71% by the ER antagonist tamoxifen. Although issues relating to the concentration of E_2 required for this *in vitro* response may be raised, these findings suggest that the short-term effects of E_2 on endothelial NO bioavailability in humans are mediated by endothelial ER (47).

III. Estrogen, NO, and Vascular Function and Disease in Animal Models

A. Estrogen, NO, and animal models of vascular disease

The impact of estrogen on the response to vascular injury has been vigorously investigated in rodent models. In studies of carotid artery injury in rats, E_2 attenuated the resulting intimal and medial hypertrophy in females but not in males, and the addition of medroxyprogesterone acetate blocked the protective effects of estrogen (48–50). Estrogen also enhances endothelial recovery after vascular injury, as indicated by greater reendothelialization and greater endothelium-dependent relaxation in previously injured arterial segments. In these studies the augmented recovery was associated with increased capacity for NO production, but it was not determined whether the effect of estrogen is mediated by an up-regulation of eNOS abundance or activity (51, 52). It has also been noted that estrogen reduces neointimal formation in mice deficient in the inducible isoform of NOS to an extent that is similar to that seen in wild-type mice. Since the inducible form of NOS and eNOS are the principal forms of the enzyme found in the vasculature, these observations suggest that eNOS is the primary isoform involved in estrogen-mediated protection (53). An additional explanation is that estrogen may primarily modify vascular injury by promoting angiogenesis (54).

Animal models have also been employed to demonstrate that estrogen has an important impact on the function of atherosclerotic arteries. In pioneering studies by Williams and colleagues (55, 56), both long-term (26 months) and brief (20 min) E_2 treatment caused enhanced Ach-mediated vasodilation in atherosclerotic coronary arteries of female cynomologus monkeys. Similarly, long-term E_2 administration in hypercholesterolemic swine led to the preservation of endothelium-dependent relaxation (57). Perhaps more importantly, there is considerable animal evidence that estrogen modifies the development of atherosclerosis. This includes studies in cholesterol-fed rabbits in which both ethinyl E_2 and conjugated equine estrogen reduced atherosclerosis by 35% in the aortic arch and by 75–80% in the thoracic and abdominal aorta (58). Similarly, E_2 administration to apolipoprotein E (apoE)-deficient mice decreased the atherosclerotic lesion area in both males and ovariectomized females. Importantly, neither the less biologically active 17α-estradiol nor tamoxifen affected lesion development in ovariectomized females in the apoE deficiency paradigm, and the beneficial effects of 17β-estradiol were only partially explained by changes in plasma lipoprotein levels (59). The latter variable has been elegantly controlled in studies of cholesterol-clamped rabbits in which the antiatherogenic properties of estrogen remain apparent. In addition, in the cholesterol-clamped rabbits the capacity of estrogen to prevent cholesterol accumulation in the vascular wall was abolished by balloon catheter injury, suggesting that an intact endothelium is required (Fig. 6) (60,

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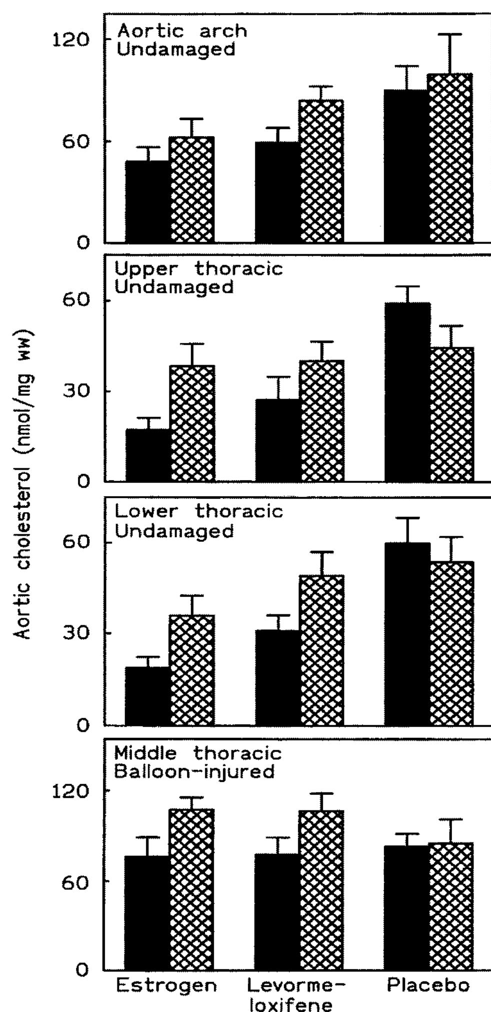

FIG. 6. Estrogen attenuates aortic cholesterol accumulation in cholesterol-clamped rabbits. Studies were performed with or without NOS antagonism with nitro-L-arginine methyl ester (L-NAME). Estrogen had an antiatherogenic effect in the undamaged aorta ($P < 0.05$ *vs.* placebo, no L-NAME by ANOVA). In contrast, this effect was abolished by balloon catheter injury (*bottom panel*). Similar findings, although not as pronounced, were obtained for levormeloxifene ($P < 0.05$ *vs.* placebo, no L-NAME by ANOVA). The atheroprotective effects of estrogen and levormeloxifene were attenuated by simultaneous NOS antagonism ($P < 0.05$ for yes L-NAME *vs.* no L-NAME). Values are mean ± SEM, n = 13–18 per group. *Solid bars,* Without L-NAME; *hatched bars,* with L-NAME. [Reprinted with permission from: P. Holm *et al.*: *J Clin Invest* 100:821–828, 1997 (62).]

61). Furthermore, in the same model NOS inhibition reduced the atheroprotection afforded by estrogen, and comparable observations were made with the SERM levormeloxifene (Fig. 6). Moreover, the antiatherogenic effect of the hormone was preceded by an increase in NO production that was followed by diminished mononuclear-endothelial cell binding. It was noted additionally that the effect of E_2 was to increase the basal release of NO, whereas NO release by Ach and NOS abundance was unaltered (62, 63). These cumulative observations suggest that the antiatherosclerotic characteristics of estrogen involve direct effects on the vascular endothelium, and that these effects may be primarily mediated by enhanced NO production unrelated to genomic effects of the hormone on eNOS expression.

B. Role of ER in animal models of vascular disease

The role of ER in the effects of estrogen on the response to vascular injury has been addressed using nonselective ER antagonism or genetically engineered mice. In models of carotid artery injury in wild-type mice, physiological levels of E_2 inhibited the increases in vascular medial area and smooth muscle cell proliferation that occur with injury (48, 64). Comparable findings were also obtained in ERα-deficient and ERβ-deficient mice (64, 65). Such observations suggested that either receptor isoform may be sufficient to mediate the effects of estrogen or that the effects are through a non-receptor-mediated process or through another unidentified ER. The first of these possibilities was addressed in recent studies of ERα,β double knockout mice. In the double knockouts, E_2 did not prevent the injury-induced increase in vascular medial area. However, VSM cell proliferation continued to be attenuated by the hormone (66). In contrast, in experiments in rats employing nonselective pharmacological ER antagonism, it has been observed that the vasoprotective effects of E_2 are completely inhibited (67). The role of ERα in the antiatherogenic features of estrogen has also been addressed in a recent report. In mice deficient in apoE alone, E_2 reduced the size of atherosclerotic lesions and also their histological complexity, as previously observed (59). Plasma cholesterol levels were decreased by E_2, but the degree of the effects of the hormone on atherogenesis could not be explained solely on the basis of changes in the plasma lipid profile. In contrast, in mice with disrupted apoE and disrupted ERα, E_2 had a more modest effect on lesion size and total plasma cholesterol was unaltered. However, the complexity of the plaques was diminished by E_2 despite the absence of ERα (68). As such, certain mechanisms whereby estrogen alters the vascular response to injury and the progression of atherosclerosis are evidently mediated by ERα or ERβ. However, the bases for multiple other processes are yet to be determined. Furthermore, in contrast to the evidence that strongly implicates an important role for eNOS in the hormonal modulation of atherogenesis, studies are yet to be performed to clarify the specific contribution of eNOS to E_2-induced changes in vascular injury responses.

C. Long-term effects of estrogen on eNOS in animal models

There is considerable evidence from investigations in intact animal models that long-term estrogen exposure enhances the capacity for endothelial NO production. For example, E_2 treatment of rabbits for 4 d caused an increase in endothelium-dependent relaxation in femoral artery rings (69). Using NOS antagonism, it has also been shown that bioassayable endothelium-derived NO is higher in thoracic aortas isolated from female *vs.* male rats (70). The same group of investigators employed ERα knockout mice to demonstrate that there is a significant association between the abundance of ER and the basal release of NO (71). In 1994 it was reported that steady-state eNOS mRNA levels are increased in guinea pig skeletal muscle during pregnancy and after E_2

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treatment. This may have been related to the induction of eNOS in endothelium, but it is notable that the cell specificity of eNOS up-regulation was not determined, and eNOS is expressed in skeletal myocytes (72, 73). The long-term effects of estrogen on eNOS expression have been demonstrated in studies of the uterine circulation in sheep. It was reported in 1996 that after 3 d of systemic E_2 treatment, there was enhanced NO-dependent, endothelium-dependent relaxation, which was related to greater NOS enzymatic activity (74). It has since been shown that eNOS abundance is up-regulated in uterine artery endothelium after both prolonged (days) and brief (2 h) E_2 treatment (75, 76). Although E_2-related effects on eNOS do not occur in certain systemic arteries in the sheep (75), studies in rats have demonstrated dramatic eNOS up-regulation in cerebral microvessels after chronic E_2 treatment. Interestingly, the eNOS responses to E_2 in the cerebral microvessels were similar in female and male rats (77). In addition, studies with knockout mice have demonstrated that the E_2-induced up-regulation in eNOS expression in the cerebral microvessels is mediated by ERα, and that parallel changes occur in the abundance of cyclooxygenase type 1, which is the rate-limiting enzyme in prostacyclin production (Fig. 7) (78). Furthermore, work in porcine coronary arteries has shown that the effects of E_2 are direct and not mediated by changes in blood flow, since isolated ring segments displayed greater NO-dependent relaxation after 18–22 h exposure to E_2 *in vitro* (79).

The capacities to up-regulate eNOS and to reverse endothelial dysfunction are not limited to endogenous forms of estrogen. In studies in sheep, the SERM raloxifene caused more than a 10-fold increase in uterine blood flow over 6 h and a 22% rise in coronary blood flow over 24 h, and these effects were at least partially NO dependent (80). Raloxifene also prevented the decrease in NOS expression which occurs with ovariectomy in rats (81). Furthermore, in rats it has been shown that the long-term administration of the phytoestogen α-zearalenol causes enhanced endothelial NO production, and the effect is fully prevented by the ER antagonist ICI 182,780, indicating that it is mediated by ER binding (82). Chronic exposure to phytoestrogen also improves endothelial dysfunction induced by ovariectomy in rats (83).

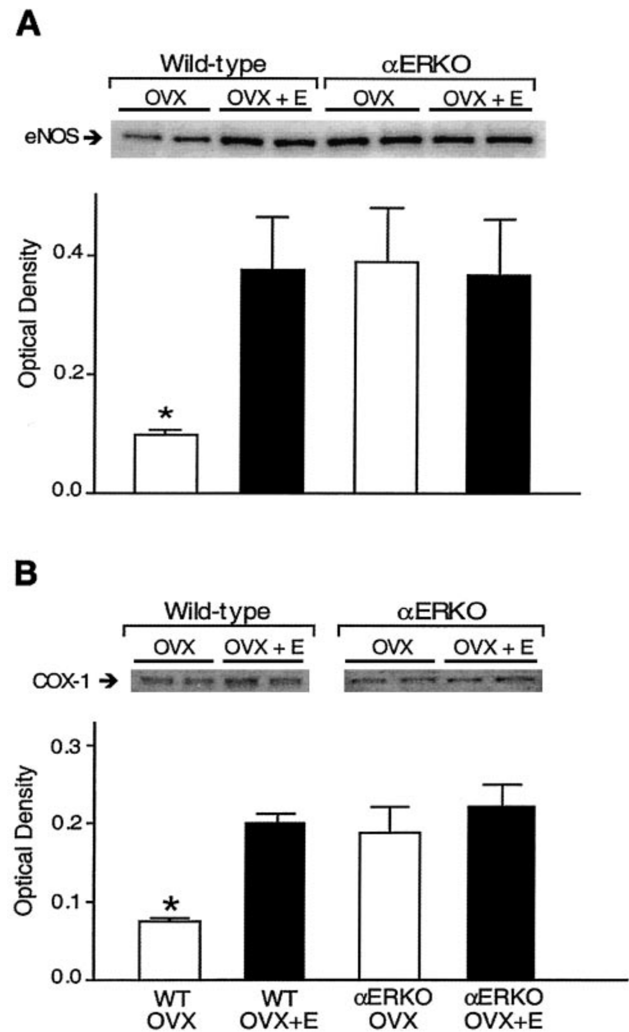

FIG. 7. Chronic estrogen treatment up-regulates eNOS and cyclooxygenase-1 (COX-1) expression in mouse cerebral microvessels by an ERα-dependent process. Levels of eNOS (A) and COX-1 protein (B) were evaluated in cerebral vessels isolated from wild-type ovariectomized (WT OVX), WT OVX with estrogen replacement (WT OVX + E), ERα knockout (αERKO) OVX, and αERKO OVX + E mice. Values are mean ± SEM. For eNOS, n = 4 experiments, each done in duplicate. For COX-1, n = 2 experiments, both done in duplicate. *, $P < 0.05$ *vs.* all other groups. [Reprinted with permission from: G. G. Geary *et al.*: *J Appl Physiol* 91:2391–2399, 2001 (78).]

D. Short-term effects of estrogen on eNOS in animal models

Experiments in isolated segments of rabbit coronary artery, rat superior mesenteric artery, and rat aorta from female animals have demonstrated that physiological levels of E_2 cause rapid, NO-dependent, endothelium-dependent relaxation or rapid, NO-dependent inhibition of platelet aggregation (84–86). However, a considerable number of additional works have alternatively demonstrated NO-independent, endothelium-independent E_2-mediated relaxation (87–89). The varying results related to endothelial NO production may be due to differences in the species or vascular beds under study, as well as varying usage of precontraction. In addition, the estrogen status of the animal is critically important. Collins and colleagues (84) demonstrated that coronary artery rings from oophorectomized, estrogen-treated, and acutely estrogen-withdrawn rabbits display robust endothelium-dependent, NO-dependent relaxation to E_2, whereas rings from oophorectomized, untreated or oophorectomized, estrogen-maintained rabbits display more modest responses (Fig. 8). Interestingly, the SERM raloxifene caused rapid NO-dependent, endothelium-dependent relaxation of coronary artery rings from both male and female rabbits studied under conditions that yielded only NO-independent responses to E_2 (88). In addition, 30-min *in vitro* exposure to phytoestrogen restored NO-mediated relaxation in pulmonary arteries isolated from chronically hypoxic rats (90). Thus, paralleling the findings related to endothelial NO-mediated responses in humans, studies in animals indicate that estrogen causes both long-term up-regulation of eNOS expression and rapid enhancement of eNOS enzyme activation.

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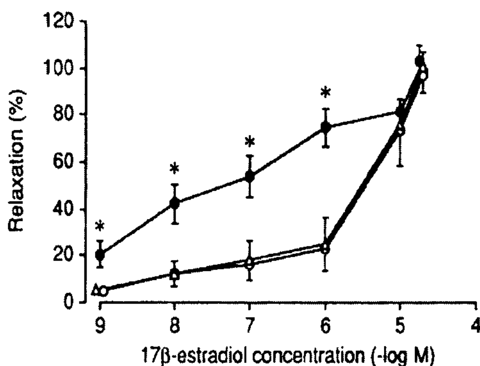

FIG. 8. Estrogen causes rapid, NO-dependent, endothelium-dependent vasodilation in rabbit coronary artery rings after estrogen withdrawal. The effects of E_2 (10^{-9} to 5×10^{-5} M) on contractile responses to prostaglandin $F_{2\alpha}$ (3×10^{-5} M) were examined in coronary arterial rings from oophorectomized, untreated (sham-operated) female rabbits (n = 5; *open circles*); oophorectomized, estrogen-maintained female rabbits (n = 5; *open triangles*); and female rabbits oophorectomized, estrogen replaced, and then deprived for 48 h (n = 6; *closed circles*). Values are mean ± SEM; *, $P < 0.01$ *vs.* untreated and estrogen-maintained groups (ANOVA). [Reprinted with permission from P. Collins *et al*: *Circulation* 90:1964–1968, 1994 (84).]

IV. Genomic Mechanisms of eNOS Regulation by Estrogen

A. Genomic regulation of eNOS expression by estrogen

The mechanisms by which long-term E_2 exposure increases eNOS abundance have been delineated in experiments in cultured endothelial cells. Studies reported in 1995 demonstrated that the capacity for NO production by human aortic endothelium is greater after 8 h or more of E_2 treatment at concentrations of approximately 10 nM or greater. eNOS protein levels were also increased (91). Additional work in human umbilical vein endothelial cells (HUVEC) demonstrated that both tamoxifen and ICI 182,780 prevent the increase in eNOS. Immunocytochemical analysis revealed ERα expression in early-passage HUVEC and diminished levels of expression with additional passage (92). Further studies in ovine endothelial cells demonstrated that E_2 induces increases in NOS enzymatic activity and eNOS protein abundance at concentrations of 0.1 nM E_2 or greater, and that these changes are accompanied by increases in eNOS mRNA levels. Transient transfection assays with a specific estrogen-responsive reporter system also demonstrated that endothelial ERs are capable of estrogen-induced transcriptional transactivation (93). Similar observations have also been made in human osteoblast-like cells, consistent with other data suggesting that NO may mediate estrogen action in bone (94). eNOS expression in neonatal and adult cardiac myocytes is also up-regulated by E_2 in an ER-dependent manner, potentially explaining some of the cardioprotective effects of the hormone (95). In addition, estrogen treatment caused an increase in eNOS mRNA abundance in LNCaP human prostate carcinoma cells within 2–4 h of exposure. Interestingly, estrone sulfate, the most abundant circulating estrogen that may serve as a prehormone for the terminal biologically active estrogen E_2 in men, also up-regulated eNOS mRNA in LNCap cells, and the response required 48 h, suggesting conversion to E_2 (96).

B. Basis for eNOS up-regulation by estrogen

Work in human endothelial EA.hy 926 cells indicates that E_2 or the more stable 17α-ethinyl E_2 enhances eNOS protein and mRNA abundance in the absence of changes in the stability of eNOS mRNA. Nuclear run-on assays showed that the increase in eNOS mRNA abundance is related to greater eNOS gene transcription. The impact of 17α-ethinyl E_2 on eNOS gene transcription was further evaluated in transient transfection studies using a 1.6-kb human eNOS promoter fragment, which lacks classical estrogen response elements (EREs). Promoter activity was enhanced to a degree comparable to the increase in eNOS mRNA levels, and EMSAs suggested greater DNA-protein complex formation involving a putative Sp1 binding element after estrogen treatment. However, immunodepletion or supershift analyses were not performed to specifically identify the nuclear protein that is involved (97). The role of ER subtypes in eNOS gene activation by E_2 has been elucidated in neonatal rat cardiac myocytes. eNOS up-regulation by E_2 was fully prevented by the ERβ-specific antagonist RR-tetrahydrochrysene (THC), indicating a primary role for that subtype (Fig. 9) (98). Further work in cultured endothelial cells indicated that eNOS down-regulation by TNFα, which is due to enhanced eNOS mRNA degradation, is prevented by E_2, and that this mechanism is also ER dependent (99). Thus, there is transcriptional regulation of eNOS expression by E_2 through mechanisms that do not involve classical ERE-mediated processes, and additional nontranscriptional mechanisms may be important under certain conditions.

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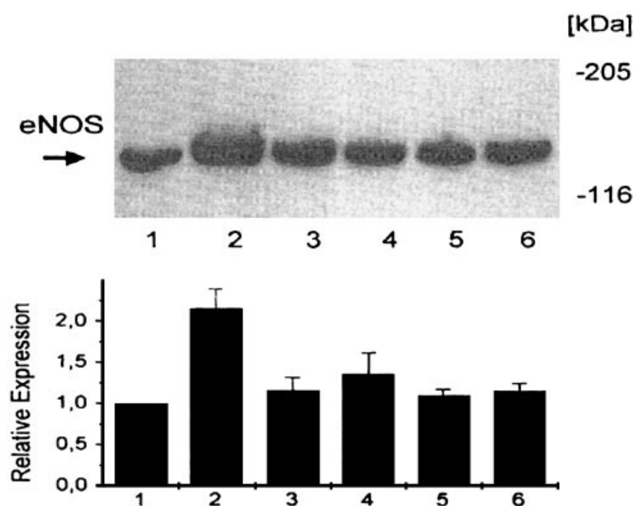

FIG. 9. ERβ mediates E_2-stimulated eNOS expression in neonatal rat cardiac myocytes. Neonatal rat cardiac myocytes were cultured in serum-free defined medium in the absence or presence of E_2 (10^{-9} M). A representative immunoblot is shown in the *upper panel*, and cumulative results for three independent experiments are displayed in the *lower panel*. The level of expression of eNOS protein is low in the absence of E_2 (control, lane 1), and it increases markedly in cells exposed to E_2 for 24 h (lane 2). Coincubation with the ERβ-selective antagonist RR-THC (10^{-5} M) completely inhibited E_2-induced expression of eNOS (lane 3). Cotreatment with ICI 182,780 (10^{-8} M) for 24 h also inhibited the estrogen-mediated increase in eNOS expression (lane 4). RR-THC (lane 5) and ICI 182,780 (lane 6) alone had no effect on protein expression. Values are mean ± SEM. [Reprinted with permission from S. Nuedling *et al*.: *FEBS Lett* 502:103–108, 2001 (98).]

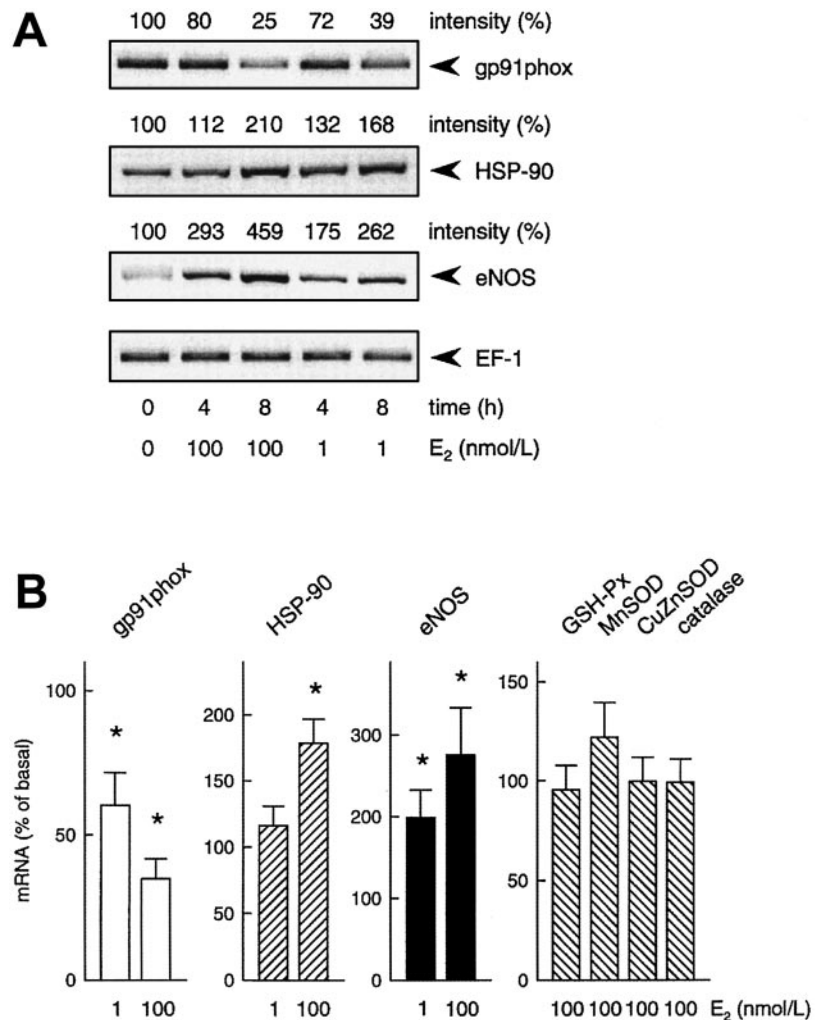

FIG. 10. Estrogen inhibits NADPH oxidase expression in endothelial cells. Time- and concentration-dependent effect of cyclodextrin-encapsulated E_2 on NADPH oxidase subunit gp91phox, HSP-90, eNOS, glutathione peroxidase (GSH-Px), SOD, and catalase mRNA expression were evaluated in cultured HUVEC. A, Typical RT-PCR analyses with the relative intensities (%), as judged by densitometry, are indicated at the *top*. The cells were incubated with E_2 (1 and 100 nmol/liter) for the indicated periods. Elongation factor-1 (EF-1) RT-PCR was performed as a control. Results are representative of two separate experiments with different batches of cells. B, Summary data are shown for HUVEC incubated for 8 h with E_2 (1 and 100 nmol/liter), calculated as percentage of expression in the presence of cyclodextrin alone (basal). Values are mean ± SEM, n = 4–8, *, $P < 0.05$ *vs.* basal. [Reprinted with permission from A. J. Wagner *et al.*: *FASEB J* 15:2121–2130, 2001 (104).]

C. Other genomic mechanisms modifying eNOS function

The eNOS enzyme is localized to endothelial cell caveolae, which are lipid-ordered domains on the plasma membrane that compartmentalize signal transduction molecules. Within caveolae, there are multiple mechanisms that modify eNOS activity, including protein-protein interaction between the enzyme and the structural and regulatory protein caveolin, which leads to attenuated eNOS activity (100). Studies in rat cerebral arterioles have demonstrated that chronic estrogen depletion (ovariectomy) causes a complete loss of Ach-induced, endothelium-dependent relaxation, and this defect is associated with not only a decrease in eNOS expression, but also an up-regulation in caveolin abundance. In addition, these changes were mimicked by simultaneous interventions that decreased eNOS and increased caveolin abundance in animals with intact ovaries, and estrogen replacement normalized the Ach responses in the ovariectomized females at the same time that it up-regulated eNOS and down-regulated caveolin (101, 102). In recent work in bovine aortic endothelial cells, eNOS expression was increased after 24 h of E_2 exposure, as seen in the intact animal; however, caveolin expression rose after 48 h of treatment. In addition, the increase in caveolin expression was prevented by ER antagonism. After E_2, NO production was elevated at 24 h but not at 48 h, which would be consistent with greater caveolin-related attenuation in eNOS activity at the later time point (103). These cumulative findings suggest that the effects of E_2 on caveolin expression may have an important impact on eNOS function, but they also emphasize that factors such as the identity of the endothelial cell target, the cellular and tissue context of the experiment, and the timing of hormone exposure must be considered.

In addition to the relative level of eNOS enzymatic activity, the ultimate bioavailability of NO as a signaling molecule is modified by the degree of scavenging by reactive oxygen species such as superoxide. In human endothelial cells, superoxide levels are dictated by the production of the radical by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and its degradation by superoxide dismutase (SOD). In recent studies in HUVEC, E_2 caused a 60% decrease in the expression of the protein and mRNA for the NADPH oxidase subunit gp91phox. There was also a less dramatic, but significant, E_2-induced decline in mRNA abundance for p22phox, which, together with gp91phox, constitute the membrane-bound cytochrome b558 portion of the NADPH oxidase complex. The two cytosolic activating fac-

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tors of the complex, p47phox and p67phox, were also down-regulated by E_2, and the net effect was an approximately 50% diminution in the capacity to produce superoxide. In addition, the effects of E_2 on NADPH oxidase subunit expression and superoxide production were prevented by ER antagonism. Heat shock protein 90 (HSP90) and eNOS expression were also up-regulated by E_2. In contrast, levels of expression of the reactive oxygen species-metabolizing enzymes glutathione-peroxidase, copper/zinc SOD, manganese SOD, and catalase were not affected by E_2 (Fig. 10 and Ref. 104). Thus, in addition to up-regulating eNOS abundance, E_2 inhibits the expression of NADPH oxidase in endothelial cells, thereby potentially increasing the bioavailability of NO through more than one process.

V. Nongenomic Regulation of eNOS by Estrogen

A. Estrogen and eNOS activation

The bases for rapid effects of E_2 on eNOS function have been elucidated in studies of cultured endothelial cells. It was shown initially that estrogen acutely (5–10 min) stimulates eNOS activity, that the response is attenuated by ER antagonism but not by inhibition of gene transcription, and that ERα is expressed in cultured endothelial cells (Fig. 11 and Refs. 105–107). It has also been shown that the overexpression of ERα in endothelial cells causes enhancement of the acute response to E_2 that is blocked by ER antagonism and specific to E_2 *vs.* other agonists (Fig. 12). In addition, the enhanced response is dependent on the ERα hormone binding domain. In COS-7 cells, which do not constitutively express ER or eNOS, the acute stimulation of eNOS by E_2 can be demonstrated after the cotransfection of ERα and eNOS cDNAs (107). Raloxifene also activates endothelial cell eNOS within 10 min, and the process is ER dependent since it is abolished by ICI 182,780 (108). eNOS activation by E_2 has also been observed in human bronchiolar epithelial cells, which also constitutively express the enzyme and ERα, suggesting that mechanisms similar to those delineated in the vasculature may play a role in hormonal modulation of airway function (109).

The signal transduction mechanisms by which E_2 activates eNOS have also been delineated. In experiments performed in ovine endothelial cells, tyrosine kinase inhibition completely prevented the response to E_2. In addition, the specific MEK inhibitor PD98059 also fully negated eNOS stimulation by E_2. Furthermore, E_2 caused a rapid increase in MAPK activity, and this effect was prevented by both tamoxifen and ICI 182,780 (Fig. 13). These data indicate that the acute stimulation of eNOS by E_2 and ERα entails the activation of tyrosine kinase/MAPK (107). There is also evidence of a role

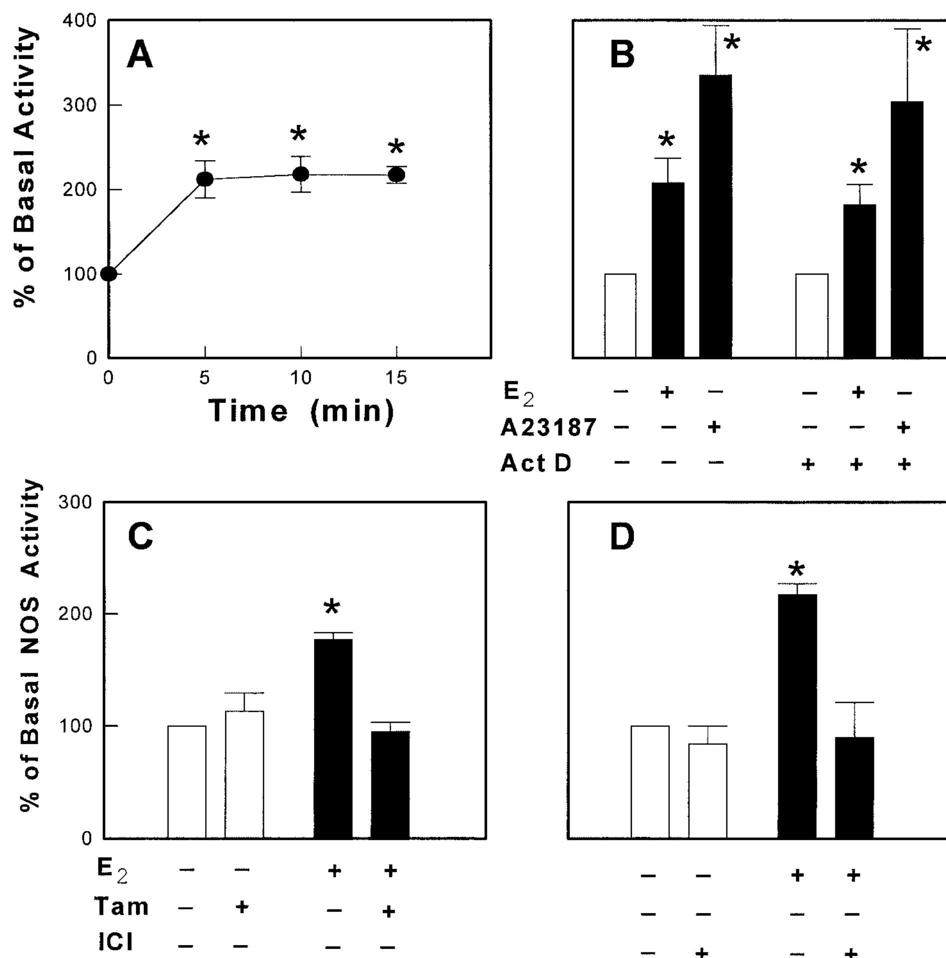

FIG. 11. Estrogen causes rapid activation of eNOS in endothelial cells. A, Effect of E_2 on eNOS activity in intact cells. ^{3}H-L-Arginine conversion to ^{3}H-L-citrulline was measured over 5–15 min in the presence of 10^{-8} M E_2. B, Effect of actinomycin D (Act D) on the rapid activation of eNOS. After 120 min preincubation in the absence or presence of 25 μg/ml Act D, 15-min incubations were done with or without continued Act D and either 10^{-8} M E_2 or the calcium ionophore A23187 (10^{-5} M). C, Effect of tamoxifen (Tam) on E_2-stimulated eNOS activity. Fifteen-minute incubations were performed in the absence or presence of 10^{-8} M E_2, with or without 10^{-6} Tam added simultaneously. Partial inhibition (50–70%) was also noted with 10^{-8} M Tam (13). D, Effect of ICI 182,780 on E_2-stimulated eNOS activity. Fifteen-minute incubations were performed in the absence or presence of 10^{-8} M E_2, with or without 10^{-5} M ICI 182,780 added simultaneously. Full inhibition was also observed with 10^{-6} M ICI 182,780 (13). Values are mean ± SEM; n = 4–6, *, $P < 0.05$ *vs.* basal. [Reprinted with permission from Z. Chen *et al.*: *J Clin Invest* 103:401–406, 1999 (107).]

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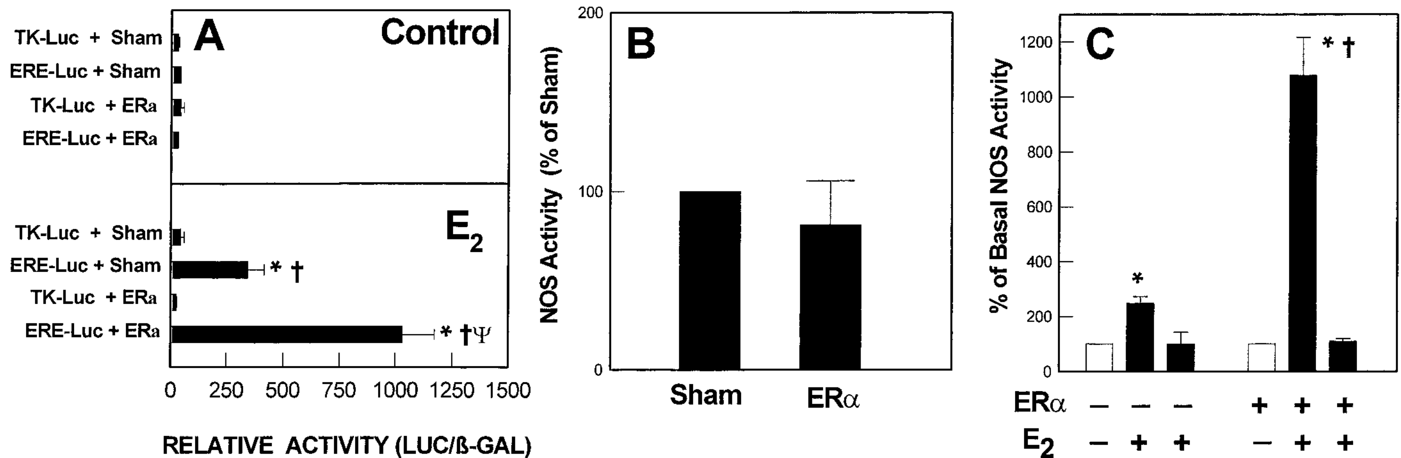

FIG. 12. Effect of ERα overexpression on transcriptional transactivation and on basal and estrogen-stimulated eNOS activity. A, Effect of ERα overexpression on ERE-mediated gene transcription in endothelial cells. Transient transfections were performed with either the estrogen-responsive reporter plasmid ERE-luciferase (Luc) or the control plasmid thymidine kinase luciferase (TK-Luc), in combination with either sham plasmid or ERα cDNA. Reporter activity was then determined in control cells (*upper panel*) and cells exposed to 10^{-8} M E_2 for 48 h (*lower panel*). Reporter activity is expressed as luciferase activity/β galactosidase activity (LUC/β-gal). *, $P < 0.05$ *vs.* TK-Luc; †, $P < 0.05$ *vs.* control cells; Ψ, $P < 0.05$ *vs.* sham. Similar findings were obtained in three independent experiments. B, Effect of ERα overexpression on basal eNOS activity in endothelial cells. Cells were transfected with sham plasmid or ERα cDNA and placed under estrogen-free conditions, and 72 h later ^{3}H-L-arginine conversion to ^{3}H-L-citrulline was measured over 15 min in nonstimulated, intact cells. C, Effect of ERα overexpression on acute eNOS activation by E_2. Endothelial cells were transiently transfected with sham plasmid or ERα cDNA as in panel B, and 72 h later ^{3}H-L-arginine conversion to ^{3}H-L-citrulline was measured in intact cells over 15 min in the absence or presence of 10^{-8} M E_2, with or without 10^{-5} M ICI 182,780 added simultaneously. Values are mean ± SEM; n = 4–6. *, $P < 0.05$ *vs.* basal; †, $P < 0.05$ *vs.* sham. [Reprinted with permission from Z. Chen *et al.*: *J Clin Invest* 103:401–406, 1999 (107).]

for the recruitment of the PI3-kinase-protein kinase B/Akt pathway. In studies of human endothelial cells, E_2-induced eNOS activation was prevented by the PI3-kinase inhibitor, LY294002, and E_2 caused rapid Akt phosphorylation on serine 473. E_2 also caused eNOS phosphorylation on serine 1177, which is a critical residue for eNOS activation and modification of the sensitivity to cellular calcium levels (Fig. 14). In addition, expression of a kinase-deficient, dominant-negative Akt prevented E_2-stimulated NO production (110–112). Additional work suggests that E_2-induced PI3-kinase/Akt signaling is mediated exclusively by ERα and not ERβ, and that it entails direct interaction between ERα and the p85 subunit of PI3-kinase (111, 112). Recent examinations of the signal transduction events by which raloxifene stimulates eNOS have also implicated involvement of MAPK and PI3-kinase/Akt signaling that is mediated by ERα (113).

The role of calcium in E_2-induced activation of eNOS has also been ascertained. In the two initial reports of eNOS stimulation in cultured endothelial cells, it was unclear whether calcium is involved. It was noted in studies in ovine endothelial cells that the removal of extracellular calcium completely prevented the response (105), yet changes in cytosolic calcium levels were not detected in parallel with eNOS activation by E_2 in HUVEC (106). Later work in bovine aortic endothelial cells and human arterial endothelial cells indicated that there is a transient rise in intracellular calcium concentration upon E_2 exposure (114, 115). Both E_2-induced Akt activation and eNOS translocation from the plasma membrane upon E_2 stimulation have been found to be calcium dependent (112, 114). These cumulative findings suggest that E_2 activation of eNOS is a calcium-dependent process, but global increases in intracellular calcium levels may not be required.

Additional mechanisms of eNOS regulation in addition to those involving protein kinase-mediated phosphorylation events and changes in calcium homeostasis have also been investigated. In studies of HUVEC, inhibitors of HSP90 function prevented E_2-stimulated NO release and cGMP production. E_2 also was found to induce HSP90-eNOS association, and this event was prevented by ER antagonism, providing further evidence for a role for HSP90 in eNOS activation by E_2 (116). Thus, the short-term effects of estrogen on eNOS that are central to cardiovascular physiology are mediated by ER functioning in a novel, nongenomic manner, and multiple signal transduction events are likely to be involved.

B. Localization of eNOS activation by estrogen to plasma membrane

Studies employing immunoidentification or conjugated estrogen have indicated that a subpopulation of ER may be associated with the cell surface in certain cell types (117, 118). In fact, immunofluorescence experiments suggest that the ligand binding domain of the ERα may actually reside on the extracellular face of the plasma membrane (119). As mentioned above, there is strong evidence that eNOS is targeted to the endothelial plasma membrane, particularly to caveolae, which are specialized, cholesterol-rich lipid-ordered domains that compartmentalize signal transduction. eNOS trafficking to caveolae is dependent upon the myristoylation and palmitoylation of the protein (100, 120–122). Initial determinations of the subcellular site of interaction between ERα and

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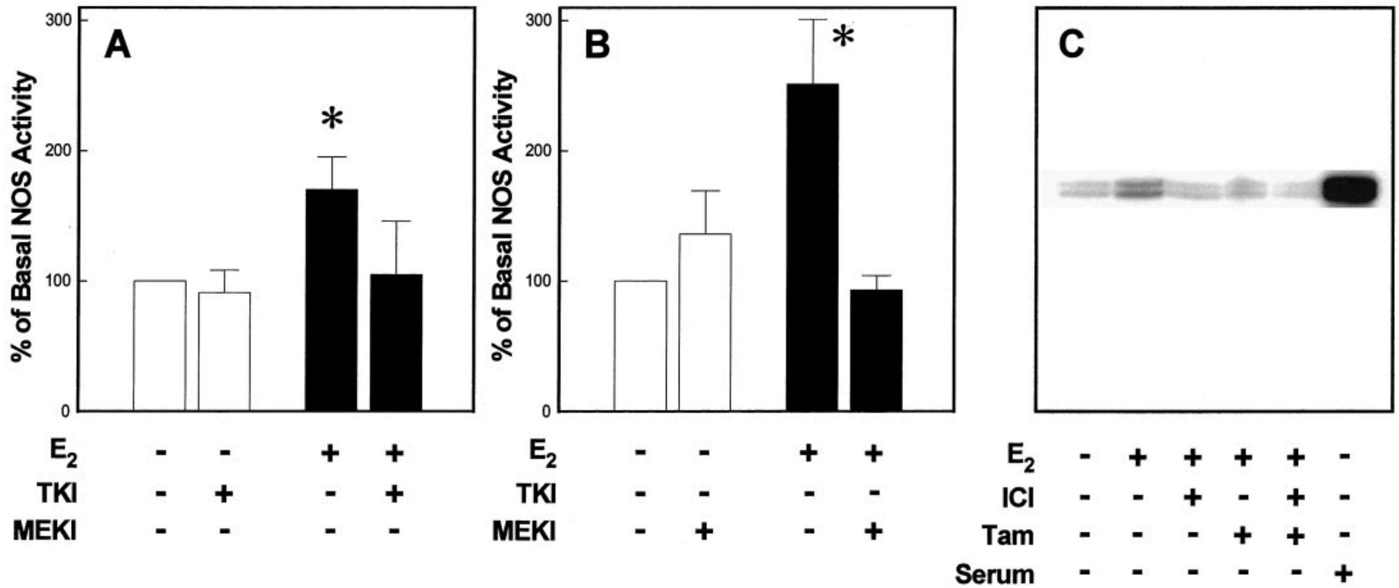

FIG. 13. Role of tyrosine kinase-MAPK signaling pathway in eNOS activation by estrogen. A, Role of tyrosine kinase in acute eNOS activation by E_2. ^{3}H-L-Arginine conversion to ^{3}H-L-citrulline was measured over 15 min in intact endothelial cells in the absence or presence of 10^{-8} M E_2, with or without treatment with the tyrosine kinase inhibitor (TKI) genistein (50 μM). Identical findings were obtained with herbimycin A. B, Role of MEK in acute eNOS activation by E_2. eNOS activity was measured in the absence or presence of 10^{-8} M E_2, with or without treatment with the MEK inhibitor (MEKI) PD98059 (50 μM). Values in panels A and B are mean ± SEM, n = 4–6; *, $P < 0.05$ *vs.* basal. C, Effect of E_2 on MAPK activity in endothelial cells. Cells were treated for 5 min with 10^{-8} M E_2 in the absence or presence of 10^{-5} M tamoxifen (Tam) or 10^{-5} M ICI 182,780, or with serum to serve as a positive control. Endogenous kinase was immunoprecipitated with anti-ERK2 antibody, and protein kinase activity was measured by evaluating the capacity to phosphorylate myelin basic protein. Quantification by phosphoimager yielded values of 1, 2.6, 1, 1, 1, and 7.5, respectively, relative to untreated cells. Results shown are representative of five independent experiments. [Reprinted with permission from Z. Chen *et al.*: *J Clin Invest* 103:401–406, 1999 (107).]

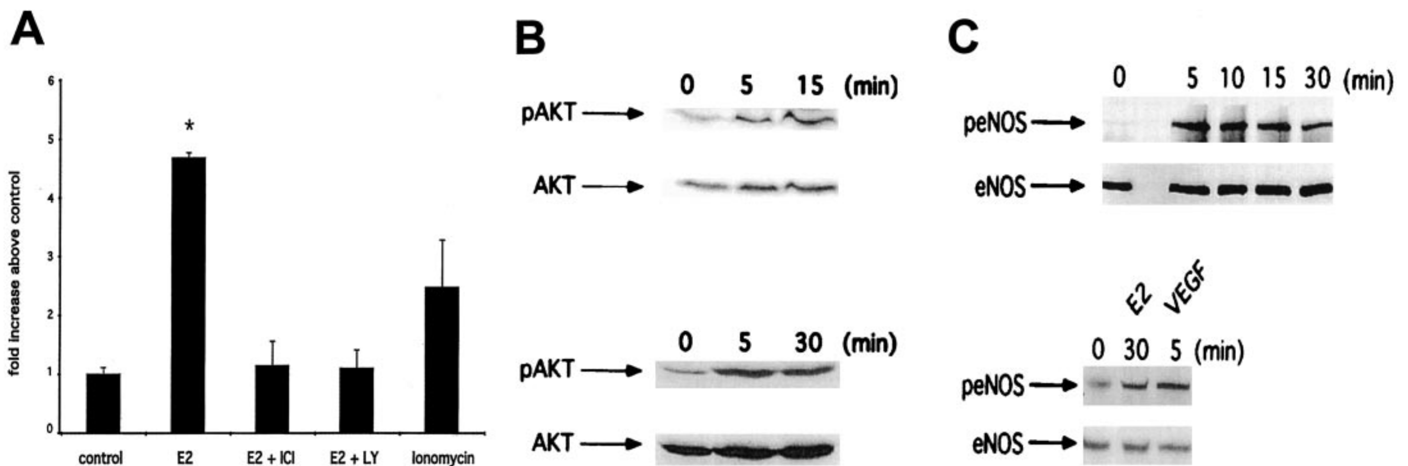

FIG. 14. PI3-kinase/Akt signaling plays a role in eNOS activation by estrogen. A, Effect of a PI3-kinase inhibitor on estrogen-induced NO release. Confluent EA.hy.926 monolayers were incubated with LY294002 (10 μM), ICI 182,780 (10 μM), or vehicle control for 1 h at 37 C. Monolayers were then stimulated with vehicle, E_2 (10 ng/ml), or ionomycin (2 μM), and the medium was collected and NO_2 content was determined by chemiluminescence. Data are mean ± SD. Results are representative of three separate experiments. *, $P < 0.01$ for E_2 *vs.* control, E_2+ICI, or E_2+LY. B, Effect of E_2 on Akt phosphorylation. Confluent monolayers of either HUVECs (*upper panel*) or EA.hy.926 cells (*lower panel*) were incubated in the presence or absence of E_2 (10 ng/ml) for the indicated time periods at 37 C. Lysates were subjected to SDS-PAGE, transferred to nitrocellulose, probed with antiphospho-Akt antibody, and reprobed with anti-Akt antibody. Densitometric analysis of HUVEC pAkt demonstrated a 3.2-fold increase at 15 min, relative to control. C, Effect of E_2 on eNOS phosphorylation. Confluent EA.hy.926 monolayers were stimulated with E_2 (10 ng/ml) for the indicated time periods (*upper panel*) or with E_2 (10 ng/ml, 30 min) or VEGF (50 ng/ml, 5 min; *lower panel*), and eNOS was immunoprecipitated. Immunoblots were probed with antiphospho-eNOS antibody and reprobed with anti-eNOS antibody. Results are representative of three separate experiments. VEGF, Vascular endothelial growth factor; pAKT, phosphorylated AKT; peNOS, phosphorylated eNOS. [Reprinted with permission from M. P. Haynes *et al.*: *Circ Res* 87:677–682, 2000 (110).]

eNOS involved studies of plasma membranes isolated from ovine endothelial cells, in which eNOS stimulation was evaluated by measuring ^{3}H-L-arginine conversion to ^{3}H-L-citrulline (123). In the absence of added calcium, calmodulin, or cofactors, 10^{-8}M E_2 (15 min) caused a 92% increase in NOS activity compared with basal levels, whereas 17α-estradiol

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had no effect. Maximal NOS activity was assessed by replacing E_2 with a mixture of calcium, calmodulin, and cofactors, yielding a 170% rise in activity compared with no additives; E_2 (10^{-8}M) did not enhance this activity (Fig. 15A). These observations indicated that all of the signal transduction machinery necessary for eNOS stimulation by E_2 is associated with the plasma membrane. Since E_2 alone activated the enzyme to approximately half of maximal levels, the response is quite robust. Time course experiments with E_2 alone further revealed a progressive, linear increase in NOS activity during the first 30 min of incubation, followed by a plateau (Fig. 15B). This contrasted with NOS activity with added calcium, calmodulin, and cofactors, which displayed linearity with time for at least 120 min (123). These data suggest that the availability of one or more of these molecules is limited in the isolated membranes; in contrast, in intact cells there are most likely mechanisms that replenish these factors in the locale of the plasma membrane.

The role of ER in E_2 activation of plasma membrane eNOS was examined using the ER antagonist ICI 182,780, and the agent was found to completely prevent E_2-stimulated NOS activity (Fig. 15C). In addition, antibody to the ligand binding domain of ERα (TE111) blocked E_2-stimulated NOS activation, whereas unrelated IgG had no effect (Fig. 15D). These observations indicated that the response to E_2 is mediated by an ER or ER-like protein associated with the endothelial cell plasma membrane. Immunoidentification experiments, which compared the plasma membrane ER with cytosolic and nuclear ER using antibodies directed against three different ERα epitopes, were then performed. Antibodies directed against amino acids 495–595 (AER320), 302–553 (TE111), or 120–170 of human ERα (AER304) all detected a single 67-kDa protein species in endothelial cell plasma membranes that was identical in size to the protein detected in nuclear and cytosolic fractions (Fig. 16A). Confirmatory studies were done involving the localization of epitope-tagged ERα (ERα-myc) transiently transfected into COS-7 cells. Whereas antibody to ERα revealed no signal in sham-

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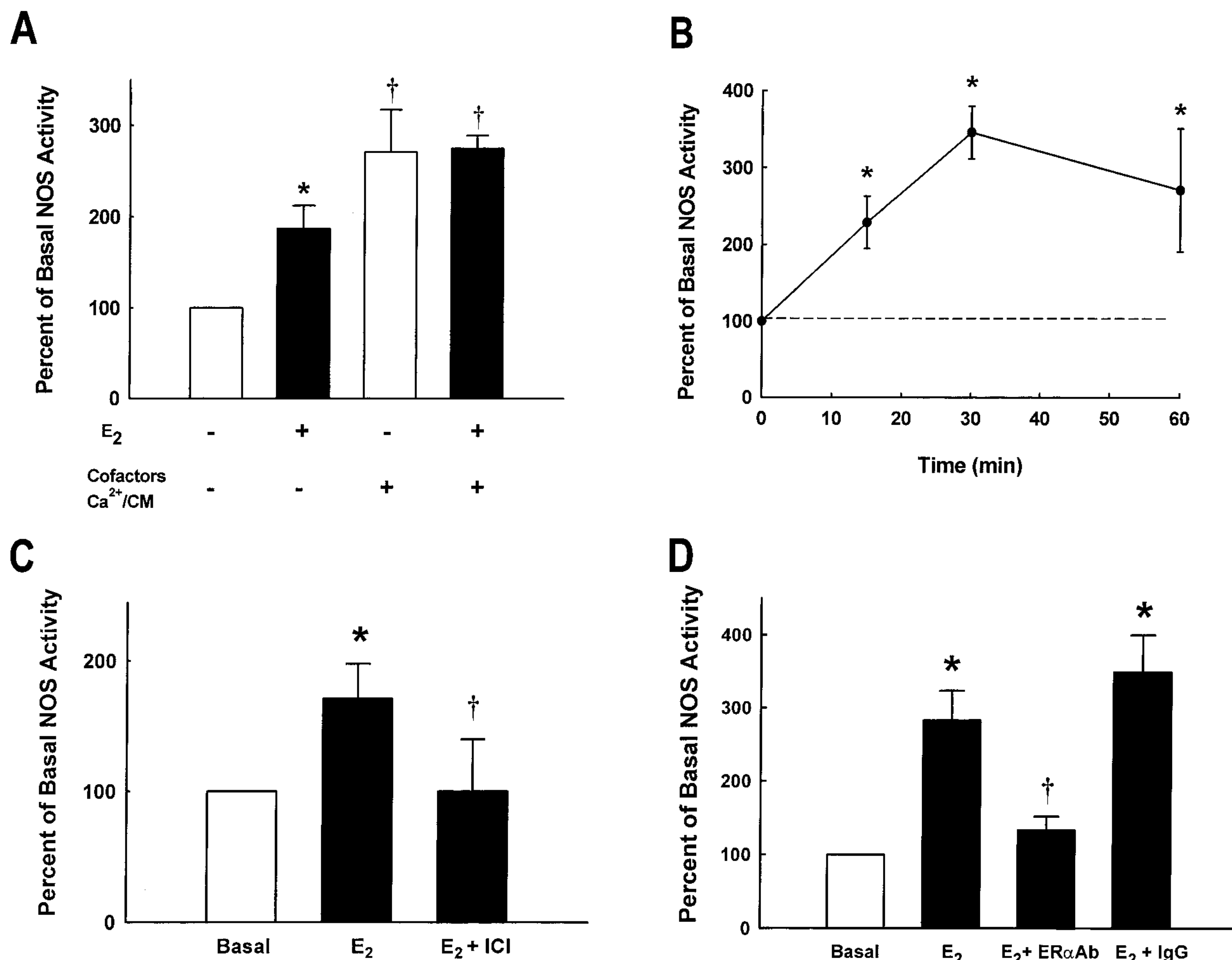

FIG. 15. Estrogen activates eNOS in isolated endothelial cell plasma membranes. A, Effect of E_2 on NOS activity. ^{3}H-L-Arginine conversion to ^{3}H-L-citrulline was measured in isolated plasma membranes in the absence (basal) or presence of 10^{-8} M E_2, and in the absence or presence of exogenous eNOS cofactors, Ca^{2+} and calmodulin (CM). B, Time course of the effect of E_2 on NOS activity. Incubations were performed without added cofactors, Ca^{2+} and CM, in the absence (basal) or presence of 10^{-8} M E_2 for 15–60 min. C, Effect of ER antagonism on response to E_2. NOS activation was measured without added cofactors, Ca^{2+} or CM, in the absence (basal) or presence of 10^{-8} M E_2, with or without 10^{-5} M ICI 182,780 added. D, Effect of ERα antibody (ERαAb) on response to E_2. Incubations were performed without added cofactors, Ca^{2+} or CM, in the absence (basal) or presence of 10^{-8} M E_2 with or without antibody to ERα (TE111) or unrelated IgG added. Values are mean ± SEM, n = 4–6, *, $P < 0.05$ *vs.* basal; †, $P < 0.05$ *vs.* E_2 alone. [Reprinted with permission from K. L. Chambliss *et al.*: *Circ Res* 87:E44–E52, 2000 (123).]

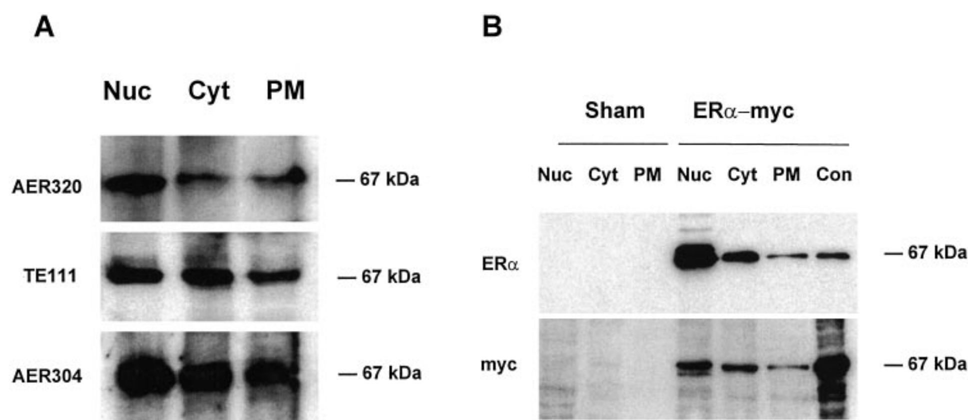

FIG. 16. Characterization of plasma membrane-associated ER. A, Immunoblot analysis for ERα in endothelial cell nucleus (Nuc), cytosol (Cyt), and plasma membrane (PM). The monoclonal antibodies employed were directed against amino acids 495–595 (AER320), 302–553 (TE111), or 120–170 (AER304) of human ERα. B, Targeting of epitope-tagged ERα to plasma membranes. After transient transfection of COS-7 cells with myc-tagged ERα, immunoblot analysis was performed for ERα and myc on cell fractions. Whole-cell lysate from a prior COS-7 cell transfection was used as a positive control (Con). Results are representative of three independent experiments. [Reprinted with permission from K. L. Chambliss *et al.*: *Circ Res* 87:E44–E52, 2000 (123).]

transfected cells, there was positive signal for ERα of comparable size in nucleus, cytosol, and plasma membrane from cells transfected with ERα-myc. In parallel, immunoblot analysis with antibody to the myc tag revealed no signal in sham-transfected cells, but a protein of similar size was detected in the nucleus, cytosol, and plasma membranes of cells expressing the tagged receptor (Fig. 16B). These cumulative findings indicate that E_2-stimulated eNOS activity is mediated by a subpopulation of ERα that is associated with the endothelial plasma membrane (123). Independent investigations demonstrating eNOS activation by membrane-impermeant E_2 conjugated to BSA have provided additional evidence of eNOS signaling by cell surface ER (110, 115).

The importance of plasma membrane colocalization for ERα-stimulated eNOS activity was evaluated in a reconstitution paradigm in COS-7 cells. Plasma membranes from cells expressing eNOS and ERα displayed rapid ER-mediated NOS stimulation, whereas membranes from cells expressing eNOS alone or ERα plus myristoylation-deficient mutant eNOS were insensitive. In fact, membranes from cells expressing myristoylation-deficient mutant eNOS and ERα displayed a decline in NOS activity with E_2 that was partially reversed by ICI 182,780. A myristoylation-deficient mutant eNOS is minimally directed to the plasma membrane but unaltered in enzymatic activity (120), these findings indicate that both normal plasma membrane targeting of eNOS and localization of ERα to that site are required for eNOS activation by E_2 (123).

C. Localization of ERα-eNOS interaction to caveolae

Since plasma membrane eNOS is exclusively localized to caveolae (120), further experiments were done to determine whether ERα protein is also associated with this subfraction of endothelial cell plasma membranes. ERα protein was detected in caveolae, and it was also detected, but to a lesser extent, in the noncaveolae fraction of the plasma membrane (Fig. 17A). Experiments were then performed to evaluate the capacity of E_2 to activate eNOS in isolated caveolae and noncaveolae fractions. In the absence of added calcium, calmodulin, or cofactors, there was no measurable NOS activity in the noncaveolae fraction under basal conditions or with E_2 added. Basal NOS activity was also below detection limits in caveolae membranes. However, 10^{-8} M E_2 caused robust activation of NOS in caveolae membranes, and this effect was prevented by ICI 182,780 (Fig. 17B). These data strongly indicate that ERα and all of the additional molecular machinery necessary for E_2-mediated activation of eNOS exist in a functional signaling module in endothelial caveolae. Since ERα was found in both caveolae and noncaveolae fractions and eNOS is solely in caveolae (120), the specificity of ERα coupling to eNOS to caveolae is evidently due to the localization of the effector, and not the receptor, in the microdomain. Furthermore, the effect of E_2 on eNOS in caveolae is prevented by calcium chelation (123), suggesting that ERα activation in caveolae modifies the local calcium environment.

D. Role of ERβ in nongenomic eNOS activation

ERα and ERβ expression have both been observed in endothelial cells in multiple paradigms (14). To determine whether endogenous ERβ plays a role in nongenomic eNOS activation, studies have been performed recently in ovine endothelial cells that display constitutive expression of both isoforms. It was found that a subpopulation of endogenous ERβ is localized to the endothelial cell plasma membrane, overexpression of ERβ in endothelial cells enhanced rapid eNOS stimulation by E_2, and the response to endogenous ER activation was inhibited by the ERβ-selective antagonist THC. In addition, eNOS activation through ERβ was reconstituted and shown to occur independent of ERα in COS-7 cells (123a).

Further experiments were done in isolated cell membranes to delineate the localization of ERβ-eNOS coupling. It was observed that THC blunts E_2 activation of eNOS in isolated endothelial cell plasma membranes. In addition, ERβ protein

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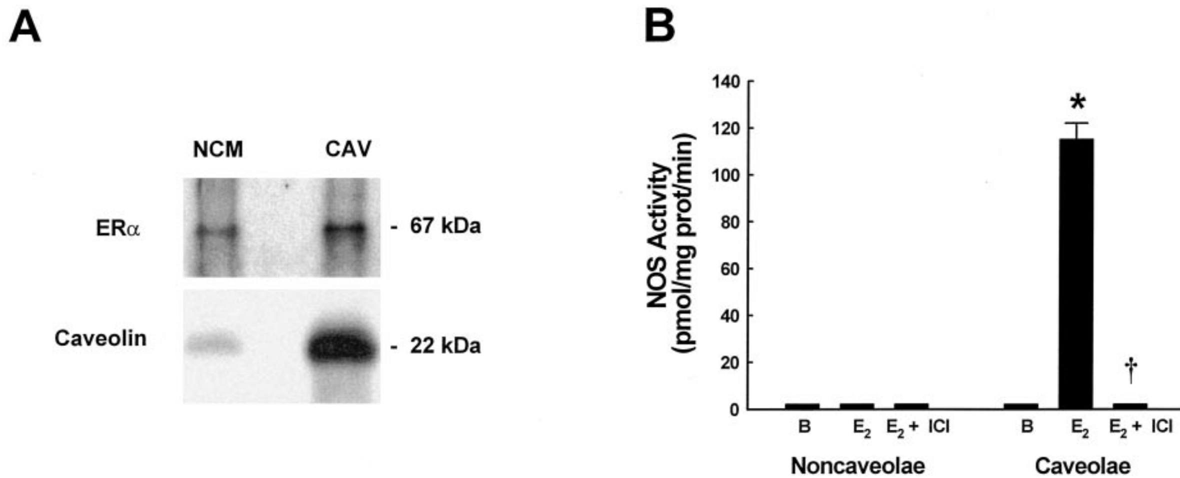

FIG. 17. Localization of ERα-eNOS interaction to caveolae. A, Immunoblot analysis for ERα and caveolin-1 in noncaveolae membranes (NCM) and caveolae membranes (CAV) obtained from endothelial cell whole-plasma membranes. Results are representative of three independent experiments. B, E_2-mediated activation of eNOS in endothelial cell caveolae membranes. ^{3}H-L-Arginine conversion to ^{3}H-L-citrulline was measured in noncaveolae and caveolae membranes obtained from endothelial cell plasma membranes. Membrane incubations were performed without added eNOS cofactors, Ca^{2+} or calmodulin, in the absence (basal, B) or presence of 10^{-8} M E_2 with or without 10^{-5} M ICI 182,780 added. NOS activity was undetectable in noncaveolae fractions in all groups, and it was also not detected in caveolae under basal conditions. Values are mean ± SEM, n = 4–6; *, $P < 0.05$ *vs.* basal; †, $P < 0.05$ *vs.* E_2 alone. [Reprinted with permission from K. L. Chambliss *et al.*: *Circ Res* 87:E44–E52, 2000 (123).]

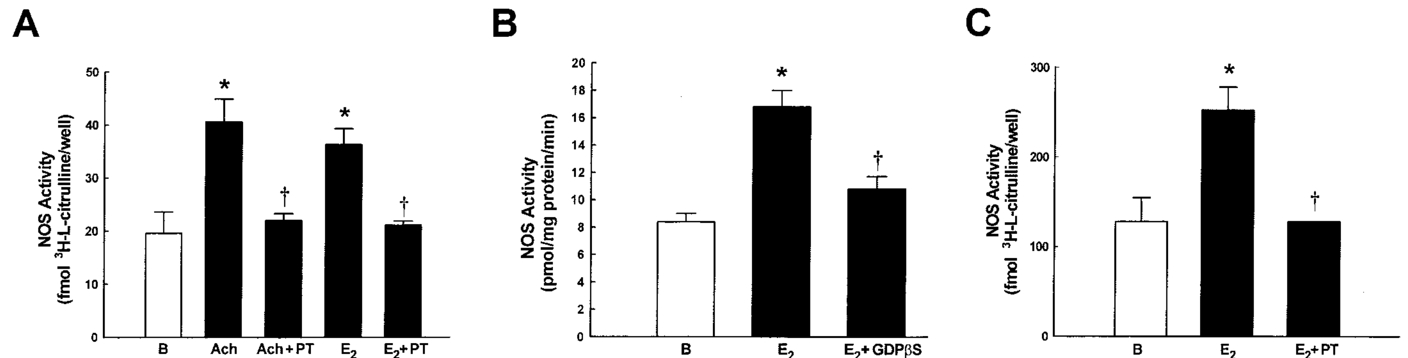

FIG. 18. G proteins play a role in estrogen stimulation of eNOS activity. A, Effect of pertussis toxin (PT) on eNOS stimulation in endothelial cells. Intact cells were pretreated with vehicle or 100 ng/ml PT, and ^{3}H-L-arginine conversion to ^{3}H-L-citrulline was assessed under basal conditions (B) or in the presence of 10^{-5} M acetylcholine (Ach) or 10^{-8}M M E_2 in the continued presence of vehicle or PT. B, Effect of exogenous GDPβS on eNOS stimulation in endothelial cell plasma membranes. The conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline was measured in purified plasma membranes incubated under basal conditions (B) or in the presence of 10^{-8} M E_2, in buffer alone or buffer plus 2 mM GDPβS. C, Effect of PT on eNOS stimulation in COS-7 cells. Cells were transfected with ERα and eNOS cDNA, and 48 h later ^{3}H-L-arginine conversion to ^{3}H-L-citrulline was measured in intact cells under basal conditions (B) or in the presence of 10^{-8} M E_2, with or without PT pretreatment. Values are mean ± SEM, n = 3. *, $P < 0.05$ *vs.* basal; †, $P < 0.05$ *vs.* no PT or no GDPβS. [Reprinted with permission from M. H. Wyckoff *et al.*: *J Biol Chem* 276:27071-27076, 2001 (128).]

was detected, THC attenuated E_2 stimulation of eNOS in isolated endothelial cell caveolae, and functional ERβ-eNOS coupling was recapitulated in caveolae from transfected cells (123a). Thus, both ERα, as described above, and ERβ have nongenomic action in endothelial cell caveolae to regulate eNOS activity in this specialized subcellular domain.

E. Coupling of plasma membrane ER to downstream signaling events

The basis by which signaling events are initiated by plasma membrane ER is perhaps best understood in the context of the known processes underlying eNOS activation by classical agonists such as Ach and bradykinin. These agents activate specific plasma membrane-associated G protein-coupled receptors (GPCRs) (124, 125). G proteins are heterotrimers of α-, β-, and γ-subunits ($G_{\alpha\beta\gamma}$) that dissociate into G_α and $G_{\beta\gamma}$ upon GPCR stimulation, after which activated G_α and/or $G_{\beta\gamma}$ modulate the activity of downstream effector molecules. Based on sequence and functional similarities, the α-subunits are divided into four subfamilies: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha 12/13}$ (126, 127). Within this context, the potential role of G proteins in the coupling of ER to downstream signaling events has been investigated in endothelial cells (128). The first approach employed was to measure E_2-stimulated eNOS activity in the absence or presence of pertussis toxin treatment, which inhibits G_α function by causing ADP ribosylation of a conserved cysteine at the fourth

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position (129). Intact endothelial cells were pretreated with vehicle or pertussis toxin and exposed to either the known GPCR agonist Ach (10^{-5} M) or E_2 (10^{-8} M) for 15 min. In the absence of pertussis toxin, Ach and E_2 caused comparable eNOS stimulation. As expected, eNOS activation by Ach was fully blocked by pertussis toxin. Similarly, eNOS stimulation by E_2 was prevented by pertussis toxin (Fig. 18A). In contrast, pertussis toxin did not prevent eNOS activation by the calcium ionophore A23187. The inhibition of ER-mediated eNOS activation by pertussis toxin implicates $G_{\alpha i}$ subfamily members only, of which $G_{\alpha i}$ is expressed in endothelial cells and $G_{\alpha o}$ is not (127, 129, 130).

To further define the role of G proteins in E_2-stimulated eNOS activation by an independent means, the effect of exogenous guanosine 5′-O-(2-thiodiphosphate) (GDPβS) on eNOS stimulation by E_2 was evaluated in isolated endothelial cell plasma membranes. When membranes were exposed to E_2 alone, eNOS activation was apparent. However, when GDPβS was added, eNOS stimulation by E_2 was inhibited by 70% (Fig. 18B). E_2-stimulated eNOS activity was not affected by GTP or GTPγS (128).

To confirm the observations made in endothelial cells and to provide a model system amenable to manipulation by cotransfection, the effect of pertussis toxin on E_2-stimulated NOS activity was determined in COS-7 cells transfected with eNOS and ERα (Fig. 18C). As observed in primary endothelial cells, E_2 treatment (10^{-8} M for 15 min) caused eNOS stimulation that was completely blocked by pertussis toxin; in contrast, cells transfected with eNOS alone were not responsive to E_2 (107). These cumulative data indicate that $G_{\alpha i}$ mediates estrogen stimulation of eNOS. Pertussis toxin also prevented E_2-mediated phosphorylation of MAPK in endothelial cells, indicating that G protein coupling occurs proximal to tyrosine kinase-MAPK activation in the series of events leading to eNOS stimulation (128).

Potential interactions between plasma membrane ERα and G_α proteins have been evaluated in coimmunoprecipitation studies using COS-7 cells transfected with ERα and either $G_{\alpha i2}$, $G_{\alpha q}$, or $G_{\alpha s}$ (Fig. 19A). Immunoprecipitation was performed with ERα antibody on plasma membranes from cells treated with vehicle or 10^{-8} M E_2 for 20 min. In plasma membranes from quiescent cells, $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$ were minimally coimmunoprecipitated with ERα. However, the association of $G_{\alpha i}$ with ERα was markedly greater after E_2 stimulation. In contrast, the association of $G_{\alpha q}$ and $G_{\alpha s}$ with ERα remained negligible after E_2 treatment. Identical findings were obtained in endothelial cells (Fig. 19B). Thus, in both an overexpression system and at constitutive levels of abundance, ERα activation by agonist leads to interaction between the receptor and Gα_i. All known GPCRs have seven-transmembrane spanning domains linked by alternating intracellular and extracellular loops, and extensive experimentation indicates that the GPCR intracellular domains function in the direct signal propagation to $G_{\alpha\beta\gamma}$ (126, 131). Since the structure of ERα is entirely different from any known GPCR, it is unlikely that ERα-$G_{\alpha i}$ interaction is direct. Alternatively, ERα may be coupled to a classical GPCR that interacts directly with $G_{\alpha i}$. There is evidence that cross-talk of this type can occur to enable non-GPCR to perform G protein-mediated functions, such as the direct protein-protein coupling of

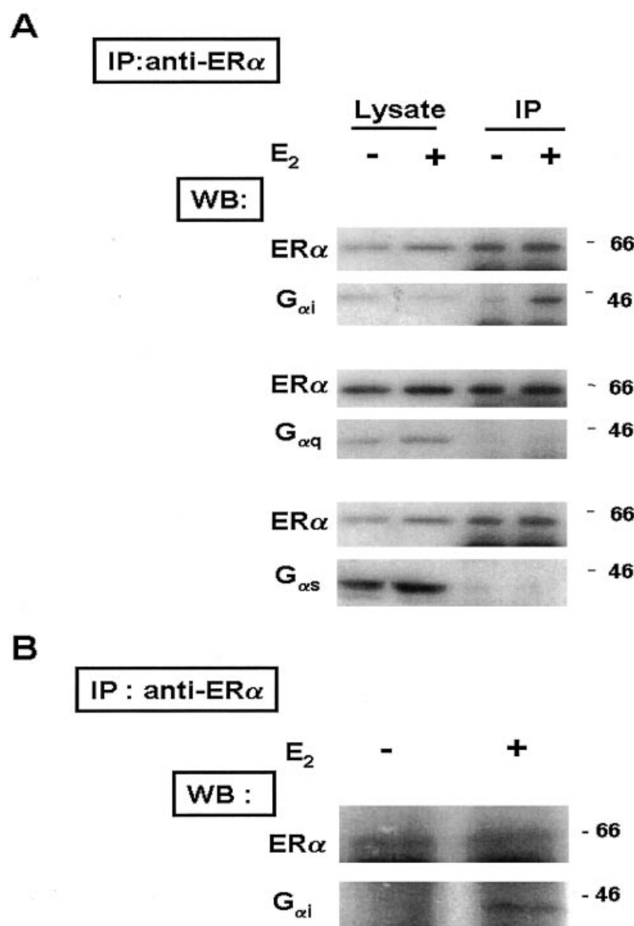

FIG. 19. Interaction of plasma membrane ERα and G_α proteins. A, Coimmunoprecipitation of ERα and $G_{\alpha i}$, $G_{\alpha q}$, or $G_{\alpha s}$ in COS-7 cell plasma membranes. Cells were transfected with ERα and either $G_{\alpha i}$, $G_{\alpha q}$, or $G_{\alpha s}$ cDNAs, and 48 h later cells were treated with vehicle or 10^{-8} M E_2 for 20 min. Plasma membranes were isolated, and immunoprecipitation (IP) was done with ERα antibody. Western blot (WB) analyses were performed on whole-cell lysates and plasma membrane immunoprecipitates for ERα and either $G_{\alpha i}$, $G_{\alpha q}$, or $G_{\alpha s}$. The band *below* the ERα or G_α band in the IP samples is IgG heavy chain. B, Coimmunoprecipitation of ERα and $G_{\alpha i}$ in endothelial cell plasma membranes. Endothelial cells were treated with vehicle or 10^{-8} M E_2 for 20 min, plasma membranes were isolated, and IP was done with ERα antibody. WB analyses were performed on immunoprecipitates for ERα and $G_{\alpha i}$. Results shown are representative of three independent experiments. [Reprinted with permission from M. H. Wyckoff *et al.*: *J Biol Chem* 276:27071-27076, 2001 (128).]

γ-aminobutyric acid A receptors to dopamine D5 receptors in hippocampal neurons. The cross-talk involves specific domains on both the non-GPCR undergoing ligand activation and the GPCR interacting with G protein (132). The third possibility is that ERα-Gαi interaction requires an intermediary protein that is not a GPCR (128).

Upon stimulation of classical GPCR, either the activated G_α- or $G_{\beta\gamma}$-subunits modulate the activity of downstream effectors (130). The potential roles of activated G_α or $G_{\beta\gamma}$ in E_2-induced activation of eNOS have been distinguished by assessing the impact of overexpression of $G_{\alpha i2}$ in COS-7 cells expressing ERα and eNOS (Fig. 20A). Whereas basal eNOS activity was unchanged by cotransfection with $G_{\alpha i2}$, $G_{\alpha i2}$

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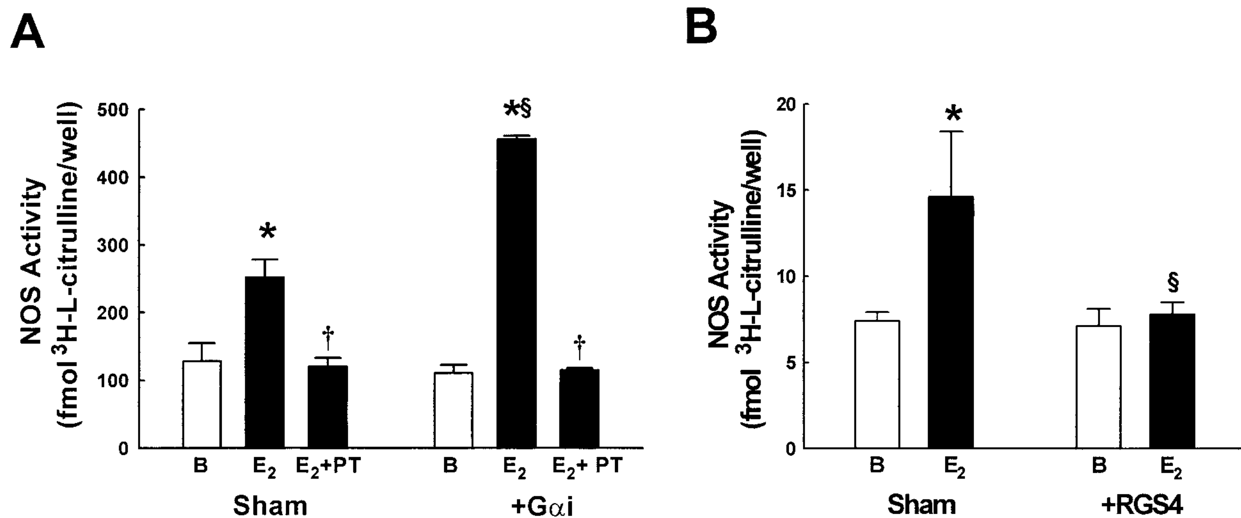

FIG. 20. Activated $G_{\alpha i}$ mediates downstream signaling leading to eNOS stimulation. A, Effect of $G_{\alpha i}$ overexpression on eNOS stimulation by E_2 in COS-7 cells. Cells were transfected with cDNAs for ERα and eNOS, and either sham vector or $G_{\alpha i}$ cDNA. ^{3}H-L-Arginine conversion to ^{3}H-L-citrulline was assessed in intact cells 48 h later under basal conditions (B) or in the presence of 10^{-8} M E_2, with or without prior pertussis toxin (PT) treatment and the continued presence of vehicle or PT. B, Effect of RGS4 overexpression on eNOS stimulation by E_2 in COS-7 cells. Cells were transfected with cDNAs for ERα and eNOS, and either sham vector or RGS4 cDNA. The conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline was measured in intact cells 48 h later under basal conditions (B) or in the presence of 10^{-8} M E_2. Values are mean ± SEM, n = 3. *, $P < 0.05$ *vs.* basal; †, $P < 0.05$ *vs.* no PT; §, $P < 0.05$ *vs.* sham. [Reprinted with permission from M. H. Wyckoff *et al.*: *J Biol Chem* 276:27071-27076, 2001 (128).]

overexpression augmented E_2-mediated eNOS stimulation by 3-fold, and pertussis toxin completely blocked the enhanced response. $G_{\alpha i1}$ overexpression had similar effects. The role of activated endogenous $G_{\alpha i}$ was further substantiated in experiments assessing the effect of overexpression of a protein regulator of G protein signaling (RGS), RGS4 (Fig. 20B). RGS4 attenuates signaling by accelerating the GTPase activity of members of the $G_{\alpha i}$ and $G_{\alpha q}$ subfamilies, but not that of $G_{\alpha s}$ or $G_{\alpha 12/13}$ (133). In COS-7 cells expressing ERα and eNOS that were cotransfected with RGS4 cDNA, E_2-mediated eNOS stimulation was decreased by 90% compared with sham-transfected cells. These cumulative observations indicate that activated $G\alpha_i$ mediates the downstream signaling processes that ultimately lead to eNOS activation. In addition to explaining the proximal events underlying nongenomic effects of estrogen in endothelial cells, these observations provide further support of a role for G proteins in nongenomic actions of estrogen in other cell types such as osteoblasts and macrophages (134, 135). There is also accumulating evidence that certain nongenomic actions of glucocorticoids, vitamin D_3, and testosterone involve steroid hormone receptors acting through pertussis toxin-sensitive mechanisms (136–139). It is proposed that the processes by which plasma membrane ERα are coupled to $G_{\alpha i}$ in endothelial cells constitute not only the basis of nongenomic estrogen action in vascular and nonvascular cells, but also a means by which other steroid hormones may have important nonnuclear effects.

VI. Summary and Future Directions

The momentum of evidence in both human studies and animal models indicates that estrogen has potent stimulatory effects on eNOS expression and activity in vascular endothelium. The effects on eNOS abundance are perhaps more predictable and are primarily transcriptionally based, and they are ER dependent but ERE independent and most probably entail an Sp1 binding element within the core eNOS promoter. Estrogen also has potent nongenomic effects on eNOS activity mediated by a subpopulation of ERα localized to caveolae in endothelial cells, where they are coupled to eNOS in a functional signaling module (Fig. 21). Emphasizing the dependence on cell surface-associated receptors, these observations provide evidence for the existence of a steroid receptor fast-action complex, or SRFC, in caveolae. Estrogen binding to ERα within caveolae leads to $G\alpha_i$ activation, which mediates downstream events. The downstream signaling includes activation of tyrosine kinase-MAPK and Akt/protein kinase B signaling, stimulation of HSP90 binding to eNOS, and perturbation of the local calcium environment, ultimately leading to eNOS phosphorylation and calmodulin-mediated eNOS stimulation. The resulting combination of genomic and nongenomic mechanisms by which estrogen modulates eNOS plays a critical role in vascular health.

The current and future challenges in this area of research are numerous. The mechanisms by which estrogen upregulates eNOS expression warrant further study. In addition to eNOS, estrogen modifies the expression of other endothelial cell genes such as cyclooxygenase type 1 (140), and the processes underlying these effects are yet to be determined. Novel endothelial cell gene targets should also be sought. Our knowledge of the nongenomic basis of estrogen action in endothelial cells is also currently limited. Although immunocytochemical analyses suggest that the ligand binding domain of cell surface-associated ERα may be extracellular (119), the orientation of plasma membrane ERα is yet to be elucidated. The mechanisms by which ERα is mem-

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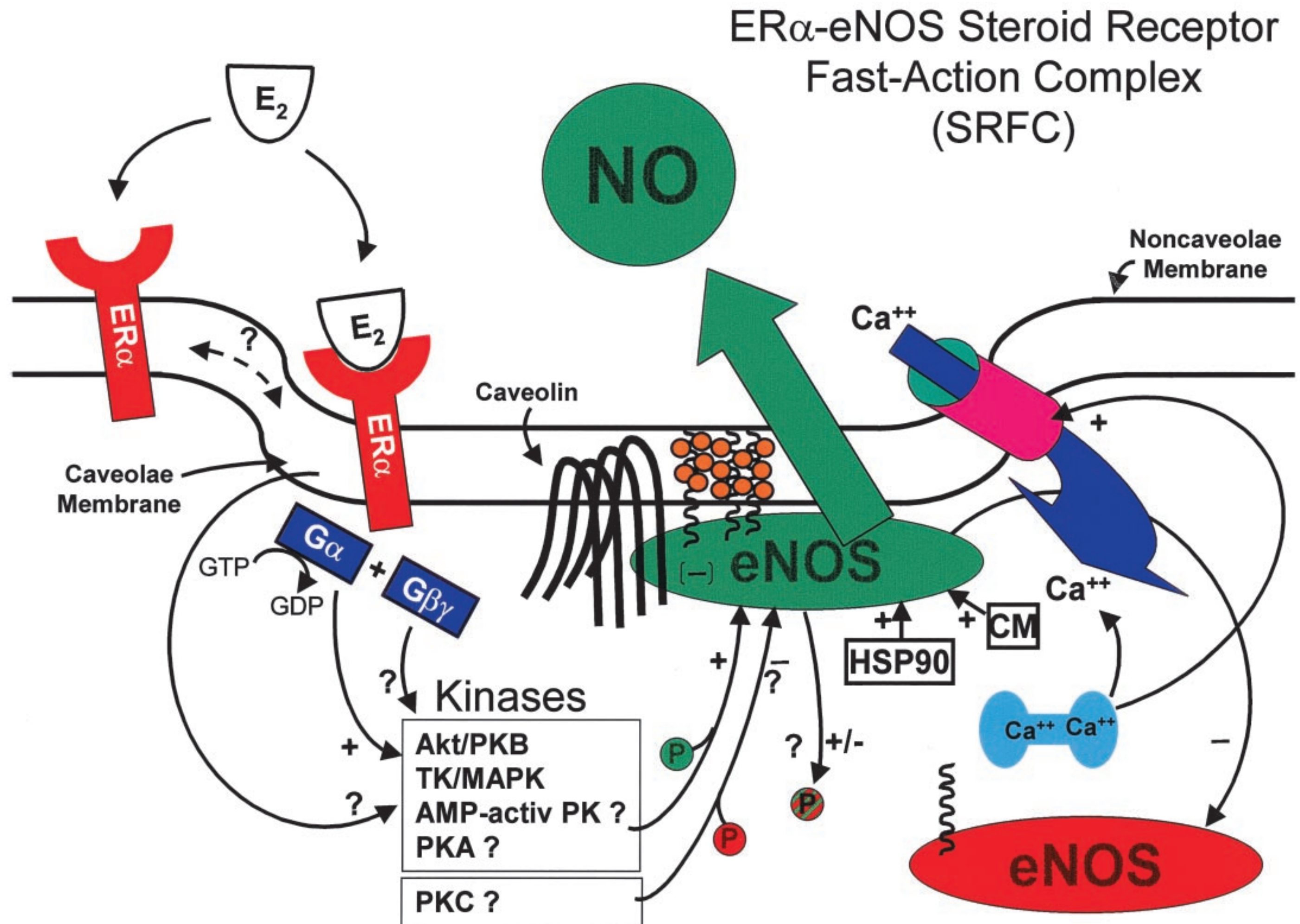

FIG. 21. Estrogen activation of eNOS involves ERα coupling to the enzyme in a SRFC in endothelial cell caveolae. eNOS localization to cholesterol-enriched (*orange circles*) caveolae is based on the myristoylation and palmitoylation of the protein (*wavy lines*), and within caveolae eNOS interaction with caveolin attenuates the activity of the enzyme. A subpopulation of ERα has also been localized to endothelial cell caveolae, and ERα is also found in noncaveolae membranes. Estrogen binding to ERα leads to $G\alpha_i$ activation, which mediates downstream events. The downstream signaling includes activation of tyrosine kinase-MAPK and Akt/protein kinase B (PKB) signaling, stimulation of HSP90 binding to eNOS, and perturbation of the local calcium environment, ultimately leading to eNOS phosphorylation and calmodulin (CM)-mediated eNOS stimulation. Soon after E_2 exposure, eNOS translocates from the membrane to intracellular sites, resulting in diminished NOS activity (change from *green* to *red*). The potential roles of other kinases and other phosphorylation/dephosphorylation events are yet to be clarified, and the mechanisms dictating the localization of ERα to plasma membrane domains are also currently unknown.

brane associated and the processes regulating the relative number of cell surface ERα are not well understood. The basis for ERα-$G\alpha_i$ interaction, which may involve a classical GPCR or an alternative intermediate protein, is also entirely unknown. Furthermore, the proximal signal transduction events following $G\alpha_i$ activation deserve in-depth study.

Our present knowledge of eNOS modulation by estrogen typifies but a fraction of what may ultimately be a total of four categories of ER action. These categories are 1) membrane-initiated, nongenomic actions; 2) membrane-initiated, genomic actions; 3) non-membrane-initiated, nongenomic actions; and 4) non-membrane-initiated, genomic actions. When one considers that there are not only ER-dependent but also ER-independent mechanisms of estrogen response in certain paradigms, there are actually eight categories of possible estrogen action. These categories should be kept foremost in mind as we probe further into the bases for estrogen effects on the vasculature and other nonreproductive target organs.

Importantly, within the eNOS realm and elsewhere, the physiological and pathophysiological impact of the different modes of estrogen and ER action is yet to be delineated in intact model systems. It is only through such focused efforts that deeper understanding will be gained about processes including novel means of genomic estrogen action and ER function in a SRFC in caveolae. It is further anticipated that we will discover that other steroid hormone responses are either fully compartmentalized or at least initiated in SRFC on the plasma membranes of other cell types.

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Address all correspondence and requests for reprints to: Philip W. Shaul, M.D., Department of Pediatrics, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas Texas 75390. E-mail: pshaul@mednet.swmed.edu

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