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Research Article

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Growth Factor Activation of the Estrogen Receptor in Vascular Cells Occurs via a Mitogen-activated Protein Kinase-independent Pathway

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Abstract

The classical estrogen receptor ER α mediates many of the known cardiovascular effects of estrogen and is expressed in male and female vascular cells. Estrogen-independent activation of ER α is known to occur in cells from reproductive tissues, but has not been investigated previously in vascular cells. In this study, transient transfection assays in human saphenous vein smooth muscle cells (HSVSMC) and pulmonary vein endothelial cells (PVEC) demonstrated ER α -dependent activation of estrogen response element-based, and vascular endothelial growth factor-based reporter plasmids by both estrogen-deficient FBS (ED-FBS) and EGF. In nonvascular cells, ER α -mediated gene expression can be activated via mitogen-activated protein (MAP) kinase-induced phosphorylation of serine 118 of ER α . However, in vascular cells, we found that pharmacologic inhibition of MAP kinase did not alter EGF-mediated ER α activation. In addition, a mutant ER containing an alanine-for-serine substitution at position 118 was activated to the same degree as the wild-type receptor by ED-FBS and EGF in both HSVSMC and PVEC. Furthermore, constitutively active MAP kinase kinase (MAPKK) activated ER α in Cos1 cells as expected, but MAPKK inhibited ER activation in PVEC. We conclude that growth factors also stimulate ER α -mediated gene expression in vascular cells, but find that this occurs via a MAP kinase-independent pathway distinct from that reported previously in nonvascular cells. (*J. Clin. Invest.* 1998; 101:2851–2861.) Key words: steroid hormone receptors • gene transcription • crosstalk • vascular smooth muscle cells • vascular endothelial cells

Introduction

Estrogen deficiency is associated with increased risk of developing coronary artery disease (1); estrogen replacement therapy attenuates this risk in postmenopausal women (2–4). While these atheroprotective effects of estrogen are mediated in part by effects of estrogens on systemic factors (5–7), the

majority of the vasculoprotective effects of estrogen appear to be due to direct effects of estrogens on vascular cells (4, 8). Direct effects of estrogens on vascular cells have been demonstrated *in vitro* and *in vivo* in both animal and human models (8–20), including effects on gene expression (17, 18), ion channel function (12, 19, 20), elaboration of and response to vasoactive substances (10, 13–16, 21, 22), as well as vascular smooth muscle cell (VSMC)¹ proliferation and migration (22–25), and endothelial cell (EC) proliferation (26). Estrogen receptor α (ER α) is expressed in human VSMC (27, 28), and we have shown that the VSMC ER is capable of altering gene expression in these cells (17, 28). ER α expression has also been demonstrated recently in human EC (29–32). The effects of estrogens on gene expression and proliferation in vascular cells are likely mediated by vascular estrogen receptors, but some of the rapid, direct effects of estrogen on vascular cells may be mediated by pathways independent of these receptors, or by estrogen receptors acting through novel, nontranscriptional pathways (10–12, 14, 15, 20, 32, 33).

ER α is expressed in VSMC derived from both women and men (28). Despite the low circulating levels of estrogen found in men, a physiologic role for ER α in males is supported by both animal and human studies. For example, estrogen administration improves vascular reactivity in some men (34–36), inhibits the response to carotid injury in castrated male rats (37), and reduces plaque formation in transgenic male mice which express high levels of apoE (38). Furthermore, disruption of the ER α gene leads to an abnormal phenotype in male mice that includes both abnormal behavior and sterility (39–42). Recent reports have also described abnormal vascular reactivity and skeletal maturation in an adult human male harboring a homozygous truncation mutation in the ER α gene (43, 44). At present, however, the mechanism(s) by which ER α mediates its vascular effects in the presence of low levels of estrogen is unclear.

Estrogen receptors, members of the large family of steroid hormone receptors that act as ligand-activated transcription factors (45, 46), activate gene expression via NH₂-terminal and/or COOH-terminal transactivation domains (AF-1 and AF-2, respectively). In nonvascular cells, alternative, hormone-independent mechanisms for receptor activation also exist. In various tumor cells, ER α -dependent transcriptional transactivation of an estrogen-response element (ERE) in the absence of estrogen has been described in response to dopaminergic stimulation, elevation of cAMP, and by specific cy-

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1. Abbreviations used in this paper: EC, endothelial cell; ECL, enhanced chemiluminescence; ED-FBS, estrogen-deficient FBS; ER, estrogen receptor; ERE, estrogen-response element; HSVSMC, human saphenous vein smooth muscle cells; MAP, mitogen-activated protein; MAPK, MAP kinase; MAPKK, MAPK kinase; PVEC, pulmonary vein endothelial cells; SFM, serum-free media; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell.

clins (47–50). EGF has also been shown to activate ER α in uterine and ovarian adenocarcinoma cells (51–53). In *Cos1* and *HeLa* cells, the mechanism for this ligand-independent ER activation pathway involves mitogen-activated protein (MAP) kinase-dependent phosphorylation of serine 118 of AF-1 in ER α (54, 55). However, hormone-independent activation of ER α has not been studied yet in vascular cells. In this study, we demonstrate that ER α also can be transcriptionally activated in vascular cells in an estrogen-independent manner, but show that hormone-independent activation of ER α in vascular cells occurs by a MAP kinase-independent pathway.

Methods

Cell culture techniques

Human saphenous vein smooth muscle cells (HSVSMC) were cultured from explants derived from surgical specimens as described (28). *Cos1* cells and *HeLa* cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in DME with 10% FBS. Rat pulmonary vein endothelial cells (PVEC), the kind gift of Paul Hassoun (New England Medical Center, Boston, MA), were maintained in RPMI. For all experiments, phenol red-free media was used. Unless otherwise noted, serum stimulation was achieved as previously described (28), by application of defined estrogen-deficient FBS (ED-FBS; Hyclone, Logan, UT) in which the estrogen content was $< 2.6 \times 10^{-11}$ M. In a subset of experiments, charcoal-stripped ED-FBS was prepared by adding PBS-rinsed Dextran-70 (Sigma Chemical Co., St. Louis, MO) and charcoal at a 1:100 (wt/vol) and 1:10 (wt/vol) dilution, respectively. The mixture was incubated at 55°C for 1 h, centrifuged, and subjected to filter sterilization. In preliminary experiments to determine the adequacy of hormone removal, 1 nM [3 H]17 β -estradiol was added to FBS before stripping and the residual radioactivity was determined after the stripping protocol. This process demonstrated removal of 98–99.6% of the 17 β -estradiol in three independent experiments.

Transfection assays

All transfections were carried out by electroporation as described (28). In experiments designed to investigate the direct effects of ED-FBS stimulation, cells were growth arrested by serum starvation for 72–96 h before transfection. VSMC, *Cos1* cells, and *HeLa* cells were arrested in DME supplemented with 0.5 μ M insulin, 0.5 μ g/ml transferrin, 0.2 mM ascorbate (56), and 10 $^{-8}$ M ICI 182780. PVEC were similarly serum-deprived in RPMI. Cellular viability was assessed extensively by trypan blue exclusion during serum deprivation in pilot studies before initiation of the studies shown.

Plasmids

The reporter plasmids ERE-*Luc*, containing three copies of the *Xenopus vitellogenin* ERE proximal to the thymidine kinase promoter-driving expression of the luciferase cDNA, and TK-*Luc*, identical to ERE-*Luc* but lacking the ERE, were the kind gift of C. Glass (University of California at San Diego, La Jolla, CA) (57). The reporter plasmids mVEGF-*Luc*, containing 1.6 kb of the 5' portion of the murine vascular endothelial growth factor (VEGF) gene (including 1.2 kb of 5'-flanking region) driving expression of the luciferase gene, and Δ Sma/VEGF-*Luc*, similar to mVEGF-*Luc* but lacking the entire 5'-flanking region) were the kind gift of P. D'Amore (Children's Hospital Medical Center, Boston, MA). The expression plasmid pCMV $_3$ -ER was constructed by cloning the full-length human ER cDNA into the CMV-driven plasmid pCDNA3.1 (Invitrogen, San Diego, CA). The plasmid pCMV $_3$ -ER-NTF was produced by inserting the coding sequence for an eight-amino acid epitope tag recognized by a commercially available mAb, M2 (Eastman Kodak, Rochester, NY) at the 5' end of the ER α coding sequence in pCMV $_3$ -ER. The expression plasmid pCMV $_3$ -ER-s118a was constructed by mutating serine 118 to alanine by the method of Kunkel et al. (58) and the mutation

was confirmed by sequencing in both directions. The expression plasmid pCMV $_3$ -ER-271 was constructed by digestion of pCMV $_3$ -ER with Xcm1 and Xho1 to remove the coding sequence distal to amino acid 271 in the D region of the receptor, followed by blunt-ending of the construct with a polymerase reaction and religation. Truncated protein of the predicted size was expressed by pCMV $_3$ -ER-271, as demonstrated by Western blotting (data not shown). The expression plasmid SR α 456-HA, driving expression of a constitutively activated form of MAP kinase (MAPK) kinase, was the kind gift of Y. Gotoh (59).

After transfection, cells were plated for 4 h in ED-FBS-containing medium to allow for cell attachment, then rinsed three times with serum-free medium, and finally placed into experimental media. In addition to ED-FBS, cells were grown in the presence or absence of the specific ER antagonist ICI 182780 (kind gift of A. Wakeling, Zeneca Pharmaceuticals, Cheshire, England), 100 ng/ml EGF (CalBiochem, La Jolla, CA), PDGF (AB isoform at 10 nM; Sigma Chemical Co.), tamoxifen (Sigma Chemical Co.), the MAP kinase kinase (MAPKK) inhibitor PD 98059 (see ref. 60; New England Biolabs, Bedford, MA) or 17 β -estradiol (E2; Sigma Chemical Co.) as described in Results. Steroid compounds were dissolved in ethanol, which was applied in appropriate concentrations to control wells to ensure a constant concentration of vehicle (< 0.02%) in all cases. Cells were harvested for determination of luciferase activity (as described in ref. 28) 48–72 h after transfection. All luciferase results presented were normalized to the activity of a cotransfected β -galactosidase expression plasmid (pCMV-LacZ), according to the manufacturer's guidelines (Galactolite; Tropix, Bedford, MA).

Immunoblotting

ER. Cells were transfected with the expression plasmid pCMV $_3$ -ER-NTF with or without the SR α 456-HA expression plasmid. Cells were plated in 100-mm dishes, exposed to experimental conditions for 48 h, and lysed as described (61) with lysis buffer modified to include 0.6 M NaCl and 10 mM Na molybdate. Equal volumes of total cellular protein were separated by SDS-PAGE and transferred to nitrocellulose. Immunoblotting was performed either with the M2 antibody or in some experiments with an antibody against the double-phosphorylated form of MAPK (Promega Biotec, Madison, WI). Immunoblots were washed and then incubated with an anti-mouse-HRP secondary antibody and developed by standard enhanced chemiluminescence (ECL) techniques. To confirm equal protein loading between lanes, membranes were stripped and reprobed with monoclonal anti-smooth muscle action antibody (Sigma Chemical Co.).

MAPK. PVEC plated in six-well plates were made quiescent by overnight serum deprivation and then incubated for 1 h in the absence or presence of the MAPKK inhibitor PD98059. The cells were then treated with vehicle or 100 ng/ml EGF for 15 min. The cells were lysed as described above, and equal volumes of total cellular protein were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with a mouse mAb against the active form of MAPK (Promega Biotec). After development by standard ECL techniques, the membrane was stripped and confirmed to be blank by repeat ECL development. The membrane was then reprobed with an anti-MAPK antibody (Upstate Biotechnology, Inc., Lake Placid, NY) and finally developed with ECL.

Proliferation assays

HSVSMC were plated in 96-well plates at a density of \sim 2,000 cells/cm 2 and rendered quiescent by serum deprivation as described above. 1 μ Ci [3 H]thymidine was then added to each well and the cells were maintained in the presence or absence of 100 ng/ml EGF or 10 nM PDGF for 24 h. The cells were then rinsed three times with PBS, lysed with 1 N HCl, and neutralized with NaHCO₃. Incorporated [3 H]thymidine was quantified by scintillation counting.

Statistical analysis

All data are presented as mean \pm SEM. Two-way comparisons were made with the Student's *t* test; multiple group comparisons were

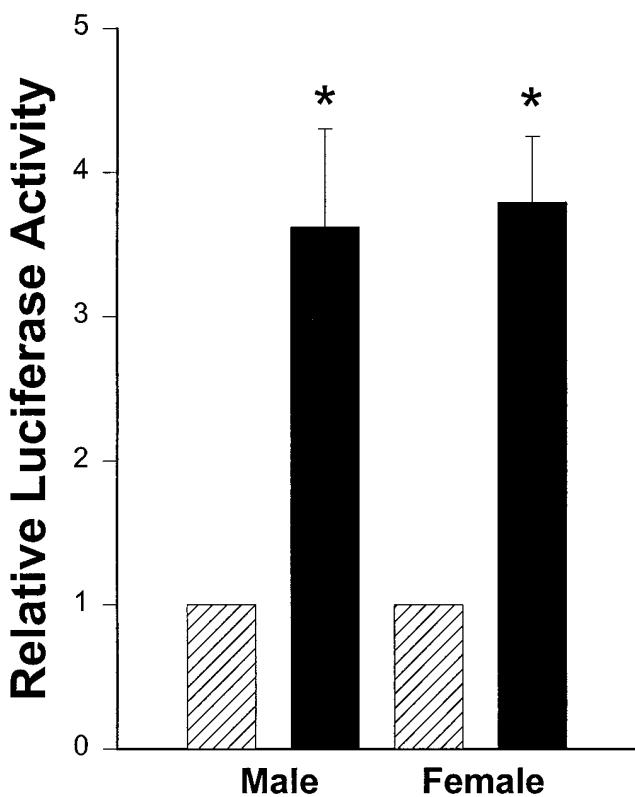


Figure 1. Estrogen-deficient serum stimulation activates an ERE in VSMC derived from males and females. Quiescent VSMC derived from saphenous veins of male or female patients were transiently transfected with an ERE-Luc plasmid and grown for 48 h in SFM (hatched bars) or in the presence of 10% ED-FBS (solid bars). In both cell types, ED-FBS treatment increased the activity of the ERE-containing reporter plasmid. Bars, mean (\pm SEM) of luciferase activity relative to the activity in quiescent cells. * $P < 0.01$ vs. SFM.

made by ANOVA. A value of $P \leq 0.05$ was considered statistically significant.

Results

Stimulation of VSMC with ED-FBS results in activation of an ERE reporter plasmid. VSMC derived from saphenous veins of men or women were growth arrested by serum starvation and then transfected with either ERE-Luc or a control reporter plasmid lacking the ERE sequences (TK-Luc). In the absence of estrogen, 48-h stimulation of male-derived HSVSMC with ED-FBS resulted in a 3.6 ± 0.7 -fold increase in activity of the ERE-Luc reporter plasmid (Fig. 1; $n = 9$; $P < 0.01$). Similar results were obtained using HSVSMC derived from women (3.8 ± 0.5 -fold, $n = 6$, $P < 0.01$). ED-FBS had no effect on the activity of the control vector TK-Luc (data not shown). Independent experiments in which VSMC were stimulated with charcoal-stripped FBS yielded similar results (data not shown).

ED-FBS-induced activation of the ERE is mediated by ER α via AF-1. To examine the role of ER in ED-FBS-induced activation of the ERE-Luc reporter, we investigated the effects of overexpression of ER α in HSVSMC on ED-FBS-mediated transactivation. As shown in Fig. 2A, transient transfection of HSVSMC with the expression plasmid pCMV₃-ER increased

activation of ERE by ED-FBS from 3.8 ± 0.7 -fold to 15.3 ± 4.4 -fold ($n = 27$; $P < 0.05$). Co-incubation of HSVSMC with the specific estrogen receptor antagonist ICI 182780 inhibited ED-FBS-mediated activation in a dose-dependent fashion in the absence (data not shown) or presence (Fig. 2A) of ER α overexpression ($P < 0.05$). Estrogen alone led to the expected activation of the ER in these studies (5.8 ± 1.1 -fold; $n = 20$; $P < 0.05$).

We also examined the effects of transfection of ER α on ED-FBS stimulation of the ERE in a second type of vascular cell, PVEC. Based on both Western blotting and transient transfection experiments, PVEC are devoid of functional ER (data not shown). In PVEC, ED-FBS treatment had no effect on ERE activation (Fig. 2B). However, ED-FBS treatment of PVEC cotransfected with pCMV₃-ER resulted in a 6.6 ± 0.9 -fold increase in ERE activation (Fig. 2B; $n = 27$; $P < 0.001$), an effect that again was completely inhibited by ICI 182780. The observed increase in ERE activation upon FBS treatment did not result from alterations in ER α protein levels, which were unchanged after FBS treatment (inset, Fig. 2B), and estrogen alone again gave the expected activation of the ER in PVEC (3.1 ± 0.4 -fold; $n = 16$; $P < 0.01$). Similar results to those of Fig. 2B also were observed in HeLa cells (3.6 ± 0.3 -fold activation; $n = 18$; $P < 0.01$ ED-FBS vs. serum-free media [SFM], data not shown).

To begin to define which domains within ER α mediate ED-FBS transactivation of the ERE, we examined the activity of an ER truncated at amino acid 271. ER271 contains the DNA binding domain and AF-1, but lacks the hormone-binding domain and AF-2. As shown previously, ED-FBS had no effect on untransfected PVEC, but caused a 5.9 ± 1.2 -fold increase in luciferase activity in PVEC transfected with wild-type ER ($n = 12$; $P < 0.01$; Fig. 2C) and a 3.1 ± 0.3 -fold increase in PVEC transfected with ER-271 ($n = 12$; $P < 0.01$). ER-271 was not activated by E2, consistent with the absence of the hormone-binding domain in this construct (Fig. 2C). These data demonstrate that AF-1 in ER α can mediate ED-FBS-induced transactivation of the ERE-Luc reporter.

ER-mediated transcriptional transactivation of a VEGF reporter. To examine whether E2-independent activation of the ER can activate transcription of a physiologically relevant gene, we examined the effect of ED-FBS on mVEGF-Luc gene expression. In human VSMC, ED-FBS treatment resulted in an 11.2 ± 4.0 -fold activation of the VEGF reporter plasmid ($n = 6$; $P < 0.05$; Fig. 3A). This activation was markedly inhibited by treatment with the ER antagonist ICI 182780, an effect partially reversed in a dose-dependent manner by co-incubation with E2 (Fig. 3A). ED-FBS had no effect on the control plasmid Δ Sma/VEGF-Luc, which lacks the 5'-flanking sequence of the VEGF gene (data not shown). These effects of ED-FBS stimulation on mVEGF-Luc were also studied in HeLa cells (an ER null cell [62]) without and with coexpression of ER α . In the absence of ER α coexpression, ED-FBS treatment had no significant effect on VEGF reporter activity in HeLa cells (Fig. 3B, first two bars). In the presence of cotransfected ER α , however, ED-FBS treatment resulted in a 2.9 ± 0.4 -fold activation of VEGF expression ($n = 24$; $P < 0.05$). As in HSVSMC, this activation also was blocked by ICI 182780, and the inhibition of ICI 182780 could be reversed by co-incubation with E2 (Fig. 3B). Hormone treatment had no effect in HeLa cells on the control vector Δ Sma/VEGF-Luc (data not shown).

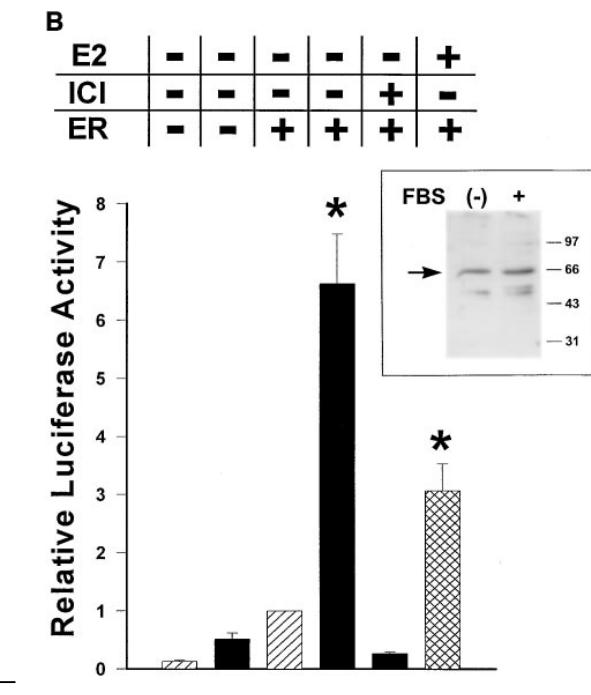
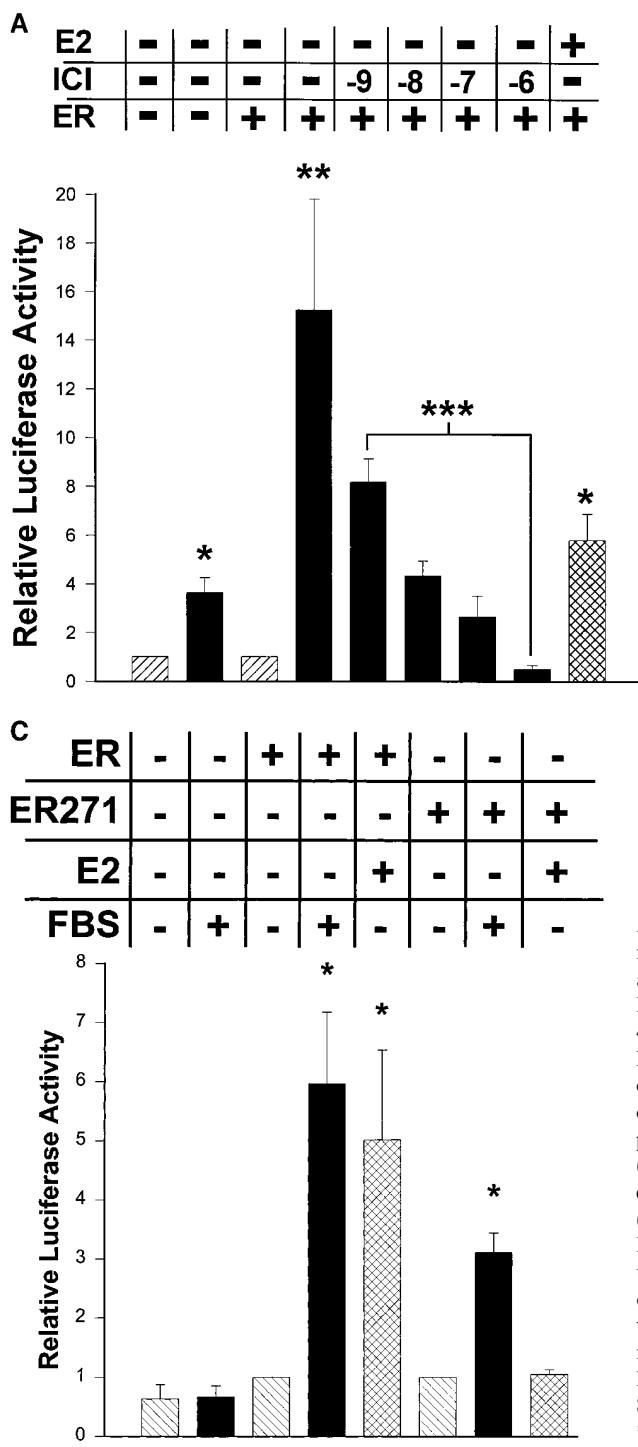


Figure 2. ED-FBS-induced activation of the ERE is mediated by ER. Serum-deprived cells were transfected with the ERE-Luc reporter plasmid along with either a control vector, or an expression plasmid for wild-type ER (ER) or the truncated ER mutant (ER271). Cells were then grown for 48 h in SFM (hatched bars) without or with (double-hatched bar) 10^{-6} M E2, or in the presence of 10% ED-FBS (solid bars) without or with addition of the ER antagonist ICI 182780 (ICI; Log M). (A) In human VSMC, ER overexpression increased ED-FBS-induced transactivation of the ERE reporter and this activation was blocked in a dose-dependent manner by ICI. (B) In PVEC, ED-FBS transactivated the ERE only when the ER was coexpressed. ED-FBS-mediated activation was again blocked by ICI (10^{-6} M). (Inset) Immunoblot for ER demonstrates no significant effect of ED-FBS stimulation on ER protein abundance in PVEC treated as in B. In untransfected cells, no ER was detectable by Western blot (data not shown; one of three similar experiments is shown). (C) In PVEC, ER271 is activated by ED-FBS, as is the wild-type ER α . Note that unlike the wild-type receptor, the truncated form is not E2 responsive. Bars, mean (\pm SEM) of luciferase activity relative to the activity in quiescent cells. *P < 0.05 vs. SFM; **P < 0.05 vs. SFM and P < 0.05 vs. ED-FBS treated ER(-) cells; ***P < 0.05 for trend.

Differential effects of anti-estrogens on ED-FBS-mediated activation of the ER. Anti-estrogens have been categorized into subtypes based largely on differences in their ability to alter activation of AF-1 and AF-2, the two transcriptional activation domains of ER α (63–65). We therefore compared the ability of two anti-estrogens, ICI 182780 (a complete antagonist) and tamoxifen (a mixed agonist/antagonist), to inhibit ED-FBS-induced activation of ER α . As Fig. 4 A shows, ICI 182780 inhibited ED-FBS-mediated activation of the ERE reporter by $90 \pm 2\%$ in HSVSMC ($n = 15$; $P < 0.001$). In con-

trast, tamoxifen acted as a weak agonist at lower doses, and no inhibitory effect was observed at any dose tested. Similar results were found in HeLa cells cotransfected with pCMV $_3$ -ER. In these cells, ICI 182780 inhibited ED-FBS-induced ERE-Luc activation $87 \pm 2\%$ ($n = 9$; $P < 0.01$) and VEGF-Luc activation ($62 \pm 3\%$; $n = 15$; $P < 0.05$), whereas tamoxifen had no significant effect, even at doses up to $1 \mu\text{M}$ (Fig. 4 B).

Effects of specific growth factors on ER activation. To investigate whether stimulation of vascular cells with specific mitogens activates the ER, the effects of EGF and PDGF on cell

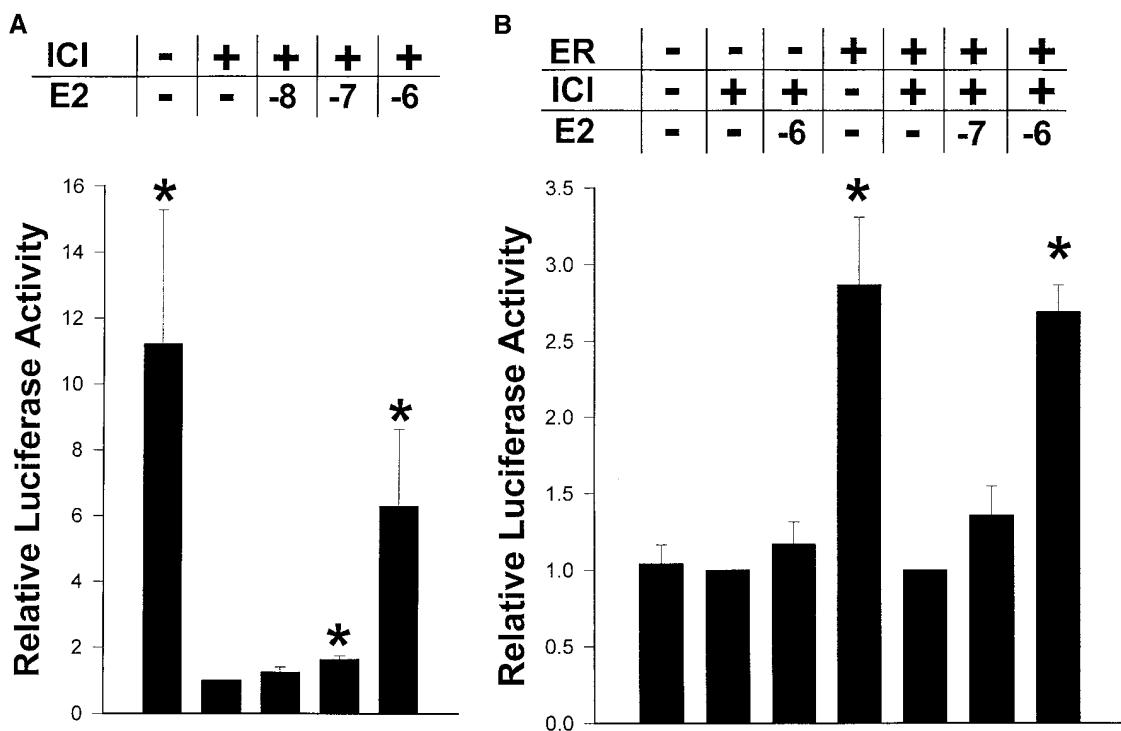


Figure 3. The estrogen receptor mediates ED-FBS-induced activation of VEGF gene expression. VSMC (A) were transfected with the reporter plasmid *mVEGF-Luc* and maintained in 10% ED-FBS in the absence or presence of either the ER antagonist ICI 182780 (ICI; 10^{-6} M) or 17 β -estradiol (E2). HeLa cells (B) were transiently transfected with the *mVEGF-Luc* reporter plasmid with or without cotransfection of the pCMV $_3$ -ER expression plasmid (ER) and maintained in 10% ED-FBS in the absence or presence of ICI (10^{-6} M), with or without addition of E2. Bars, mean (\pm SEM) of luciferase activity relative to the activity in ICI-treated cells. * $P < 0.05$ vs. ICI.

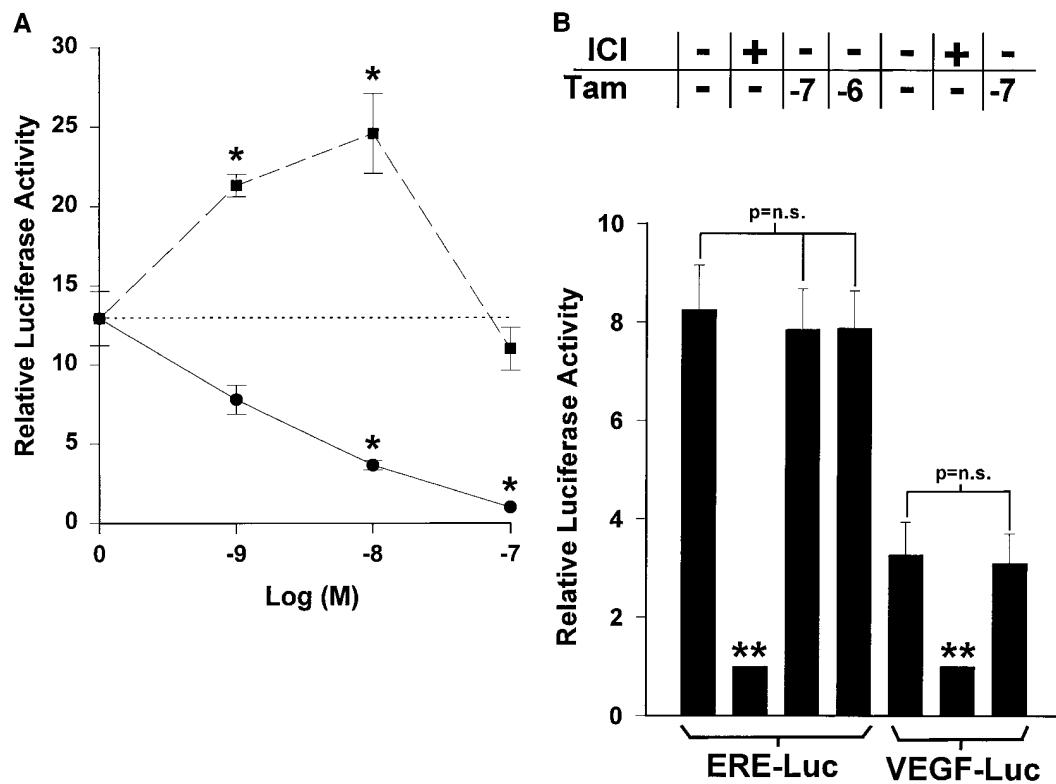


Figure 4. ED-FBS-mediated activation of the ER is blocked by ICI 182780, but not by tamoxifen. (A) Human VSMC transfected with the ERE-Luc reporter plasmid were treated with 10% ED-FBS in the presence or absence of ICI 182780 (circles) or tamoxifen (boxes). (B) HeLa cells transfected with the pCMV $_3$ -ER expression plasmid, and either the ERE-Luc or mVEGF-Luc reporter plasmids, and treated with 10% ED-FBS in the presence or absence of ICI or tamoxifen. Bars, mean (\pm SEM) of luciferase activity relative to the activity in ICI-treated cells. * $P < 0.05$ vs. ED-FBS alone; ** $P < 0.01$ vs. ED-FBS alone.

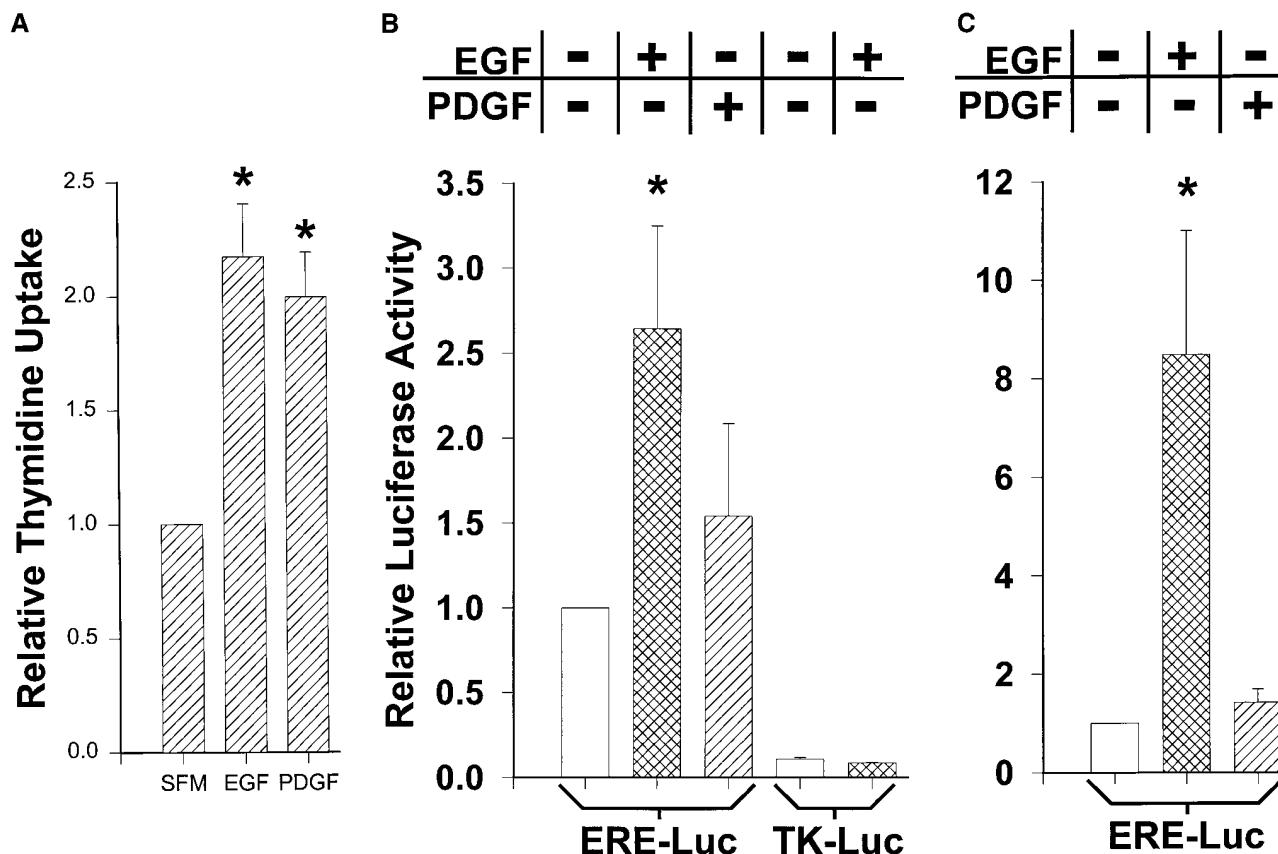


Figure 5. Differential effects of mitogenic stimulation on ER activation in vascular cells. (A) Quiescent VSMC were stimulated with 100 ng/ml EGF or 10 nM PDGF in the presence of [³H]thymidine. Cells were harvested after 24 h and quantified after the incorporation of [³H]thymidine. Both EGF and PDGF treatment increased [³H]thymidine uptake (2.2 ± 0.3 -fold; $n = 16$; * $P < 0.05$, and 2.0 ± 0.2 -fold, $n = 8$; $P < 0.05$, respectively). Quiescent PVEC (B) and VSMC (C) were transiently transfected with the ERE-Luc reporter or the control vector TK-Luc and an ER α expression plasmid and maintained in SFM or stimulated with 100 ng/ml EGF or 10 nM PDGF for 48 h. In both vascular cell types, EGF, but not PDGF stimulation, significantly increased ERE transactivation but had no effect on the TK-Luc control. Bars, mean (\pm SEM) of luciferase activity relative to the activity in quiescent cells. * $P < 0.01$ vs. SFM.

proliferation and ERE-Luc reporter activity were studied. Stimulation of HSVSMC by EGF or PDGF resulted in a mitogenic response (assessed by incorporation of [³H]thymidine; Fig. 5 A). EGF stimulation was accompanied by an 8.5 ± 2.5 -fold increase in ERE-Luc activity in HSVSMC ($n = 9$; $P < 0.01$; Fig. 5 B) and a 2.6 ± 0.6 -fold increase in ERE-Luc activity in PVEC ($n = 24$; $P < 0.01$; Fig. 5 B). However, in contrast to the effects of EGF, PDGF had no significant effect on ERE activity in either cell type.

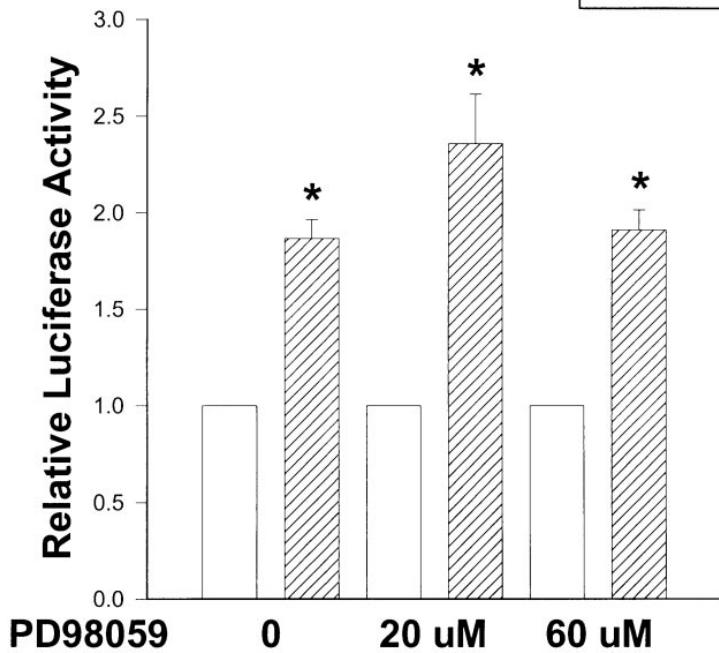
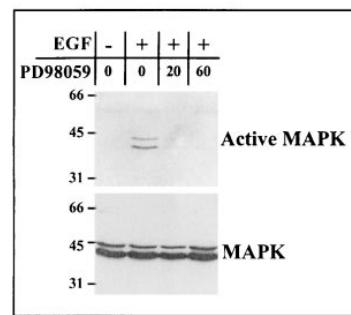
MAP kinase inhibition does not alter EGF-mediated ER activation in vascular cells. In nonvascular cells like Cos1 and HeLa, ligand-independent activation of the ER occurs via the MAP kinase pathway (54, 55). We therefore examined the effect of the specific MAPKK inhibitor PD98059 on EGF-mediated ER activation in PVEC. Neither 20 nor 60 μ M PD98059 interfered with EGF-mediated activation of ER α in PVEC (Fig. 6 A), suggesting ER activation in these vascular cells proceeds via a MAP kinase independent-pathway. Immunoblot analysis of protein lysates from PVEC demonstrated that these concentrations of PD98059 fully inhibited EGF-mediated activation of MAPK (Fig. 6 A, inset).

Hormone-independent activation of the ER in vascular cells does not depend on serine 118. In Cos1 and HeLa cells, phos-

phorylation of serine 118 of the ER is critical for ER activation by MAP kinase (54, 55). We therefore investigated the role of serine 118 in mitogenic stimulation of ER activation in vascular cells. A mutant ER, in which an alanine replaces the serine normally found at position 118 (ERs118a) was studied in PVEC, stimulated either with ED-FBS or EGF. ED-FBS treatment resulted in a 5.9 ± 1.2 -fold increase in activation of the wild-type ER in PVEC ($n = 21$; $P < 0.01$ vs. SFM; Fig. 6 B) and, in contrast to nonvascular cells, a 4.2 ± 0.7 -fold activation of the mutant ERs118a ($n = 15$; $P = \text{n.s.}$ vs. wild type ER). EGF treatment also induced activation of both the wild-type receptor (2.3 ± 0.5 -fold; $n = 30$; $P < 0.01$ vs. SFM) and ERs118a to comparable levels (2.1 ± 0.5 -fold; $n = 30$; $P = \text{n.s.}$ vs. wild-type ER; Fig. 6 B). Results were similar in parallel experiments in VSMC (data not shown).

MAPKK activates ER in Cos1 cells, but inhibits ER activation in vascular cells. To investigate further the effect of MAP kinase activation on the ER, we cotransfected constitutively active MAPKK into Cos1 cells and vascular cells. As previously reported (55), in Cos1 cells, cotransfection of activated MAPKK resulted in a dose-dependent increase in transcriptional activity of the wild-type ER (maximal activation = 12.0 ± 2.5 -fold; $n = 20$; $P < 0.01$; Fig. 7 A), but not the s118a

A



B

ER-WT	+	-	+	-	+	-
ER-118a	-	+	-	+	-	+

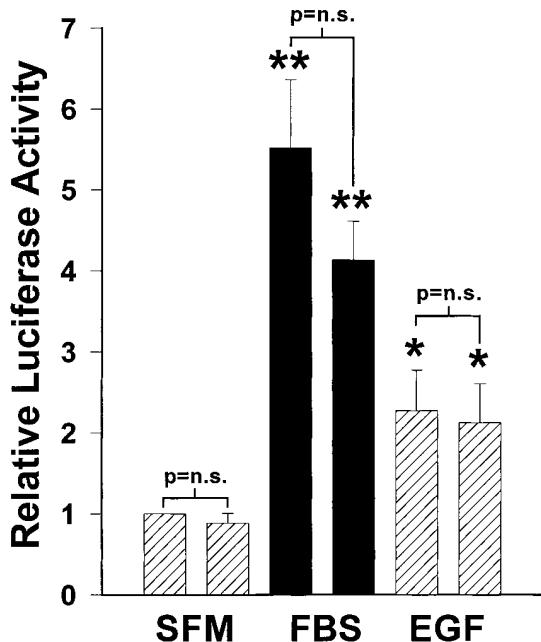


Figure 6. MAP kinase inhibitor does not block ligand-independent ER activation and ED-FBS and EGF activate the wild-type ER and the s118a mutant ER similarly in vascular cells. (A) Quiescent PVEC were maintained in the absence (open bars) or presence of 100 ng/ml EGF (hatched bars) with 20 or 60 μ M of the MAPKK inhibitor PD98059. PD98059 had no effect on the magnitude of EGF-induced ER activation. Bars, mean (\pm SEM) luciferase activity relative to cells maintained without EGF ($n = 16$ –48 each bar); * $P < 0.01$ vs. EGF. (Inset) Quiescent PVEC were treated with 100 ng/ml EGF for 15 min either with or without pretreatment with 20 or 60 μ M PD98059, and protein lysates were immunoblotted for either active MAPK or total MAPK. PD98059 effectively inhibited EGF-induced MAPK activation. (B) PVEC were transfected with the ERE reporter plasmid and either wild-type pCMV₃-ER (ER-WT) or the serine 118-to-alanine mutant ER (ER-s118a), and maintained in SFM (hatched bars), 10% ED-FBS (solid bars), or SFM supplemented with EGF (EGF; 100 ng/ml). ED-FBS- and EGF-mediated activation of the ERE both were significant, but did not differ between the wild-type and the s118a receptors for either agonist. Bars, mean (\pm SEM) of luciferase activity relative to the activation in the wild-type transfected quiescent cells. * $P < 0.05$ vs. SFM; ** $P < 0.01$ vs. SFM.

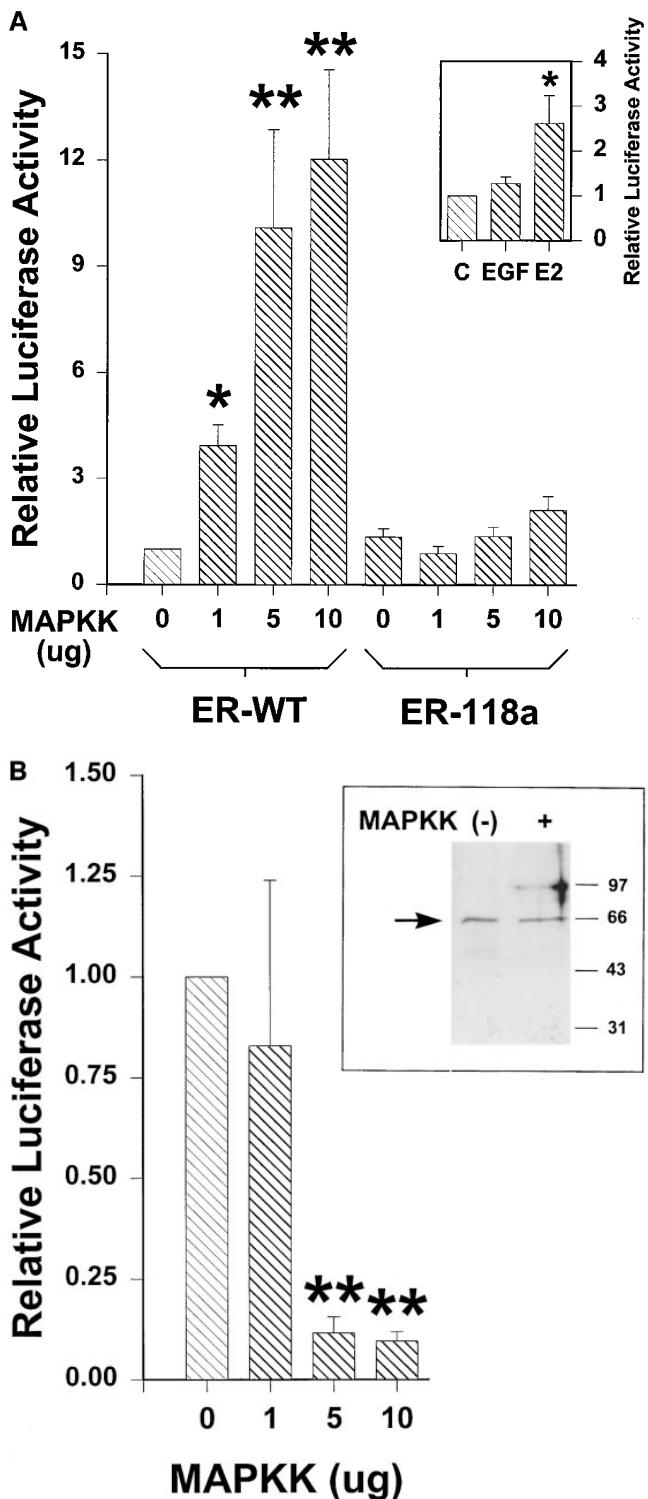


Figure 7. Coexpression of activated MAPK activates the wild-type ER in *Cos1* cells, but inhibits ER activation in vascular cells. *Cos* cells (A) or PVEC (B) transfected with either pCMV₃-ER or pCMV₃-ERs118a and the ERE-Luc reporter plasmid and increasing amounts of constitutively active MAPKK expression plasmid in serum free medium. MAPKK expression resulted in a dose-dependent activation of the wild-type ER in *Cos1* cells, but had no effect on the s118a mutant (A). The s118a mutant ER also was not activated by EGF in *Cos1* cells, but remained responsive to E2 (inset). In PVEC, in contrast to *Cos1* cells, overexpression of MAPKK inhibited ER activation (B). Bars, mean (\pm SEM) of luciferase activity relative to the ac-

mutant (Fig. 7 A). Also as expected, ER-s118a in *Cos1* cells responds to E2, but is not activated by EGF (Fig. 7 A, inset; 2.6 ± 0.6 -fold activation; $P < 0.05$). However, cotransfection of the MAPKK expression plasmid in PVEC somewhat surprisingly resulted in a dose-dependent decrease in ER α activation (maximal level of inhibition = $90 \pm 2\%$; $n = 12$; $P < 0.01$; Fig. 7 B). The level of ER α protein was unchanged by cotransfection of MAPKK in PVEC (Fig. 7 B, inset), and overexpression of ER α had no effect on MAP kinase activation as determined by immunoblotting (data not shown). Furthermore, the inhibitory effect of MAPKK expression in PVEC did not result from toxicity to the transfected cells as assessed by trypan blue and β -galactosidase activity studies (data not shown).

Discussion

This study demonstrates estrogen-independent activation of the estrogen receptor in vascular cells and shows further that this pathway in vascular cells is independent of MAP kinase and thus distinct from that observed in nonvascular cells. The role of ER α in mediating ED-FBS-induced ERE activation in vascular cells is supported by several observations including: (i) augmentation of the ED-FBS effect in VSMC by cotransfection of an ER expression plasmid; (ii) inhibition of ED-FBS-induced activation by ICI 182780; and (iii) the requirement for cotransfection of an ER α expression plasmid for the ED-FBS effect in ER null cells (PVEC and *HeLa*). Although it is possible that the defined FBS used in the majority of these studies contains very small amounts of estrogen, this alone cannot account for our observations, as hormone-independent ER activation is also observed when the cells are stimulated with either EGF in serum-free medium or with charcoal-stripped serum. ED-FBS-mediated activation of ER-271, which lacks the hormone-binding domain, also supports the hormone-independent nature of this pathway. We also found that ER α can mediate serum-induced activation of VEGF gene expression, suggesting that estrogen-independent activation of the ER can regulate expression of a physiologically relevant gene. We chose to study the VEGF gene for several reasons. VEGF, a secretory product of VSMC (66), acts as a highly specific mitogen for endothelial cells (67, 68) and has been implicated in the regulation of neovascularization (69–71) and reendothelialization after vascular injury (72, 73). In addition, VEGF production is induced by mitogenic and other stimuli in VSMC (74–76) and by estrogen in uterine cells (77) and VSMC (78).

EGF-mediated activation of ER α has been demonstrated previously in nonvascular cells (51–54). This study extends these findings by demonstrating coupling of mitogenic and steroid receptor-mediated signaling pathways in vascular cells. Though ligand-independent activation of ER α has been previously reported (48–50, 54, 55), the molecular pathways that mediate these effects are not well understood. Since two previous reports demonstrated MAPK mediates ligand-independent ER α activation in nonvascular cells (54, 55), we hypothesized first that the ED-FBS and EGF-mediated activation of

tivity in cells not transfected with MAPKK. * $P < 0.05$ vs. 0 MAPKK; ** $P < 0.01$ vs. 0 MAPKK. (B, inset) Immunoblot analysis demonstrating ER protein abundance in PVEC in the absence or presence of the MAPKK plasmid. Cells were treated as in B.

the ER we observe in vascular cells might be due to activation of AF-1 via the MAPK pathway. However, we found that in vascular endothelial and vascular smooth muscle cells: (i) inhibition of MAPK does not block growth factor-induced ER α activation; (ii) the s118a mutant ER is activatable, and (iii) overexpression of MAPKK inhibits ER activation. Several potential mechanisms for the differential effects of MAPK on ER activation in vascular versus nonvascular cells can be envisioned. MAPK substrates may differ between these cell types, such that Cos1 cells may express ER-associated proteins that act as transcriptional activators (79–87) after MAPK-mediated phosphorylation, whereas vascular cells may express different ratios of receptor-associated proteins or associated proteins that act as transcriptional repressors (88–91) after MAPK activation. Alternatively, MAPKK could activate different isoforms of MAPK in vascular cells recruiting distinct downstream effector proteins. Another potentially important difference between the vascular cells used in these studies and the nonvascular cells in which ER α is activated in response to MAPKK is that the nonvascular cells are either tumor-derived (*HeLa* cells) or transformed with large T antigen (Cos1 cells), and thus likely have altered intracellular signaling pathways. Large T antigen, for example, binds several critical cellular proteins (for example, Rb, p53) that themselves interact with known ER α -associated proteins such as the transcriptional integrator p300/CBP (92–94). Indeed, we now have firm evidence that overexpression of large T antigen in VSMC reverses the inhibitory effects of MAP kinase on ER-mediated transactivation, and alters their phenotype to that of Cos1 cells for this pathway (R.H. Karas, manuscript in preparation).

In summary, we have demonstrated hormone-independent transcriptional activation of the estrogen receptor in vascular cells after stimulation with estrogen-deficient serum or EGF. Although this vascular cell activation pathway involves the AF-1 domain of ER α , it does not appear to proceed via activation of the MAPK pathway. These data underscore the cell-specific nature of steroid hormone action, and suggest an alternate estrogen receptor activation pathway in vascular cells that may be relevant in both men and women.

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