

Specific Contribution of Estrogen Receptors on Mitogen-Activated Protein Kinase Pathways and Vascular Cell Activation

Pedro Geraldès, Martin G. Sirois, Jean-François Tanguay

Abstract—Randomized clinical trials have not provided conclusive data that hormone replacement therapy confers cardioprotection against coronary artery disease in postmenopausal women. However, other studies have shown that estrogens can induce beneficial effects on the vasculature. Nevertheless, the specific contribution of estrogen receptors (ERs) α and β on vascular cells is not well characterized. Therefore, we used an antisense gene therapy approach to investigate the contribution of ER α and ER β on p38 and p42/44 mitogen-activated protein kinase (MAPK) activation and on vascular cell responsiveness. Treatment of porcine smooth muscle cells (PSMCs) with platelet-derived growth factor-BB induced p38 and p42/44 MAPK activation and their migration and proliferation. These effects were prevented by pretreatment with 17 β -estradiol (17 β E). The inhibitory effects of 17 β E on PSMCs were abrogated by the downregulation of ER β protein expression with selective ER β mRNA antisense oligomers, whereas the downregulation of ER α had no effect. However, treatment of porcine aortic endothelial cells with 17 β E promoted p38 and p42/44 MAPK phosphorylation and their migration and proliferation. These effects were ER α dependent, as defined by antisense gene therapy. These results suggest that in PSMCs, 17 β E reduces p42/44 and p38 MAPK activity through ER β stimulation, whereas in contrast, in porcine aortic endothelial cells, 17 β E induces p42/44 and p38 MAPK through ER α activation. 17 β E may contribute to the vascular healing process and to the prevention of restenosis by improving reendothelialization through ER α activation and by decreasing smooth muscle cell migration and proliferation through ER β stimulation. (*Circ Res.* 2003;93:399-405.)

Key Words: 17 β -estradiol ■ estrogen receptors ■ vascular cells ■ mitogen-activated protein kinase

Cardiovascular diseases (CVDs) are the leading cause of mortality for postmenopausal women in industrialized countries, accounting for >30% of deaths.¹ Epidemiological studies over the past years have suggested a protective effect of hormone replacement therapy (HRT).² The beneficial effects of estrogens were initially attributed to a decreased level of LDL cholesterol and to an increased level of HDL cholesterol. However, the positive effects of estrogens on lipid profile account for only approximately one third of the observed reduction in the risk of mortality from CVD among HRT users.³ Other studies have demonstrated that estrogens have direct actions on the blood vessel wall.⁴ Physiological concentrations of estrogens can inhibit platelet and monocyte aggregations and stimulate NO production and reendothelialization.⁵ Despite the beneficial effects of estrogens, randomized double-blind studies have reported no overall benefit from HRT.^{6,7} A better understanding of the effects of estrogen on vascular cells might contribute to optimization of the vascular healing process.

Estrogen receptors (ERs) α and β are members of the steroid/thyroid hormone receptor superfamily of ligand-acti-

vated transcription factors.⁸ Estrogen receptors contain DNA and ligand binding domains, which are critically involved in regulating vascular structures and functions.⁹ Receptor-ligand interactions trigger a cascade of events, including dissociation from heat shock proteins, receptor dimerization, phosphorylation, and the association of the hormone-activated receptor with specific regulatory elements in target genes.⁹ ER α and ER β are expressed in vascular endothelial cells (ECs) and smooth muscle cells (SMCs), and their activation may lead to distinct biological activities even though they share many functional characteristics.¹⁰ In a previous study, Pare et al¹¹ showed in ER α and ER β knockout mice that the protective effects of estrogens to vascular injury are ER α dependent. However, the exact contribution played by ER β remains to be clarified. Previous experiments have shown that compared with wild-type mice, ER β -deficient mice exhibit higher vasoconstriction and blood pressure.¹² However, several limitations exist when using knockout animal preparations, whereas the disruption of a gene may influence the response of estrogens.

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Recently, we reported that local delivery of 17 β -estradiol (17 β E) after coronary angioplasty in pigs promotes the vascular healing process by reducing neointimal formation and by improving the reendothelialization process and endothelial NO synthase (eNOS) expression.^{13,14} Classically, ERs act as transcriptional factors by regulating gene expression. However, other specific effects of estrogens may induce nongenomic signaling pathways and may interact with intracellular second messengers, such as mitogen-activated protein kinase (MAPK).¹⁵ Under in vitro conditions, we showed that 17 β E prevents SMC proliferation and migration by inhibiting p42/44 and p38 MAPK activation, whereas it promotes these events in ECs.¹⁶ However, the specific contributions of ER α and ER β on these events remain unknown. We used an antisense gene therapy approach to regulate the protein expression of ER α and ER β and to better understand the specific contribution of each ER. In the present study, we report that 17 β E promotes p42/44 and p38 MAPK phosphorylation through ER α stimulation on ECs, whereas on SMCs, the inhibitory effects of 17 β E on p42/44 and p38 MAPK phosphorylation are mediated by ER β activation.

Materials and Methods

Cell Culture

Porcine aortic ECs (PAECs) and porcine SMCs (PSMCs) were isolated from freshly harvested aortas, cultured, and characterized as described previously.¹⁶ PAECs and PSMCs were used between passages 3 and 8.

Antisense Oligonucleotide Gene Therapy

To distinguish the role played by ER α and ER β in the migration and proliferation of PSMCs and PAECs as well as in the activation of p38 and p42/44 MAPKs, we treated the cells with antisense oligonucleotide sequences complementary to porcine ER α and ER β mRNA (GenBank accession Nos. Z37167 and AF164957, respectively). A total of four different antisense oligodeoxyribonucleotide phosphorothioate sequences were used: two targeted porcine ER α mRNA sequences (antisense 1 [AS1-ER α], 5'-CTC GTT GGC TTG GAT CTG-3'; antisense 2 [AS2-ER α], 5'-GAC GCT TTG GTG TGT AGG-3'), and two targeted porcine ER β mRNA sequences (antisense 1, [AS1-ER β], 5'-GTA GGA GAC AGG AGA GTT-3'; antisense 2 [AS2-ER β], 5'-GCT AAA GGA GAG AGG TGT-3'). Two scrambled oligodeoxyribonucleotide phosphorothioate sequences (scrambled ER α [SCR-ER α], 5'-TGT AGC TCG GTT CTG TCG-3'; scrambled ER β [SCR-ER β], 5'-GAG TGG ACG TGA AGA AGT-3') were also used as negative controls. These sequences were selected because they had no more than three consecutive guanines and had no capacity or minimal capacity to dimerize together and to form hairpins. All sequences were synthesized at the Armand Frappier Institute (Laval, Quebec, Canada). On synthesis, the oligonucleotides were dried, resuspended in sterile water, and quantified by spectrophotometry.

Western Blot Analyses of ER α and ER β Expression and p42/44 and p38 MAPK Phosphorylation

The efficiency and specificity of our antisense oligomers to prevent the expression of targeted proteins were evaluated by Western blot analyses. Culture media of confluent PAECs and PSMCs (100-mm tissue culture plate) were removed, and the cells were rinsed with DMEM (Life Technologies Inc) and trypsinized (trypsin-EDTA, Life Technologies). The cells were resuspended in DMEM containing 5% FBS (Hyclone Laboratories) and antibiotics (penicillin and streptomycin, Sigma), and the cell count was obtained with a Coulter counter Z1 (Coulter Electronics). Cells were seeded at 1×10^6 cells per 100-mm tissue culture plate (Becton-Dickinson) and stimulated

for 24 hours in DMEM, 5% FBS, and antibiotics with or without antisense oligomers (10^{-7} , 5×10^{-7} , and 10^{-6} mol/L). LipofectAmine (5 μ g/mL, Life Technology Inc) was used to improve the cellular uptake of antisense oligomers in PSMCs. Go synchronization was achieved by starving the cells for 48 hours in DMEM, 0.1% FBS, and antibiotics with or without antisense oligomers (10^{-7} , 5×10^{-7} , and 10^{-6} mol/L) added daily. The cells were then grown to confluence for 16 or 72 hours in DMEM, 1% FBS, and antibiotics with or without antisense oligomers (10^{-7} , 5×10^{-7} , and 10^{-6} mol/L) added daily and starved for 7 hours in DMEM, 0.1% FBS, and antibiotics with or without antisense oligomers (10^{-7} , 5×10^{-7} , and 10^{-6} mol/L) to induce an upregulation of ER expression. Culture medium was removed, and the cells were rinsed. PSMCs and PAECs were then stimulated with or without 17 β E as previously described.¹⁶ Briefly, PSMCs were incubated on ice in DMEM with or without 17 β E (10^{-8} mol/L) for 30 minutes and incubated at 37°C for 30 minutes. Cells were then rinsed, incubated in DMEM with platelet-derived growth factor (PDGF)-BB (10 ng/mL) for 30 minutes on ice, and incubated at 37°C for 5 or 30 minutes. PAECs were incubated on ice in DMEM with or without 17 β E (10^{-8} mol/L) for 30 minutes and then incubated at 37°C for 5 or 30 minutes. Total proteins were isolated by the addition of 500 μ L lysis buffer containing 10 μ g/mL leupeptin (Sigma), 1 mmol/L phenylmethylsulfonyl fluoride (Sigma), 30 μ g/mL aprotinin (Sigma), and 1 mmol/L NaVO₃ (Sigma). Plates were incubated at 4°C for 30 minutes and scraped, and the protein concentration was determined with a protein assay kit (Bio-Rad). Proteins (100 μ g) were separated by a 10% gradient SDS-PAGE (Protein II kit, Bio-Rad) and transblotted onto 0.45- μ m polyvinylidene difluoride membranes (Millipore Corp). The membranes were blocked in 5% Blotto-TTBS (5% nonfat dry milk [Bio-Rad], 0.05% Tween 20, 0.15 mol/L NaCl, and 25 mmol/L Tris-HCl, pH 7.4) for 1 hour at room temperature with gentle agitation and incubated overnight in 0.5% Blotto-TTBS containing the desired antibody (rabbit polyclonal anti-human ER α or anti-human ER β , 1:5 000 dilution, Santa Cruz Biotechnology, Santa Cruz, Calif, or rabbit polyclonal anti-phospho-p42/44 MAPK, 1:10 000 dilution, or anti-phospho-p38 MAPK, 1:5 000 dilution, New England BioLabs). Membranes were washed three times with TTBS and incubated with a horseradish peroxidase goat anti-rabbit IgG antibody (1:10 000 dilution, Santa Cruz Biotechnology) in 0.5% Blotto-TTBS for 30 minutes. Membranes were washed with TTBS, and horseradish peroxidase bound to secondary antibody was revealed by chemiluminescence (Renaissance kit, NEN Life Science Products). Kaleidoscope molecular weight and SDS-PAGE broad-range marker proteins (Bio-Rad) were used as standards. Membranes were stripped with Re-Blot Plus (Chemicon International Inc), and total p42/44 and p38 MAPK expression was performed using the desired antibody (rabbit polyclonal anti-p42/44 MAPK, 1:5 000 dilution, or anti-p38 MAPK, 1:5 000 dilution, New England BioLabs). Digital image densitometry (PDI Bioscience) was performed to determine the relative expression of ER α and ER β proteins. Western blot analyses were performed in triplicate, and results of image densitometry are representative of these experiments.

Mitogenic Assay

Confluent PAECs and PSMCs were rinsed with DMEM and trypsinized. Cells were resuspended in 10 mL DMEM, 5% FBS, and antibiotics, and a cell count was obtained using a Coulter counter Z1. PAECs and PSMCs were initially seeded at 1×10^4 cells per well in 24-well tissue culture plates, stimulated for 24 hours in DMEM, 5% FBS, and antibiotics with or without antisense oligomers (10^{-6} mol/L), and starved for 48 hours in DMEM, 0.1% FBS, and antibiotics with or without antisense oligomers (10^{-6} mol/L daily) for Go synchronization. The cells were stimulated for 72 hours in DMEM, 1% FBS, and antibiotics with or without antisense oligomers (10^{-6} mol/L daily) and with or without 17 β E (10^{-8} mol/L). After trypsinization, cell number was determined using a Coulter counter Z1.

Chemotactic Assay

Cell migration was evaluated using a modified Boyden 48-well microchamber kit (NeuroProbe). Near-confluent PAECs and PSMCs were rinsed with DMEM and trypsinized. Cells were resuspended in DMEM, 5% FBS, and antibiotics, and a cell count was obtained. PAECs and PSMCs were seeded at 2.5×10^5 cells per well in 6-well tissue culture plates, stimulated for 24 hours in DMEM, 5% FBS, and antibiotics with or without antisense oligomers (10^{-6} mol/L), and starved for 48 hours in DMEM, 0.1% FBS, and antibiotics with or without antisense oligomers (10^{-6} mol/L daily) and with or without $17\beta\text{E}$ (10^{-8} mol/L). Cells were harvested by trypsinization and resuspended in DMEM, 1% FBS, and antibiotics at a concentration of 2.5×10^4 cells/mL. Fifty microliters of this cell suspension with or without antisense oligomers (10^{-6} mol/L) treated with or without $17\beta\text{E}$ (10^{-8} mol/L) was added to the higher chamber of the modified Boyden chamber apparatus, and the lower chamber was filled with DMEM, 1% FBS, and antibiotics plus the desired concentration of agonist, either $17\beta\text{E}$ (10^{-8} mol/L) or PDGF-BB. The two sections of the system were separated by a porous polycarbonate filter (5- μm pores size), pretreated with a gelatin solution (1.5 mg/mL), and assembled. Five hours after incubation at 37°C , the nonmigrated cells were scraped with a plastic policeman, and the migrated cells were stained using a Quick-Diff solution (Shandon Inc). The filter was then mounted on a glass slide, and migrated cells were counted using a microscope adapted to a video camera to obtain a computer-digitized image. Because of a slight variation of basal cell migration between experiments, data were reported as relative mean migrating cells compared with baseline.

Statistical Analysis

Data are mean $\pm$ SEM. Statistical comparisons were determined by ANOVA, followed by an unpaired Student *t* test with the Bonferroni correction for multiple comparisons. A value of $P < 0.05$ was considered to be significant.

Results

Modulation of ER α and ER β Protein Expression by Antisense Oligonucleotide Gene Therapy

To evaluate the potency of antisense oligonucleotides to prevent the expression of targeted proteins, PSMCs and PAECs were treated either with antisense or scrambled oligomers (as described in Materials and Methods) after 72 hours of stimulation with 1% FBS, and the expression of each receptor was determined by Western blot analysis. In PSMCs, we observed a basal ER α protein expression, which was inhibited by a treatment with antisense oligomers (10^{-6} mol/L) targeting porcine ER α mRNA. The antisense oligomers AS1-ER α and AS2-ER α suppressed ER α protein expression by 88% and 89%, respectively, in PSMCs (Figure 1A). Similar treatment with antisense oligomers (AS1-ER β and AS2-ER β , 10^{-6} mol/L) directed against ER β mRNA also reduced the basal ER β protein expression in PSMCs by 84% and 92%, respectively (Figure 1A). The same series of experiments was conducted in PAECs. The antisense oligomers AS1-ER α and AS2-ER α (10^{-6} mol/L) suppressed PAEC ER α protein expression by 94% and 95%, respectively (Figure 1C), and AS1-ER β and AS2-ER β (10^{-6} mol/L) downregulated ER β protein expression by 90% and 97%, respectively (Figure 1C). Treatment with scrambled oligomers (SCR-ER α and SCR-ER β , 10^{-6} mol/L) had no significant effect on basal ER α and ER β protein expression (Figures 1A and 1C). We also performed a new set of experiments in which we showed that treatment with

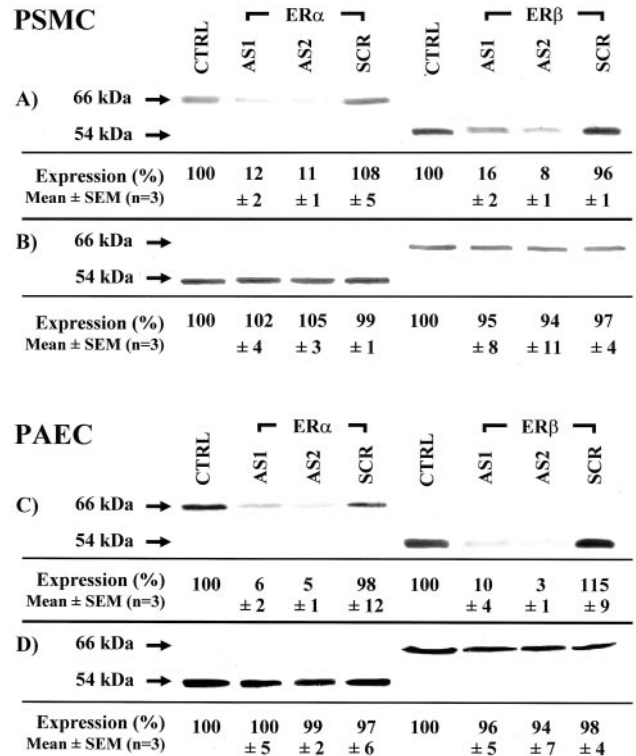


Figure 1. Antisense (AS) regulation of ER α and ER β expression on PSMCs and PAECs. PSMCs and PAECs were seeded at 1×10^6 cells per 100-mm culture plate and grown to confluence. Cells were treated with PBS (CTRL) or either with AS or scrambled (SCR) oligomers, as described in Materials and Methods. In PSMCs and PAECs, treatment with AS oligomers against ER α and ER β mRNA decreased ER α (66-kDa) and ER β (54-kDa) protein expression, respectively (A and C). These effects were specific, inasmuch as the AS oligomers against ER α did not alter ER β protein expression and vice versa (B and D). Image densitometry results are given as relative expression (%) compared with CTRL cells.

PDGF-BB (for 5 hours) did not alter the protein expression of ER α and ER β (data not shown).

To ensure that the antisense oligomers designed to downregulate the expression of ER α would not affect ER β expression and vice versa, we performed additional Western blot analyses to evaluate the specificity of our antisense oligomers. Treatment with antisense oligomers targeting ER α mRNA (10^{-6} mol/L) did not affect ER β basal protein expression, whereas the antisense oligomers directed against ER β mRNA (10^{-6} mol/L) did not alter the basal protein expression of ER α on PSMCs and PAECs (Figures 1B and 1D).

Contribution of ER α and ER β on PSMC Proliferation

Because the expressions of ER α and ER β were specifically blocked by antisense oligomers, we investigated the contribution of both receptors on PSMC proliferation. Stimulation of quiescent PSMCs with DMEM containing 1% FBS for 72 hours increased PSMC proliferation by 88%, from 5432 ± 680 to 10216 ± 546 cells per well (Figure 2). Treatment with $17\beta\text{E}$ (10^{-8} mol/L) prevented by 95% the PSMC proliferation mediated by 1% FBS. Treatment of PSMCs with AS1-ER β and AS2-ER β prevented the inhibitory effects of $17\beta\text{E}$ on PSMC proliferation ($P < 0.05$), whereas the antisense oli-

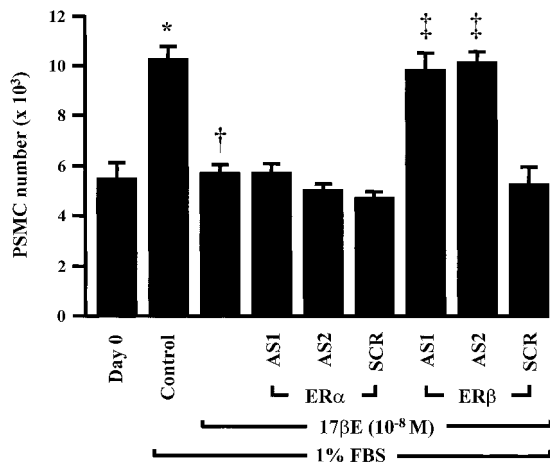


Figure 2. Contribution of ER α and ER β on PSMC proliferation. PSMCs were seeded at 1×10^4 cells per well and stimulated with or without AS oligomers as described in Materials and Methods. Cells were then stimulated with or without 17β E (10^{-8} mol/L), and a cell count was achieved 72 hours after treatment. Values are means of cell counts obtained from 6 wells for each treatment. * $P < 0.05$ compared with day 0; † $P < 0.05$ compared with control (1% FBS); and ‡ $P < 0.05$ compared with cells treated with 17β E (10^{-8} mol/L).

gomers directed against ER α mRNA did not influence 17β E activity (Figure 2). Treatment with scrambled oligomers did not affect the inhibitory activity of 17β E on PSMC proliferation (Figure 2).

Antichemotactic Effect of 17β E on PSMCs: Role of ER α and ER β

Using a modified Boyden chamber assay, we observed that compared with treatment with 1% FBS, treatment with PDGF-BB (10 ng/mL) for 5 hours increased the basal migration of PSMCs by 141% (Figure 3). Treatment with 17β E (10^{-8} mol/L) completely inhibited the chemotactic effect of PDGF-BB (10 ng/mL) (Figure 3). To evaluate the contribution of each ER subtype on the antichemotactic effect of 17β E on PSMCs, the cells were treated with antisense oligomers targeting either ER α or ER β mRNA. Treatment with antisense against ER α mRNA (10^{-6} mol/L) did not alter the effect of 17β E on PSMC migration induced by PDGF-BB. However, treatment with AS1-ER β and AS2-ER β directed against ER β mRNA abolished the antichemotactic effect of 17β E on PSMCs ($P < 0.05$). Treatment with scrambled oligomers did not influence the 17β E antichemotactic activity on PSMCs (Figure 3).

Role of ER α and ER β on p42/44 and p38 MAPK Phosphorylation in PSMCs

Because 17β E can influence p42/44 and p38 MAPK phosphorylation in PSMCs, we evaluated the specific contribution of ER α and ER β in this regard. Treatment of PSMCs with PDGF-BB increased p42/44 (Figure 4A) and p38 (Figure 4B) MAPK phosphorylation, which was reversed by a 30-minute pretreatment with 17β E (10^{-8} mol/L). Treatment of PSMCs with antisense oligomers targeting ER α mRNA did not affect the inhibitory effect of 17β E at preventing the p42/44 and p38 MAPK phosphorylation induced by PDGF-BB. In con-

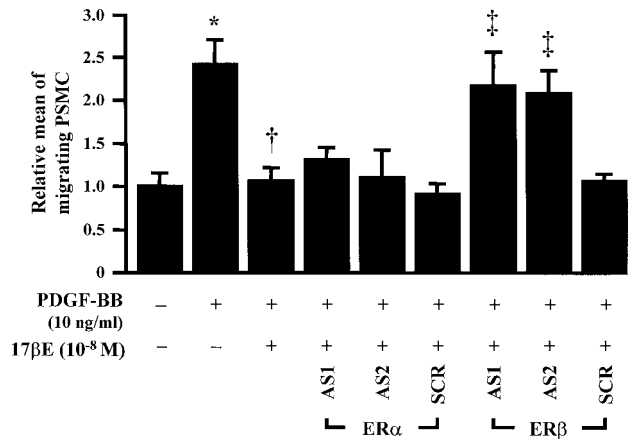


Figure 3. Contribution of ER α and ER β on PSMC migration. PSMCs were trypsinized and resuspended in DMEM; 2.5×10^5 cells per well of a 6-well tissue culture plate were stimulated with or without AS oligomers as described in Materials and Methods. Cells (2.5×10^4) were added to the higher compartment of the modified Boyden chamber apparatus with or without AS oligomers, and the lower chamber was filled with DMEM containing 1% FBS and antibiotics with or without PDGF-BB (10 ng/mL). Five hours after incubation at 37°C , the migrated cells were fixed, stained, and counted using a microscope adapted to a digitized video camera. Values are represented as relative mean of migrating cells from 6 chambers for each treatment. * $P < 0.05$ compared with unstimulated cells; † $P < 0.05$ compared with cells treated with PDGF-BB; and ‡ $P < 0.05$ compared with cells treated with 17β E (10^{-8} mol/L).

trast, a treatment with antisense oligomers directed against ER β mRNA significantly blocked the effects of 17β E on p42/44 and p38 MAPK phosphorylation ($P < 0.05$) (Figures 4A and 4B). In the same series of experiments, scrambled oligomers did not alter 17β E activity on these MAPKs (Figures 4A and 4B).

Contribution of ER α and ER β on PAEC Proliferation

Stimulation of PAECs with DMEM containing 1% FBS increased their proliferation by 83%, from 7427 ± 423 to 13566 ± 1931 cells per well within 3 days. Compared with treatment with 1% FBS, the addition of 17β E (10^{-8} mol/L) enhanced the proliferation of PAECs by 123% (Figure 5). To investigate the selective contribution of ER α and ER β on the positive mitogenic effect of 17β E on endothelial cells, PAECs were treated with antisense oligomers targeting ER α or ER β mRNA. AS1-ER α and AS2-ER α significantly reduced the mitogenic effects of 17β E by 80% and 100%, respectively ($P < 0.05$). Treatment with antisense oligomers directed against ER β mRNA failed to alter the mitogenic activity of 17β E on PAECs. Again, PAEC proliferation induced by 17β E was not influenced by treatments with scrambled antisense oligomers (Figure 5).

Antichemotactic Effects of 17β E on PAECs: Role of ER α and ER β mRNA

Compared treatment with 1% FBS, treatment of PAECs with 17β E (10^{-8} mol/L) for 5 hours promoted their migration by 363% ($P < 0.05$) (Figure 6). Treatment with antisense oligomers (10^{-6} mol/L) directed against ER α mRNA prevented the chemotactic activity of 17β E (10^{-8} mol/L) on PAECs by

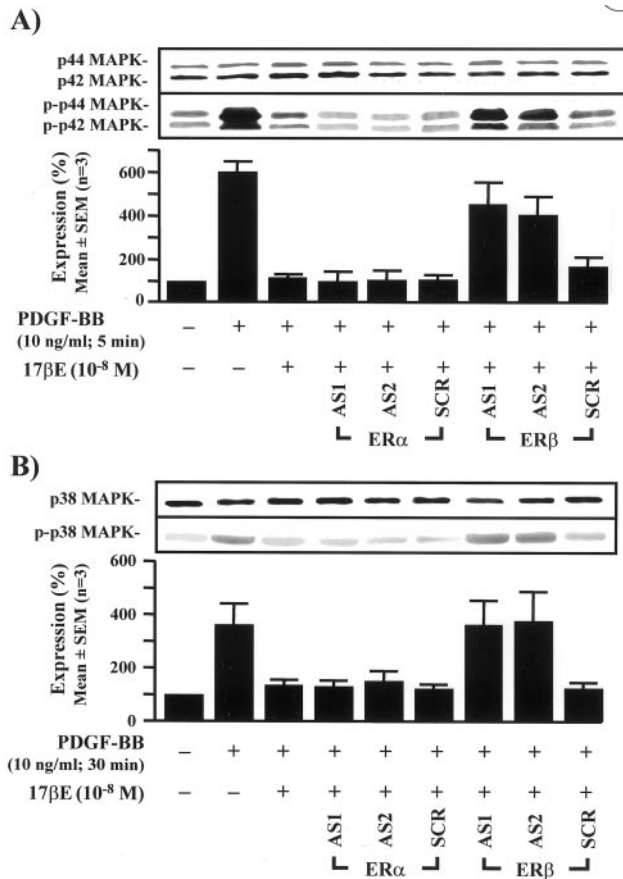


Figure 4. Contribution of ER α and ER β on p42/44 and p38 MAPK activation in PSMCs. PSMCs were seeded at 1×10^6 cells per 100-mm culture plate and grown to confluence. Cells were treated either with AS or SCR oligomers as described in Materials and Methods. Cells were then treated with or without 17 β E (10^{-8} mol/L) for 30 minutes and stimulated for 5 minutes (p42/44 MAPK, A) or 30 minutes (p38 MAPK, B) with PDGF-BB. p-p42 MAPK indicates phospho-p42 MAPK; p-p44 MAPK, phospho-p44 MAPK. Proteins were detected by Western blot analyses. Image densitometry results are given as relative expression (%) compared with control PBS-treated cells.

75% and 76%, respectively ($P < 0.05$) (Figure 6), whereas the inhibition of ER β protein expression did not prevent the 17 β E activity on PAECs (Figure 6). Treatment with scrambled oligomers did not alter the chemotactic activity of 17 β E (Figure 6).

Role of ER α and ER β on p42/44 and p38 MAPK Phosphorylation in PAECs

We have previously demonstrated that 17 β E induces a marked increase of p42/44 and p38 MAPK phosphorylation in PAECs. To determine the contribution of ER α and ER β on these intracellular mechanisms, PAECs were treated with antisense oligomers targeting ER α or ER β mRNA. PBS-treated PAECs showed a basal phosphorylation of p42/44 (Figure 7A) and p38 MAPK (Figure 7B). Stimulation with 17 β E (10^{-8} mol/L) for 5 minutes increased p42/44 MAPK phosphorylation by 317%, and 30 minutes of stimulation with 17 β E increased p38 MAPK phosphorylation by 254%. Treatment of PAECs with AS1-ER α and AS2-ER α prevented the

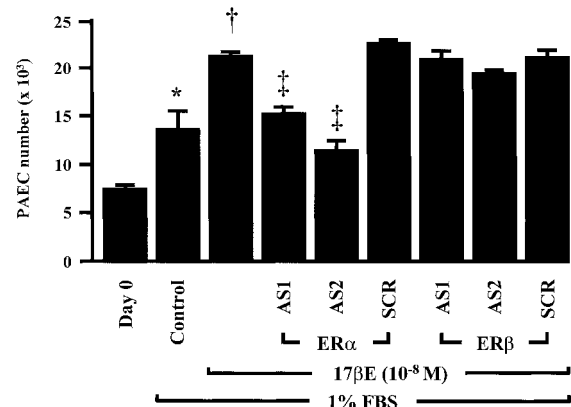


Figure 5. Contribution of ER α and ER β on PAEC proliferation. PAECs were seeded at 1×10^4 cells per well and stimulated with or without AS oligomers as described in Materials and Methods. Cells were then stimulated with or without 17 β E (10^{-8} mol/L), and a cell count was achieved 72 hours after treatment. Values are means of cell counts obtained from 6 wells for each treatment. * $P < 0.05$ compared with day 0; † $P < 0.05$ compared with control (1% FBS); and ‡ $P < 0.05$ compared with cells treated with 17 β E (10^{-8} mol/L).

p42/44 and p38 MAPK phosphorylation induced by 17 β E (Figures 7A and 7B). In contrast, treatment with antisense oligomers targeting ER β mRNA did not significantly reduce the p42/44 and p38 MAPK phosphorylation mediated by 17 β E. Treatment with scrambled oligomers did not influence 17 β E activity on p42/44 and p38 MAPK phosphorylation (Figures 7A and 7B).

Discussion

Previous studies have demonstrated that the disruption of ER α in mice reduces the cardioprotective effects of estrogens

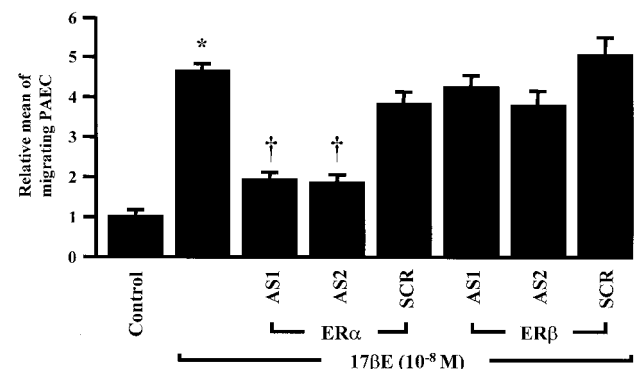


Figure 6. Contribution of ER α and ER β on PAEC migration. PAECs were trypsinized and resuspended in DMEM; 2.5×10^5 cells per well of a 6-well tissue culture plate were stimulated with or without AS oligomers as described in Materials and Methods. Cells (2.5×10^4) were added to the higher compartment of the modified Boyden chamber apparatus with or without AS oligomers, and the lower chamber was filled with DMEM containing 1% FBS and antibiotics with or without 17 β E (10^{-8} mol/L). Five hours after incubation at 37°C, the migrated cells were fixed, stained, and counted using a microscope adapted to a digitized video camera. Values are represented as relative mean of migrating cells per mm² from 6 chambers for each treatment. * $P < 0.05$ compared with unstimulated cells; † $P < 0.05$ compared with cells treated with 17 β E (10^{-8} mol/L).

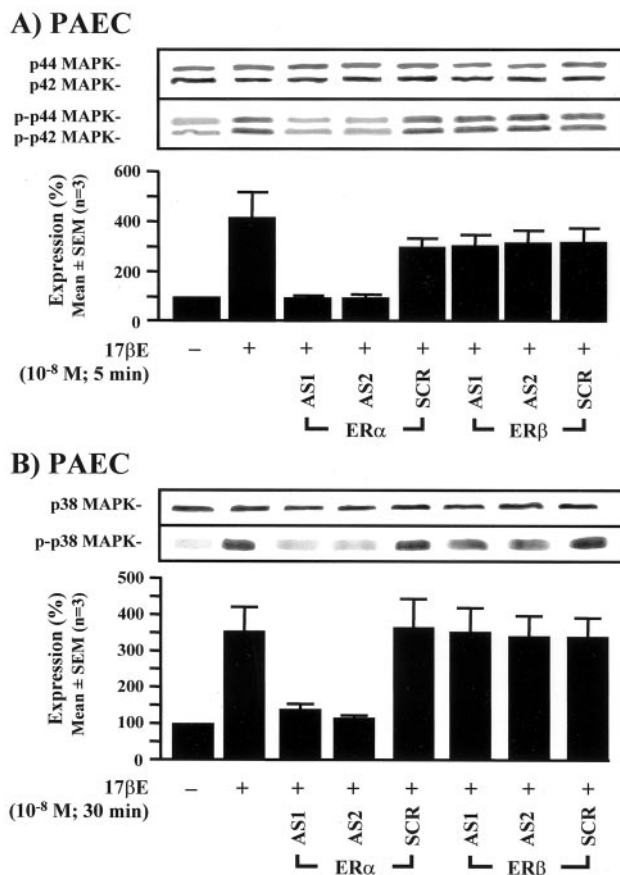


Figure 7. Contribution of ER α and ER β on p42/44 and p38 MAPK activation in PAECs. PAECs were seeded at 1×10^6 cells per 100-mm culture plate and grown to confluence. Cells were treated either with AS or SCR oligomers as described in Materials and Methods. Cells were then treated with or without 17 β E (10^{-8} mol/L) for 5 minutes for p42/44 MAPK activation (A) or 30 minutes for p38 MAPK stimulation (B). Proteins were detected by Western blot analyses. Image densitometry results are given as relative expression (%) compared with control PBS-treated cells.

on restenosis.¹¹ However, other investigators have indicated that ER β , the major ER expressed within the vasculature, might contribute to the beneficial effects of estrogens.¹⁷ Previously, we demonstrated that local delivery of 17 β E on a porcine coronary angioplasty reduces restenosis by improving the reendothelialization process, eNOS expression, and vascular healing.^{13,14} In addition, we showed under in vitro conditions that the beneficial effects of 17 β E on restenosis may be explained by a reduction of PSMC p38 and p42/44 MAPK phosphorylation, migration, and proliferation combined with a positive effect of these mechanisms in PAECs.¹⁶ To the best of our knowledge, the specific contribution of each ER (ER α and ER β) on MAPK phosphorylation and vascular cell migration and proliferation remained unknown. In the present study, we demonstrated that these effects of 17 β E on PAECs are mediated through ER α activation, whereas in PSMCs, 17 β E activities are mediated through ER β stimulation.

Regulation of ER α and ER β Protein Expression by Antisense Gene Therapy

We used an antisense gene therapy approach to prevent selectively the protein expression of ER α or ER β , which allowed us to evaluate separately the contribution of ER α and ER β on intracellular pathways in native ECs and SMCs. Other investigators have used antisense gene therapy to decrease brain ERs.¹⁸ In their experiments, the intraventricular infusion of antisense decreased ER protein expression by 65% at 6 hours after infusion. In the present study, we observed that treatment of PSMCs or PAECs with selective antisense oligomers (10^{-6} mol/L) for 4 days decreased ER α and ER β protein expression up to 97% (Figure 1). ER α and ER β can form homodimers and heterodimers in living cells.¹⁹ By downregulating ER α or ER β , we observed that 17 β E can still induce selective effects on vascular cells, suggesting that heterodimerization is not necessarily required in the biological activities studied.

Biological Activities of 17 β E Are Mediated Through ER β in PSMCs

Usually activated by growth factors and cytokines, SMC proliferation and migration remain important targets for the prevention of in-stent restenosis. Many studies have indicated that estrogens prevent restenosis formation by inhibiting SMC proliferation and migration after balloon injury. We have previously demonstrated that local delivery of 17 β E prevents restenosis on an angioplasty.¹³ In the present study, we observed that treatment with 17 β E (10^{-8} mol/L) inhibits the PSMC migration and proliferation induced by PDGF-BB. In addition, the downregulation of ER β protein expression reduced the inhibitory effects of 17 β E on PSMC proliferation and migration. Our results support other studies suggesting that gene knockout of ER β leads to hyperproliferative disease.²⁰ Recently, we have reported that treatment of PSMCs with 17 β E reduces the p42/44 and p38 MAPK phosphorylation induced by PDGF-BB.¹⁶ To further evaluate the contribution of ER α and ER β on PSMCs, we demonstrated that treatment with antisense oligomers targeting ER β mRNA abrogates the inhibitory effects of 17 β E on the p42/44 and p38 MAPK phosphorylation mediated by PDGF-BB. These results support previous observations that ER β may be responsible for abnormal vascular contraction, ion channel dysfunction, and hypertension in mice deficient in ER β .¹² Lindner et al²¹ have also demonstrated that ER β mRNA expression is induced after vascular injury, supporting a direct contribution of this receptor in the vascular effects of estrogen. In contrast to ER β , the absence of ER α protein expression did not influence the inhibitory effects of 17 β E on p42/44 and p38 MAPK phosphorylation in PSMCs.

ER α Activation by 17 β E Induces MAPK Phosphorylation in PAECs

Various conditions, such as hypercholesterolemia, hypertension, inflammation, and estrogen deficiency, have been associated with endothelial dysfunction.⁴ Vessel wall impairment may contribute to the development of atherosclerosis and CVD. Several animal and in vitro studies have shown that estrogens improve endothelial function. We have demon-

strated that local delivery of 17 β E improves vascular healing and reendothelialization by promoting EC proliferation and migration and eNOS expression. However, the respective contribution of ER α and ER β to these effects of 17 β E has not been specifically evaluated. In the present study, we showed that the beneficial effects of 17 β E on PAEC migration and proliferation are mediated through ER α stimulation. Our results are in agreement with the study of Bouchet et al,²² who observed that ER α is required for estrogen-accelerated reendothelialization in an electric injury model. Estrogens can also interact with the MAPK pathway,²³ and we have previously demonstrated that 17 β E significantly induces p42/44 and p38 MAPK activation on ECs.¹⁶ In the present study, we showed that the inhibition of ER α protein expression reduces p42/44 and p38 MAPK phosphorylation induced by 17 β E. These results support previous work demonstrating a strong relationship between ER α activation by estrogens and MAPK activity in breast cancer cells.²⁴ Furthermore, our results confirm that the principal actions of estrogen on ECs are not mediated through ER β . Ihionkhan et al²⁵ have postulated that estrogens upregulate ER α expression in ECs, supporting an important role for ER α in the biological effects of 17 β E on the endothelium.

In conclusion, the properties of 17 β E to promote p38 and p42/44 MAPK activation and the migration and proliferation of PAECs are directly mediated through ER α stimulation. In contrast, 17 β E inhibits these same events in PSMCs, which are mediated through ER β activation. Our results suggest that in different vascular cell types but on the same mechanisms, the effects of 17 β E are not mediated through the same ER, which may explain the distinct biological activity of estrogens. The present study provides new insight into our understanding of the specific contribution of estrogens to the vascular healing process.

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