Estrogen Regulation of Endothelial and Smooth Muscle Cell Migration and Proliferation
Role of p38 and p42/44 Mitogen-Activated Protein Kinase

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Objective—Restenosis is a major limitation of percutaneous coronary intervention. Migration and proliferation of vascular cells remain a cornerstone in neointimal formation. The cardioprotection of estrogen is well recognized, but the intracellular mechanisms related to these beneficial effects are not completely understood.

Methods and Results—We investigated the effects of 17β-estradiol (17βE) on mitogen-activated protein kinase (MAPK) activity and the migration and proliferation of porcine aortic endothelial cells (PAECs) and porcine smooth muscle cells (PSMCs). Treatment with 17βE (10⁻⁸ mol/L) abrogated p38 and p42/44 MAPK phosphorylation mediated by platelet-derived growth factor-BB as well as the migration and proliferation of PSMCs. In contrast, treatment with 17βE (10⁻⁸ mol/L) induced the phosphorylation of p38 and p42/44 MAPK and the migration and proliferation of PAECs. Interestingly, the effects of 17βE on PSMCs and PAECs were reversed by selective estrogen receptor antagonists (tamoxifen, 4-OH-tamoxifen, and raloxifen). These results suggest that in PSMCs, 17βE inhibits chemotactic and mitogenic effects of platelet-derived growth factor-BB as well as p38 and p42/44 MAPK phosphorylation. In contrast, 17βE promotes in PAECs the phosphorylation of p42/44 and p38 MAPK as well as the migration and proliferation of these cells.


Key Words: 17β-estradiol ■ smooth muscle ■ endothelium ■ mitogen-activated protein kinase

Restenosis, occurring in 20% to 40% of patients, is currently the primary limitation of percutaneous transluminal coronary angioplasty.1 Estrogens play an important role in bone maintenance, in the cardiovascular system, and in the growth, differentiation, and biological activity of various tissues.2 Moreover, numerous in vivo studies in various animal models have demonstrated that the neointimal formation induced after balloon injury is increased in the absence of estrogen but is decreased in its presence.3 The protective effects of 17β-estradiol (17βE) are related to favorable changes in the plasma lipid profile,4 to the inhibition of vascular smooth muscle cell (VSMC) proliferation5 and migration,6 to the relaxation of coronary vessels through endothelial NO synthase (eNOS) activity,7 and to the reduction of platelet and monocyte aggregation,8 tumor necrosis factor-α release,9 and extracellular matrix synthesis.10 We have shown that local delivery of 17βE reduces the neointimal thickness produced by coronary balloon injury in a porcine model.11

Estrogen can bind 2 estrogen receptors (ERs), ERα and ERβ, which are expressed in all vascular cell types.12 The classic genomic mechanism, or long-term effect of estrogen on vascular tissues, is dependent on a change in gene expression. Most recently, a second mechanism related to the direct effect of estrogen has been identified.13 Several studies have demonstrated that 17βE can activate many intracellular signaling responses.14 The mitogen-activated protein kinase (MAPK) cascade plays a central role in the cellular signal transduction pathway in response to vascular stimuli.15 Well-characterized subfamilies of the MAPKs, extracellular signal-regulated kinase and p38 MAPK pathways, are involved in chemotactic and mitogenic activity in a variety of cell types.16 These MAPKs are stimulated after arterial injury.17 Therefore, we hypothesized that an acute administration of 17βE may influence these MAPK activities in vascular cells. Estrogens can modulate intracellular events through other ligand receptors18 and reduce neointimal formation after injury in ERαβ knockout mice.19 These results suggest that estrogen may provide a protection against endothelial injury in the absence of ERαβ.

In the present study, we evaluated the activity of 17βE on endothelial and smooth muscle cell (SMC) proliferation and
migration and p42/44 and p38 MAPK phosphorylation. Furthermore, we examined whether the actions of 17βE on p42/44 and p38 MAPK signal transduction pathways are ER-dependent.

**Methods**

**Cell Culture**

Porcine aortic endothelial cells (PAECs) and porcine SMCs (PSMCs) expressing ERα and ERβ were isolated from freshly harvested aortas and cultured in DMEM (Life Technologies Inc) containing 5% FBS (HyClone Laboratories) and antibiotics (penicillin and streptomycin, Sigma Chemical Co). PAECs were characterized by their cobblestone monolayer morphology. PSMCs were characterized by anti-smooth muscle α-actin monoclonal antibodies and by specific morphology for SMCs. PAECs and PSMCs between the third and eighth passages were used.

**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 with Coulter counter Z1 (Coulter Electronics). PAECs and PSMCs were initially seeded at 1.5×10^4 cells per well of 24-well tissue culture plates (Becton-Dickinson), stimulated for 24 hours in DMEM, 5% FBS, and antibiotics, and starved for 48 hours in DMEM, 0.1% FBS, and antibiotics. The initial cell number for growth was determined by using a Coulter counter. The cells were stimulated for 72 hours in DMEM, 1% or 5% FBS, and antibiotics with or without 17βE (10^-7 mol/L), Tam (10^-7 mol/L), Tam (10^-8 mol/L), or Ral (10^-7 mol/L). ER antagonists were added 5 minutes before 17βE. Total proteins were prepared by the addition of 500 μL lysis buffer containing phenylmethylsulfonyl fluoride (1 mmol/L), leupeptin (10 μg/mL), aprotinin (30 μg/mL), and NaVO₃ (1 mmol/L, Sigma). Proteins were separated on SDS-PAGE gels, transferred to polyvinylidene difluoride membranes (Millipore Corp), and blotted with antibodies (anti-γ-tubulin or anti-actin) before detection with enhanced chemiluminescence. Data are mean ± SEM. Statistical comparisons were performed by ANOVA, followed by an unpaired Student t test. A value of P<0.05 was considered significant.

**Western Blot Analysis of p38 and p42/44 MAPK Phosphorylation**

Confluent PAECs and PSMCs were starved for 7 hours in DMEM and antibiotics. Culture medium was removed, and the cells were rinsed twice with ice-cold DMEM. PAECs were incubated on ice in DMEM with or without 17βE (10^-8 mol/L) for 30 minutes, incubated at 37°C for 5, 10, 15, and 30 minutes, and then brought back on ice. Cells were then rinsed with cold DMEM, incubated on ice in DMEM, BSA (1 mg/mL), and PDGF-BB (10 ng/mL) for 30 minutes, incubated at 37°C for 5 minutes, and then brought back on ice. PAECs were incubated on ice in DMEM with or without 17βE (10^-8 mol/L) for 30 minutes, incubated at 37°C for 5, 10, 15, and 30 minutes, and then brought back on ice. For all the experiments on PSMCs and PAECs, Tam (10^-7 mol/L), 4-OHT (10^-7 mol/L), or Ral (10^-7 mol/L) was added 5 minutes before 17βE treatment. Total proteins were prepared by the addition of 500 μL lysis buffer containing phenylmethylsulfonyl fluoride (1 mmol/L), leupeptin (10 μg/mL), aprotinin (30 μg/mL), and NaVO₃ (1 mmol/L, Sigma). Plates were incubated at 4°C for 30 minutes and scraped, and the protein concentration was determined with a protein kit (Bio-Rad). The same protein quantity for each cell type and condition was dissolved in Laemmli buffer, boiled for 5 minutes in reducing conditions, separated by 10% gradient SDS-PAGE (Protean II kit, Bio-Rad), and transblotted onto 0.45-μm polyvinylidene difluoride membranes (Millipore Corp). The membranes were blocked in 5% Blotto-TTBS (containing 5% nonfat dry milk [Bio-Rad], 0.05% Tween 20, 0.15 mol/L NaCl, and 25 mmol/L Tris-HCl, pH 7.5) for 1 hour at room temperature with gentle agitation and incubated overnight at 4°C in 0.5% Blotto-TTBS with the addition of anti-phospho-p38 MAPK (1:1000 dilution, Cell Signaling) or anti-phospho-p42 MAPK (1:1000 dilution, Cell Signaling) affinity-purified antibodies. Membranes were rinsed with TTBS and incubated at room temperature with an anti-rabbit IgG antibody coupled to horseradish peroxidase (dilution 1:10 000 to 1:20 000, 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 for SDS-PAGE. Molecular mass was determined by x-ray film to determine the relative phosphorylation of p42/44 and p38 MAPK. All Western blot analysis was performed in triplicate, and the results of image densitometry are representative of these experiments.

**Statistical Analysis**

Data are mean ± SEM. Statistical comparisons were performed by ANOVA, followed by an unpaired Student t test. A value of P<0.05 was considered significant.

**Results**

**Effects of 17βE on PSMC Proliferation**

First, we evaluated the effect of 17βE on PSMC proliferation. Treatment with 17βE (10^-8 mol/L) abrogated by 88% and 90% the PSMC proliferation mediated by 1% and 5% FBS, respectively (data not shown). Then, we investigated how different estrogen antagonists may interfere with 17βE activity. Treatment of quiescent PSMCs with DMEM and 1% and 5% FBS significantly increased their proliferation from 9894±714 cells per well to 16 258±1441 and 42 500±2889 cells per well, respectively. Treatment with 17βE (10^-8 mol/L) completely inhibited the 1% FBS mitogenic activity (Figure 1). Treatment with 17βE (10^-7 mol/L) completely inhibited the 1% FBS mitogenic activity (Figure 1). The antimitogenic activity of 17βE on PSMCs was reversed by Tam (10^-7 mol/L), 4-OHT (10^-8 mol/L and 10^-7 mol/L), and Ral (10^-7 mol/L) by 75%, 81%, and 100%, respectively (Figure 1). In the absence of 17βE, treatment of PSMCs with these ER antagonists did not alter the mitogenic activity of 1% FBS (data not shown).
These ER antagonists in the absence of 17βE did not modify the effect of PDGF-BB on PSMC migration (data not shown).

**Effects of 17βE on PSMC p42/44 and p38 MAPK Phosphorylation**

Because PDGF-BB can induce SMC p42/44 and p38 MAPK phosphorylation, we investigated whether treatment with 17βE might influence the phosphorylation of these MAPKs mediated by PDGF-BB. Treatment of PSMCs with PDGF-BB induced a rapid and transient phosphorylation of p42/44 MAPK within 5 minutes, which decreased below the basal level within 15 minutes (data not shown). Pretreatment with 17βE (10^-8 mol/L) inhibited time-dependently, with maximum inhibition at 30 minutes, the phosphorylation of p42/44 MAPK induced by 5-minute stimulation with PDGF-BB (Figure 3). Pretreatment with Tam, 4-OHT, or Ral (10^-7 mol/L) 5 minutes before stimulation with 17βE (30 minutes) reversed by 54%, 79% (P<0.05), and 100% (P<0.05), respectively, the inhibitory effect of 17βE on PDGF-BB-mediated p42/44 MAPK phosphorylation (Figure 3).

The same series of experiments was performed on p38 MAPK phosphorylation induced by PDGF-BB. Stimulation of PSMCs with 10 ng/mL PDGF-BB (compared with PBS) induced the phosphorylation of p38 MAPK, which was maximal within 30 minutes as (data not shown). Treatment of PSMCs with 17βE (10^-8 mol/L) before stimulation with PDGF-BB (30 minutes) decreased the phosphorylation of p38 MAPK in a time-dependent manner and produced up to 85% inhibition of phosphorylation at 30 minutes after PDGF-BB treatment (Figure 3). Pretreatment with Tam, 4-OHT, and Ral (10^-7 mol/L) reversed by 51%, 53%, and 32%, respectively, the effect of 17βE (10^-8 mol/L) on p38 MAPK induced by 30-minute stimulation with PDGF-BB (Figure 3). In the absence of PDGF-BB stimulation, treatment with 17βE alone or with ER antagonists alone did not alter the basal phosphorylation of p42/44 and p38 MAPK on PSMCs (data not shown).

**Effects of 17βE on PAEC Proliferation**

Quiescent PAECs were stimulated with DMEM and 1% FBS, which raised the basal cell count from 13 328±560 to 24 244±843 cells per well. The addition of 17βE (10^-10 to 10^-7 mol/L) induced a dose-dependent proliferation of PAECs, with a maximum induction at 10^-8 mol/L (data not shown). To investigate how ER antagonists may interfere with the positive effect of 17βE on endothelial cell proliferation, quiescent PAECs were stimulated with DMEM and 1% FBS, which raised the cell count from 10 512±832 to 29 138±870 cells per well in 72 hours. Treatment of PAECs with 17βE (10^-8 mol/L) induced the proliferation of PAECs by 37% over 1% FBS treatment (Figure 4). Pretreatment with Tam, 4-OHT, and Ral (10^-7 mol/L) completely inhibited the 17βE mitogenic activity in PAECs (Figure 4). Treatment of these cells with ER antagonists in the absence of 17βE did not affect the mitogenic effect of 1% FBS (data not shown).

**Effects of 17βE on PAEC Migration**

Compared with treatment with 1% DMEM, treatment with bFGF at 1, 5, and 10 ng/mL dose-dependently induced the
migration of PAECs by 46%, 124%, and 114%, respectively, in 5 hours (P<0.05, data not shown). In another series of experiments, compared with bFGF (10 ng/mL) treatment alone, a combined treatment with 17βE (10⁻⁸ mol/L) significantly stimulated (by 121%) PAEC migration (Figure 5). Pretreatment of PAECs with ER antagonists (10⁻⁷ mol/L) 5 minutes before the addition of 17βE completely prevented the chemotactic activity of 17βE (10⁻⁸ mol/L) on PAECs (Figure 5). We also investigated whether the ER antagonists in the absence of 17βE had an effect on bFGF chemotactic activity. The ER antagonists did not alter the chemotactic activity of bFGF (10 ng/mL) on PAECs (data not shown).

**Effects of 17βE on PAEC p42/44 and p38 MAPK Phosphorylation**

Considering that 17βE can stimulate the proliferation and migration of PAECs, we evaluated the effect of 17βE on the phosphorylation of p42/44 and p38 MAPK in PAECs. Control (PBS-treated) PAECs showed a basal phosphorylation of p42/44 MAPK. Stimulation with 17βE (10⁻⁸ mol/L) increased time-dependently at 5, 10, 15, and 30 minutes the phosphorylation of p42/44 MAPK by 1122%, 1074%, 1420%, and 1835%, respectively (data not shown). Pretreatment with Tam, 4-OHT, or Ral (10⁻⁷ mol/L) 5 minutes before the addition of 17βE decreased by 36% (P<0.05), 44% (P<0.05), and 66% (P<0.05) the phosphorylation of p42/44 MAPK mediated by a 5-minute treatment with 10⁻⁸ mol/L 17βE (Figure 6). Similar to the results for p42/44 MAPK, treatment of PAECs with 17βE at 10⁻⁸ mol/L (compared with unstimulated [PBS-treated] PAECs) induced a time-dependent phosphorylation of p38 MAPK, with a maximum stimulation at 30 minutes (data not shown). Pretreatment with ER antagonists (10⁻⁷ mol/L) 5 minutes before the addition of 17βE inhibited by 84%, 81% (P<0.05), and 98% (P<0.05) the phosphorylation of p38 MAPK induced by a 30-minute treatment with 17βE at 10⁻⁸ mol/L (Figure 6). In the absence of 17βE stimulation, treatment with the ER antagonists did not alter the basal phosphorylation of PAEC p42/44 and p38 MAPK (data not shown).
Discussion

Several studies have shown that estrogen treatment may have beneficial effects on the cardiovascular system by reducing postinjury neointimal formation in animal carotid arteries and by improving some aspects of endothelial function in postmenopausal women. We have previously demonstrated that a local delivery of 17β-E on a porcine coronary angioplasty reduces the degree of restenosis by up to 50% and improves the reendothelialization, eNOS expression, and vascular healing. In the present study, we observed that treatment with 17β-E stimulates PAECs but reverses PSMC phosphorylation of p42/44 and p38 MAPK. Our results also suggest that these effects of 17β-E are at least partially ER dependent.

Antimitogenic and Antichemotactic Effects of 17β-E in PSMCs

VSMCs contribute to the pathological formation of restenosis by migrating from the media to the intima, proliferating, and depositing extracellular matrix proteins. PDGF, which is secreted from platelets and macrophages recruited at the early inflammatory lesion, has been described as playing an important role in restenosis. In animal experiments, PDGF-BB has been associated with SMC proliferation and migration. In the present study, treatment with 17β-E inhibited the proliferation and migration of PSMCs by stimulating the p38 MAPK pathway, and it decreased the phosphorylation of p42/44 MAPK and p38 MAPK activity. PAECs were pretreated with 17β-E for 5 minutes with Tam, 4-OHT, or Ral and then stimulated with 17β-E for 5 minutes (for p42/44 MAPK) and for 30 minutes (for p38 MAPK). Proteins were detected by Western blot analysis. Image densitometry results are given as relative expression (percentage) compared with PBS-treated cells.

17β-E Promotes Reendothelialization by Increasing the Proliferation and Migration of PAECs

Reendothelialization plays a critical role in restenosis. The improvement of endothelium regeneration will accelerate vascular healing after balloon injury and will reduce neointimal formation. A previous study has noted that the administration of estrogen in healthy young men is associated with enhanced arterial endothelial function. We have shown that local delivery of 17β-E improves reendothelialization and eNOS expression after angioplasty. In the present study, we propose that 17β-E increases reendothelialization by increasing the proliferation and migration of PAECs. Previous studies have demonstrated that estrogen can rapidly induce ER-mediated rapid activations of p42/44 and p38 MAPK activity. PAECs were pretreated with ER antagonists before treatment with 17β-E. We showed that pretreatment of PSMCs with ER antagonists reversed the effect of 17β-E, ie, prevention of the phosphorylation of p42/44 and p38 MAPK induced by PDGF-BB. These ER antagonists are also selective ER modulators and may have potential positive effects on cardiovascular diseases. In contrast, we have not observed any beneficial effect of Tam, 4-OHT, or Ral alone in the prevention of PSMC proliferation and migration and MAPK activity mediated by PDGF-BB.
17βE. Interestingly, treatment of PAECs with ER antagonists alone did not affect the proliferation, migration, or MAPK activity of these cells.

In conclusion, an acute administration of 17βE activates p42/44 and p38 MAPK, thus promoting the proliferation and migration of PAECs, and in contrast, it inhibits these events in PSMCs. Our results suggest that the beneficial effects of treatment with 17βE on restenosis may be explained by a reduction of PSMC migration and proliferation combined with positive endothelial cell migrating and proliferating activity. These effects of 17βE appear to be at least partially ER dependent.

References
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