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Estradiol Attenuates Directed Migration of Vascular Smooth Muscle Cells *in Vitro*

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Although the cardiovascular benefits of the hormone estrogen are at least, in part, mediated by its antiproliferative effect on vascular smooth muscle, its action on the migration of these cells is unknown. To explore this relationship, female rat aortic smooth muscle cells were grown in hormone-free medium, and the effect of various concentrations of β-estradiol on directed cellular migration was measured in vitro using a microwell Boyden chamber apparatus. Migration of smooth muscle cells to the known chemoattractants platelet-derived growth factor, insulin-like growth factor-1, and fibronectin (all at peak doses for migratory activity) was attenuated by β-estradiol (0.5 to 10 ng/ml) in a concentration-dependent manner relative to control cells treated with vehicle (0.01% ethanol). This response was insensitive to pretreatment with indomethacin and was stereospecific (17α-estradiol lacked response). Like β-estradiol, the synthetic estrogen diethylstilbestrol attenuated directed smooth muscle cell migration whereas the male hormone testosterone was ineffective. Additional studies showed that β-estradiol-mediated suppression of migration was inhibited by the anti-estrogen ICI 164,384 and the gene transcription inhibitor actinomycin D. These are the first results demonstrating a reduction in directed smooth muscle cell migration by β-estradiol. The mechanism of this estrogen-mediated response appears to involve conventional estrogen receptors. (Am J Pathol 1996, 148:969–976)

Although the clinical cardiovascular benefits of estrogen replacement therapy in postmenopausal women have become apparent,[1–5] the mechanisms whereby estrogen affects target cells of the blood vessel wall are poorly understood. Experimental studies suggest an anti-atherogenic effect of estrogen independent of its action on cardiovascular risk factors associated with atherosclerosis.[6–8] Estrogen-binding receptors have been identified on both vascular smooth muscle cells (SMCs)[9–12] and endothelium,[13] consistent with the possibility that vascular function, at least in part, is under direct hormonal control.

Estrogen is thought to limit atherosclerosis and neointimal formation after balloon arterial injury due to its antiproliferative effect on vascular smooth muscle.[14,15] Intimal migration of arterial SMCs may also play an integral role in the development of arterial lesions after vascular injury.[16,17] It is therefore conceivable that estrogen could decrease SMC migration as another potential vasculoprotective mechanism in women. Thus, the aim of the present study was to assess the *in vitro* effects of β-estradiol on SMC migration and to determine whether these responses were attributed to genomic or nongenomic actions of estrogen.

Materials and Methods

Reagents and Hormones

Estradiol-17β, estradiol-17α, dithethylstilbestrol, testosterone, and indomethacin were obtained from

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Sigma Chemical Co., (St. Louis, MO). The mouse monoclonal antibody to human estrogen receptor was purchased from Affinity Bioreagents (Neshanic Station, NJ). Fetal bovine serum (FBS) and medium 199 (M199) were purchased from Gibco Laboratories (Grand Island, NY). Charcoal/dextran-treated FBS was obtained from Hyclone (Logan, UT). Recombinant human platelet-derived growth factor (PDGF-BB) and insulin-like growth factor (IGF-1) were purchased from Collaborative Research (Bedford, MA). Purified fibronectin from rat plasma was from Biomedical Technologies (Stoughton, MA). The anti-estrogen, *N*-(*n*-butyl)-11-[3,17β-dihydroxyestra-1,3,5(10)-trien-7α-yl]*N*-methylundecanamide (ICI 164,384) was kindly provided by M. Y. Farhat (Department of Physiology, Georgetown University, Washington, DC). Actinomycin D was purchased from Boehringer Mannheim (Indianapolis, IN).

Solutions of hormones were prepared fresh in absolute ethanol. Indomethacin (10 mmol/L) was dissolved in 100 mmol/L Na_2CO_3 and diluted in phosphate-buffered saline PBS. The specific estrogen antagonist ICI 164,384 was prepared as a stock solution in 100% ethanol and stored at −20°C.

Cell Culture

Rat aortic SMCs were prepared from adult female Sprague-Dawley rats (Hazelton, PA) weighing 200 to 250 g by the explant method of Cole et al.[18] Briefly, aortic explants were obtained from the thoracic aorta, the adventitial layer was dissected away with a scalpel blade, and the endothelium was removed with a cotton swab. The tissue explants were maintained in medium 199 supplemented with 10% FBS, 4 mmol/L L-glutamine, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate in a humidified atmosphere of 5% CO_2/95% air. The medium was replaced every third day. SMCs were allowed to grow out from the tissue, which was subsequently removed after 9 to 11 days. After confluency, the cells were subcultured using 0.05% trypsin and 0.53 mmol/L EDTA. SMCs were characterized by immunofluorescence using a monoclonal antibody directed against α-SMC actin (Sigma).[19] Negative staining for factor VIII using a polyclonal anti-von Willebrand factor (vWF; Atlantic Antibodies, Stillwater, MN) confirmed that the cultures were not contaminated with endothelium.[20] Cells were routinely subcultured at a 1:5 ratio and used between passages 4 and 6.

Smooth muscle cells were cultured in hormone/phenol-free media for at least 48 hours before the experiments. Because phenol functions as a weak estrogen antagonist,[21] growth medium was prepared using phenol-free M199 (Gibco) containing 10% charcoal/dextran-treated fetal bovine serum. Serum treated in this manner lacks several biologically active molecules in addition to endogenous estrogen.[22]

Cell Migration Assay

Migration of SMCs was assayed in a 48-well chemotaxis chamber (Neuro Probe, Cabin John, MD). Briefly, cultured SMCs were trypsinized and suspended at a concentration of 5.0×10^5 cells/ml in phenol-free M199 with 10% charcoal/dextran-treated FBS. In the standard assay, a 50-μl volume of SMC suspension was placed in the upper chamber and 25 μl of M199 containing a migration factor (PDGF-BB, IGF-1, or fibronectin) was placed in the lower chamber. Chemoattractants were diluted in M199. β-Estradiol (0.1 to 10 ng/ml), other test agents (hormones/inhibitors), or vehicle (0.01% ethanol) were added to both the upper and lower chambers at the same concentrations. Assays were performed in which the total number of cells migrating through gelatin-coated polyvinylpyrrolidone-free polycarbonate membranes (8-μm pores; Nucleopore Corp., Pleasanton, CA) were quantified. For assay of SMC migration, chambers were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air for 4 hours. After incubation, nonmigrating cells were wiped off the filters. The filters were then fixed in methanol and stained with Gill-3 hematoxylin (Shandon, Pittsburgh, PA). Migrated cells were counted using image analysis software (IP Lab Spectrum, Signal Analytics Corp., Vienna, VA). Random migration was assessed by quantifying cell migration in response to medium alone. The data are presented as mean ± SEM of triplicate assays and represent the total number of migrating SMCs per four high power fields (×200 HPF).

For experiments testing the effects of the antiestrogen ICI 164,384 and the RNA polymerase inhibitor actinomycin D, cell were pretreated in suspension at the indicated concentrations for 1 hour and 30 minutes, respectively, at 37°C. SMCs were then prepared for migration studies as described above. For cyclooxygenase inhibitor studies, cells in suspension were incubated with 5 μmol/L indomethacin for 20 minutes before migration assays.

Cell Adhesion Assay

Cell adhesion was measured similarly to the methods of Shimokado et al.[23] Briefly, SMCs in suspen-

sion (10^5 cells/well) were added to gelatin-coated 24-well plates. β-Estradiol (5 ng/ml) was added directly to the wells; control wells received 0.01% ethanol. After various times (30 minutes and 1, 2, and 4 hours) nonadherent cells were removed by washing (three times) with Hanks' balanced salt solution. Cells were trypsinized and counted with a Coulter counter (model ZM). Experiments were performed in triplicate.

Data Analysis

Random migration of cells determined in the absence of stimulatory agonists was minimal, ranging from three to seven cells per HPF. The data presented were not adjusted for random migration. The final results are reported as mean ± SEM of the total migrating SMCs. Group comparisons were made using one-way analysis of variance, and statistical differences were determined by Scheffé's test (StatView for the Macintosh, Abacus Concepts, Berkeley, CA). Differences with a value of $P < 0.05$ were considered statistically significant.

Results

Inhibitory Effects of β-Estradiol on Directed Smooth Muscle Migration

PDGF-BB, IGF-1, and fibronectin are known chemoattractants of vascular SMCs and their effects on migration of SMCs *in vitro* are shown in Figure 1. Maximal migration activity was observed at 10 ng/ml for both PDGF-BB and IGF-1 (PDGF-BB response being approximately twice that of IGF-1) whereas maximal migration with fibronectin occurred at 10 μg/ml, an approximate 1000-fold increase in concentration relative to the response to either PDGF-BB or IGF-1.

Responses to varying concentrations of β-estradiol were assessed in cells maximally stimulated with either PDGF-BB, IGF-1, or fibronectin. A β-estradiol-mediated concentration-dependent decrease in SMC migration was observed in cells maximally stimulated with all three selected polypeptides (Figure 2). Significant suppression of migration occurred at β-estradiol concentrations of 1 ng/ml or more and peaked at concentrations of 10 ng/ml. Migration activity at 10 ng/ml β-estradiol in PDGF-BB-, IGF-1-, and fibronectin-stimulated SMCs was 48, 55, and 31%, respectively, of control wells. β-Estradiol alone in concentrations up to 10 ng/ml did not affect random migration or cell adhesion relative to SMCs

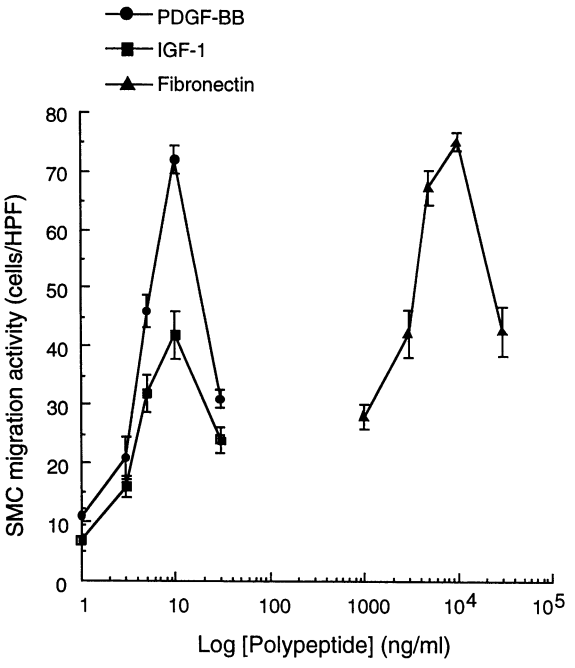

Figure 1. *Graph showing a dose-dependent increase in rat aortic SMC migration activity by the chemoattractant polypeptides PDGF-BB, insulin-like growth factor-1, and rat plasma fibronectin. SMC migration was examined in the micro-well Boyden chamber, as described in Materials and Methods. SMC suspensions were placed in the upper chamber, and M199 containing the indicated concentrations of the chemoattractant polypeptide was placed in the lower chamber. The incubation time for all experiments was 4 hours. Each point represents the increase in migration activity as mean ± SEM of triplicate observations for the number of cells observed per HPF (×200) from three separate experiments.*

treated with 0.01% ethanol (data not shown). In addition, β-estradiol-mediated suppression of PDGF-induced SMC migration was unchanged by pretreatment with 5 μmol/L indomethacin (number of cells per HPF at maximal β-estradiol dose was 46 ± 0.7 *versus* 48 ± 0.8 ($P = 0.3$) with and without indomethacin, respectively).

Effect of 17α-Estradiol, Testosterone, and Diethylstilbestrol on Smooth Muscle Migration

To determine the stereoselectivity and specificity of the β-estradiol-mediated response, the effects of 17α-estradiol, the nonsteroidal estrogen diethylstilbestrol, and the androgen testosterone on smooth muscle migration was examined. Unlike β-estradiol, the enantiomer 17α-estradiol at similar concentrations had no effect on cell migration toward PDGF-BB, indicating that the response to estradiol was stereospecific (Figure 3). As with β-estradiol, the synthetic estrogen diethystilbestrol equally suppressed smooth muscle migration whereas testosterone was ineffective (Figure 3).

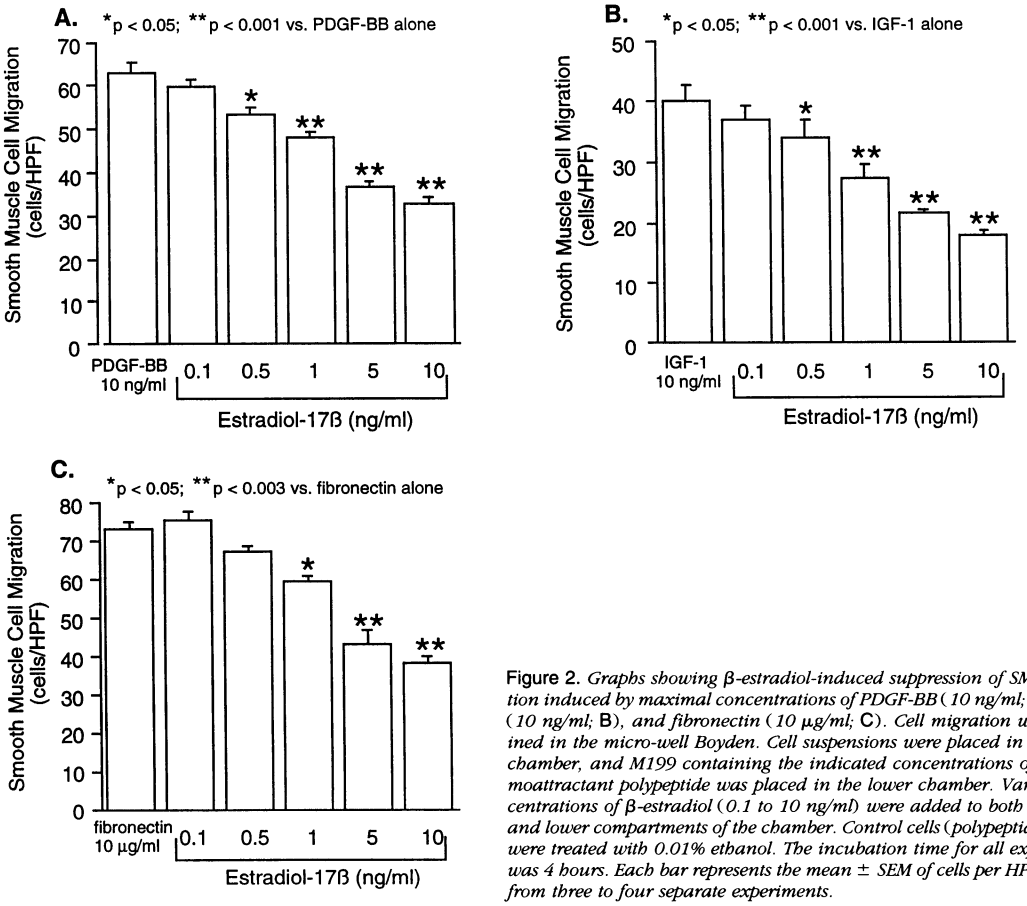

Figure 2. *Graphs showing β-estradiol-induced suppression of SMC migration induced by maximal concentrations of PDGF-BB (10 ng/ml; A), IGF-1 (10 ng/ml; B), and fibronectin (10 μg/ml; C). Cell migration was examined in the micro-well Boyden. Cell suspensions were placed in the upper chamber, and M199 containing the indicated concentrations of the chemoattractant polypeptide was placed in the lower chamber. Various concentrations of β-estradiol (0.1 to 10 ng/ml) were added to both the upper and lower compartments of the chamber. Control cells (polypeptides alone) were treated with 0.01% ethanol. The incubation time for all experiments was 4 hours. Each bar represents the mean ± SEM of cells per HPF (×200) from three to four separate experiments.*

Neutralization of Estrogen-Induced Suppression of Smooth Muscle Migration by the Anti-Estrogen ICI 164,384 or the Gene Transcription Blocker Actinomycin D

To determine whether the response to β-estradiol is receptor mediated, cells were treated with the anti-estrogen ICI 164,384, an agent that decreases estrogen receptor expression[24] (Figure 4A). Cells pretreated with 1.0 μmol/L ICI 164,384 for 1 hour at 37°C before the migration assay did not alter migration responses toward PDGF-BB and resulted in a complete reversal of the inhibitory effect of estrogen.

The importance of gene transcription in mediating suppression of smooth muscle migration was also assessed. Before the migration assay, SMCs were pretreated (30 minutes) with actinomycin D (10 μg/ml) to inhibit RNA polymerase and gene transcription. Pretreatment with actinomycin D had no effect on smooth muscle migration toward PDGF-BB; however, it completely prevented the suppression of migration induced by β-estradiol (Figure 4B).

Discussion

The present series of experiments demonstrates that β-estradiol, at the upper range of physiological concentrations, inhibits *in vitro* directed migration of vascular SMCs. Suppression of cell migration by β-estradiol was observed between 0.5 and 10 ng/ml hormone and was independent of the applied chemotactic stimuli. The response to β-estradiol was insensitive to indomethacin and stereospecific as the enantiomer α-estradiol showed no activity. The inhibitory effects of β-estradiol were reproduced by the synthetic estrogen diethylstilbestrol whereas the androgen testosterone had no significant effect. Reduced migration by β-estradiol appears to be mediated by conventional (gene-activating nuclear) estrogen-estrogen receptor complexes as both the anti-estrogen ICI 164,384 and the gene transcription inhibitor actinomycin D blocked its activity. These data suggest that suppression of directed SMC migration by β-estradiol involves estrogen receptors and gene transcription, providing evidence of a novel genomic action of estrogen.

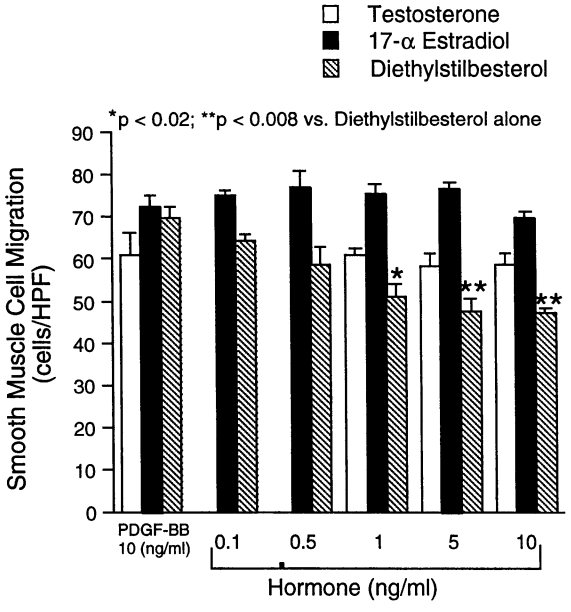

Figure 3. *Graph showing the dose-dependent effects of the synthetic estrogen diethylstilbestrol, 17α-estradiol, and testosterone on SMC migration toward PDGF-BB. Results are expressed as the mean ± SEM of cells per HPF (×200) from four separate experiments. Increasing concentrations of 17α-estradiol and the male hormone testosterone did not effect cell migration induced by PDGF-BB, whereas the effects of 17β-estradiol were mimicked by the synthetic nonsteroidal estrogen diethylstilbestrol.*

Previous experimental studies suggest a protective action of estrogen on atherosclerosis and restenosis independent of its effects on risk factors for coronary heart disease. Therefore, the effects of estrogen are most likely mediated through a direct activation of cells of the arterial wall.[25–26] The presence of estrogen receptors in vascular SMCs suggest that SMCs are indeed estrogen sensitive.[9–12] However, little is known of the functional significance of the expression of estrogen receptors on vascular cells. Thus far, experimental studies have focused only on the effect of estrogen on SMC growth[14,28,29] and not on cell migration, which has been shown in the rat carotid balloon injury model to involve as many as 50% of cells within the intima.[27]

In vivo studies demonstrating an estrogen-induced reduction in neointimal formation in animal models of atherosclerosis and restenosis after balloon arterial injury are well described in the literature.[6–8,15] However, only one study has examined the effects of estrogen on SMC function.[15] In a rabbit model of balloon arterial injury, chronic estrogen therapy (22 to 24 days) reduced intimal hyperplasia by 50 to 70%. Total [^{3}H]thymidine incorporation and DNA content were also significantly decreased in balloon-injured arteries of estrogen-treated animals. Measurement of cell proliferation, however, was performed at 3 days after vascular injury, a time point at which neointimal proliferation is minimal.[30] It is therefore difficult to conclude from these data whether suppression of neointimal formation with estrogen treatment results from decreased SMC proliferation, migration, or both.

A number of polypeptides (PDGF-BB being the most potent) stimulate directed migration of vascular smooth muscle *in vitro*.[31–34] In *in vivo* animal studies, infusion of PDGF-BB after arterial balloon injury accelerates intimal lesion formation with little effect on SMC proliferation.[35–37] These investigations indicate that PDGF may promote directional migration of medial SMCs into the intima after vascular injury.

Chemotactic responses in the presence of estrogen have been previously studied in both polymorphonuclear leukocytes and endothelial cells.[38,39] Unlike its effects on SMC migration, β-estradiol alone was shown to promote chemotaxis in bovine polymorphonuclear leukocytes. In human umbilical vein endothelial cells, β-estradiol stimulated angiogenic activity and enhanced migration toward endothelial cell growth supplement.[39] Similar to our findings, the estrogenic effects in vascular endothelium appear to involve activation of estrogen receptors such that responses were inhibited by the specific estrogen receptor antagonist ICI 182,780.

In the present study, the concentrations of estrogen routinely tested ranged from 0.1 to 10 ng/ml (3.6×10^{-10} to 3.6×10^{-8} mol/L). Estrogen concentrations >10 ng/ml had no additional effect on PDGF-induced migration (data not shown). In premenopausal nonpregnant women, estrogen concentrations are in the range of 0.03 to 0.4 ng/ml.[40] The inhibition of estrogen in our study was apparent at concentrations of 0.5 ng/ml (1.8 nmol/L) or greater, which is at the upper end of the physiological range. Maximal suppression of migration induced by β-estradiol was approximately 30 to 50% depending on the migratory stimuli. The effects of estrogen were studied only in SMCs maximally stimulated for migration. Perhaps using submaximal concentrations of migratory stimuli would have resulted in greater effects of estrogen at lower, more physiological concentrations.

Estrogen has been shown to increase prostaglandin synthesis in arterial SMCs.[41] Furthermore, prostaglandins E_2 and $F_{2\alpha}$, at nanomolar concentrations, have been shown to inhibit both PDGF- and interleukin-8-induced smooth muscle migration.[34] In view of this inhibitory effect, we speculated that estrogen may decrease PDGF-induced migration secondarily by release of endogenous prostaglandins. Such a response was not apparent in our culture system because pretreatment of cells with the cy-

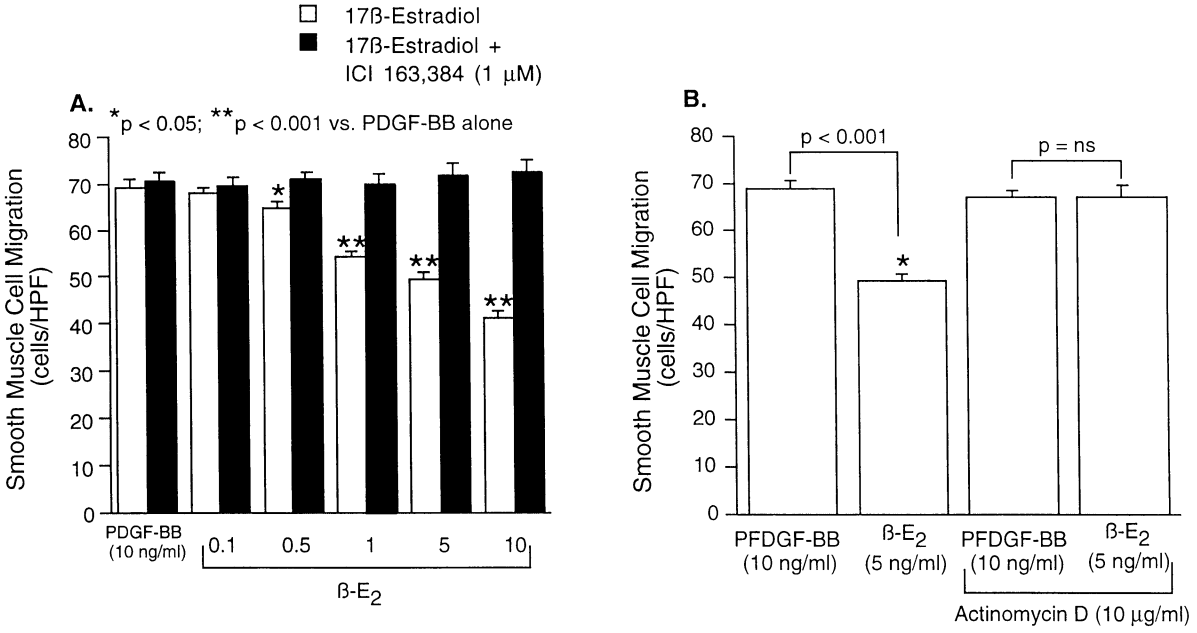

Figure 4. *Graphs showing the effects of the anti-estrogen ICI 164,384* (A) *and the RNA synthesis inhibitor actinomycin D* (B) *on β-estradiol-mediated suppression of smooth muscle cell migration toward PDGF-BB. Hormone (β-estradiol; 5 ng/ml) was placed in both the upper and lower chamber. Before assay, cells were either pretreated with ICI 164,384 (1 μmol/l) at 37°C for 1 hour or actinomycin D (10 μg/ml) for 30 minutes at 37°C; control cells were treated with either 0.01% ethanol or phosphate-buffered saline, respectively. Results are expressed as the mean ± SEM of cells per HPF (×200) from at least four separate experiments. Migration responses to 17β-estradiol were blocked by both ICI 164,384 and actinomycin D, providing evidence of a genomic effect of estrogen.*

clooxygenase inhibitor indomethacin did not inhibit the suppression of migration mediated by β-estradiol.

The precise mechanism of β-estradiol interference with SMC migration is unknown. In nonvascular smooth muscle such as the uterus, polypeptide growth factors and steroid hormones act synergistically through shared membrane receptors and/or other transcription factors to elicit cell growth and differentiation.[42] The mechanism by which the actions of growth factors and estrogen converge does not appear to apply to vascular SMCs, in which estrogen interferes with growth factor signaling pathways known to cause migration.

The regulation of SMC migration by β-estradiol appears to be mediated by the estrogen receptor, which functions as a transcription factor. The presence of estrogen receptors in rat aortic smooth muscle has been demonstrated by radioligand binding assays,[9] reverse transcriptase polymerase chain reaction,[10] and immunocytochemistry.[10] Transcriptional activation of the estrogen receptor has been shown to enhance c-*fos* mRNA transcription in SMCs, further suggesting that effects of estrogen are receptor mediated.[10] Additional support for this hypothesis is provided by the present study, in which the specific estrogen receptor antagonist ICI 164,384 blocked estrogenic suppression of PDGF-induced migration *in vitro*. The anti-estrogen ICI 164,384 is a purer antagonist, unlike tamoxifen, the more conventional estrogen receptor blocker that possesses partial agonistic effects.[43] Treatment with ICI 164,384 is thought to cause a rapid depletion of estrogen receptors from estrogen-responsive target tissue.[44] This decrease in estrogen receptor expression by ICI 164,384 results from an increase in estrogen receptor protein turnover[24] and a disruption of nucleoplasmic shuttling of the estrogen receptor.[45] Therefore, treatment with ICI 164,384 results in an inadequate amount of estrogen receptors to bind to the native ligand and elicit agonist responses.

Estradiol-induced suppression of SMC migration toward PDGF was also preventable by actinomycin D, an inhibitor of DNA-dependent RNA polymerase, further implicating receptor activation and gene transcription as a mechanism for this estrogenic response. Contrary to previous studies of fibroblasts and bovine SMCs, in our investigations actinomycin D did not inhibit PDGF-induced migration.[31,46] In other cell systems, actinomycin D has been shown to inhibit neutrophil chemotaxis toward formyl-methionyl-leucyl-phenylalanine,[47] whereas others have shown no effect on neutrophil migration with this agent.[48,49] Interestingly, in our studies, cell migration toward PDGF was inhibited only by cycloheximide (unpublished observation), which is in agreement

with the aforementioned studies of fibroblasts and smooth muscle cells, further confirming a requirement for continuous protein synthesis for cell migration. Such a mechanism seems reasonable in that the regulation of actin filament disassembly and assembly is critical for cell translocation, and actinomycin D does not appear to interfere with this mechanism.

The discrepancy of the relation of the effects of actinomycin D in our study and previous studies is unknown. Whether the cell type, phase of the cell cycle passage in culture, or species differences play a role in the response to actinomycin D is unclear. Recent studies have identified potential signal transduction pathways associated with PDGF-induced migration of arterial SMCs.[50,51] These investigations provide evidence of a localized time-dependent change in actin filament assembly and disassembly regulated by levels of phosphatidylinositol bisphosphate and calcium and activation of protein kinase A. Unlike the mitogenic response induced by PDGF, which requires DNA replication and transcription, the mechanisms associated with PDGF-induced migration of arterial SMCs appear to be fully activated by second messenger pathways independent of nuclear signaling. Whether estrogen interferes with PDGF-induced smooth muscle migration directly or through signal transduction pathways linked to gene activation is yet to be determined.

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