# Both estrogen and raloxifene cause G1 arrest of vascular smooth muscle cells

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## Abstract

The proliferation of vascular smooth muscle cells (VSMC) is a crucial pathophysiological process in the development of atherosclerosis. Although estrogen is known to inhibit the proliferation of VSMC, the mechanism responsible for this effect remains to be elucidated. In addition, the effect of raloxifene on VSMC remains unknown. We have shown here that 17β-estradiol ($E_2$) and raloxifene significantly inhibited the platelet-derived growth factor (PDGF)-stimulated proliferation of cultured human VSMC. Flow cytometry demonstrated that PDGF-stimulated S-phase progression of the cell cycle in VSMC was also suppressed by $E_2$ or raloxifene. We found that PDGF-induced phosphorylation of retinoblastoma protein (pRb), whose hyperphosphorylation is a hallmark of the G1–S transition in the cell cycle, was significantly inhibited by $E_2$ and raloxifene. These effects were associated with a decrease in cyclin D1 expression, without a change in cyclin-dependent kinase 4 or cyclin-dependent kinase inhibitor, $p27^{kip1}$ expression. ICI 182,780 abolished the inhibitory effects of $E_2$ and raloxifene on PDGF-induced pRb phosphorylation. Next, we examined which estrogen receptor (ER) is necessary for these effects of $E_2$ and raloxifene. Since VSMC express both ERα and ERβ, A10, a rat aortic smooth muscle cell line that expresses ERβ but not ERα, was used. The dose-dependent stimulation of A10 cell proliferation by PDGF was not inhibited by $E_2$ or raloxifene in contrast to the results obtained in VSMC. Moreover, $E_2$ and raloxifene significantly inhibited the PDGF-induced cyclin D1 promoter activity in A10 cells transfected with cDNA for ERα but not in the parental cells. These results suggested that $E_2$ and raloxifene exert an antiproliferative effect in VSMC treated with PDGF, at least in part through inhibition of pRb phosphorylation, and that the inhibitory effects of $E_2$ and raloxifene may be mainly mediated by ERα.

*Journal of Endocrinology* (2003) **178,** 319–329

## Introduction

The proliferation of vascular smooth muscle cells (VSMC) is a crucial pathophysiological process in the development of atherosclerosis and of restenosis after coronary angioplasty (Ross 1995, Schwarts *et al.* 1995, Hanke 1996, Braun-Dullaeus *et al.* 1998). VSMC are normally quiescent and display contractile properties. This contractility contributes to vasoconstriction and vasodilatation in the regulation of blood pressure and flow volume. Mechanical and/or chemical stresses alter the VSMC phenotype from the contractile to the synthetic state, and synthetic VSMC then migrate into the intima (Ross 1995).

Many epidemiological and basic studies demonstrated that estrogen affords cardioprotection in postmenopausal women (Stampfer *et al.* 1991, Mendelsohn & Karas 1999). Although the results of the Women's Health Initiative's (WHI) large prospective randomized controlled study seem to raise questions about the cardioprotective effect of estrogen (Writing Group for the Women's Health Initiative Investigators 2002), interim reports on women on estrogens alone did not show adverse effects on cardiovascular disease or breast cancer, and this part of the trial continues. Thus, the cardioprotective effect of estrogen itself was not necessarily disproven by the results of the WHI study. Moreover, there is a need for an ideal estrogen that does not increase the risk of breast cancer (Cummings *et al.* 1999). Raloxifene is a non-steroidal benzothiophene that has been classified as a selective estrogen receptor modulator based on the fact that it produces both estrogen-agonistic effects on bone and lipid metabolism and estrogen-antagonistic effects on uterine endometrium and breast tissue. Although we found that raloxifene rapidly induces nitric oxide synthase activation in vascular


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endothelial cells (Hisamoto *et al.* 2001*a*), its ability to protect against cardiovascular disease has yet to be demonstrated and is now being tested in the Raloxifene Use for the Heart trial.

Several researchers have recently focused on the direct effects of estrogen on the endothelium and VSMC (Sullivan *et al.* 1995, Hisamoto *et al.* 2001*b*). We previously reported that estrogen rapidly induces nitric oxide synthase activation in vascular endothelial cells (Hisamoto *et al.* 2001*b*). Nitric oxide is secreted from normal endothelium and has important roles in maintaining normal vasculature, such as relaxation of vascular smooth muscle and inhibition of platelet activation (Moncada & Higgs 1993). Platelet-derived growth factor (PDGF) is released from platelets, macrophages and endothelial cells, and is important in triggering VSMC proliferation and migration (Ross 1986, Schwartz *et al.* 1995). Some investigators have reported that estrogen inhibits PDGF-induced proliferation and migration of VSMC *in vitro* (Dai-Do *et al.* 1996, Kolodgie *et al.* 1996, Suzuki *et al.* 1996, Somjen *et al.* 1998). However, the mechanism responsible for this effect is not yet fully understood.

Recently, VSMC proliferation has been reported to be inhibited by a reduction of retinoblastoma protein (pRb) phosphorylation and by modulation of cell cycle regulators (Kintscher *et al.* 2000, Marra *et al.* 2000, Wakino *et al.* 2001). The status of pRb phosphorylation is critical for cell proliferation. In quiescent cells, pRb is present in a hypophosphorylated state in which it is able to bind to and sequester members of the E2F family of transcription factors (Weinberg 1995). Phosphorylation of pRb at multiple sites induces a conformational change that releases E2F, which in turn activates the transcription of genes required for further cell cycle progression (Nevins 1992, La Thangue 1996). Therefore, we hypothesized that the antiproliferative effect of estrogen in VSMC might be due to inhibition of pRb phosphorylation.

We report here that 17β-estradiol ($E_2$) exerts an antiproliferative effect through inhibition of pRb phosphorylation in PDGF-induced VSMC, and that the inhibitory effect of estrogen is mainly mediated by estrogen receptor α (ERα).

## Materials and Methods

### *Materials*

Raloxifene analogue LY117018 was a kind gift from Eli Lilly Research Laboratories (Indianapolis, IN, USA). $E_2$ and propidium iodide were purchased from Sigma Chemical Co. (St Louis, MO, USA). ICI 182,780 was obtained from Tocris (Ballwin, MO, USA). PDGF was obtained from R&D Systems, Inc. (Minneapolis, MN, USA). ECL Western blotting detection reagents were from Amersham Pharmacia Biotech (Arlington Heights, IL, USA). The Cell Titer 96 Aqueous One Solution Cell Proliferation Assay kit was purchased from Promega (Madison, WI, USA). Mouse anti-pRb antibody was obtained from PharMingen (San Diego, CA, USA). Rabbit polyclonal anti-phospho pRb Ser807/811 was purchased from New England Biolabs (Beverly, MA, USA). Anti-cyclin D1, -β-actin, -cyclin dependent-kinase (cdk) 4 and -$p27^{kip1}$ were obtained from Santa Cruz LipofectAMINE plus was obtained from Invitrogen (Carlsbad, CA) Biotechnology, Inc. (Santa Cruz, CA, USA). The PicaGene dual sea pansy system was purchased from Toyo Inc. (Tokyo, Japan).

### *Constructs*

The human ERα expression vector, pIE, was a kind gift from Dr D J Shapiro (University of Illinois, Urbana, IL, USA). The cyclin D1 (D1 pro-1749) promoter plasmid construct, which contains the cyclin D1 regulatory region ($-1749$ to $+135$) fused to a luciferase reporter gene, was kindly provided by Dr T Shiozawa (Shinshu University School of Medicine, Matsumoto, Japan).

### *Cell culture*

Primary cultures of human aortic smooth muscle cells (VSMC) (Clonetics, Walkersville, MD, USA) and primary rat aortic smooth muscle cells (A10) (American Type Culture Collection, Rockville, MD, USA) were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a water-saturated atmosphere of 95% air and 5% $CO_2$. VSMC cultured for at most ten passages were used for the following studies.

### *Cell proliferation assay*

Five thousand cells were seeded per well in a 96-well plate, incubated for 24 h in DMEM with 10% FBS, and then starved in phenol red-free DMEM with 0·4% charcoal–dextran-treated FBS for 48 h. $E_2$ ($10^{-6}$ to $10^{-8}$ M) or vehicle was added 1 h before VSMC or A10 were challenged with PDGF for 48 h. Cell proliferation was assessed by measuring the absorbance at 490 nm of the dissolved formazan product formed after addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) and incubation for 1 h as described by the manufacturer (Promega). All experiments were carried out in quadruplicate and proliferation was expressed as the ratio of the absorbance of treated cells to that of untreated quiescent cells cultured in 0·4% FBS. For cell number experiments, VSMC were plated at a density of $5 \times 10^3$ cells per well in a 24-well plate and allowed to attach overnight. The cells were starved as described above for 48 h, and were then

treated with fresh preparation of medium containing $E_2$ or raloxifene every 48 h for 6 days, and counted each time the medium was exchanged. A Neubauer chamber was used to count the cell number and the trypan-blue exclusion test was carried out to determine the cell viability.

### *Western blotting analysis*

VSMC were starved as described above for 48 h and then treated with the various agents indicated in the text. They were then washed twice with phosphate-buffered saline (PBS) and lysed in ice-cold lysis buffer (20 mM Tris, pH 7·4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2·5 mM sodium pyrophosphate, 1 mM β-glycerol-phosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride). The extracts were centrifuged to remove cellular debris, and the protein content of the supernatants was determined using the Bio-Rad protein assay reagent. Lysate samples containing 40 μg protein were boiled and the proteins were separated by electrophoresis on a 10% glycine–SDS–polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane (Hybond-P; Amersham Pharmacia Biotech, Amersham, Bucks, UK). The membrane was incubated with blocking buffer for 1 h at room temperature, probed with primary antibody with gentle rocking at 4 °C overnight, and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (at 1:2000 dilution) for 1 h at room temperature. The signal was detected by chemiluminescence with ECL Western blotting detection reagents (Amersham Pharmacia Biotech). All Western blot experiments were repeated at least three times with identical results.

### *Cell cycle distribution*

Quiescent VSMC were pretreated for 1 h with $10^{-8}$ M $E_2$ or vehicle (ethanol), and then treated with 5 ng/ml PDGF. After 24 h, cells were trypsinized, centrifuged at 1200 r.p.m. for 5 min, washed with PBS, and then treated with RNase A (20 μg/ml). DNA was stained with propidium iodide (100 μg/ml) for 30 min at 4 °C in the dark. The DNA content of the cells was analyzed using a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). DNA histogram analysis was performed using ModFitLT software (Becton Dickinson).

### *RT-PCR experiments*

Total RNA was extracted from VSMC and A10 cells with Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. Total RNA (5 μg) was used as a template for

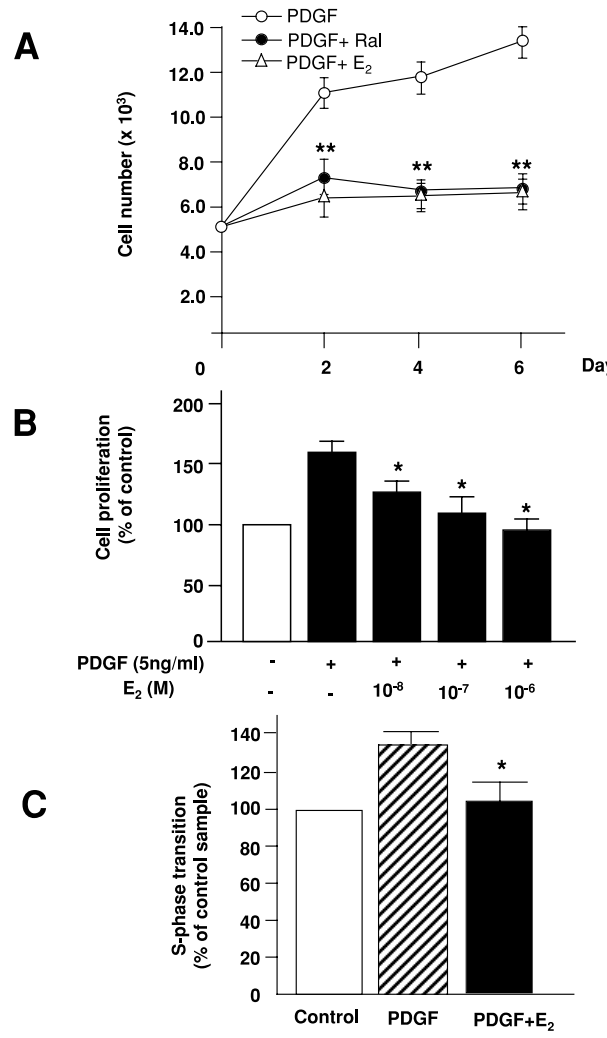


**Figure 1** $E_2$ and raloxifene (Ral) inhibit PDGF-induced cell proliferation in VSMC. (A) VSMC were starved for 48 h, and then cultured with 5 ng/ml PDGF alone, or with PDGF in the presence of $E_2$ ($10^{-8}$ M) or raloxifene ($10^{-8}$ M), and then treated with a fresh preparation of medium containing $E_2$ or raloxifene every 48 h for 6 days, and counted each time the medium was exchanged. The viable cell number was determined by the trypan-blue exclusion test. Data are shown as the means ± S.E.M. from at least three separate experiments. $^{**}P<0{\cdot}01$ vs VSMC treated with PDGF alone. (B) VSMC were pretreated with various concentrations of $E_2$ or vehicle for 1 h, and then treated with 5 ng/ml PDGF. After 48 h of incubation with PDGF, cell proliferation was determined by the MTS assay. Cell proliferation is expressed as a percentage of that in the non-stimulated condition (control). Data are shown as the means ± S.E.M. from at least three separate experiments. $^{*}P<0{\cdot}05$ vs VSMC treated with PDGF alone. (C) Quiescent VSMC were pretreated with $10^{-8}$ M $E_2$ or vehicle, and then stimulated with 5 ng/ml PDGF for 24 h. DNA was stained with propidium iodine and cells were analyzed by flow cytometry. The percentage of cells in S phase in the VSMC treated with PDGF+$E_2$ relative to that in the VSMC treated with PDGF alone is shown. Data are shown as the means ± S.E.M. from at least three separate experiments. $^{*}P<0{\cdot}05$ vs VSMC treated with PDGF alone.

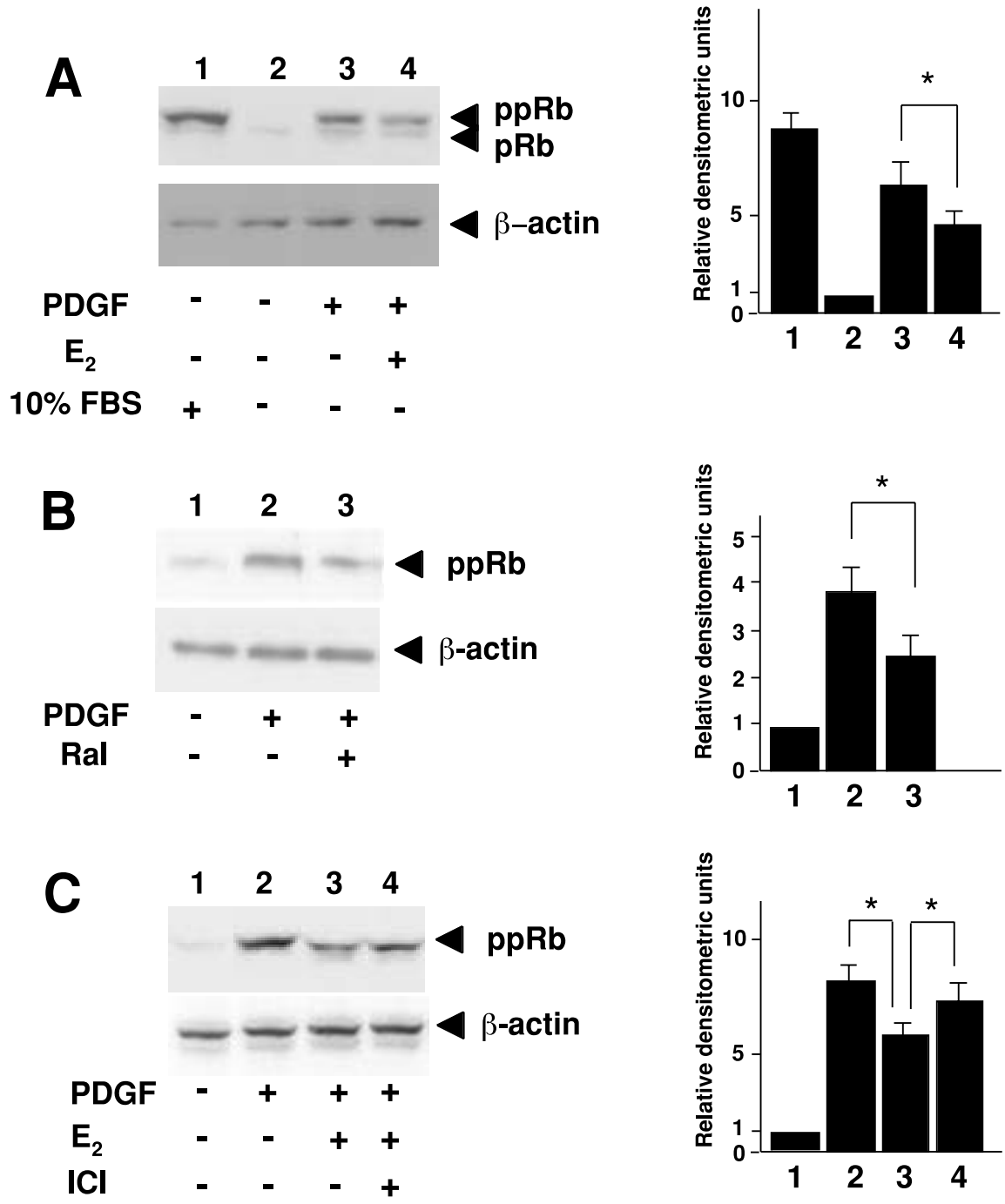


**Figure 2** Effects of $E_2$ and raloxifene on the phosphorylation of pRb. Quiescent VSMC were pretreated with $10^{-8}$ M $E_2$ (A) or $10^{-8}$ M raloxifene (Ral) (B) for 1 h before treatment with PDGF (5 ng/ml) for 24 h. Control VSMC were cultured in growth medium (DMEM with 10% FBS). To analyze pRb phosphorylation, extracted proteins were resolved by 8% SDS-PAGE and subjected to Western blotting with anti-pRb antibody (A, upper panel) or antiphospho pRb ser807/811 antibody (B, upper panel). (C) VSMC were pretreated with 1 μM ICI 182,780 (ICI) or vehicle for 15 min, and then treated with $10^{-8}$ M $E_2$ or vehicle for 1 h. The cells were then treated with 5 ng/ml PDGF for 24 h. Extracted proteins were subjected to 8% SDS-PAGE followed by Western blotting with antiphospho pRb ser807/811 antibody. The positions of the hypophosphorylated (pRb) and hyperphosphorylated (ppRb) forms of pRb protein are indicated on the right. β-Actin analyzed similarly by immunodetection with anti-β-actin antibody served as a loading control (A–C, lower panel). Relative densitometric units of the ppRb bands are shown in the right panels, with the density of the control bands set arbitrarily at 1·0 (A–C). Data are shown as the means ± S.E.M. from at least three separate experiments. *$P < 0{\cdot}05$.

first-strand cDNA synthesis. cDNA was amplified using an RNA PCR kit obtained from TaKaRa (Tokyo, Japan) according to the manufacturer's instructions. Primers designed based on the published gene sequences were obtained from Amersham Pharmacia Biotech Japan (Tokyo, Japan) and Hitachi Instruments Service Co. Ltd (Tokyo, Japan). The sequences of the PCR primers were as follows: rat ERα: 5′-AATTCTGACAATCGACGC CAG-3′ (sense), 5′-GTGCTTCAACATTCTCCCTCC TC-3′ (antisense); rat ERβ: 5′-TTCCCGGCAGCAC CAGTAACC-3′ (sense), 5′-TCCCTCTTTGCGTTTG GACTA-3′ (antisense); human ERα: 5′-AATTCAGAT AATCGACGCCAG-3′ (sense), 5′-GTGTTTCAACAT TCTCCCTCCTC-3′ (antisense); human ERβ: 5′-GTT GCGCCAGCCCTGTTAC-3′ (sense), 5′-CTCGTCG GCACTTCTCTGTCTC-3′ (antisense). To check the cDNA integrity, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified in parallel. All transcripts were analyzed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The amplification profile consisted of 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 59 °C for 1 min, and extension at 72 °C for 1 min. PCR products were electrophoresed on a 1·5% agarose gel and visualized by ethidium bromide staining under UV illumination.

*Cell transfections and reporter gene assays*

Approximately $2 \times 10^5$ A10 cells were plated per well of a six-well tissue culture plate 24 h before transfection. For analysis of the effects of estrogen on reporter gene activity, transfection complexes containing a total of 1·55 μg plasmids (1 μg D1 pro-1749, 0·5 μg pIE and 0·05 μg pRL-CMV (ToYo Ink, Tokyo, Japan) as an internal control) were added to each well of A10 cells as recommended by the manufacturer (Invitrogen). After 3 h, the transfection cocktail was replaced with growth medium, and the cells were cultured for 24 h. The growth medium was replaced with DMEM containing charcoal–dextran-stripped 0·4% FBS to induce the A10 cells to become quiescent and the cells were cultured in this medium for 24 h. The quiescent A10 cells were pretreated for 1 h with $10^{-7}$ M $E_2$ or vehicle (ethanol), and then treated with 5 ng/ml PDGF. After 24 h, the cells were harvested and luciferase assays were performed with the PicaGene dual sea pansy system. Firefly-luciferase activity and sea pansy-luciferase activity were measured as relative light units with a luminometer (Lumat LB9507; EG&G, Berthold, Bad Wildbad, Germany). The firefly-luciferase activity was then normalized relative to the sea pansy-luciferase activity to determine the transfection efficiency.

*Statistics*

Statistical analysis was performed by Student's *t*-test, and $P < 0{\cdot}05$ was considered significant. Data are expressed as the means ± S.E.M.

## Results

*Effects of $E_2$ and raloxifene on VSMC proliferation*

The effects of raloxifene and $E_2$ on the proliferation of VSMC were evaluated (Fig. 1A). Both $10^{-8}$ M $E_2$ and $10^{-8}$ M raloxifene significantly inhibited the growth of VSMC. Pathological proliferation of VSMC plays a major role in the development of atherosclerotic lesions (Braun-Dullaeus *et al.* 1998). PDGF released from platelets, macrophages and endothelium is a major trigger of VSMC proliferation. Therefore, we evaluated the effect of $E_2$ or raloxifene on the proliferation of PDGF-stimulated VSMC. When quiescent VSMC were treated with 5 ng/ml PDGF in 0·4% FBS for 48 h, the proliferation of the VSMC was stimulated by about 50% compared with that of the quiescent VSMC. PDGF-stimulated VSMC proliferation was significantly inhibited by $E_2$ in a concentration-dependent manner in the range of $10^{-8}$ to $10^{-6}$ M (Fig. 1B) and also by raloxifene (data not shown). We also determined the effect of $E_2$ or raloxifene on PDGF-induced cell cycle progression by flow cytometry. Subconfluent VSMC were synchronized by culturing with 0·4% FBS for 48 h, pretreated with $E_2$ or vehicle, and then treated with 5 ng/ml PDGF. Both $10^{-8}$ M $E_2$ (Fig. 1C) and $10^{-8}$ M raloxifene (data not shown) reduced the proportion of VSMC that progressed to S phase.

*Effects of $E_2$ and raloxifene on hyperphosphorylation of pRb*

Because cell cycle progression is regulated by hyperphosphorylation of pRb, we examined the effects of $E_2$ and raloxifene on pRb hyperphosphorylation. In quiescent cells, most pRb is hypophosphorylated (Weinberg 1995). Hyperphosphorylation of pRb is required for S-phase progression in most mammalian cells (Weinberg 1995). Anti-pRb antibody, which recognizes both hyper- and hypophosphorylated Rb protein, was used to assess the phosphorylation status of pRb. Hyperphosphorylated pRb (ppRb) was increased in cells cultured with 5 ng/ml PDGF (Fig. 2A, lane 3) as well as in cells cultured with 10% FBS (Fig. 2A, lane 1) for 24 h. $E_2$ clearly attenuated the PDGF-induced increase of ppRb (Fig. 2A, lane 4). To investigate whether raloxifene also decreases the PDGF-induced ppRb, phospho-specific antibody was used to assess the phosphorylation status at Ser807/811 of pRb, which is important for cdk-dependent function. ppRb was increased in cells cultured with 5 ng/ml PDGF (Fig. 2B, lane 2) and raloxifene clearly decreased the PDGF-induced increase of ppRb (Fig. 2B, lane 3). To investigate whether the inhibitory effect of $E_2$ or raloxifene on pRb phosphorylation is ER-mediated, the effect of ICI 182,780, a potent ER antagonist, was examined. Antiphospho-specific pRb Western blotting analysis showed that 1 μM ICI 182,780 clearly attenuated the inhibitory effects of $E_2$ (Fig. 2C, lane 4) and raloxifene

(data not shown) on the PDGF-induced hyperphosphorylation of pRb. This result suggested that ER is involved in the antiproliferative effects of $E_2$ and raloxifene in PDGF-stimulated VSMC.

### *Effects of $E_2$ and raloxifene on the protein expression of G1 regulators*

The phosphorylation of pRb is catalyzed, at least in part, by the D-type cyclins and their associated catalytic partners, cdk4 and cdk6, as well as by cyclin E and its catalytic partner, cdk2. We therefore examined the effects of $E_2$ and raloxifene on the expression of cyclin D1 (Fig. 3). Treatment of cells with 5 ng/ml PDGF induced the expression of cyclin D1 (Fig. 3A and B, lane 2). $E_2$ at $10^{-8}$ M (Fig. 3A, lane 4) and raloxifene at $10^{-8}$ M (Fig. 3B, lane 4) significantly decreased the PDGF-induced expression of cyclin D1. On the other hand, neither $E_2$ nor raloxifene affected the PDGF-induced expression of cdk4 (data not shown). Induction of cdk inhibitors has been shown to accompany cell cycle arrest in response to various antiproliferative stimuli. Therefore, the effect of $E_2$ on the level of $p27^{kip1}$ was examined. Neither $E_2$ nor raloxifene had any effect on the expression of $p27^{kip1}$ (data not shown).

### *Antiproliferative effects of $E_2$ and raloxifene are mediated by ERα*

Next, we examined which ER is necessary for these effects of $E_2$. RT-PCR analysis showed that VSMC expressed both ERα and ERβ mRNAs (Fig. 4). Although we confirmed that rat uterus expressed ERα (data not shown), A10 cells, which are derived from the thoracic aorta of embryonic rats and are a commonly used model of VSMC (Weiss *et al.* 2000), expressed ERβ but not ERα (Fig. 4). We therefore used A10 cells to examine whether or not ER is involved in the antiproliferative effect of $E_2$. A10 cells that had been induced to enter a quiescent state by incubation in low-serum medium proliferated in response to PDGF stimulation in a dose-dependent manner (Fig. 5A). In contrast to the effect of $E_2$ (Fig. 1B) and raloxifene in VSMC, $E_2$ (Fig 5B) and raloxifene (data not shown) did not significantly inhibit the PDGF-induced proliferation of A10 cells, suggesting that the growth inhibition by estrogen and raloxifene may not be mediated by ERβ.

We next sought to determine whether $E_2$ and raloxifene inhibit the PDGF-induced cyclin D1 promoter activity. A cyclin D1 promoter ($-1749$ to $+135$ bp)–luciferase reporter construct was transiently transfected into A10 cells. As shown in Fig. 6, addition of 5 ng/ml PDGF for 24 h enhanced the luciferase activity. To examine whether the inhibition of the PDGF-induced cyclin D1 promoter activity by $E_2$ or raloxifene is mediated by ERα, A10 cells were transiently transfected

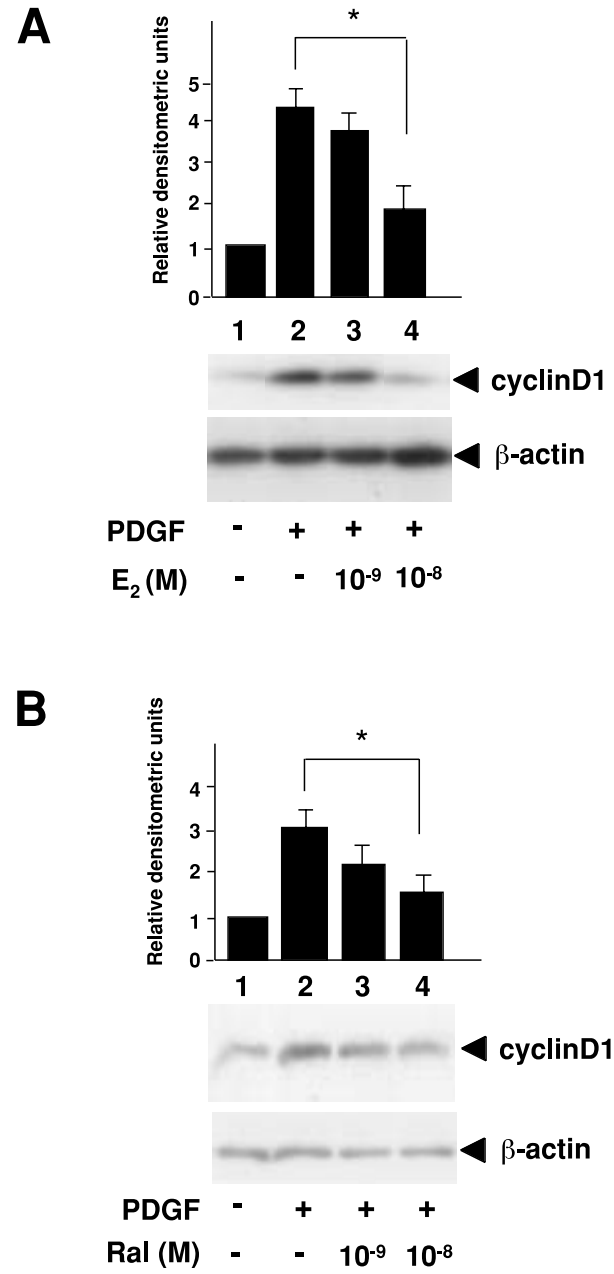


**Figure 3** Effects of $E_2$ and raloxifene on cyclin D1 expression. Quiescent VSMC were pretreated with $E_2$ (A) or raloxifene (Ral) (B) for 1 h and then treated with 5 ng/ml PDGF for 6 h. Immunoblotting of cell extracts was performed to detect cyclin D1 (A and B, middle panel). β-Actin analyzed similarly by immunodetection with anti-β-actin antibody served as a loading control (A and B, lower panel). Relative densitometric units of the cyclin D1 bands are shown in the upper panels, with the density of the control bands set arbitrarily at 1·0 (A and B). Data are shown as the means ± S.E.M. from at least three separate experiments. *$P < 0.05$.

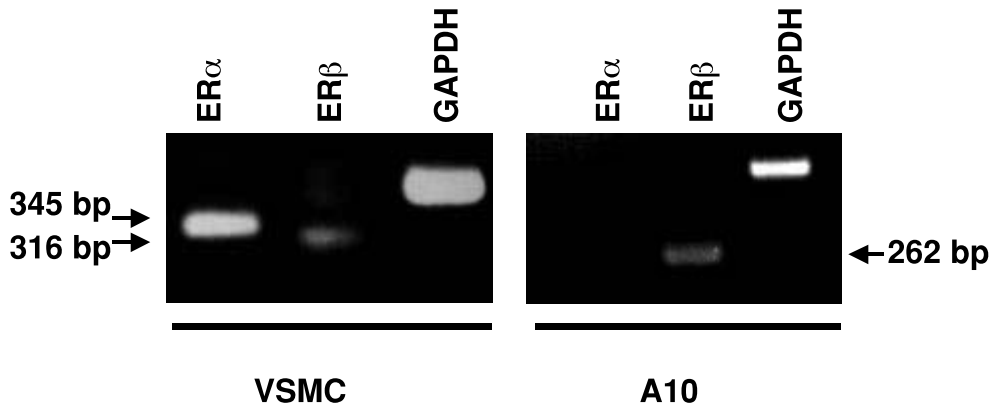


**Figure 4** Expression of ERα and ERβ in VSMC and A10 cells. Total RNA samples were isolated from cultured VSMC and A10 cells. RT-PCR was performed as described in Materials and Methods. The PCR products were resolved by electrophoresis on a 1·5% agarose gel and stained with ethidium bromide.

with an ERα expression vector in addition to the cyclin D1 reporter construct. Although $E_2$ and raloxifene had no effect on the PDGF-induced cyclin D1 promoter activity in A10 cells, transfection of the ERα expression vector rendered the PDGF-induced cyclin D1 promoter activity sensitive to inhibition by $E_2$ and raloxifene (Fig. 6). These results indicated that ERα is required for the inhibition of the PDGF-induced cyclin D1 promoter activity by $E_2$ and raloxifene.

## Discussion

We have demonstrated here that $E_2$ and raloxifene inhibit PDGF-induced VSMC proliferation, at least in part by reducing the PDGF-induced pRb phosphorylation. Protective effects of estrogen against cardiovascular disease in postmenopausal women are well documented (Forrester *et al.* 1991, Belchetz 1994). Putative mechanisms of these effects involve effects on endothelium-dependent and -independent vasodilation (Williams *et al.* 1990, 1992, Jiang *et al.* 1991, Rosano *et al.* 1993, Gilligan *et al.* 1994) and improvement in the lipid profile (Belchetz 1994). Estrogen stimulates endothelial nitric oxide synthase (eNOS) activity *in vivo* (Weiner *et al.* 1994) and *in vitro* (Hisamoto *et al.* 2001*b*), and inhibits smooth muscle cell proliferation by increasing the endothelium-derived nitric oxide (Garg & Hassid 1989). Proliferation and migration of VSMC are believed to significantly contribute to intimal thickening in atherosclerosis and venous bypass graft disease (Calcagno *et al.* 1992). PDGF is known to stimulate both proliferation and migration of VSMC, and the importance of this growth factor in the pathophysiology of intimal hyperplasia has been established. The mechanism of VSMC proliferation and migration is related to the stimulation of mitogen-activated protein kinase (MAP kinase) (Claesson-Welsh 1994, Graves *et al.* 1996), and the resultant increase of the transcription of many immediate early genes (Chen *et al.* 1992, Seth *et al.* 1992), including cyclin D1, which is required for transition from the G0 to the G1 phase in the cell cycle (Abrieu *et al.* 1996, Lavoie *et al.* 1996). Although phytoestrogen and estrogen inhibit VSMC proliferation by reducing MAP kinase activity (Dubey *et al.* 1999, 2000, Hwang *et al.* 2002), the mechanism of this inhibition by estrogen is not fully understood.

Mitogen-induced VSMC proliferation is inhibited by numerous agents such as sodium salicylate (Marra *et al.* 2000), doxazocin (Kintscher *et al.* 2000), retinoids (Wakino *et al.* 2001), 3-hydroxyl-3-methylglutraryl co-enzyme A (HMG-CoA) reductase inhibitor (Yang *et al.* 2000), parathyroid hormone-related protein (Stuart *et al.* 2000) and dexamethasone (Reil *et al.* 2000). Studies of the inhibitory effects of these agents have mainly focused on the inhibition of pRb phosphorylation. pRb is hypophosphorylated in quiescent cells and binds to transcriptional factor E2F. E2F activates the transcription of genes required for S-phase DNA synthesis, including the genes for thymidine kinase dihydrofolate reductase, cell division cycle 2 and cyclin A (Nevins 1992, La Thangue 1996). Thus, pRb phosphorylation is among the most crucial steps regulating progression of the cell cycle. In this study, $E_2$ and raloxifene significantly inhibited the PDGF-stimulated pRb phosphorylation (Fig. 2). The inhibitory effect of $E_2$ on pRb phosphorylation was significant and similar to the effects reported for various other agents that inhibit the proliferation of VSMC (Kintscher *et al.* 2000, Marra *et al.* 2000, Yang *et al.* 2000, Wakino *et al.* 2001). In addition, some reports have shown that $E_2$ inhibited [$^3$H]thymidine incorporation into PDGF-stimulated VSMC and decreased [$^3$H]thymidine uptake rates to approximately 60–70% of the control rates (Suzuki *et al.* 1996, Dubey *et al.* 2000). Collectively, the findings of the present study together with the published data indicate

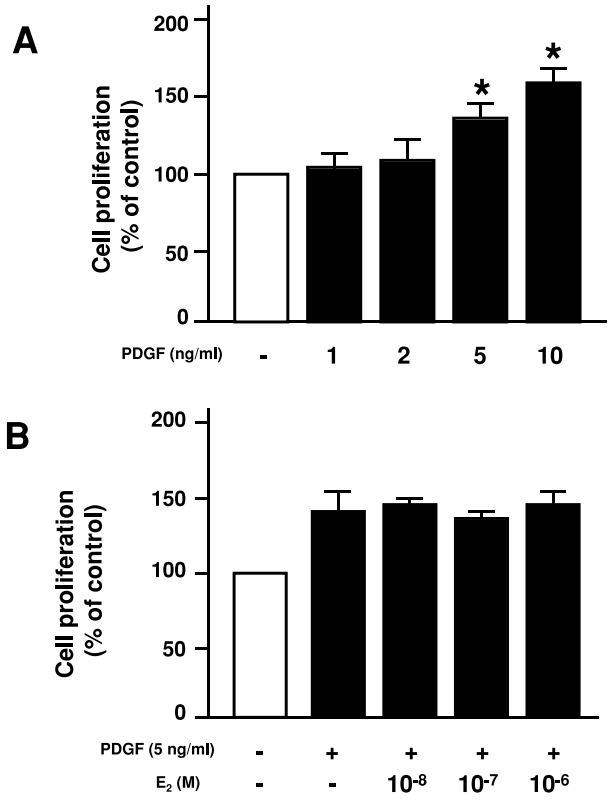


**Figure 5** $E_2$ does not inhibit PDGF-stimulated proliferation of A10 cells. A10 cells were stimulated with various concentrations of PDGF (A) or pretreated with various concentrations of $E_2$ for 1 h followed by PDGF (5 ng/ml) stimulation (B). Cell proliferation is expressed as a percentage of that of A10 cells without PDGF stimulation. Data are shown as the means ± S.E.M. from at least three separate experiments. *$P < 0{\cdot}05$ vs A10 cells without PDGF stimulation.

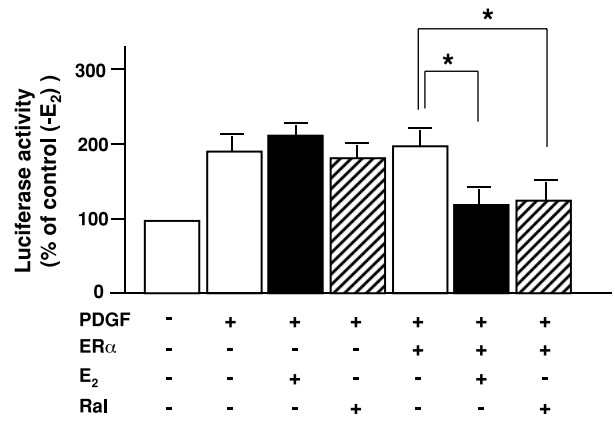


**Figure 6** Cyclin D1 promoter activity is suppressed by $E_2$ or raloxifene (Ral) via ERα. A10 cells were transfected with a plasmid containing a cyclin D1 promoter–luciferase gene fusion construct (D1 pro-1749) together with an ERα expression vector (pIE) (+) or vector alone ( − ) and pRL-CMV internal control plasmid, as described in Materials and Methods. Transfected cells were pretreated with $10^{-8}$ M $E_2$ or $10^{-8}$ M raloxifene for 1 h and then stimulated with 5 ng/ml PDGF for 24 h. Firefly-luciferase activity, normalized by sea pansy-luciferase activity, was expressed as a percentage of the activity obtained in the absence of $E_2$ or raloxifene in cells transfected with pIE or vector alone. Data are shown as the means ± S.E.M. from at least three separate experiments. *$P < 0{\cdot}05$.

that the inhibitory effect of $E_2$ on S-phase transition is partial rather than complete in VSMC.

We investigated whether or not $E_2$-induced G1 arrest was dependent upon ER. Pretreatment with ICI 182,780, a specific ER antagonist, restored the status of pRb phosphorylation from the hypophosphorylated to the hyperphosphorylated state (Fig. 2C). This result indicated that the inhibitory effect of $E_2$ on pRb phosphorylation was mediated by ER. The expression of two ER subtypes, ERα and ERβ, is observed in VSMC; however, which subtype is responsible for the atheroprotective effect of $E_2$ has not hitherto been determined. A10 cells, a primary rat embryonic thoracic aorta cell line, have commonly been used as a model of VSMC. We found that A10 cells expressed ERβ, but not ERα (Fig. 4). Although the proliferation of A10 cells was increased by PDGF similarly to that of VSMC, $E_2$ could not inhibit the PDGF-induced proliferation of A10 cells (Fig. 5). Moreover, although $E_2$ did not inhibit the PDGF-induced cyclin D1 promoter activity in A10 cells, $E_2$ significantly inhibited the PDGF-induced cyclin D1 promoter activity in A10 cells transfected with ERα (Fig. 6). These results suggested that the inhibitory effect of $E_2$ on the PDGF-induced proliferation of VSMC was mediated by ERα. However, the fact that ERβ is the predominantly expressed form of ER in VSMC suggests that the protective effects of estrogens in the cardiovascular system may be due to the genomic effects of ERβ in vascular tissue (Hodges *et al.* 2000). In addition, it was reported that estrogen inhibited VSMC proliferation in response to vascular injury in knockout mice lacking ERα (Iafrati *et al.* 1997). However, ERα may be involved in the protective effects because this knockout mouse expressed a variant ERα (Kos *et al.* 2002). Our data are in agreement with the recent finding that ERα mediates the protective effects of estrogen against vascular injury (Pare *et al.* 2002).

Estrogen is now believed to exert rapid membrane effects independently of the classical gene activation pathway of steroid action. Recently we (Hisamoto *et al.* 2001*a*,*b*) and other groups (Haynes *et al.* 2000, Simoncini *et al.* 2000) have shown that estrogen induces the activation of eNOS in a non-genomic manner in vascular endothelial cells. In VSMC, inhibition of $Ca^{2+}$ influx via L-type $Ca^{2+}$ channels by estrogen is one of estrogen's acute, non-genomic vasodilator actions (Ruehlmann *et al.* 1998), estrogen inhibits lysophosphatidylcholine-induced proliferation via a non-genomic antioxidant mechanism

(Yoon *et al.* 2001), and estrogen inhibits angiotensin II-induced proliferation via the activation and induction of phosphatases through non-genomic as well as genomic signaling (Takeda-Matsubara *et al.* 2002). Moreover, it was reported that in MCF-7 cells estrogen rapidly stimulates a p85-regulated phosphatylinositol kinase and Akt, and increases the expression and promoter activity of cyclin D1 as well as the entry of cells into S phase (Castoria *et al.* 2001). We recently found that $E_2$- and raloxifene-induced inhibition of VSMC growth is in part due to induction of apoptosis through the p38 cascade with a non-genomic mechanism (authors' unpublished data). However, it remains unclear whether the inhibitory effects of estrogen on the PDGF-induced cyclin D1 promoter activity, pRb phosphorylation and S-phase entry observed in the present study are mediated in a genomic or non-genomic manner. These inhibitory effects of $E_2$ and raloxifene were not as rapid as the effects of estrogen and raloxifene on eNOS activation (Hisamoto *et al.* 2001*a*,*b*). Moreover, actinomycin D, an inhibitor of gene transcription, was tested to rule out the influence of non-genomic events, and it inhibited the effects of $E_2$ and raloxifene observed in the present study (data not shown). We therefore consider it likely that the effects of $E_2$ and raloxifene which we observed here mainly depend on the genomic effects of estrogen mediated by ERα.

Although estrogen enhances the proliferation of breast or uterine cells and has anti-apoptotic effects in vascular endothelial cells (Razandi *et al.* 2000), estrogen inhibits the proliferation of VSMC. In addition, it has been reported that estrogen induces apoptosis in an erythroid cell line (Blobel & Orkin 1996) and induces apoptosis and G1 cell cycle arrest of human multiple myeloma cells (Treon *et al.* 1998, Wang *et al.* 2001). Thus, $E_2$ modulates diverse cell functions in a cell- and tissue-specific manner. Although the mechanism of this heterogeneity of estrogen's actions remains unknown, it is possible that it involves 'cross-talk' between membrane-mediated events and nuclear receptor activation.

VSMC proliferation is regulated by direct cell cycle-specific effects as well as indirect effects via the regulation of VSMC mitogen production by endothelial cells (Kourembanas *et al.* 1998). Thus, it is possible that the inhibitory effects of $E_2$ and raloxifene on VSMC proliferation are mediated by direct cell cycle-specific effects. It was reported recently that VSMC express telomerase activity when stimulated to proliferate (Minamino & Kourembanas 2001). We are therefore now investigating the effects of $E_2$ and raloxifene on telomerase activity.

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Received 3 March 2003
Accepted 15 May 2003