Estrogen and raloxifene induce apoptosis by activating p38 mitogen-activated protein kinase cascade in synthetic vascular smooth muscle cells

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Abstract

Proliferation of vascular smooth muscle cells (VSMC) plays a major role as an initiating event of atherosclerosis. Although estrogen directly inhibits the proliferation of VSMC, the mechanism has not been firmly established. In addition, the effect of raloxifene on VSMC remains unknown. 17β -Estradiol (E_2) and raloxifene significantly inhibited the growth of VSMC under growth-stimulated conditions. Since mitogen-activated protein (MAP) kinases have been implicated in VSMC proliferation, the role of MAP kinases in both the E_2 - and raloxifene-induced growth inhibition of VSMC was studied. Both E_2 and raloxifene caused rapid, transient phosphorylation and activation of p38 that was not affected by actinomycin D and was blocked by ICI 182,780. In contrast with p38 phosphorylation, extracellular signal-regulated protein kinase (ERK) phosphorylation was significantly inhibited and c-Jun N-terminal kinase (JNK) phosphorylation was

not changed by E_2 . Because VSMC expressed both estrogen receptor (ER) α and ER β , it is not known which of them mediates the E_2 -induced phosphorylation of p38. Although E_2 did not affect the p38 phosphorylation in A10 smooth muscle cells, which express ER β but not ER α , transfection of ER α expression vector into A10 cells rendered them susceptible to induction of p38 phosphorylation by E_2 . We then examined whether E_2 and raloxifene induce apoptosis through a p38 cascade. Both E_2 and raloxifene induced apoptosis under growth-stimulated conditions. The p38 inhibitor SB 203580 completely blocked the E_2 -induced apoptosis. Our findings suggest that both E_2 - and raloxifene-induced inhibition of VSMC growth is due to induction of apoptosis through a p38 cascade whose activation is mediated by ER α via a nongenomic mechanism.

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Introduction

Many epidemiological studies and basic studies have shown that estrogen has the significant function in the vasculature of preventing the primary development of cardiovascular disease in women (Stampfer *et al.* 1991, Mendelsohn & Karas 1999). In the Women's Health Initiative's (WHI), a large prospective randomized controlled study, although women on combined estrogen–progestin had an increase in the relative risk of cardiovascular events, interim reports on women on estrogen alone did not show adverse effects on the risk of cardiovascular disease (Writing Group for the Women's Health Initiative Investigators 2002). Thus, the cardioprotective effect of estrogen itself was not ruled out by the results of the WHI study. It is possible that progestin has adverse effects on the cardiovascular system (Wakatsuki *et al.* 2001). Therefore, there is a need for an ideal estrogen analog that does not require the addition of progestin

to protect the uterine endometrium. Raloxifene is a nonsteroidal benzothiophene that has been classified as a selective estrogen receptor modulator (SERM) based on the fact that it produces estrogen-agonistic effects on bone (Ross & Glomset 1973) and lipid metabolism (Truss & Beato 1993) and estrogen-antagonistic effects on uterine endometrium (Ross & Glomset 1973) and breast tissue (Pietras & Szego 1977, Falkenstein & Wehling 2000). However, its ability to protect against cardiovascular disease has yet to be proven, and is now being tested in the RUTH (Raloxifene Use for The Heart) trial.

Pathological proliferation of vascular smooth muscle cells (VSMC) plays a major role in the development of atherosclerotic lesions (Kindy & Sonenshein 1986). The growth of VSMC is stimulated by the presence of a high concentration of serum (10% FBS) or platelet-derived growth factor (PDGF) (Nilsson *et al.* 1985), and this growth is accompanied by a change from the contractile to the synthetic phenotype of VSMC (Nilsson *et al.* 1985,

Kindy & Sonenshein 1986), and thus represents an atherosclerotic model *in vitro*.

In vertebrates, the actions of many stimuli resulting in proliferative or hypertrophic growth converge on a set of cellular kinase cascades that are collectively called the mitogen-activated protein (MAP) kinase cascades (Force & Bonventre 1998). These MAP kinases have been implicated in VSMC proliferation and hypertrophy and in responses that are central to the pathophysiology of hypertension (Force & Bonventre 1998). Dubey *et al.* (2000) reported that 17 β -estradiol (E_2) inhibits the growth of VSMC partly by reducing extracellular signal-regulated protein kinase (ERK) 1/2 activity. We (Hisamoto *et al.* 2000) and other groups (Simoncini *et al.* 2000) also showed that estrogen induces the activation of endothelial nitric oxide synthase (eNOS) in a nongenomic manner in vascular endothelial cells. However, the range of the biological consequences of the signal transduction cascades activated in VSMC by estrogen in a nongenomic manner has not been defined.

Therefore, these considerations led us to examine whether there are other E_2 -induced signaling pathways involved in the inhibition of VSMC growth, and whether raloxifene, like estrogen, also inhibits VSMC growth, and the mechanism of the growth inhibition. We report here the novel observation that both E_2 and raloxifene induce the phosphorylation and activation of p38, which is a member of the MAP kinase family, in VSMC. This signal transduction cascade substantially induces apoptosis under growth-stimulated conditions. These findings implicate the non-genomic actions of both E_2 and raloxifene in the anti-atherogenic effects of the hormones.

Materials and Methods

Materials

E_2 and actinomycin D were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Raloxifene analog (LY 117018) was a kind gift from Eli Lilly Research Laboratories (Indianapolis, IN, USA). ICI 182,780 was obtained from Tocris (Ballwin, MO, USA). SB 203580 was obtained from CALBIOCHEM (La Jolla, CA, USA). Platelet-derived growth factor (PDGF)-BB was obtained from PeproTech (Rocky Hill, NJ, USA). The Cell Titer 96 AQueous One Solution Cell Proliferation Assay kit was obtained from Promega (Madison, WI, USA). Rabbit polyclonal anti-(phospho- and total) c-Jun N-terminal kinase (JNK), p38, and ERK1/2 antibodies, and a p38 activity assay kit including ATF-2 fusion protein and anti-phospho-ATF-2 antibody were obtained from New England Biolabs (Beverly, MA, USA). Rabbit polyclonal anti-estrogen receptor (ER) α antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Human aortic smooth muscle cells (VSMC) were obtained from Clonetics (Walkersville, MD, USA). The A10 cell

line, which was derived from the thoracic aorta of embryonic rats, was obtained from American Type Culture Collection (Rockville, MD, USA).

Constructs

The human ER α expression vector, pCMV-fER, was a kind gift from Dr D J Shapiro (University of Illinois, Urbana, IL, USA) (Zhang *et al.* 1999).

Cell viability assays

After 5.0×10^3 cells were seeded per well in a 96-well plate, they were incubated for 24 h in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), and then starved in DMEM with 0.4% FBS for 48 h. Cell viability was assessed after a 48-h incubation with E_2 or raloxifene with or without a high concentration of serum (10% FBS) or 25 ng/ml PDGF-BB. The number of surviving cells was measured by determination of the absorbance at 490 nm of the dissolved formazan product after addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) for 1 h as described previously (Hayakawa *et al.* 2000).

Western blots

Cells were washed with phosphate-buffered saline (PBS), and total cell lysates were prepared in lysis buffer (20 mM Tris (pH 7.4), 150 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM sodium orthovanadate, 1 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Twenty micrograms cell lysates were analyzed by SDS-PAGE followed by the chemiluminescence Western blotting method using the indicated antibodies, as described previously (Hayakawa *et al.* 2000).

Assay of p38 activity

Two hundred micrograms cell lysate were incubated with immobilized phospho-p38 monoclonal antibody. The immunoprecipitated products were resuspended in 50 μ l kinase buffer (25 mM Tris, pH 7.5, 10 mM MgCl₂, 5 mM β -glycerolphosphate, 0.1 mM sodium orthovanadate, and 2 mM dithiothreitol) containing 200 μ M ATP and 2 μ g ATF-2 fusion protein. The kinase reaction was allowed to proceed at 30 °C for 30 min and stopped by the addition of sample buffer. Reaction products were resolved by SDS-PAGE and then subjected to Western blotting with anti-phospho-ATF-2 antibody.

Apoptosis assay

Five thousand cells/well were placed into extracellular matrix-coated chamber slides in DMEM with 10% FBS,

and then starved for 48 h in DMEM with 0.4% FBS. After starvation, some cells were treated with various materials. Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining using an Apoptosis In Situ Detection kit (Wako, Osaka, Japan), and the cells undergoing programmed cell death were counted in five separate fields/experiment. Alternatively, cells were analyzed for apoptosis by flow cytometry (FACSCalibur, Becton Dickinson, Mountain View, CA, USA). Following the treatment described above, trypsin-detached VSMC were pooled and washed once with PBS and fixed in 70% cold ethanol. After fixation, cells were stained with PBS, propidium iodide (50 µg/ml), RNase A (50 µg/ml), 0.1% Triton X-100 and 0.1 mM EDTA for 24 h. The DNA content of VSMC was analyzed by flow cytometry using a FACSCalibur flow cytometer. At least 10 000 events were analyzed, and the percentage of cells in the sub-G₁ population was calculated. Both types of apoptosis assays were repeated three times with consistent results.

Reverse transcriptase-polymerase chain reaction (RT-PCR) experiments

Total RNA was extracted from VSMC and A10 cells with Tri-Reagent (Molecular Research Center, Inc, Cincinnati, OH, USA) according to the manufacturer's instructions. Total RNA (5 µg) was used as a template for first-strand synthesis. An RT-PCR kit obtained from TakaRa (Tokyo, Japan) was used according to the manufacturer's instructions. Primers were designed from published gene sequences. The sequences of the PCR primers were as follows: rat ERα: 5'-AATTCTGACAATCGACGCC-3' and 5'-GTGCTTC AACATTCTCCCTCCTC-3'; rat ERβ: 5'-TTCCC GGCAGCACCAGTAACC-3' and 5'-TCCCTCTTTG CG TTTGGACTA-3'; human ERα: 5'-GGCCAGT ACCAATGACAAGGGAAG-3' and 5'-CCAGCAAGC ATGTCGAAGATCTCC-3'; human ERβ: 5'-GTTGC GCCAGCCCTGTTC-3' and 5'-CTCGTCGGCA CTTCTCTGTCTC-3'. To check cDNA integrity, fragments of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified in parallel. The amplification conditions consisted of 35 cycles of incubation at 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min. PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining under UV illumination.

Assay of p38 phosphorylation using a transient expression system

A10 cells cultured in 100-mm dishes were transfected with 1 µg ERα expression vector (pCMV-*ER*) using Lipofectamine Plus as described previously (Hisamoto *et al.* 2000). Seventy-two hours after transfection, serum-

deprived cells were incubated with 10⁻⁸ M E₂ and cell lysates were analyzed by SDS-PAGE, followed by Western blotting using a specific antibody against phosphorylated p38.

Statistics

Statistical analysis was performed by Student's *t*-test, and *P* < 0.05 was considered significant. Data are expressed as means ± S.E.

Results

Effects of E₂ and raloxifene on cell growth of VSMC

We evaluated the effect of E₂ on VSMC under growth-stimulated and non-stimulated conditions. No growth inhibitory effect by estrogen at 10⁻¹⁰ to 10⁻⁷ M was observed under quiescent conditions (0.4% FBS) (Fig. 1A). Treatment with 10% FBS significantly stimulated the proliferation (by 1.53-fold) (Fig. 1B), as did treatment with 25 ng/ml PDGF-BB (by 1.33-fold) (Fig. 1C). Significant, dose-dependent growth inhibition by E₂ under the growth-stimulated (10% FBS) conditions was observed at concentrations of 10⁻⁹ M to 10⁻⁷ M (Fig. 1B and C). Raloxifene at 10⁻⁹ M also caused a significant growth inhibition in cells treated with 10% FBS (Fig. 1D). These data suggest that physiological concentrations of E₂ and raloxifene inhibit the cell growth of VSMC with the synthetic phenotype.

Effect of E₂ and raloxifene on MAP kinase family members in VSMC

Since mitogen-activated protein (MAP) kinases have been implicated in VSMC proliferation, the role of MAP kinases in the E₂- and raloxifene-induced growth inhibition of VSMC was studied. To evaluate whether MAP kinases are activated by E₂, cells were treated with 10⁻⁸ M of E₂ under growth-stimulated (10% FBS) or unstimulated (0.4% FBS) conditions. Cell extracts were prepared and subjected to Western blot analysis using antibodies against the phosphorylated forms of various MAP kinase family members, namely ERK (p-ERK), JNK (p-JNK), and p38 (p-p38). As reported previously (Dubey *et al.* 2000), ERK phosphorylation was significantly inhibited by E₂ in a dose-dependent manner under the growth-stimulated conditions (Fig. 2A, upper and middle panels). Although JNK was phosphorylated in response to UV (30 min) and anisomycin treatment, JNK phosphorylation was not changed detectably by E₂ (Fig. 2B, upper and middle panels). The total amounts of ERK (Fig. 2A, lower panel) and JNK (Fig. 2B, lower panel) proteins were not changed by E₂. Although E₂ did not affect the phosphorylation of p38 under quiescent conditions (0.4% FBS), E₂ induced p38 phosphorylation

Figure 1 E₂ and raloxifene inhibit VSMC proliferation induced by 10% FBS or by PDGF. After starvation, cell growth was promoted by 0.4% FBS (A), 10% FBS (B and D) or 25 ng/ml PDGF (C). Growth-promoted cells were treated with various concentrations of E₂ (A, B, C) or 10⁻⁹ M raloxifene (D). After 48 h incubation, cell growth was evaluated by the MTS assay as described in Materials and Methods. Cell growth is expressed as a percentage of the absorbance obtained in the non-stimulated condition. Data are expressed as means ± S.E.M. (n=12). ***P<0.0001 compared with 0.4% FBS; **P<0.01 compared with no treatment with E₂ or raloxifene.

under growth-stimulated conditions, such as in medium containing 10% FBS (Fig. 2C). Raloxifene at 10⁻⁹ M also induced the phosphorylation of p38 (Fig. 2D, upper and middle panels). The total amount of p38 was not changed by E₂ (Fig. 2C, lower panel) or raloxifene (Fig. 2D, lower panel).

E₂-induced phosphorylation and activation of p38

To evaluate the dose dependency of E₂-induced phosphorylation of p38 in VSMC under the growth-stimulated

conditions, cells were treated with various concentrations of E₂ for 10 min. In the range of 10⁻⁹ M to 10⁻⁶ M, E₂ induced the phosphorylation of p38 in a dose-dependent manner (Fig. 3A, upper and middle panels). The time course of E₂-induced phosphorylation of p38 was also evaluated in VSMC under the growth-stimulated conditions. The increase in the phosphorylation of p38 induced by 10⁻⁸ M E₂ reached a maximum at 10 min and rapidly declined thereafter (Fig. 3B, upper and middle panels). E₂ did not change the total amount of p38 protein (Fig. 3A and 3B, lower panels).

Figure 2 Effect of E₂ and raloxifene on MAP kinase family members. VSMC were treated for 10 min with the indicated concentrations of E₂ with 10% FBS (A), or with UV for 30 min, 10⁻⁶ M anisomycin for 10 min or 10⁻⁸ M E₂ for 10 min (B). VSMC were treated for 10 min with or without 10⁻⁸ M E₂ with 0.4% FBS or 10% FBS (C). VSMC were treated for 10 min with or without 10⁻⁹ M raloxifene with 10% FBS (D). Equal amounts of protein were analyzed by Western blotting using a specific antibody against phosphorylated ERK1/ERK2 (A, middle panel), phosphorylated JNK (B, middle panel), phosphorylated p38 (p-p38) (C and D, middle panels), or total ERK1/ERK2, JNK, p38 antibody (A, B, C and D, lower panels). Relative densitometric units of the p-ERK (A), p-JNK (B), and p-p38 (C and D) bands are shown in the upper panels, with the density of the control bands set arbitrarily at 1.0. Values shown represent the mean ± S.E. from at least three separate experiments. Significant differences are indicated: **P* < 0.01.

To determine if this response involves ER, the effect of concomitant treatment with the highly specific ER antagonist ICI 182,780 was determined (Fig. 3C). ICI 182,780 completely abolished the induction of p38 phosphorylation by E₂. Moreover, the effect of actinomycin D, an inhibitor of gene transcription (Hisamoto *et al.* 2000) was tested to examine the influence of genomic events mediated by nuclear ERs (Fig. 3D). Although actinomycin D inhibited the E₂-induced transcriptional transactivation of an estrogen-responsive reporter plasmid encoding estrogen response element-thymidine kinase-luciferase in VSMC (data not shown), it did not detectably affect the induction of p38 activation by E₂. This result suggests that the phosphorylation and activation of p38 by E₂ mainly depend on non-genomic effects of estrogen

via ER. The total amount of p38 was not changed by E₂ (Fig. 3C and 3D, lower panels).

Effect of ERα expression on E₂-induced p38 phosphorylation

Because VSMC express both ERα and ERβ mRNA (Fig. 4A) as reported previously (Karas *et al.* 1994), it is not known which estrogen receptor mediates the phosphorylation of p38 in VSMC. The A10 cell line is derived from the thoracic aorta of embryonic rats and is a commonly used model of vascular smooth muscle cells (Weiss *et al.* 1998). ERα mRNA was not detected by RT-PCR (Fig. 4A) and ERα was not detected by Western blotting (Fig. 4B, lower panel) in A10 cells (Fig. 4A), in agreement with a previous report (Smith *et al.* 1998). We therefore

Figure 3 E₂-induced p38 phosphorylation and activation. VSMC were treated for 10 min with various concentrations of E₂ (A), and with 10⁻⁸ M E₂ for the indicated times (B). (C) VSMC were pre-treated with 10⁻⁶ M ICI 182,780 (ICI) for 30 min (lane 3), then treated for 10 min with (lanes 2 and 3) or not treated with (lane 1) 10⁻⁸ M E₂. Equal amounts of protein were analyzed by the Western blotting method using a specific antibody against phosphorylated p38 (p-p38) (A, B, and C, middle panels) or total p38 (A, B, C and D, lower panels). (D) VSMC were treated with 10⁻⁸ M E₂ with 10% FBS for 10 min (lane 2), or with 25 µg/ml actinomycin D (Act-D) for 120 min, and then treated for 10 min with 10⁻⁸ M E₂ (lane 3). Lysates were subsequently subjected to immunoprecipitation with immobilized anti-p38 antibody and the kinase reaction was carried out in the presence of GSK-ATF2 fusion protein, followed by Western blotting with an anti-phospho-ATF2 antibody (D, middle panel). Relative densitometric units of the p-p38 (A-C) and p-ATF2 (D) bands are shown in the upper panels, with the density of the control bands set arbitrarily at 1.0. Values shown represent the mean ± S.E. from at least three separate experiments. Significant differences are indicated: **P* < 0.01.

examined the effect of E₂ on the phosphorylation of p38 in A10 cells and A10 cells transfected by ERα expression vector. The expression of ERα in A10 cells transfected with ERα expression vector was confirmed by Western blotting

(Fig. 4B, lower panel). Although E₂ had no effect on the p38 phosphorylation in A10 cells, transfection of ERα expression vector caused an increase in E₂-induced p38 phosphorylation (Fig. 4B, upper and middle panels).

Figure 4 Effect of ER α expression on E₂-induced p38 phosphorylation. (A) Total RNA samples were isolated from cultured VSMC and A10 cells. RT-PCR was performed as described in Materials and Methods. Amplification products of 345 and 262 bp correspond to ER α and ER β cDNA respectively. (B) Cells were transfected (lanes 3 and 4) or not transfected (lanes 1 and 2) with ER α expression vector (pCMV-ER), and after 72 h were treated for 10 min with 10⁻⁸ M E₂ with 10% FBS. Equal amounts of protein were analyzed by Western blotting using a specific antibody against phosphorylated p38 (middle panel) or ER α (lower panel). Relative densitometric units of the p-p38 bands are shown in the upper panel, with the density of the control bands set arbitrarily at 1.0. Values shown represent the mean $\pm$ S.E. from at least three separate experiments. Significant differences are indicated: **P* < 0.01. N.S., not significant.

These results suggest that ER α is required for the E₂-induced p38 phosphorylation.

E₂ and raloxifene induce apoptosis in VSMC

The signaling cascade of p38 is known to be involved in cellular processes involving apoptosis (Xia *et al.* 1995) and to activate apoptosis-related transcription factors such as ATF2 or Elk-1 in many kinds of cells (Schaeffer & Weber 1999). In addition, it was reported that activation of p38 by E₂ is coupled to ER-induced apoptosis in HeLa cells transfected with ER (Zhang & Shapiro 2000). Therefore, we next examined whether E₂ induces apoptosis in VSMC. VSMC were grown in the presence of 10% FBS or 25 ng/ml PDGF-BB with or without 10⁻⁸ M E₂. Cultured cells were stained by the TUNEL method (Gavrieli *et al.* 1992). Apoptotic cells were stained brown (Fig. 5A), and were counted by inspection under a light microscope. E₂ induced apoptosis in VSMC cultured in medium containing 10% FBS (Fig. 5B) or 25 ng/ml PDGF-BB (Fig. 5C), while E₂ did not induce apoptosis without these growth stimulants (0.4% FBS) (Fig. 5B). These data suggest that estrogen induced apoptosis in VSMC only under growth-stimulated conditions. Furthermore, a specific antagonist of ER, ICI 182,780 and a specific inhibitor of p38, SB 203580, significantly inhibited the E₂-induced apoptosis (Fig. 5B and 5C). These data suggest that the signaling cascade of p38 mediates the E₂-induced apoptosis of VSMC via a mechanism involving ER.

Moreover, we examined whether raloxifene induces apoptosis in VSMC. TUNEL staining analysis showed that raloxifene at 10⁻⁹ M induced apoptosis in VSMC cultured in medium containing 10% FBS (Fig. 5D).

We also performed cell cycle analysis to examine the effects of E₂ and raloxifene on serum-stimulated cell cycle progression in VSMC. In the presence of 10⁻⁸ M E₂ (Fig. 5E) or 10⁻⁹ M raloxifene (data not shown), the entry of VSMC into S phase from G₀/G₁ phase was partially blocked, while there was a concomitant increase in the percentage of apoptotic cells to a level about five-fold higher than that under non-stimulated conditions, suggesting that E₂ induced the cells to undergo apoptosis.

Discussion

Although recent evidence suggests that direct vascular effects of estrogen play a crucial role in the atheroprotective properties of the hormone (Mendelsohn & Karas 1999), the mechanisms are not well understood. Smooth muscle cells are the major cellular constituent of the medial layer of the aorta. These cells play a key role in the progression of atherosclerosis. The major finding of the present study is that non-genomic signaling mediates

Figure 5 Effects of E_2 and raloxifene on apoptosis of VSMC. Cells were grown on a chamber slide. (A–C) VSMC were treated for 1 h with or without 10^{-8} M E_2 under quiescent conditions (0.4% FBS) or growth-stimulated conditions (10% FBS or 25 ng/ml PDGF). In some groups, cells were pre-treated with 10^{-6} M ICI 182,780 (ICI) or 10^{-6} M SB 203580 (SB) for 30 min. (D) VSMC were treated for 1 h with or without 10^{-9} M raloxifene under growth-stimulated conditions (10% FBS). Cells were stained by the TUNEL method using a kit for detection of apoptosis *in situ*. (A, left panel) FBS 0.4% with 25 ng/ml PDGF. (A, right panel) FBS 0.4% with 25 ng/ml PDGF and 10^{-8} M E_2 . Apoptotic cells were stained and were counted by visual inspection under a light microscope. Methyl green solution (0.5%) was also used for counterstaining. Magnification: $\times 200$. Bar=100 μ m. The percentage of apoptotic cells is shown in B, C, and D, with the proportion of apoptotic cells in the cultures treated with vehicle set arbitrarily at 100%. Values shown represent the mean $\pm$ S.E. from five separate experiments. Significant differences are indicated: $*P < 0.05$. (E) VSMC were growth-stimulated by 10% FBS or were not stimulated (0.4% FBS), and were then incubated with or without E_2 for 24 h. All cells were subjected to FACS analysis to measure the DNA content. The apoptotic cells included the sub- G_0/G_1 fraction. Values are the mean $\pm$ S.E.M. of three independent experiments. $*P < 0.05$ compared with the vehicle.

the estrogen- and raloxifene-induced growth inhibition of human VSMC in the pathologically proliferative state. VSMC grown under proliferative conditions *in vitro* undergo a change from the contractile to the synthetic phenotype and are considered a model for thickening of the arterial intimal layer as an initiating event of atherosclerosis *in vivo* (Kindy & Sonenshein 1986).

MAP kinases have been implicated in the signaling cascades involved in the proliferation and hypertrophy of smooth muscle cells (Force & Bonventre 1998). These kinases include the ERK and the stress-activated MAP kinases, JNK and p38. One of the mechanisms involved in these effects was previously reported to be inhibition of ERK activity by estrogen in human VSMC (Dubey *et al.* 2000), which we also confirmed (Fig. 2A). We further

explored the signaling cascade that underlies the estrogen-induced inhibition of VSMC proliferation, and found evidence that estrogen rapidly activates the p38 signaling cascade and induces apoptosis via ER in VSMC. Activation of p38 may be an upstream step regulating other proteins involved in cell death signaling (Xia *et al.* 1995). A specific inhibitor of p38, SB 203580, blocked apoptosis, and a specific antagonist of ER, ICI 182,780, blocked both apoptosis and the induction of p38 phosphorylation by E_2 in VSMC. To our knowledge, although there is evidence that docosahexaenoic acid induces apoptosis in rat mesenteric VSMC via the activation of p38 (Diep *et al.* 2000), this is the first study showing that E_2 induces apoptosis via a p38-dependent mechanism in human VSMC.

In the present study, we demonstrated that E₂ induced apoptosis through a p38 signaling cascade in synthetic VSMC. Although estrogen was reported to have an anti-apoptotic effect in vascular endothelial cells (Razandi *et al.* 2000), it was reported that estrogen induced apoptosis in an erythroid cell line (Blobel & Orkin 1996) and induced apoptosis and G₁ cell cycle arrest of human multiple myeloma cells (Wang *et al.* 2001). Moreover, E₂ stimulates the growth of MCF-7 cells and does not induce p38 phosphorylation or apoptosis, while activation of p38 by E₂ is coupled to ER-induced apoptosis in HeLa cells transfected with ER (Zhang & Shapiro 2000). Thus, the ability of E₂ to modulate such diverse cell functions appears to be the result of its ability to modulate signaling cascades in a cell- and tissue-specific manner. Hence, the balance among activities of the ERK, the JNK, and the p38 cascades may be an important factor in the decision of different types of cells whether to proliferate, remain quiescent, or undergo apoptosis when they are stimulated by estrogen. Hayashi *et al.* (1999) reported that a change in the balance between the phosphoinositide 3-kinase (PI3-K)/Akt cascade and the ERK/p38 MAP kinase cascade could determine the phenotypes of VSMC. In this study, we have shown that E₂ affects the balance between the ERK and p38 MAP kinase cascades: E₂ enhanced p38 phosphorylation and inhibited ERK phosphorylation. Because E₂ stimulates the PI3-K/Akt pathway in human vascular endothelial cells (Hisamoto *et al.* 2000, Simoncini *et al.* 2000), it might also affect the balance of the PI3-K/Akt pathway in VSMC.

One report suggested that commercially available E₂-BSA did not mimic E₂ and is not an appropriate ligand for investigating non-genomic events mediated by a membrane ER (Steviss *et al.* 1999). However, the E₂ effects in the present study were very rapid, and a specific ER antagonist could prevent the signaling. Moreover, actinomycin D, an inhibitor of gene transcription, was also tested to rule out the influence of genomic events mediated by nuclear ER, and it did not affect the activation of p38. Therefore, we believe that the effects of E₂ which we observed here mainly depend on non-genomic effects of estrogen mediated by ER.

The two known estrogen receptors, ER α and ER β , mediate the effects of estrogen in all target tissues, including blood vessels. Because both are expressed in VSMC (Fig. 4A), it is not known which of them mediates the phosphorylation of p38 in VSMC. We therefore examined the effect of E₂ in A10 rat aortic smooth muscle cells, which express ER β but not ER α (Fig. 4A). Although E₂ had no effect on the p38 phosphorylation in A10 cells, transfection of ER α expression vector caused an increase in E₂-induced p38 phosphorylation (Fig. 4B, upper panel). These results suggest that ER α is involved in the induction of p38 phosphorylation by E₂ in VSMC. Recently, it was reported that VSMC isolated from ER β -deficient mice showed multiple abnormalities of ion

channel function (Zhu *et al.* 2002). Thus, the function mediated by ER α might be different from that mediated by ER β in VSMC. In addition, Karas *et al.* (2001) showed that E₂ did not inhibit an increase in the vascular medial area following injury in ER α , β (double) knockout mice, but did inhibit VSMC proliferation of injured carotid arteries in ER α , β (double) knockout mice. Therefore, it is possible that there are other receptor-independent mechanisms or unidentified receptor(s) for estrogen.

We conclude that E₂ and raloxifene inhibit the proliferation of VSMC by increasing apoptosis induced by p38 activation, and by reducing ERK MAP kinase activity under growth-stimulating conditions. These effects of E₂ and raloxifene on VSMC may mainly depend on non-genomic effects mediated by ER, although the participation of other mechanisms cannot be ruled out. This effect on VSMC may partly account for the anti-thickening effect of E₂ on the arterial intimal layer. Moreover, our data support the possibility that raloxifene has favorable effects on vasculature (Barrett-Connor *et al.* 2002). Since E₂ induces apoptosis and G₁ cell cycle arrest in human multiple myeloma cells (Wang *et al.* 2001) and apolipoprotein E inhibits the PDGF-induced proliferation of VSMC by suppressing cyclin D1 expression (Ishigami *et al.* 1998), it is possible that E₂ affects the cell cycle in synthetic VSMC. This possibility is currently under investigation.

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