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Responses of vascular smooth muscle cells to estrogen are dependent on balance between ERK and p38 MAPK pathway activities

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Abstract

Objective: To investigate the mechanisms underlying the dual effects of estrogen on vascular smooth muscle cells (VSMC).
Methods: MTT assay, ELISA, flow cytometry and Western analysis were used to investigate the effects of 17β-estradiol (E_2) on proliferation, apoptosis, cell cycle progression, ERK and p38 activities of subcultured rat VSMC with or without chemical block of MEK or p38 kinases.
Results: E_2-promoted VSMC proliferation was accompanied with an increased phosphorylation of ERK1/2, which could be blocked by MEK inhibitor U0126; the E_2-induced VSMC apoptosis, which appeared mainly in the G2/M phase, was related with the activation of p38 and could be blocked by p38 inhibitor SB203580. More interestingly, MEK inhibition in E_2-treated VSMC led to an enhanced p38 phosphorylation and a shift of apoptosis from G2/M phase-predominant to G0/G1 phase-predominant; whereas block of p38 increased the E_2-induced ERK1/2 phosphorylation and proliferation of the VSMC. This reciprocal phenomenon was related with cross-talk between ERK and p38 pathways which might be mediated by MKP-1 and PP2A. The effects of E_2 on proliferation and apoptosis, and their related pathways could be separately induced by the specific agonists of estrogen receptor (ER) α and β alone and inhibited or eliminated by the ER blocker ICI 182,780.
Conclusion: The dual effects of estrogen on VSMC involve concurrent activations of ERK and p38 pathways by ER α and β respectively, and the fates of VSMC are determined by the dynamic balance between these two pathways.

Keywords: Estrogen; Vascular smooth muscle cell; Proliferation; Apoptosis; MAP kinase

1. Introduction

It is well documented that the incidence of cardiovascular diseases is much lower in women before menopause, and this is due to a beneficial effect of estrogen on the cardiovascular system. However, the mechanisms of the hormone's cardiovascular-protective actions are still not very clear [1].

The unbalance of proliferation and apoptosis of vascular smooth muscle cells (VSMC) plays an important role in the development of proliferative vascular diseases such as primary atherosclerosis, postangioplasty restenosis and transplant vasculopathy. Although numerous studies have suggested that in vivo, administration of estrogen significantly suppresses injury-induced arterial intimal hyperplasia through inhibiting VSMC proliferation [2–6], data from in vitro experiments are conflicting with some studies showing that estrogen inhibits cultured VSMC replication and migration [7–14], and others that the hormone promotes ^{3}H-thymidine incorporation or proliferation of the cells [15–18]. A recent study using aromatase-knockout (ArKO) mouse as an estrogen-deficient animal model showed that VSMC cultured from the ArKO mice had a much lower proliferative response to serum or PDGF-BB than those from the wild-type animals; and the thickness of the aortic medial smooth muscle was approximately 10% less in the ArKO mice than WT male mice at the age of 1 year [15]. This implicates that endogenous estrogen may play an

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important role in maintaining of a normal proliferative response that is necessary for homeostasis of the vascular wall.

The reason for the disparity is unknown, but studies by Mori-Abe et al. have shown that estrogen not only affects the proliferation, but also induces apoptosis of VSMC [19], which may complicate its effects on VSMC growth. A recent study in our laboratory further demonstrated that the estrogen-induced apoptosis of VSMC appeared mainly in the G2/M phase with an accelerated G1 to S phase transition of the cell cycle, suggesting that the hormone triggers apoptosis of the cells that are undergoing replication [20].

It is known that proliferation and apoptosis are two associated, but mutually exclusive cellular events. In most cases, a factor that enhances proliferation of a cell inhibits its apoptosis, and vice versa. It is also well documented that two mitogen-activated protein kinase (MAPK) pathways are involved in these two cellular events: the extracellular signal-regulated kinase (ERK) pathway that is related to cell proliferation [21,22] and the p38 MAPK pathway, to apoptosis [23,24].

Why is estrogen able to concurrently promote such two opposing cellular courses? To answer this question, the present study investigated the effects of the hormone on the activities of both ERK and p38 in cultured VSMC, and the responses of the cell under the same conditions after blocking either one of these two MAPK pathways.

2. Materials and methods

The animal use protocol in the present investigation is approved by the Institutional Animal Care and Use Committee of Wuhan University.

2.1. Materials

The 17β-estradiol (E_2) (E4389; Sigma; MO, USA), estrogen receptor (ER) isoform α and β specific agonists PPT (1426) and DPN (1494), ER antagonist ICI 182,780 (1047; Tocris Cookson Incorporation, Missouri, USA) and Phenol red-free RPMI 1640 (SH30197.01; Hyclone; Utah, USA) were commercially obtained. Antibodies against ERK1 (sc-94), ERK2 (sc-154), phospho-ERK1/2 (sc-7383), p38 (sc-7149), phospho-p38 (sc-7973), MAPK phosphatase (MKP)-1 (sc-370) and protein phosphatase (PP) 2A (sc-6110) were purchased from Santa Cruz Biotechnology (CA, USA). MEK inhibitor U0126 (V1121) and p38 kinase inhibitor SB203580 (V1161) were purchased from Promega Corporation (Wisconsin, USA), and ELISA Cell Death Detection kit and Annexin V-FITC kit were obtained from the Roche Diagnostics Corporation (Germany) and Jingmei Biotech Company (Guangzhou, China), respectively.

2.2. Isolation and culture of VSMC

VSMC were enzyme-dispersed from the aortas of the mature and non-pregnant female Sprague–Dawley rats (200 g±20 g) using a modified method originally described by Campbell et al. [25]. The cells were cultured in phenol red-free RPMI 1640 containing 10% FCS, 100 units/ml penicillin, 100 pg/ml streptomycin and 4 mmol/L L-glutamine at 37 °C in 5% CO_2. The purity of the VSMC was examined by an immunocytochemical staining with anti-α SM actin. Passages of 2 to 4 were used for this study.

2.3. Cell proliferation assay

The effect of E_2 on VSMC proliferation was measured by methyl thiazolyl tetrazolium (MTT) assay. The cells were plated in 96-well culture plates (10^3 cells per well) and incubated for 72 h in the medium containing 10^{-8} M E_2 with or without pre-administration of 10^{-6} M ER antagonist ICI 182,780 for 30 min. After treatment, the cells were washed with PBS for 3 times, incubated with 0.5% MTT in RPMI 1640 at 37 °C for 4 h and dissolved in 150 μl DMSO. The absorbance of deoxidized MTT, which represents the levels of succinic acid dehydrogenase of mitochondria in the live cells, was measured at 492 nm using a microplate reader (GENios; TECAN; Austria) against a background control. Data were expressed as means of absorbance from 5 wells in each treatment.

2.4. Detection of cell apoptosis

To detect the E_2-induced apoptosis, a measurement of mono- and oligo-nucleosomes in the cytoplasmatic fraction of the VSMC was performed with ELISA. The cells treated as above were prepared according to the protocol provided by the manufacturer and analyzed on the plate reader at 492 nm. Data were expressed as means of absorbance from 5 wells in each treatment.

2.5. Analysis of relationship between VSMC apoptosis and cell cycle progression

The relationship between E_2-induced apoptosis and the cell cycle progression was studied by a bivariate analysis of DNA content (PI staining) and Annexin V binding with flow cytometry. After a 24 h serum deprivation for cell cycle synchronization, the VSMC were incubated for 72 h in the medium containing 10^{-8} M E_2. After the treatment, the cells were harvested, incubated with 5 μl Annexin V-FITC for 1 h at RT, fixed with ice-cold acetone for 10 min and stained with 20 μg/ml PI solution for 30 min at RT. Triplicate samples with 1×10^6 cells for each treatment were analyzed by flow cytometry under an excitation light of 488 nm. The green FITC fluorescence was detected at 510–550 nm wavelengths, and the red PI, at 610 nm. The assay above was repeated in three culture plates.

2.6. Detection of activities of MAPK pathways

To investigate the possible involvement of MAPK signaling pathways in the effects of estrogen on VSMC, levels of total and phosphorylated ERK and p38 were analyzed with western blot. The VSMC were treated with 10^{-8} M E_2 for different times (5 min, 10 min, 30 min, and 60 min) or with different concentrations (10^{-11} M–10^{-8} M) of E_2 for 30 min. To extract the total proteins, the cells were rinsed twice with ice-cold PBS, lysed in ice-cold lysing buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 100 mM NaF, 1 mM sodium orthovanadate, 5 mM EDTA, 0.1% Triton X-100, and 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin) and centrifuged at 8000 rpm for 10 min at 4 °C. The supernatants were analyzed with BCA Protein Assay kit (Beyotime Biotechnology, Haimen, China) to check the concentrations of gross proteins and stored at −70 °C until use. Equal amounts of gross protein extracts (15 μg/lane) were subjected to SDS-PAGE on a 10% separating gel and electrophoretically transferred onto PVDF membrane. After blocking with 5% skim milk powder in 0.1% Tween 20 in TBS, the membranes were incubated with antibodies against ERK1, ERK2, phospho-ERK1/2, p38, or phospho-p38 for 2 h, followed by a horseradish peroxidase-conjugated secondary antibody for 1 h at RT. The reaction was visualized by DAB staining. The blot was scanned and the integrated intensity of all pixels in each band was analyzed with Scion Image software (Scion Corporation, Frederick, MD, USA). The relative phosphorylation levels of each protein were expressed as means of phospho-/total-protein × 100%. All experiments were performed in twice with three separate cultures.

2.7. MAPK signal transduction block tests

To further confirm the involvements of ERK pathway in VSMC proliferation and p38 MAPK pathway in apoptosis, the VSMC were incubated for 30 min with either a MEK inhibitor (U0126) or a p38 kinase inhibitor

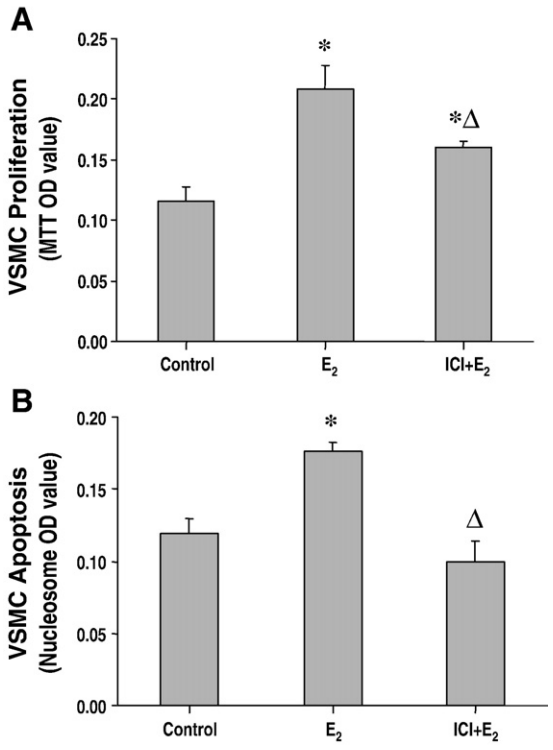

Fig. 1. Effects of E_2 on VSMC proliferation (A: MTT assay) and apoptosis (B: ELISA analysis for cytoplasmatic fractions of mono- and oligo-nucleosomes). The cells were treated with vehicle (Control), 10^{-8} M E_2 (E_2) for 72 h or 10^{-6} M ICI 182,780 for 30 min followed by 10^{-8} M E_2 for 72 h (ICI+E_2). Data were expressed as means±SD of absorbance. $N=5$; $^{*}P<0.05$, compared to control group; $^{\Delta}P<0.05$, compared to E_2 group.

(SB203580) at different concentrations before adding 10^{-8} M E_2. After the treatments, the cells were prepared for analyses of proliferation, apoptosis, ERK1/2 and p38 phosphorylation, or protein phosphatases MKP-1 and PP2A expressions with MTT, ELISA, flow cytometry or western blot as mentioned above.

2.8. Detection of functional difference of ER isoforms in VSMC

To investigate if the dual actions of E_2 on VSMC are related with different ERs, additional experiments were performed with two specific ER isoform agonists, PPT for ERα and DPN for ERβ. The VSMC were treated with PPT or DPN in different concentrations (10^{-11} M–10^{-7} M), or with 10^{-7} M PPT (or DPN) plus 10^{-6} M ICI 182,780. After the treatments, the cells were prepared for analyses of proliferation and apoptosis by MTT and ELISA. The activities of corresponding intracellular signaling (phosphorylations of ERK1/2 and p38) were analyzed with western blot as mentioned above.

2.9. Statistical analysis

Data were statistically analyzed by SPSS 11.0 software (SPSS Inc. IL, USA) with the Student–Newman–Keuls (S–N–K) multiple comparison test after a one-way ANOVA. A difference was considered statistically significant when $P<0.05$.

3. Results

3.1. Effects of E_2 on proliferation and apoptosis of VSMC

In our previous observations [18,20], we have shown that E_2 displayed a dual (growth-promoting and apoptosis-inducing) effect on VSMC in a concentration-dependent manner with a maximal-effective concentration of E_2 at 10^{-8} M. Here, as demonstrated by MTT assay (Fig. 1A) and ELISA analysis (Fig. 1B), E_2 at this maximal-effective concentration caused a 0.80-fold increase in proliferation and a 0.40-fold increase in apoptosis of the same cells, which could be significantly inhibited or totally eliminated by treatment of 10^{-6} M ICI 182,780 respectively. Flow cytometry analysis further confirmed that the 10^{-8} M E_2-enhanced proliferation of VSMC was due to an accelerated transition of the cells from G1 to S phase (Table 1; compare E_2 group with control). Although, at its maximal-effective concentration, E_2-induced apoptosis appeared in both S and G2/M phases, it was considerably higher in G2/M phase (Table 2; compare E_2 group with control).

3.2. Effects of E_2 on MAPK in VSMC

Western analysis showed that the expressions of total-ERK1/2 proteins in the VSMC treated with 10^{-8} M E_2 were unchanged, but levels of phospho-ERK1/2 increased from 10 min after treatment, keeping a high-plateaued value of phospho-/total-ERK till 30 min (Fig. 2A). Similarly, the total-p38 expression was unchanged in E_2-treated VSMC, while the levels of phospho-p38 increased with a peak value at 30 min (Fig. 2B). When the cells were treated with different concentrations of E_2 for 30 min, both phospho-ERK1/2 and phospho-

Table 1
Cell cycle progression of VSMC (% of total cells; means±SD; $N=9$).

VSMC	G0/G1 (%)	S (%)	G2/M (%)	RI (%)
Control	77.66±0.65	20.73±0.88	1.60±0.27	22.34±0.65
E_2	72.42±1.27*	26.30±1.30*	1.28±0.13	27.58±1.26*
U0	80.48±0.89*	18.82±0.78	0.70±0.26*	19.52±0.89*
U0+E_2	79.65±0.72$^{*\Delta}$	19.1±0.60$^{\Delta}$	1.25±0.31	21.02±0.46$^{*\Delta}$
SB	77.65±1.13	20.71±1.58	1.30±0.70	22.02±1.66
SB+E_2	66.62±0.87$^{*\Delta}$	32.08±0.87$^{*\Delta}$	1.29±0.72	33.37±0.87$^{*\Delta}$

The subcultured VSMC were stained by PI after treatment with vehicle (Control), 10^{-8} M E_2 (E_2) for 72 h, 10 μM U0126 (U0) or 10 μM SB203580 (SB) alone for 30 min, or 10 μM U0126 or 10 μM SB203580 for 30 min followed by 10^{-8} M E_2 for 72 h (U0+E_2 or SB+E_2).
$^{*}P<0.05$, compared with control group; $^{\Delta}P<0.05$, compared with E_2 group.

Table 2
Apoptosis of VSMC during cell cycle progression x (% of total cells; means±SD; $N=9$).

VSMC	G0/G1	S	G2/M	SUM
Control	1.85±0.12	1.49±0.15	1.19±0.07	4.53±0.13
E_2	1.72±0.12	1.98±0.34*	3.12±0.34*	6.82±0.24*
U0	3.59±0.28*	1.67±0.15	1.24±0.11	6.50±0.30*
U0+E_2	4.09±0.31$^{*\Delta}$	1.70±0.11	1.38±0.27$^{\Delta}$	7.17±0.36*
SB	1.66±0.11	1.57±0.31	0.97±0.22	4.20±0.37
SB+E2	1.77±0.14	1.54±0.15	1.06±0.09$^{\Delta}$	4.38±0.33$^{\Delta}$

The subcultured VSMC were stained by PI after treatment with vehicle (Control), 10^{-8} M E_2 (E_2) for 72 h, 10 μM U0126 (U0) or 10 μM SB203580 (SB) alone for 30 min, or 10 μM U0126 or 10 μM SB203580 for 30 min followed by 10^{-8} M E_2 for 72 h (U0+E_2 or SB+E_2).
$^{*}P<0.05$, compared with control group; $^{\Delta}P<0.05$, compared with E_2 group.

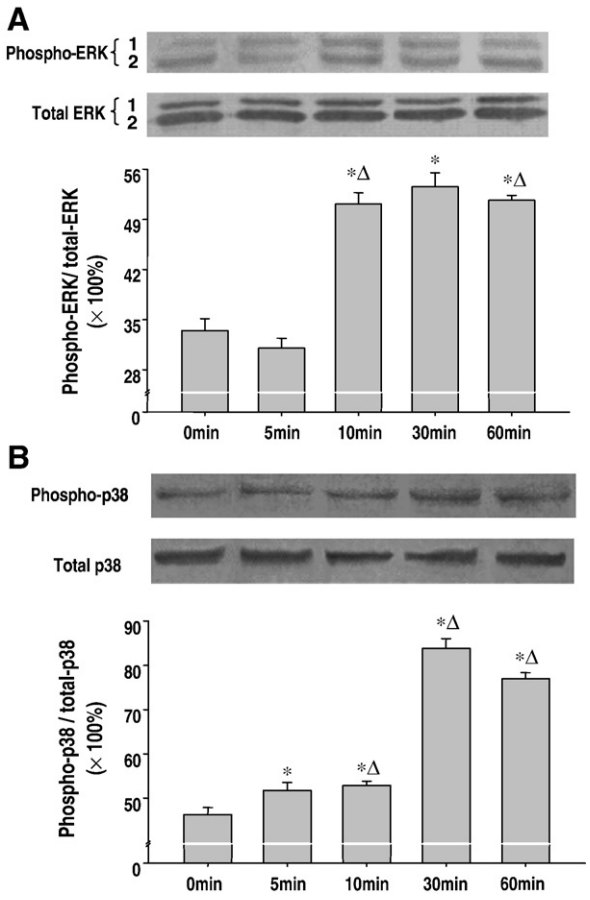

Fig. 2. Western blot analysis on phosphorylation of ERK 1/2 (A) and p38 (B) in subcultured VSMC treated with 10^{-8} M E_2 for indicated times. The relative phosphorylation levels of each protein were expressed as phospho-/total-protein × 100%. Data were presented as means ± SD; $N=6$; *$P<0.05$, compared to 0 min group; $^{\Delta}P<0.05$, compared to previous adjacent group.

p38 levels increased with the raise of E_2 concentration from 10^{-11} M to 10^{-8} M (Fig. 3). When the VSMC were treated with 10^{-6} M ICI 182,780, the E_2-enhanced phosphorylations of ERK1/2 and p38 were obviously inhibited, but not completely eliminated (Fig. 3; compare group ICI + 10^{-8} M E_2 with control).

3.3. E_2-induced VSMC proliferation and apoptosis after ERK or p38 pathway block

To further confirm the involvement of MAPK pathways in the E_2's dual effect, the VSMC were treated with either MEK inhibitor U0126 or p38 kinase inhibitor SB203580 before adding 10^{-8} M E_2. As shown by MTT assay and ELISA, treatment of the cells with U0126 led to a concentration-dependent inhibition of E_2-enhanced VSMC proliferation with a maximal inhibition concentration of U0126 at 10 μM (Fig. 4A). Similarly, treatment of SB203580 caused a concentration-dependent inhibition of E_2-enhanced apoptosis with a maximal inhibition concentration at 10 μM (Fig. 4B). More interestingly, MEK inhibition by 10 μM U0126 not only led to a block of VSMC proliferation in both E_2-treated and untreated cultures (Fig. 5A), but also significantly increased the apoptotic cells (Fig. 5B; compare U0 or U0 + E_2 group with control group). However, U0126 did not significantly further increase the E_2-induced apoptosis (Fig. 5B; compare U0+E_2 group with E_2 group). Similarly, 10 μM p38 kinase inhibitor SB203580 not only blocked the apoptosis in both E_2-treated and untreated cultures (Fig. 6A), but also obviously enhanced the growth-promoting action of E_2 (Fig. 6B). However, SB203580 alone had no effect on the proliferation of the cells (Fig. 6B). The bivariate analysis revealed that the MEK inhibition by U0126 led to a partial block of G1 to S transition (cell numbers increased in G0/G1 phase and decreased in S phase) (Table 1) with significantly increased apoptotic cells in G0/G1 phase (Table 2) in both E_2-treated and untreated cultures in comparison with control cells. What more important is that although E_2 alone induced an increased apoptosis predominant in G2/M phase, the inhibition of MEK in E_2-treated cultures did not further increase the total number of apoptotic cells, but caused a shift of the apoptosis from G2/M phase-predominant to G0/G1 phase-predominant (Table 2; compare U0 + E_2 group with E_2 group). Inhibition of p38 MAPK

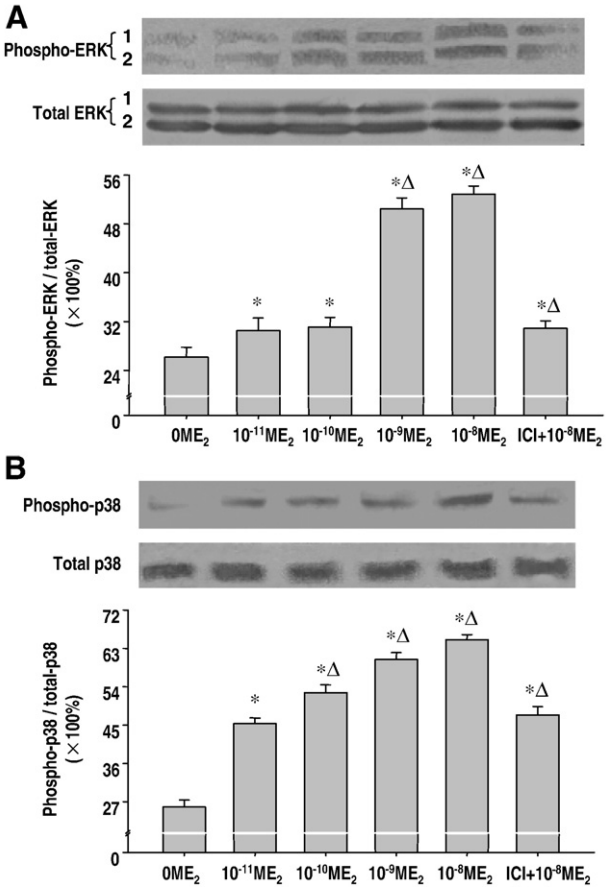

Fig. 3. Western blot analysis on phosphorylation of ERK 1/2 (A) and p38 (B) in subcultured VSMC treated with indicated concentrations of E_2 for 30 min. The relative phosphorylation levels of each protein were expressed as phospho-/total-protein × 100%. Data were presented as means ± SD. $N=6$; *$P<0.05$, compared to 0 M E_2 group; $^{\Delta}P<0.05$, compared to previous adjacent group.

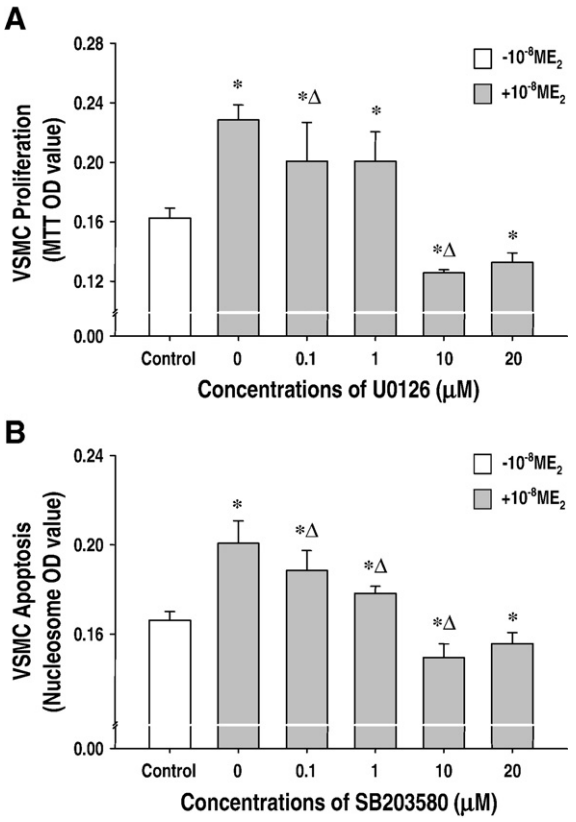

Fig. 4. E_2-induced VSMC proliferation and apoptosis after inhibition of ERK or p38 MAPK pathway. A: MTT assay for proliferation of VSMC. The cells were treated with vehicle (Control) or indicated concentrations of U0126 for 30 min followed by 10^{-8} M E_2 for 72 h. B: ELISA analysis for cytoplasmatic mono- and oligo-nucleosomes of VSMC. The cells were treated with vehicle (Control) or indicated concentrations of SB203580 for 30 min, followed by 10^{-8} M E_2 for 72 h. Data were expressed as means±SD of absorbance. $N=5$; $^{*}P<0.05$, compared to control group; $^{\Delta}P<0.05$, compared to previous adjacent group.

pathway accelerated G1-S phase transition (Table 1) and eliminated E_2-induced apoptosis in G2/M phase (Table 2).

3.4. Cross-talk between ERK and p38 pathways in the presence of E_2

Western analysis revealed that block of ERK or p38 MAPK pathway induced a reciprocal change between ERK1/2 and p38 phosphorylations. Administration of 10 μM U0126 caused an inhibition of ERK phosphorylation and a promotion of p38 phosphorylation in both E_2-treated and untreated cells (Fig. 7A, B; compare the lanes U0 with Control, and U0+E_2 with E_2). Whereas inhibition of p38 by 10 μM SB203580 led to a decrease in phospho-p38 and an increase in phosphorylation of ERK 1/2 (Fig. 7B, A; compare the lanes SB with Control, and SB+E_2 with E_2). To investigate the possible mechanisms underlying the cross-talk between E_2 activated ERK and p38 signaling pathways, the levels of two key protein phosphatases MKP-1 and PP2A were analyzed with Western blot. As shown in Fig. 8, in the presence of 10^{-8} E_2, the expressions of both MKP-1 and PP2A were significantly increased in comparison with control cells. Block of ERK pathway with 10 μM U0126 caused a significantly decreased expression of MKP-1 accompanied by an increased PP2A level. In contrast, inhibition of p38 with 10 μM SB203580 led to a decreased expression of PP2A with an increased MKP-1 level.

3.5. Effects of ER isoform specific agonists on VSMC

To investigate if the dual effects of E_2 are related with different ER isoforms, the actions of two specific ER agonists (PPT for ERα and DPN for ERβ) on VSMC were observed. As shown by MTT and ELISA analysis, a 72 h treatment of the VSMC with PPT caused a dose-dependent increase in proliferation (Fig. 9A), but had no effect on apoptosis (Fig. 9B). In contrast, administration of DPN had no obvious effect on VSMC proliferation (Fig. 10A), but led to a significantly increased apoptosis (Fig. 10B). The effects of both ER agonists could be inhibited or eliminated by 10^{-6} M ICI 182,780 (Figs. 9 and 10). Western blot analysis showed that a 30 min treatment of the VSMC with 10^{-7} M PPT had no effect on p38 phosphorylation, but significantly increased the level of phospho-ERK1/2 (Fig. 11B, A; compare the lanes PPT with Control). In contrast, a 30 min treatment with 10^{-7} M DPN did

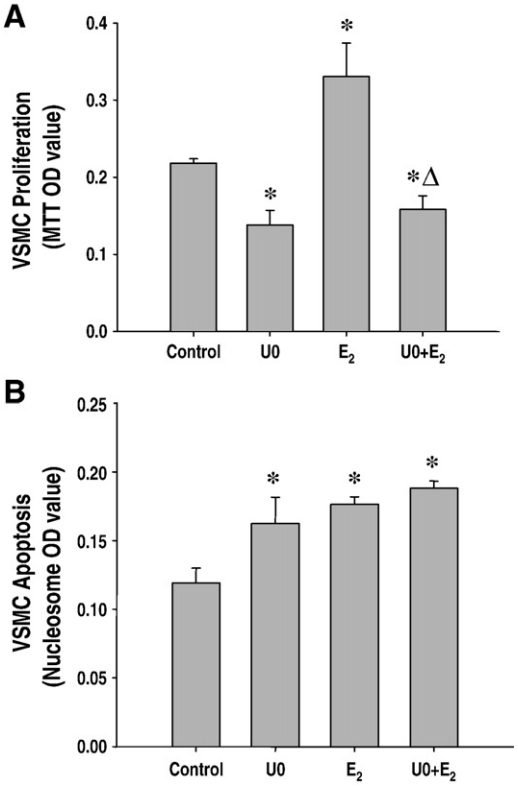

Fig. 5. Blocking test of ERK pathway. Subcultured VSMC were treated with vehicle (Control), 10^{-8} M E_2 for 72 h (E_2), 10 μM U0126 alone for 30 min (U0), or 10 μM U0126 for 30 min followed by 10^{-8} M E_2 for 72 h (U0+E_2). A: VSMC proliferation evaluated by MTT assay. B: VSMC apoptosis evaluated by ELISA analysis for cytoplasmatic mono- and oligo-nucleosomes. Data were expressed as means±SD of absorbance. $N=5$; $^{*}P<0.05$, compared to control group; $^{\Delta}P<0.05$, compared to E_2 group.

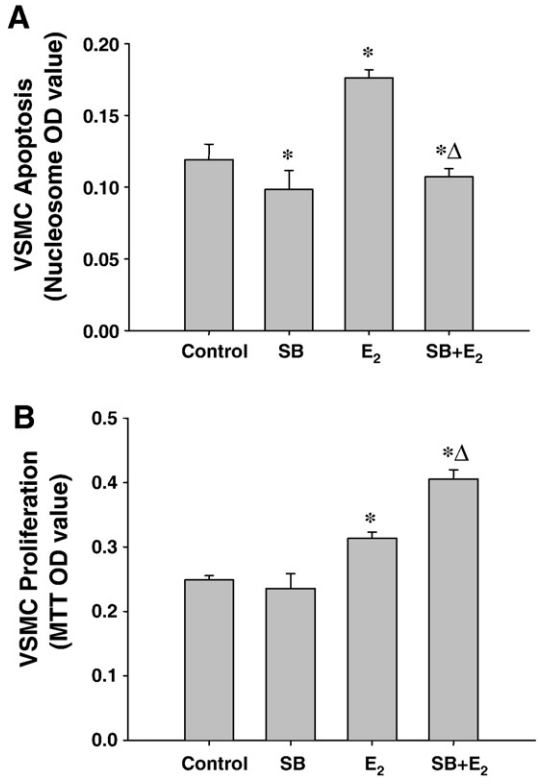

Fig. 6. Blocking test of p38 pathway. Subcultured VSMC were treated with vehicle (Control), 10^{-8} M E_2 for 72 h (E_2), 10 μM SB203580 alone for 30 min (SB), or 10 μM SB203580 for 30 min followed by 10^{-8} M E_2 for 72 h (SB+E_2). A: VSMC proliferation evaluated by MTT assay. B: VSMC apoptosis evaluated by ELISA analysis for cytoplasmatic mono- and oligonucleosomes. Data were expressed as means±SD of absorbance. $N=5$; $^{*}P<0.05$, compared to control group; $^{\Delta}P<0.05$, compared to E_2 group.

not affect the level of phospho-ERK1/2, but enhanced the phosphorylation of p38 (Fig. 11A, B; compare the lanes DPN with Control).

4. Discussion

The dual actions of estrogen on cellular proliferation and apoptosis have been found in various cell types. For example, E_2 was reported to promote not only proliferation, but also apoptosis of breast cancer cells [26], cervical epithelial and stromal cells [27], and mammary epithelial cells [28]. A similar phenomenon was also found in VSMC in a recent study in our laboratory, in which E_2 promoted the subcultured VSMC growth through up-regulation of Cyclin D1 and Cdk4; and simultaneously, it triggered apoptosis of the cells through a p38 pathway-related up-regulation of bax [20]. Here, as an extension, the present study further demonstrated that the dual effects of estrogen on the VSMC involved a concurrent activation of both ERK and p38 MAPK pathways; block of ERK inhibited the cell's proliferative response to E_2 and block of p38 eliminated the hormone-induced apoptosis.

The finding that E_2 could simultaneously affect proliferation and apoptosis of the synthetic VSMC was first reported by Mori-Abe et al. [19]. In their study, however, the E_2-induced apoptosis appeared only in the VSMC growing in 10% FCS, and it was accompanied with an inhibition of ERK phosphorylation and a partial block of G1 to S transition. It seems difficult to explain why E_2 induces apoptosis of SMC only when the cells are growth-stimulated. In addition, a growth-arrested cell is more liable to spontaneous apoptosis, therefore, it is difficult to identify if the apoptosis is induced directly by E_2 or is a secondary phenomenon due to the partial G1-S block. By using a bivariate analysis of apoptosis and cell cycle progression with flow cytometry, we have previously shown that the E_2-induced VSMC apoptosis appeared predominantly in the G2/M phase, following an accelerated G1-S transition [20]. Here, the present study further demonstrated that when the E_2-treated cells were partially growth-arrested in G0/G1 phase by the MEK inhibitor U0126, the E_2-induced increase of apoptotic cells in G2/M phase was eliminated, suggesting that E_2 selectively induced apoptosis of the cells that were undergoing mitosis. The apoptosis caused by MEK inhibition appeared mainly in

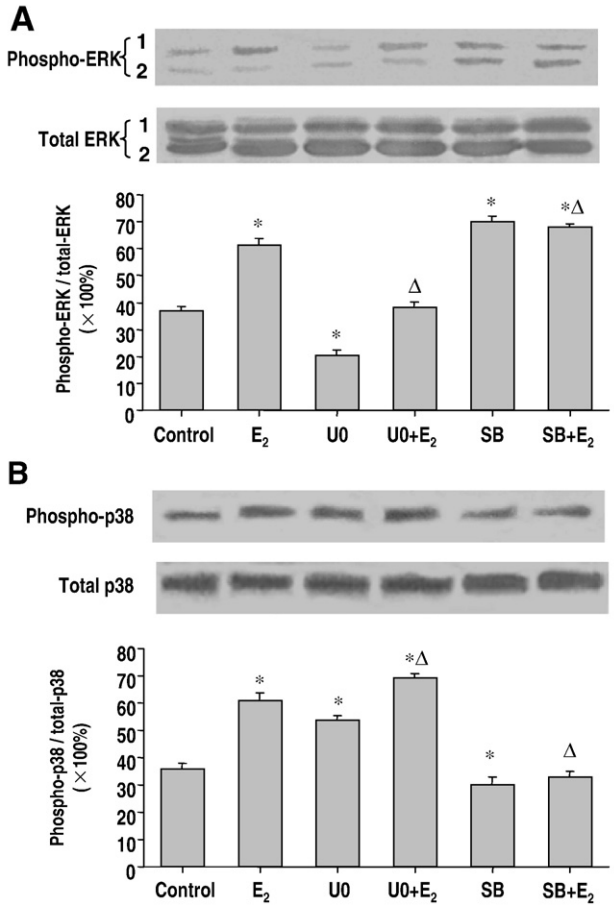

Fig. 7. Western blot analysis for phosphorylation of ERK 1/2 (A) and p38 (B) in VSMC after blocking ERK or p38 pathway. Subcultured VSMC were treated with vehicle (Control), 10^{-8} M E_2 (E_2), 10 μM U0126 (U0) or 10 μM SB203580 (SB) alone for 30 min, or 10 μM U0126 or 10 μM SB203580 for 30 min followed by 10^{-8} M E_2 for another 30 min (U0+E_2 or SB+E_2). The relative phosphorylation levels of each protein were expressed as phospho-/total-protein×100%. Data were presented as means±SD. $N=6$; $^{*}P<0.05$, compared to control group; $^{\Delta}P<0.05$, compared to E_2 group.

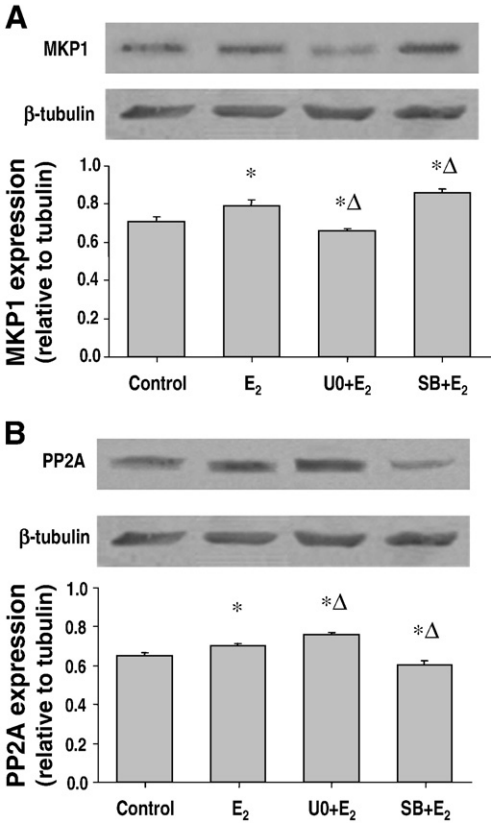

Fig. 8. Western blot analysis for MKP1 (A) and PP2A (B) expressions in VSMC after blocking ERK or p38 pathway. Subcultured VSMC were treated with vehicle (Control) or 10^{-8} M E_2 (E_2) for 30 min, or 10 μM U0126 or SB203580 for 30 min followed by 10^{-8} M E_2 (U0+E_2 or SB+E_2) for 30 min. The relative levels of each protein were expressed as MKP1 or PP2A/tubulin×100%. Data were presented as means±SD. $N=6$; *$P<0.05$, compared to control group; $^{\Delta}P<0.05$, compared to E_2 group.

the G0/G1 phase regardless of the presence or absence of E_2; thus it should be a secondary phenomenon due to the partial G0/G1 arrest, and E_2 might have no pro-apoptotic effects on VSMC in G0/G1 phase. This provides a reasonable explanation for why the hormone induces apoptosis of VSMC only when the cells are growth-stimulated.

How does estrogen exert its actions on both ERK and p38 MAPK signal pathways? A possible explanation is that VSMC express two isoforms of ER (α and β) that may evoke different intracellular signal transductions. As demonstrated in the present study, the dual effects of E_2 could be dissected by specific ER isoform agonists. Activation of ERα by PPT had no effect on apoptosis, but enhanced proliferation of the VSMC via ERK pathway; whereas, stimulation of ERβ by DPN only induced p38 activity and apoptosis of VSMC. This is consistent with the previous observations on several cancer cell lines, in which ERα rapidly activated multiple signal transduction pathways (i.e., ERK/MAPK, PI3K/AKT), whereas ERβ induced the phosphorylation of p38/MAPK [29]. In the present study, the ICI 182,780, a blocker of both ERα and β, at the suggested block concentration of 10^{-6} M [30], failed to totally eliminate the E_2-induced phosphorylations of both ERK1/2 and p38. In addition, although 10^{-6} M ICI 182,780 blocked E_2's apoptotic effect, it did not inhibit the E_2-promoted proliferation to control levels. Therefore, we do not exclude that there might be an additional non-receptor mechanism [31] under the hormone's effects. It was recently reported that some locally produced metabolic products of E_2 could directly act on cells without mediation by either ERα or ERβ [32].

Although the concurrent activations of both ERK and p38 MAPK signal transduction may explain why estrogen is able to promote two opposing cellular courses (the proliferation and apoptosis of VSMC), a key question is still remained as to why the same cells, the VSMC, in different experimental conditions have diverse responses to estrogen, such as an inhibited [7,11] or promoted proliferation [16,17], an inhibited proliferation with an increased apoptosis [19] or a promoted proliferation with an increased apoptosis [20]. To answer this question, the present study employed the subcultured VSMC in passages 3–4 as a model and investigated the responses of the cells to E_2 after their ERK or p38 MAPK signal transduction was blocked with specific inhibitor U0126 or SB203580. The VSMC in passages 3–4 are stably in the synthetic phenotype, which avoids the interference with the result by the cell's phenotypic

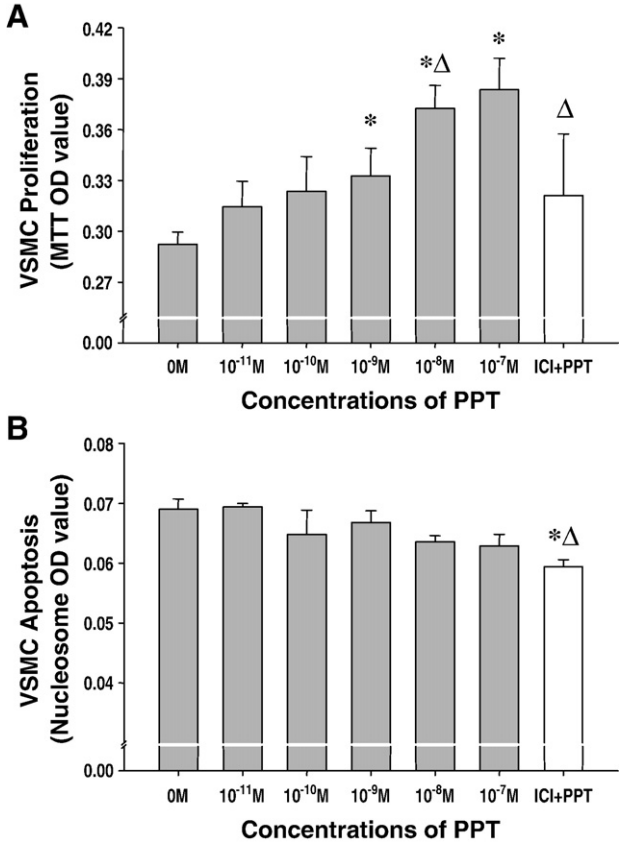

Fig. 9. Effects of specific ERα agonist PPT on VSMC proliferation (A: MTT assay) and apoptosis (B: ELISA analysis for cytoplasmatic fractions of mono- and oligo-nucleosomes). The subcultured VSMC were treated with vehicle (Control), indicated concentrations of PPT for 72 h or 10^{-6}M ICI 182,780 for 30 min followed by 10^{-7} M PPT for 72 h (ICI+PPT). Data were expressed as means±SD of absorbance. $N=5$; *$P<0.05$, compared to 0 M group; $^{\Delta}P<0.05$, compared to previous adjacent group.

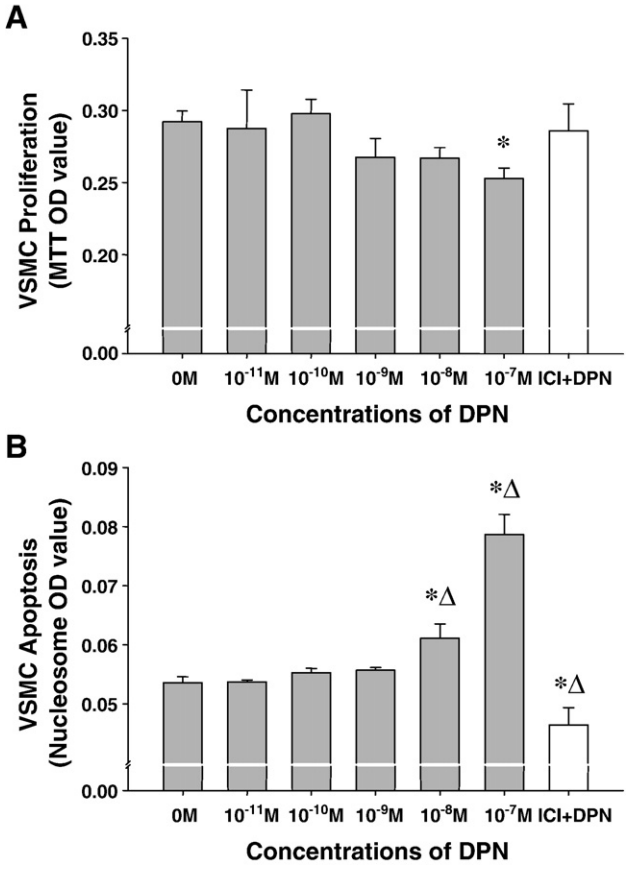

Fig. 10. Effects of specific ERβ agonist DPN on VSMC proliferation (A: MTT assay) and apoptosis (B: ELISA analysis for cytoplasmatic fractions of mono- and oligo-nucleosomes). The subcultured VSMC were treated with vehicle (Control), indicated concentrations of DPN for 72 h or 10^{-6} M ICI 182,780 for 30 min followed by 10^{-7} M DPN for 72 h (ICI + DPN). Data were expressed as means ± SD of absorbance. $N=5$; *$P<0.05$, compared to 0 M group. $^{\Delta}P<0.05$, compared to previous adjacent group.

modulation [18,25]. In addition, the subcultured VSMC are much closer in growth property to their in vivo counterpart (especially the intimal VSMC) in comparison with the immortalized VSMC cell lines. U0126 and SB203580 are widely used as a pharmacological tool to dissect the role of MAPK pathway in various physiological processes [33–35]. The U0126 is a potent and specific blocker of ERK cascade, which selectively inhibits MEK-1 and MEK-2, preventing downstream phosphorylation [33,36]. The SB203580 belongs to a class of pyridinyl imidazoles that are highly potent and selective inhibitors of p38, but not of other mitogen-activated protein kinases [37].

As shown by the Western blot analyses, a reciprocal cross-talk between ERK and p38 MAPK pathways in the VSMC in response to E_2 was observed. The MEK inhibitor U0126 caused not only a decrease in phosphorylated ERK, but also an increase in phosphorylated p38. This led to an inhibition of the cell's proliferative response to E_2 and a secondary apoptosis due to the partial G0/G1 growth arrest. In contrast, the p38 kinase inhibitor SB203580 caused a decrease of phosphorylated p38 with an increase of phosphorylated ERK, leading to an enhanced proliferative response of the cells to E_2 and an elimination of E_2-induced G2/M phase-predominant apoptosis. These findings suggest that the fates of VSMC in response to estrogen may be, at least partly, dependent upon the relative activities between the ERK and p38 MAPK pathways.

Similar reciprocal changes in activities between ERK and p38 MAPK pathways have been observed in various cell types [38–41]. However the exact underlying mechanism is still not very clear. In general, the cross-talk between MAPK pathways is mediated by a complex network of protein phosphatases [42]. These phosphatases are negative feed-back regulators that are usually induced by the upstream activators at different levels and cross-react with substrates in more than one signaling channels. Thus, reductions of the phosphatases due to an inhibited signaling in one channel may lead to an amplification of signaling in other channels. In the present study, we analyzed the effects of E_2 on the expressions of two widely studied protein phosphatases (MKP-1 and PP2A) with or without inhibition of ERK or p38 signaling. As shown by the western analysis, the concurrent activations of ERK and p38 MAPK pathways by E_2 were accompanied with increased expressions of both MKP-1

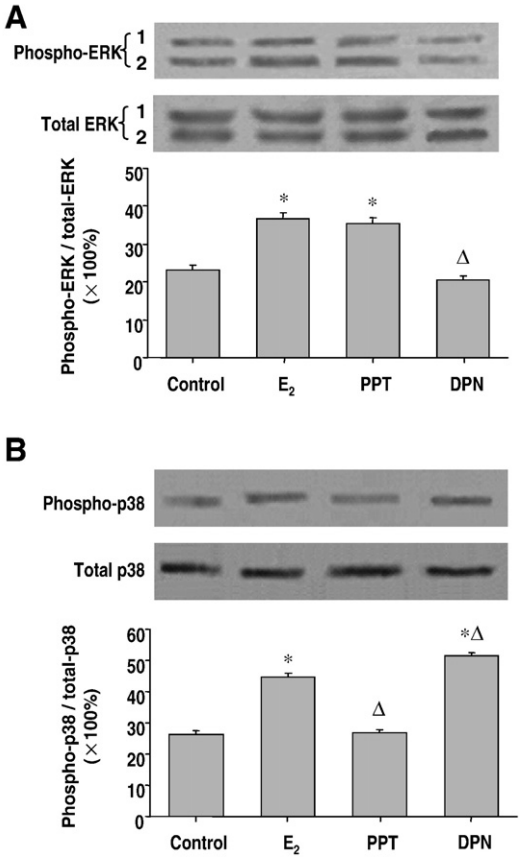

Fig. 11. Western blot analysis for phosphorylation of ERK 1/2 (A) and p38 (B) in VSMC after treatment with specific ER agonist PPT or DPN. The subcultured VSMC were treated with vehicle (Control), 10^{-8} M E_2 (E_2), 10^{-7} M PPT (PPT), or 10^{-7} M DPN (DPN) for 30 min. The relative phosphorylation levels of each protein were expressed as phospho-/total-protein × 100%. Data were expressed as means ± SD. $N=6$; *$P<0.05$, compared to control group; $^{\Delta}P<0.05$, compared to E_2 group.

and PP2A. Inhibition of MEK1/2 reduced the E_2-induced increase of MKP-1, but enhanced the expression of PP2A. Whereas, inhibition of p38 led to a decreased expression of PP2A with an increased MKP-1 level. The MKP-1 is a widely accepted key negative regulator of signaling through three MAPK pathways [43]. However, recent studies with the cells from mice lacking MKP-1 provided compelling evidence that the enzyme plays an impotent role in preferentially attenuating p38 and JNK signaling [44], and promoting cell survival [45]. The PP2A is reported to be an inhibitor of activated MEK1/2 and ERK, and is activated mainly by p38 [41]. Thus, the reduced expression of MKP-1 after MEK inhibition in the present study may be responsible for the amplification of p38 pathway activity, which in turn increases the expression of PP2A. Whereas, the decreased expression of PP2A after inhibition of p38 may be the reason for the enhanced ERK pathway activity.

In summary, the present study reveals that the dual effects of estrogen on VSMC proliferation and apoptosis involve a concurrent activation of two MAPK signaling pathways, ERK pathway by ERα and p38, by ERβ; the fates of the cell in response to the hormone may be, at least partly, dependent upon the dynamic balance between the ERK and p38 MAPK pathways. A VSMC with prominent ERK activity may have an enhanced proliferation; a VSMC with prominent p38 activity may be growth-arrested in G1 phase and then undergo apoptosis; and a VSMC with compromised activities between these two pathways may have a mildly promoted proliferation with some cells going into apoptosis during mitosis.

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