Resveratrol enhances proliferation and osteoblastic differentiation in human mesenchymal stem cells via ER-dependent ERK1/2 activation

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Abstract

In the present study, we investigated the in vitro effect of resveratrol (RSVL), a polyphenolic phytoestrogen, on cell proliferation and osteoblastic maturation in human bone marrow-derived mesenchymal stem cell (HBMSC) cultures. RSVL (10^{-8}–10^{-5} M) increased cell growth dose-dependently, as measured by [\textsuperscript{3}H]-thymidine incorporation, and stimulated osteoblastic maturation as assessed by alkaline phosphatase (ALP) activity, calcium deposition into the extracellular matrix, and the expression of osteoblastic markers such as \textit{RUNX2/CBFA1}, \textit{Osterix} and \textit{Osteocalcin} in HBMSCs cell cultures. Further studies found that RSVL (10^{-6} M) resulted in a rapid activation of both extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) signaling in HBMSCs cultures. The effects of RSVL were mimicked by 17\beta-estradiol (10^{-8} M) and were abolished by estrogen receptor (ER) antagonist ICI182780. An ERK1/2 pathway inhibitor, PD98059, significantly attenuated RSVL-induced ERK1/2 phosphorylation, consistent with the reduction of cell proliferation and osteoblastic differentiation as well as expression of osteoblastic markers. In contrast, SB203580, a p38 MAPK pathway blocker, blocked RSVL-induced p38 phosphorylation, but resulted in an increase of cell proliferation and a more osteoblastic maturation. These data suggest that RSVL stimulates HBMSCs proliferation and osteoblastic differentiation through an ER-dependent mechanism and coupling to ERK1/2 activation.

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Introduction

Osteoporosis associated with estrogen deficiency after menopause is the most common cause of age-related bone loss (Kanis et al., 1994). Hormone replacement therapy (HRT) could effectively prevent postmenopausal osteoporosis (Lindsay et al., 1984) and reduce the incidence of fractures (Blank and Bockman, 1999). However, HRT also increases the risk of breast and endometrial cancer, in addition to other undesirable side effects (Vihtamaki et al., 1999). An alternative therapy of the classical HRT is needed. The phytoestrogens, therefore, are potentially important in the prevention of postmenopausal osteoporosis.

Resveratrol (3,4’,5-trihydroxystilbene, RSVL), a known phytoestrogen, is a phenolic compound that occurs naturally in mulberries, peanuts and the berry skins of most grape cultivars (Jang et al., 1997) and
possesses estrogenic activity (Gehm et al., 1997). Several biological effects of RSVL have been reported, including platelet aggregation attenuation (Wang et al., 2002), cardiovascular protection (Cao and Li, 2004), and anticancer activity (Savouret and Quesne, 2002). Recent studies show that RSVL stimulates the proliferation and differentiation of osteoblastic MC3T3-E1 cells in vitro (Mizutani et al., 1998), and inhibited bone loss in ovariectomized rats in vivo (Mizutani et al., 2000). Most recently, RSVL was proved to act against Cyclosporin A (CsA) inhibition of proliferation and osteoblastic differentiation in mouse bone mesenchymal stem cells (Song et al., 2006). However, how RSVL positively regulates bone metabolism and cell functions in human bone marrow-derived mesenchymal stem cell (HBMSC) cultures remains unclear.

There is evidence that the biological effects of RSVL were associated with MAPK signaling pathways. RSVL was reported to induce apoptosis in thyroid cancer cell lines via a MAPK- and p53-dependent mechanism (Shih et al., 2002), and reduce cell oxidation and proliferation in human retinal pigment epithelial cells via extracellular signal-regulated kinases (ERK) inhibition (King et al., 2005). Although some recent studies showed that MAPK pathways were involved in human bone metabolism, including the commitment of human bone marrow-derived mesenchymal stem cells (HBMSCs) to the osteogenic and adipogenic lineages (Jaiswal et al., 2000) and mechanical stimulus-induced cell proliferation and matrix mineralization of HBMSCs (Simmons et al., 2003; Riddle et al., 2006), whether the effect of RSVL on bone metabolism occurs via MAPK signaling is still unknown. In the current study, we investigated the in vitro effect of RSVL on proliferation and osteoblastic maturation of HBMSCs cultures, and explored the role of the MAPK signaling pathway in RSVL-mediated anabolic cell responses.

Materials and methods

Reagents

Alpha minimum essential medium (α-MEM), phenol red-free α-MEM, fetal bovine serum (FBS), and sodium dodecyl sulfate (SDS) were obtained from GIBCO BRL (Grand Island, NY, USA). Resveratrol (RSVL), 17β-estradiol (E2), trypsin, ascorbic acid, β-glycerophosphate, p-nitrophenyl phosphate (p-NPP), p-nitrophenol (p-NP), diethanolamine, dextran-coated charcoal, PD98059 and SB203580 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). ICI182,780 was purchased from Tocris Cookson Inc. Ltd. (Avonmouth Bristol BS11 8TA, UK). [3H]-thymidine ([3H]-TDR) was purchased from Shanghai Institute of Nuclear Research (Shanghai, China). Bio-Rad reagent for protein assay was obtained from Bio-Rad Laboratories (Hercules, CA, USA). PhosphoPlus® p38 MAP Kinase (Thr180/ Tyr182) and p44/42 MAP Kinase (Thr202/Tyr204) Antibody Kits were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). iQ™ SYBR® Green Supermix was obtained from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals were of analytical grade and were obtained from Shanghai Biotech Co. Ltd. (Shanghai, China).

Cell cultures

Human fetal bone marrows were obtained from the limb bones of a 5-month-old aborted fetus (Hunan Maternal and Child Health Hospital, Changsha, China), which was allowed by the parents and in accordance with the ethical standards of the Hunan Ethics Committee. Mononucleate cells were isolated by density gradient centrifugation using Histopaque® solution (Sigma) and subsequently seeded in α-MEM, 15% FBS (inactivated), and maintained in a humidified incubator with 5% CO2 and 95% air at a temperature of 37°C. Passages of three to five HBMSCs were used in this study. At subconfluence, HBMSCs were detached with 0.25% trypsin, seeded in 24-well plastic dishes at a density of 5 × 10^4 cells/l and cultured in osteogenic differentiation medium, consisting of phenol red-free α-MEM plus 15% FBS (dextran-coated charcoal stripped, DCS) supplemented with dexamethasone (10^-8 M), ascorbic acid (50 mg/l) and β-glycerophosphate (10^-2 M). Treatments were added respectively and the medium was replaced every 3 days thereafter.

[3H]-thymidine incorporation assay

[3H]-thymidine incorporation of HBMSCs were measured as described previously (Song et al., 2006). Radioactivity was determined by scintillation counting and expressed as count per minute (CPM).

Alkaline phosphatase (ALP) activity

Cellular alkaline phosphatase (ALP) activity was assayed according to our previous study (Xiao et al., 2001). Enzyme activity was determined colorimetrically using p-NPP as the substrate at pH 10.3 after incubation at 37°C for 30 min and the optical density was read at 405 nm. ALP activity was normalized to the protein content and is expressed as μmol/h/mg protein.

Quantitation of calcium deposition

Calcification of HBMSCs was assessed by a modification of the Wada procedure (Xiao et al., 2001). Calcium
kit was purchased from Shanghai Kehua Biotech Co. Ltd. (Shanghai, China). The calcium content of the cell layer was normalized to the protein content. Calcium deposition is expressed as \( \mu g/mg \) protein.

**MAPK activation studies**

HBMSCs were seeded and allowed to adhere for 24 h in serum-containing medium. To synchronize the cells and reduce basal MAPK activity, the cells were then maintained for 48 h in basal medium containing 0.5% FBS, and finally serum-starved for 4 h prior to and during the indicated application as described previously (Simmons et al., 2003). Whole cell extracts were then prepared by harvesting cells in 1 \( \times \) SDS-PAGE loading buffer (2% w/v SDS, 50 mM dithiothreitol, 10% glycerol, 62.5 mM Tris–HCl (pH 6.8), 0.1% w/v bromphenol blue). Proteins were separated on SDS-PAGE, electrophoretically transferred to a PVDF membrane, and probed with a specific rabbit anti-human ERK1/2 or p38 antibody according to the manufacturer’s protocols. Immunoreactivity was determined using the ECL chemiluminescence reaction.

**MAPK inhibition studies**

The ERK1/2 pathway was blocked with PD98059, an inhibitor of the upstream kinase MAPK-ERK kinase 1 (MEK1), while p38 MAPK was specifically inhibited with SB203580. Preliminary experiments showed that the optimal concentrations for inhibition of ERK1/2 and p38 without cytotoxicity were \( 10^{-5} \) M PD98059 and \( 5 \times 10^{-6} \) M SB203580, respectively (data not shown). ICI182,780 (10 \(-7\) M) was also applied to investigate the interactions between ER and MAPK pathway. To assess the effects of RSVL or E2 on MAPK activity, these inhibitors were added to the serum-free cell culture media 1 h prior to application of RSVL or E2, then the cells were co-incubated with RSVL or E2 for its peak time, and the lysates were collected for immunoblot analysis. To examine whether the effects of RSVL or E2 on cell proliferation and osteogenic differentiation through MAPK pathway, RSVL or E2 were co-incubated with or without the MAPK inhibitors in osteogenic differentiation medium, \([^{3}H]\)-thymidine incorporation, ALP activity and calcium deposition assays were performed in HBMSCs culture as described above (Song et al., 2006).

**Density analysis of results in western blotting**

Band densitometry was quantified using Quantity One Analysis software (version 4.5.2, BioRad Laboratories, CA, USA) and data of phosphorylated MAPK signaling were expressed as relative expression (Mean ± SD) normalized to unphosphorylated bands.

**Real-time RT-PCR**

Total RNA was isolated from the HBMSCs by the single-step method using Trizol reagent for quantitative real-time RT-PCR as described previously (Pan et al., 2005). The threshold cycle (Ct) of tested-gene product from the indicated genotype was normalized to the Ct for GAPDH.

**Statistical analysis**

The data were expressed as the Mean ± S.D. and statistically analyzed by repeated measures or one-way ANOVA. Values of \( p < 0.05 \) were considered to be statistically significant.

**Results**

**Effects of RSVL on cell proliferation and osteogenic differentiation in HBMSCs cultures**

There was a time-dependent effect of RSVL on ALP activity and calcium deposition in HBMSC cultures. The peak stimulation of ALP activity was found at day 12 in osteogenic differentiation medium, both E2 and RSVL treatment significantly enhanced ALP activity as a function of time in HBMSCs cultures (data not shown). In contrast, the peak accumulation of calcium deposition was observed at day 16, which accords with a previous report (Jonsson et al., 1999). RSVL and E2 markedly promoted the calcium accumulation only at day 16 of HBMSCs cultures (data not shown). Therefore, in the following experiments, we chose Day 12 and 16 as the corresponding time point to examine the changes of ALP activity and calcium deposition, respectively.

As shown in Table 1, RSVL dose-dependently increased the \([^{3}H]\)-thymidine incorporation, ALP activity and calcium deposition in HBMSCs cultures. For \([^{3}H]\)-thymidine incorporation and calcium deposition, the maximally stimulated concentration of RSVL was \( 10^{-3} \) M, whereas RSVL with \( 10^{-4} \) M turned out to have an inhibitory effect on HBMSCs proliferation and calcium accumulation (Table 1). For ALP activity, there was a slight trend towards up-regulation in the cultures treated by different RSVL concentrations, but only \( 10^{-6} \) M RSVL achieved both statistical significance and maximal response as compared with control group (Table 1). Based on these results, we employed \( 10^{-6} \) M RSVL to continue the proceeding experiments because it is the optimal concentration to sufficiently induce
the proliferation and osteogenic differentiation of HBMSCs.

Next, the increases of [3H]-thymidine incorporation, ALP activity and calcium deposition induced by RSVL (10^{-6} M) were abolished by co-incubation with a pure ER antagonist ICI182,780 (10^{-7} M), and E2 (10^{-8} M) showed similar effects in the absence or presence of ICI182,780 (10^{-7} M) (Fig. 1), indicating that ER mediated RSVL-induced anabolic responses in HBMSCs cultures. ICI182,780 alone had no effect on these indices (Fig. 1).

Effects of RSVL on the activation of ERK1/2 and p38 in HBMSCs cultures

RSVL (10^{-6} M) alone quickly activated both ERK1/2 and p38 activity in 5 min in HBMSCs cultures, which lasted about 15 min, then returned to basal levels (Fig. 2). Further studies revealed that RSVL- or E2-induced activation of ERK1/2 and p38 could be inhibited by PD98059 (10 \mu M) and SB203580 (5 \mu M) employed 1 hour prior to RSVL application, respectively (Fig. 3). Moreover, ER antagonist ICI182,780 (10^{-7} M) completely abolish the RSVL- or E2-induced phosphorylation of ERK1/2 and p38 in HBMSCs cultures (Fig. 4), suggesting that ER mediated RSVL-induced MAPKs activation.

To further explore whether ERK1/2 signaling is involved in RSVL- or E2-induced growth and osteoblastic differentiation, we used PD98059 (10^{-5} M) to block ERK1/2 activity in HBMSCs culture. As shown in Fig. 5, both RSVL and E2 significantly induced growth and osteoblastic differentiation of HBMSCs, evidenced by an increase of [3H]-thymidine incorporation as well as ALP activity and calcium deposition. In the presence of PD98059, we observed a conspicuous decrease in the expression of osteogenic markers in control groups, as shown by about 30% decrease in ALP activity at day 12 (Fig. 5B) and a 64% reduction in mineral deposition at day 16 (Fig. 5C). However, PD98059 itself had no significant effect on proliferation (Fig. 5A). Concurrent incubation with either RSVL or E2, PD98059 markedly blocked RSVL- or E2-induced proliferation (Fig. 5A) and osteoblastic differentiation (Figs. 5B and C), indicating that ERK1/2 signaling plays an important role in RSVL- or E2-induced growth and osteoblastic differentiation of HBMSCs.

Conversely, p38 inhibitor SB203580 alone resulted in about a 1.5 fold increase in [3H]-thymidine incorporation

### Table 1. Stimulatory effect of RSVL on the proliferation and osteogenic differentiation of HBMSCs

<table>
<thead>
<tr>
<th>Group</th>
<th>[3H]-thymidine incorporation (CPM)</th>
<th>ALP activity (µmol/h/mg protein)</th>
<th>Calcium deposition (µg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>126 ± 7.3</td>
<td>22.1 ± 0.94</td>
<td>11.0 ± 1.69</td>
</tr>
<tr>
<td>E2 (10^{-8} M)</td>
<td>258 ± 11.1a</td>
<td>29.7 ± 1.63a</td>
<td>18.5 ± 2.28a</td>
</tr>
<tr>
<td>RSVL 10^{-8} M</td>
<td>187 ± 16.2a</td>
<td>20.5 ± 0.71</td>
<td>10.5 ± 1.91</td>
</tr>
<tr>
<td>RSVL 10^{-7} M</td>
<td>213 ± 22.1a</td>
<td>23.9 ± 1.91</td>
<td>14.6 ± 2.50</td>
</tr>
<tr>
<td>RSVL 10^{-6} M</td>
<td>243 ± 39.8a</td>
<td>25.5 ± 1.78a</td>
<td>17.7 ± 3.06a</td>
</tr>
<tr>
<td>RSVL 10^{-5} M</td>
<td>371 ± 50.1a</td>
<td>25.0 ± 2.00b</td>
<td>30.4 ± 3.60a</td>
</tr>
<tr>
<td>RSVL 10^{-4} M</td>
<td>41 ± 7.0a</td>
<td>17.4 ± 3.26a</td>
<td>22.2 ± 3.01a</td>
</tr>
</tbody>
</table>

Mean ± S.D., n = 6.  
^a^p<0.01 vs. control.  
^b^p<0.05.
Fig. 2. RSVL rapidly activated ERK1/2 and p38 in HBMSCs cultures. HBMSCs were treated with 10^{-6} M RSVL and the time points indicated that whole cell lysates were subjected to western blotting analysis to detect the phosphorylated (upper panel) or unphosphorylated (lower panel) forms of ERK1/2 and p38 MAPKs (A, C). The relative expressions of phosphorylated ERK1/2 and p38 (pERK1/2 and pp38) were quantified by Quantity One density analysis and normalized to corresponding unphosphorylated ones from triplicate independent experiments (B, D). Data are expressed as Mean ± S.D., n = 4. *p < 0.05 vs. control (0').

Fig. 3. PD98059 and SB203580 attenuated RSVL-induced phosphorylations of ERK1/2 and p38 in HBMSCs cultures, respectively. HBMSCs were cultured in phenol red–free α-MEM in the presence or absence of RSVL (10^{-6} M) or E2 (10^{-8} M) with or without the MAPK inhibitors. Western blotting analysis for the phosphorylated (upper panel) or unphosphorylated (lower panel) forms of the ERK1/2 (A) and p38 (C). Relative expression of pERK1/2/ERK1/2 (B) and pp38/p38 (D) derived from Quantity One density analysis. Data are expressed as Mean ± S.D., n = 4. *p < 0.01 vs. control; b p < 0.05 vs. E2; c p < 0.05 vs. RSVL.

(Fig. 5A) and a 1.6 fold increase in ALP activity (Fig. 5B), but had little effect on calcium deposition (Fig. 5C) as compared with control groups. Concurrent treatment with RSVL or E2, SB203580 further increased [3H]-thymidine incorporation (50% increase, Fig. 5A) and calcium deposition (4.1 fold increase, Fig. 5C) in
HBMSCs cultures compared to SB203580 alone groups, but there were no further inductions in ALP activity (Fig. 5B). These data suggested that blocking of p38 signaling enhanced RSVL- or E2-induced proliferation and osteoblastic differentiation of HBMSCs.

**Effects of RSVL on mRNA expression of** RUNX2, Osterix and Osteocalcin **in HBMSCs**

Effects of RSVL on the expressions of RUNX2/ CBFA1, Osterix and Osteocalcin were evaluated by real-time RT-PCR analysis in RNA preparations from HBMSCs cultures at day 8. RSVL (10^{-6} M) significantly increased RUNX2/CBFA1 mRNA message as well as its downstream genes Osterix and Osteocalcin (Table 2) as compared with vehicle control. ICI182,780 (10^{-7} M) and PD98059 (10^{-5} M) reversed the up-regulation of RSVL-induced Runx2/Cbfal, as well as its downstream genes Osterix and Osteocalcin expressions. In contrast, incubation with SB203580 (5 \times 10^{-6} M) did not affect RSVL-induced increments of these osteogenic markers. In addition, ICI182,780 or SB203580 alone had no effect on these genes expressions (Table 2), whereas PD98059 itself markedly reduced these osteogenic markers in HBMSCs cultures, E2 (10^{-8} M), served as positive control, displayed the similar results as RSVL did (Table 1). These data are consistent with ERK1/2 signaling involved in RSVL- or E2-induced osteoblastic differentiation.

**Discussion**

In our previous study, we found that phytoestrogens Genistein and RSVL had estrogen-like activity and stimulated the proliferation and osteoblastic differentiation through ER-dependent NO/cGMP pathway in mouse BMSCs cultures (Pan et al., 2005; Song et al., 2006). In the present study, we extended our observations to examine the effects of phytoestrogens on human bone marrow cultures, to explore other possible mechanisms such as MAPKs pathways in RSVL-induced stimulations. Firstly, we observed that RSVL (10^{-8}\sim10^{-5} M) stimulated the cell proliferation and osteoblastic differentiation in a time- and dose-dependent manner, evidenced by increasing the [^{3}H]-thymidine incorporation, ALP activity and calcium deposition in HBMSCs cultures (Table 1). Secondly, we found that RSVL-induced stimulations were associated with ER signaling and increasing ERK1/2 activity (Figs. 1 and 5). Finally, we demonstrated that RSVL-induced ER signaling and ERK1/2 activity increased osteogenic genes RUNX2/CBFA1, a well-known critical transcription factor in the osteoblast recruitment and differentiation (Ducy et al., 1997), together with Osterix and
which are osteogenic markers and RUNX2/CBFA1 concentration of 10^{-6}M required in bone formation. However, RSVL at the excesses high concentration (Basly et al., 2000). ER antagonist ICI182,780 markedly inhibited the RSVL- or E2-induced MAPK activation, consistent with previous study showing that RSVL and E2 rapidly activated MAPK signaling through ER in endothelial cells (Klinge et al., 2005). Moreover, RSVL-induced responses were mainly mediated by ERK1/2 signaling, evidenced by PD98059 (an ERK1/2 signaling inhibitor) but not SB203580 (a p38 signaling inhibitor) blocked RSVL-induced anabolic responses in HBMSCs cultures (Figs. 4 and 5).

Our current findings accord with previous observations that ERK1/2 signaling served as a common mediator for a variety of anabolic stimuli in BMSCs and bone cell cultures, including mechanical strain and fluid flow (Jessop et al., 2002), soluble signals (Jaiswal et al., 2000) and cell-matrix interactions (Lai et al., 2001). In addition, unlike other previous observation that ERK1/2 mainly mediated the proliferation rather than differentiation in MC3T3-E1 (Suzuki et al., 2002), we found that ERK1/2 also played critical roles in osteoblastic differentiation as well as proliferation in HBMSCs cultures (Fig. 5). The reason for this discrepancy could be either the differential ERK1/2 inhibitors, which PD98059 seems to modulate more in cell differentiation than U0126 (Dang and Lowik, 2004), or different stimuli in different cell culture conditions.

### Table 2. Effects of ICI182,780, SB203580 and PD98059 on the RSVL (10^{-6}M)-induced RUNX2, Osterix and Osteocalcin expression of HBMSCs by real-time PCR

<table>
<thead>
<tr>
<th>Group</th>
<th>RUNX2</th>
<th>Osterix</th>
<th>Osteocalcin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0±0.18</td>
<td>1.0±0.21</td>
<td>1.0±0.23</td>
</tr>
<tr>
<td>RSVL</td>
<td>2.9±0.16a</td>
<td>2.7±0.17a</td>
<td>4.3±0.34a</td>
</tr>
<tr>
<td>ICI182,780</td>
<td>1.0±0.16</td>
<td>0.92±0.19</td>
<td>1.2±0.22</td>
</tr>
<tr>
<td>RSVL + ICI182,780</td>
<td>1.1±0.21b</td>
<td>1.2±0.17b</td>
<td>0.97±0.23b</td>
</tr>
<tr>
<td>SB203580</td>
<td>0.96±0.18</td>
<td>1.2±0.17</td>
<td>1.3±0.23</td>
</tr>
<tr>
<td>RSVL + SB203580</td>
<td>3.2±0.25</td>
<td>2.9±0.23</td>
<td>4.6±0.32</td>
</tr>
<tr>
<td>PD98059</td>
<td>0.75±0.14a</td>
<td>0.71±0.13a</td>
<td>0.66±0.11a</td>
</tr>
<tr>
<td>RSVL + PD98059</td>
<td>1.2±0.18b</td>
<td>1.1±0.19b</td>
<td>0.94±0.22b</td>
</tr>
<tr>
<td>E2</td>
<td>2.9±0.19b</td>
<td>3.4±0.26a</td>
<td>4.9±0.35a</td>
</tr>
<tr>
<td>E2 + ICI182,780</td>
<td>1.1±0.16c</td>
<td>1.2±0.17c</td>
<td>0.98±0.21c</td>
</tr>
</tbody>
</table>

Mean ± S.D., n = 4. *p < 0.01 vs. control (Me2SO).
*p < 0.01 vs. RSVL.
*p < 0.01 vs. E2.

RSVL-stimulated proliferation and osteoblastic differentiation was via ER signaling pathway in HBMSCs cultures. Compared with E2-treated groups, RSVL displayed similar effects on cell growth and osteoblastic differentiation in HBMSCs cultures (Table 1 and Fig. 1), and ICI182,780, a complete antagonist of ER, abolished the effects induced by RSVL or E2. These data are consistent with that RSVL bound ERβ and ERα (Bowers et al., 2000) and competed with E2 for ER in vitro to activate transcription of estrogen-responsive reporter genes (Gehm et al., 1997), indicating that ER mediates RSVL- or E2-induced anabolic stimulation in HBMSCs cultures.

Osteocalcin (Table 2), the downstream genes of RUNX2/CBFA1 which are osteogenic markers and required in bone formation. However, RSVL at the concentration of 10^{-6} M presented a strong suppressing effect, which may result from the cytotoxicity of the excessively high concentration (Basly et al., 2000).
Besides ERK1/2 activation, the p38 MAPK pathway was also activated by RSVL (Figs. 2 and 3). Our current investigation showed that blocking p38 pathway increased cell proliferation and osteoblastic differentiation in the control groups (Fig. 5), suggesting p38 pathway plays a negative role in HBMSCs cultures. This was also supported by other studies showing that p38 signaling played an inhibitory role in cyclic strain-induced osteoblastic differentiation of HBMSCs (Simmons et al., 2003) and BMP2-induced osteogenic differentiation of multipotent mouse C2C12 cells (Vinals et al., 2002). Moreover, we found that inhibition of p38 pathway displayed additive effects on cell proliferation and osteogenic differentiation when SB203580 was concurrently treated with RSVL or E2 (Fig. 5). It has been reported that activation of p38 could repress the ERK1/2 signaling cascade in several cell lines (Zhang et al., 2001). Moreover, inhibition of p38 signaling could also enhance ERK1/2 phosphorylation (Singh et al., 1999). These findings provided considerable evidence that p38 MAPK might negatively regulate ERK1/2 pathway through more complicated mechanisms.

RSVL-induced ER signaling and MAPK activations result in osteogenic genes expression in HBMSCs cultures. As shown in Table 2, RSVL or E2 alone significantly increased RUNX2/CBFA1 mRNA message as well as its downstream genes Osterix and Osteocalcin expressions compared with vehicle control. Similarly, ER antagonist ICI182,780 reversed these inductions by RSVL or E2, PD98059 but not SB203580 blocked RSVL-induced osteogenic genes increments in HBMSCs cultures, suggesting that RSVL-stimulated osteogenic responses via ER signaling and ERK1/2 activations. There is compelling data showing that both ERK1/2 (Rubin et al., 2003) and p38 MAPK (Zhou et al., 2006) pathways are involved in nitric-oxide synthase (NOS) activation in artery endothelial cells and osteoprogenitor stromal cells. Our previous studies found that Genistein and RSVL enhanced the expression of the osteogenic markers via ER/NO/cGMP pathway in mouse BMSCs culture (Pan et al., 2005; Song et al., 2006). The activation of ERK1/2 leads to increased expression and DNA-binding activity of Runx2/Cbfa1 by phosphorylation (Xiao et al., 2000). Therefore, RSVL could be through ER/MAPK/NOS/cGMP to regulate cell growth, osteoblastic differentiation, and osteogenic genes expression in HBMSCs cultures. Further studies need to be done to figure out these complicated signaling pathways.

In conclusion, our present study is the first to demonstrate that RSVL directly stimulates cell proliferation, osteoblastic differentiation, and osteogenic gene expressions through mechanisms involving the ER-dependent MAPK pathway in HBMSCs cultures. Activation of this pathway may mediate the anabolic effects of RSVL on bone cells and serve as an important mechanism for the effectiveness of this stilbene as an anti-osteoporotic treatment.

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