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Inhibition of Oxidized-Phospholipid-Induced Vascular Smooth Muscle Cell Proliferation by Resveratrol Is Associated with Reducing Cx43 Phosphorylation

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ABSTRACT: Abnormal proliferation of vascular smooth muscle cells (VSMCs) is an important factor during the progression of atherosclerosis. In this study, we investigated the effects of resveratrol on atherosclerosis-associated proliferation of VSMCs. We utilized an oxidized phospholipid, 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphorylcholine (POVPC) to induce abnormal proliferation of VSMCs. Our results showed the treatments with resveratrol dose-dependently abolished POVPC-induced VSMC proliferation, as evidenced by the decreased [^{3}H]thymidine incorporated into VSMCs and reduced percentage of 5-ethynyl-2′-deoxyuridine (EdU)-positive VSMCs. Cell cycle analysis demonstrated that resveratrol inhibited POVPC-induced increase in the S phase cell population and DNA synthesis. Our study further indicated that POVPC-induced VSMC proliferation was associated with a significant increase in the phosphorylation of Cx43, which was a consequence of activation of MAPK signaling. Interestingly, treatment with resveratrol abolished POVPC-induced phosphorylation of Cx43 as a result of inhibiting activation of Src, MEK, and ERK1/2. Our results provided a novel mechanism by which resveratrol may contribute to cardiovascular protection.

KEYWORDS: *oxidized phospholipids, resveratrol, vascular smooth muscle cell, proliferation, connexin 43*

INTRODUCTION

Proliferation of vascular smooth muscle cell (VSMC) plays an important role in the progression of atherosclerosis.[1−4] During atherogenesis, minimally modified low density lipoproteins (LDL), the oxidative products of LDL, accumulate in blood vessel walls.[5] Oxidized phospholipids (OxPLs) are components of minimally modified LDL[6,7] and have been considered to be an important factor in the formation of atherosclerotic lesions.[8,9] A number of OxPLs are known to induce vascular inflammation.[5,10−12]

There is a growing body of evidence suggesting that OxPLs enhances lipid accumulation and oxidation, promotes key changes in VSMC phenotype, and triggers the progression of the atherosclerosis.[13] Atherosclerotic lesions have been shown to associate with a number of specific OxPLs, for example, 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphorylcholine (POVPC).[14,15]

The gap junction protein connexin 43 (Cx43) has been shown to be involved in VSMCs proliferation and the development of atherosclerosis.[16] During human coronary atherosclerosis, the expression level of Cx43 in intimal SMCs was increased at early stages of the disease but reduced in advanced atheroma.[17] Neointima formation, macrophage infiltration, and VSMC migration after acute vascular injury in Cx43$^{\pm}$LDLR$^{-/-}$ mice was found to be markedly decreased in comparison with Cx43$^{+/+}$LDLR$^{-/-}$ mice.[18] The reduced Cx43 expression was associated with an inhibition on the formation of atherosclerotic lesion in LDLR$^{-/-}$ mice.[19] Furthermore, MAPK-phosphorylated Cx43 could interact with cell cycle regulating protein cyclin E and promote VSMC proliferation.[20] In addition, recent studies suggested that POVPC-promoted VSMC proliferation was associated with enhanced phosphorylation of Cx43 at S279/282.[21] Collectively, these results underscore the important role of Cx43 in the pathogenesis and progression of atherosclerosis and suggest that suppression of Cx43 may have a therapeutic potential for atherosclerosis.

Resveratrol, a polyphenolic compound found in grapes, has various beneficial effects on cardiovascular diseases.[22] Previous studies have demonstrated that resveratrol protected the vascular walls against oxidation, inflammation, platelet aggregation, thrombus formation, and vascular smooth muscle cell proliferation.[23,24] However, the underlying molecular mechanisms of such protective effects of resveratrol on the vascular system have not been fully understood. In the present study, we determined the inhibitory effects of resveratrol on POVPC-induced vascular smooth muscle cell proliferation and identified the signal pathways associated with the inhibitory effects.

MATERIALS AND METHODS

Materials. Resveratrol and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from

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GIBCO BRL, Invitrogen Co. (Carlsbad, CA). POVPC was purchased from Cayman Chemical Co. (Ann Arbor, MI). Rabbit polyclonal antibodies for ERK1/2, phospho-ERK1/2, MEK1/2, phospho-MEK1/2, Src, phospho-Src, and GAPDH were obtained from Cell Signaling Technology (Beverly, MA). DMSO was used as a vehicle to deliver compounds to the cell culture at 0.1%.

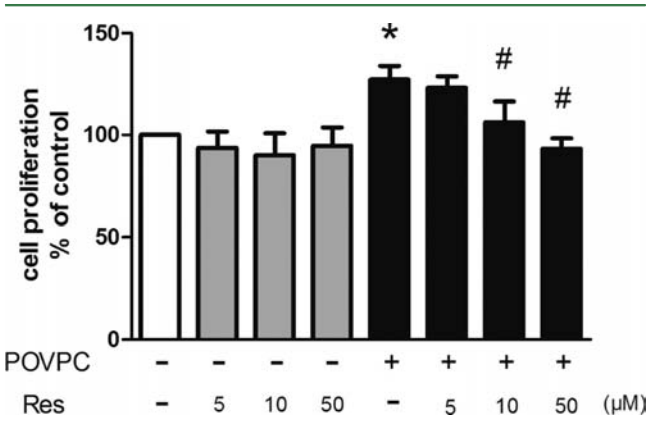

Figure 1. Resveratrol caused a dose-dependent suppression on POVPC-induced increase in [^{3}H]thymidine incorporation in VSMCs. VSMCs were pretreated for 0.5 h with 5, 10, or 50 μM of resveratrol and then incubated with 5 μg/mL of POVPC for 24 h. Cell proliferation was assessed by the CCK-8 assay. [^{3}H]Thymidine incorporation assay was performed. Data represent the mean ± SD of three independent experiments; * indicates $p < 0.05$ compared to the control group, and # indicates $p < 0.05$ compared to the POVPC-treated positive control group.

Cell Culture. VSMCs were isolated from the thoracic aorta of Sprague−Dawley rats, as previously described.[25] The isolated cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Cells grown to 80%−90% confluence were used for all experiments.

Cell Viability Assay. The cell viability was measured by the Cell Counting Kit-8 assay (Dojindo Molecular Technologies Inc., Shanghai, China) following the manufacturer's instruction. Briefly, cells were plated at 2000 cells/well in 96-well plates. After 24 h of treatment with POVPC and various resveratrol doses, 10 μL of CCK-8 solution was added to each well and further incubated for 1 h. The optical density at 570 nm of each well was then measured using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA). All data were from at least three independent replications of each experiment.

Cell Proliferation Assay. VSMCs, grown to 80%−90% confluence in six-well culture plates, were treated with a combination of 5-ethynyl-2′-deoxyuridine (EdU) (10 μM) plus POVPC (5 μg/mL) with or without resveratrol for 24 h. The same final concentration of DMSO was used in place of these reagents to treat the control cells. Cells were then fixed in 3.7% formaldehyde and permeabilized using 0.5% Triton X-100. EdU was conjugated to Alexa Fluor 488-azide using the Click-IT reaction kit (Invitrogen). Nuclear was visualized by Hoechst 33342 staining. Positive cells were counted under a fluorescent microscope and normalized with the total number of cells observed in the field.

Cell Cycle Analysis. VSMCs were pretreated with resveratrol for 30 min and were then cultured with POVPC and resveratrol in 10%

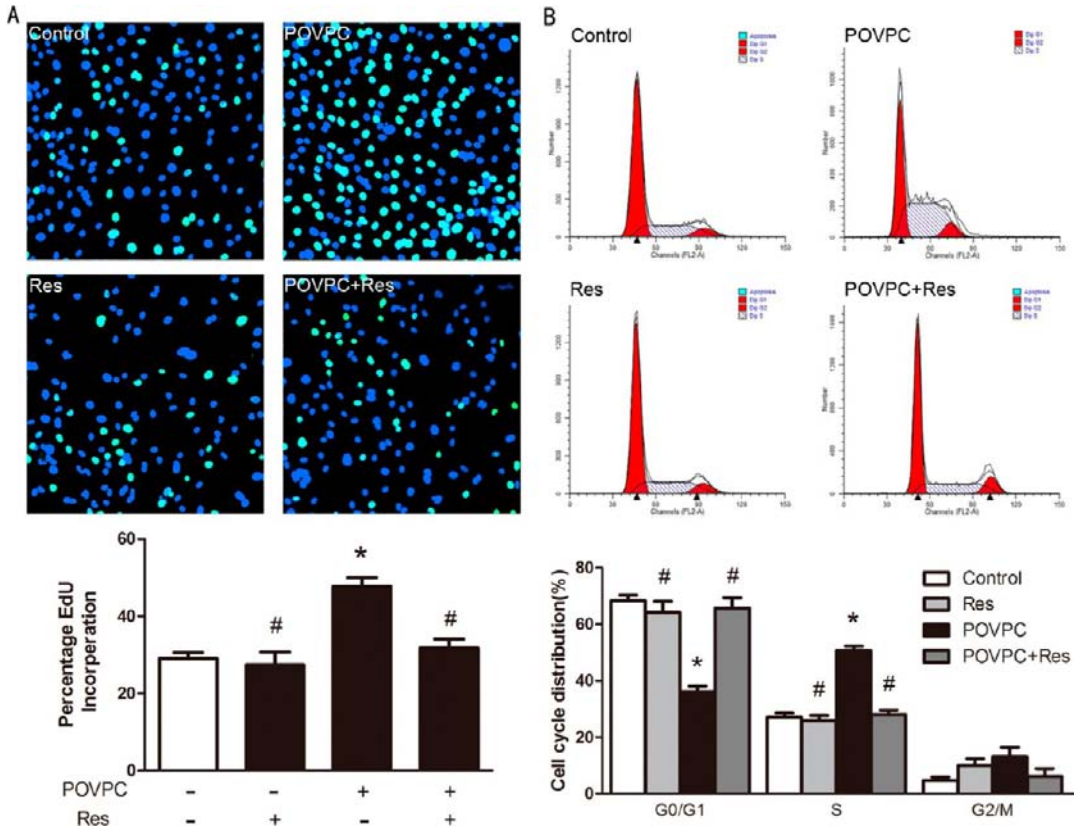

Figure 2. Inhibition of POVPC-induced VSMC proliferation by resveratrol. (A) Treatment of VSMCs with 5 μg/mL POVPC for 24 h caused a marked increase in proliferation, which was attenuated by pretreating VSMCs with 50 μM of resveratrol for 30 min. In each image, blue represents nuclei stain, and green represents EdU stain. The percentages of EdU-positive cells were quantified. Results are representatives of three independent experiments; ** indicates $p < 0.01$, compared to the control group and ## indicates $p < 0.01$ compared to the POVPC-treated group. (B) Analysis of cell cycle distribution by flow cytometry. The histograms represent the percentages of each phase in cell cycle. Results are representative of three independent assays; * indicates $p < 0.05$ compared to the control group and # indicates $p < 0.05$ compared to the POVPC-treated group.

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FBS DMEM for 24 h. The VSMCs were harvested for cell-cycle analysis using FCM (flow cytometry). After being washed with PBS, the cells were fixed with 75% ice-cold ethanol and maintained overnight at 4 °C. The cells were collected; resuspended in PBS containing 40 μg/mL of PI, 0.1 mg/mL of RNase, and 5% of Triton X-100; and then incubated at 37 °C for 30 min. The cells were then analyzed by a FACS Calibur flow-cytometer (Becton Dickinson, Franklin Lakes, NJ). At least 10 000 counts were conducted for each sample. The percentage distribution in the cell cycle phases was analyzed using CellQuest. The percentages of cells in the G0/G1, S, and G2/M phases were calculated using Multicycle software (Phoenix Flow Systems, San Diego, CA).

Western Blot Analysis. Western blot analysis was performed to measure the relative protein levels of Cx43, p-Cx43, ERK1/2, p-ERK1/2, MEK, p-MEK, Src, and p-Src. VSMCs were grown in 10 cm dishes until 80%−90% confluence. Cells were pretreated with resveratrol for 0.5 h and were then cultured with POVPC plus resveratrol for another 0.5 h. Cells were harvested and lysed into lysis buffer. The protein concentration of each cell lysate was determined by BCA reagents (Pierce, Rockford, IL). The total protein was separated by 10% SDS−PAGE gels and electrophoretically transferred to polyvinylidene difluoride membranes. After blocking for 1 h with 5% skimmed milk in TBS buffer (10 mM Tis, 150 mM NaCl), the membranes were washed three times for 10 min with TBST buffer (10 mM Tris, 150 mM NaCl, and 0.1% Tween-20). The membranes were incubated with the specific primary antibody and then with the peroxidase-conjugated secondary antibody. The bands were visualized using the ECL system (Bio-Rad Laboratories, Hercules, CA), and the band density was determined by Image J software (NIH).

Statistical Analysis. All data were expressed as the mean ± SD of three independent experiments. The statistical analysis was conducted using one-way ANOVA; $p < 0.05$ was considered statistically significant.

RESULTS

Resveratrol Inhibited OxPL-Induced VSMC Proliferation. To quantify the effects of POVPC (an OxPL) and resveratrol on the proliferation of VSMCs, we utilized two different methods. As shown in Figure 1, stimulation with POVPC (5 μg/mL) significantly increased the amount of [^{3}H]thymidine incorporated into VSMCs by 27.1% in comparison with the control cells. This POVPC-induced increase in cell proliferation was dose-dependently abolished by the pretreatment of resveratrol. For example, pretreatment of resveratrol at 10 and 50 μM decreased the amount of [^{3}H]thymidine incorporated into VSMCs by 21.0% and 34.0%, respectively. Our results also demonstrated that resveratrol alone at 5−50 μM did not significantly affect cell viability.

Next, we utilized the Click-iT EdU assay to detect and quantify proliferating VSMCs. As shown in Figure 2A, after stimulation with POVPC (5 μg/mL) for 24 h, 47.7% of VSMCs showed high proliferation, which was 1.7-fold higher than that of the control cells (29.0%). Interestingly, pretreatment of resveratrol at 50 μM for only 30 min abolished the POVPC-induced increase in proliferation. No significant difference was observed between the control cells and the cells treated with resveratrol + POVPC. Resveratrol alone at 50 μM did not affect the basal level of EdU incorporation.

To further understand the inhibitory effects of resveratrol on POVPC-induced VSMC proliferation, cell cycle analysis was conducted. As shown in Figure 2B, in comparison with the control cells, POVPC induced a 26.9% increase in cell population at the S phase, which was accompanied by a 27.7% decrease in cell population at the G0/G1 phase. Interestingly, pretreatment with resveratrol abolished the POVPC-caused changes on cell cycle distribution; i.e., resveratrol increased G0/G1 phase cell population by 20.3% and decreased S phase cell population by 23.9% in comparison with the cells treated with POVPC. Consequentially, resveratrol-treated cells showed cell cycle distribution similar to that of the control cells. Resveratrol alone at 50 μM did not affect the cell cycle distribution of VSMC.

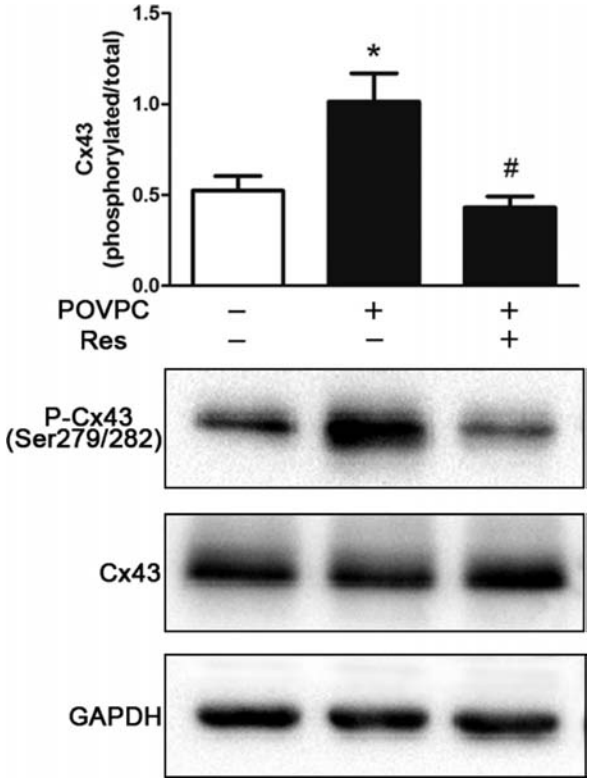

Figure 3. Inhibition of POVPC-induced phosphorylation of Cx43 by resveratrol. Cells were pretreated with 50 μM of resveratrol for 30 min prior to incubation with 5 μg/mL of POVPC for 30 min. After treatment, cells were harvested and subject to Western blotting analysis for Cx43 and p-Cx43 following the procedure described in the Materials and Methods section. The band intensity was measured and calculated relative to the controls. The data are expressed as mean ± SD; * indicates $p < 0.05$ in comparison to the control group, and # indicates $p < 0.05$ in comparison to the POVPC-treated group. The results are representative of three independent experiments.

Resveratrol Inhibited OxPL-Induced Phosphorylation of Cx43. Previous studies have shown that the level of phosphorylated Cx43 was elevated in response to POVPC, and Cx43 was involved in proliferation of VSMCs.[21] Therefore, we examined the effects of resveratrol on POVPC-induced Cx43 phosphorylation in VSMCs. VSMCs were pretreated with 50 μM of resveratrol for 30 min and then stimulated with 5 μg/mL of POVPC for 0.5 h. Western blotting assay showed that the level of p-Cx43 (Ser 279/282) in the POVPC-stimulated VSMCs was increased to about 2-fold of that of the control cells (Figure 3). The results further demonstrated that pretreatment of resveratrol completely reversed the POVPC-induced increase in the level of p-Cx43 (Ser 279/282).

Src, MEK, and ERK1/2 Were Involved in VSMC Proliferation Induced by OxPL. Several lines of evidence have demonstrated that oxLDL increased the levels of p-MEK and p-ERK1/2. Aurélie et al.[3] showed that Src family kinases were also involved in VSMCs proliferation. To determine the

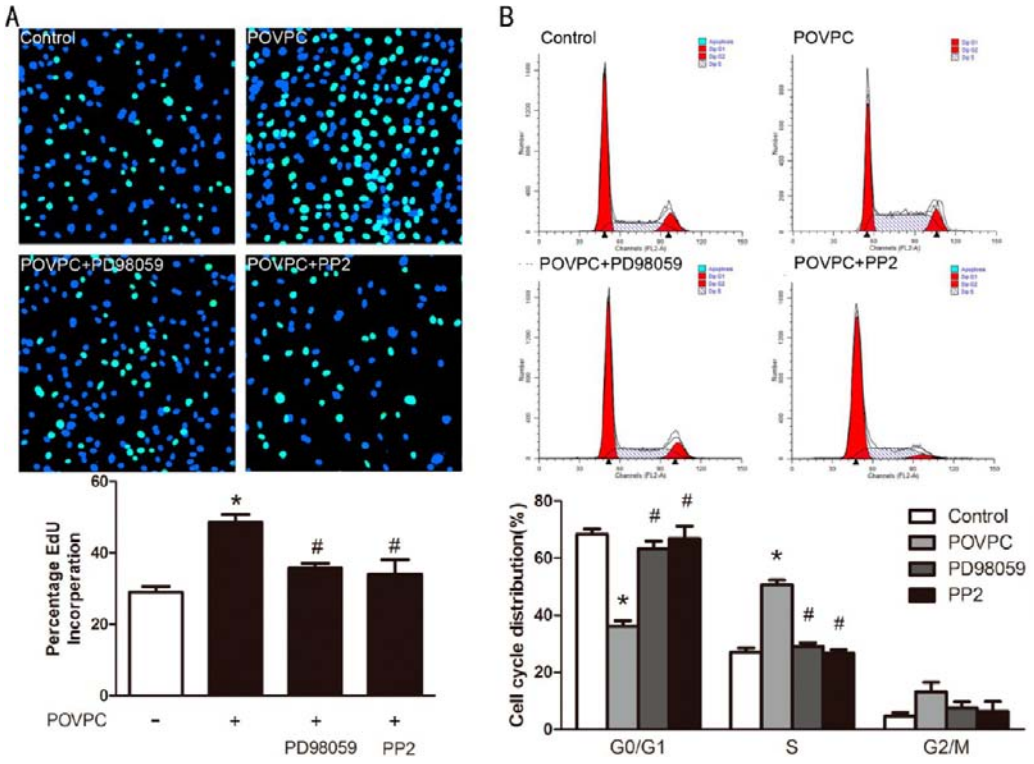

Figure 4. Effect of MAPK inhibitors on POVPC-induced VSMC proliferation. (A) PD98059 and PP2 attenuate POVPC-induced VSMCs proliferation. The VSMCs were preincubated with PD98059 (50 μM) or PP2 (20 μM) for 0.5 h before treatment of POVPC and then harvested at 24 h and analyzed for EdU incorporation assay. The cells were double labeled for EdU (green) and nuclei (blue). In the histogram, ** indicates $p < 0.01$, compared to the control group, and # indicates $p < 0.05$ compared to the POVPC-treated group. (B) Effects of MAPK inhibitors on POVPC-induced changes in cell cycle distribution of VSMCs. Cell cycle distributions are shown in the histograms; * indicates $p < 0.05$, compared to the control group, and # indicates $p < 0.05$ compared to the POVPC-treated group.

role of these signaling proteins in POVPC-induced VSMC proliferation, we used two kinase inhibitors, PP2 and PD98059, to block Src family tyrosine kinase and MEK1 activity, respectively, which in turn can inhibit phosphorylation of ERK1/2.[26] Each inhibitor was incubated with VSMCs for 30 min prior to POVPC stimulation. After 24 h of stimulation with POVPC, Click-iT EdU assay and cell cycle analysis were utilized to quantify and characterize VSMC proliferation. As shown in Figure 4A, stimulation with POVPC caused a significant increase in VSMC proliferation, and this POVPC-induced increase was greatly diminished by the pretreatment of PP2 and PD98059. Flow cytometric analysis demonstrated that PD98059 and PP2 treatment abolished POVPC-induced changes in cell cycle distribution of VSMCs, i.e., increased G0/G1 phase cell population and decreased S phase cell population in comparison with the cells treated with POVPC (Figure 4B). Consequentially, PD98059- and PP2-treated cells showed cell cycle distribution similar to that of the control cells.

Src, MEK, and ERK1/2 Were Involved in Cx43 Phosphorylation Induced by OxPL. As shown in Figure 2, our results showed that stimulation with POVPC induced phosphorylation of Cx43 in VSMCs. Next we determined whether Src kinase and MEK/ERK1/2 signaling were required for Cx43 phosphorylation induced by POVPC. As shown in Figure 5, stimulation with POVPC caused an marked increase in the level of phosphorylation of Cx43 in VSMCs. Pretreatment with PD98059 or PP2 completely abolished POVPC-induced phosphorylation of Cx43. Actually, the levels of Cx43 phosphorylation in the cells treated with PD98059 or PP2 were even lower than that of the negative control cells.

Resveratrol Inhibited OxPL-Induced Phosphorylation of Src, MEK, and ERK1/2. We examined the effects of POVPC treatment on the phosphorylation of Src, MEK, and ERK1/2. As shown in Figure 6, it is clear that stimulation with POVPC led to marked increases in the phosphorylation of Src, MEK, and ERK1/2. In comparison with the control cells, POVPC caused 1.6-, 1.9-, and 1.6-fold increase in the phosphorylation levels of Src, MEK, and ERK1/2, respectively. Our results further showed that pretreatment with resveratrol completely abolished the POVPC-induced phosphorylation of Src, MEK, and ERK1/2.

DISCUSSION

Cardiovascular disease is one of major pathological causes of human death. Natural products have long been used to prevent and treat chronic diseases including cardiovascular disease. As one of the most studied dietary natural products, resveratrol has been shown to offer protective effects against cardiovascular disease.[27] However, the exact mechanisms underlying these protective effects have not been fully understood. Previous studies have showed that abnormal proliferation of VSMCs is an important factor during the progression of atherosclerosis.[1−4] Herein, we investigated the effects of resveratrol on atherosclerosis-associated proliferation of VSMCs. We utilized POVPC to induce proliferation of VSMCs, since POVPC, one of the pro-atherosclerotic OxPLs, has been shown to promote proliferation of VSMCs.[14,15] Our results indicated that POVPC indeed significantly enhanced the proliferation of VSMCs, as

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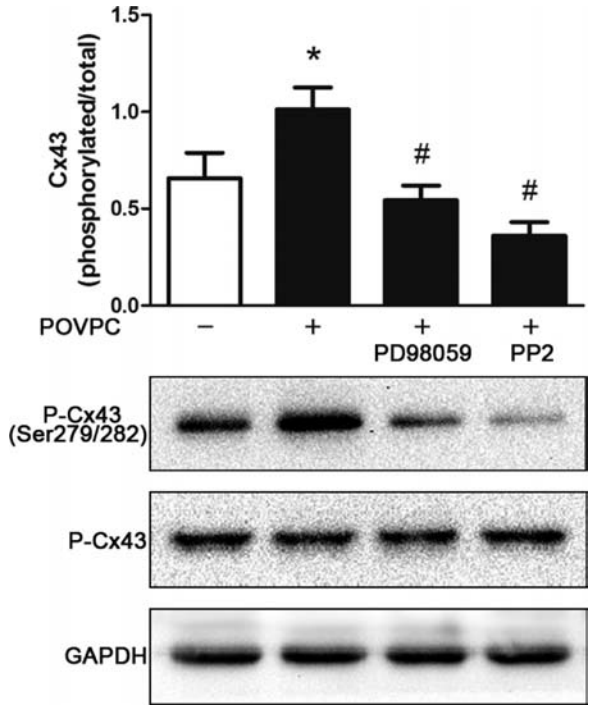

Figure 5. The effects of MAPK inhibitors on the POVPC-induced Cx43 phosphorylation in VSMCs. VSMCs were pretreated with the specific ERK1/2, MEK, or Src inhibitors, PD98059 (50 μM) or PP2 (20 μM), for 30 min prior to treatment with 5 μg/mL of POVPC for 30 min. Cells were then harvested, and the levels of Cx43 phosphorylation were determined by Western blot analysis. The results are representative of three independent experiments. The data are expressed as mean ± SD; * indicates $p < 0.05$ compared to the control group, and # indicates $p < 0.05$ compared to the POVPC-treated positive control group.

evidenced by the increased [^{3}H]thymidine incorporated into VSMCs and increased percentage of EdU-positive VSMCs after treatment with POVPC (Figures 1 and 2). Our results further demonstrated that the treatments with resveratrol dose-dependently abolished POVPC-induced increase of [^{3}H]thymidine incorporation and POVPC-caused increase of EdU-positive cells. This is the first report that resveratrol inhibited POVPC-induced proliferation of VSMCs, which is consistent with previous studies by us and other investigators where resveratrol was shown to inhibit VSMC proliferation induced by different stimulators including angiotensin II, FBS, and platelet-derived growth factor.[28−30]

We further characterized the POVPC-induced proliferation of VSMCs and found that POVPC caused a marked increase of VSMC cell population in the S phase and decrease in the G0/G1 phase of cell cycle. This finding suggested that POVPC increased DNA synthesis of VSMCs and potentially promoted mitosis of VSMCs. On the other hand, treatment with resveratrol completely reversed POVPC-induced changes in cell cycle distribution of VSMCs, which indicated that resveratrol may have inhibited POVPC-induced DNA synthesis and associated mitosis. It has been shown that resveratrol caused G1 cell cycle arrest in VSMCs.[31] However, our results showed that resveratrol did not cause G1 cell cycle arrest in primary VSMCs but blocked POVPC-stimulated VSMCs entering the S phase from the G1 phase by inhibiting their DNA synthesis.

Recent studies have shown that Cx43, the principal gap junction protein in VSMCs, plays important roles in the progression of atherosclerosis.[18,21,32] It has been reported that Cx43 expression level was upregulated in VSMCs during early atherosclerosis.[33] A decreased expression level of Cx43 was associated with reduced SMC migration and proliferation.[18,19] Furthermore, phosphorylation of Cx43 has been associated with VSMC proliferation in atherosclerosis.[21] We studied the effects of POVPC on Cx43 in VSMCs and found that POVPC caused a significant increase in the phosphorylation of Cx43 without affecting the total expression level of Cx43 (Figure 3). These results suggested that increased phosphorylation of Cx43 may be involved in POVPC-induced VSMC proliferation. One potential mechanism by which Cx43 promotes VSMC proliferation is by interaction with cycline E after Cx43 phosphorylation, and this interaction can promote cell cycle progression and cell proliferation.[20] Our results showed that treatment with resveratrol abolished POVPC-induced phosphorylation of Cx43 (Figure 3). This finding reinforced the potential importance of phosphorylation of Cx43 during POVPC-induced VSMC proliferation, since resveratrol inhibited POVPC-induced VSMC proliferation (Figures 1 and 2). Cycline E plays an important role at the G0/G1 to S check point of the cell cycle. Our results support the notion that resveratrol inhibits POVPC-induced VSMC proliferation by inhibiting phosphorylation of Cx43, which in turn blocks cell cycle progression at the G0/G1 to S check point.

MAP kinase pathway has been shown to play a crucial role in OxPLs-induced VSMC proliferation.[21] OxPLs can induce phosphorylation and activation of ERK1/2.[34] OxLDL-induced activation of ERK1/2 and DNA synthesis in VSMCs was found to be dependent on activation of MEK1/2, an upstream kinase of ERK1/2.[35] Furthermore, activation of Src was reported to be involved in VSMC proliferation induced by oxLDL.[3] In order to determine the extent to which the MAPK pathway mediates OxPL-induced VSMC proliferation, we determined the effects of POVPC on phosphorylation of ERK1/2, MEK, and Src in VSMCs and found that POVPC significantly increased phosphorylation levels of these three kinases (Figure 6). Our results demonstrated that PD98059 and PP2 (MEK1 and Src specific inhibitors, respectively) abolished POVPC-induced VSMC proliferation and increase in the S phase cell population (Figure 4). Furthermore, PD98059 and PP2 also significantly decreased the phosphorylation level of Cx43 induced by POVPC (Figure 5). Our findings suggested that MAPK signaling is involved in the POVPC-induced VSMC proliferation, and phosphorylation of Cx43 is likely a consequence of activation of MAPK signaling by POVPC. Our results also demonstrated that treatment with resveratrol abolished POVPC-induced phosphorylation of Src, MEK, and ERK1/2 (Figure 6). These findings indicated that downregulation of MAPK signaling by resveratrol plays an important role in the resveratrol-elicited inhibition of VSMC proliferation induced by POVPC.

In conclusion, the present study demonstrated that resveratrol inhibits POVPC-induced pro-atherosclerotic VSMC proliferation, which is associated with the inhibition of MAPK-mediated phosphorylation of Cx43. Our results provided a novel mechanism by which resveratrol may contribute to cardiovascular protection, which warrants future in vivo study to validate the mechanism.

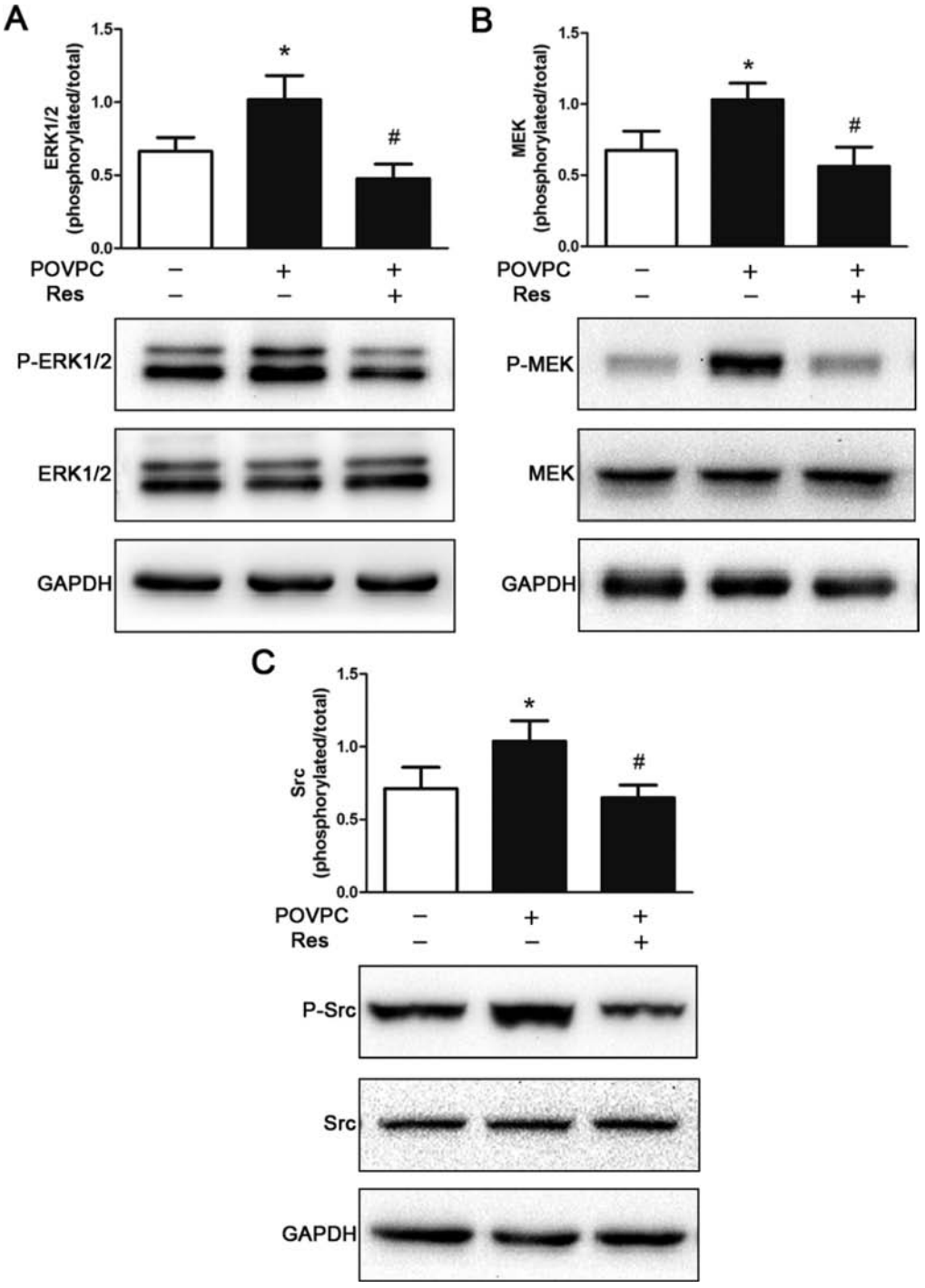

Figure 6. Inhibition of POVPC-induced phosphorylation of ERK1/2 (A), MEK (B), and Src kinase (C) by resveratrol. Cells were pretreated with resveratrol (50 μM) for 30 min, and then incubated with POVPC for another 30 min. Cells were harvested and subjected to Western blot analysis for p-ERK1/2, p-MEK, and p-Src. The results are representative of three independent experiments. The data are expressed as mean ± SD; * indicates $p < 0.05$ compared to the control group, and # indicates $p < 0.05$ compared to the POVPC-treated positive control group.

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Author Contributions

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

POVPC, 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphorylcholine; Res, resveratrol; OxPLs, oxidized phospholipids; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; VSMCs,

vascular smooth muscle cells; PI, propidium iodine; Cx43, connexin 43.

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