Resveratrol prevents endothelial progenitor cells from senescence and reduces the oxidative reaction via PPAR-γ/HO-1 pathways

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Abstract. Increasing evidence suggests endothelial progenitor cells (EPCs) improve neovascularization and endothelium regeneration. Resveratrol (RSV) is a natural polyphenolic compound, which has been demonstrated to exert multiple protective effects on the cardiovascular system, including inhibition of platelet adhesion and aggregation, reduction of myocardial ischemia-reperfusion injury, and suppression of neointimal hyperplasia of injured vascular tissue. The present study investigated the role of RSV on levels of oxidative stress and senescence of EPCs, and the effects of RSV on vascular-promoting and/or vascular-healing capacity of EPCs. It was demonstrated that EPCs could promote the repair of endothelium of the injured artery. RSV reduced the oxidative reaction of EPCs and inhibited EPC senescence, and these effects may occur via the peroxisome proliferator-activated receptor-γ/heme oxygenase-1 signaling pathways.

Introduction

Cardiovascular disease is currently the leading cause of mortality, and the incidence continues to increase, in spite of advancements in diagnosis and treatment of cardiovascular disease. Thus, novel types of therapy are under development for patients who are suffering from cardiovascular disease.

Increasing evidence suggests endothelial progenitor cells (EPCs) improve neovascularization and endothelium regeneration, suggesting there may be potential for cell therapy in the future to promote endothelial repair and reendothelialization of ischemic tissue (1-4). Resveratrol (RSV) is a natural polyphenolic compound, modern medical research has demonstrated RSV exerts multiple protective effects on the cardiovascular system, including reducing platelet adhesion and aggregation, protecting myocardial cells from ischemia-reperfusion injury, and suppression of neointimal hyperplasia of injured vascular tissue (5-7). A previous study indicated that RSV prevented the onset of EPC senescence and enhanced telomerase activity via the phosphatidylinositol-4,5-bisphosphate 3-kinase-Akt signaling pathway (8). The present study hypothesized that RSV protects EPCs from senescence via improving pathological factors that induce EPC dysfunction *in vivo*. In addition, they may also enhance the properties of EPCs that are important for cell therapy. The aim of the present study was to investigate whether RSV could prevent EPCs from senescence, and to investigate the effects of RSV on the potential repair and re-endothelialization capacity of EPCs in injured vessels.

Materials and methods

Cell isolation and culture. The technique to culture EPC subpopulations was conducted as described previously (9,10). The blood samples were obtained from healthy volunteers subsequent to obtaining informed, written consent (n=20; age, 28±4 years; 10 male, 10 female), and were treated, observed and analyzed individually in independent experiments. The peripheral blood mononuclear cells (PBMNCs) were isolated by density-gradient centrifugation with Ficoll separating solution (Cedarlane Laboratories Ltd., Burlington, Ontario, Canada). The study was approved by the ethics committee of Zhejiang University (Hangzhou, China). PBMNCs were plated into fibronectin-coated six-well plates in 1.5 ml EGM-2MV medium (Lonza Group, Basel, Switzerland) at a density of $2x10^5/cm^2$, containing 10% fetal bovine serum, vascular endothelial growth factor, fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor, and ascorbic acid. Adherent MNCs cultured for 7-21 days in the above conditions

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were used to derive late outgrowth EPCs and the colonies exhibit a cobblestone morphology.

Determination of reactive oxygen species (ROS) production. The membrane permeable indicator H_2DCF-DA (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to determine ROS production by EPCs. EPCs were loaded with 10 μmol/l H_2DCF-DA in serum-free EGM-2MV medium at 37˚C for 30 min and then washed twice with phosphate-buffered saline (PBS). Following treatment with various concentrations of RSV (0.01, 0.1, 1, 5 and 10 μmol/l; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) for 48 h, the cells were analyzed with a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. ROS production was determined by comparing the changes in fluorescence intensity with those of the control. CellQuest Pro software, version 3.1 (BD Biosciences) was used for analysis.

Measurement of NADPH oxidase activity. To evaluate NADPH oxidase activity in EPCs, the lucigenin-derived enhanced chemiluminescence assay (Beyotime Institute of Biotechnology, Shanghai, China) was employed. EPCs were starved in serum-free EGM-2MV medium for 24 h and treated with RSV (0.01, 0.1, 1, 5 and 10 μmol/l), and subsequently washed twice with ice-cold PBS (pH 7.4) and centrifuged at 2,000 x *g* for 5 min at 4˚C. The cells were re-suspended in ice-cold buffer (pH 7.0) containing 1 mmol/l ethylene glycol tetraacetic acid, protease inhibitors, and 150 mmol/l sucrose, and lysed. A Bradford assay was used to determine the total protein concentration, which was adjusted to 1 mg/ml. Every 100 μl of protein sample, including 2.5 μmol/l lucigenin, was measured over 6 min in quadruplicate using NADPH (100 μmol/l) as a substrate in a luminometer counter (Centro LB 960; Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany).

Evaluation of nitric oxide (NO) production. EPCs were starved in serum-free medium for 48 h and treated with RSV (0.01, 0.1, 1, 5 and 10 μmol/l). The production of NO was evaluated by identifying the concentration of NO in the culture supernatant. The effect of RSV on NO generation of EPCs was determined with the NO Assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Briefly, EPCs were seeded onto 96-well plates at a density of $2x10^4$ cells/well, and pre-treated with or without lipopolysaccharides (1 μg/ml) for 4 h. The cells were then stimulated with or without different concentrations of RSV for 24 h. Subsequently, Griess reagent I and Griess reagent II from the kit were added into the cell supernatants, and the optical density was measured at 540 nm using the abovementioned luminometer counter.

Determination of senescence-associated β-galactosidase (SA-β-gal) activity. Following treatment with RSV (0.01, 0.1, 1, 5 and 10 μmol/l) for 48 h, EPC subpopulations were harvested and SA-β-gal activity was measured according to a previously described method (11). The SA-β-gal Activity Assay kit was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Briefly, EPCs were washed with PBS, fixed in

fixative solution (2% paraformaldehyde, 0.2% glutaraldehyde, PBS) for 10 min at room temperature, and cultivated overnight at 37˚C (no CO_2) with fresh SA-β-gal staining solution [1 mg/ml 5-bromo-4-chloro-3-indyl β-D-galactopyranoside (X-gal), 5 mmol/l potassium ferrocyanide, 5 mmol/l potassium ferricyanide, 150 mmol/l NaCl, 2 mmol/l $MgCl_2$, 40 nmol/l citric acid/sodium phosphate (pH 6.0)]. A light microscope was used to observe the cells stained blue, and all the cells were stained with 4',6-diamino-2-phenylindole for 10 min in order to count the total cell numbers.

Western blot analysis. Following RSV treatment, equal amounts (20~30 μl) of cellular proteins were obtained. The cellular proteins were extracted using lysis buffer containing 1% NP-40, 150 mmol/l NaCl, 50 mmol/l Tris-HCl, pH 8.0, 0.5% sodium deoxycholate and 0.1% SDS. The concentration of total proteins was measured using the bicinchoninic acid assay (Pierce Biotechnology, Inc., Rockford, IL, USA). and separated by 10% SDS-polyacrylamide gel and then electrotransferred to a polyvinylidene difluoride membrane. Prior to incubation of the membranes overnight at 4˚C with the primary antibodies, they were blocked in blocking solution (Tris-buffered saline; TBS) containing 0.1% (v/v) Tween 20 and 5% (v/v) bovine serum albumin (Sigma-Aldrich; Merck Millipore) for 1 h at room temperature. The primary antibodies (1:1,000) used were as follows: Anti-telomerase reverse transcriptase (TERT; BS60032; Bioworld Technology, Inc., St. Louis Park, MN, USA), anti-peroxisome proliferator-activated receptor-γ (PPAR-γ; #2430; Cell Signalling Technology, Inc.), anti-heme oxygenase-1 (HO-1; #5853; Cell Signalling Technology, Inc.), anti-sirtuin-1 (#2496; Cell Signalling Technology, Inc.) and anti-GAPDH (#2118; Cell Signalling Technology, Inc.). Subsequently, the membranes were washed extensively in TBS containing 0.1% (v/v) Tween 20 3 times, and incubated for 1 h at room temperature with mouse anti-rabbit (#3677) and rabbit anti-mouse (#58802) secondary antibodies conjugated to horseradish peroxidase (1:5,000; Cell Signalling Technology, Inc.). Enhanced chemiluminescence (ECL) solution (ECL Protein Biotinylation System; GE Healthcare Life Sciences, Chalfont, UK) was used to visualize the protein bands on the membranes. The western blotting results were analyzed using Multi-gauge software, version 3.11 (Fujifilm, Tokyo, Japan).

Animal study. EPCs were isolated and cultured from the peripheral blood of New Zealand rabbits (n=25; weight, 2.3-2.9 kg; 12 male and 13 female; 3-4 months old) as described above. The rabbits were purchased from the Animal Center of Zhejiang University (Hangzhou, China) and were individually housed with access to a high-fiber pelleted laboratory diet and water, maintained at 23±1˚C, with 30-70% relative humidity and a 12:12 h light:dark cycle (6 am-6 pm). All rabbits were distributed into 5 groups: i) Carotid injury group without transplantation of EPCs; ii) carotid injury group with transplantation of EPCs but without intervention; iii) carotid injury group with transplantation of EPCs pretreated with RSV; v) control group with sham procedure, which involved anesthesia with ketamine, an anterior midline incision made to expose the left common carotid artery and the internal and external carotid arteries, which was then closed after ~10 min. The carotid injury induced in the rabbits in the carotid injury groups was induced by a balloon catheter.
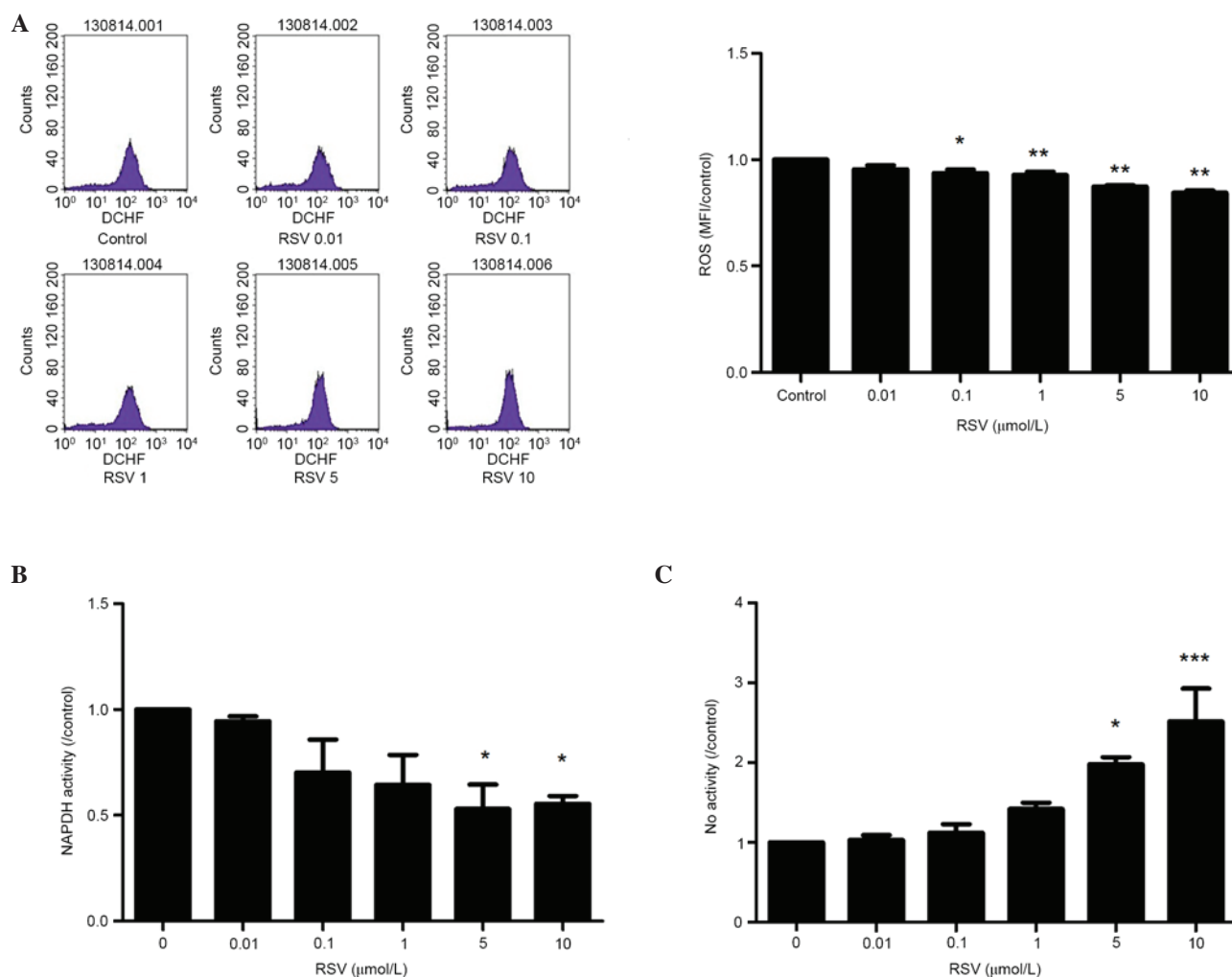
Figure 1. Effect of RSV on the oxidative reaction of endothelial progenitor cells. (A) RSV decreased DCF fluorescence in a concentration-dependent manner. Representative flow cytometry results indicated that with the increase of the concentration of RSV, ROS production gradually reduced. (B) Pretreatment with RSV significantly reduced the NADPH oxidative activity in a concentration-dependent manner. (C) Stimulation with RSV led to a concentration-dependent increase of NO production. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. the control. RSV, resveratrol; ROS, reactive oxygen species; NO, nitric oxide.

Rabbits were anesthetized with 35 mg/kg ketamine, and an anterior midline incision was made to expose left common carotid artery, internal and external carotid arteries. The distal end of the external carotid artery was ligated and the proximal common carotid artery and internal carotid artery were temporarily interrupted. A 3F Fogarty balloon catheter was inserted into the common carotid artery via the incision of the external carotid artery (~3 cm) and then inflated with the pressure of 10 atmospheres. The balloon catheter was withdrawn then reentered 3 times to injury the artery. The EGM-2 suspension containing $1x10^6$ EPCs cultured *in vitro* or alone was injected locally into the arterial lumen and maintained there for ~5 min. The distal end of the external carotid artery was ligated and the blood flow to the common carotid was restored by release of the ligatures in the proximal common carotid artery and internal carotid artery, and the wound was closed. No adverse neurological or vascular effects were observed in any animal undergoing this procedure. Rabbits were sacrificed after 1 week via injection of 10 ml air into the ear vein under ketamine anesthesia, and the target carotid artery was removed for the measurement of re-endothelialization and morphological study. Repair of the artery was evaluated by staining with Evans Blue

dye (Sigma-Aldrich; Merck Millipore) according to a method described previously (12). Briefly, at 30 min prior to sacrifice, 5 ml of saline containing 5% Evans blue was injected into the ear vein. The artery was fixed with a perfusion of 4% paraformaldehyde for 45 min. The area stained in blue indicates endothelium injury and the ratio between the area free from stain and the total injured carotid artery area was calculated. For histological with hematoxylin and eosin (H&E) analyses, a segment of each artery was perfusion fixed with 4% paraformaldehyde at physiological pressure (110 mmHg) and subsequently processed.

Statistical analysis. Values are expressed as the mean ± standard error in the text and figures. Data were analyzed using independent t-tests and one-way analysis of variance using GraphPad Prism, version 4 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

RSV reduces the oxidative reaction of EPCs. To determine the effect of RSV on the oxidative reaction of EPCs, the
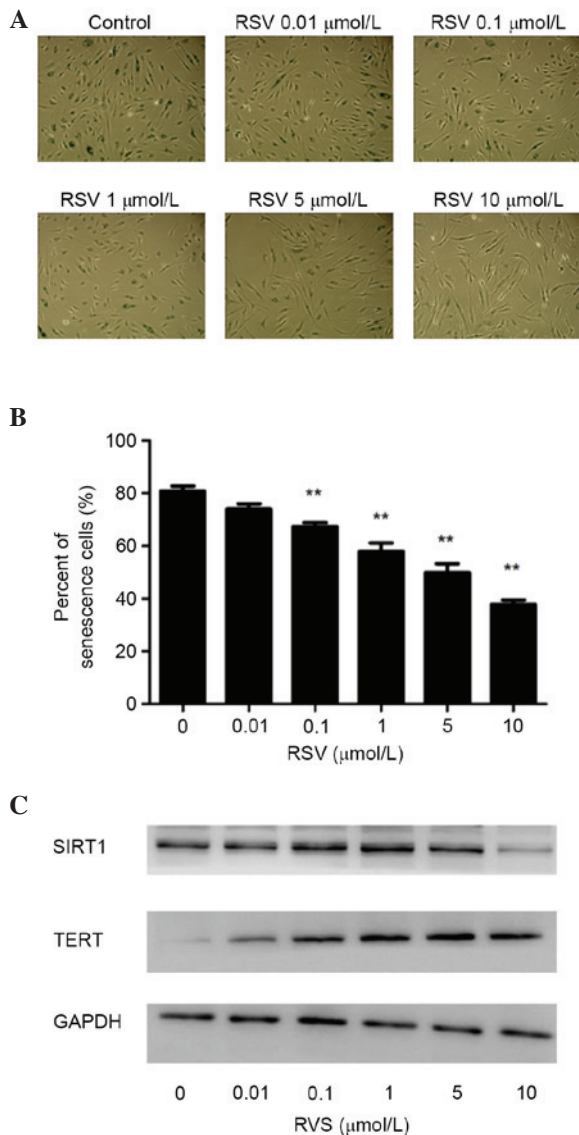
Figure 2. Effect of RSV inhibiting premature senescence in EPCs with the increased expression of h-TERT and suppression of SIRT1 expression. (A) Representative photomicrographs demonstrated senescence-associated β-galactosidase positive cells (blue) in EPCs treated with RSV. Magnification, x40. (B) Data is presented as mean ± standard error of the mean. (C) RSV increased the h-TERT protein expression levels in a dose-dependent manner, and the expression of SIRT1 was reduced. **P<0.01 vs. the control. EPC, endothelial progenitor cells; h-TERT, human telomerase reverse transcriptase; SIRT1, sirtuin 1.

RSV inhibits premature senescence in endothelial progenitor cell with increased expression of human (h)-TERT. To investigate the effect of RSV on the senescent phenotype in EPCs, cells were treated with different concentrations of RSV for ~48 h, and it was demonstrated that treatment with RSV significantly inhibited the senescent phenotype using the SA-β-gal assay with concentrations of 0.1-10 μmol/l (P<0.01; Fig. 2A and B), which has been indicated previously (13). To investigate the mechanism by which RSV prevents premature EPC senescence, the present study evaluated the involvement of TERT expression. As Fig. 2C indicates, the expression levels of h-TERT protein markedly increased by RSV in a dose-dependent manner.

RSV activates PPAR-γ/HO-1 signaling pathways. Treatment with RSV significantly increased the PPAR-γ and HO-1 protein expression levels with concentrations of 0.1-10 μmol/l in a concentration-dependent manner (P<0.01; Fig. 3).

Effects of RSV on the re-endothelialization capacity of EPCs. The H&E staining of the left common carotid artery indicated that each layer of the normal carotid artery could be clearly observed. The endovascular surface was covered with a layer of flat endothelial cells. However, in the animal model with the balloon-injured artery, the intima of the carotid artery was injured and endothelial cells had disappeared (data not shown). As presented in Fig. 4, the EPCs repaired the injured artery. However, the high concentration of RVS enhanced the re-endothelialization capacity of EPCs. EPCs treated with RSV were demonstrated to improve re-endothelialization ability compared to EPCs without RSV treatment (Fig. 4).

Discussion

In the present study, it was demonstrated that RSV could prevent EPCs from senescence, and reduce the expression of NAPDH oxidase and the oxidative reaction of EPCs, likely via the PPAR-γ/HO-1 signaling pathways. In animal studies, the re-endothelialization capacity of EPCs was significantly enhanced by RSV.

RSV (3,5,4'-trihydroxystilbene) is a stilbenoid, a type of natural phenol, and a phytoalexin produced naturally by a number of plants in response to injury or when the plant is under attack by pathogens, such as bacteria or fungi (14). RSV is found in numerous foods, including the skin of grapes, blueberries, raspberries, and mulberries (15). Extensive studies on its activity, predominantly on cellular models, demonstrates that RSV promotes cell proliferation, enhances differentiation, and induces apoptosis and autophagy (16-20). The compound also generates angiogenesis and inflammation. The potential chemotherapeutical capacity of RSV was confirmed by investigations into the effect of RSV on implanted cancers and chemically induced tumors. Similarly, a study indicated that RSV may positively influence the progression and generation of chronic diseases including type 2 diabetes, obesity, coronary heart disease, metabolic syndrome, and neurodegenerative pathologies in animal models (21).

Previous studies have demonstrated that RSV enhanced proliferation, migration and angiogenesis of EPCs, and

changes in ROS production, NAPDH oxidase activity and NO production were observed. The intracellular ROS levels were measured using a flow cytometer following staining of EPCs with the ROS-sensitive fluorescent probe, H_2DCF-DA. As Fig. 1A demonstrates RSV decreased DCF fluorescence in a concentration-dependent manner (P<0.01). The NADPH oxidase activity with lucigenin-enhanced chemiluminescence was measured. Pretreatment with RSV significantly reduced the NADPH oxidative activity in a dose-dependent manner (P<0.01 when the concentration of RSV was ≥1 μmol/l; Fig. 1B). Furthermore, the present study observed the NO production of EPCs with or without RSV pretreatment. Stimulation with RSV resulted in a concentration-dependent increase of NO production (P<0.05; Fig. 1C).
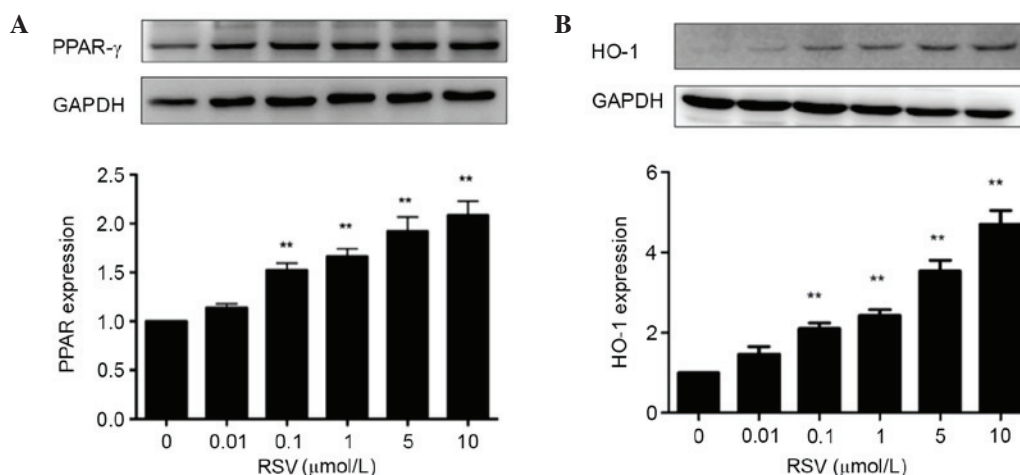
Figure 3. Effects of RSV on the PPAR-γ/HO-1 signaling pathway. Treatment with RSV increased (A) PPAR-γ and (B) HO-1 protein expression levels in a dose-dependent manner. **P<0.01 vs. the control. PPAR-γ, peroxisome proliferator-activated receptor-γ; HO-1, heme oxygenase-1; RVS, resveratrol.
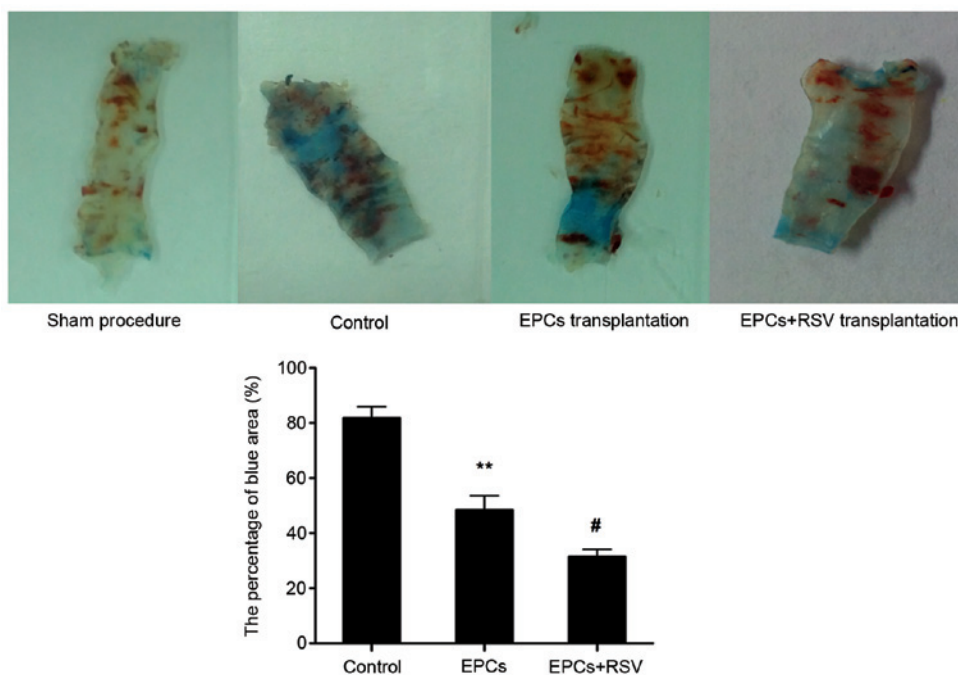

Figure 4. Effect of RSV on the recruitment of EPCs. The EPCs repaired the injured artery. However, the high concentration of RSV enhanced the re-endothelialization capacity of EPCs. Data were presented as the mean ± standard error of the mean. **P<0.01 vs. the control, #P<0.05 vs. the EPCs transplantation. EPC, endothelial progenitor cell; RSV, resveratrol.

prevent TNF-α-induced EPC apoptosis (14,22). Our previous study suggested that RSV inhibited the onset of EPCs senescence may via telomerase (23). Cellular senescence is the most important cause of EPCs dysfunction and death. In populations with cardiovascular disease, EPC senescence is observed, and oxidized low density lipoprotein (ox-LDL), homocysteine and angiotension II may inhibit the function of EPCs via inducing cellular senescence (11,24,25). Oxidative stress may be the one of the most important mechanisms of EPC aging, diabetes can increase the reactive oxygen species level in EPCs, resulting in impaired vascular-healing capacity by EPCs (26). Ox-LDL and angiotensin II (AngII) increase the expression of NADPH oxidase subunit gp91phox in EPCs, and induced peroxynitrite formation via inhibition

of telomerase activity, which may accelerate EPC senescence (11,24,25). Antioxidation is an important mechanism by which RSV exerts its biological activity. It is reported that RSV attenuated the expression of NADPH oxidase subunit gp91phox, Ras-related C3 botulinum toxin substrate 1 and p47phox, and inhibited endothelial cells NADPH oxidase activation and the increase of ROS levels, which were induced by AngII, ox-LDL or homocysteine (26,27). In addition, RSV suppresses the production of O_2^-, promotes the expression of NO, and improves endothelium-dependent vasodilation via inhibition NADPH oxidase activation of thoracic aorta of diabetes mellitus mice (28). A previous study demonstrated that PPAR-γ agonist rosiglitazone can decrease the activity of NADPH oxidase of EPCs in

patients with diabetes, reduce the production of peroxides, improve the biological activity of NO, and improve the EPCs endothelial repair capacity (26), suggesting that PPAR-γ has an important influence on the activity of EPCs and oxidative damage. In a randomized, double-blind clinical trial, pioglitazone can enhance the number and function of EPCs in patients with coronary artery disease and normal glucose tolerance, and reduce NADPH oxidase, suggesting the effect of on EPCs may be independent from the hypoglycemic effect (29). Imanishi *et al* (30) demonstrated that pioglitazone inhibits AngII-induced expression of NADPH oxidase gp91phox, prevent telomerase inactivation and EPC senescence. Another previous study demonstrated that the PPAR-γ agonist telmisartan may also induce proliferation of human EPCs (31), indicating the effect of the PPAR-γ agonist on EPCs is not specific to thiazolidinediones. These results suggest that PPAR-γ is important in regulating the anti-oxidative effect of EPCs and maintaining cytoativity. In the present study, RSV activated the expression of PPAR-γ in a concentration-dependent manner, suggesting RSV prevents EPC senescence and enhances re-endothelialization of EPCs possibly via the activation of PPAR-γ.

It has been demonstrated that PPAR-α and PPAR-γ transcriptionally regulated the expression of HO-1, suggesting a mechanism for the anti-inflammatory and anti-proliferative effects of PPAR ligands via upregulation of HO-1 (32). HO-1 catalyzes hemoglobin into biliverdin and carbon monoxide, and it is a key line of defence against oxidative stress in the cardiovascular system (33). Previous studies have demonstrated that HO-1 inhibits the activity of NADPH oxidase, decreases the level of ROS, and improves endothelial function in endothelial cells of diabetic rats (34). A previous study demonstrated that RSV upregulated the expression of HO-1 in coronary artery endothelial cells and myocardial cells of rats, reduced the myocardial infarction area following anterior descending coronary artery ligation, and promoted the recovery of cardiac function; however, HO-1 antagonist tin protoporphyrin IX blocks these effects, suggesting HO-1 is important in the anti-oxidative effect of RSV (35,36). Deshane *et al* (37) reported that HO-1 mediated EPCs migration and angiogenesis induced by stromal cell-derived factor-1. Wu *et al* (38) demonstrated that HO-1 could increase the number of circulating EPCs, enhance the colony formation ability of EPCs, and promote re-endothelialization (38). All of these results indicated that HO-1 may be the downstream element of the regulation of EPCs oxidative reaction and cytoactivity by PPAR-γ. The present study also demonstrated that RSV increases the expression levels of PPAR-γ and HO-1, and the re-endothelialization of EPCs, which may indicate that RSV restores the cardiovascular repair capacity of EPCs via the PPAR-γ and HO-1 signaling pathways.

However, the current study has certain limitations. Firstly, the mechanism by which RSV activates the PPAR-γ and HO-1 signaling pathway was not illustrated clearly, further studies are required to elucidate the detailed underlying mechanisms. Secondly, type II diabetes and gene knockdown animal models are required to further confirm the effect of RSV. Animal models of type II diabetes will be used in future study by our laboratory to further investigate the effect of RSV.

In conclusion, RSV prevents EPCs from senescence and reduces the oxidative reaction of EPCs. PPAR-γ and HO-1 signaling pathways are activated by RSV and RSV also promotes the re-endothelialization capacity of EPCs.

Acknowledgements

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