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

XIAOHUA SHEN, MEIHUI WANG, XUKUN BI, JIEFANG ZHANG, SHAOXIANG WEN, GUOSHENG FU and LIANG XIA

Department of Cardiology, Biomedical Research (Therapy) Center, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang 310058, P.R. China

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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.

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 (PBMCs) 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). PBMCs 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

Correspondence to: Dr Liang Xia, Department of Cardiology, Biomedical Research (Therapy) Center, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, 3 East Qingchun Road, Hangzhou, Zhejiang 310058, P.R. China
E-mail: icewater20042005@aliyun.com

Abbreviations: RSV, resveratrol; EPC, endothelial progenitor cell; PBMCs, peripheral blood mononuclear cells; ECFCs, late outgrowth EPCs; SA-β-gal, senescence-associated β-galactosidase

Key words: resveratrol, senescence, endothelial progenitor cell, oxidative reaction
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$_2$DCF-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$_2$DCF-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 24 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 abovementioned instructions were used to determine NO production by EPCs. 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; iv) 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.
Rabbits were anesthetized with 35 mg/kg ketamine, and an anterior midline incision was made to expose the 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 and reinserted 3 times to injure 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 analysis 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
changes in ROS production, NADPH 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$_2$DCF-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).

**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 NADPH 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...
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, 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

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; RSV, 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.
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 cytotoxicity. 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.

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