

Resveratrol inhibits angiotensin II-induced proliferation of A7r5 cells and decreases neointimal hyperplasia by inhibiting the CaMKII-HDAC4 signaling pathway

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Received September 29, 2017; Accepted March 26, 2018

DOI: 10.3892/mmr.2018.9056

Abstract. Resveratrol has been reported to inhibit vascular smooth muscle cell proliferation and neointimal hyperplasia following arterial injury; however, the underlying mechanisms remain unclear. The present study was designed to investigate the effects of resveratrol on angiotensin II (AngII)-induced proliferation of A7r5 cells and explore the molecular mechanisms responsible for the observed effects. Resveratrol inhibited cell proliferation and migration, and decreased the AngII-induced protein expression of α -smooth muscle actin (α -SMA), proliferating cell nuclear antigen (PCNA) and cyclin-dependent kinase 4 (CDK4). Resveratrol inhibited AngII-induced activation of intracellular Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) and histone deacetylases 4 (HDAC4), as well as blocking AngII-induced cell cycle progression from the G_0/G_1 to S-phase. *In vivo*, 4-weeks of resveratrol treatment decreased the neointima area and the neointima/media area ratio in rats following carotid balloon injury. Resveratrol also inhibited the protein expression of total and phosphorylated CaMKII and HDAC4 in the injured arteries. In conclusion, the present study demonstrated that resveratrol attenuated AngII-induced cell proliferation and neointimal hyperplasia by inhibiting the CaMKII-HDAC4 signaling pathway. These findings suggest that resveratrol may potentially prevent arterial restenosis.

Introduction

Coronary heart disease poses a major health problem worldwide. It is often caused by coronary atherosclerosis or by vasospasm stenosis or obstruction. Although there has been remarkable progress in basic and clinical research on coronary heart disease, patient outcome is still not optimal. For example, percutaneous coronary intervention (PCI) is an important therapeutic strategy to treat coronary heart disease. However, restenosis of the coronary arteries occurs in roughly 5-10% of individuals after stent placement (1). Two important vascular smooth cell processes-proliferation and migration-are the main factors that drive restenotic vascular remodeling as well as atherosclerosis after PCI (2). Therefore, there is an urgent need to develop novel approaches to prevent and treat coronary restenosis.

Resveratrol (3,5,4'-trihydroxy-trans-stilbene), a non-flavonoid polyphenolic compound (3), is considered a phytoalexin because it is produced by plants under stress conditions (4). Resveratrol has been shown to exert several beneficial effects in the treatment of cardiovascular diseases, including hypertension, atherosclerosis, ischemic heart diseases, heart failure, arrhythmia, and stroke (5). Resveratrol inhibits vascular smooth muscle cell (VSMC) proliferation (6-9) and neointimal hyperplasia after arterial injury (10,11), but the mechanism is not fully understood. The present study investigated the mechanism underlying resveratrol's inhibitory effects on VSMC proliferation and migration and neointimal hyperplasia in a carotid balloon injury model in rats.

More specifically, the present study was designed to gain better insight into resveratrol's potential effects on the Ca^{2+} /calmodulin-dependent protein kinase II and histone deacetylases 4 (CaMKII-HDAC4) pathway. CaMKII is a multimeric enzyme, and its activity is regulated by Ca^{2+} /calmodulin (CaM) binding, which activates its protein kinase activity and promotes intrasubunit autophosphorylation (12). CaMKII is strongly expressed in VSMCs (13) and is critical for VSMC proliferation (14), migration (15), and neointima proliferation after vascular injury (16,17). Interestingly, resveratrol prevents diabetes-induced retinal neuronal cell death via

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Key words: resveratrol, A7r5, neointimal hyperplasia, Ca^{2+} /calmodulin-dependent protein kinase II

downregulation of CaMKII (18). It also reduces the expression and phosphorylation of CaMKII induced by aortic banding in rats (19).

Histone deacetylases (HDACs) play a central role in the epigenetic regulation of gene expression (20). HDAC4 controls platelet derived growth factor-BB (PDGF-BB)-mediated increases in VSMC proliferation and migration (20). HDAC4 activity is regulated by CaMKII in various cell types (13,21).

Therefore, in the present study, we used *in vitro* and *in vivo* models to test the hypothesis that resveratrol's regulation of the CaMKII-HDAC4 pathway is critical for its inhibitory effects on VSMCs. Our study provides a rationale for the use of resveratrol to prevent and treat coronary restenosis.

Materials and methods

Cell culture. The VSMC line A7r5 was purchased from the Cell Bank of Chinese Academy of Sciences, Shanghai, China. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ at 37°C. Upon reaching 50–60% confluence, the cells were treated with angiotensin II (AngII) (1 µM) or resveratrol at various concentrations (10, 25, and 50 µM) dissolved in dimethyl sulfoxide (DMSO) alone or in combination. The concentrations used in the present study were based on the previous report (8).

Cell growth and proliferation assay. Cell growth was measured using the MTT assay. In brief, cells (10⁴ cells/well) were seeded and cultured in 96-well plates overnight. After treatment with AngII or resveratrol for indicated periods, the MTT solution (20 µl, 5 mg/ml) was added to each well and incubated at 37°C for 4 h; 150 µl of DMSO was then added. The absorbance at 490 nm was measured with a microplate reader. Our initial study showed that 50 µM resveratrol induced the greatest inhibition of cell growth; thus, this concentration was chosen for subsequent experiments.

The EdU incorporation assay was used to determine the cell proliferation rate. The cells (10⁴ cells/well) were cultured in 96-well plates, and then incubated with 50 nM of EdU for an additional 2 h at 37°C, fixed with 4% formaldehyde for 15 min at room temperature, and then treated with 0.5% Triton X-100 for 20 min at room temperature to permeabilize the cells. The cells were washed three times with phosphate buffered saline (PBS) and then incubated in a 1X Apollo reaction cocktail (100 µl/well) for 30 min. DNA was stained with 10 µg/ml Hoechst 33342 solution (100 µl/well) for 20 min and visualized with fluorescence microscopy. Five fields were randomly selected from each sample image; the EdU-positive cells were counted, and the relative positive ratio was calculated.

Cell migration assay. Cell migration was analyzed using the Transwell system (Corning, Inc., Corning, NY, USA), per the manufacturer's instructions. In brief, cells (10⁵/well) were seeded on the upper chamber with serum-free DMEM containing resveratrol (10, 25, and 50 µM) or DMSO. DMEM supplemented with AngII (1 µM) was added into the lower chamber. The apparatus was incubated for 8 h. The cells that had not migrated were removed from the top of the insert. Those cells that did migrate were fixed in methanol and then

stained with crystal violet (0.5%). The migrated cells were manually counted using phase-contrast microscopy.

Western blot analysis. Total protein was isolated using a protein extraction kit (Beyotime, Shanghai, China), according to the manufacturer's instructions. Protein concentration was quantified using the bicinchoninic acid protein assay (Beyotime). Equal amounts of protein from all samples were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% fat-free milk in Tris-buffered saline with Tween-20 (TBST) and then incubated with primary antibodies at 4°C overnight and subsequently incubated with secondary antibodies at room temperature for 1 h. The protein bands were detected using an enhanced chemiluminescence dependent detection system. The following dilutions were used for the primary antibodies: Anti-CaMKII antibody (1:1,000; SAB Biotherapeutics, Inc., Sioux Falls, SD, USA), anti-phosphorylated (p)-CaMKII antibody (1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-HDAC4 antibody (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-p-HDAC4 antibody (1:1,000; SAB Biotherapeutics, Inc.), anti- α -smooth muscle actin (α -SMA) antibody (1:1,000; Abcam, Cambridge, MA, USA), anti-proliferating cell nuclear antigen (PCNA) antibody (1:1,000; Cell Signaling Technology, Inc.), anti-cyclin-dependent kinase-4 (CDK4) antibody (1:1,000; Abcam), and anti- β -actin antibody (1:1,000; Cell Signaling Technology, Inc.).

Cell cycle analysis. Cells were cultured in DMEM without serum for 24 h to induce synchronization in G0-phase of the cell cycle. DMEM containing serum and resveratrol was then added to the cells to stimulate cell cycle progression. 48 h later, the cells were trypsinized, collected, and centrifuged at 1,250 x g for 5 min. To stain DNA, propidium iodide (PI) (50 µg/ml) was added at 37°C for 30 min; flow cytometry was used to analyze cell cycle.

Rat carotid artery balloon injury and treatment. All the experimental procedures involving animals were approved by the Animal Care and Use Committee of South China Agricultural University. All the animals were fed with a standard diet of rat chow and housed in a well-controlled environment (21–26°C; 40–70% humidity; 12/12-h light/dark cycle). Eighteen male Sprague-Dawley rats (weighing 400–450 g) were randomly divided into three groups: Sham, injury, and resveratrol-treated groups (n=6 for each group). The rats were anesthetized by an intraperitoneal injection of pentobarbital (30 mg/kg) before operation. Balloon injury of the left common carotid artery was done as previously published (22). Briefly, after an intravenous injection of heparin sodium (100 U/kg), the left common, external, and internal carotid arteries were exposed. Microvascular clips were used to temporarily block blood flow in the common and internal carotid arteries. We partially clipped the external carotid artery with microscissors at ~2 mm distal to the carotid bifurcation. A balloon angioplasty catheter (diameter=1.25 mm; length=10 mm; Boston Scientific, Marlborough, MA, USA) was placed in the

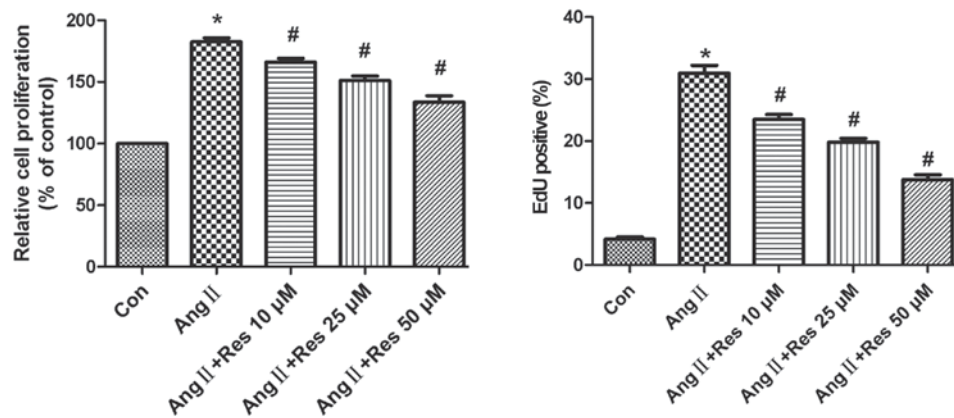


Figure 1. Inhibitory effect of resveratrol on AngII-induced cell proliferation. A7r5 cells were pre-treated with the indicated concentrations of resveratrol for 30 min prior to being stimulated with 1 μ M of AngII. MTT and EdU incorporation assays were performed to measure cell proliferation. Data are presented as the mean \pm standard error of the mean of 4 independent experiments. * P <0.05 vs. the control group; # P <0.05 vs. the AngII group. AngII, angiotensin II; EdU, 5-ethynyl-2'-deoxyuridine; Con, control; Res, resveratrol.

common carotid artery by way of the external carotid artery. The balloon was then inflated to create moderate resistance and pulled back and forth three times. After the catheter was removed, the external carotid branch was ligated and blood flow in the common and internal carotid arteries was restored. The same surgical procedure was applied to the sham group, but without balloon insertion. The rats in the resveratrol group were treated with 2 ml resveratrol (50 mg/kg/day) by gavage for 4 weeks. The sham and injury control groups were treated with the same volume of physiological saline.

Morphometric analysis. Rats were anesthetized with pentobarbital (200 mg/kg) prior to sacrifice. We carefully dissected the injured carotid arteries and then fixed them in 4% paraformaldehyde. Three cross-sections, 6- μ m in thickness, were cut from each sample at the approximate middle of the injured artery, stained with hematoxylin and eosin (H&E), and observed under a microscope. The internal and external elastic lamina, lumen, neointimal, and medial areas were analyzed by Image-Pro Plus 6.0 software, and the ratio of intima/media areas for each sample was calculated.

Statistical analysis. Data are expressed as mean \pm standard error of the mean. We used one-way analysis of variance (ANOVA) to analyze differences between groups, with Tukey post hoc test when necessary. P <0.05 was considered to indicate a statistically significant difference. All data and statistical analyses were performed using the SPSS Statistical software (version 17.0; SPSS, Inc., Chicago, IL, USA).

Results

Resveratrol inhibits AngII-induced cell proliferation. To assess the inhibitory effects of resveratrol on AngII-stimulated cell proliferation, serum-starved A7r5 cells were pre-treated with various concentrations of resveratrol for 30 min and then incubated with AngII (1 μ M) for 24 h. As shown in Fig. 1, the results from the MTT assay indicated that AngII induced growth of A7r5 cells, which was significantly prevented by resveratrol treatment in a concentration-dependent manner. The results from the EdU incorporation assay confirmed

that AngII significantly increased the amount of DNA, an index of increased cell proliferation. Furthermore, the results showed that resveratrol significantly inhibited cell proliferation in a concentration-dependent manner. These data suggest that resveratrol exerts a potent anti-proliferative effect on AngII-induced cell proliferation.

Resveratrol attenuates AngII-induced cell migration. In the present study, cell migration was measured using the Transwell chamber assay. As shown in Fig. 2, AngII stimulated cell migration, compared to the unstimulated control group, and resveratrol significantly inhibited migration in a concentration-dependent manner.

Resveratrol prevents AngII-induced S-phase entry. As shown in Fig. 3, pretreatment with resveratrol (50 μ M) increased the number of cells arrested in G₀/G₁, accompanied by a concurrent decrease in the number of cells in S-phase, compared to the AngII-treated group. These results indicate that resveratrol inhibits AngII-induced G₁-S progression. These results also suggest that resveratrol may prevent AngII-induced S-phase entry in A7r5 cells as a mechanism for inhibiting cell growth, as observed above.

Resveratrol upregulates α -SMA and suppresses CDK4 and PCNA protein expression. To investigate the underlying mechanism of resveratrol's inhibitory effect on AngII-induced cell proliferation, α -SMA, CDK4, and PCNA protein expression were measured by western blot analysis. As shown in Fig. 4, stimulation with AngII (1 μ M) for 24 h decreased α -SMA protein expression, compared to the control group. Pretreatment with various concentrations of resveratrol for 30 min prior to AngII stimulation reversed this effect in a concentration-dependent manner, compared to the AngII-stimulated group. AngII-induced CDK4 and PCNA expression was also significantly inhibited by resveratrol in a concentration-dependent manner compared to the control.

The CaMKII-HDAC4 pathway is involved in resveratrol's inhibition of cell proliferation and migration. To examine the

potential role of the CaMKII-HDAC4 pathway in resveratrol's inhibition of AngII-induced proliferation and migration, A7r5 cells were pretreated with resveratrol for 30 min and then stimulated with AngII (1 μ M) for another 30 min. AngII stimulation significantly increased p-CaMKII levels, which was reversed by resveratrol (Fig. 5). AngII similarly increased HDAC4 activation, which was also reversed by resveratrol. No changes in total CaMKII or HDAC4 protein expression were detected among the three groups.

Resveratrol attenuates neointimal hyperplasia in balloon-injured arteries. To investigate the effect of resveratrol on neointimal hyperplasia, rats were treated with resveratrol (50 mg/kg/day) by gastric gavage at 3 days before balloon injury and 28 days after balloon injury. As shown in Fig. 6, significant neointimal hyperplasia was observed 28 days after balloon injury. The rats treated with resveratrol showed a remarkable reduction in neointimal hyperplasia, and the ratio of intima/media areas was significantly decreased by over 64%, compared to the injured group.

The CaMKII-HDAC4 pathway is involved in the inhibitory effect of resveratrol on balloon injury. To explore the possible effect of resveratrol on the CaMKII-HDAC4 pathway after balloon injury, total CaMKII, p-CaMKII, total HDAC4, and p-HDAC4 were measured in tissue samples using western blot analysis. Total CaMKII, p-CaMKII, total HDAC4, and p-HDAC4 protein levels were all increased after balloon injury compared to the sham group, and the effect on protein expression was reversed by resveratrol treatment (Fig. 7).

Discussion

The major finding of this study is that resveratrol attenuated AngII-induced VSMC proliferation and migration via inhibiting CaMKII-HDAC4 signaling, which also resulted in decreased neointimal formation in balloon-injured carotid arteries.

Changes in VSMC proliferation and migration are largely reported to contribute to neointimal hyperplasia and restenosis after angioplasty (23). It has been demonstrated that environmental factors, such as PDGF, endothelin, transforming growth factor, and inflammatory mediators, can induce synthetic smooth muscle cell phenotypic differentiation (24). AngII is a well-documented activator of VSMC proliferation and migration, which promotes atherosclerosis and vessel stenosis (25-27). Here, we showed that resveratrol inhibited AngII-induced proliferation and migration in A7r5 cells. These results are consistent with previous studies that demonstrated the anti-proliferative effects of resveratrol on VSMC proliferation following serum (24) or PDGF-BB (28) stimulation.

Our results indicate that resveratrol's inhibitory effect on AngII-induced cell proliferation might be associated with its effects on α -SMA, PCNA, and CDK4 expression. α -SMA is a marker of VSMC differentiation, PCNA is an indicator of cell proliferation, and CDK4 plays a role in cell cycle regulation. The Cyclin D/Cdk4 complex is integral for cell cycle progression from G1 to S phase. Our results demonstrated that resveratrol increased α -SMA expression and decreased PCNA

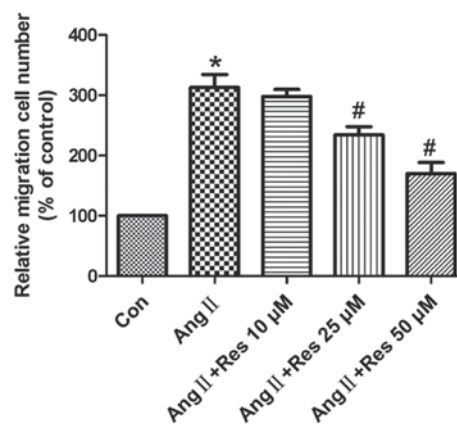


Figure 2. Inhibitory effect of resveratrol on AngII-induced cell migration. Following incubated with the indicated concentrations of resveratrol for 30 min, A7r5 cells were stimulated with 1 μ M of AngII. The cell migration rate was determined using the Transwell chamber assay. Data are presented as the mean \pm standard error of the mean of 4 independent experiments. *P<0.05 vs. the control group; #P<0.05 vs. the AngII group. AngII, angiotensin II; Con, control; Res, resveratrol.

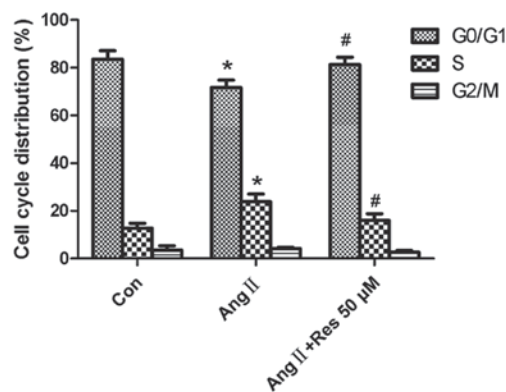


Figure 3. Inhibitory effect of resveratrol on AngII-induced cell G1-S progression. Following incubated with 50 μ M of resveratrol, A7r5 cells were stimulated with 1 μ M of AngII. Cell cycle was analyzed by flow cytometry. Data are presented as the mean \pm standard error of the mean of 4 independent experiments. *P<0.05 vs. the control group; #P<0.05 vs. the AngII group. AngII, angiotensin II; Con, control; Res, resveratrol.

and CDK4 expression in A7r5 cells stimulated by AngII, suggesting that resveratrol inhibits cells proliferation through regulating α -SMA, PCNA, and CDK4.

The CaMKII-HDAC4 pathway plays an important role in VSMC proliferation and migration. It has been shown that resveratrol influences CaMKII activity in various vascular diseases, such as diabetes-induced retinal neuronal cell death (20) and aortic banding in rats (21). In the present study, we demonstrated that resveratrol inhibited AngII-induced phosphorylation of CaMKII and HDAC4, suggesting that resveratrol inhibits A7r5 proliferation, and migration maybe mediated by the CaMKII-HDAC4 pathway.

In the present study, we attempted to confirm the above *in vitro* results with *in vivo* experiments. Neointimal hyperplasia is a common pathological process in vascular diseases like atherosclerosis and restenosis, and is largely driven by increased VSMC proliferation. We further demonstrated that resveratrol decreased neointima

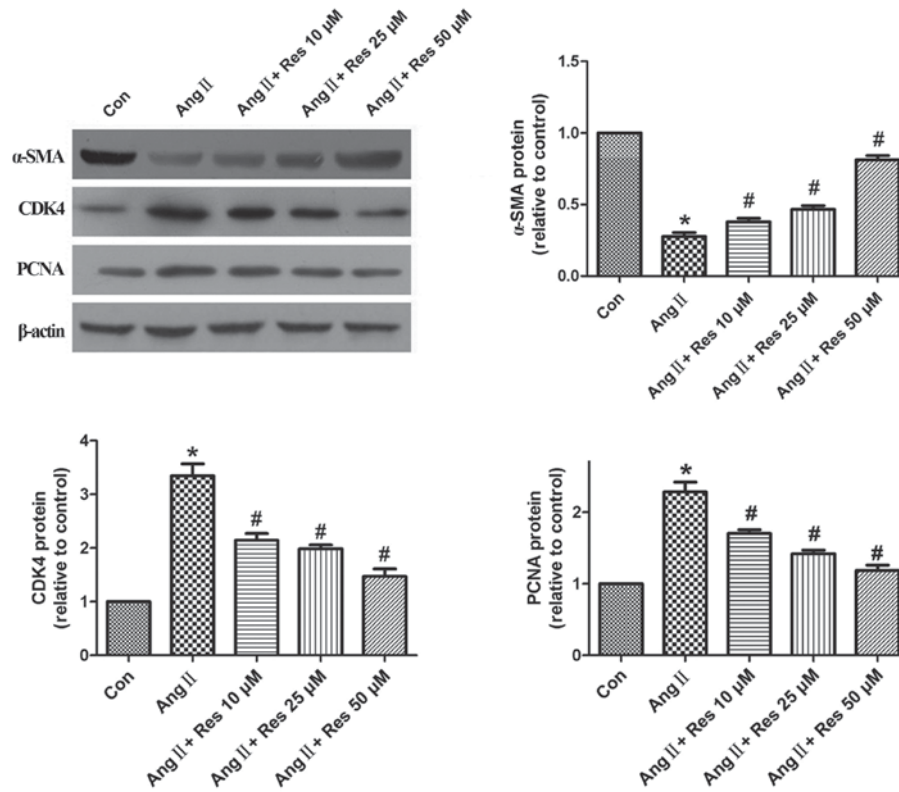


Figure 4. Effect of resveratrol on α -SMA, CDK4 and PCNA protein expression following AngII stimulation. A7r5 cells were pretreated with various concentrations of resveratrol for 30 min and then stimulated with 1 μ M AngII for 24 h. α -SMA, CDK4 and PCNA protein levels were detected by western blot analysis. Data are presented as the mean \pm standard error of the mean of 3 independent experiments. * P <0.05 vs. the control group; # P <0.05 vs. the AngII group. AngII, angiotensin II; Con, control; Res, resveratrol; SMA, smooth muscle actin; CDK, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen.

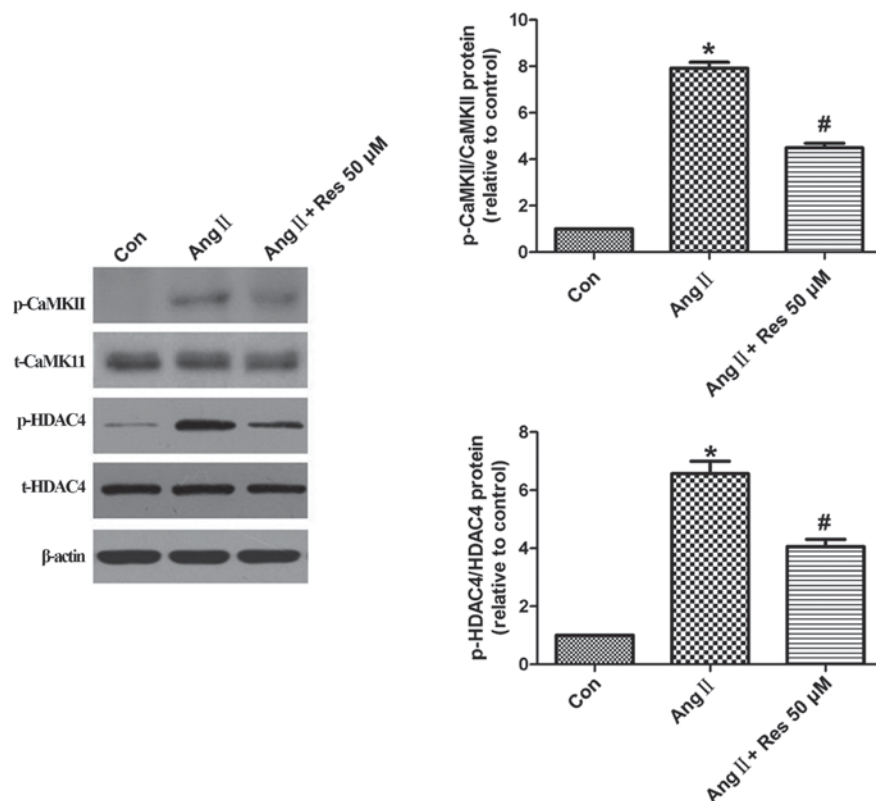


Figure 5. Effect of resveratrol on the CaMKII-HDAC4 signaling pathway. A7r5 cells were pretreated with various concentrations of resveratrol for 30 min and then stimulated with 1 μ M of AngII for 30 min. Total CaMKII, p-CaMKII, total HDAC4 and p-HDAC4 were detected by western blot analysis. Data are presented as the mean \pm standard error of the mean of 3 independent experiments. * P <0.05 vs. the control group; # P <0.05 vs. the AngII group. AngII, angiotensin II; Con, control; Res, resveratrol; p-, phosphorylated; t-, total; CaMKII, Ca^{2+} /calmodulin-dependent protein kinase II; HDAC4, histone deacetylases 4.

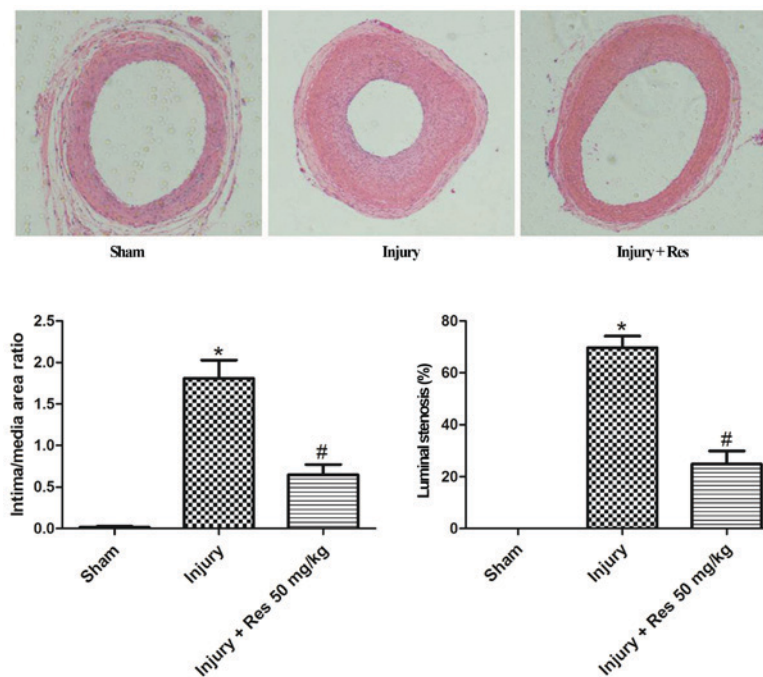


Figure 6. Inhibitory effect of resveratrol on neointimal hyperplasia induced by balloon injury. Rats were treated with resveratrol (50 mg/kg/day) by gastric gavage at 3 days before balloon injury and 28 days following balloon injury. The ratio of intima area to media area was analyzed by H&E staining. Magnification, $\times 100$. Data are presented as the mean \pm standard error of the mean from 6 rats. * $P < 0.05$ vs. the sham group; # $P < 0.05$ vs. the injury group. Con, control; Res, resveratrol.

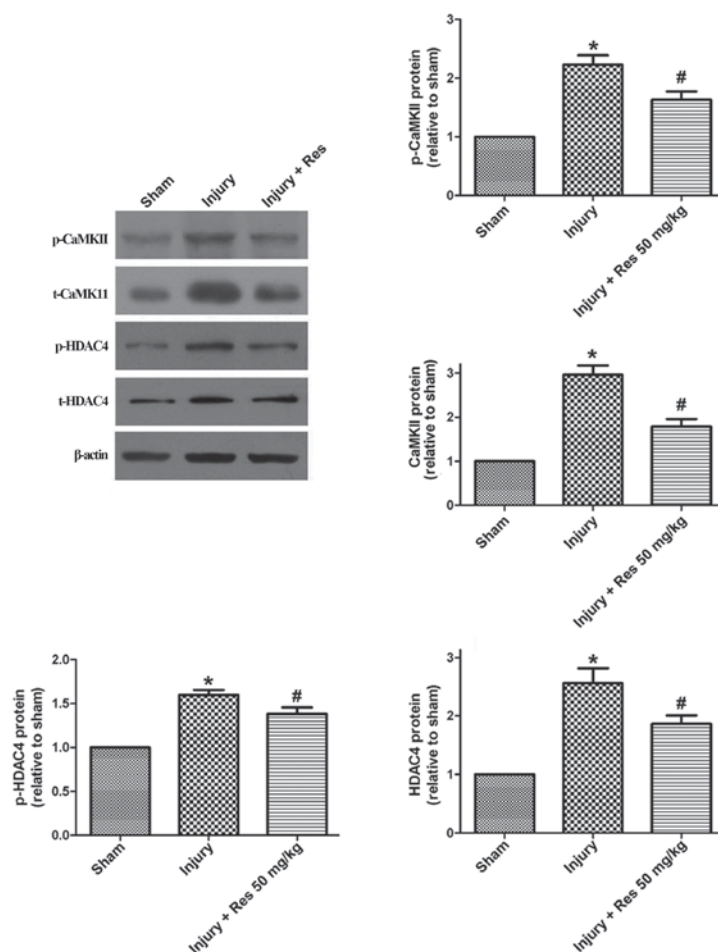


Figure 7. Inhibitory effect of resveratrol on the CaMKII-HDAC4 signaling pathway following balloon injury. Rats were treated with resveratrol (50 mg/kg/day) by gastric gavage at 3 days prior to 28 days following balloon injury. Total and p-CaMKII, and total and p-HDAC4 protein levels were measured by western blot analysis. Data are presented as mean \pm standard error of the mean of 4 independent samples. * $P < 0.05$ vs. the sham group; # $P < 0.05$ vs. the injury group. Res, resveratrol; p-, phosphorylated; t-, total; CaMKII, Ca^{2+} /calmodulin-dependent protein kinase II; HDAC4, histone deacetylases 4.

formation and the ratio of intima to media at 4 weeks after surgery. Meanwhile, resveratrol decreased expression of total and phosphorylated CaMKII and HDAC4 in injured arteries. In summary, our findings suggest that resveratrol may attenuate neointimal hyperplasia via regulating the CaMKII-HDAC4 pathway.

Our study demonstrated that resveratrol inhibits VSMC cell proliferation and migration *in vitro*, as well as decreases neointimal hyperplasia *in vivo* through regulating the CaMKII-HDAC4 pathway. The present study supports future development of resveratrol as a therapeutic agent for the treatment of proliferative vascular diseases, such as atherosclerosis and restenosis after angioplasty.

Acknowledgements

The animal experimental procedures were performed at the South China Agricultural University (Guangzhou, China). The authors would like to thank the Animal Use and Care Committee of South China Agricultural University for reviewing and approving the experimental protocols and monitoring the study for animal welfare issues. The authors would also like to thank the professional staff in the animal center of the University for their excellent assistance during the study.

Funding

The present study was supported by the Medical and Health Science and Technology Project of Guangzhou (grant no. 20161A011072), the Science and Technology Planning Project Of Guangdong Province (grant no. 2013B021800179), The Key Medical Disciplines and Specialties Program of Guangzhou (2017-2019), The Young Innovation Talents Project from The Department of Education of Guangdong Province (grant no. 2016KQNCX130) and The National Natural Science Foundation of China (grant no. 81570259).

Availability of data and materials

All the data generated or analyzed during this study have been included in this published article. Original data can be verified upon request.

Authors' contributions

XL and SL conceived and designed the experiments and wrote the paper. XL, JZ, PM, CC, CL and QH performed the experiments. BL and WO analyzed the data.

Ethics approval and consent to participate

All of the experimental procedures involving animals were approved by the Animal Care and Use Committee of South China Agricultural University (Guangdong, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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