Resveratrol Attenuates Oxidative Stress Induced by Balloon Injury in the Rat Carotid Artery Through Actions on the ERK1/2 and NF-Kappa B Pathway

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Key Words
Resveratrol • Neointimal hyperplasia • Oxidative stress • Vascular smooth muscle cells • Nuclear factor-kappa B • Extracellular regulated protein kinase

Abstract

\textbf{Background/Aim:} Oxidative stress plays a critical role in pathogenesis of the neointimal arterial hyperplasia. The aim of the study was to evaluate effects of resveratrol (RSV) on the vascular hyperplasia stimulated by oxidative damage. \textbf{Methods:} Balloon vascular injury was induced in rats that were intraperitoneally exposed to resveratrol (1 mg/kg) on 7 or 14 days after surgical procedure. Animals were euthanized on 7 or 14 days after operation. The blood level of 8-iso-prostaglandin $\alpha_2$, arterial morphology as well as expression of monocyte chemotactic protein-1 and interleukin-6 in carotid wall were measured. Vascular smooth muscle cells (VSMCs) were isolated from the thoracic aorta. Cellular proliferation and migration assays, reactive oxygen species (ROS), superoxide dismutase (SOD) and NADPH oxidative activity, protein level of $\beta$-actin, histone H3, NF-$\kappa$B p65, I$\kappa$B, ERK1/2, phospho-ERK1/2, phospho-p38 as well as NF-$\kappa$B transcription activity were evaluated in-vitro after angiotensin II stimulation and resveratrol (50-200 µmol/L) treatment. \textbf{Results:} Significant decreases in neointimal/medial area, serum prostaglandin level and genes expression were found in rats treated with resveratrol, when compared to the control group. Significant changes were also revealed for proliferation and migration rates, ROS level, as well as SOD, NADPH oxidase, ERK1/2 phosphorylation and NF-$\kappa$B transcriptional activity in cell cultures exposed to highest dose of resveratrol. Insignificant changes were also observed for NF-$\kappa$B p65 translocation and I$\kappa$B degradation, p38 phosphorylation in MAPK pathway. \textbf{Conclusion:} Resveratrol significantly suppressed the neointimal hyperplasia after balloon injury through inhibition of oxidative stress and inflammation by blocking the ERK1/2/NF-$\kappa$B B pathway.
Introduction

Coronary heart disease is the major cause of death in developed countries [1]. Percutaneous coronary intervention (PCI) is an important means of clinical treatment for coronary heart disease, but the higher incidence of restenosis after surgery has a serious impact on the long-term benefits [2]. While the use of drug-eluting stents has lowered the in-stent restenosis rates, the clinical restenosis incidence remains at 5%-10% [3]. In the United States alone, more than 20 million patients require repeated revascularization each year due to restenosis [4]. Thus, understanding the mechanisms that induce restenosis has important clinical significance and will enable the development of better therapeutic options.

The restenosis rate is regulated by multiple complex pathological processes, including the natural biological response, the effect of arterial contributions, the type of stent used, and implantation factors [5]. Implantation factors include incomplete stent expansion, geographic issues, and deployment of agents in the clot-laden arterial segment. Oxidative stress induced by excessive reactive oxygen species (ROS) generation after vascular injury plays an important role in the initiation and development of restenosis [6]. Excessive ROS generation accelerated vascular remodeling after artery injury related with promotion of migration and proliferation of medial VSMCs [7]. In a rabbit iliac artery injury model, ROS levels increased 20 times immediately after injury and remain more than 5 times increased at 7 days after injury [8]. Neointimal formation could be induced by an early injection with oxidative glutathione or cystine and could be inhibited with anti-oxidants [9-11]. A variety of anti-oxidant gene therapy strategies have shown success in lowering restenosis rates in the clinic [6]. These results suggest that excessive ROS production plays a critical role in vascular pathological changes. Antagonizing oxidative stress may be an optimal strategy to inhibit vascular post-angioplasty restenosis and improve the physiological functions after vessel injury. Resveratrol, as a non-flavonoid polyphenol compound, has anti-tumor, anti-inflammatory and anti-oxidant pharmacological effects [12]. Resveratrol significantly attenuates ROS generation induced by a variety of stimuli, inhibits inflammation processes and facilitates intracellular expression of several anti-oxidant enzymes [13, 14]. However, the possible roles and molecular mechanisms of resveratrol to inhibit oxidative stress after vascular injury have not yet been determined.

Angiotensin II (Ang II), a multifunctional hormone involved in cell growth, apoptosis, migration, inflammation, and fibrosis [15, 16], is robustly stimulated after vascular injury [17, 18]. In the current study, we hypothesized that resveratrol would antagonize vascular remodeling induced by excessive ROS after artery injury. This hypothesis was tested by using a carotid artery balloon injury model in rat and mechanisms of resveratrol actions were explored in Ang II-stimulated VSMCs.

Materials and Methods

Balloon injury model of rat carotid artery

All animals used in this study were provided and cared by Animal Center of Renmin Hospital of Wuhan University. The experimental procedures and animal care were approved by the Animal Care and Use Committee of Wuhan University. All animals were given the conventional diet until sacrificed. Sixty male Sprague-Dawley rats weighing 350-400 g were randomly divided into five groups (n=12 rats in each group). The groups were: 1) sham group; 2) 7 days saline group; 3) 7 days resveratrol group; 4) 14 days saline group; and 5) 14 days resveratrol group.

The animals were anesthetized with 2% sodium pentobarbital (Sigma, USA) at a dose of 40 mg/kg (intraperitoneal injection). The superficial fascia and muscles were separated bluntly layer by layer, until the left common carotid artery, internal carotid, and external carotid artery were exposed. After intravenous injection of 100 U/kg of heparin sodium, a balloon catheter (balloon diameter 1.25 mm, balloon length 15 mm; Medtronic, USA) was introduced into the common carotid through the external carotid artery cut under temporary occlusion of the internal and common carotid arterial blood flow. The balloon was
inflated and dragged back and forth with rotation three times. The sham group was exposed to the surgery without artery injury. The resveratrol group was given resveratrol (Sigma, USA) at a dose of 1 mg/kg/d by intraperitoneal injection, beginning from one day after surgery. Rats in the saline group received an equal volume of phosphate buffered saline (saline) as the control.

**Histomorphological detection**

After 7 or 14 days, rats were sacrificed and the injured and control common carotid arteries were collected. An approximately 2 cm long segment of the vessel was fixed in 4% paraformaldehyde and embedded in paraffin. Hematoxylin and eosin stained sections were observed by light microscopy. The blood vessel lumen and neointimal and medial area were analyzed using image analysis software (Image Pro Plus 6.0), and the intima/media (I/M) ratios were calculated.

**Quantitative real-time RT-PCR assay**

Total RNA was extracted from common carotid arteries following 7 days after injury using the picopure RNA isolation kit (Applied Biosystems, USA). After purification, the isolated RNA was reverse transcribed into cDNA using first-strand synthesis system (Invitrogen, USA). The cDNA was added to the ABI Prism 7500 sequence detection system (PE Applied Biosystems, USA) with primer sequences and Sybgreen supermix kit (Bio-Rad, USA) according to manufacturer’s instruction. The PCR cycling condition included pre-denaturing at 95°C for 5 min, 40 cycles of denaturation at 95°C, annealing at 60°C, and extension at 72°C for 30s each. GAPDH was used for normalization and amplification results were analyzed by the 2^-∆∆Ct method. The primer sequences in this study are shown in Table 1.

**ELISA assay**

Blood samples were collected when rats were sacrificed. After centrifugation at 3000 rpm for 10 min, the serum was isolated and used to determine the level of 8-iso-PGF2α using a competitive enzyme-linked immunoassay kit (Cell Biolabs, USA) according to manufacturer’s instruction.

**Cell culture**

Primary VSMCs were isolated from the thoracic aorta of male Sprague-Dawley rats (180-200g; n=3) as described previously [19]. When the cells reached 80-90% confluence, primary cells were passaged and grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Hycolon, USA). Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells at passages 3-5 were used for these experiments.

**Cell proliferation assay**

Cell proliferation was assessed using the CCK-8 kit (Dojindo Laboratories, Japan). In each well of a 96-well plate, 8,000 VSMCs were seeded and cultured for 12 h in 10% FBS. After synchronization with DMEM containing 0.5% FBS for 24 h, VSMCs were pre-treated with resveratrol at three different concentrations (50, 100, 200 µmol/L) for 30 min and stimulated with 1 µmol/L Ang II (Sigma, USA) for 24 h. Following CCK-8 treatment, OD values were determined at 450 nm using a microplate spectrophotometer (Bio-Rad, USA).

**Cell migration assay**

Cell migration was measured in a 24-well transwell chamber (Corning, USA). In brief, 1x10⁵ cells were seeded into the upper chamber that contained 200 µL serum-free DMEM and 200 µmol/L resveratrol. The lower chamber was filled with 600 µL DMEM with 1 µmol/L Ang II. After 8 h, cotton swabs were used to remove cells remaining in the upper chamber. The lower surface was fixed in methanol for 15 min and
stained with 0.1% crystal violet for 8 min. The filter was washed 3 times, and the number of cells on the upper surface of the filter was quantified by microscopic evaluation. The total number of cells was measured in 5 random fields at a magnification of x100.

Reactive oxygen species detection

The level of intracellular reactive oxygen species was evaluated with the fluorescent probe DCFH-DA (Beyotime, China). VSMCs were seeded onto a 24-well plate at a density of 1.5x10^5 cells per well. After synchronization for 24 h, the cells were pre-treated with 200 µmol/L resveratrol 30 min and stimulated with 1 µmol/L Ang II for 1 h. The fluorescent probe DCFH-DA was added to each well at a final concentration of 10 µmol/L and incubated at 37°C for 20 min. The plate was washed 3 times with saline and immediately photographed under fluorescent microscopy. Image pro plus 6.0 was used to measure mean density between the groups.

Determination of NADPH oxidase activity

NADPH oxidase activity was estimated by the lucigenin chemiluminescent method as described previously [20, 21]. The cells were pre-treated with 200 µmol/L resveratrol for 30 min followed by stimulation with 1 µmol/L Ang II for 1 h. The VSMCs were collected and incubated on ice in 500 µL cell lysis buffer (1 mM EDTA, 10 mg/ml aprotinin, 0.5 mg/ml leupeptin, 0.7 mg/ml pepstatin, and 0.5 mM PMSF, pH=7.0). After centrifugation at 14000 rpm for 5 min, 200 µl of the supernatant is added to 800 µl buffer (1 mM EDTA, 150 mM sucrose, 100 mM lucigenin, and 100 mM NADPH, pH=7.0; Sigma, USA). Luminescence was measured immediately, and the NADPH oxidase activity was expressed as each second luminous value per 1 mg of protein.

Superoxide dismutase activity assay

Superoxide dismutase (SOD) activity was assessed by the oxidase system (Cell Biolabs, USA). Cells were incubated with resveratrol and Ang II as described above and protein was extracted in lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 0.5% Triton-100). After centrifugation at 12000 g for 10 min, 70 µL supernatant of each sample was tested according to manufacturer’s instruction.

Immunoblotting analysis

Immunoblotting was performed as previously described [22]. The nuclear and cytoplasmic proteins were sequentially isolated according to instructions of the protein extraction kit (Beyotime, China). Protein concentration was detected by the bicinchoninic acid protein assay (Beyotime, China). Total protein was separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to PVDF membranes. Blots were probed with antibodies against β-actin (Santa Cruz, USA), histone H3, NF-κB p65, IkB, ERK1/2, phospho-ERK 1/2 or phospho-p38 (CST, USA). Protein expression was identified with horseradish peroxidase-conjugated secondary antibody (Santa Cruz, USA) and ECL plus detection kit (Pierce, USA). Histone H3 and β-actin served as the internal loading controls.

Luciferase reporter gene assay

NF-κB transcription activity was determined by the luciferase reporter gene assay kit (Beyotime, China) as reported previously [23]. Cells at passages 3-5 were seeded in a 24-well plate at 2x10^5 cells per well. When the adherent cells reached 70% confluence, pGL6-NF-xB-Luc and pRL-TK plasmids were transfected into the VSMCs using Lipofectamine 2000 (Invitrogen, USA). After 6 h of transfection, cells were treated with resveratrol and Ang II as described above. Cells were collected and samples were processed. The luciferase activity was normalized to pRL-TK group and reported as a fold change compared to the control group.

Statistical analysis

Data was represented as mean±SD. All statistical analysis was performed with Statistical Product and Service Solutions 13.0 software (SPSS 13.0). Statistical analysis was performed by one-way ANOVA, followed by Student-Newman-Keuls post-test. A P<0.05 was considered as a statistically significant.
**Results**

**Resveratrol attenuated neointimal hyperplasia after balloon-injury**

Histomorphological results revealed that balloon injury induced significant intimal hyperplasia in the injured common carotid artery. VSMCs were disarrayed and the vessel lumen suffered a serious concentric or eccentric loss in the saline groups. Likewise, the neointimal hyperplasia was more severe at 14 days after surgery, compared to the 7 days time point. Compared to the saline groups, resveratrol dramatically suppressed neointimal formation and increased lumen area (Fig. 1A). In addition, the intimal/medial area ratio was reduced in resveratrol-treated arteries compared to the saline groups at both 7 and 14 days after injury (Fig. 1B).

**Resveratrol inhibited mRNA expression of MCP-1 and IL-6 in injured arteries**

In order to examine the effect of resveratrol on pro-inflammatory cytokine levels, monocyte chemotactic protein-1 (MCP-1) and interleukin-6 (IL-6) mRNA expression were measured in injured carotid arteries. After 7 days of injury, MCP-1 and IL-6 mRNA expression increased 3-fold and 1.59-fold in the saline group compared to the sham group, whereas resveratrol prevented this increase and actually decreased the MCP-1 and IL-6 mRNA expression by 0.70-fold and 0.52-fold compared to the sham group (Fig. 2).

**Resveratrol reduced the serum levels of 8-iso-PGF2α after vessel injury**

After 7 and 14 days of balloon injury, serum concentrations of 8-iso-PGF2α were markedly increased in saline groups, and resveratrol reduced these levels (Table 2).

**Resveratrol inhibited VSMCs proliferation and migration induced by Ang II**

Proliferation was markedly induced in VSMCs treated with 1 µmol/L Ang II for 24h, compared to the unstimulated control group (Fig. 3). Pre-treatment with resveratrol at three
different concentrations for 30 min all showed decreased proliferation rates compared to the Ang II stimulated group, with the maximal effect seen at a dose of 200 µmol/L. Consisted with the results of the CCK-8 kit, the total cell numbers counted in five random sections increased after stimulation with 1 µmol/L Ang II for 8h (Fig. 4). Resveratrol at the dose of 200 µmol/L significantly suppressed the migratory effects induced by Ang II.

Table 2. Resveratrol reduced 8-iso-PGF2α levels in serum induced by arterial injury. Values are expressed as mean±SD; n=12 per group. *P<0.05 vs. the sham group, **P<0.05 vs. the saline group.

<table>
<thead>
<tr>
<th>Group</th>
<th>7 days</th>
<th>14 days</th>
</tr>
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<tbody>
<tr>
<td>Sham group (pg/ml)</td>
<td>51.43±26.67</td>
<td>51.43±26.67</td>
</tr>
<tr>
<td>Saline group (pg/ml)</td>
<td>465.01±119.38</td>
<td>558.18±160.95*</td>
</tr>
<tr>
<td>RSV group (pg/ml)</td>
<td>67.97±39.86**</td>
<td>141.80±108.29**</td>
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Fig. 3. Effect of resveratrol on VSMC proliferation induced by Ang II. (R1): 50 µmol/L resveratrol pre-treatment, (R2):100 µmol/L resveratrol pre-treatment, and (R3): 200 µmol/L resveratrol pre-treatment. Three independent experiments were done and the data are expressed as mean±SD. * * P<0.05 vs. the Ang II stimulated group, * P<0.05 vs. the control unstimulated group.

Fig. 4. Effect of resveratrol on VSMC migration induced by Ang II. Total cell number was calculated at a magnification of x100 in 5 random fields. All values are expressed as mean±SD of three separate experiments. * P<0.05 vs. the control unstimulated group, ** P<0.05 vs. the Ang II group.

Fig. 5. Effect of resveratrol on intracellular reactive oxygen species induced by Ang II. (A) Representative fluorescent images in each group (x 100 magnifications). (B) Fluorescent mean density measured with Image pro plus 6.0. Values are expressed as mean±SD; n=3 per group. * P<0.05 vs. the control unstimulated group, ** P<0.05 vs. the Ang II group.
Zhang/Chen/Yang/Xu/Pu/Ding/Jiang: Resveratrol Inhibits Neointimal Formation

Resveratrol suppressed generation of intracellular reactive oxygen species induced by Ang II

Intracellular ROS production in VSMCs was remarkably increased in the presence of Ang II for 1 h (Fig. 5A). However, resveratrol pre-treatment showed a significant decrease in ROS generation. In VSMCs treated with 200 µmol/L resveratrol, the fluorescence intensity decreased approximately 70% of the Ang II group levels (Fig. 5B). In accordance with the ROS results, NADPH oxidase activity was down-regulated and SOD activity was markedly elevated by resveratrol (Fig. 6).

Resveratrol inhibited ERK1/2 phosphorylation induced by Ang II

Compared to the control group, the relative protein expression of phospho-ERK increased by 3.3-fold in the Ang II-treated group. Resveratrol exerted a distinct inhibitory influence on ERK1/2 phosphorylation. In cells pre-treated with 200 µmol/L resveratrol,
the phospho-ERK relative expression decreased by 61%. No changes in total ERK1/2 or phospho-p38 protein expression were observed among the three groups (all \( p = \) not significant; Fig. 7).

Resveratrol suppressed Ang II-induced NF-\( \kappa \)B transcriptional activity

Ang II strongly promoted NF-\( \kappa \)B luciferase activity, showing a 4.4-fold increase versus the basal activity of unstimulated cells. Resveratrol attenuated this increase, increasing only 2.2-fold compared to unstimulated cells (Fig. 8). Resveratrol had no effect on Ang II-induced NF-\( \kappa \)B translocation or \( \kappa \)B degradation (Fig. 9).

Discussion

In the present study, we have shown that resveratrol could attenuate oxidative damage and inflammation induced by vascular endothelial injury. The major findings of this study were that resveratrol 1) attenuated the oxidative damage stimulated by vessel injury and inhibit neointimal hyperplasia; 2) inhibited Ang II-induced VSMCs proliferation, migration,
and excessive ROS generation; and 3) inhibited ERK1/2 phosphorylation and NF-κB transcriptional activity in VSMCs stimulated by Ang II. These results indicate that resveratrol may be a therapeutic option for vessel hyperplasia therapy.

Medial VSMCs produce a large amount of ROS under various stimuli, including inflammatory factors, cytokines, and hemodynamic alterations, all of which are induced following vessel injury [24]. Excessive ROS activates quiescent VSMCs to proliferate and migrate from the medial to the intimal. VSMCs also release matrix metalloproteinases and inflammatory factors, which initiate neointimal formation [25-27]. The results in our study agree with previous research that showed 8-iso-PGF2α, a biological indicator of oxidative damage in vivo [28], significantly increased after balloon injury, which is accompanied by increases in MCP-1 and IL-6.

Resveratrol suppresses the inflammatory response, attenuates excessive ROS generation and blocks the cross-talk between inflammation and oxidative stress in several diseases and individual cell types [29-31]. It is, however, still unclear whether resveratrol has similar inhibitory capacity on VSMCs. In VSMCs, NADPH oxidase (NOX) is the major contributor of ROS generation [6]. NOX oxidizes NADPH to NADP+ with the concomitant generation of peroxides, including H₂O₂ [32]. In contrast, SOD serves as a crucial anti-oxidant enzyme that plays an important role in scavenging oxygen free radicals and protecting tissue from ROS-induced oxidative stress injury [33]. Under normal circumstances, NOX and SOD are maintained in dynamic balance, as the excessive activation of NOX and consumption of SOD synergistically stimulate ROS generation. In this study, we found that resveratrol inhibited the excessive activation of NOX and enhanced the SOD activity. Thus, the increase of ROS production induced by Ang II was dually antagonized by resveratrol.

Endogenous ROS serves as a second messenger in the cascading effect of multiple signaling pathways [34]. Similar to protein tyrosine kinase, JNK, p38/MAPK, ERK5 and ERK1/2 MAPK kinase are all ROS-sensitive regulators [35, 36]. We found that resveratrol treatment reduced ERK1/2 phosphorylation without affecting total ERK expression. Likely, the downstream transcription factors c-fos and Elk-1 were suppressed, and the cell cycle was maintained in the quiescent stage.

Interestingly, there were no statistical differences in p38/MAPK phosphorylation detected among the experimental groups. It has not been clear whether Ang II can activate p38 and resveratrol can regulate the p38/MAPK pathway. Haider et al. [37] demonstrated that resveratrol did not decrease the phospho-p38 expression stimulated by Ang II. However, Kim et al. [38] has proposed that resveratrol can attenuate p38/MAPK phosphorylation to disturb high-glucose stimulatory effects on human vein endothelial cells. These investigations imply that the ability of resveratrol to regulate MAPK kinase is dependent on cell type, drug dose, and stimulus type, to name a few.

Previous studies have recognized that NF-κB is another important ROS-sensitive transcription factor, and it plays a critical role in cross-talk between oxidative stress and inflammation [39, 40]. NF-κB binds to a wide variety of gene promoter and enhancer sequences to stimulate transcription. The inactive form of NF-κB is composed of a p65-p50 heterodimer attached to IκBα in the cytoplasm. In the presence of stimuli, IκBα is dissociated from the polymer after phosphorylation and the p65-p50 subunit is released to become the bioactive form. Subsequently, the active dimer translocates into nucleus and binds to cognate DNA regulatory element to adjust transcriptional activity of target gene [41]. Unexpectedly, resveratrol demonstrated no effect on NF-κB p65 nuclear translocation and IκBα degradation. Nevertheless, the inhibitory effects on ROS generation and pro-inflammatory factor expression indicate that NF-κB transcriptional activity is regulated by resveratrol. NF-κB transcriptional activity is associated with numerous factors, including DNA binding activity, RelA phosphorylation, and transcriptional coactivation and repression [42-47]. In fact, our own results in the transient luciferase reporter assay revealed that resveratrol dramatically decreased NF-κB transcriptional activity despite having no effect on nuclear translocation. Certainly, the specific regulation mechanism cannot be ignored and still needs further study.
Taken together, our present study suggests that resveratrol could inhibit neointimal hyperplasia after balloon injury by down-regulating excessive ROS generation and inflammation. Furthermore, there is a possible molecular mechanism linked to the inhibition of ERK1/2 phosphorylation and NF-κB transcriptional activity. As such, we believe that resveratrol might be a novel therapeutic strategy for occlusive vascular diseases. Auxiliary application of resveratrol consisted with PCI treatment, would alleviate vascular local and even systemic inflammatory response and inhibit restenosis, which significantly reduce patient's pain and the risk of repeated revascularization.

At last, we would like to stress this study was conducted on animal model and for such reason, the obtained data cannot be directly transferred to human. Moreover, other non-rodent studies are recommended to support the final conclusion and usefulness of resveratrol in prevention of the neointimal arterial hyperplasia.

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References


