Naringenin Inhibits Angiotensin II-Induced Vascular Smooth Muscle Cells Proliferation and Migration and Decreases Neointimal Hyperplasia in Balloon Injured Rat Carotid Arteries through Suppressing Oxidative Stress

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Proliferation and migration of vascular smooth muscle cells (VSMCs) play pivotal roles in the development of restenosis after angioplasty and oxidative stress involves both processes. Naringenin, a flavanone compound found in citrus fruits, has been widely evaluated for antioxidant activity. This study was designed to explore whether naringenin could inhibit angiotensin II-induced VSMCs proliferation and migration and decrease neointimal hyperplasia in balloon injured rat carotid arteries. VSMCs were treated with or without naringenin before stimulation with 1 µM angiotensin II and twenty-four rats were subjected to carotid arteries injury and the carotid arteries were harvested at 14d after balloon injury. The results showed naringenin led to a significant inhibition of angiotensin II-induced VSMCs proliferation and migration. Naringenin significantly attenuated the reactive oxygen species production, increased the superoxide dismutase activity and decreased the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, reduced phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) and the nuclear translocation of nuclear factor (NF)-κB p65 in angiotensin II-treated VSMCs. Moreover, naringenin decreased the ratio of neointima to media by 63.8% in balloon injured rat carotid arteries, and the serum level of 8-isoprostaglandin F2α in naringenin-treated rats was significantly decreased. These results indicated naringenin exhibited antioxidant activity on angiotensin II-treated VSMCs and balloon injured rat carotid arteries and could be a potential protective agent for restenosis after angioplasty.

Key words naringenin; vascular smooth muscle cell; oxidative stress; neointimal hyperplasia; angiotensin II

Coronary heart disease (CHD) remains the leading cause of mortality in most developed countries and many developing countries. Percutaneous coronary intervention with or without intracoronary stent placement is widely used for treatment of symptomatic CHD and has greatly improved the survival rate. However, restenosis after angioplasty, which happen in 5–10% patients, is still a major clinical problem. Studies have shown that abnormal proliferation and migration of vascular smooth muscle cells (VSMCs) play pivotal roles in the development of restenosis after angioplasty, as well as in atherosclerosis. Inhibition of proliferation and migration of VSMCs could remarkably attenuate the neointimal hyperplasia in animal studies.

Naringenin, a flavanone compound found in citrus fruits, such as oranges and grapes, has been pharmacologically evaluated for antioxidant activity. Although some studies showed that naringenin could inhibit VSMCs proliferation and migration in vitro, the effect and mechanism of naringenin on vascular injury are still far from clear. In this investigation, we attempted to evaluate whether naringenin could inhibit angiotensin II-induced VSMCs proliferation and migration in vitro and neointimal hyperplasia in balloon injured rat carotid arteries in vivo.

MATERIALS AND METHODS

Primary VSMCs Culture and Naringenin Treatment VSMCs were isolated from the thoracic aorta of male Sprague-Dawley rats (6–8 weeks old) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). The purity of VSMCs was assessed by fluorescent immunostaining with anti-smooth muscle (SM) α-actin antibody (Sigma-Aldrich, U.S.A.) and 4′,6-diamidino-2-phenylindole (DAPI) (Life Technology, U.S.A.). The cell purity was calculated with the ratio of SM α-actin positive cells to the DAPI stained cells in the same field of microscope. The purity of VSMCs was more than 95% and the third to fifth passages were used in the present study. Naringenin was purchased from Sigma-Aldrich (St. Louis, U.S.A.) and resolved in dimethyl sulfoxide (DMSO). DMSO alone was used as a control and DMSO treatment alone did not alter cell viability, proliferation, migration, or the related molecular mechanism.

Cell Proliferation Assay The cell proliferation assay was performed using Cell Counting Kit-8 (Dojindo, Japan) as described previously. Briefly, VSMCs were seeded in 96-well plates at a density of 1 × 10^4 cell per well in 200 µL culture medium. After synchronization by DMEM with 0.5% FBS overnight, cells were treated with normal growth medium with various final concentrations of naringenin (0, 10, 50, 100 µM) for 30 min, and then the cells were stimulated with 1 µM angiotensin II and incubated for another 48 h. At the end of the incubation, 20 µL of the kit was added and measured for absorbance at 450 nm. Cell viability was assessed by trypan blue staining. No significant difference of cell viability was detected among all groups.

Cell Migration Assay Transwell chamber system (Corning, U.S.A.) was used to measure VSMCs migration activity following the manufacturer’s instruction. Briefly, 10^5 cells per
well were seeded into the upper chamber in the serum-free medium with or without 100 μM naringenin, and the lower chamber was filled with DMEM containing 1 μM angiotensin II. After incubation for 8 h, non-migrated cells on the top of the membrane were moved and the cells on the bottom of the membrane were fixed with methanol, stained with 0.5% crystal violet. The number of migrated cells was manually counted in nine independent, randomly chosen visual fields on a phase contrast microscope.

Detection of Reactive Oxygen Species (ROS) ROS generation by VSMCs was measured with ROS assay kit (Beyotime, China) according to the manufacturer’s instruction. In brief, VSMCs were cultured in 6-well plates and synchronized by DMEM without serum overnight. VSMCs were treated with 100 μM naringenin for 30 min and then exposed to 1 μM angiotensin II for 1 h. Cells were then incubated with 10 μM 2,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min in dark at room temperature. Fluorescent images were acquired at an excitatory wavelength of 495 nm. The mean densities of the fluorescent images were quantified with Image-Pro Plus 6.0 software, by dividing integral optical density (IOD) by area, from three random fields per well.

Superoxide Dismutase (SOD) and Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase Activities Assay NADPH oxidase and SOD are the major regulators of intracellular ROS. To determine whether naringenin could change the activities of SOD and NADPH oxidase, VSMCs were treated with 100 μM naringenin for 30 min and then stimulated with 1 μM angiotensin II for another 1 h. For detection of SOD activity, total SOD activity was investigated by using the SOD activity assay kit (BeyoVision, U.S.A.) according to the manufacturer’s instruction. For detection of NADPH oxidase activity, lucigenin chemiluminescence assay was used as described previously. Briefly, the total protein was extracted and incubated with 5 μM lucigenin (Sigma-Aldrich, U.S.A.) for 10 min at 37°C in assay buffer with the final system volume of 1 mL. After adding NADPH, luminescence was recorded for 180 s and NADPH oxidase activity was calculated as relative light units per 1 mg protein for each minute.

Western Blot Analysis Total, nuclear and cytosolic proteins were obtained with lysis buffer according to the manufacturer’s protocol (BeyoVision, U.S.A.). Protein concentration was determined by the bicinchoninic acid protein assay (Beyotime, China). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. For immunoblotting, PVDF membranes were blocked and probed with antibodies against p-extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Cell Signaling, U.S.A.), ERK1/2 (Cell Signaling), p38 mitogen-activated protein kinase (MAPK) (Cell Signaling), p38 MAPK (Cell Signaling), nuclear factor (NF)-κB p65 (Santa Cruz, U.S.A.), inhibitor of NF-κB (IκB) (Santa Cruz), β-actin (Santa Cruz), and Histone H3 (Abcam, U.S.A.) overnight at 4°C. After three washes, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Pierce, U.S.A.) for 1 h at room temperature, and subsequently analyzed by an enhanced chemiluminescence dependent detection system.

Rat Carotid Artery Balloon Injury and Naringenin Treatment The protocols for animal care and this experiment were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University and conformed to the Guideline for the Care and Use of Laboratory Animals published by the U.S.A. National Institutes of Health (NIH Publication, revised 1996). Twenty-four male Sprague-Dawley rats weighing 450 g to 500 g (Wuhan University, Wuhan, China) were randomly assigned into three groups, including sham group, control group and naringenin group (n=8 each). The rats in naringenin group and control group were anesthetized with pentobarbital (30 mg/kg intraperitoneal injection) and subjected to balloon injury of the left common carotid artery as reported previously. In brief, after intravenous injection of 100 U/kg of heparin sodium, the left common, external and internal carotid arteries were exposed. Blood flow was temporarily interrupted by ligation of the common and internal carotid arteries using microvascular clips and the external carotid artery was partially cut at about 2 mm from the arterial bifurcation with microscissors. A balloon angioplasty catheter (balloon diameter 1.25 mm, balloon length 20 mm, Medtronic, U.S.A.) was inserted through the external carotid into the common carotid artery. The balloon was then inflated to produce moderate resistance, and gradually withdrawn for three times. Then the catheter was removed, and the external carotid branch was ligated and the blood flow of the common and internal carotid arteries was restored. The rats in the sham group underwent the same procedures without the step of balloon insertion. After the procedure, the naringenin group was treated with naringenin (25 mg/kg/d) by gavage for 14 d. The other two groups were treated with saline as control. At the end of treatment, rats were euthanized and the injured arteries were harvested and fixed with 4% paraformaldehyde.

Morphometric Analysis For morphologic analysis of neointimal hyperplasia, three round cross-sections (6 μm thickness) were cut from the approximate middle of the injured artery, stained with hematoxylin-eosin, photographed, and analyzed using Image-Pro Plus 6.0 software. The intimal and medial cross-sectional areas of the carotid arteries were measured by the same investigator blind to the assignment, and the intima/media ratios were calculated.

Immunohistochemical Staining Antibodies against proliferating cell nuclear antigen (PCNA) and NF-κB p65 were used for immunohistochemical staining. The procedure was performed as previously. Data were calculated as the percentage of total cells within a given area positive for the specific antigen.

Detection of Serum Level of 8-iso-Prostaglandin F2alpha (8-iso-PGF2α) To assess the oxidative stress in vivo, the levels of 8-iso-PGF2α, an oxidative stress marker, were investigated. Blood samples from external jugular vein were collected 14 d post balloon injury. The levels of 8-iso-PGF2α were analyzed by a commercial ELISA kit (Cell Biolabs, U.S.A.) according to the manufacturer’s instruction.

Statistical Analysis Data were presented as mean±standard error of the mean (S.E.M.). Data were analyzed using one-way ANOVA followed by Newman–Keuls test. As the homogeneity of variances of the level of 8-iso-PGF2α among groups was not assumed, Dunnett’s T3 post hoc test was used for multiple comparisons. Analysis was performed with SPSS 13.0 for Windows. A value of p<0.05 was considered statistically significant.
RESULTS

**Naringenin Inhibits Angiotensin II-Induced Proliferation and Migration of VSMCs**

VSMCs were successfully isolated and cultured with high purity. As shown in Fig. 1A, angiotensin II significantly increased the proliferation of VSMCs, while naringenin (100 µM) alone didn’t affect the proliferation of VSMCs. However, naringenin (10, 50, or 100 µM) inhibited angiotensin II-induced proliferation of VSMCs in a concentration-dependent manner (all *p*<0.05). In addition, naringenin could inhibit the migration of VSMCs induced by angiotensin II by 56.6% (Fig. 1B).

**Naringenin Decreases Angiotensin II-Induced ROS Generation in VSMCs**

ROS plays an important role in the proliferation of VSMCs and induces inflammation in the vessel wall.23 The effect of naringenin on angiotensin II-induced ROS generation in VSMCs was investigated. Compared with the control group, angiotensin II significantly increased ROS generation in VSMCs (0.250±0.033 vs. 0.110±0.017, *p*<0.05, Fig. 2A). However, naringenin (100 µM) could obviously inhibit the increased intracellular ROS generation induced by angiotensin II (0.179±0.016 vs. 0.250±0.033, *p*<0.05, Fig. 2A).

**Naringenin Increases SOD Activity and Decreases NADPH Oxidase Activity in Angiotensin II-Treated VSMCs**

SOD activity and NADPH oxidase activity were investigated in angiotensin II-treated VSMCs pretreated with or without naringenin (100 µM). As shown in Fig. 2B and C, naringenin increased SOD activity and decreased NADPH oxidase activity in angiotensin II-treated VSMCs (both *p*<0.05, *p*<0.05 compared to control VSMCs and angiotensin II-treated VSMCs, respectively).

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Fig. 1. Naringenin Inhibits Angiotensin II-Induced VSMCs Proliferation and Migration

Angiotensin II could significantly promote VSMCs proliferation and pretreatment of naringenin could dose-dependently inhibit the proliferation of VSMCs induced by angiotensin II (A). Meanwhile, 100 µM naringenin could effectively decrease the angiotensin II-induced migration of VSMCs (B). Experiments were repeated three times with similar results. *p*<0.05 compared to control group. #*p*<0.05 compared to angiotensin II-treated VSMCs.

Fig. 2. Naringenin Decreases Intracellular ROS and NADPH Oxidase Activity and Increases SOD Activity in Angiotensin II-Treated VSMCs

VSMCs were treated with or without 100 µM naringenin for 30 min and then exposed to 1 µM angiotensin II for 1 h. The ROS were detected by incubating with DCFH-DA. Angiotensin II could increase the intracellular ROS and pretreatment of naringenin can decrease intracellular ROS (A, ×100). In addition, naringenin could increase the SOD activity and decrease NADPH oxidase activity in angiotensin II-treated VSMCs (B and C). Experiments were repeated three times with similar results. *p*<0.05 compared to control VSMCs. #*p*<0.05 compared to angiotensin II-treated VSMCs.
VSMCs  The SOD activity was decreased and NADPH oxidase activity was increased in VSMCs treated with 1 µM angiotensin II for 1 h, as shown in Figs. 2B and 2C. However, pretreatment of naringenin could significantly, but partially, reverse both changes (both \( p < 0.05 \)).

**Naringenin Blocks Angiotensin II-Induced Activation of MAPK-NF-κB Signaling Pathway in VSMCs**  As shown in Fig. 3, angiotensin II can increase the phosphorylation of ERK1/2 and p38 MAPK without affecting the total level of ERK1/2 and p38 MAPK. Also, angiotensin II increased the expression of nuclear NF-κB p65 and decreased the expression of cytoplasmic IκB, indicating the increased NF-κB translocation to nuclear in angiotensin II-treated VSMCs. However, pretreatment of 100 µM naringenin can significantly block these changes (all \( p < 0.05 \)).

**Naringenin Inhibits Neointimal Hyperplasia in the Balloon Injured Carotid Arteries**  Fourteen days after balloon injury, the common carotid arteries of the control group underwent serious stenosis (Fig. 4A). However, naringenin could significantly decrease intima area and the ratio of intima area to media area (0.338 ± 0.057 vs. 0.935 ± 0.153, \( p < 0.01 \)). The immunohistochemical staining showed naringenin could decrease the expression of PCNA and NF-κB p65 in the neointima of the injured carotid arteries as well (Fig. 4B).

**Naringenin Decreases the Serum Level of 8-iso-PGF2α in Balloon-Injured Rats**  As shown in Fig. 4C, balloon injury could significantly increase serum level of 8-iso-PGF2α at 14d post the surgery (\( p < 0.01 \)). Naringenin, which has the antioxidant activity, could significantly decrease the serum level of 8-iso-PGF2α compared to the control group (169.30±17.16 vs. 479.75±50.36 pg/mL, \( p < 0.01 \)).

**DISCUSSION**  In the present study, we have demonstrated that naringenin inhibits angiotensin II-induced VSMCs proliferation, migration, and the activation of ERK1/2, p38 MAPK and NF-κB. Further, we show that naringenin can reduce angiotensin II-induced ROS production, and increase SOD activity and decrease NADPH oxidase activity in angiotensin II-treated VSMCs. Moreover, we find that naringenin could inhibit neointimal hyperplasia in balloon-injured carotid arteries and decrease oxidative stress in vivo.

Abnormal proliferation and migration of VSMCs are the basic mechanism of neointimal hyperplasia in restenosis after angioplasty. Angiotensin II is a strong stimulator for proliferation and migration of VSMCs and participates in the process of atherosclerosis and vascular stenosis. In agreement with those studies, our results show that angiotensin II increases the proliferation and migration of VSMCs. Meanwhile, 100 µM naringenin can inhibit angiotensin II-induced proliferation, and migration. These results are consistent with previous studies, which showed naringenin could inhibit the proliferation of VSMCs stimulated by platelet derived growth factor-BB (PDGF-BB) or tumor necrosis factor (TNF)-α. Yang et al. reported that naringenin reduced the binding probability of transforming growth factor beta 1 (TGF-β1) to its specific receptor TβRII, thus inhibiting the receptor dimerization and activation for the signaling complex formation, so it’s possible that naringenin inhibited the effects of angiotensin II on VSMCs in the present study by suppressing the binding probability of angiotensin II to AT1 receptor or AT2 receptor and further study is needed. Since others’ results combined with our result reveal that naringenin could inhibit VSMCs proliferation and migration induced by different stimulators, naringenin may also affect the critical step of initiation and/or de-
Oxidative stress involves the stimulation of VSMCs proliferation and migration and plays a critical role in the process of various vascular diseases, such as atherosclerosis and hypertension. It is well known that angiotensin II is a vital mediator of oxidative stress and can increase generation of ROS in the vessel wall during the development of these diseases. Angiotensin II-stimulated ROS production subsequently induced MAPK pathway and the redox-regulated transcription factor NF-κB. In the present study, angiotensin II increased the activity of ERK1/2, p38 MAPK and NF-κB p65, while naringenin can block these changes. Chen et al. showed that naringenin could inhibit TNF-α-induced activation of ERK/MAPK pathway. However, Lee et al. demonstrated that naringenin didn’t affect PDGF-BB-induced activation of ERK1/2 and Akt and the antiproliferative effect of naringenin involved the suppression of DNA synthesis via a G0/G1 arrest. We speculate that this discrepancy might be attributed to different stimulators and further research is needed.

Neointimal hyperplasia is a major process of proliferative vascular disease, including restenosis after angioplasty. In the present study, we further studied whether naringenin could decrease the neointimal hyperplasia in a rat carotid artery balloon injury model, which is highly characterized and commonly used for investigating the gross morphological, cellular and molecular components of the response to arterial injury. As expected, naringenin decreased the area of intima and the ratio of intima to media 14d post surgery. Also, naringenin...
significantly decreased the ratio of positive cells stained with PCNA and NF-κB p65. These findings and in vitro findings support each other. Furthermore, Cayci et al.54 found naringenin could inhibit neointimal hyperplasia following arterial reconstruction with interpositional vein graft. In their study, this effect happened at 4 weeks, not at 2 weeks. This difference may be due to different animal models.

CONCLUSION

All in all, this study demonstrated that naringenin could inhibit VSMCs proliferation and migration in vitro and neo-intimal hyperplasia following carotid balloon injury in vivo by regulating oxidative stress and MAPK/NF-κB pathway. Naringenin, a flavonone compound, may have therapeutic effect on proliferative vascular diseases, such as atherosclerosis and restenosis after angioplasty.

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