Sulfur Dioxide Inhibits Extracellular Signal-regulated Kinase Signaling to Attenuate Vascular Smooth Muscle Cell Proliferation in Angiotensin II-induced Hypertensive Mice

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Abstract

Background: Clarifying the mechanisms underlying vascular smooth muscle cell (VSMC) proliferation is important for the prevention and treatment of vascular remodeling. Previous research has shown that the gaseous signaling molecule sulfur dioxide (SO₂) inhibits VSMC proliferation, but the mechanism for the inhibition of the angiotensin II (AngII)-induced VSMC proliferation by SO₂ has not been fully elucidated. This study was designed to investigate if SO₂ inhibited VSMC proliferation in mice with hypertension induced by AngII.

Methods: Thirty-six male C57 mice were randomly divided into control, AngII, and AngII + SO₂ groups. Mice in AngII group and AngII + SO₂ group received a capsule-type AngII pump implanted under the skin at a slow-release dose of 1000 ng·kg⁻¹·min⁻¹. In addition, mice in AngII + SO₂ received intraperitoneal injections of SO₂ donor. Arterial blood pressure of tail artery was determined. The thickness of the aorta was measured by elastic fiber staining, and proliferating cell nuclear antigen (PCNA) and phosphorylated-extracellular signal-regulated kinase (P-ERK) were detected in aortic tissues. The concentration of SO₂ in serum and aortic tissue homogenate supernatant was measured using high-performance liquid chromatography with fluorescence determination. In the in vitro study, VSMC of A7R5 cell lines was divided into six groups: control, AngII, AngII + SO₂, PD98059 (an inhibitor of ERK phosphorylation), AngII + PD98059, and AngII + SO₂ + PD98059. Expression of PCNA, ERK, and P-ERK was determined by Western blotting.

Results: In animal experiment, compared with the control group, AngII markedly increased blood pressure (P < 0.01) and thickened the aortic wall in mice (P < 0.05) with an increase in the expression of PCNA (P < 0.05). SO₂, however, reduced the systemic hypertension and the wall thickness induced by AngII (P < 0.05). It inhibited the increased expression of PCNA and P-ERK induced by AngII (P < 0.05). In cell experiment, PD98059, an ERK phosphorylation inhibitor, blocked the inhibitory effect of SO₂ on VSMC proliferation (P < 0.05).

Conclusions: ERK signaling is involved in the mechanisms by which SO₂ inhibits VSMC proliferation in AngII-induced hypertensive mice via ERK signaling.

Key words: Angiotensin II; Hypertension; Proliferation; Sulfur Dioxide; Vascular Smooth Muscle Cell

Introduction

Vascular smooth muscle cell (VSMC) proliferation is the common pathological basis of vascular remodeling-related diseases such as hypertension, atherosclerosis, and vascular restenosis. Clarifying the mechanisms underlying VSMC proliferation is important for the prevention and treatment of vascular remodeling and the reverse of hyperplastic lesions. Angiotensin II (AngII) is produced from AngI by the action of Ang-converting enzyme through hydrolysis of peptides. AngII exerts its physiological effects by binding to the angiotensin II type 1 receptor (AT1R). After coupling to heterotrimeric G protein complexes, the activation of classical downstream effectors (phospholipase C, phospholipase A2, phospholipase D, phospholipase A2, phospholipase C), the initiation of intracellular signal transduction pathways is triggered. In the case of vascular smooth muscle cells, the activation of heterotrimeric G protein complexes, the activation of classical downstream effectors, and the new creations are licensed under the identical terms.

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and phospholipase D) can be promoted by AT1R, and in the meantime, its activation can also mediate signaling via β-arrestin recruitment or transactivation of tyrosine kinase receptors (RTKs). These activate the mitogenic signaling including extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase. After the activated ERK was translocated into the nucleus, transcription factors can be phosphorylated to regulate the gene expression of cycle-related proteins and other growth factors, such as platelet-derived growth factor (PDGF). The effects of AngII on VSMC include arteriole contraction, increase in arterial blood pressure, and VSMC proliferation.

Previously, sulfur dioxide (SO₂) was considered a toxic gas, but recently, it was found to be generated endogenously in vivo from sulfur-containing amino acids. L-cysteine is first oxidized to L-cysteinesulfinate by cysteine dioxygenase. L-cysteinesulfinate can develop through transamination by glutamate-oxaloacetate transaminase into β-sulfinylpyruvate, which decomposes spontaneously to pyruvate and SO₂.

Previous studies showed that SO₂ could be endogenously produced in multiple apparatus and systems of the organism, such as the cardiovascular system, the nervous system, the respiratory system, the digestive system, the urinary system, and the immune system. The results of the study in the cardiovascular system showed that SO₂ could be produced in vascular endothelial cells, VSMCs, vascular fibroblasts, and myocardial cells. With high water solubility, SO₂ in physiological environments was soluble in water to form the sulfite and bisulfate. SO₂ gas, the form of sulfite and bisulfate, coexisted in human circulating blood.

Previous research by our team has shown that the gaseous signaling molecule SO₂ inhibits VSMC proliferation, but the mechanisms by which SO₂ inhibits AngII-induced VSMC proliferation have not been fully elucidated. Cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) can initiate apoptosis by inducing p53 and p21 transcriptions in the suppression of human aortic smooth muscle cells, and it can also prevent growth factor-stimulated Erk/mitogen-activated protein kinase (MAPK) from being activated in VSMCs. c-Raf kinase is the target of cAMP/PKA signaling in the Erk/MAPK pathway. After the Ser43, Ser233, Ser259, and Ser621 sites are phosphorylated in c-Raf by PKA, the Erk/MAPK cascade is blocked. Therefore, this study was undertaken to explore whether SO₂ inhibited VSMC proliferation via ERK signaling pathway.

**Methods**

**Materials**

Na₂SO₃ and NaHSO₃ were purchased from Sigma-Aldrich (St. Louis, MO, USA), AngII from Phoenix Biotech Co., Ltd. (Beijing, China), immunohistochemical staining kits from Wuhan Boster Biological Engineering Co., Ltd., proliferating cell nuclear antigen (PCNA) from Shanghai Baili Biological Technology Co., Ltd. (Shanghai, China), and ERK, phosphorylated-ERK (P-ERK), and PD98059 (an inhibitor of ERK phosphorylation) was purchased from Shanghai Beyotime Institute of Biological Technology Co., Ltd. (Shanghai, China). A7R5 cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Hyclone, South Logan, UT, USA) with 10% fetal bovine serum (FBS) (Gibco BRL Co., Ltd., Gaithersburg, USA), 1% L-glutamine, and 1% penicillin with streptomycin (Invitrogen, USA).

**Preparation of animal model**

Eight-week-old specific pathogen-free healthy male mice of an inbred strain, weighing 20–30 g, were purchased from the Beijing Vital River Experimental Animal Technology Co., Ltd. (Beijing, China) and kept in the First Hospital of Peking University Laboratory Animal Center. Animals experiments was approved by Peking University First Hospital Ethics Committee (ethical approval number: J201335), and the mice license for No. 11400700106308 SCXK (Beijing), 2012-0001. The mice were allowed to feed freely, with a 12 h/12 h circadian rhythm. Thirty-six mice were randomly divided into a control group \((n = 10)\), an AngII group \((n = 14)\), and an AngII + SO₂ group \((n = 12)\). In the AngII and AngII + SO₂ groups, a capsule-type AngII pump was implanted subcutaneously in the back of each mouse; the slow-release dose was set to 1000 ng kg⁻¹ min⁻¹. The mice in AngII + SO₂ group also received a once-daily intraperitoneal injection of the SO₂ donor sodium sulfite mixed with sodium bisulfite (Na₂SO₃/NaHSO₃, molar ratio 3:1, dose 85 mg/kg); the control group and AngII group received abdominal cavity injections of the same dose of normal saline daily for 2 weeks. At the beginning of the experiment and after 1 week and 2 weeks, we measured the arterial pressure of the mice by the caudal artery method. Two weeks later, the mice were sacrificed; part of the aortic tissue was used to prepare paraffin sections and the other part was analyzed as a homogenized supernatant.

**Determination of sulfur dioxide content**

We used high-performance liquid chromatography with fluorescence determination (HPLC-FD) to measure the total sulfite concentration of serum and aortic tissue, which reflects the SO₂ content. First, as a standard, Na₂SO₃ was diluted into different concentrations to draw a standard curve. Then, we measured the samples (the aortic tissue homogenized supernatant) to get the peak area values and put them into the formula from the standard curve, and calculated the concentration of SO₂⁻².

**Elastic fiber staining**

Paraffin sections of aortic tissue were stained using Hart’s modified elastic fiber staining method. Weigert’s elastic fiber staining kits were purchased from Beijing Leagene Biological Technology Co., Ltd. (Beijing, China). The thickness of the thoracic aortic wall was measured on each tissue section of 12, 3, 6, and 9 points at the same magnification, and the mean values were obtained.

**Immunohistochemical staining**

The mice were anesthetized and perfused. Aortic tissues were obtained, placed in a freezing microtome at an...
optimal cutting temperature, and serially sliced into 10 µm thick coronal section. The section was fixed with formal acetic fixative solution. The kit in this experiment was purchased from Boster Biotech Co., Ltd., China. After dewaxing, endogenous peroxidase activity was inhibited by three 5-min washes in phosphate-buffered saline (PBS), then blocked by 5% BSA blocking solution. After microwave reparation, the sections were incubated overnight at 4°C with PCNA antibody (concentration 1:50, Shanghai Bioleaf Biotech Co., Ltd., China) and p-ERK (concentration 1:100, Shanghai Beytime Biotech Co., Ltd., China). The next day, after washing in PBS, the sections were incubated with secondary antibody (37°C for 40 min). We observed PCNA and p-ERK staining on aortic tissue paraffin sections in four perpendicular fields of vision for each sample. Brown granules seen around or overlapping the cell nucleus represented PCNA expression. p-ERK-positive expression was also the brown granules seen around or overlapping the cell nucleus.[11] We calculated the ratio of the number of smooth muscle cells positive for p-ERK to the total number of smooth muscle cells.

**Western blotting analysis**

We used Western blotting to determine the expression of PCNA, ERK, and p-ERK. Equal amounts of protein (from tissue and cells) were boiled and separated by sodium dodecyl polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. To block nonspecific binding sites, membranes were incubated in tris-buffered saline solution containing 0.1% Tween-20 (Sinopharm Chemical Reagent Co. Ltd., China) and 5% skim milk (Applygen Technologies Co. Ltd., China). The primary antibody dilutions were 1:1000 for PCNA, 1:1000 for ERK (Shanghai Beytime Biotech Co., Ltd., China), 1:1000 for P-ERK, and 1:3000 for β-actin (Santa Cruz, USA). Secondary antibodies were used at a dilution of 1:4000. The X-ray film used for the Western blotting was manufactured by Kodak (Rochester, NY, USA). Other chemicals and reagents were of analytical grade. The developed signal was visualized using an enhanced chemiluminescence detection kit and quantified with AlphaImager (Alpha Innotech, San Leandro, CA, USA).[12]

**Cell experiment**

VSMCs were grown in DMEM with 10% FBS, 1% L-glutamine, and 1% penicillin with streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, and the serum starved for 24 h in DMEM with 0.1% FBS when they reached 80% confluence. The cells were then divided into six groups: control, AngII, AngII + SO₂, PD98059, AngII + PD98059, and AngII + SO₂ + PD98059. Administered concentrations were 100 µmol/L for SO₂ (Na₂SO₃/NaHSO₃, molar ratio 3:1), 1 µmol/L for AngII, and 20 µmol/L for PD98059.

**Statistical analysis**

All data were expressed as the mean ± standard error (SE). Statistical differences were evaluated by one-way analysis of variance (ANOVA) followed by a post hoc test (least-square difference). P < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 17.0 (IBM, Armonk, NY, USA).

**Results**

**Sulfur dioxide reduces hypertension caused by angiotensin II**

At the beginning of the experiment and after 1 week and 2 weeks, we measured the blood pressure of the mice in all groups. We found that the blood pressure in the AngII group was higher than that in control group (P < 0.01); however, the blood pressure in the AngII + SO₂ group was lower than that in AngII group (P < 0.01) [Figure 1]. This suggested that SO₂ reduced hypertension caused by AngII in mice.

**Angiotensin II reduces sulfur dioxide concentration in serum and tissue**

We measured SO₂ content in serum and aortic tissue homogenized supernatant by HPLC-FD. We found that the SO₂ concentration in AngII group was lower than that in control group (P < 0.05), but the SO₂ concentration in AngII + SO₂ group was higher than that in AngII group (P < 0.05) [Figure 2].

**Sulfur dioxide inhibits the thickness of the thoracic arteries**

Paraffin sections of aortic tissue were stained using Hart’s modified elastic fiber staining method. We found that the mean thickness of the thoracic aortic wall in AngII group was greater than that in control group; however, the mean thickness of the thoracic aortic wall in AngII + SO₂ group was thinner than that in AngII group [Figure 3].

**Sulfur dioxide inhibits the expression of proliferating cell nuclear antigen and phosphorylated-extracellular signal-regulated kinase**

The results of immunohistochemical staining showed that more cells had higher PCNA expression in AngII group than those in control group, which indicated that AngII promoted VSMC proliferation. However, fewer cells had higher PCNA expression in AngII + SO₂ group than those in AngII group, which suggested that SO₂ inhibited the effect of AngII [Figure 4].

At the same time, we found that the expression of P-ERK cells around the nucleus in AngII group was higher than that in control group, but the expression of P-ERK cells in AngII + SO₂ group was lower than that in AngII group [Figure 5].

**Sulfur dioxide inhibits the expression of proliferating cell nuclear antigen and phosphorylated-extracellular signal-regulated kinase in tissue protein by Western blotting**

Western blotting analysis of aortic tissue protein showed...
Figure 1: Systolic blood pressure of AngII-infused C57 mice measured every week by tail-cuff plethysmography. Blood pressure of mice before the experiment (a), blood pressure in mice infused with AngII for 1 week, (b) and blood pressure in mice infused with AngII for 2 weeks (c). Control group, \( n = 10 \); AngII group, \( n = 14 \); AngII + sulfur dioxide group, \( n = 12 \). *\( P < 0.01 \) compared with control group; †\( P < 0.01 \) compared with AngII group. AngII: Angiotensin II.

Figure 2: The content of sulfur dioxide in serum (a) and aorta (b) (\( \mu \)mol/L). *\( P < 0.05 \) compared with control group; †\( P < 0.01 \) compared with control group; ‡\( P < 0.05 \) compared with AngII group. AngII group was lower than that in control group \( (P < 0.05) \), but higher in AngII + sulfur dioxide group than that in AngII group \( (P < 0.05) \). (a) Aortic tissue supernatant sulfur dioxide in AngII group was lower than that in control group \( (P < 0.01) \), but higher in AngII + sulfur dioxide group than that in AngII group \( (P < 0.05) \) (b). *\( P < 0.05 \), ‡\( P < 0.05 \), †\( P < 0.01 \). AngII: Angiotensin II. 1mmHg = 0.133kPa.

Figure 3: Hart’s modified elastic fiber staining showed the pathological structural changes in mice thoracic aortic wall in each group. (a) Control group, (b) AngII group, and (c) AngII + sulfur dioxide group. Thoracic aortic wall in AngII group (b) was thicker than that in control group (a), but thinner in AngII + sulfur dioxide group (c) than that in AngII group (b). Original magnification: ×400. AngII: Angiotensin II.

that the expression of PCNA in the AngII group was higher than that in control group \( (P < 0.05) \), but the expression in the AngII + SO\(_2\) group was lower than that in AngII group \( (P < 0.05) \). At the same time, the expression of P-ERK in AngII group was higher than that in control group \( (P < 0.05) \), but the expression in AngII + SO\(_2\) group was lower than that in AngII group \( (P < 0.05) \) [Figure 6].

PD98059, an extracellular signal-regulated kinase phosphorylation inhibitor, blocks the inhibitory effect of sulfur dioxide on vascular smooth muscle cell proliferation

Western blotting analysis of cell protein showed that the expression of PCNA in the AngII group was higher than that in control group \( (P < 0.05) \), but the expression in the AngII + SO\(_2\) group was lower than that in AngII
Figure 4: Immunohistochemistry of PCNA expression in mice thoracic aortic wall in each group. (a) Control group, (b) AngII group, (c) AngII + sulfur dioxide group, and (d) negative control group. The brown granules around or in the cell nucleus indicate positive expression of PCNA. More cells had higher PCNA expression in AngII group (b) than those in control group (a), but lower expression in AngII + sulfur dioxide group (c) than that in AngII group (b). Original magnification: ×400. The positive expression is marked by “↓” AngII: Angiotensin II; PCNA: Proliferating cell nuclear antigen.

Figure 5: Immunohistochemistry of P-ERK expression in mice thoracic aortic wall in each group. (a) Control group, (b) AngII group, (c) AngII + sulfur dioxide group, and (d) negative control group. The brown granules around or in the cell nucleus indicate positive expression of P-ERK. More cells had higher P-ERK expression in AngII group (b) than those in control group (a), but lower in AngII + sulfur dioxide group (c) than those in AngII group (b). Original magnification: ×400. The positive expression is marked by “↓” AngII: Angiotensin II; P-ERK: Phosphorylated-extracellular signal-regulated kinase.

Figure 6: Protein expression of PCNA and the P-ERK in aortic tissue. (a) PCNA expression; and (b) P-ERK expression. PCNA expression was higher in AngII group than that in control group (P < 0.05), but lower in AngII + sulfur dioxide group than that in AngII group (P < 0.05) (a). P-ERK expression was higher in AngII group than that in control group (P < 0.05), but lower in AngII + sulfur dioxide group than that in AngII group (P < 0.05) (b). Values are expressed as mean ± standard error (n = 3). *P < 0.05 versus control group; †P < 0.05 versus AngII group. AngII: Angiotensin II; PCNA: Proliferating cell nuclear antigen; P-ERK: Phosphorylated-extracellular signal-regulated kinase.

Figure 7: P-ERK expression was lower in AngII + sulfur dioxide group than that in AngII group (P < 0.05). There was no difference in P-ERK between AngII + PD98059 group and AngII + sulfur dioxide + PD98059 group (P > 0.05). PCNA expression was higher in AngII group than that in control group (P < 0.05), but lower in AngII + sulfur dioxide group than that in AngII group (P < 0.05). There was no difference in PCNA expression between AngII + PD98059 group and AngII + sulfur dioxide + PD98059 group (P > 0.05). (a) ERK phosphorylation; and (b) PCNA protein expression. Values are expressed as mean ± standard error (n = 3). *P < 0.05. AngII: Angiotensin II; PCNA: Proliferating cell nuclear antigen; P-ERK: Phosphorylated-extracellular signal-regulated kinase. PD98059: An inhibitor of ERK phosphorylation.
groups (all $P > 0.05$) [Figure 7]. This indicated that after the administration of PD98059, the inhibitory effect caused by SO$_2$ was blocked.

**Discussion**

Hypertension is one of the most common chronic cardiovascular diseases, with vascular complications that can be life-threatening and reduce the life quality of the affected individuals.$^{[13]}$ Abnormal proliferation of VSMC is an important pathological basis of hypertension, atherosclerosis, and coronary restenosis after angioplasty.$^{[14]}$ Clarifying the mechanism responsible for VSMC proliferation is, therefore, important for the prevention and treatment of vascular remodeling.

SO$_2$ has been considered harmful to the human body, but recently, there has been increasing interest in the physiological and pathophysiological roles of SO$_2$ as a gaseous signaling molecule in the cardiovascular system, with functions such as inhibiting smooth muscle cell proliferation, relaxing blood vessels, reducing vascular inflammation and oxidative stress, and protecting myocardium.$^{[15,16]}$ Our previous research demonstrated that SO$_2$ could be endogenously generated in vascular tissues from sulfur-containing amino acid metabolism through transamination, and it could regulate vascular activities.$^{[17,18]}$ However, the mechanisms underlying the inhibition of smooth muscle cell proliferation by SO$_2$ have not been fully elucidated.

In our previous studies on spontaneously hypertensive rat model, it was proved that SO$_2$ could reduce the blood pressure and inhibit VSMC proliferation, which in cellular level proved the fact that SO$_2$ could inhibit VSMC proliferation with serum and PDGF stimulation.$^{[19,20]}$ The present study used a mouse model with hypertension induced by AngII. In systemic hypertension, vascular structural remodeling and excessive VSMC proliferation developed in AngII-infused mice. We found that the SO$_2$ content in serum and aorta was decreased with AngII treatment. This indicated a downregulated SO$_2$ production in the development of hypertensive VSMC proliferation.

To determine whether the downregulated SO$_2$ pathway was involved in the development of hypertensive vascular smooth muscle proliferation, an AngII + SO$_2$ group was included in the experiment design. Na$_2$SO$_2$ and NaHSO$_3$ in the ratio of 3:1 (mmol/kg) were used as SO$_2$ donors in mice of the AngII + SO$_2$ group.$^{[21,22]}$ With AngII infusion, mice of the AngII + SO$_2$ group showed an increased SO$_2$ content in serum and aorta, meanwhile blood pressure was obviously lowered by the administration of an exogenous SO$_2$ donor. Exogenous SO$_2$ also inhibited thoracic aortic wall thickening and AngII-induced PCNA expression. Such data demonstrated that the downregulated SO$_2$ pathway was involved in the development of hypertensive VSMC proliferation.

AngII promotes VSMC proliferation by binding to its receptor, AT1R. The receptor activation can mediate signaling via β-arrestin recruitment or transactivation of RTK. These often lead to the activation of ERK/MAPK signaling. Activated ERKs translocate to the nucleus to phosphorylate transcription factors and thereby regulate gene expression of cycle-related proteins and other growth factors. Activation of these downstream proteins regulates cellular function associated with cell proliferation.$^{[23]}$

In the animal experiment, we found that AngII could reduce the SO$_2$ content in serum and tissue, and previous studies reported that endoplasmic reticulum stress could inhibit mRNA transcription of AAT1, and at the same time, AngII could induce endoplasmic reticulum stress.$^{[24]}$ Therefore, we deduced that AngII inhibited the expression of AAT by promoting endoplasmic reticulum stress, and then the production of endogenous SO$_2$ was inhibited.

In the present study, immunohistochemistry showed that SO$_2$ inhibited phosphorylation of ERK in the aorta from AngII-infused mice. Moreover, SO$_2$ could also suppress phosphorylation of ERK in AngII-treated VSMC. The ERK pathway is an important part of the MAPK signaling pathway involved in cell proliferation, and P-ERK is the main active constituent.$^{[25]}$ Our observations suggested that SO$_2$ might inhibit VSMC proliferation in association with inhibiting the phosphorylation of ERK in this pathway.$^{[26]}$ In our *in vitro* experiment, the inhibitory role of SO$_2$ in VSMC proliferation was attenuated when phosphorylation of ERK was blocked by PD98059. Thus, the SO$_2$-inhibited VSMC proliferation in mice with hypertension induced by AngII might be associated with inhibiting ERK phosphorylation. cAMP/PKA can prevent growth factor-stimulated ERK/MAPK from being activated in VSMC. The target of cAMP/PKA signaling on the ERK/MAPK pathway is c-Raf kinase. Our previous study showed that SO$_2$ could stimulate the cAMP/PKA pathway to block c-Raf activation, whereas the Ser259 site on c-Raf played an important role in SO$_2$-induced suppression of ERK/MAPK pathway. However, until now, it is not clear whether SO$_2$ inhibits AngII-induced ERK activation through cAMP/PKA signaling.$^{[5]}$ However, these findings identify another important role for SO$_2$ as a gaseous signaling molecule in regulating the cardiovascular function and it is of significance in further clarifying the mechanisms for VSMC proliferation for the prevention and the treatment of vascular remodeling and attenuation of hyperplastic lesions.

The present study also had limitations. We did not perform the studies with the activator since there was no suitable ERK phosphorylation activators available.

In conclusion, the present and the previous study justified that endogenous SO$_2$ played an important role in regulating physiological and pathophysiological cardiovascular function of mammals. Supplementing SO$_2$ donor or intervening the generation system of SO$_2$ could improve the pathological state of the cardiovascular system to some extent. The findings provided a new direction in researching the pathogenesis of cardiovascular diseases, opened a new train of thought in the prevention and treatment of cardiovascular...
disease, and promoted the related pharmacological research progress of drugs used in cardiovascular system diseases.

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Conflicts of interest
There are no conflicts of interest.

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