Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Piperazine ferulate exerts antihypertensive effect and improves endothelial function *in vitro* and *in vivo* via the activation of endothelial nitric oxide synthase

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Received December 10, 2018; **Accepted** March 29, 2019; **Published** March 31, 2019

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Doi: http://dx.doi.org/10.14715/cmb/2019.65.3.18

Abstract: To investigate the effect of piperazine ferulate (PF) on hypertension and endothelial function, and to assess the possible underlying mechanism. Human umbilical vein endothelial cells (HUVEC), adult male Wistar Kyoto (WKY) rats aged 12 to 14 weeks, and spontaneously hypertensive (SH) and Sprague Dawley (SD) rats were used for this study. Cell viability, activities of angiotensin-converting enzyme (ACE) and heme oxygenase-1 (HO-1), *in vivo* NO synthesis, arterial systolic blood pressure, vascular function, expressions of endothelial NO synthase (eNOS) and phosphorylated-eNOS (p-eNOS) were determined or assessed as appropriate. The results of MTT assay showed the number of viable cells were significantly increased with increase in PF concentration ($p < 0.05$). The level of expression of ACE was significantly reduced with increase in PF concentration ($p < 0.05$), while the level of HO-1 expression significantly increased ($p < 0.05$). Results of DAF-FM fluorescent staining showed that the amounts of NO synthesized *in vivo* was significantly higher in aortic rings of SH and SD rats treated with PF than in the corresponding control groups ($p < 0.05$). Treatment with PF *in vivo* significantly improved impaired acetylcholine-induced aortic relaxation in SH rats. Total eNOS expression was significantly increased after treatment with PF ($p < 0.05$). The expressions of total eNOS and p-eNOS in both groups were not affected by PF when compared to the control group. These results indicate that PF exerts antihypertensive effect and improves endothelial function *in vitro* and *in vivo* via the activation of eNOS.

Key words: Hypertension; Piperazine ferulate; Endothelial function; Endothelial nitric oxide synthase; Systolic blood pressure.

Introduction

Hypertension is a chronic cardiovascular disease (CVD) characterized by damage to target organs such as heart, brain and kidney, and adverse effects on the quality of life of sufferers (1, 2). Oxygen free radicals and oxidative stress are involved in the pathogenesis of a number of diseases such as atherosclerosis, coronary artery disease (CAD), cardiovascular diseases, cancer and aging (3 - 5). Reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radical which are continually generated by metabolic activities are responsible for oxidative stress (6). Under normal physiological conditions, low concentrations of ROS regulate the activities of vascular cells, thereby maintaining normal functions of blood vessels. However, when ROS are produced in excess or are not cleared in time, they cause damage to vessel tissue cells, leading to hypertension (7). The stimulation of endothelial cells by external factors results in the secretion of inflammatory factors such as TNF-α, adhesion factor and chemokines, resulting in vascular inflammation that can lead to CVDs (8, 9).

Nitric oxide (NO) is an endothelium-derived relaxation factor that regulates a number of biological processes such as vascular tone, cardiac and vascular remodeling, platelet aggregation, neural development and proliferation of vascular smooth muscle cells (10 - 12). It is synthesized from L-arginine by the catalytic actions of eNOS, neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) (13). In the endothelium, NO is synthesized in a reaction catalyzed by eNOS (14).

Piperazine ferulate (piperazine 3-(4-hydroxy-3-methoxyphenyl) acrylate, PF) is a synthetic drug with a range of pharmacological effects such as relief of vasospasm, anticoagulation and antiplatelet aggregation effects, and dilatation of microvessels (15, 16). The aim of this study was to investigate the effect of PF on

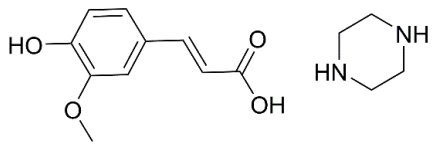

Figure 1. Structure of piperazine ferulate (PF).

hypertension and endothelial function, and to assess the possible underlying mechanism.

Materials and Methods

Materials and chemicals

Human umbilical vein endothelial cells (HUVECs) were obtained from Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd.; fetal bovine serum (FBS) was a product of Hyclon (USA), while 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate was obtained from Invitrogen Co., Ltd. (USA). Piperazine ferulate, phenylephrine, L-NAME, and Tween-20 were products of Sigma Chemicals (USA). Sodium chloride (NaCl) was purchased from Calbiochem Merck (Germany); magnesium sulfate (Mg_2SO_4), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), glucose and calcium chloride ($CaCl_2$) were purchased from BDH Laboratory Supplies (UK). Bovine serum albumin (BSA) was a product of Santa Cruz (USA); optimal cutting temperature (OCT) compound was obtained from Sakura Finetik, AJ Alphen AAN Den Rijn (Netherlands), while cryostat was purchased from Thermo Fisher Scientific (USA). Fluorescence microscope was purchased from Leica Microsystems (Germany); NIBP Monitoring System was purchased from IITC Inc (USA), and force transducer was obtained from Grass Instrument Co. (USA). PowerLab recording system was a product of AD Instruments (Australia); Lowry's assay kit was a product of Bio-Rad Laboratories (USA), while polyvinylidene fluoride membrane was purchased from Millipore, Billerica (USA). Western Blotting assay reagent was obtained from Amersham (UK); eNOS primary antibody was purchased from BD Transduction Laboratory (UK) and p-eNOS ser1176 was a product of Abcam (UK). ImageJ analysis software was purchased from NIH Co. Ltd. (USA)

Cell lines and culture

The HUVECs were cultured in Ham's F12 medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS) in a humidified incubator at 37 ℃ and 5 % CO_2 atmosphere. Cells in exponential growth were used for this study. The cells were randomly assigned to eight groups: control group, TNF-α group (10 µg/L), and six PF groups which were treated with increasing concentrations of PF ranging from 0.5 - 40 µM. Tumor necrosis alpha (TNF-α) was used to establish an endothelial cell model used *in vitro* study.

Experimental rats

Adult male Wistar Kyoto (WKY) rats aged 12 to 14 weeks, spontaneously hypertensive (SH) and Sprague Dawley (SD) rats were used for the *in vivo* study, while L-NAME was used as a NOS inhibitor. The rats were housed in metal cages at controlled temperature (22 - 25 °C) and 12 h light/12 h dark cycle, with free access to standard feed and water. The rats were handled according to the guidelines of Animal Research Reporting of *in vivo* Experiments (ARRIVE). The WKY and SH rats were divided into four groups: WKY control, WKY + PF (PF, 10 µM/ kg body weight, (bwt)/day, for 4 weeks) SH control and SH + PF. The SD rats were randomly divided into four groups: SD control, SD + PF, L-NAME control (L-NAME, 60 mg/kg bwt/ day) and L-NAME + PF. The L-NAME rats were treated with L-NAME for two weeks before and during treatment with PF. The study protocol was approved by the Zhejiang University Animal Care and Ethics Committee.

Collection of blood samples

At the end of the treatment period, the rats were sacrificed by dislocation according to the rules of the ethics committee and blood was collected through cardiac puncture, and centrifuged at 3000 g for 10 min to obtain serum which was used for biochemical analyses. The thoracic aortas were also excised and used for the determination of arterial blood pressure and assessment of vascular function.

MTT assay

The cells (1×10^5 cells/ml) were seeded into 96-well plates and pretreated with various concentrations of PF ranging from 0.5 to 40 µM for 24 h, and then treated with TNF- α for 8 h. This was followed by the addition of 20 µl of 0.5 % MTT solution within 4 h, after which the culture medium was changed. Dimethysulfoxide (DMSO, 150 µl) was added in drops to each well, and the wells were placed on an oscillator for 10 min to completely dissolve the formazan crystals. The control wells contained culture medium, MTT solution and DMSO only, and were treated same way as the sample wells. Each well was incubated in the dark for 2 h and absorbance was measured at 490 nm using Sunrise enzyme labeling instrument. The procedure was repeated 5 times, and cell viability calculated thus:

Cell viability (%) = $(1 - Abs) / Abc \times 100\ \%$

where Abs = absorbance of sample well; and Abc = absorbance of control well.

Determination of the activities of ACE and HO-1

The activities of ACE and HO-1 were determined in rat serum using their respective enzyme-linked immunosorbent assay (ELISA) kits.

Determination of in vivo NO synthesis

This was performed using DAF-FM (17). Isolated aortas from the rats were embedded in OCT compound until frozen. The frozen aortic segments were cut into 5-µm thick sections using a cryostat, and incubated at 37 °C in normal physiological saline containing 5 µM DAF-FM diacetate. After 15 min, they were subjected to fluorescence microscopy at an excitation wavelength of 495 nm and an emission wavelength of 515 nm in a Leica LAS-AF software (2.6.0).

Determination of arterial systolic blood pressure

The mean systolic blood pressure of the rats were measured before treatment and 1 week after the start of treatment using the tail-cuff method. The rats were subjected to constraint training prior to measurement. They were immobilized in a preheating chamber (28 - 30 °C) for at least 30 min before each blood pressure measurement. Measurements were made consecutively at least 6 to 7 times and the mean taken (18).

Assessment of vascular function

The excised thoracic aortas were placed in a modified

Krebs physiological saline consisting of NaCl (118.93 mM), sodium bicarbonate ($NaHCO_3$, 25 mM, $MgSO_4$ (1.18 mM), KCl (4.69 mM), KH_2PO_4 (1.03 mM), glucose (11.1 mM), and $CaCl_2$ (2.38 mM). The blood vessels were cut into 3 - 4 mm long sections; some fragments were snap frozen in liquid nitrogen and stored at -80 °C for subsequent processing. The aortic segments for isometric tension measurements were suspended in an organ chamber containing 5 ml of control solution at 37 °C and continuously inflated with 95 % O_2 and 5 % CO_2. The loop was attached to a force transducer and changes in isometric tension were recorded using a PowerLab recording system. After equilibration for 60 min at 1.0 g resting tension, the viability of the loop was tested by the addition of 60 mM KCl until a steady contraction was achieved. The loop was then washed thrice with the control solution and phenylephrine (PE, 0.3 - 1 μM) was added to induce stable contraction. A cumulative concentration was thereafter obtained which was a response to the endothelium-dependent relaxation agonist, acetylcholine (ACh, 0.3 - 10 μM) and the endothelium-dependent vasodilator, sodium nitroprusside (0.1 - 10 μM). Relaxation was expressed as a percentage of the level of pre-shrinkage obtained with norepinephrine.

Western blotting

Portions of the excised aortas were homogenized in ice-cold 1X RIPA buffer and the lysate was centrifuged at 15,000 g for 30 min at 4 ° C, and the resultant supernatant was used for Western blotting. The protein concentration of the supernatant was determined using a modified Lowry's assay. A portion of total tissue protein (20 μg) from each sample was separated on a 12 % sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 ° C for 120 min. Subsequently, 3 % BSA in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at room temperature and incubated to block non-specific binding of the blot. Thereafter, the blot was incubated with eNOS primary antibody (1; 500) and p-eNOS ser1176 at 4 °C overnight. Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using ImageJ analysis software. Respective protein expression levels were normalized to that of β-actin which was used as a standard reference.

Statistical analysis

Data are expressed as mean ± SD, and the statistical analysis was performed using GraphPad Prism (6.0). Groups were compared using Student's *t*-test and Bonferroni's Multiple Comparison tests. Values of $p < 0.05$ were considered statistically significant.

Results

Effect of PF on cell viability

There were significantly less viable cells in TNF-α group ($p < 0.001$), relative to control group. However, the number of viable cells were significantly increased with increase in the concentration of PF used to pretreat the cells ($p < 0.05$: Figure 2).

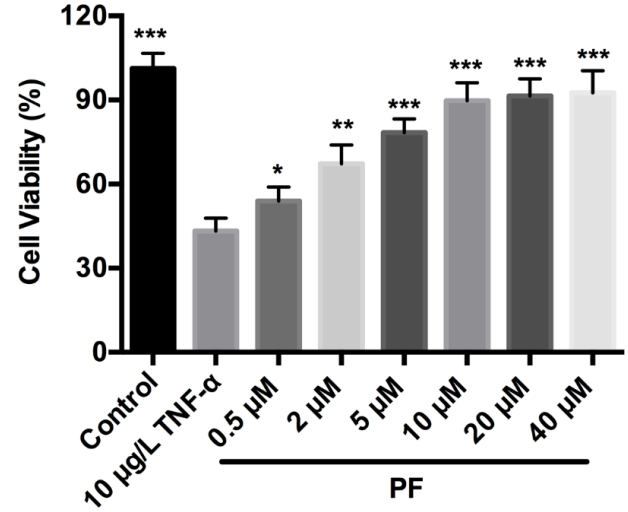

Figure 2. Effects of different concentrations of PF on cell viability. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, when compared to the TNF-α group.

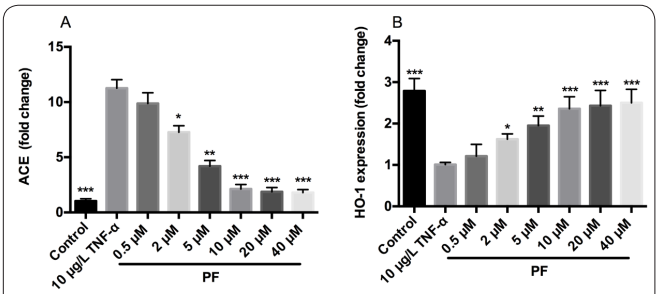

Figure 3. Comparison of effect of PF on TNF α- induced expressions of ACE and HO-1 in HUVECs. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, when compared to TNF-α group. A: expression of ACE in HUVEC; and B: expression of HO-1 in HUVEC.

Effect of PF on TNF α- induced HUVEC expressions of ACE and HO-1

The level of expression of ACE was significantly higher in TNF-α group than in control group, and was significantly reduced with increase in PF concentration ($p < 0.05$). However, the level of expression of HO-1 was significantly lower in the TNF-α group than in control group, and was significantly increased with increase in the concentration of PF ($p < 0.05$). The expression of ACE in TNF-α group was 11.22 times higher than that of control group, and the inhibitions in the PF-treated groups at PF concentrations of 0.5, 2, 5, 10, 20 and 40 μM were 12.3, 35.4, 63.0, 81.2, 83.3, and 84.1 %, respectively. The inhibition was highest at 10 μM (Figure 3A and B).

Effect of PF on the level of NO in vivo

The results of DAF-FM fluorescent staining showed that the amounts of NO synthesized *in vivo* was significantly higher in aortic rings of SH and SD rats treated with PF than in the corresponding control groups ($p < 0.05$). However, there were no significant differences in the amounts of NO synthesized *in vivo* in WKY and L-NAME rats treated with PF, when compared to the corresponding control groups ($p > 0.05$). The results are shown in Figures 4A and 4B.

Effect of PF on systolic blood pressure

Basal systolic blood pressure was significantly higher in SH rats than in WKY rats, but was significantly reduced after 4 weeks of treatment with PF ($p < 0.05$). There was no significant difference in basal systolic blood pressure between L-NAME and the control groups, but after 1 week of L-NAME treatment, the systolic blood pressure was significantly increased and was not affected by treatment with PF ($p < 0.05$). There was no significant difference in systolic blood pressure between the WKY and SD rats, after treatment with PF (Figures 5A and B).

Effect of PF on relaxation in aortic rings of rats

Relaxation was significantly shorter in aortic rings of SH and L-NAME rats than in the corresponding control groups ($p < 0.05$). Treatment with PF *in vivo* significantly improved impaired acetylcholine-induced aortic relaxation in SH rats. However, the effect was not significant in the aortic rings of L-NAME-treated rats (Figures 6A and 6B).

Expressions of eNOS and p-eNOS in PF treated rats

The expression of total eNOS was significantly lower in the aorta of SH rats than in WKY rats, but was significantly increased after treatment with PF ($p < 0.05$). However, the expression of p-eNOS was significantly higher in the aorta of SH rats than in WKY rats, and increased up to the 4th week of treatment with PF ($p < 0.05$). However, there were no significant differences in the expressions of total eNOS and p-eNOS between SD and L-NAME rats ($p > 0.05$). The expressions of total eNOS and p-eNOS in SD and L-NAME groups were not significantly affected by PF, when compared to the control group ($p > 0.05$). The results are shown in Figures 7A and 7B.

Discussion

Cardiovascular diseases (CVDs) are characterized by symptoms of hypertension and atherosclerosis. Oxidative stress causes damage to the vascular endothelial system thereby leading to vascular diseases. Vascular dysfunction is caused by an imbalance in the release of endothelium-derived relaxing factors and vascular endothelium-derived contractile factors (19, 20). Endothelium-derived relaxing factors include NO and prostacyclin (PGI2), while vascular endothelium-derived contractile factors comprise endothelin (ET), angiotensin II and thromboxane (TXA2) (21). Nitric oxide (NO) increases cyclic guanosine monophosphate (cGMP) levels in smooth muscle cells via the activation of guanylate cyclase which stimulates adenylate cyclase. This increases the intracellular levels of cyclic adenosine monophosphate (cAMP), thus, relaxing smooth muscle cells and dilating blood vessels (22). Reduced levels of these endothelium-derived vasodilators as seen in hypertension and diabetes, increase the levels of endothelium-derived contractile factors in blood vessels, thereby aggravating vascular endothelial dysfunction (23).

In the present study, TNF-α was used to establish an endothelial cell model for studying the antioxidant and vasodilation effects of PF. The results showed there were few viable cells in TNF-α group, relative to control group. However, the number of viable cells were significantly increased with increase in the concentration of PF used to pretreat the cells. These results suggest that PF may directly exert concentration-dependent protective effect on endothelial cells. The level of expression of ACE was significantly higher in TNF-α group than in control group, and was significantly reduced with increase in the concentration of PF. These results appear to suggest that PF may play the role of a vasodilator which slows/prevents the development of hypertension. The level of expression of HO-1 was significantly lower in the TNF-α group than in control group, and was significantly increased with increase in PF concentration. This is an indication that PF may significantly increase the expression of HO-1 which was hitherto inhibited by TNF-α in a concentration-dependent manner. It appears that PF may have antioxidant properties or it may en-

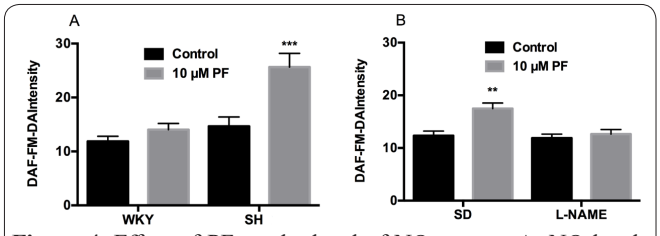

Figure 4. Effect of PF on the level of NO *in vivo*. A: NO levels in the aortic tissues of WKY and SH rats treated with PF; B: NO levels in the aortic tissues of SD and L-NAME rats treated with PF. $^{**}p < 0.05$, $^{***}p < 0.01$, when compared to control.

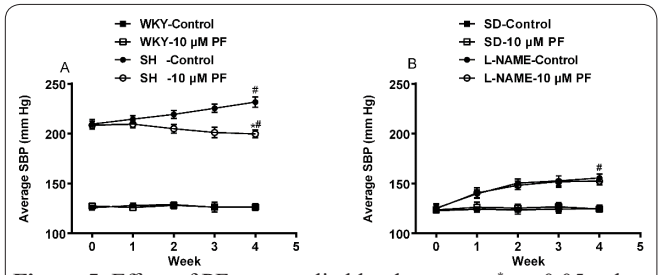

Figure 5. Effect of PF on systolic blood pressure. $^{*}p < 0.05$, when compared to control group; $^{\#}p < 0.05$, when compared to SH group.

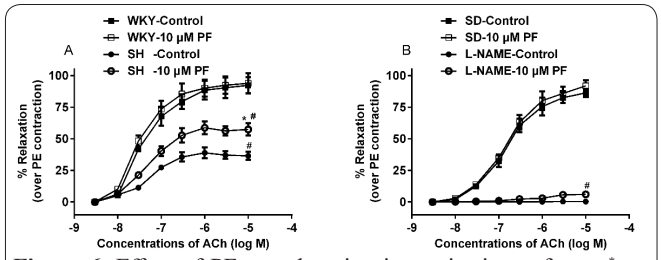

Figure 6. Effect of PF on relaxation in aortic rings of rats. $^{*}p < 0.05$, when compared to control group; $^{\#}p < 0.05$, when compared to SH group.

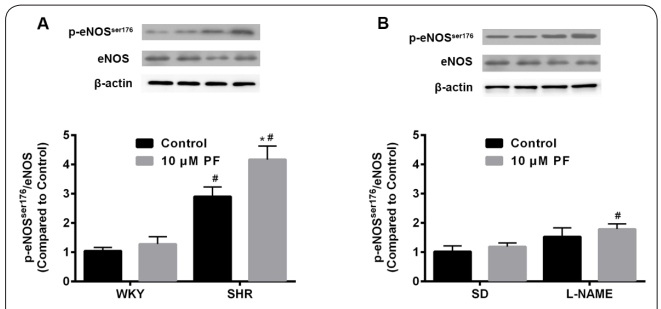

Figure 7. Effect of PF on the expressions of eNOS and p-eNOS in aortic rings of rats. $^{*}p < 0.05$, when compared to control group; $^{\#}p < 0.05$, when compared to SH group.

hance the antioxidant capacity of cells, thereby protecting vascular endothelial cells from oxidative damage and preventing hypertension.

Studies have shown that NO plays an important role in antihypertensive processes (24-25).

In this study, the results of DAF-FM fluorescent staining showed that the amounts of NO synthesized *in vivo* was significantly higher in aortic rings of SH and SD rats treated with PF than in the corresponding control groups. However, there were no significant differences in the amounts of NO synthesized *in vivo* in WKY and L-NAME rats treated with PF when compared to the corresponding control groups. These results suggest that PF exerts some beneficial effects on rats with essential hypertension. Basal systolic blood pressure was significantly higher in SH rats than in WKY rats, but was significantly reduced after 4 weeks of treatment with PF. There was no significant difference in basal systolic blood pressure between L-NAME and the control groups, but after 1 week of L-NAME treatment, the systolic blood pressure was significantly increased and was not affected by treatment with PF. There was no significant difference in systolic blood pressure between the WKY and SD rats after treatment with PF. Relaxation was significantly shorter in aortic rings of SH and L-NAME rats than in the corresponding control groups. Treatment with PF *in vivo* significantly improved impaired acetylcholine-induced aortic relaxation in SH rats. However, the effect was not significant in the aortic rings of L-NAME treated rats. The inability of PF to significantly lower blood pressure in some of the rats may be due to the fact that the blood pressure of L-NAME-treated SD rats did not reach the same levels as those of other rats. Several factors can result in reduced NO synthesis, including changes in the expressions of mRNAs of related synthases and reduced levels of the proteins, post-translational modifications, interactions with endogenous modulators, substrate availability and the levels of cofactors (26).

In this study, the expression of total eNOS was significantly lower in the aorta of SH rats than in WKY rats, but was significantly increased after treatment with PF. However, the expression of p-eNOS was significantly higher in the aorta of SH rats than in WKY rats and increased up to the 4th week of treatment with PF. However, there were no significant differences in the expressions of total eNOS and p-eNOS between SD and L-NAME-induced rats. The expressions of total eNOS and p-eNOS in both groups were not affected by PF when compared to the control group. These results suggest that the antihypertensive effect of PF may involve activation of eNOS, and that increased endogenous NO synthesis may be associated with increased PF concentration. Treatment with PF may have up-regulated eNOS expression, thereby promoting its activity.

The results of this study indicate that PF exerts antihypertensive effect and improves endothelial function *in vitro* and *in vivo* via a mechanism involving the activation of eNOS.

Author's contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Chen Huiting; Lin Bin, Lin Wenhui, Chen Yunpeng, Fan Chenrong, Zheng Lin collected and analysed the data; Shao Jianzhi and Wang Qizeng wrote the text and all authors have read and approved the text prior to publication.

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