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Original Article

The protective effects of *Polygonum multiflorum* stilbeneglycoside preconditioning in an ischemia/reperfusion model of HUVECs

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Aim: To investigate the protective effects of preconditioning human umbilical vein endothelial cells (HUVECs) with *Polygonum multiflorum* stilbeneglycoside (PMS) under anoxia/reoxygenation (A/R), and the mechanism of protection.
Methods: Prior to A/R, HUVECs were incubated with PMS (0.6×10^{-11}, 1.2×10^{-11}, or 2.4×10^{-11} mol/L) for 3 h. Cell injury was subsequently evaluated by measuring cell viability with an MTT assay and lactate dehydrogenase (LDH) release, whereas lipid peroxidation was assayed by measuring malondialdehyde (MDA) content. Antioxidant capacity was quantified by superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity. Nitric oxide (NO) production was determined by nitrite accumulation. Endothelial NO synthase (eNOS) and inducible NOS (iNOS) protein expression was detected by Western blotting. Guanylate cyclase activity and cyclic GMP (cGMP) activity were assessed by an enzyme immunoassay kit.
Results: PMS incubation attenuated A/R-induced injury in a concentration-dependent manner, as evidenced by a decrease in LDH activity and an increase in cell viability. PMS exerted its protective effect by inhibiting the A/R-mediated elevation of MDA content, as well as by promoting the recovery of SOD and GSH-Px activities. Additionally, PMS incubation enhanced NO and cGMP formation by increasing iNOS expression and guanylate cyclase activity. The protective effects of PMS were markedly attenuated by NOS inhibitor *L*-NAME, soluble guanylate cyclase inhibitor ODQ or PKG inhibitor KT5823.
Conclusion: PMS preincubation resulted in the enhancement of antioxidant activity and anti-lipid peroxidation. The NO/cGMP/cGMP-dependent protein kinase (PKG) signaling pathway was involved in the effect of PMS on HUVECs.

Keywords: *Polygonum multiflorum* stilbeneglycoside; anoxia/reoxygenation; human umbilical vein endothelial cells; NO/cGMP/PKG pathway

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Introduction

Although restoration of blood flow to an ischemic organ is essential to prevent irreversible tissue injury, reperfusion may result in a local and systemic inflammatory response that may further increase tissue injury. Reperfusion of ischemic tissues results in both a local and a systemic inflammatory response that, in turn, may result in widespread microvascular dysfunction and an altered tissue barrier function[1]. A variety of studies have shown that the vascular endothelium is a crucial site that is affected by ischemia/reperfusion (I/R) injury[2–5]. I/R results in the formation of reactive oxygen species (ROS). As potent oxidizing and reducing agents, ROS directly damage the endothelium cellular membrane through lipid peroxidation[1, 6]. ROS also up-regulate the expression of cell adhesion molecules, induce transcription of cytokines, and subsequently stimulate the activation and chemotaxis of neutrophils, which can injure the endothelium[1].

However, brief intermittent periods of ischemia and reperfusion, termed ischemic preconditioning (IPC), can increase endothelial resistance to ischemic injury[4, 5]. IPC has been shown to decrease ROS generation and maintain the normal structure and function of the vascular endothelium. Whereas the actual mechanism of IPC protection remains unclear, previous studies have shown that nitric oxide (NO) plays

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a prominent role in the signaling pathway of ischemic and drug-induced preconditioning[7–9]. Recent studies have demonstrated that the NO produced by inducible NO synthases (iNOS) can protect the heart, and that this effect involves K^+_{ATP} channels and contractile fibers[10, 11]. The fundamental signaling role of NO is to stimulate soluble guanylate cyclase, which leads to the production of cyclic GMP (cGMP) and the activation of cGMP-dependent protein kinases (PKG)[12]. It is noteworthy that the NO/cGMP/PKG signaling pathway is a survival signal that is intimately involved in the protective action in I/R injury[13].

In the past decade, several endogenous and exogenous substances that mimic the protective effect of IPC have been identified[14]. In this study, we investigated the ability of traditional Chinese medicines to mimic the beneficial effects of IPC. Our goal was to discover a novel therapeutic approach for the treatment of ischemic disorders, including peripheral vascular disease, myocardial infarction and stroke.

Polygonum multiflorum Thunb (PM), the root tuber of the polygonum plant *Polygonum multiflorum*, is one of the most famous tonics among traditional medicines in China and Japan[15]. PM has been shown to have a protective effect on the cardiovascular system. Studies have indicated that PM can enhance cellular antioxidant activity (*eg* the activity of superoxide dismutase, SOD), significantly inhibit the formation of oxidized lipids[16] and repress lipid peroxidation in rat heart mitochondria[17]. The medicinal effects of PM for the treatment of cardiovascular diseases, including atherosclerosis and hypertension, are most likely due to its antioxidant capacity. Chen *et al*[18] identified that gallic acid, catechin, and stilbeneglycoside in the ethyl acetate fraction of PM extracts showed strong antioxidant activities. Among these molecules, *Polygonum multiflorum* stilbeneglycoside (2,3,5,4′-tetrahydroxystilbene-2-*O*-β-*D*-glucoside, $C_{20}H_{22}O_9$, PMS, Figure 1) is an important active component. So far, several studies have demonstrated PMS's antioxidant activity. Zhang *et al* found that PMS decreased ROS generation, resulting in protection against doxorubicin-induced cardiotoxicity[19]. Lv *et al* demonstrated that PMS could increase the activities of SOD and glutathione peroxidase (GSH-Px) in the serum and organs (liver, heart, and brain) of senile rats and decrease the level of 2-thiobarbituric acid-reactive substances (TBARS) *in vivo*[20]. Additionally, PMS was found to possess an anti-atherosclerotic and hypolipidemic activity[21]. Therefore, PMS may have protective effects against I/R injury.

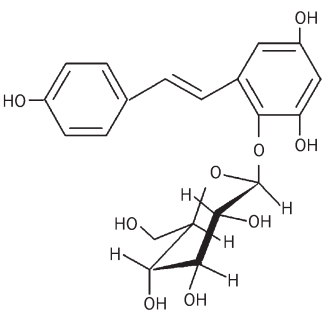

Figure 1. Chemical structure of *Polygonum multiflorum* stilbeneglycoside (2,3,5,4′-tetrahydroxystilbene-2-*O*-β-*D*-glucoside, PMS). PMS ($C_{20}H_{22}O_9$, molecular weight 406) is an active component extracted from *Polygonum multiflorum Thunb*.

In order to examine the beneficial effects of PMS in an I/R injury model, we cultured human umbilical vein endothelial cells (HUVECs) under anoxia/reoxygenation (A/R) conditions *in vitro*[22]. Specifically, we studied the NO/cGMP/PKG signaling pathway to determine the mechanism of PMS protection in response to I/R.

Materials and methods

This study was performed in accordance with the guidelines of the Declaration of Helsinki, as revised in 2000, and was approved by the Ethics Committee of the Nanchang University School of Medicine.

Drugs and reagents

PMS (purity: HPLC>98%) was purchased from Chengdu Biological Co Ltd (Chengdu, China) and was dissolved in distilled water. Cell culture products were obtained from Life Technologies (Paisley, Scotland). N^G-nitro-*L*-arginine methyl ester (*L*-NAME), ^{1}H-[1,2,4] oxadiazolo [4,3-α]quinoxalin-1-one (ODQ), KT5823 and other chemical agents were purchased from Sigma Chemical Company (St Louis, MO, USA). *L*-NAME was dissolved in distilled water and ODQ and KT5823 were dissolved in dimethyl sulfoxide (DMSO). Anti-iNOS and anti-endothelial NOS (eNOS) primary antibodies, as well as the HRP-linked secondary antibody, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The cGMP enzyme immunoassay Kit, SOD and GSH-Px activity assay kits were purchased from the Cayman Chemical Company (Cayman Chemical Co, Ann Arbor, MI, USA).

HUVEC cell culture

HUVECs were isolated from fresh human umbilical cord veins by collagenase digestion according to a modified technique described by Jaffe *et al*[23]. Briefly, HUVECs were cultured at 37 °C in a 95% O_2–5% CO_2 humidified atmosphere in Iscove's modified Dulbecco's medium supplemented with 20% fetal bovine serum, 100 μg/mL streptomycin, 100 IU/mL penicillin, 50 μg/mL heparin and 20 ng/mL endothelial cell growth factor. The HUVECs were subcultured nine to ten days later and all experiments were performed on cells from the third passage.

Establishment of the A/R model

The A/R model was established and run according to the method described by Koyama *et al*[24] with slight modifications. Briefly, anoxic conditions were created by a small enclosed humidified plexiglass chamber filled with 95% N_2 and 5% CO_2 (Changjing Biotech Co, Jiangsu, China) at 37 °C. Reoxygenation was achieved by exposing the cells to a normal atmosphere (CO_2 incubator). Subconfluent HUVECs were grown in a mimic hypoxic solution (0.9 mmol/L NaH_2PO_4, 6.0

mmol/L $NaHCO_3$, 1.0 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 20.0 mmol/L HEPES, 98.5 mmol/L NaCl, 10.0 mmol/L KCl, 40.0 mmol/L sodium lactate, pH 6.8), exposed to anoxic conditions for 3 h and then exposed to normoxia for 2 h at 37 °C in normal tyrode solution (0.9 mmol/L NaH_2PO_4, 20.0 mmol/L $NaHCO_3$, 1.0 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 20.0 mmol/L HEPES, 129.5 mmol/L NaCl, 5.0 mmol/L KCl, 5.5 mmol/L glucose, pH 7.4).

Drug treatment

At the start of each experiment the HUVECs were washed with phosphate buffered saline and incubated for 3 h in fresh medium with or without various concentrations of PMS (0.6×10^{-11}, 1.2×10^{-11}, or 2.4×10^{-11} mol/L) prior to A/R. In a different series of experiments, 100 μmol/L *L*-NAME (an NOS inhibitor), 20 μmol/L ODQ (a soluble guanylate cyclase inhibitor), or 0.2 μmol/L KT5823 (a PKG inhibitor) were administered to the cell cultures 30 min before and during treatment with 2.4×10^{-11} mol/L of PMS. The same final concentration of solvent used to dissolve the various compounds was included in all of the respective control experiments. Cell viability, lactate dehydrogenase (LDH) activity, SOD activity, GSH-Px activity, and the MDA concentration were measured at the end of the reoxygenation times. The other parameter measurements were performed after 3 h of incubation with PMS.

Cell viability assay[25]

Cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide (MTT) assay. The HUVECs were seeded in 96-well plates at 1×10^5 cells/well. After the experimental treatment, the HUVECs were washed with warm phosphate buffered saline and incubated with 0.5 mg/mL MTT in phosphate buffered saline for 4 h at 37 °C. The reaction was stopped by the addition of 150 μL diphenylamine solution and the absorbance of the blue formazan derivative was read at 570 nm using a microplate reader (Bio-Rad Laboratories, CA, USA).

Measurement of LDH activity

To measure LDH activity in the culture supernatant of HUVECs, 0.1 mL of culture supernatant was taken after experimental treatment and analyzed with an automatic biochemical analyzer (Hitachi 7060, Japan).

MDA content assay

The MDA content was measured according to the method of Niehauss and Samuelsson[26] and was assessed to indicate the level of lipid peroxidation. Briefly, the supernatant was incubated with trichloroacetic acid-thiobarbituric acid plus 0.5 mmol/L Trolox for 15 min at 100 °C. The absorbance of the supernatant was subsequently measured at 535 nm.

SOD and GSH-Px activity

The antioxidant potential of PMS was determined by measuring the activities of SOD and GSH-Px using commercial assay kits from the Cayman Chemical Company. The SOD and GSH-Px assays were performed using the nitroblue tetrazolium (NBT)[27] and 5,5′-dithiobis-2-nitrobenzoic acid (DNTB) methods[28], respectively.

Nitrite production

To evaluate NO production, the nitrite accumulation in the culture medium was measured by the Griess reaction[29]. Briefly, after 3 h of PMS treatment, the culture medium was collected and centrifuged at 179×*g* to remove dead HUVECs. The supernatant was subsequently incubated with a 1:1 mixed solution of α-naftilethilen-diamine (stock solution 0.1% *w/v* in water) and sulphanilamide (stock solution 1% *w/v*, in 5% H_3PO_4) for 10 min and kept in the dark at 23 °C. Nitrite production was recorded with a Perkin-Elmer LS 55 luminescence spectrometer (Perkin-Elmer Ltd, Beaconsfield, England) by monitoring the absorbance at 550 nm. A $NaNO_2$ standard curve was used to quantify the nitrite production obtained during these experiments.

Determination of eNOS and iNOS expression

NOS protein expression levels in HUVECs were assessed by Western blotting. Briefly, HUVECs were lysed with lysis buffer containing 50 mmol/L Tris–HCl (pH 7.5), 100 mmol/L NaCl, 5 mmol/L EDTA, 1% (*v/v*) TritonX-100, 1 mmol/L NaF, 1 mmol/L Na_3VO_4, 0.2 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin. Equivalent amounts of protein were resolved on a 10% sodiumdodecylsulfate-polyacrylamide gel with electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking with 5% non-fat milk, the membranes were blotted with a polyclonal anti-eNOS or anti-iNOS antibody (1:1000 dilution) and detected with a horseradish peroxidase-conjugated secondary antibody (1:2000 dilution). The specific protein bands were detected with chemiluminescence reagents and exposure to a chemiluminescence film. Densitometric analysis of the Western blot was performed with a GDS-8000 UVP photo scanner and LAB WOEK45 Image software (Bio-rad). The results were normalized to the β-actin expression level.

Determination of guanylate cyclase activity[9]

After PMS treatment, the HUVECs were lysed by sonication for 45 s and kept on ice in 25 mmol/L HEPES buffer (pH 7.5) containing dithiothreitol (1 mmol/L), phenylmethylsulphonyl fluoride (10 μg/mL), trypsin inhibitor (10 μg/mL), leupeptin (10 μg/mL), antipain (10 μg/mL), chymostatin (10 μg/mL) and pepstatin (10 μg/mL). The lysate solution was then centrifuged at 78 000×*g* for 20 min at 4 °C to obtain supernatant for the guanylate cyclase activity assay. The supernatant (100 μL or 1 μg protein) was added to a prewarmed (37 °C) buffer (25 mmol/L Tris-HCl, pH 7.2, 3 mmol/L GTP, 5 mmol/L $MgCl_2$, and 1 mmol/L 3-isobutyl-L-methylxanthine) and incubated for 20 min. The reaction was terminated by boiling for 3 min and the amount of cGMP in the mixture was measured by the enzyme immunoassay kit. The guanylate cyclase activity was expressed as pmol/min per mg of protein.

Determination of intracellular cGMP[9]

After PMS treatment, the HUVECs were lysed in 0.1 mol/L hydrochloric acid. Aliquots (50 μL) of the cell lysate were transferred into 96-well plates and cGMP was quantified using a colorimetric cGMP enzyme immunoassay kit according to the manufacturer's instructions. The absorbance was measured on a microplate reader at a wavelength of 415 nm. cGMP levels were corrected by measurements of protein concentration and the data are shown as pmol/mg protein.

Statistical analyses

The data are expressed as the mean±SEM. One-way analysis of variance followed by the Student-Newman-Keuls test was applied to calculate the statistical significance between the various groups. A value of $P<0.05$ was considered to be statistically significant.

Results

The effect of PMS on A/R injury in HUVECs

LDH release and cell viability were used in this study as indicators of vascular endothelium injury. As shown in Figure 2A, the viability of HUVECs subjected to A/R was only 45.6% compared to the untreated cells (control). However, pretreatment with PMS (0.6×10^{-11}, 1.2×10^{-11}, or 2.4×10^{-11} mol/L) significantly increased the viability of HUVECs to 75.3%, 81.5%, and 89%, respectively, indicating a dose-dependent protection against A/R. When 100 μmol/L of *L*-NAME (a NOS inhibitor), 20 μmol/L ODQ (a soluble guanylate cyclase inhibitor) or 0.2 μmol/L KT5823 (a PKG inhibitor) was administered 30 min before and during 2.4×10^{-11} mol/L PMS pretreatment, the protective effect of PMS was abolished (Figure 2B). PMS incubation also significantly decreased the LDH activity (Figure 3A) and this effect was abolished in the presence of 100 μmol/L *L*-NAME, 20 μmol/L ODQ or 0.2 μmol/L KT5823 (Figure 3B). Additional experiments showed that *L*-NAME, ODQ, KT5823, or DMSO (the solvent for ODQ and KT5823) had no toxic effects on HUVECs (data not shown). These results indicate that the NO/cGMP/PKG signaling pathway is involved in the mechanism of PMS protection.

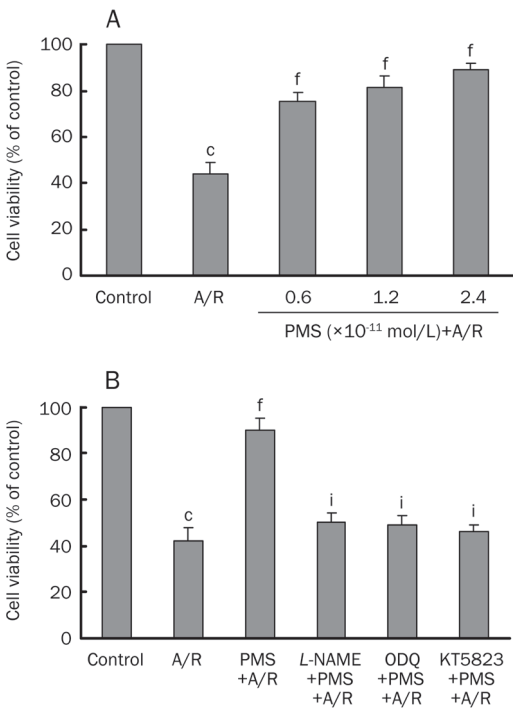

Figure 2. Effects of PMS and combination with *L*-NAME, ODQ or KT5823 on viability of HUVECs subjected to A/R. (A) HUVECs were incubated for 3 h with or without various concentrations of PMS (0.6×10^{-11}, 1.2×10^{-11}, 2.4×10^{-11} mol/L), and then subjected to A/R. (B) HUVECs were incubated 3 h with PMS (2.4×10^{-11} mol/L) in the presence or absence of NOS inhibitor *L*-NAME (100 μmol/L), soluble guanylate cyclase inhibitor ODQ (20 μmol/L) or PKG inhibitor KT5823 (0.2 μmol/L), followed by A/R. Cell viability was measured by MTT assay as described in the Materials and methods section. All data are presented as mean±SEM. $n=8$. $^{c}P<0.01$ *vs* control; $^{f}P<0.01$ *vs* A/R; $^{i}P<0.01$ *vs* PMS+A/R.

Effects of PMS on MDA, SOD, and GSH-Px activity of HUVECs subjected to A/R

When HUVECs were subjected to A/R injury, the content of MDA rose remarkably and was accompanied by a decrease in SOD and GSH-Px activities. PMS pretreatment inhibited the A/R-induced elevation of MDA contents and promoted the recovery of SOD and GSH-Px activities in a dose-dependent manner as shown in Table 1. However, the protective effect of PMS toward antioxidant enzymes and lipid peroxidation lev-

Table 1. Effects of PMS and combination with *L*-NAME, ODQ or KT5823 on MDA contents, SOD and GSH-Px activity of HUVECs subjected to A/R. (A) HUVECs were incubated for 3 h with or without various concentrations of PMS (0.6×10^{-11}, 1.2×10^{-11}, 2.4×10^{-11} mol/L), and then subjected to A/R. (B) HUVECs were incubated 3 h with PMS (2.4×10^{-11} mol/L) in the presence or absence of NOS inhibitor *L*-NAME (100 μmol/L), soluble guanylate cyclase inhibitor ODQ (20 μmol/L) or PKG inhibitor KT5823 (0.2 μmol/L), followed by A/R. Mean±SEM. $n=8$. All data are presented as mean±SEM. $n=8$. $^{a}P>0.05$, $^{c}P<0.01$ *vs* control. $^{e}P<0.05$ *vs* A/R. $^{f}P<0.01$ *vs* PMS+A/R.

Groups	MDA (μmol/mg protein)	SOD (U·min^{-1}·mg^{-1} protein)	GSH-Px (U·min^{-1}·mg^{-1} protein)
A			
Control	3.56±0.21	8.17±0.56	55.27±3.55
A/R	10.79±1.82^{c}	3.45±0.28^{c}	28.67±4.05^{c}
PMS (0.6×10^{-11} mol/L)	6.06±0.61^{e}	6.04±0.46^{e}	42.28±4.56^{e}
PMS (1.2×10^{-11} mol/L)	5.43±0.65^{e}	7.89±0.66^{e}	48.36±5.06^{e}
PMS (2.4×10^{-11} mol/L)	4.22±0.25^{ae}	9.08±0.65^{ae}	60.8±5.97^{ae}
B			
Control	3.41±0.30	8.36±0.47	56.19±3.09
A/R	10.99±1.93^{c}	3.35±0.28^{c}	29.14±3.72^{c}
PMS+A/R	4.38±0.25^{e}	10.01±0.49^{e}	59.96±6.03^{e}
L-NAME+PMS+A/R	8.29±0.97^{f}	5.03±0.33^{f}	35.43±3.86^{f}
ODQ+PMS+A/R	9.18±1.87^{f}	5.01±0.61^{f}	36.10±3.22^{f}
KT5823+PMS+A/R	8.92±1.37^{f}	4.89±0.51^{f}	32.73±3.16^{f}

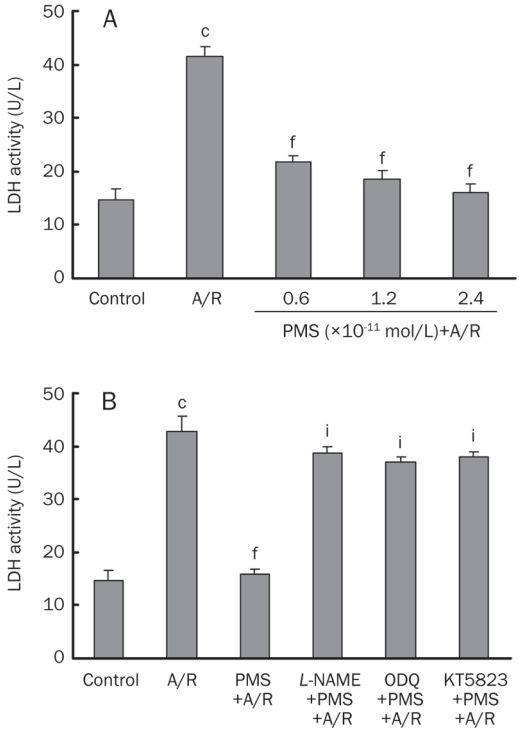

Figure 3. Effects of PMS and combination with *L*-NAME, ODQ or KT5823 on LDH activity in HUVECs subjected to A/R. (A) HUVECs were incubated for 3 h with or without various concentrations of PMS (0.6×10^{-11}, 1.2×10^{-11}, 2.4×10^{-11} mol/L), and then subjected to A/R. (B) HUVECs were incubated 3 h with PMS (2.4×10^{-11} mol/L) in the presence or absence of NOS inhibitor *L*-NAME (100 μmol/L), soluble guanylate cyclase inhibitor ODQ (20 μmol/L) or PKG inhibitor KT5823 (0.2 μmol/L), followed by A/R. All data are presented as mean±SEM. n=8. ^{c}P<0.01 *vs* control; ^{f}P<0.01 *vs* A/R; ^{i}P<0.01 *vs* PMS+A/R.

els was abolished by the addition *L*-NAME, ODQ or KT5823. These data also suggest that the anti-peroxidative effect of PMS is mediated by the NO/cGMP/PKG signaling pathway.

Effects of PMS on NO production in HUVECs

To confirm the potential involvement of the NO/cGMP/PKG signaling pathway in the protective effect of PMS against A/R injury, the effect of PMS on NO production was measured. The data presented in Figure 4 show that PMS caused a concentration-dependent increase in NO production, which was seen as a significant increase in nitrite production.

Effect of PMS on the expression of iNOS and eNOS in HUVECs

We further examined whether the activation of NOS was mediated by the up-regulation of relevant protein expression, which was determined by Western blot analysis. Although the expression of eNOS was not significantly altered by PMS treatment, a concentration-dependent increase in iNOS expression was observed in PMS-treated HUVECs compared to the untreated HUVECs (Figure 5).

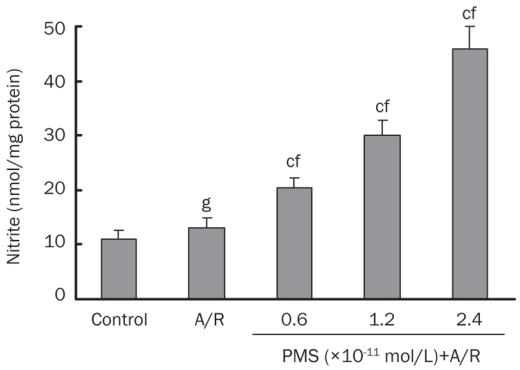

Figure 4. Effects of PMS on NO production in HUVECs. HUVECs were incubated for 3 h with or without various concentrations of PMS (0.6×10^{-11}, 1.2×10^{-11}, 2.4×10^{-11} mol/L). NO production was measured as described in the Materials and methods section. All data are presented as mean±SEM. n=8. ^{c}P<0.01 *vs* control; ^{f}P<0.01 *vs* A/R; ^{g}P>0.05 *vs* control.

Effect of PMS on soluble guanylate cyclase activity and cGMP formation in HUVECs

We also investigated the effects of PMS on soluble guanylate cyclase activity and cGMP levels in HUVECs. As shown in Figure 6, PMS incubation increased the soluble guanylate cyclase activity (Figure 6A) and the formation of cGMP (Figure 6B) in a concentration-dependent manner.

Discussion

To our knowledge, this is the first study to demonstrate the protective effect of PMS via the NO/cGMP/PKG signal pathway toward endothelial cells subjected to oxidative stress generated from A/R injury. It is widely accepted that ROS play a major role in the injury caused by A/R. Generally, prolonged ischemia leads to an increase in ROS generation and in response, cells will typically generate more antioxidants to avoid free-radical damage. The SOD and GSH-Px enzymes assist cells in repairing damaged membranes are the most important antioxidants utilized by the cells to survive oxidative damage[30, 31]. However, the burst of free radical production that occurs during reperfusion overwhelms the cellular capacity for protection against free radical-mediated damage by depleting natural sources of antioxidants[32]. Excessive ROS generation results in lipid peroxidation, oxidation of proteins, and DNA damage[33]. In the present study, a significant decrease in SOD and GSH-Px activities was seen after HUVECs were exposed to A/R, indicating impairment of the antioxidant defenses. The results showed that an increased intracellular level of MDA was associated with an increase in cellular LDH release and decrease in cell viability. A/R led to the most profound decrease in intracellular antioxidant enzyme activity and increase in MDA. Therefore, during I/R injury, administration of free-radical scavengers or antioxidants could protect cells by neutralizing reactive oxygen species. Thus, antioxidants have been suggested as a protection strategy against I/R injury[34].

In this study, preincubation with PMS attenuated

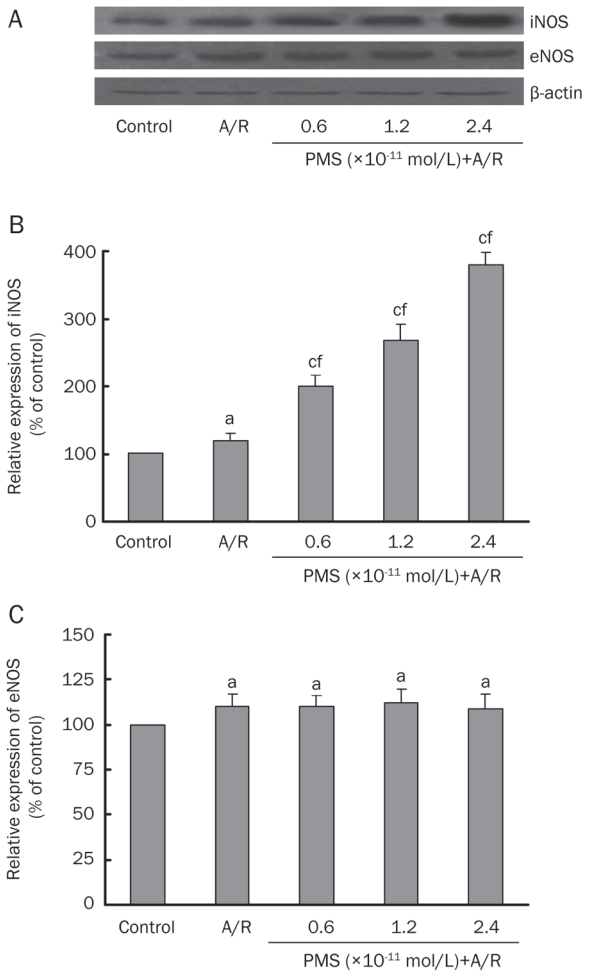

Figure 5. Effect of PMS on the expression of iNOS and eNOS in HUVECs. HUVECs were incubated for 3 h with or without various concentrations of PMS (0.6×10^{-11}, 1.2×10^{-11}, 2.4×10^{-11} mol/L). The expression of iNOS and eNOS were assessed by Western blotting as described in the Materials and methods section. (A) A representative Western blot for iNOS and eNOS; (B) Relative levels of iNOS protein as assessed by densitometry; (C) Relative levels of eNOS protein as assessed by densitometry. Quantitations were normalized to value obtained for β-actin protein expression. All data are presented as mean±SEM. n=8. ^{a}P>0.05, ^{c}P<0.01 *vs* control; ^{f}P<0.01 *vs* A/R.

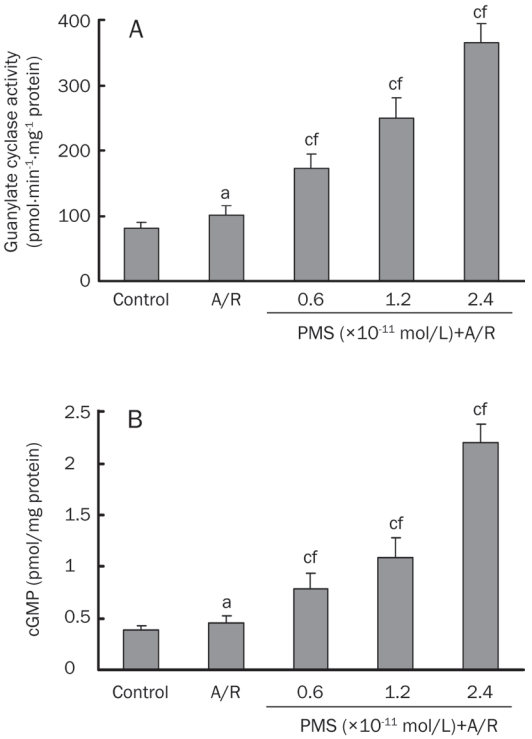

Figure 6. Effects of PMS on guanylate cyclase activity (A) and cGMP formation (B) in HUVECs. HUVECs were incubated for 3 h with or without various concentrations of PMS (0.6×10^{-11}, 1.2×10^{-11}, 2.4×10^{-11} mol/L). Guanylate cyclase activity and cGMP formation were measured as described in the Materials and Methods section. All data are presented as mean±SEM. n=8. ^{a}P>0.05, ^{c}P<0.01 *vs* control; ^{f}P<0.01 *vs* A/R.

A/R-induced injury in a concentration-dependent manner, as was evident by a decrease in LDH activity and increase in cell viability. These results indicate that PMS pretreatment has an obvious protective effect against A/R-mediated damage in HUVECs. Additionally, PMS pretreatment also led to the enhancement of cellular SOD and GSH-Px activities and a decrease in MDA production in a concentration-dependent manner. The strong positive correlations between GSH-Px and SOD activities with cell viability, and LDH with MDA, jointly suggest that enhancement of endogenous antioxidant capacities and attenuation of lipid peroxidation may represent the major mechanism of PMS's cellular protection.

Furthermore, we found that PMS up-regulated the expression of iNOS in HUVECs, resulting in an increase of NO production. This protective effect by PMS could be blocked by *L*-NAME, which is a specific inhibitor of NOS. The effect of PMS on NO production may play a central role in the mechanism for cell protection. Previously, we have observed that NO activates the cGMP-mediated signal transduction pathway to prevent oxidative stress-induced injury[35–38]. Estrogen induces NOS1/NO/cGMP/PKG to activate the signaling pathway and results in the expression of thioredoxin and MnSOD, crucial components of the estrogen-mediated multifaceted neuroprotective mechanisms[35]. Cuong *et al*[36] reported that anoxic preconditioning induced iNOS expression and activated the NO/cGMP/PKG pathway, which may play an important role in maintaining mitochondrial K^{+}_{ATP} channels in an open state during A/R. This would then protect myocytes against oxidative stress by regulating the mitochondrial respiratory chain and balancing the levels of ROS and reactive nitrogen species during reperfusion. Jang *et al*[37] demonstrated that exogenous NO mobilizes intracellular Zn^{2+} via a cGMP/PKG-dependent signal pathway in rat cardiomyocytes and prevents mitochondrial oxidative damage. NO also protects cells against oxidative stress-induced apoptosis via the cGMP/PKG pathway in SH-SY5Y cells[38]. According to these previous studies, PMS increased NO production and increased the activity of soluble guanylate cyclase in a concentration-dependent manner, which led to an increased expression of cGMP. Experiments

utilizing ODQ, a soluble guanylate cyclase inhibitor, proved that cGMP could be regulated by PMS in HUVECs. Additionally, the protective effects of PMS were completely abolished by KT5823, a PKG inhibitor. Thus, NO activates guanylyl cyclase to produce cGMP, which in turn stimulates PKG. Our studies here show that PMS signals through or affects the NO/cGMP/PKG pathway and provides an antioxidant protection to A/R-treated HUVECs. However, it should be noted that PMS increased iNOS expression and NO production in a concentration-dependent manner, but had no effect on eNOS expression levels. This result suggests that iNOS expression may play a central role in the effects of PMS against A/R damage in HUVECs.

In conclusion, our study provides clear evidence for the protective effect of PMS against A/R damage to HUVECs. The protective effect was related to the up-regulation of cellular antioxidants (*eg* SOD and GSH-Px) and subsequently the decrease in MDA concentration, which indicates that PMS attenuated lipid peroxidation. We also observed that the NO/cGMP/PKG signaling pathway is involved in the mechanism of PMS protection. iNOS, rather than eNOS, might be the key regulation point for future therapy targeted toward this signaling pathway.

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Author contribution

Ming HE, Yao-fang YANG designed research; Li-ping LIU, Zhang-ping LIAO, Dong YIN, Wei-dong LI, Dan LIU, Qing LI performed research; Dong YIN contributed new reagents or analytic tools; Zhang-ping LIAO, Qi-ren HUANG analyzed data; Zhang-ping LIAO, Dong YIN wrote the paper.

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Correction

The epoxyeicosatrienoic acid-stimulated phosphorylation of EGF-R involves the activation of metalloproteinases and the release of HB-EGF in cancer cells. Acta Pharmacologica Sinica 2010; 31(2): 211–218.

In the footnote the first sentence "The first two authors contributed equally to this work" should be deleted. The first author is Li-ming CHENG and the second author is Jian-gang JIANG.

The authors felt sorry to make such a mistake.