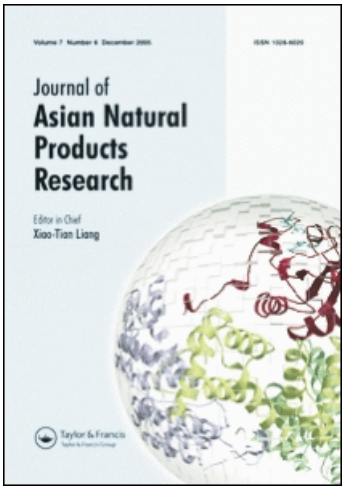

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Puerarin (**1**) is a major effective ingredient extracted from the traditional Chinese medicine Ge-gen (*Radix Puerariae*, RP). Recently, puerarin has been used to treat patients with coronary artery diseases (CAD). However, the mechanisms of puerarin on CAD are still not very clear. In this study, we investigated the role of puerarin on serum nitric oxide (NO) concentration, myocardial endothelial nitric oxide synthase (eNOS) gene expression, the protein expression of eNOS and inducible nitric oxide synthase (iNOS), as well as the level of protein kinase B (Akt/PKB) phosphorylation in rats with myocardial infarction. We found that puerarin (120 mg/kg/day, i.p.) could increase serum nitrite concentration in rat with myocardial ischemia (MI). It also induced the gene expression or activation of eNOS, protein expression of eNOS, and the Akt/PKB phosphorylation. From these results, we suggested that puerarin could increase serum nitric oxide level of rat with myocardial infarction, which should be one of the mechanisms of the therapeutic effect of puerarin on CAD. The increased expression of eNOS and the Akt/PKB pathway may be the underlying mechanism by which puerarin stimulates NO production.

Keywords: puerarin; endothelial nitric oxide synthase; inducible nitric oxide synthase; protein kinase B

1. Introduction

Puerarin (**1**) (Figure 1) is a major active ingredient extracted from the traditional Chinese medicine Ge-gen (*Radix Puerariae*, RP). The uses of Ge-gen described in pharmacopeias and in traditional systems of medicine are for the treatment of fever, pain, diabetes, measles, acute dysentery, diarrhea, etc. Compound **1** has been shown to be able to block the β-adrenoreceptor of isolated organs and in the whole animal. In addition, it possesses anticonvulsive activity. It suppresses alcohol intake, attenuates the hyperthermia produced by 2,4-dinitrophenol, and improves retinal functions. In particular, it has long been used to treat cardiovascular diseases including coronary artery diseases (CAD), arrhythmia, and hypertension.

There is evidence that reduced nitric oxide (NO) availability may play an important role in the pathophysiology both after experimental myocardial ischemia (MI)[1] and in patients with MI.[2] Inhibition of NO production results in impaired endothelium-dependent vasodilation, reduces myocardial neovascularization, augments myocardial remodeling,[4] and impairs myocardial perfusion and efficiency.[3] Furthermore, NO rapidly and reversibly inhibits mitochondrial respiration and limits oxygen consumption by the tissue. The constitutive isoform of endothelial nitric oxide synthase (eNOS) plays a significant role in the regulation of tissue oxygen supply and consumption in cardiac and skeletal muscle microcirculations.[4] eNOS-deficient mice developed more severe left ventricular (LV) dysfunction and remodeling after MI than wild-type (WT) mice did, and vice versa.[5] Endothelial overexpression of eNOS has been shown to attenuate LV dysfunction in mice after MI.[6]

Our and other studies have confirmed that **1** is efficacious to CAD. However, the exact mechanisms of **1** on CAD are still not very clear. We hypothesized that **1** treatment may profoundly increase NO availability after MI, leading to the therapeutic effect.

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2. Results and discussion

2.1 Determination of serum nitrites

Serum nitrite concentration increased in the rats that underwent left anterior descending (LAD) coronary artery occlusion when compared with that in the sham-operated rats ($p < 0.05$). Serum nitrite concentration increased in **1** treatment group when compared with that of the model group ($p < 0.05$) (Figure 2).

2.2 RT-PCR

eNOS mRNA expression was investigated in the cardiac tissue of the sham-operated group, the non-ischemic and ischemic zones of the model group, and the treatment group. In the ischemic zones, eNOS mRNA expression increased significantly in the model and treatment groups when compared with that of the sham-operated group ($p < 0.05$ or $p < 0.01$). The expression of the gene was significantly higher in the treatment group than model group ($p < 0.01$). In the non-ischemic zones, eNOS mRNA expression increased significantly in the model and treatment groups when compared with that of the sham-operated group ($p < 0.05$ or $p < 0.01$). The expression of eNOS increased slightly, but no significant difference was observed in the treatment group when compared with the model group ($p < 0.05$) (Figures 3 and 4).

2.3 Western blot

eNOS, iNOS protein expressions and the level of AKT phosphorylation were investigated in the non-ischemic and ischemic zones of the sham-operated group, the model group, and the treatment group. In the ischemic and non-ischemic zones, the eNOS mRNA expression and the level of AKT phosphorylation were significantly increased in the model group and the treatment group when compared with that of the sham-operated group ($p < 0.01$). The expression of these proteins was significantly higher in the treatment group than the model group ($p < 0.05$ or $p < 0.01$). No significant difference was observed in iNOS protein expression among the sham-operated, model, and treatment groups ($p > 0.05$) (Figures 5 and 6).

2.4 Discussion

Myocardial production of NO is one element in a constellation of physiological regulators of normal cardiac contraction or among the pathogenic mediators of its degradation toward heart failure. NO can modulate most other major inotropic intervention and virtually, all regulatory steps of endothelial cell (EC) coupling.

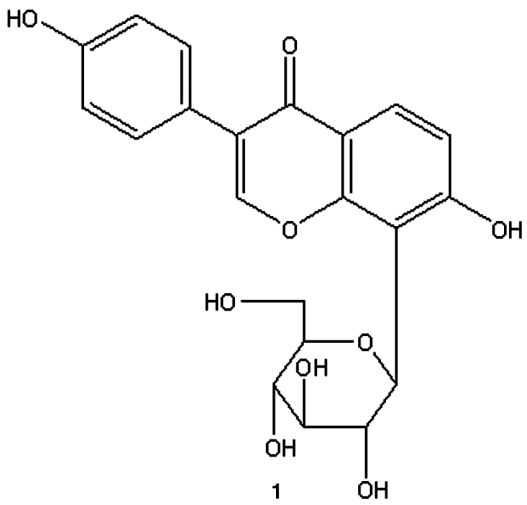

Figure 1. The chemical structure of puerarin.

In addition, the production of NO by eNOS has been shown to play an important role in angiogenesis in many different vascular beds, e.g., fetal myocardium, ischemic hind limb, wound healing, and coronary collateral development. NO is a critical regulatory molecule for physiological angiogenesis that constitutes a naturally occurring, compensatory response to ischemia. Guo *et al.* showed that an exogenous administration of a novel NO donor stimulated the proliferation of cultured rat aortic ECs.[7] Ziche *et al.* suggested that NO may play a role in the angiogenesis elicited by substance P and vascular endothelial growth factor (VEGF).[8]

We recently demonstrated that **1** has a protective effect on MI. Furthermore, **1** can induce angiogenesis *in vivo*[9] and *in vitro*.[10] In this study, we examined whether **1** could accelerate the production of NO in rats with myocardial infarction. We found that **1** could increase the serum nitric oxide level of rats with myocardial infarction. We suggested that the mechanisms of the therapeutic effect of **1** on CAD may be correlated with

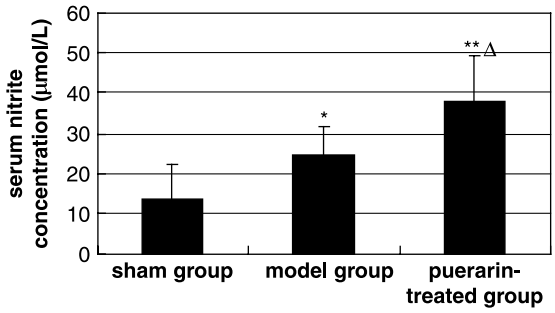

Figure 2. Serum nitrite concentrations in the sham-operated, model and puerarin-treated groups ($n = 7$). Results were expressed as mean ± SD; *$p < 0.05$ and **$p < 0.01$ compared with the sham group, and $^{\Delta}p < 0.05$ compared with the model group.

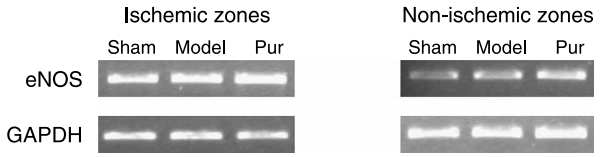

Figure 3. Electrophoresis of RT-PCR fragments of GAPDH and myocardial eNOS mRNA in 1% agarose gel, respectively, in the ischemic and non-ischemic zones. Sham, sham group; Model, model group; Pue, puerarin-treated group.

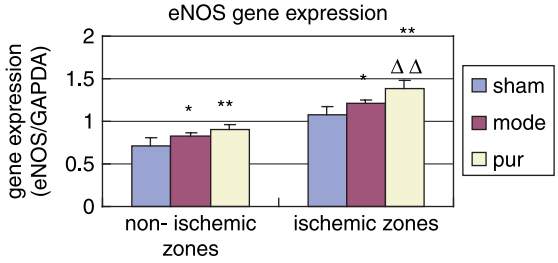

Figure 4. Densitometric data from RT-PCR analysis demonstrating eNOS mRNA expression in the respective ischemic and non-ischemic zones of the sham-operated, model, and puerarin-treated groups. Sham, sham group; Model, model group; Pue, puerarin-treated group. The data were expressed as mean ± SD; $n = 5$, $*p < 0.05$, $**p < 0.01$ compared with the sham operation, $^{\triangle}p < 0.05$ compared with the model.

NO. Synthesis of NO is regulated by a family of isoenzymes, NO synthase (NOS), which share in common the property of converting L-arginine to L-citrulline, yielding free NO gas. Three isoforms of NOS have been identified so far: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Both nNOS and eNOS are constitutively expressed, and their activities are regulated by calcium and calmodulin, whereas iNOS is expressed in response to stimulation by inflammatory cytokines. The eNOS isoform, which was originally characterized in large conduit vessel endothelium, is expressed within the heart in the endocardium and the endothelium of the coronary vasculature, including capillary and venular endothelia.

Protection yielded *via* transgenic overexpression of eNOS could also be credited to the enhanced control of myocardial oxygen consumption.[11] Additional reports have suggested that eNOS-mediated increase in cGMP downregulated adhesion molecule expression and subsequent leukocyte recruitment.[12] In correlation, increased eNOS expression has been shown to be a powerful agent in preconditioning against future ischemic episodes.[13] Cardiomyocyte-specific eNOS overexpression resulting in increased cardiac NO significantly decreases the infarct size beyond that of the systemic overexpression. Site-specific targeting of gene therapy may be more advantageous in limiting myocardial infarction–reperfusion (MI–R) injury and subsequent cardiac dysfunction.[14]

Experiments performed in the animal model indicate that eNOS is essential for angiogenesis in ischemic tissues *in vivo*.[15] Spontaneous angiogenesis developed in response to limb ischemia was severely attenuated in mice lacking the eNOS gene (eNOS-/-).[15]

Previous studies have shown the effect of **1** on eNOS expression and NO production. **1** has been shown to significantly attenuate the decrease of eNOS expression in the aorta of rats fed with high-cholesterol diet[16] and stimulate NO production in the lipopolysaccharide-activated macrophage.[17]

In this study, we investigated the eNOS and iNOS gene and protein expressions in ischemic and non-ischemic myocardia of the rat myocardial infarction model. We found that eNOS gene and protein expressions were induced by **1** in ischemic and non-ischemic myocardia of the rats, but no significant difference was observed in iNOS protein expression. The induction of eNOS expression might explain the principal mechanism through which **1** increased the serum nitric oxide level of rat with myocardial infarction. According to our results, the increase of NO could not be attributed to inflammation, which should induce iNOS expression.

Initial evidence suggested that the activation of eNOS by a sequential activation of phosphoinositide 3-kinase (P13K) and the protein kinase B (Akt/PKB) pathway is the underlying mechanism by which shear stress stimulates NO production in a calcium/calmodulin insensitive manner.[18] Akt is activated by a number of growth factors and cytokines in a phosphatidylinositol-3 kinase-dependent manner and serves as a multifunctional regulator of cell biology, glucose metabolism, and protein synthesis. Akt can be thought of as a major hub in cellular signaling, a central component that connects diverse upstream signaling to even more diverse physiological outputs. Recently, studies have shown

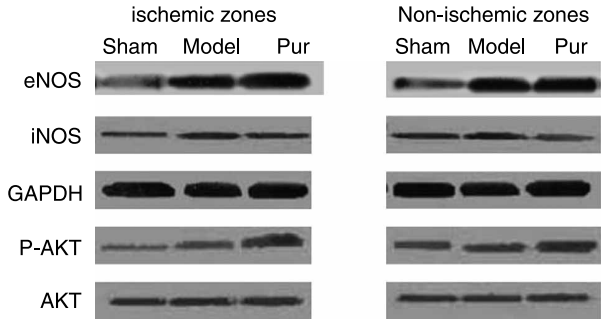

Figure 5. Western blot analysis of eNOS, iNOS, P-AKT, AKT, and GAPDH protein expressions, respectively, in the ischemic and non-ischemic myocardial zones. Sham, sham group; Model, model group; Pue, puerarin-treated group.

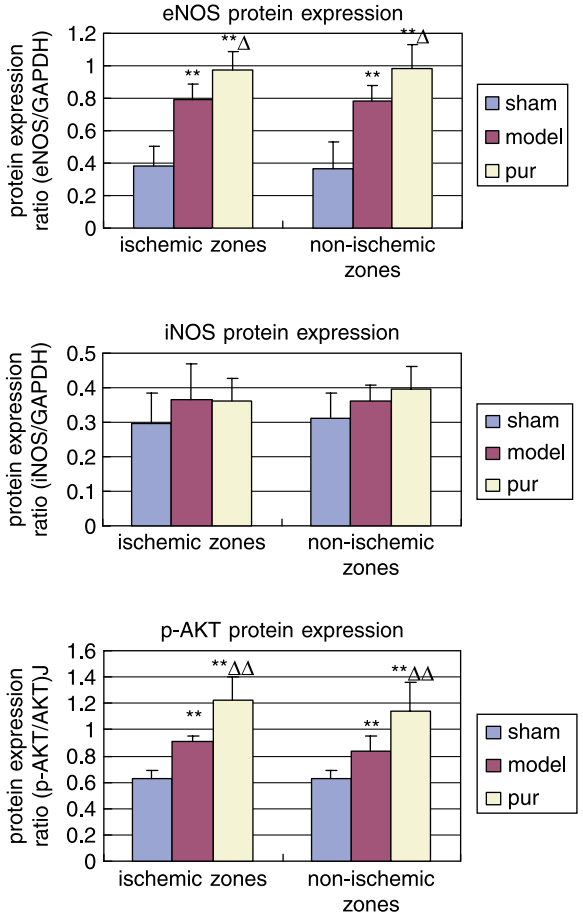

Figure 6. Densitometric data from western blot analysis demonstrating eNOS, iNOS, P-AKT protein expressions, respectively, in the ischemic and non-ischemic myocardial zones of the sham-operated, model, and puerarin-treated groups. Sham, sham group; Model, model group; Pue, puerarin-treated group. The data were expressed as mean ± SD; $n = 5$; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the sham operation, $^{\triangle}p < 0.05$ compared with the model.

that Akt functions downstream of VEGF to promote the survival of ECs and mediates NO production through the direct phosphorylation of eNOS.[19]

We demonstrated earlier that VEGF and eNOS expressions were induced by **1** in myocardium of the rat myocardial infarction model.[9] In this study, we also studied the AKT protein phosphorylation level and found that it was induced by **1** in ischemic and non-ischemic myocardia of the rats with myocardial infarction. We hypothesized that eNOS expression may attribute to the induction of the AKT protein phosphorylation.

Taken together, these results indicated that **1** could increase the serum nitric oxide level of rat with myocardial infarction, which should be one of the mechanisms of the therapeutic effect of **1** on CAD. The increased expressions of eNOS and the Akt/PKB pathway may be the underlying mechanism by which **1** stimulates NO production.

The effect of **1** on NO has not been investigated previously. The finding that **1** stimulates NO production in myocardium of rat with MI is novel and preliminary. The explicit mechanisms of how **1** induces NO production will be further investigated.

3. Materials and methods

3.1 Preparation method of puerarin injection

1.2 g puerarin was dissolved in 100 ml propylene glycol solution (150 g/100 ml), the final concentration was 12 mg/ml. The sample was sonicated for 30 minutes and filtered through a 0.22 μm membrane, then kept in abeyance.

3.2 Rat myocardial infarction model

All experiments were performed on Sprague–Dawley male rats weighing 250–350 g and kept under standardized housing condition. The temperature in the chamber was maintained at 22–24°C. All animals were maintained on a 12 h light/dark cycle.

Rats were anesthetized with urethane (1 g/kg i.p.). The surgical procedure was performed according to the method of Grzegorz Heba *et al.*[20] In brief, the trachea was incised longitudinally and cannulated. The chest was opened under ventilation with room air (Rodent Ventilator TKR-200C) by left thoracotomy. After opening the pericardium, the LAD coronary artery was ligated near its origin by a 6.0 prolene suture. Thereafter, the chest and the skin were closed.

3.3 Treatment

After surgery, the rats were randomly divided into three groups. The model and sham groups consisted of rats treated with propylene glycol solution (150%). The treatment group consisted of rats treated with **1** (Beijing Union Pharmaceutical Factory, purity ≧ 99.80%) at the dosage of 120 mg/kg/day ip. **1** was administered for 30 consecutive days after surgery. The rats in the sham group underwent the same procedure except the LAD ligation. All the rats were killed 30 days after surgery. The hearts were removed and the blood was treated as described below.

3.4 Spectrophotometric determination of serum nitrite concentration

Nitrite (NO_2^-) and nitrate (NO_3^-) are the primary oxidation products of NO; therefore, the concentration

of NO_2^- plus NO_3^- in serum was used as an indicator of the total NO synthesis. Serum nitrate/nitrite level was measured after the enzymatic conversion of NO_3^- to NO_2^- by nitrate reductase according to NO kit instructions (Jingmei BioTech Co. Ltd). Briefly, serum samples were incubated with nitrate reductase and phosphate buffer for 1 h at 37°C. Subsequently, reagents III and IV were added and the mixture was incubated at room temperature for 10 min. Optical density at 530 nm (OD_{530}) was measured and total nitrite concentration for each sample was calculated by comparison with the OD_{530} of a standard nitrite calibration curve.

3.5 RT-PCR for eNOS

The non-ischemic and ischemic cardiac tissues were obtained from the rats of sham-operated, model, and **1** treatment groups. Ischemic zones consisted of normal appearing myocardium surrounding the infarct zone. Non-ischemic zones are normal zones distant from the infarct and peri-infarct zones. The infarct zone could be readily distinguished from the normal myocardium surrounding it by its distinctive pale coloration. In the sham-operated rats, the entire ventricle was quick-frozen.

RNA was isolated from cardiac tissue by the TRIZOL Reagent (Invitrogen). Transmural samples (~100 mg) of ventricle after 30 days of surgery were excised and cleaned of adherent fat in ice-cold 0.9% NaCl solution. RNA was extracted, precipitated, and washed according to the manufacturer's instruction, and stored in DEPC-treated H_2O at −80°C until analysis.

Total RNA (5 μg) was reverse-transcribed in 20 μl volumes using SuperScript™ III Reverse Transcriptase (Invitrogen) with 0.5 μg oligo dT15 (Promega). For each RT product, aliquots (1–2 μl) of the final reaction volume were amplified in two parallel PCR using eNOS- or GAPDH-specific primers and Taq DNA polymerase (Fermentas). The sequences of the primers used were as follows:

eNOS forward: 5′-ATGGCGAAGCGTGTGAAG-3′,
reverse 5′-ATTGTGGCTCGGGTGGAT-3′;
GAPDH forward: 5′-CAACTCCCTCAAGATTGT-CAGC-3′,
reverse 5′-CCCTGTTGCTGTAGCCATATTC-3′.

Reactions were run on an ABI Thermoblock thermocycler. Following an initial denaturation at 94°C for 3 min, eNOS were subjected to 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C (58°C for GAPDH) for 45 s, primer extension at 72°C for 1 min, and a final extension at 72°C for 7 min.

PCR products were analyzed with electrophoresis in TBE buffer with ethidium bromide at 120 V in 0.8% agarose gel. The product sizes of eNOS and GAPDH were 797 and 548 bp, respectively.

The level of gene expression was assessed by densitometric measurement of the amount of PCR products on scanned agarose gels. The units of expression were calculated as the ratio of the amount of PCR product of eNOS mRNA to the amount of PCR product of the constitutively expressed housekeeping gene GAPDH, the amount of which, was assumed to be expressed constantly in the cell. The measurements were made with a Bio-Rad analysis system.

3.6 Western blot for eNOS, iNOS, p-AKT, and AKT protein expressions

Western blot was used to detect the protein expression. Briefly, tissue samples were homogenized in lysis buffer [5 mM EDTA, 1% TritonX-100, 10 mM Tris, 0.1% SDS, 100 mM NaCl, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate (Na_2VO_4), 1 mM PMSF, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 1 μg/ml aprotinin], and the protein content of the lysate was determined using the Coomassie blue protein assay. Forty microgram of protein was separated *via* size filtration using 10% SDS–PAGE and transferred to polyvinylidene difluoride membranes in a semi-dry apparatus.

The membranes were blocked in a 20 mM Tris (pH 7.6), 137 mM NaCl, 0.1% Tween-20 (TBST), and 4% non-fat dry milk for 12 h at 4°C. Membranes were first incubated for 3 h at room temperature in TBST containing primary antibody. The polyclonal rabbit anti-AKT and anti-p-AKT antibodies (cell signaling) were applied at a dilution of 1:1000, the polyclonal rabbit anti-eNOS antibody (BD Transduction Laboratories) and the polyclonal mouse anti-inducible NOS (iNOS) antibody (BD Transduction Laboratories) at 1:1000 dilution, and the anti-GADPH antibody at 1:5000 dilution. Then it was washed three times for 10 min at room temperature in TBST, and finally incubated for 3 h at room temperature in TBST containing secondary antibody (1:5000 dilution).

Following three washes in TBST, immunoreactive bands were detected using the enhanced chemiluminescence (ECL) detection system from PIERCE (Pierce Biotechnology, Inc.) according to the manufacturer's instruction. The protected membrane was placed in a film cassette with the protein side facing up. With all the lights turned off, the film (KODAK, BioMax Light film) was carefully placed on the top of the membrane. The exposure time was adjusted to achieve the optimal results. Finally, the film was developed using the appropriate developing and fixing solution (KODAK).

The level of protein expression was assessed from densitometric data by western blot analysis. The unit of

expression was calculated as the ratio of the amount of western blot analysis of eNOS and iNOS to that of the housekeeping gene GAPDH, the amount of which was assumed to be constant in the cell. The level of AKT phosphorylation was calculated as the ratio of expression of *p*-AKT to that of AKT. The measurement was made with the BIO-RAD analysis system.

3.7 Statistical analysis

All values were given as the mean ± SD. Differences between the groups were calculated by analysis of variance (ANOVA) followed by Duncan's multiple range test or Student's *t*-test, where appropriate. A value of $p < 0.05$ was considered significant.

Acknowledgements

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