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Vasodilatory and anti-inflammatory effects of the 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (PGG) via a nitric oxide–cGMP pathway

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Abstract

Vasorelaxant and anti-inflammatory effects of a 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (PGG) isolated from the root barks of *Paeonia suffruticosa* and possible mechanisms responsible were investigated. PGG induced a concentration-dependent relaxation of the phenylephrine-precontracted rat aorta. This effect disappeared with the removal of functional endothelium. Pretreatment of the aortic tissues with either N^G-nitro-L-arginine methyl ester (L-NAME) or 1H-[1,2,4]-oxadiazole-[4,3-α]-quinoxalin-1-one (ODQ) inhibited the relaxation induced by PGG. Incubation of human umbilical vein endothelial cells (HUVECs) or carotid arteries isolated from rats with PGG increased the production of cGMP in a dose-dependent manner, but this effect was blocked by pretreatment with L-NAME and ODQ, respectively. PGG treatment attenuated tumor necrosis factor-α (TNF-α)-induced nuclear factor-kappaB (NF-κB) p65 translocation in human umbilical vein endothelial cells. In addition, PGG suppressed the expression levels of adhesion molecules including intracellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) induced by TNF-α. TNF-α-induced monocyte chemoattractant protein-1 (MCP-1) expression was also attenuated by addition of PGG. PGG treatment inhibited cellular adhesion of U937 cells onto human umbilical vein endothelial cells induced by TNF-α. Taken together, the present study suggests that PGG dilates vascular smooth muscle and suppresses the vascular inflammatory process via endothelium-dependent nitric oxide (NO)/cGMP signaling.

Keywords: PGG (1,2,3,4,6-penta-*O*-galloyl-β-D-glucose); Vasorelaxation; Nitric oxide/cGMP (guanosine 3′, 5′,-cyclic monophosphate); Cell adhesion

1. Introduction

Endothelial cells respond to various neurohumoral and physical stimuli by releasing endothelium-dependent vasodilators such as endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980), prostacyclin (Jaffe, 1985), and endothelium-derived hyperpolarizing factor (EDHF) (Beny and Brunet, 1988). EDRF has been identified as nitric oxide (NO), which is produced in a reaction catalyzed by nitric oxide synthase (NOS) using L-arginine as a substrate (Palmer et al., 1988). NO activates soluble guanylyl cyclase and thereby, indirectly increases the production of guanosine 3′, 5′,-cyclic monophosphate (cGMP). This leads to protein kinase G activation, which inhibits Ca^{2+} influx and decreases the sensitivity of contractile elements to Ca^{2+} (Sato et al., 1988). In addition to its well-known vasodilating activity, endothelial NO inhibits platelet aggregation, thrombogenesis, leukocyte adhesion, and proliferation of vascular smooth muscle cells (Cohen, 1995). Endothelial dysfunctions that are closely related with impaired NO may represent an early stage of vasculopathy that can lead to atherosclerotic cardiovascular disorders (Egashira et al., 1993). Impaired release of NO from vascular beds results in increased leukocyte–endothelium interactions

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via an up-regulation of endothelial cell adhesion molecules including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (De Caterina et al., 1995; Cartwright et al., 1997). Under these conditions, numerous leukocytes adhere to vascular endothelium, transmigrate the endothelium, and aggravate endothelial dysfunction and tissue injury (Nakache et al., 1989). On the contrary, systemic administration of NO donors to NO-deficient animals preserves endothelial function and attenuates pathological interactions between circulating leukocytes and vascular endothelium (Granger and Kubes, 1994; De Caterina et al., 1995).

While conducting an in vitro screening of active components isolated from Chinese medicinal plants, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (PGG) was found to exhibit a distinct vasorelaxant activity. PGG, a polyphenolic compound, has been known to be a major constituent of the root cortex of *Paeonia suffruticosa* ANDREWS, which is an important traditional Chinese crude drug possessing the effects of curing inflammatory diseases and invigorating blood circulation. Growing epidemiological evidences suggest the existence of a negative correlation between consumption of polyphenol-rich foods and the incidence of cardiovascular diseases (Renaud and de Lorgeril, 1992; Osakabe et al., 2000). Recently, Oh et al. (2003) demonstrated that PGG suppresses interleukin-8 (IL-8) expression through the inactivation of the proinflammatory transcription factor nuclear factor-kappaB (NF-κB). However, to our best knowledge, the effects of PGG on the vascular system have not been examined previously. The present study, therefore, was designed to examine the effects of PGG on the vascular tone and expression levels of cytokine-induced proinflammatory and adhesion molecules.

2. Materials and methods

2.1. Isolation of PGG

PGG (Fig. 1) was isolated from the root cortex of *P. suffruticosa* ANDREWS and the structure of PGG was identified by the comparison of spectral properties (MS, [^{1}H] NMR, and [^{13}C] NMR) with those reported in the literature (Oh et al., 2001).

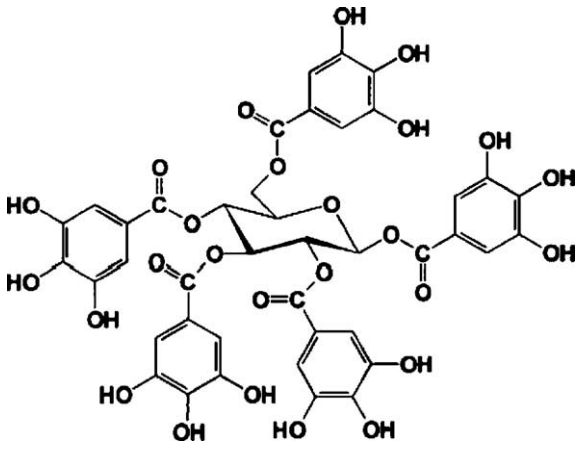

Fig. 1. Chemical structure of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (PGG) isolated from the root cortex of *P. suffruticosa* ANDREWS.

2.2. Preparation of aortic rings

The animal procedures were in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Utilization Committee for Medical Science of Wonkwang University. Male Sprague–Dawley rats were purchased from Korean Experimental Animals Co. (Daejeon, Korea). The rats (weighing 250–300 g) were sacrificed by decapitation. The thoracic aortae were rapidly and carefully dissected and placed into ice-cold Krebs solution (pH 7.4) containing 118 mmol/l NaCl, 4.7 mmol/l KCl, 1.1 mmol/l $MgSO_4$, 1.2 mmol/l KH_2PO_4, 1.5 mmol/l $CaCl_2$, 25 mmol/l $NaHCO_3$, and 10 mmol/l Glucose. The aortae were removed free of connective tissue and fat, and then cut into rings with a width of approximately 3 mm. All dissecting procedures were done with extreme care to protect the endothelium from inadvertent damage. In some aortic rings, the endothelial layer was mechanically removed by gently rubbing the luminal surface of the aortic ring back and forth several times with plastic tubing. Endothelial integrity or functional removal was verified by the presence or absence, respectively, of the relaxant response to 3×10^{-6} M acetylcholine on phenylephrine (3×10^{-6} M) contracted vessels.

2.3. Recording of isometric vascular tone

The aortic rings were suspended, by means of two L-shaped stainless-steel wires inserted into the lumen, in a tissue bath containing Krebs solution (pH 7.4) at 37 °C. 95% O_2–5% CO_2 was continuously bubbled through the bath. The baseline load placed on the aortic rings was 2.0 g. Changes in isometric tension were recorded using a force–displacement transducer (Grass FT 03, Quincy, MA, USA) connected to a Grass polygraph recording system (Model 7E). In the first set of experiments, the aortic rings were contracted with phenylephrine (3×10^{-6} M) to obtain a maximal response. The aortic rings were then washed every 20 min with Krebs solution until the tension returned to the basal level. A concentration-dependent response curve to acetylcholine (10^{-9}–10^{-5} M) was generated as a positive control for endothelium-intact aortic rings contracted by 3×10^{-6} M phenylephrine. The rings were then exposed to various drugs for 30 min and aortic relaxation was carried out by the cumulative addition of PGG. The effect of vehicle, <0.2% dimethylsulfoxide (DMSO), was also tested. After each test, the aortic rings were washed three times with fresh Krebs solution and allowed to equilibrate for 30 min.

2.4. Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated as described previously (De Martin et al., 1993). The

cells were grown in a gelatin-coated 75-cm^2 flask in RPMI-1640 (GIBCO-BRL, Eggenstein, Germany) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 mg/ml streptomycin, and 5 units/ml heparin at 37 °C under 5% CO_2 and 95% air. The cells used in this study were between passages 3 to 6. Cells grew to confluence in about 2 days. Cells were then serially passaged with 0.25% trypsin/1 mM EDTA (GIBCO-BRL). U937 cells (American Type Culture Collection, Bethesda, MD, USA) were also grown in a gelatin-coated 75-cm^2 flask in RPMI-1640 (GIBCO-BRL) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 mg/ml streptomycin, and 5 units/ml heparin at 37 °C under 5% CO_2 and 95% air.

2.5. Measurement of cGMP production

To determine the effect of PGG on cGMP production in human umbilical vein endothelial cells, human umbilical vein endothelial cells were incubated for 10 min with PGG in Dulbecco's modified Eagle's medium (DMEM) containing 0.1 mM 3-isobutyl-1-methylxanthine (IBMX). Removing the supernatant stopped the incubation. The cells were then immediately extracted with 0.7 ml ice-cold trichloroacetic acid (6%) and scraped off. The cell suspension was then ultrasonicated for 10 s. before being centrifuged for 5 min at 8000×*g*. Supernatants were extracted with two volumes of water-saturated diethylether and the samples were vacuum dried. To determine the effect of PGG on cGMP production in carotid arteries, after equilibration of the vessels for 30 min in Krebs solution gassed with 95% O_2–5% CO_2, rings were incubated in 1 ml of fresh Krebs solution containing 0.1 mM IBMX, at shaking constant temperature water bath (37 °C). The vessels were then allowed to equilibrate for an additional 30 min before addition of phenylephrine (3×10^{-6} mol/L). After PGG or vehicles were subjected for 10 min, reactions were stopped by removal of the tissues. The tissues were frozen rapidly and then homogenized with Polytron homogenizer in 20 mmol/l Tris–HCl buffer (pH 7.4). The homogenates were centrifuged at 3000×*g* for 10 min, and the protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard. The supernatants were extracted four times with two volumes of water-saturated diethylether and then concentrated with speed-vac concentrator (Sarvant Instrument, Farmingdale, NY, USA). cGMP content was measured by an equilibrated radioimmunoassay (RIA) as described previously (Kim et al., 1998). In brief, standards or samples were introduced in a final volume of 100 μl of 50 mM sodium acetate buffer (pH 4.8). Then, 100 μl of diluted cGMP antiserum (Calbiochem-Novabiochem Co., San Diego, CA, USA) and iodinated cGMP (10,000 cpm/100 μl, specific activity 2,200 Ci/mM, Dupont-New England Nuclear, Wilmington, DE, USA) were added in succession and incubated for 24 h at 4 °C. The bound form was separated from the free form by charcoal suspension. Results were expressed as picomoles of cGMP generated per milligram of protein (pmol/mg of protein).

2.6. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from 1×10^6 cells according to the manufacture's instructions (Chomczynski and Sacchi, 1987). Briefly, Trizol (Sigma, Missouri, USA) was added to the plates to lyses the cells, and then the cells were transferred to a microcentrifuge tube. Chloroform was added and total RNA was collected in the aqueous phase after centrifugation. Finally, RNA was precipitated by isopropyl alcohol, and then re-dissolved in diethyl pyrocarbonate (DEPC)-treated water. The OD260 and OD260/280 values were measured with a spectrophotometer to determine the RNA concentrations. Reverse transcription was performed at 42 °C for 60 min and followed by incubation at 95 °C for 5 min. The reaction mixture (50 μl of total volume) consisted of 5 μg of total RNA, 5 mM of $MgCl_2$, 10 mM of Tris–HCl, pH 9.0, 50 mM of KCl, 0.1% Triton X-100, 1 mM of dNTP Mixtures, 1 units/μl recombinant RNasin ribonuclese inhibitor, 15 U/μg of avian myeloblastosis virus (AMV) reverse transcriptase and 0.5 μg of oligo$(dT)_{18}$ primer. cDNA samples were analyzed for the specific cDNA of VCAM-1, ICAM-1, monocyte chemoattractant protein-1 (MCP-1), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) by PCR amplification using specific primers (Table 1). 5 μl of cDNA was added to 50 μl of PCR mixture containing 33.5 μl of H_2O, 1 μl of 5′ primer (10 pM), 1 μl of 3′ primers, 2 μl of dNTPs (2.5 mM), 5 μl of 10×PCR buffer (the final Mg^{2+}), 0.5 μl of Taq DNA polymerase (5 U/μl). The following conditions were used for PCR amplification: 35 cycles×1 min 94 °C, 1 min at 62 °C, and 1 min at 72 °C for VCAM-1; 30 cycles×1 min 94 °C, 1 min at 64 °C, and 1 min at 72 °C for ICAM-1; 30 cycles×30 s 94 °C, 30 s at 60 °C, and 30 s at 72 °C for MCP-1; 35 cycles×1 min 94 °C, 1 min at 55 °C, and 1 min

Table 1
Primers for RT-PCR amplification of cDNA fragments

Gene	Primer sequences	Size (bp)	References
VCAM-1	5′-CCCTTGACCGGCTGGAGATT-3′ 5′-CTGGGGGCAACATTGACATAAAGTG-3′	241	Zapolska-Downar et al., 2004
ICAM-1	5′-CAGTG ACCATCTACAGCTTT CCGG-3′ 5′-GCTGCTACCACAGTGATGATGACAA-3′	555	Madan et al., 2001
MCP-1	5′-ATGAAAGTCTCTGCCGCC-3′ 5′-TTG CTTGTCCAGGTGGTC-3′	290	Oh et al., 2003
GAPDH	5′- TCA TTG ACC TCA ACT ACA TG-3′ 5′-CAA AGT TGT CAT GGA TGA CC-3′	460	Chen et al., 2004

at 72 °C for GAPDH. 15 μl of PCR product from each sample were electrophoresed on a 1.2% agarose gel containing 0.5 μg/ml ethidium bromide. Gels were visualized and photographed by a Chemi-Doc image analyzer (Bio-Rad, Hercules, CA, USA). The housekeeping gene GAPDH was used for normalization. The ratios of the emissions incorporated into the PCR products of the tested gene to the GAPDH products were calculated to evaluate relative changes in the mRNA expression levels of the tested genes.

2.7. Preparation of nuclear extracts and Western blot analysis

Human umbilical vein endothelial cells were washed twice with ice-cold PBS and scraped in 1 ml of the same buffer. After centrifugation at 10,000×*g*, the cell pellet was suspended in ice-cold hypotonic lysis buffer (10 mmol/l HEPES pH 7.9, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l KCl 0.2 mmol/l phenylmethylsulphonylfluoride, 0.5 mmol/l dithiothreitol), vortexed for 10 s and then centrifuged at 10,000×*g* for 5 min. The packed cells were suspended in ice-cold hypotonic lysis buffer in the presence of 50 μl of 10% Nonidet P-40 and then kept on ice for 25 min. The nuclear fraction was precipitated by centrifugation at 10,000×*g* for 15 min. The supernatants, corresponding to the cytosolic fraction, were transferred to fresh tubes and assayed for protein content by the Bradford (1976) method. The nuclei pellet was resuspened in 50–100 μl of low salt extraction buffer (20 mmol/l HEPES pH 7.9, 1.5 mmol/l $MgCl_2$, 25% glycerol, 20 mmol/l KCl 0.2 mmol/l, EDTA 0.2 mmol/l phenylmethylsulphonylfluoride, 0.5 mmol/l dithothreitol) and added to an equal volume of high salt extraction buffer (20 mmol/l HEPES pH 7.9, 1.5 mmol/l $MgCl_2$, 25% glycerol, 80 mmol/l KCl 0.2 mmol/l EDTA 0.2 mmol/l phenylmethylsulphonylfluoride, 0.5 mmol/l dithothreitol) in a dropwise fashion, and then incubated under continuous shaking at 4 °C for 45 min. The sample was centrifuged for 20 min at 10,000×*g*. The nuclear extract was aliquoted and store at −80 °C. Protein samples (50 μg) were electrophoretically fractionated with a discontinuous system consisting of 10% polyacrylamide resolving gels and 5% stacking gels, and then transferred to nitrocellulose membranes (Amersham, Buckinghamshire, England) at 20 V and 100 mA (current constant) overnight. The membranes were washed, blocked, and then incubated with primary antibodies (1:2000 dilution) against NF-κB p65 and β-actin proteins (Santa Cruz Biotechnology, USA), respectively. The bound horseradish peroxidase-conjugated secondary antibody was detected by an enhanced chemiluminescence (Amersham, Buckinghamshire, England) procedure. Protein expression levels were determined by analyzing the signals captured on the nitrocellulose membranes (Amersham, Buckinghamshire, England) using a Chemi-doc image analyzer (Bio-Rad, Hercules, CA, USA).

2.8. Cell adhesion assay

The cell adhesion assay was modified as described (Yang et al., 2004). Briefly, regularly passaged U937 cells were labeled with 10 μg/ml 2′,7′-bis(carboxylethyl)-5(6)-carboxyfluorescein acethoxymethyl ester (BCECF/AM, Sigma) at 10 μM final concentration in RPMI 1640 medium containing 10% FBS at 37 °C for 30 min. The labeled cells were harvested by centrifugation and washed three times with phosphate-buffered saline (PBS) before suspension in the medium, and added to human umbilical vein endothelial cells in six-well culture plates at 4×10^5 cells/ml. The co-incubation was done at 37 °C for 30 min and unbound U937 cells were removed by stringent washing two times with PBS. U937cells bound to human umbilical vein endothelial cells were lysed with 0.1% SDS and fluorescence was measured on a spectrofluorometer at 485 nm excitation and 530 nm emission wavelength.

2.9. Reagents

Acetylcholine chloride, phenylephrine HCl, N^G-nitroarginine methyl ester (L-NAME), methylene blue hydrate, 1H-[1,2,4]-oxadiazole-[4,3-α]-quinoxalin-1-one (ODQ), indomethacin, glibenclamide, tetraethylammonium (TEA) chloride, 3-isobutyl-1-methylxanthine (IBMX), (±)-verapamil HCl, atropine, and (±)-propranolol HCl were purchased from Sigma Chemical Co.(St. Louis, MO, USA). Acetylcholine, phenylephrine, L-NAME, methylene blue, IBMX, and atropine were dissolved in distilled water. Stock solutions of indomethacin, ODQ, TEA, propranolol, and verapamil were dissolved in

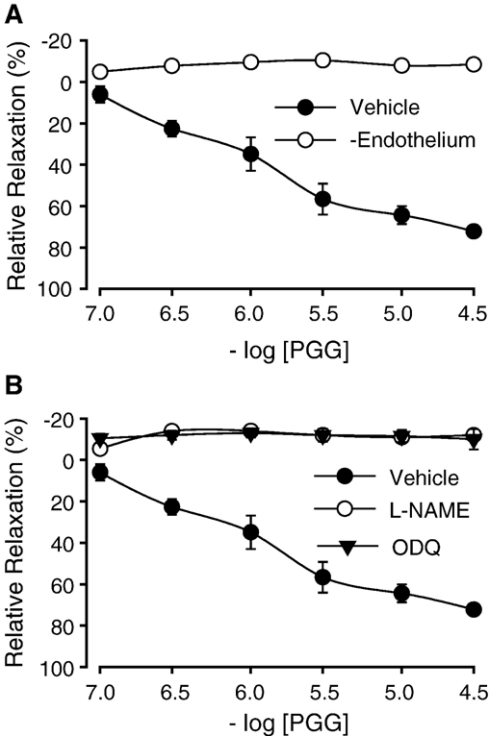

Fig. 2. Concentration–response curves for the relaxant effect of PGG in phenylephrine contracted endothelium-intact or endothelium-denuded aortic ring (-endothelium) (A). Concentration–response curves for the relaxant effect of PGG in phenylephrine contracted endothelium-intact (vehicle) aortic rings in the absence (vehicle) or presence of 1×10^{-5} M N^G-nitro-L-arginine methyl ester (L-NAME) or 1×10^{-6} M 1H-[1,2,4]-oxadiazole-[4,3-α]-quinoxalin-1-one (ODQ) (B). Each value shows mean ± S.E.M. of six experiments.

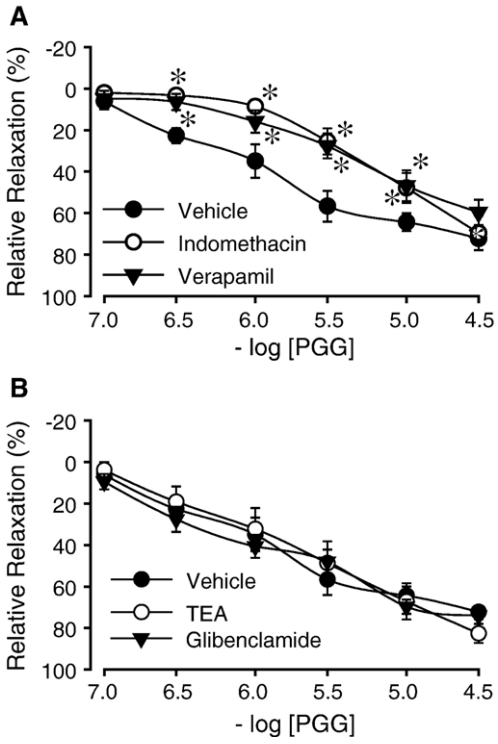

Fig. 3. Concentration–response curves for the relaxant effect of PGG in the endothelial intact aortic rings in the presence of indomethacin (1×10^{-5} M) or verapamil (1×10^{-6} M) (A), and glibenclamide (1×10^{-6} M) or tetraethylammonium (TEA) (1×10^{-4} M) (B). Each value shows mean±S.E.M. of six experiments. *$P<0.01$, vs. vehicle.

dimethylsulfoxide (DMSO); working solutions were made in Krebs solution. Control experiments demonstrated that the highest DMSO level (0.2%) had no effect on vascular smooth muscle contraction.

2.10. Statistical analysis

Relaxant responses are expressed as percentage relaxation from phenylephrine precontraction levels unless otherwise described in the figure legends. Results were expressed as means±S.E.M. The statistical significance of the difference between the group means was determined using the one-way ANOVA and Student's *t*-test.

3. Results

3.1. Effect of PGG on vascular tone

With endothelium-intact aortic preparations, PGG relaxed phenylephrine (3×10^{-6} M) precontracted aortic rings in a dose-dependent manner (Fig. 2A). The maximal relaxant effect of PGG was 77.2±2.2% (vs. phenylephrine contraction) under the concentration of 3×10^{-5} mol/l. The relaxant effect of PGG in aortic tissue was completely abolished by denudation of the endothelial layer. Pretreatment of aortic tissue with L-NAME (1×10^{-5} M) completely inhibited the PGG-induced relaxation (Fig. 2B). ODQ (1×10^{-6} M), an inhibitor of soluble guanylyl cyclase, also completely blocked the concentration-dependent relaxation induced by PGG. PGG-induced endothelium-dependent vascular relaxation was attenuated by addition of verapamil (1×10^{-6} M), a L-type Ca^{2+} channel blocker, or indomethacin (1×10^{-5} M) (Fig. 3A). On the contrary, the relaxant effect of PGG was not altered by pretreatment with TEA (1×10^{-4} M), a nonselective K^+-channel blocker, or glibenclamide (1×10^{-5} M), an ATP-sensitive K^+-channel blocker (Fig. 3B).

3.2. Effect of PGG on the production of cGMP in carotid arteries and HUVECs

To confirm whether a NO/cGMP pathway is involved in the PGG-induced endothelium-dependent relaxation, the effect of PGG on the accumulation of cGMP in the carotid arteries and human umbilical vein endothelial cells was determined. As shown in Fig. 4A, incubation of carotid arteries with PGG increased the accumulation of cGMP in a dose-dependent manner, but this effect was blocked by pretreatment of carotid arteries with L-NAME (1×10^{-5} M) or ODQ (1×10^{-6} M) (Fig. 4). Incubation of human umbilical vein endothelial cells with PGG also increased the accumulation of cGMP in a dose-dependent manner, but this effect was blocked by pretreatment of human umbilical vein endothelial cells with L-NAME (1×10^{-5} M) or ODQ (1×10^{-6} M) (Fig. 5).

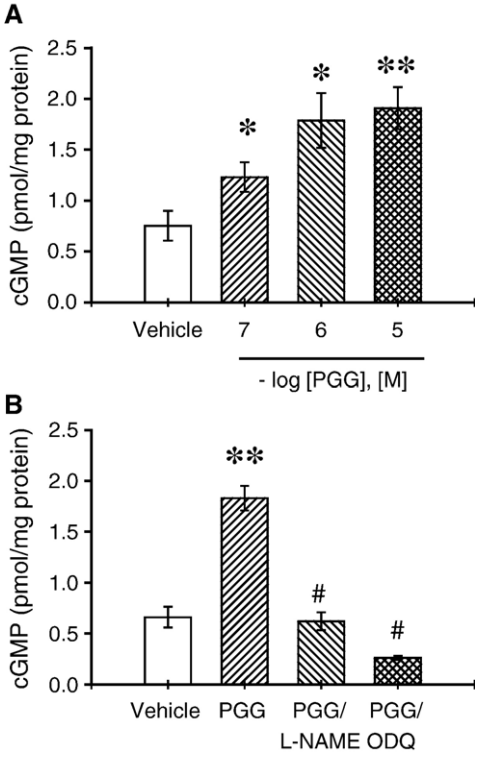

Fig. 4. Concentration-dependent cGMP production by PGG (A) and effects of L-NAME (1×10^{-5} M) and ODQ (1×10^{-6} M) on PGG (1×10^{-5} M)-induced cGMP production (B) in carotid arteries isolated from rats. Each value shows mean±S.E.M. of six experiments. *$P<0.05$, **$P<0.01$ vs. vehicle group. #$P<0.01$, vs. PGG-treated group (PGG).

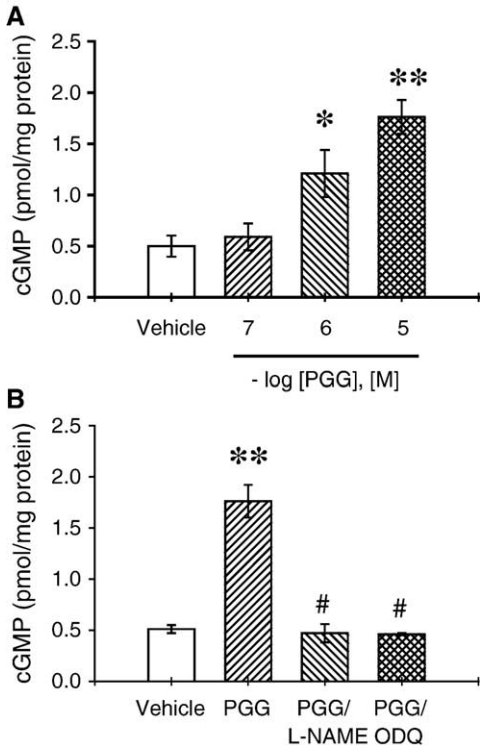

Fig. 5. Concentration-dependent cGMP production by PGG (A) and effects of L-NAME (1×10^{-5} M) and ODQ (1×10^{-6} M) on PGG-induced cGMP production (B) in human umbilical vein endothelial cells. Each value shows mean±S.E.M. of six experiments. *$P<0.05$, **$P<0.01$ vs. vehicle group. $^{\#}P<0.01$, vs. PGG-treated group (PGG).

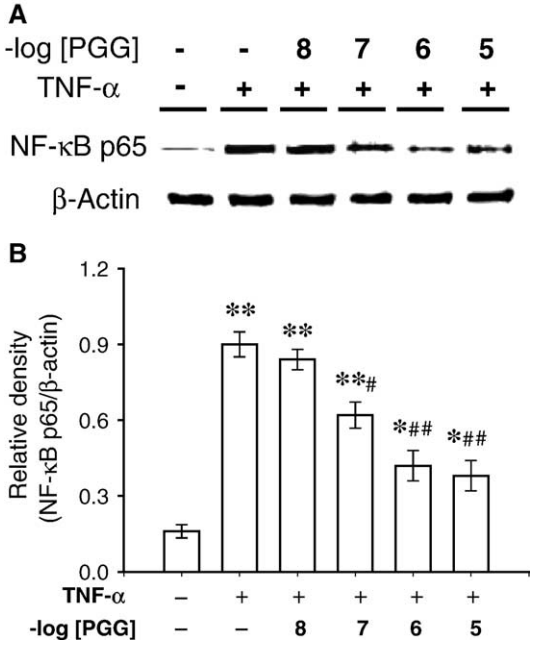

Fig. 6. Representative Western blot (A) and densitometric analyses (B) of inhibitory activity of PGG on the TNF-α-induced NF-κB p65 translocation in the nuclear fraction of human umbilical vein endothelial cells. Each value shows mean±S.E.M. of four experiments. *$P<0.05$, **$P<0.01$ vs. vehicle group. $^{\#}P<0.05$, $^{\#\#}P<0.01$, vs. TNF-α (10 ng/ml)-treated group.

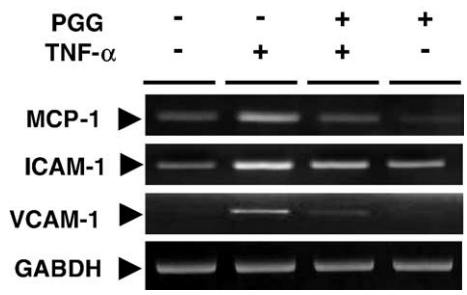

Fig. 7. Representative RT-PCR analysis demonstrating the effect of PGG (1×10^{-5} M) on the TNF-α-induced MCP-1, ICAM-1, and VCAM-1 mRNA expressions in human umbilical vein endothelial cells.

3.3. Effects of PGG on the expression levels of tumor necrosis factor-α (TNF-α)-induced NF-κB, MCP-1, ICAM-1, and VCAM-1 in human umbilical vein endothelial cells

To study the effect of PGG on TNF-α-induced NF-κB activation by human umbilical vein endothelial cells, Western blot analysis of the nuclear fraction of cell lysates was performed. As shown in Fig. 6, the expression levels of NF-κB in the nuclear fractions of human umbilical vein endothelial cells were increased by treatment with TNF-α (10 ng/ml). However, pretreatment of human umbilical vein endothelial cells with PGG attenuated TNF-α-induced nuclear NF-κB expression by human umbilical vein endothelial cells in a dose-dependent manner. The expression level of MCP-1 in human umbilical vein endothelial cells, determined by RT-PCR, was also augmented by treatment with TNF-α. Pretreatment of human umbilical vein endothelial cells with PGG (1×10^{-4} mol/l) blocked a TNF-α-induced increase of MCP-1 expression (Fig. 7). TNF-α-induced expression levels of VCAM-1 and ICAM-1 in human umbilical vein endothelial cells were also attenuated by pre-treating with PGG (Fig. 7).

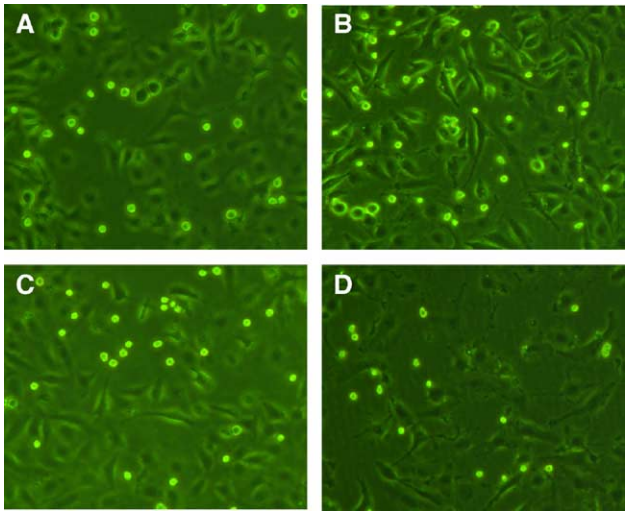

Fig. 8. Effect of PGG on the adhesion of U937 cells to TNF-α-stimulated human umbilical vein endothelial cells. (A) Vehicle; (B) TNF-α (10 ng/ml) treated; (C) co-treated with TNF-α (10 ng/ml) and PGG (1×10^{-6} M); and (D) co-treated with TNF-α (10 ng/ml) and PGG (1×10^{-5} M) in human umbilical vein endothelial cells.

3.4. PGG inhibits adhesion of U937 cells to TNF-α-stimulated human umbilical vein endothelial cells

To explore the effect of PGG on endothelial cell-leukocyte interaction, we examined the adhesion of U937 cells to TNF-α-activated human umbilical vein endothelial cells under static conditions. Control confluent human umbilical vein endothelial cells showed minimal binding to U937, but adhesion was markedly increased when the human umbilical vein endothelial cells were treated with TNF-α. Pretreatment of human umbilical vein endothelial cells with 1×10^{-6} or 1×10^{-5} mol/l PGG reduced the number of U937 cells adhering to TNF-α-stimulated human umbilical vein endothelial cells (Fig. 8).

4. Discussion

The present study showed that PGG exerts a vasorelaxant effect on phenylephrine-contracted aortic rings from Sprague–Dawley rats. Removing functional endothelium abolishes the relaxant response to PGG, suggesting that the vasorelaxation caused by PGG was endothelium-dependent. To verify the involvement of endothelium-derived vasodilators, the effects of various inhibitors on PGG-induced vascular relaxation were examined. Pretreatment of aortic tissues with L-NAME, a nitric oxide synthase inhibitor, abolished the PGG-induced vascular relaxation. The present study also showed that pretreatment with ODQ, a soluble guanylyl cyclase inhibitor, completely blocked the vascular relaxation induced by PGG. These results suggest that the PGG-induced vascular relaxation is closely related with the activation of a NO-cGMP pathway. To determine whether prostacyclin is involved in PGG-induced vasorelaxation, we also assayed the effects of indomethacin on PGG-induced relaxation. The endothelium-dependent relaxation caused by PGG in aortic rings was attenuated by indomethacin, indicating that vasoactive prostacyclin (PGI_2) may contribute to the PGG-induced relaxation (Gordon and Martin, 1983). Since calcium and potassium channels play an essential role in NO synthesis and release in endothelial cells (Loeb et al., 1988; Standen et al., 1989), we tested the effects of calcium and potassium channel blockers on PGG-induced vascular relaxation. Because PGG-induced relaxation was not inhibited by glibenclamide or tetraethylammonium (TEA), our results indicate that ATP- or TEA-sensitive K^+ channels do not play a significant role. However, pretreatment with verapamil, a voltage-gated calcium channel blocker, attenuated the relaxant response to PGG in aortic tissues. Therefore, PGG-induced relaxation may involve L-type Ca^{2+} channel function.

Relaxation of vascular smooth muscle by NO-cGMP signaling involves a sequence of steps. Nitric oxide is formed in the endothelium by the activation of nitric oxide synthase using L-arginine as a substrate. Once formed, nitric oxide diffuses out of the endothelium with some entering the underlying vascular smooth muscle where it binds to and activates soluble guanylyl cyclase. This enzyme catalyzes the conversion of GTP to cGMP. cGMP-activated protein kinase G inhibits Ca^{2+} influx, augments Ca^{2+} sequestration and decreases the sensitivity of contractile elements to Ca^{2+} (Sato et al., 1988). The present study shows that exposing endothelium-intact aortic rings to PGG increases the tissue accumulation of cGMP. This increase was abolished by pretreatment with either L-NAME or ODQ, suggesting a significant role for endothelium/NO-cGMP signaling in PGG-induced vascular relaxation. These findings are consistent with the hypothesis that PGG-induced vascular relaxation is due to the activation of a vascular NO/cGMP system, which may be causally related with L-type Ca^{2+} channels (Loeb et al., 1988).

In addition to its vasodilating activity, it has been well documented that an endothelial NO/cGMP system serves as an endogenous defense mechanism against vascular inflammation. NO inhibits platelet aggregation, thrombogenesis, leukocyte adhesion, and proliferation of vascular smooth muscle cells (Cohen, 1995). Impaired release of NO from vascular beds results in increased leukocyte–endothelium interactions via the up-regulation of endothelial cell adhesion molecules including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (De Caterina et al., 1995; Cartwright et al., 1997). Under these conditions, numerous leukocytes adhere to vascular endothelium and transmigrate the endothelium, thus aggravating the endothelial dysfunction and tissue injury leading to atherosclerotic cardiovascular disorders (Nakache et al., 1989; Zeiher et al., 1995). Systemically administering NO donors to NO-deficient animals preserves endothelial function and attenuates pathological interactions between circulating leukocytes and vascular endothelium (Granger and Kubes, 1994; De Caterina et al., 1995). If PGG-induced vascular relaxation is mainly due to the activation of NO/cGMP signaling, PGG may be useful in preventing the vascular inflammatory process. In the present study, we found that PGG treatment blocks NF-κB p65 translocation in human umbilical vein endothelial cells in a dose-dependent manner. NF-κB is a critical signal molecule for the inflammatory process and a ubiquitously expressed multiunit transcription factor that is activated by diverse signals, possibly via phosphorylation of the I-κB subunit and its dissociation from the inactive cytoplasmic complex, followed by translocation of the active dimmer, p50 and p65, to the nucleus (Gosh and Baltimore, 1990; Geng, 2001).

In addition, we demonstrated that PGG inhibits VCAM-1 and ICAM-1 expressions in human umbilical vein endothelial cells. These adhesion molecules play a critical role in adhesion and recruitment of mononuclear leukocytes during times of vascular inflammation, and have been linked to the early phase of atherosclerosis (O'Brien et al., 1993). Expression of adhesion molecules can be regulated by endothelial NO; increased levels of NO are associated with decreased expression of the adhesion molecules. Indeed, exogenous NO donors might down-regulate adhesion molecules in vitro (Khan et al., 1996) or in vivo (Ahluwalia et al., 2004). Regulation of adhesion molecule expression occurs at the transcriptional level and is mediated via the transcription factor NF-κB. From this point of view, inhibition of adhesion molecule expression by PGG might in part be due to enhanced NO/cGMP signaling via inhibition of NF-κB expression in human umbilical vein endothelial cells.

Since monocyte chemoattractant protein 1 (MCP-1) plays a key role in the subendothelial recruitment of monocytes or lymphocytes and plays an essential pathogenic role in vascular diseases including atherosclerosis (Nelken et al., 1991; Kataoka et al., 2004), we tested whether PGG modulates the expression of MCP-1 in cultured human umbilical vein endothelial cells. Treatment of human umbilical vein endothelial cells with PGG blocked a TNF-α-induced increase of MCP-1 expression. Previous reports have shown that NO could down-regulate MCP-1 expression in endothelial cells (Zeiher et al., 1995; Desai et al., 2003; Kataoka et al., 2004). Another report suggests that putative binding sites for NF-κB exists in the 5′-flanking region of the MCP-1 gene, suggesting that MCP-1 transcription is regulated by NF-κB (Shyy et al., 1993). Our data suggest that the attenuation of MCP-1 expression by PGG was due to the activation of NO/cGMP signaling and the suppression of NF-κB activation.

To elucidate whether PGG could suppress adhesion between lymphocytes and endothelial cells, we examined the adhesion of U937 cells to TNF-α-activated human umbilical vein endothelial cells. Pretreatment of human umbilical vein endothelial cells with PGG reduced the number of U937 cells adhering to TNF-α-stimulated human umbilical vein endothelial cells. This finding is consistent with the results showing that the expression levels of the proinflammatory and adhesion molecules were suppressed by treatment with PGG.

Taken together, the present data suggest that PGG dilates vascular smooth muscle via activating NO/cGMP signaling. PGG treatment not only suppresses a TNF-α-induced increase of proinflammatory and adhesion molecules in human umbilical vein endothelial cells, but also reduces the adhesion between U937 cells and human umbilical vein endothelial cells. PGG might have the potency to dilate vascular tissues and suppress the vascular inflammatory process, which may be closely related with the activation of vascular NO/cGMP signaling.

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