Anti-atherosclerotic Activity of Platycodin D Derived from Roots of Platycodon grandiflorum in Human Endothelial Cells

Jingtao Wu,a,b,# Guiwen Yang,a,# Wenxing Zhu,a Wujun Wen,a Fumiao Zhang,a Jinduo Yuan,a and Liguo An,a,*

a Shandong Provincial Key Laboratory of Animal Resistance Biology, College of Life Science, Shandong Normal University; Jinan 250014, China; and b Department of Food Science and Nutrition, University of Jinan; Jinan 250002, China. Received July 10, 2011; accepted April 2, 2012

This study examined the effects of platycodin D (PD), a triterpene saponin from the root of Platycodon grandiflorum A.DC on human umbilical vein endothelial cells (HUVECs) in vitro, which were pretreated with PD (0.01, 0.15, 0.25 mg/mL), respectively, and treated with 50 mg/L oxidized low-density lipoprotein (oxLDL). The levels of nitric oxide (NO) and malonaldehyde (MDA) in the culture medium, vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) mRNA expression in endothelial cells and the adhesion of monocytes to endothelial cells were measured. The results showed that PD increased NO concentration and decreased MDA level induced by oxLDL in the medium of endothelial cells. Moreover, PD significantly inhibited the oxLDL-induced increase in monocyte adhesion to endothelial cells as well as decreasing mRNA expression levels of VCAM-1 and ICAM-1 on these cells. Based on these results, it is suggested that PD is a promising anti-atherosclerotic activity, which is at least in part the result of its increasing NO concentration, reducing the oxLDL-induced cell adhesion molecule expression in endothelial cells and the endothelial adhesion to monocytes.

Key words Platycodon grandiflorum; platycodin D; adhesion molecule; endothelial cell

Platycodi Radix is the root of Platycodon grandiflorum A.DC, which is commonly known as Jiegeng in China. In China as well as other Asian countries, Platycodi Radix has been widely used as a food material and for the treatment of many chronic inflammatory diseases in traditional oriental medicine for such conditions as bronchitis, asthma and pulmonary tuberculosis. Chemical analyses of Platycodi Radix have revealed that triterpenoid saponins are its main chemical components. And earlier studies examining the roots of Platycodon grandiflorum A.DC have reported on the isolation and structural elucidation of these triterpenoid saponins, in which platycodins A, C, and D were determined as the primary saponins. Among the many compounds that have been isolated from Platycodi Radix, platycodin D has been found to possess the most potent biological activities, including immunological adjuvant, anti-inflammation and immunomodulation, a protective effect on ischemia/reperfusion injury in the gerbil hippocampus, induction apoptosis through nuclear factor-κB activation in immortalized keratinocytes, a cholesterol-lowering effect, an antinociceptive profile, an antileukemia activity, benefits in cancer chemotherapy and so on.

Atherosclerosis is a chronic inflammatory process resulting from increased oxidative stress. Studies have demonstrated that oxidized low-density lipoprotein (oxLDL) plays an important role in the initiation and progression of atherosclerosis. OxLDL may promote atherosclerosis by direct cytotoxicity of inhibition of nitric oxide (NO) synthesis by endothelium, transformation of macrophages to foam cells, arterial smooth muscle cell proliferation and migration. The oxidation theory of atherosclerosis implies that agents effectively inhibiting low-density lipoprotein (LDL) oxidation and lesion development are potential antiatherogenic compounds.

The key stages in the development of atherosclerosis are the activation of the vascular endothelium, the increased adhesion of circulating monocytes to the injured endothelial layer, following by their infiltration into the vessel wall and differentiation into macrophages. Endothelial cells recruit monocytes by selectively expressing cell surface adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1). Proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), commonly found in atherosclerotic lesions, can induce chemotactic factors, other cytokines and cell adhesion molecules, all of which can contribute to the inflammatory process.

The present study examined the effects of platycodin D (PD) on an oxLDL-induced oxidative injury by using an oxLDL-induced endothelial cells model to determine the basis of its resistance to atherosclerosis by a variety of aspects: (1) nitric oxide (NO) and malonaldehyde (MDA); (2) vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) mRNA expression; and (3) the adhesion states of monocytes to endothelial cells.

MATERIALS AND METHODS

Preparation of PD The species, Platycodon grandiflorum was collected from Lu-mountain (Zibo, China) and identified by Professor Shoujin Fan at College of Life Sciences, Shandong Normal University (Jinan, China). Dried roots of Platycodon grandiflorum (5 kg) were extracted with methanol at room temperature for 24 h and ultrasonically treated three times at 45°C for 30 min. Concentration of the solvent gave a brown syrup extract (1.4 kg) which was suspended in water and defatted with ether. The water layer portion was extracted with the n-BuOH until n-BuOH layer became colorless. Then these layers were concentrated to dryness (721 g), then suspended in H2O (2 L) and poured onto a D101 resin column (Mitsubishi Chemical Corporation, Japan) chromatography (Φ=5×100 cm), which was stabilized with H2O. The column

* To whom correspondence should be addressed. e-mail: an.liguo58@gmail.com © 2012 The Pharmaceutical Society of Japan
was washed with H₂O (2L) and then eluted with MeOH (5L) and the fractions eluted at 60–80% of methanol were concentrated under reduced pressure to give a crude saponins mixture (70g).

The crude saponins (50g) were further purified by repeated silica gel (Merck, Germany) chromatography eluting with a stepwise gradient of ascending polarity of CHCl₃:MeOH (9:1–7:1–5:1–3:1–1:1) to ensure separation of saponins from compounds of similar polarity. The process was repeated several times until a sufficient quantity of PD was obtained (7.5 g). The purified PD was identified on the basis of thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), electrospray ionization-mass spectrometry (ESI-MS, =1225.38) and ¹³C-NMR spectra and compared with the authentic PD (Jilin Agriculture University, Fig. 1) and those previous reports. HPLC showed this material to be 92.28% chemically pure. The PD was dissolved in the culture media.

**Cell Culture** Experimental cells were obtained and cultured as described previously. Human umbilical vein endothelial cells were obtained from human umbilical cord. In brief, the cord was filled with 0.25% trypsin (Shanghai Yaxin, China) and incubated at 37°C for 20 min. After being harvested in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, New York, U.S.A.), endothelial cells were seeded into culture flasks and incubated at 37°C. The medium was changed every 2–3 d until the cells had grown to confluence. U937 cells, human monocytic leukemia cells, were obtained from the American Type Culture Collection (Bethesda, Maryland, U.S.A.). The endothelial cells and monocyteic cells were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies) at 37°C in a 5% CO₂ humidified incubator.

**Assays of Cellular Viability and Integrity** Three different assays were used to assess the effect of the extract on cellular viability and integrity: (1) the Trypan blue exclusion test to determine cell viability; (2) the [di-methylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay to monitor mitochondrial respiration; and (3) the lactate dehydrogenase (LDH) assay to assess plasmallemma integrity.

**Trypan Blue Exclusion Assay** The trypan blue exclusion assay is widely used to determine cellular integrity. Endothelial cells (2×10⁴ cells/well) were incubated with 0 mg/mL, 0.01 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL and 0.75 mg/mL PD, respectively, for 24 h at 37°C. The cells were then exposed to the dye (Shanghai Jahe, China) and the number of cells that taking up the dye was counted in a NucleoCounter (Astori, Italy). The proportion of dye-containing cells to the ones that did not absorb it was then calculated.

**MTT Assay** The MTT assay is a test of metabolic competence and assesses mitochondrial performance. It is a colometric assay that relies on the conversion of yellow tetrazolium bromide (MTT) to the purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. Briefly, endothelial cells (2×10⁴ cells/well) were first incubated with 0.01 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL and 0.75 mg/mL PD, respectively, for 24 h at 37°C. They were then incubated in serum-free medium to which 10 µL 0.5 mg/mL MTT (Roche, Switzerland) had been added. Following 3.5 h incubation, 100 µL acidic isopropanol (0.1 mol/L HCl in absolute isopropanol) was added to dissolve the formazan crystals and the absorbance was determined in an ELISA reader (Thermo, Hudson, New Hampshire, U.S.A.) at 490 nm. The number of metabolically competent cells was determined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells that served as control.

**LDH Assay** The presence of the LDH in the cell culture medium was indicative of cell membrane damage. Briefly, endothelial cells (2×10⁴ cells/well) were first incubated with 0.01 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL and 0.75 mg/mL PD, respectively, for 24 h at 37°C. Upon the completion of the incubation, 50 µL of the upper medium was collected from each well. The untreated cells were then lysed with a cell lysis solution for 40 min at room temperature and the lysate collected. LDH activity was measured using a CytoTox 96 Nonradioactive Cytotoxicity Assay Kit (Promega, Madison, Wisconsin, U.S.A.) in accordance with the manufacturer’s instructions. The percentage of LDH released from the cells was determined using the formula: (absorbance of supernatant)/(absorbance of supernatant+absorbance of lysate)×100.

**Measurement of NO and Malondialdehyde (MDA) Released by oxLDL-Induced Endothelial Cells** Endothelial cells were seeded onto 24-well plates. The cells were divided into six groups: group CON (control group, pure medium); group MOD (model group, medium and 50 mg/L oxLDL); group A (0.10 mg/mL PD and 50 mg/L oxLDL); group B (0.15 mg/mL PD and 50 mg/L oxLDL); group C (0.25 mg/mL PD and 50 mg/L oxLDL) and group SIM (simvastatin group, 0.2 mg/mL simvastatin and 50 mg/L oxLDL). Endothelial cells were pre-incubated with PD and simvastatin for 6 h, and then cultured with oxLDL (Sigma, St. Louis, Missouri, U.S.A.) for 24 h. The level of NO as well as MDA in culture media was measured using a commercial kit (Nanjing, Jiancheng, China).

**VCAM-1 and ICAM-1 mRNA Expression** The endothelial cells were pretreated with PD for 1 h and stimulated with oxLDL for 24 h, which was followed by washing twice with phosphate buffered saline (PBS). The total RNA was isolated from the endothelial cells using the Trizol reagents according to the procedure of the supplier (Invitrogen, Carlsbad, California, U.S.A.). TRizol reagents were used as per manufacturer’s guidelines. The integrity of the extracted RNA was checked by electrophoresis on a 1% agarose gel. RNA was extracted

---

Fig. 1. Structure of Platycodin D
Platycodin D is a triterpenoid bidesmoside, composed of an aglycone moiety, 3-glucose and 28-O-arabinosyl-rhamnosyl-xyllosyl-apiosyl.
by precipitation using 2.5 volumes of 100% ethanol and 0.1 volume of 3 mol/L sodium acetate at pH 5.2. The RNA pellet was washed with 70% ethanol and dissolved in diethyl pyrocarbonate-treated water. cDNA synthesis was done using a cDNA synthesis kit as per manufacturer's instructions (Bio-Rad, Hercules, California, U.S.A.).

LightCycler Probe Design software 2.0 was used to design primers for the target genes (Table 1) VCAM-1, ICAM-1 and the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Applied Biosystems 7900HT Fast Real-Time PCR System (ABI), TOYOBO-Realtime PCR Master Mix (SYBR Green, Toyobo, Japan), standard procedures and SDS software 2.3 were used to assess the mRNA expression of VCAM-1, ICAM-1 and GAPDH mRNA. The reaction parameters were as follows: 40 cycles of 95°C denaturation for 15 s, 60°C annealing and extension for 60 s.

Monocyte-Endothelial Cells Adhesion Assay The endothelial cells were grown to confluence in 48-well plates, pre-treated with PD for 1 h, and stimulated with oxLDL for 24 h; they were then washed twice with PBS. The monocyte cells were labeled with 10 µM 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF, AM, Life Technologies) for 1 h at 37°C and washed twice with the growth medium. This was followed by adding 2.5×10^6 of the labeled cells to the endothelial cells and incubating them in a CO₂ incubator for 1 h. The non-adherent cells were removed from the plate by washing with PBS, and the monocyte cells bound to the endothelial cells were lysed with 50 mM/L Tris–HCl, pH 8.0, containing 0.1% SDS. The fluorescent intensity was measured using a spectrofluorometer (Varioskan, Finland) at an excitation and emission wavelength of 488 nm and 535 nm, respectively. The adhesion data were represented in terms of the fold change compared with the control values.

Statistical Analysis The results were presented as mean±S.E. Significance of difference among the groups was determined by one-way analysis of variance using 13th SPSS, then the differences among means were analyzed using Fisher's protected LSD multi-comparison test. Values of p<0.05 were considered statistically significant.

RESULTS

The Effect of PD on the Integrity of Endothelial Cells First, the effect of the increasing concentrations of PD on the viability of cultured endothelial cells was examined using the trypan blue exclusion test. It was found that the extract had no evident effect on cellular viability following 24h incubation with PD (Fig. 2).

The MTT assay to assess the effect of the different concentrations of PD on the mitochondrial respiration of cultured endothelial cells was used. At 0.05–0.25 mg/mL the aqueous extract boosted mitochondrial respiration (p<0.01). At the concentrations of 0.01 mg/mL and 0.5 mg/mL, the extract had a slight effect on mitochondrial respiration while at higher concentrations 0.75–1 mg/mL, the extract inhibited the respiration (p<0.01) (Fig. 3).

The LDH assay was then used to determine the effect of PD on the integrity of the plasmalemma of cultured endothelial cells. At low concentrations, 0.01–0.25 mg/mL, the extract had no effect on the LDH leakage following 24h incubation. At higher concentrations of 0.5–1 mg/mL, the extract increased cellular LDH efflux (p<0.01) (Fig. 4).

Based on these findings, three concentrations of the extract, namely, 0.10 mg/mL, 0.15 mg/mL and 0.25 mg/mL, were then used to evaluate the effect on the intracellular biochemical parameter.

Effects of PD on the Level of NO and MDA The effect of PD on concentration of NO and MDA levels in the culture media of endothelial cells was examined. Compared with the control group, the concentration of NO of endothelial cells in culture media decreased from 161.03±27.37 to 13.10±3.95 µmol/mL, and the concentration of MDA in the culture media increased from 0.31±0.00 to 18.90±3.19 µmol/L, and the concentration of MDA in the culture media increased from 0.38±0.06 to 6.12±0.38 nmol/mL in the model group. NO and MDA concentrations in the

Table 1. The Primers Sequence Used for Real-Time PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
<th>Amplified fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAM-1</td>
<td>GGTAGAGTGGGCTGGAGAAGGG</td>
<td>GGCAGGTATTAAAGGAGG</td>
<td>106 bp</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>TGGGATGACCCAAACCAAT</td>
<td>TGCAGTTCCACCCGTTC</td>
<td>90 bp</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCATGGGAGAAGGCTGGGG</td>
<td>CAAAGTGTACAGGATGACC</td>
<td>96 bp</td>
</tr>
</tbody>
</table>
culture media were significantly affected by the exposure to simvastatin ($p<0.01$). PD (0.15 mg/mL and 0.25 mg/mL) protected the cells against the oxLDL-induced depletion of NO ($p<0.05$). MDA in the culture media was significantly decreased by pre-incubation with PD (0.15 mg/mL and 0.25 mg/mL) ($p<0.01$) and PD (0.10 mg/mL) ($p<0.05$) (Figs. 5, 6).

### Effects of PD on VCAM-1 and ICAM-1 mRNA Expression

The effects of PD on the expression of cell adhesion molecules in endothelial cells were confirmed by RT-PCR analysis in order to examine the mechanism responsible for the changes in the amount of cell adhesion molecules. Compared with the control group, VCAM-1 and ICAM-1 mRNA expression of endothelial cells increased markedly ($p<0.01$) in the model group. But pretreating the cells with PD (0.15 mg/mL, 0.25 mg/mL PD) significantly ($p<0.01$) inhibited the oxLDL-induced VCAM-1 and ICAM-1 expression, respectively (Fig. 7).

### PD Inhibits Monocyte Adhesion to oxLDL-Induced Endothelial Cells

The effect of PD on the adhesion of monocytes to endothelial cells was examined. The endothelial cells were pretreated with PD for 1h before treating them with oxLDL for 6h, which was followed by an adhesion process and the quantification of adhered fluorescent monocytes. As shown in Fig. 8, oxLDL significantly increased the adhesion of monocytes to the endothelial cells compared with the untreated cells. However, PD (0.10 mg/mL, 0.15 mg/mL and 0.25 mg/mL) significantly inhibited the adhesion of monocytes to the endothelial cells in a dose-dependent manner ($p<0.01$).

### DISCUSSION

The aim of this paper was to assess the effects of PD on anti-atherosclerotic activity. The study found that PD had no effect on cellular integrity at low (0.01–0.25 mg/mL) concentrations. At higher concentrations (0.75–1 mg/mL), the extract...
was toxic to cells because it inhibited mitochondrial respiration and increased cellular LDH efflux. Both methods, the trypan blue exclusion test and LDH assay, reflect injury of the plasma membrane, but during our 24 h experiment, the cells treated with a higher concentration of PD may have been dissolved after staining by trypan blue, so they were not observed in the visual field; LDH could be measured accordingly. Therefore, the appropriate concentrations of PD were used for the cell experiments, namely 0.10 mg/mL, 0.15 mg/mL and 0.25 mg/mL. Several reports have shown that PD induces apoptosis in immortalized keratinocytes and leukemia cells at lower concentrations, but we did not study the effect of low concentration PD on apoptosis in endothelial cells. From the previous results of the trypan blue exclusion test and LDH assay, effects of a low concentration of PD on cell status were not obvious.

*Platycodon grandiflorum* has beneficial effects on inflammatory diseases and previous studies have demonstrated that PD isolated from *Platycodon grandiflorum* has anti-inflammatory effects. However, it is unclear whether PD increases the level of NO of oxLDL-induced endothelial cells, inhibits adhesion molecule mRNA expression of oxLDL-induced endothelial cells, reduces the adhesion of monocytes to endothelial cells or affects the oxidation state of endothelial cells. The above problems were investigated in this study. The results suggest that pretreatment with PD significantly suppressed the expression of VCAM-1 and ICAM-1, the adhesion to monocytes and the level of MDA in cultured endothelial cells, and increased the level of NO as well. These results suggest that PD can inhibit vascular inflammation and prevent atherogenesis in vitro. Several reports have shown that PD inhibits NO production in RAW264.7 cells at the lower concentrations, but our results showed that PD increased NO concentration induced by oxLDL in the medium of endothelial cells. This is consistent with the results of our previous research. Dysfunction of the endothelium is a hallmark of an early atherosclerotic lesion. NO is now considered as a major endogenous biological modulator with numerous diverse functions. As NO determines the antiatherosclerotic properties of the endothelium, one deficiency of it plays an important role in dysfunction of the endothelium. A number of studies suggest that oxLDL may downregulate the availability of NO. PD effectively increased NO production in the presence of oxLDL indicating that PD acted to improve dysfunction of the endothelium.

MDA is an end product of lipid peroxidation. Its concentration in culture media can be used to quantify lipid peroxidation lesions in endothelial cells. PD decreased the MDA concentration in the culture media of oxLDL-induced endothelial cells, indicating that it could inhibit dysfunction of the endothelium induced by oxLDL. Simvastatin is known to have an antiatherosclerotic effect and antioxidant activity against oxLDL. The study showed that PD and simvastatin had similar effects on NO and MDA in the culture media. One of the earliest events in atherogenesis is the adhesion of monocytes to the endothelium, which is followed by their infiltration and differentiation into macrophages. This key step is mediated by an interaction between the monocytes and the molecules expressed on the endothelial cell surface. These cell adhesion molecules primarily mediate the adhesion of monocytes specifically found in atherosclerosis lesions to the vascular endothelium. It was found that oxLDL-induced VCAM-1 and ICAM-1 expression at the mRNA level were blocked by pretreating the cells with PD in a dose-dependent manner. Moreover, the adhesion of monocytes to the endothelial cells was markedly inhibited. Expression of VCAM-1 and ICAM-1 mRNA is reduced at 0.1 mg/mL concentration of PD. Although the decrease was not obvious, its expression significantly inhibited the oxLDL-induced increased in monocyte adhesion to endothelial cells. An examination of the cytotoxicity of PD in the endothelial cells indicated that it did not adversely affect the cell viability. Therefore, inhibition of the adhesion of monocytes on the endothelial cells by PD was not the result of its cytotoxicity against the cells.

It has been demonstrated that atherosclerosis is a chronic inflammatory disease associated with increased oxidative stress in the vascular endothelium. Oxidative stress upregulates the expression of the cell adhesion molecules via the activation of redox-sensitive transcriptional factors such as NF-κB. NF-κB is involved in the development of atherosclerosis as well as in the signal transduction pathways for the TNFα-induced cell adhesion molecules such as VCAM-1 and ICAM-1. Current results showed that the activation of oxLDL-stimulated VCAM-1 and ICAM-1 in endothelial cells was evidently inhibited by PD. However, further studies are needed to define its mechanism: whether or not is it some inhibitory effect on the NF-κB pathways specific to oxLDL-stimulated induction of VCAM-1 and ICAM-1 expression.

In conclusion, PD inhibits the oxLDL-induced expression of the cell adhesion molecules and the adhesion of monocytes to endothelial cells through its antioxidant properties. The findings suggest a new insight into the mechanism responsible for the antiinflammatory and anti-atherosclerotic properties of PD.

**Acknowledgement** This study was financially supported by the Fund of University of Jinan (project number: xky0903).

**REFERENCES**

7. Choi JH, Yoo KY, Park OK, Lee CH, Won MH, Hwang IK, Ryu SY, Kim YS, Yi JS, Bae YS, Kang IJ. Platycodon D and 2'-O-acetylpolygalacin D2 isolated from *Platycodon grandiflorum* protect...


