Naunyn-Schmied Arch Pharmacol (2009) 380:399–406
DOI 10.1007/s00210-009-0450-8

ORIGINAL ARTICLE

Inhibitory effect of reinioside C on monocyte–endothelial cell adhesion induced by oxidized low-density lipoprotein via inhibiting NADPH oxidase/ROS/NF-κB pathway

Yong-Ping Bai · Chang-ping Hu · Mei-Fang Chen · Kang-Ping Xu · Gui-Shan Tan · Rui-Zhen Shi · Yuan-Jian Li · Guo-Gang Zhang

Received: 19 April 2009 / Accepted: 22 August 2009 / Published online: 3 September 2009

Abstract Monocyte adhesion to activated vascular endothelial cells is the critical event in the initiation of atherosclerosis. Adhesion molecules are inflammatory markers, which are upregulated by oxidized low-density lipoprotein (ox-LDL) and play a pivotal role in atherogenesis. In present study, the effect of reinioside C, a major compound of *Polygala fallax* Hemsl., on adhesion of monocytes to endothelial cells induced by ox-LDL was investigated. The results showed that incubation of endothelial cells with ox-LDL (100 μg/mL) for 24 h markedly increased the expression of ICAM-1 and P-selectin and enhanced the adhesion of monocytes to endothelial cells. Pretreatment with reinioside C (1, 3, or 10 μM) dose-dependently decreased ox-LDL-induced upregulation of expression of ICAM-1 and P-selectin and the enhanced adhesion of monocytes to endothelial cells. To determine the role of NADPH oxidase/reactive oxygen species (ROS)/nuclear factor-κB (NF-κB) pathway, endothelial cells were treated with ox-LDL (100 μg/mL) for 2 h, and NADPH oxidase subunit (Nox 2 and $p22^{phox}$) mRNA expression, intracellular ROS level, and NF-κB activity were measured. The results showed that reinioside C attenuated ox-LDL-induced NADPH oxidase subunit (Nox 2 and $p22^{phox}$) mRNA expression, generation of ROS, and activation of NF-κB in endothelial cells in a dose-dependent manner; the two latter effects were inhibited by pyrollidine dithiocarbamate, the inhibitor of NF-κB. These findings suggest that reinioside C attenuates ox-LDL-induced expression of adhesion molecules (P-selectin and ICAM-1) and the adhesion of monocytes to endothelial cells by inhibiting NADPH oxidase/ROS/NF-κB pathway.

Keywords Reinioside C · *Polygala fallax* Hemsl. · Adhesion · Adhesion molecules · NADPH oxidase · Nuclear factor-κB

Sources of Funding: This study was supported by a grant from the Natural Science Foundation of Hunan Province, China (No. 07JJ3045) and the Graduate degree thesis Innovation Foundation of Central South University (no. 2008yb029 and 2960-71131100011).

Y.-P. Bai · M.-F. Chen · R.-Z. Shi · G.-G. Zhang (✉)
Department of Cardiovascular Medicine, Xiangya Hospital,
Central South University,
Xiang-Ya Road 87#,
Changsha 410008, China
e-mail: xyzgg2006@sina.com

C.-p. Hu · Y.-J. Li
Department of Pharmacology, School of Pharmaceutical Sciences,
Central South University,
Changsha 410078, China

K.-P. Xu · G.-S. Tan
Department of Medicinal Chemistry, Chemistry Section,
School of Pharmaceutical Sciences, Central South University,
Changsha 410013, China

Introduction

Atherosclerosis is a chronic inflammatory disorder. Many studies have shown that the first step in atherogenesis is the adhesion of monocytes to the vascular endothelium (Ross 1999). The mechanisms by which monocyte adhesion to endothelium occurs involve the expression of monocyte chemoattractant protein-1 (MCP-1) and adhesion molecules such as P-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (Blankenberg et al. 2003). It has been reported that P-selectin and ICAM-1 expression are upregulated in atherosclerotic lesions in both humans and experimental animals, suggesting that they may play a significant role in the pathogenesis of atherosclerosis

(Davi et al. 1998; Parissis et al. 2001). Studies have also shown that a variety of stimuli including oxidized low-density lipoprotein (ox-LDL), homocysteine, and angiotension II induce P-selectin and ICAM-1 expression in endothelial cells (Cernuda-Morollón and Ridley 2006). Thus, these adhesion molecules can be considered to be potent mediators of atherogenesis.

The abnormal lipid metabolism is a critical factor in the initiation and development of atherosclerosis. One of the abnormalities in lipid metabolism is the increase of LDL in plasma and the enhanced oxidation of LDL. Ox-LDL exhibits numerous biological effects, including inducing endothelial dysfunction, stimulating inflammatory cytokines release, promoting monocyte and T-cell recruitment, and fostering formation of monocyte and smooth muscle cell-derived foam cells (O'Byrne et al. 2001; Davignon and Ganz 2004). Studies have shown that ox-LDL activates lectin-like oxidized low-density lipoprotein receptor (LOX)-1 (a specific receptor that facilitates the uptake of ox-LDL in endothelial cells then enhances monocyte adhesion to endothelium; Francone et al. 2009). Previous studies have also demonstrated that some anti-atherogenetic drugs such as simvastatin inhibit ox-LDL-induced adhesion of monocytes to endothelial cells via decreasing adhesion molecule levels (Dje N'Guessan et al. 2009). Modulation of monocyte adhesion to endothelium could be an important target in the therapy of atherosclerosis.

Polygala fallax Hemsl., a commonly used Chinese medicinal herb, has been used to treat some diseases such as infective inflammation and hypercholesterolemia. Reinioside C (Fig. 1) is the main component extracted from *P. fallax* Hemsl (Xu et al. 2006). Recently, it has been reported that reinioside C exerts hypolipidemic effect in hyperlipidemic mice (Li et al. 2008). Our previous studies have shown that reinioside C inhibits the elevated expression of LOX-1 induced by ox-LDL in endothelial cells (Bai et al. 2006) and the increase in TNF-α induced by exogenous asymmetric dimethylarginine (ADMA) through inhibiting reactive oxygen species (ROS)/nuclear factor-κB (NF-κB) pathway in monocytes (Zhang et al. 2008). NADPH oxidases are a major source of ROS generation in endothelial cells (Ushio-Fukai 2006). In the present study, we therefore studied the effect of reinioside C on monocyte–endothelial cell adhesion induced by ox-LDL and whether this effect is related to regulation of NADPH oxidase/ROS/NF-κB pathway.

Materials and methods

Reagents

Dulbecco's modified essential medium (DMEM), RPMI 1640, penicillin, and streptomycin were obtained from Gibco-BRL. Fetal bovine serum (FBS) was obtained from Sijiqing Biological Engineering Materials (Hangzhou, China). Pyrollidine dithiocarbamate (PDTC) was purchased from Sigma. Reinioside C (purity was 99.0% by HPLC, and the structure was elucidated on the basis of spectral evidences) was extracted from *P. fallax* Hemsl. (School of Pharmaceutical Sciences, Central South University, China). Polycolonal antibodies to ICAM-1 and P-selectin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [γ-^{32}P] ATP was obtained from Furui Biological Engineering Institute (Beijing, China). ROS detection kits were purchased from Beyotime Company (Jiangsu, China). Enzyme-linked immunosorbent assay (ELISA) kits for measurement of sICAM-1 and P-selectin were obtained from Senxiong Biological Limited Corporation (Shanghai, China). Gel shift assay system for determination of NF-κB activity and the probe of NF-κB were obtained from Promega. All other biochemicals used were of the highest purity available.

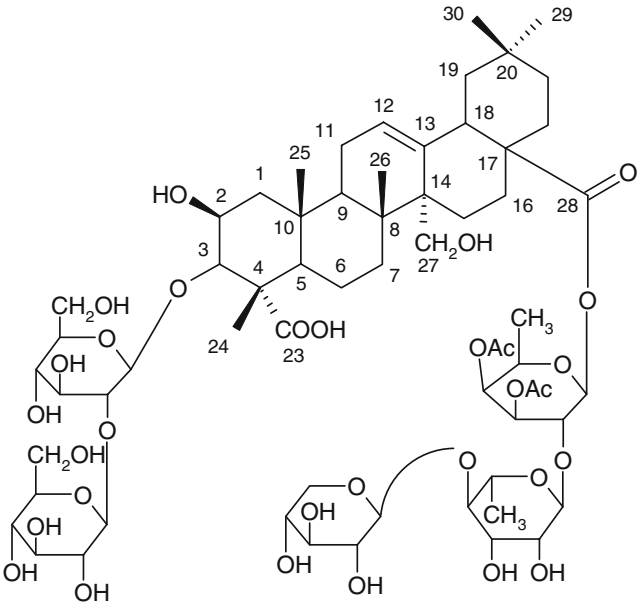

Fig. 1 Chemical structure of reinioside C

Cell culture

Human umbilical vein endothelial cells (HUVECs) and human monocytoid cells (THP-1) were originally purchased from American Type Culture Collection. HUVECs were cultured according to the standard procedure as described previously (Jiang et al. 2007). Cells were seeded at a density of 3×10^5 per 100-mm dish in DMEM, supplemented with 20 mM HEPES and 20% FBS. The cultures were maintained at 37°C with a humidified atmosphere enriched with 5% CO_2. All media were supplemented with 5 U/ml heparin, 100 U/ml penicillin,

and 100 μg/ml streptomycin. Endothelial cells of the forth to sixth passages in the actively growing condition were used for experiments. THP-1 cells were cultured in RPMI medium 1640 containing 15% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The viability of monocytic cells was assessed before experiment using trypan blue exclusion and was always >95%. When the cells had reached subconfluence, they were passaged into six-well culture dishes at an optimal density of 10^6 cells/ml.

To study the effect of reinioside C on adhesion of monocytes to endothelial cells, endothelial cells were pretreated with reinioside C (1, 3, or 10 μM) for 1 h and then cultured with ox-LDL (100 μg/mL) for 24 h. To further examine the role of NADPH oxidase/ROS/NF-κB pathway in the expression of adhesion molecules induced by ox-LDL, endothelial cells were pretreated with reinioside C (1, 3, or 10 μM) or PDTC (100 μM) for 1 h then exposed to ox-LDL (100 μg/mL) for 2 h; the mRNA expression of NADPH oxidase subunits (Nox 2 and $p22^{phox}$), intracellular ROS level, and NF-κB activity were determined.

Preparation of LDL and LDL oxidation by copper

Native LDL (nLDL) was isolated from pooled plasma of healthy donors through sequential density gradient ultracentrifugation in sodium bromide density solutions in the density range of 1.019–1.063 kg/L as previously described (Jiang et al. 2005). Then, isolated LDL was dialyzed under nitrogen for 24 h at 4°C against phosphate-buffered saline (PBS, 5 mM phosphate buffer and 125 mM NaCl, pH7.4). LDL was oxidized by dialysis for 24 h at 37°C against 10 μM $CuSO_4$ in PBS, as previously described in detail, and then the oxidized LDL was dialyzed for 24 h at 4°C against PBS containing 0.3 mM EDTA. Protein concentration was measured by Lowry's method. The levels of thiobarbituric acid reactive substance (TBARS), reflecting the extent of LDL oxidation, were measured by previously described methods (Jiang et al. 2005). The levels of TBARS were 6.15±0.88 and 25.12±6.78 μM/g protein for LDL and ox-LDL, respectively.

Monocyte–endothelial cells adhesion assays

HUVECs were seeded into 35-mm diameter wells on six-well plates for 3 days prior to assays, and confluence was confirmed prior to binding studies. Cultured THP-1 cells were added to wells containing confluent endothelial monolayer. The six-well plates were transferred to a rocked platform for 30 min; after nonadherent cells were removed, the plates were shaken for an additional 5 min with fresh binding buffer. Binding buffer was then replaced with Hank's buffered salt solution containing 2% glutaraldehyde to fix the remaining cells. Adherent cells were enumerated by video microscopy using a computer-aided image analysis system. The mean number of THP-1 cells per high power field was counted and calculated. Data were expressed as percentage of control.

Western blotting analysis of P-selectin, ICAM-1, and IκB-α protein

The cellular levels of P-selectin, ICAM-1, or IκB-α protein were determined by Western blotting analysis. Briefly, cell proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by electrophoretic transfer of proteins from the gel to a nitrocellulose membrane. After incubation in blocking solution (4% nonfat milk, Sigma), membranes were incubated with 1:1,000 dilution primary antibody (monoclonal antibody to P-selectin, ICAM-1, or IκB-α) overnight at 4°C. Membranes were washed and then incubated with 1:2,000 dilution second antibody for 1 h, and the bands corresponding to P-selectin, ICAM-1, or IκB-α were visualized using enhanced chemiluminescence reagents and analyzed with a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst version 1.1).

ELISA for P-selectin and sICAM-1 in the medium of HUVECs

The levels of P-selectin and sICAM-1 in the endothelial cell-conditioned medium were measured by ELISA. The measurement of both P-selectin and sICAM-1 was performed step by step based on the protocol booklet of ELISA kits (Senxiong Biotech Co., Ltd.).

Real-time quantitative reverse-transcriptase polymerase chain reaction for NADPH oxidase subunits

The mRNA expression of NADPH oxidase subunits ($p22^{phox}$, Nox 2) in the HUVECs was evaluated by real-time quantitative reverse-transcription polymerase chain reaction. Briefly, total RNA was extracted from HUVECs using Trizol reagent. Aliquot of 1 μg RNA from each sample was reverse-transcribed. Quantitative analysis using the SYBR Green method of NADPH oxidase subunits $p22^{phox}$ and Nox 2 mRNA expression was performed by using the ABI 7300 real-time PCR system. Sequences of primers were as follows: $p22^{phox}$, 5′-GACGCTTCACGCA GTGGTACT-3′ (sense) and 5′-CACGACCTCATCTGTCA CTGG-3′ (antisense); Nox 2, 5′-AAAGGAGTGCCCAGTA CCAAAGT-3′ (sense) and 5′-TACAGGAACATGGGAC CCACTAT-3′ (antisense); GAPDH, 5′-CTGCACCACCAA CTGCTTAG-3′ (sense) and 5′-AGGTCCACCACTGACA CGTT-3′ (antisense). A 25-μL reaction mixture containing

1 μL cDNA template, 12.5 μL SYBR Master mix, and 0.20 μL of each primer was amplified using the following thermal parameters: denaturing at 95°C for 10 min and 45 cycles of the amplification step (denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min). All amplification reactions were performed in triplicate, and the averages of the threshold cycles (Cts) were used to interpolate curves using 7300 System SDS Software. Results were expressed as the ratio of $p22^{phox}$ or Nox 2 mRNA with GAPDH mRNA, respectively.

Determination of ROS

Changes in intracellular ROS levels were determined by measuring the oxidative conversion of cell permeable 2′,7′-dichlorofluorescein diacetate (H_2DCF) to fluorescent dichlorofluorescein (DCF) in fluorospectrophotometer (F4000, Japan) as described previously (Korge et al. 2008). The medium was aspirated, and then cells were washed twice with PBS and incubated in 1 ml medium without Fetal calf serum (FCS). H_2DCF was added at a final concentration of 10 μM and incubated for 20 min at 37°C. The cells were then washed once with PBS and maintained in 1 ml culture medium. The fluorescence was monitored using a fluorescence microscope equipped with an flourescein isothiocyanate filter, and the average intensity values were measured at ×200 magnification in five randomly chosen fields of each nine replicates from four independent experiments.

Electrophoretic mobility shift assay

Nuclear protein extraction was carried out as our previous methods (Zhang et al. 2008). The electrophoretic mobility shift assay for determining the NF-κB DNA-binding activity was performed by incubating aliquots of nuclear extracts containing 15 μg total protein with γ-^{32}P-labeled double-stranded NF-κB-specific oligonucleotide probe (sense: 3′-TCAACTCCCCTGAAAGGGTCCG-5′; antisense: 5′-AGTTGAGGGGACTTTCCCAGGC-3′) by T4 polynucleotide kinase. The labeled probe was purified through Sephadex G-25. After 10 min of incubation at room temperature, the mixture was run on a 4% non-denaturing polyacrylamide gel in 0.5× Tris base–boric acid–EDTA buffer. After electrophoresis, the gels were dried, and the DNA–protein complexes were detected by autoradiography.

Statistic analysis

Results are expressed as means ± SEM. The data were analyzed by ANOVA followed by Newmann–Keuls–Student test for multiple comparisons. The statistical significance was considered if $P<0.05$.

Results

Effect of reinioside C on ox-LDL-induced monocyte–endothelial cells adhesion

Treatment with ox-LDL (100 μg/ml) for 24 h caused a significant increase in the adhesion of monocytes to endothelial cells. Reinioside C (1, 3, or 10 μM) significantly attenuated the increase in adhesion of monocytes induced by ox-LDL in a concentration-dependent manner (Fig. 2). The increased adhesion of monocytes induced by ox-LDL was also attenuated by treatment with PDTC (100 μM). Reinioside C (10 μM) or PDTC (100 μM) itself had no effect on the adhesion of monocytes to endothelial cells. Higher concentrations of reinioside C (20, 40 μM) were also tested in the monocyte adhesion assay, and they did not significantly improve the protection (data not shown). We therefore used reinioside C at the concentrations of 1, 3, or 10 μM for the rest of the study.

Effect of reinioside C on the expression of adhesion molecules induced by ox-LDL

In accordance with previous observations (Jiang et al. 2007), treatment with ox-LDL (100 μg/ml) for 24 h significantly increased the expression of ICAM-1 and P-selectin in endothelial cells. Pretreatment of cells with reinioside C (1, 3, or 10 μM) concentration-dependently

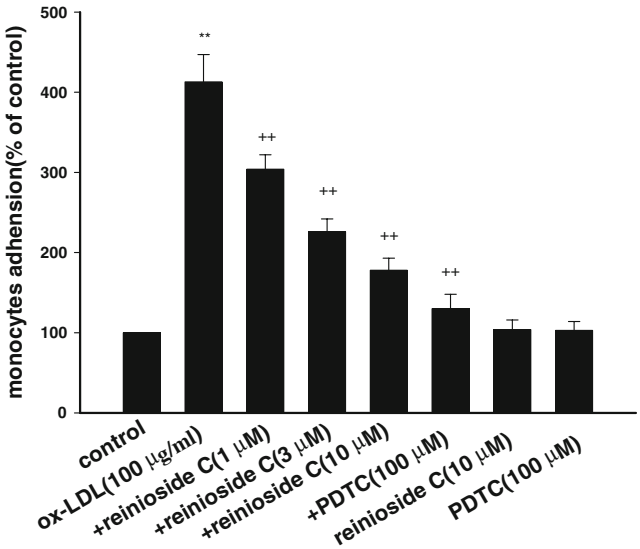

Fig. 2 Effect of reinioside C on elevated adhesion induced by ox-LDL. Cells were preincubated with different concentrations of reinioside C (1, 3, or 10 μM), PDTC (100 μM) for 1 h, and then 100 μg/ml ox-LDL was added for an additional 24-h period. Data are expressed as percentage (mean ± SEM) of control. $n=6$. Compared with control, $^{**}P<0.01$. Compared with ox-LDL, $^{++}P<0.01$. *+reinioside C* ox-LDL + reinioside C; *+PDTC* ox-LDL + PDTC

reduced the expression of ICAM-1 and P-selectin in endothelial cells (both Western blotting and ELISA; Fig. 3a, b). The increased expression of adhesion molecules (ICAM-1 and P-selectin) induced by ox-LDL was also attenuated by treatment with PDTC (100 μM). Reinioside C (10 μM) or PDTC (100 μM) itself had no effect on the expression of adhesion molecules.

Effect of reinioside C on NADPH oxidase/ROS/NF-κB pathway

We investigated whether NADPH oxidase/ROS/NF-κB pathway is involved in the effect of reinioside C on ox-LDL-induced expression of adhesion molecules and monocyte–endothelial cell adhesion. In accordance with previous observations from our laboratory (Zhang et al. 2008), treatment with ox-LDL (100 μg/ml) for 2 h significantly increased the NADPH oxidase subunit (Nox 2 and $p22^{phox}$) mRNA expression (Fig. 4a) and intracellular ROS level (Fig. 4b) and activated the NF-κB activity (Fig. 5a). The results also showed that reinioside C (1, 3, or 10 μM) concentration-dependently reduced NADPH oxidase subunit (Nox 2 and $p22^{phox}$) mRNA expression, intracellular ROS elevation, and NF-κB activation induced by ox-LDL (100 μg/ml, 2 h). Furthermore, pretreatment with PDTC (100 μM; NF-κB inhibitor) prevented ox-LDL-induced intracellular ROS production and NF-κB activation but had no effect on NADPH oxidase subunit (Nox 2 and $p22^{phox}$) mRNA expression. Reinioside C (10 μM) or PDTC (100 μM) itself had no effect on intracellular ROS level and NF-κB translocation in endothelial cells.

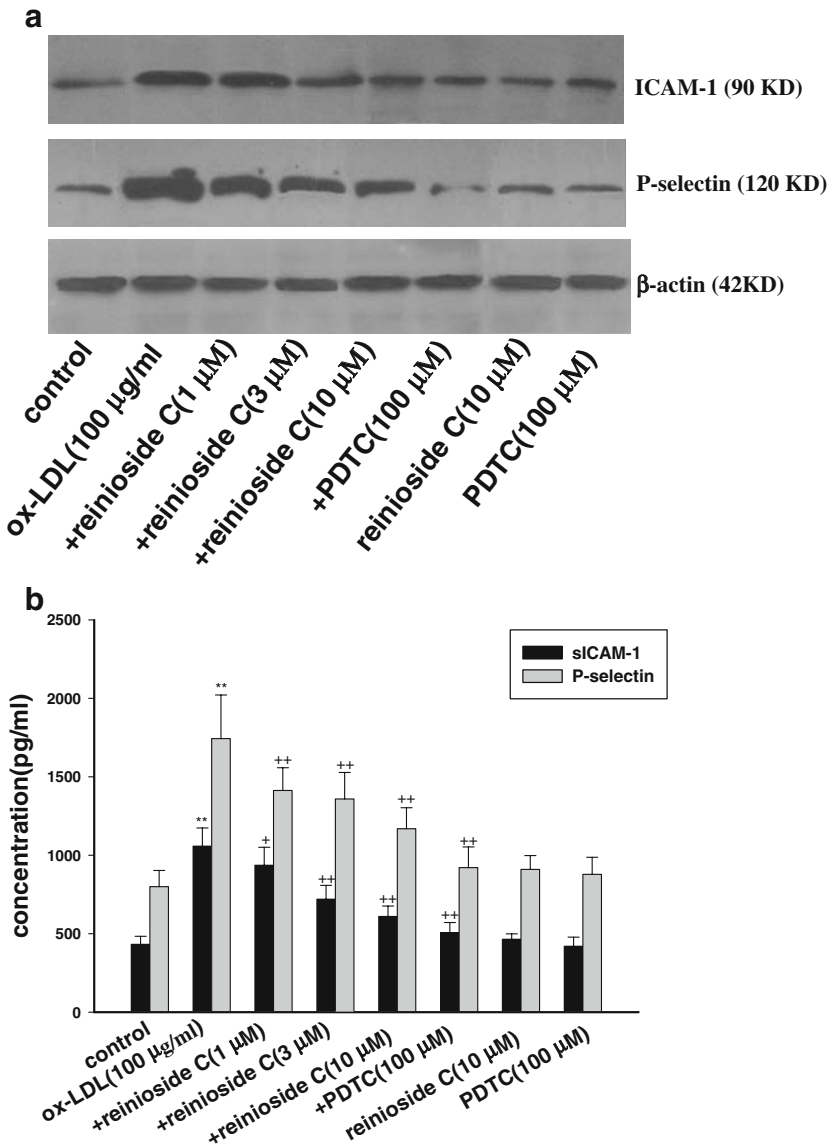

Fig. 3 Effect of reinioside C on elevated level of ICAM-1 and P-selectin induced by ox-LDL in cultured HUVECs. Cells were preincubated with different concentrations of reinioside C (1, 3, or 10 μM) or PDTC (100 μM) for 1 h, and then 100 μg/ml ox-LDL was added for an additional 24-h period. **a** The protein expression of ICAM-1 and P-selectin by Western blotting analysis. **b** The levels of sICAM-1 and P-selectin in the medium with ELISA. Data are expressed as means ± SEM, $n=6$. Compared with control, $^{**}P<0.01$; compared with ox-LDL, $^{+}P<0.05$, $^{++}P<0.01$. *+reinioside C* ox-LDL + reinioside C; *+PDTC* ox-LDL + PDTC

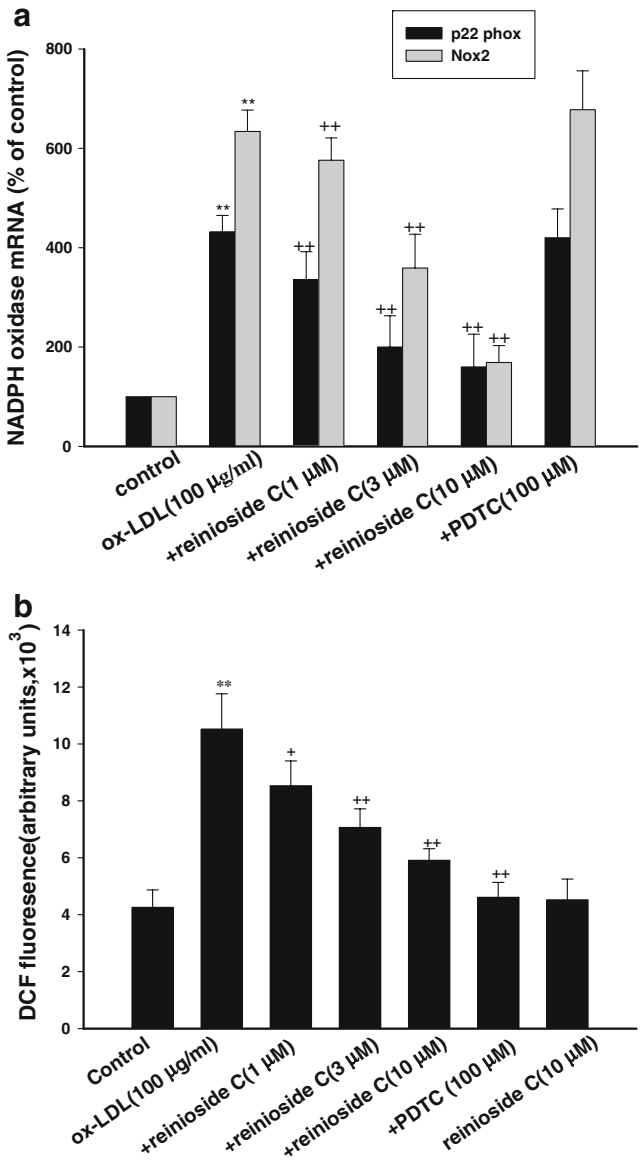

Fig. 4 Effect of reinioside C on elevated expression of NADPH oxidase and level of intracellular ROS induced by ox-LDL in cultured HUVECs. Cells were preincubated with different concentrations of reinioside C (1, 3, or 10 μM) or PDTC (100 μM) for 1 h, and then 100 μg/ml ox-LDL was added, and cells were incubated for an additional 2-h period. **a** The mRNA expression of NADPH oxidase subunits (Nox 2 and $p22^{phox}$) by real-time RT-PCR analysis. **b** The level of intracellular ROS as shown by DCF fluorescence. Data are expressed as means ± SEM, $n=6$. Compared with control, $^{**}P<0.01$; Compared with ox-LDL, $^{+}P<0.05$, $^{++}P<0.01$. *+reinioside C* ox-LDL + reinioside C; *+PDTC* ox-LDL + PDTC

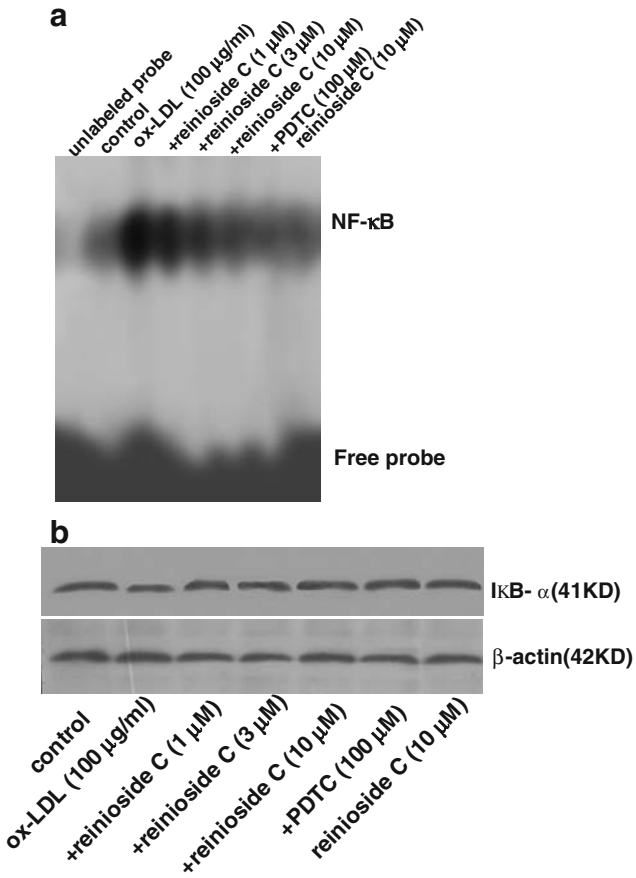

Fig. 5 Effect of reinioside C on ox-LDL-induced NF-κB/DNA-binding activity (**a**) and the phosphorylation status of IκB-α (**b**) in cultured HUVECs. Cells were preincubated with different concentrations of reinioside C (1, 3, or 10 μM) or PDTC (100 μM) for 1 h, and then 100 μg/ml ox-LDL was added, and cells were incubated for an additional 2-h period. Competition experiment where unlabeled NF-κB probe was added at 100-fold excess concentrations to the γ-^{32P}-labeled probe was performed

We also investigated the effect of reinioside C on the phosphorylation status of IκB-α induced by ox-LDL. As shown in Fig. 5b, reinioside C significantly inhibited the decrease in IκB-α level induced by ox-LDL (100 μg/ml); the same effects were also observed when the cells pretreated with PDTC (100 μM). Reinioside C (10 μM) or PDTC (100 μM) itself had no effect on IκB-α phosphorylation in endothelial cells.

Discussion

The major findings in the present study were that: (1) this study for the first time to our knowledge determined that reinioside C, a major compound of *P. fallax* Hemsl., significantly attenuated the expression of P-selectin and ICAM-1 and inhibited adhesion of monocytes to endothelial cells induced by ox-LDL; (2) these effects of reinioside C were related to the inhibition of NADPH oxidase/ROS/NF-κB pathway.

There is growing evidence that ox-LDL plays a key role in the development of atherosclerotic lesions. Enhanced adhesion of monocytes to endothelium induced by ox-LDL

in the arterial wall is one of the earliest signs of atherogenesis. P-selectin is thought to mediate monocytes to attach to and to roll along the activated vascular endothelium, and ICAM-1 is believed to be associated with adhesion and transendothelial migration of monocytes (Takei et al. 2001). It has been shown that the levels of soluble forms of P-selectin and sICAM-1 are elevated in hypercholesterolemic humans or in cultured endothelial cells (Takei et al. 2001). Increased expression of P-selectin and ICAM-1 has been shown in atherosclerotic plaque taken from animals or humans. The atherosclerotic lesions are markedly decreased by blocking ICAM-1 action using specific antibodies in hypercholesterolemic mice (Andrea and Hubbard 2000). Knockout of ICAM-1 results in attenuation of atherosclerotic process in the apo-E knockout mice (Bourdillon et al. 2000). Administration of anti-P-selectin antibody causes a decrease in leukocyte rolling and attachment to vascular endothelium (Thorlacius et al. 1997), and deficiency of P-selectin in mice shows a complete absence of leukocyte rolling and reduced atherosclerotic lesion formation (Dong et al. 2000; Collins et al. 2000). These observations collectively suggest that inhibition of adhesion molecule expression and adhesion of monocytes to endothelium can reduce atherosclerosis. In the present study, we found that reinioside C attenuated the expression of P-selectin and ICAM-1 and inhibited adhesion of monocytes to endothelial cells induced by ox-LDL. Recently, it has been reported that reinioside C exerts hypolipidemic effect in hyperlipidemic mice (Li et al. 2008). Our previous study has shown that reinioside C inhibits the elevated expression of LOX-1 mRNA and protein induced by ox-LDL in endothelial cells (Bai et al. 2006) and also attenuates the increase in TNF-α induced by exogenous ADMA through inhibition ROS/NF-κB pathway in monocytes (Zhang et al. 2008).

Accumulating evidence indicates that oxidative stress plays an important role in the pathogenesis of endothelial dysfunction and atherosclerosis, and NADPH oxidase is the major source of ROS in endothelial cells (Jacobi et al. 2005). Recently, ROS has been considered as second messengers leading to NF-κB activation in response to extracellular stimuli and then upregulation of the gene expression of pro-inflammatory cytokines including TNF-α, P-selectin, and ICAM-1. An important link between ox-LDL and inflammation is NF-κB. The expression of adhesion molecules, including P-selectin and ICAM-1, is regulated principally at the level of transcription (de Winther et al. 2005). In the endothelial cells, there are several transcriptional factor-binding sites in cytokine promoter including NF-κB. Activated NF-κB can bind to cytokine promoter, which is critically involved in cytokine gene regulation induced by ox-LDL (Wilson et al. 2000). Previous investigations have shown that a single injection of LDL induces inflammatory reactions such as activation of NF-κB and the expression of sICAM-1 and TNF-α in the arterial walls in rats (Niemann-Jonsson et al. 2000). More recently, it has been reported that ox-LDL activates NF-κB in monocytes/macrophages, endothelial cells, and vascular smooth muscle cell and consequently results in the upregulation of pro-inflammatory cytokines such as MCP-1, interleukin-6, and interleukin-8. Inhibition of activation of this transcription factor may be a reasonable approach to interfere with atherogenic processes. It has been demonstrated that NF-κB is redox-sensitive and can be activated by ROS, and activation of NF-κB can be inhibited by antioxidants (Bar-Shai et al. 2008). Our study demonstrated that ox-LDL significantly increased intracellular ROS production as shown by DCF fluorescence intensity, induced the enhanced adhesion of monocyte to endothelial cells, and upregulated the expression of P-selectin and ICAM-1 in endothelial cells, which were abolished by pretreatment with PDTC. Although PDTC is a well-known inhibitor of NF-κB, there is evidence that PDTC suppresses NF-κB activation through its antioxidant property (Yokoo and Kitamura 1996), which possibly accounts for the reduced ROS formation by PDTC in our experiment. In the present study, we also found that reinioside C attenuated the enhanced adhesions and down-regulated the expression of P-selectin and ICAM-1 concomitantly with reduction of intracellular ROS levels and NF-κB activity induced by ox-LDL. Interestingly, reinioside C, but not PDTC, also decreased the enhanced mRNA expression of NADPH oxidase subunits (Nox 2 and $p22^{phox}$) induced by ox-LDL. These findings suggest that reinioside C attenuates the upregulated expression of adhesion molecules and the adherence of monocytes to endothelial cells via inhibiting NADPH oxidase/ROS/NF-κB pathway. Reinioside C may be a potential anti-atheroscleric compound, and further study is needed to define its effect in in vivo experiment with hypercholesterolemic animals.

A variety of natural products have similar activity with reinioside C, such as glabridin, panax notoginseng saponins, ginsenoside Rb1, theaflavin, and probucol. By comparing the chemical structures of these compounds, hydroxyl seems to be crucial in maintaining the anti-atherosclerosis effect. Since there are no previous reports of similar structures related to the same activity, it is difficult to discuss the sites that may be involved in the responses examined in the present study. It has been well documented that saponins (ginsenoside Rb_1, Rg_1, Re, and panax notoginseng saponins R_1) can inhibit monocyte–endothelial cell adhesion (Kim et al. 2006; Chai et al. 2008; Kim and Cho 2009). From these findings, the structure–activity relationships seem to be based on the position of sugar linkage attached to the aglycone and on the number of sugar residues in the saponin molecule. We, therefore,

postulate that sugar linkage position and sugar residues number may play a role in the anti-atherosclerosis activity of reinioside C. We recognize that further investigations need to be done to address the structure–activity relationship.

Reference

Andrea K, Hubbard RR (2000) Intercellular adhesion molecule-1 (ICAM-1) expression and cell signaling cascades. Free Radic Biol Med 28:1379–1386

Bai YP, Chen MF, Shi RZ, Tang GS, Li YJ, Zhang GG (2006) Inhibitory effect of reinioside C on LOX-1 expression induced by ox-LDL. Zhong Nan Da Xue Xue Bao Yi Xue Ban 31:659–662

Bar-Shai M, Carmeli E, Ljubuncic P, Reznick AZ (2008) Exercise and immobilization in aging animals: the involvement of oxidative stress and NF-kappaB activation. Free Radic Biol Med 44:202–214

Bourdillon MC, Poston RN, Covacho C, Chignier E, Bricca G, McGregor JL (2000) ICAM-1 deficiency reduces atherosclerotic lesions in double-knockout mice (ApoE(−/−)/ICAM-1(−/−)) fed a fat or a chow diet. Arterioscler Thromb Vasc Biol 20:2630–2635

Cernuda-Morollón E, Ridley AJ (2006) Rho GTPases and leukocyte adhesion receptor expression and function in endothelial cells. Circ Res 98:757–767

Chai H, Wang Q, Huang L, Xie T, Fu Y (2008) Ginsenoside Rb1 inhibits tumor necrosis factor-alpha-induced vascular cell adhesion molecule-1 expression in human endothelial cells. Biol Pharm Bull 31:2050–2056

Collins RG, Velji R, Guevara NV, Hicks MJ, Chan L, Beaudet AL (2000) P-selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice. J Exp Med 191:189–194

Davì G, Romano M, Mezzetti A, Procopio A, Iacobelli S, Antidormi T, Bucciarelli T, Alessandrini P, Cuccurullo F, Bittolo Bon G (1998) Increased levels of soluble P-selectin in hypercholesterolemic patients. Circulation 97:953–957

Davignon J, Ganz P (2004) Role of endothelial dysfunction in atherosclerosis. Circulation 109:27–32

de Winther MP, Kanters E, Kraal G (2005) Nuclear factor κB signaling in atherogenesis. Arterioscler Thromb Vasc Biol 25:904–914

Dje N'Guessan P, Riediger F, Vardarova K, Scharf S, Eitel J, Opitz B, Slevogt H, Weichert W, Hocke AC, Schmeck B, Suttorp N, Hippenstiel S (2009) Statins control oxidized LDL-mediated histone modifications and gene expression in cultured human endothelial cells. Arterioscler Thromb Vasc Biol 29:380–386

Dong ZM, Brown AA, Wagner DD (2000) Prominent role of P-selectin in the development of advanced atherosclerosis in ApoE-deficient mice. Circulation 101:2290–2295

Francone OL, Tu M, Royer LJ, Zhu J, Stevens K, Oleynek JJ, Lin Z, Shelley L, Sand T, Luo Y, Kane CD (2009) The hydrophobic tunnel present in LOX-1 is essential for oxidized LDL recognition and binding. J Lipid Res 50:546–555

Jacobi J, Kristal B, Chezar J (2005) Exogenous superoxide mediates pro-oxidative, pro-inflammatory, and pro-coagulatory changes in primary endothelial cell cultures. Free Radic Biol Med 39:1238–1124

Jiang JL, Zhu HQ, Chen Z, Xu HY, Li YJ (2005) Angiotensin-converting enzyme inhibitors prevent LDL-induced endothelial dysfunction by reduction of asymmetric dimethylarginine level. Int J Cardiol 101:153–155

Jiang JL, Wang S, Li NS, Zhang XH, Deng HW, Li YJ (2007) The inhibitory effect of simvastatin on the ADMA-induced inflammatory reaction is mediated by MAPK pathways in endothelial cells. Biochem Cell Biol 85:66–77

Kim BH, Cho JY (2009) Regulatory role of ginsenoside Rp1, a novel ginsenoside derivative, on CD29-mediated cell adhesion. Planta Med 75:316–320

Kim JY, Kim DH, Kim HG, Song GY, Chung YC, Roh SH, Jeong HG (2006) Inhibition of tumor necrosis factor-alpha-induced expression of adhesion molecules in human endothelial cells by the saponins derived from roots of Platycodon grandiflorum. Toxicol Appl Pharmacol 210:150–156

Korge P, Ping P, Weiss JN (2008) Reactive oxygen species production in energized cardiac mitochondria during hypoxia/reoxygenation: modulation by nitric oxide. Circ Res 103:873–880

Li H, Wang QJ, Zhu DN, Yang Y (2008) Reinioside C, a triterpene saponin of Polygala aureocauda Dunn, exerts hypolipidemic effect on hyperlipidemic mice. Phytother Res 22:159–164

Niemann-Jonsson A, Dimayuga P, Jovinge S (2000) Accumulation of LDL in rat arteries is associated with activation of tumor necrosis factor-alpha expression. Arterioscler Thromb Vasc Biol 20:2205–2211

O'Byrne D, Devaraj S, Islam KN, Collazo R, McDonald L, Grundy S, Jialal I (2001) Low-density lipoprotein (LDL)-induced monocyte–endothelial cell adhesion, soluble cell adhesion molecules, and autoantibodies to oxidized-LDL in chronic renal failure patients on dialysis therapy. Metabolism 50:207–215

Parissis JT, Venetsanou KF, Mentzikof DG, Kalantzi MV, Georgopoulou MV, Chrisopoulos N (2001) Plasma levels of soluble cellular adhesion molecules in patients with arterial hypertension. Eur J Int Med 12:350–356

Ross R (1999) Atherosclerosis: an inflammatory disease. N Engl J Med 340:115–126

Blankenberg S, Barbaux S, Tiret L (2003) Adhesion molecules and atherosclerosis. Atherosclerosis 170:191–203

Takei A, Huang Y, Lopes-Virella MF (2001) Expression of adhesion molecules by human endothelial cells exposed to oxidized low density lipoprotein. Influences of degree of oxidation and location of oxidized LDL. Atherosclerosis 154:79–86

Thorlacius H, Lindbom L, Raud J (1997) Cytokine-induced leukocyte rolling in mouse cremaster muscle arterioles in P-selectin dependent. Am J Physiol 272:H1725–H1729

Ushio-Fukai M (2006) Redox signaling in angiogenesis: role of NADPH oxidase. Cardiovasc Res 71:226–235

Wilson SH, Caplice NM, Simari RD, Holmes DR Jr, Carlson PJ, Lerman A (2000) Activated nuclear factor-kappaB is present in the coronary vasculature in experimental hypercholesterolemia. Atherosclerosis 148:23–30

Xu KP, Huang W, Tan GS (2006) Study on the antihyperlipidemia effective constituen t of *Polygala fallax* Hemsl. Zhong Yao Cai 29:16–18

Yokoo T, Kitamura M (1996) Antioxidant PDTC induces stromelysin expression in mesangial cells via a tyrosine kinase-AP-1 pathway. Am J Physiol 270:F806–F811

Zhang GG, Bai YP, Chen MF, Shi RZ, Jiang DJ, Fu QM, Tan GS, Li YJ (2008) Asymmetric dimethylarginine induces TNF-alpha production via ROS/NF-kappaB dependent pathway in human monocytic cells and the inhibitory effect of reinioside C. Vascul Pharmacol 48:115–121