Inhibitory effects of paeoniflorin on lysophosphatidylcholine-induced inflammatory factor production in human umbilical vein endothelial cells

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Abstract. Lysophosphatidylcholine (LPC) plays an important role in atherosclerosis through initiation of endothelial inflammation response. Paeoniflorin (PEF), isolated from the dry root of *Paeonia*, has been reported to exert an anti-inflammatory effect, but the exact mechanism is not fully understood. The aim of this study was to investigate the inhibitory effects of PEF on LPC-induced inflammatory factor production and the underlying mechanisms. In human umbilical vein endothelial cells (HUVECs), different concentrations (1, 10 or 100 μmol/l) of PEF were added 2 h prior to exposure to LPC (10 mg/l) for 24 h. The results showed that PEF significantly inhibited LPC-induced inflammatory factor production. In addition, PEF was also able to suppress the enhanced high mobility group box-1 (HMGB1) expression and release, upregulated expression of receptor for advanced glycation end product (RAGE), Toll-like receptor (TLR)-2 and TLR-4, and increased nuclear factor-κB (NF-κB) activity induced by LPC. Our results suggest that PEF suppresses LPC-induced inflammatory factor production through inhibition of the HMGB1-RAGE/TLR-2/TLR-4-NF-κB pathway.

Introduction

It is well recognized that atherosclerosis is a chronic inflammatory disease (1). Vascular inflammatory response plays an important role in the onset, development and evolution of atherosclerosis (2). Many reports have demonstrated that vascular inflammatory response is closely associated with endothelial dysfunction (3,4). Endothelial cells can release several inflammatory factors in response to a variety of harmful stimulations including increased lysophosphatidylcholine (LPC) (5). LPC is the major phospholipid component of oxidized low-density lipoprotein (ox-LDL) (6), and it is well known for its ability to mimic the effects of ox-LDL (7). Increased plasma ox-LDL is an independent risk factor in atherosclerosis and plays a key role in initiation of the inflammatory response (8,9). Recently, it has been reported that ox-LDL-induced inflammation in endothelial cells is related to stimulation of high mobility group box-1 (HMGB1) release (10).

HMGB1 is a non-histone DNA-binding nuclear protein that can be positively released from immune-activated cells or passively released from necrotic cells (11). There is growing evidence that extracellular HMGB1 is a very potent pro-inflammatory mediator (12,13). HMGB1 can stimulate the expression and release of numerous inflammatory factors, such as tumor necrosis factor-α (TNF-α), interleukins, intercellular adhesion molecule-1 (ICAM-1) and selectins (14,15). It has been reported that the pro-inflammatory effect of HMGB1 is mediated mainly through binding to its specific receptors, including receptor for advanced glycation endproduct (RAGE), Toll-like receptor (TLR)-2 and TLR-4 (16). The binding of HMGB1 to its specific receptors ultimately results in the activation of nuclear factor-κB (NF-κB), and then upregulation of inflammatory factor expression and release (17,18). Therefore, the HMGB1-RAGE/TLR-2/TLR-4-NF-κB pathway may be an important inflammatory signaling pathway.

Paeoniflorin (PEF), a monoterpene glucoside, is the primary active ingredient extracted from the dry root of *Paeonia*, which is a traditional Chinese herbal medicine extensively used in China for more than 1000 years to treat numerous diseases, such as virus hepatitis, anemia, systemic lupus erythematosus and gynecological diseases (19). In recent years, it has been reported that PEF exhibits anti-inflammatory properties. In Sprague-Dawley rats, both pretreatment and post-treatment with PEF alleviated ischemia/reperfusion-induced cerebral injury, at least in part, through inhibition of inflammatory factor production (20). In addition, PEF was able to protect against lipopolysaccharide-induced acute lung injury in mice

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by alleviating inflammatory cell infiltration and microvascular permeability (21). However, the exact anti-inflammatory mechanisms of PEF remain unclear.

In the present study, we investigated whether PEF has an inhibitory effect on LPC-induced inflammatory factor production using cultured human umbilical vein endothelial cells (HUVECs), and whether the anti-inflammatory effect is related to the inhibition of the HMGB1-RAGE/TLR-2/TLR-4-NF-κB pathway.

Materials and methods

Materials. HUVECs were obtained from the Tumor Research Institute of Peking University (Peking, China). PEF (purity ≥98%) was purchased from the Yangling Dongke Pharmaceutical Division (Shanxi, China). LPC was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were provided by Gibco-BRL (Grand Island, NY, USA). TRIzol reagent was a product of Invitrogen Corp. (Carlsbad, CA, USA). The First Strand cDNA Synthesis kit was purchased from MBI Fermentas Inc. (Vilnius, Lithuania). RAGE, TLR-2, TLR-4 and β-actin antibodies were from Abcam (Cambridge, UK). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Beyotime Biotechnology (Jiangsu, China). ICAM-1, MCP-1, IL-6, TNF-α, HMGB1 and NF-κBp65 ELISA kits were obtained from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Cell culture and treatment. HUVECs were cultured in DMEM containing 10% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37˚C. Cells were passaged by trypsinization and seeded at ~10^5 cells/ml. When cells reached 80% confluence, the culture medium was replaced with serum-free medium for 24 h, and then cells were pretreated with various concentrations (1, 10 or 100 μmol/l) of PEF 2 h prior to exposure to LPC (10 mg/l) for 24 h. LPC was dissolved in ethanol. The final concentration of ethanol was less than 0.1% (v/v). Cells used in the experiments were from 5 to 8 passages.

Cell viability assay. MTT was used to determine cell viability. Briefly, HUVECs were seeded at a density of $1x10^4$ cells/well in a 96-well culture plate. After drug treatment, the cells were washed twice with PBS to remove the medium, and 10 μl of MTT (0.5 mg/ml) was added to each well and incubated for an additional 4 h at 37˚C. Subsequently, 100 μl of dimethy sulfoxide (DMSO) was added to dissolve the MTT, and the absorbance at 490 nm was read on a microplate reader. Data are expressed as a percentage of the control, which was considered to be 100% viable.

ELISA. After drug treatment, cell culture supernatants were collected and centrifuged at 3,000 x g for 10 min to remove debris. The nuclear lysates were prepared as described by Wu *et al* (22). Briefly, cells were washed with cold PBS and then scraped from the well. Cells were treated with hypotonic buffer and centrifuged. The pellet was collected and treated with cell extraction buffer, vortexed, centrifuged and the supernatants (nuclear lysates) were stored at -70˚C. The concentrations of ICAM-1, MCP-1, IL-6, TNF-α and HMGB1 in cell culture supernatants and NF-κBp65 in nuclear lysates were determined by commercially available ELISA kits according to the manufacturer's instructions.

Quantitative real-time PCR. Total RNA was isolated from HUVECs using TRIzol reagent and quantified by measuring the optical density at 260 nm. cDNA was synthesized from 1 μg of total RNA, which was the used for quantitative real-time PCR. Quantitative analysis of mRNA expression was performed using the ABI 7300 real-time PCR system with the Power SYBR-Green PCR Master Mix kit. PCR primers were as follows: HMGB1 (forward, 5'-ATGTTGCGAAGAAACTGG-3' and reverse, 5'-TTCAGCCTTGACAACTCC-3'); RAGE (forward, 5'-AAGCCCCTGGTGCCTAATGAG-3' and reverse, 5'-CACCAATTGGACCTCCTCCA-3'); TLR-2 (forward, 5'-ATCCTCCAATCAGGCTTCTCT-3' and reverse, 5'-ACACCTCTGTAGGTCACTGTTG-3'); TLR-4 (forward, 5'-ATATTGACAGGAAACCCCATCCA-3' and reverse, 5'-AGAGAGATTGAGTAGGGGCATTT-3'); GAPDH (forward, 5'-CAATGACCCCTTCATTGA-3' and reverse, 5'-GACAAGCTTCCCGTTCTCAG-3'). The PCR amplification profiles consisted of denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing at 60˚C for 60 sec. All amplification reactions for each sample were carried out in triplicates, and the relative expression values were normalized to the expression value of GAPDH.

Western blot analysis. The protein expression of RAGE, TLR-2 and TLR-4 was determined by western blotting. Briefly, after drug treatment, cells were lysed with ice-cold lysis buffer [0.33 mol/l Tris/HCl, 10% SDS (wt/vol), 40% glycerol (vol/vol) and 50 mmol/l DTT containing bromophenol blue], and the protein content of the lysates was measured using the bicinchoninic acid (BCA) method. Equal amounts of protein were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. After being blocked with TBST containing 5% bovine serum albumin, the membranes were incubated with the primary antibody for RAGE, TLR-2, TLR-4 and β-actin overnight at 4˚C, and then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The protein bands were quantitated by video densitometry, and the results were normalized to β-actin expression.

Statistical analysis. Data are expressed as means ± SEM. All values were analyzed by analysis of variance followed by the Student-Newman-Keuls test. $P<0.05$ was regarded as indicative of statistical significance.

Results

Effect of PEF on cell viability induced by LPC. MTT assay showed that treatment of HUVECs with LPC (10 mg/l) for 24 h significantly decreased the cell viability. Pretreatment with PEF 2 h prior to exposure to LPC concentration dependently inhibited the decrease in cell viability induced by LPC. However, PEF alone had no effect on cell viability (Fig. 1).

Effect of PEF on inflammatory factor production induced by LPC. ELISA showed that treatment with LPC significantly

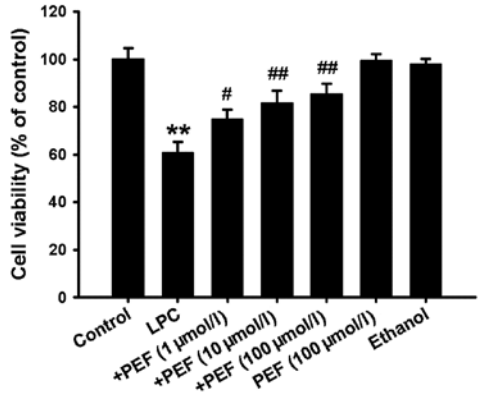
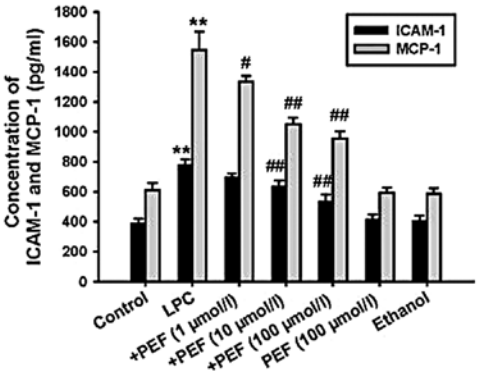


Figure 1. Effect of PEF on LPC-induced cell viability in HUVECs. Data are expressed as means ± SEM and represent three independent experiments. **P<0.01 vs. control; #P<0.05, ##P<0.01 vs. LPC.



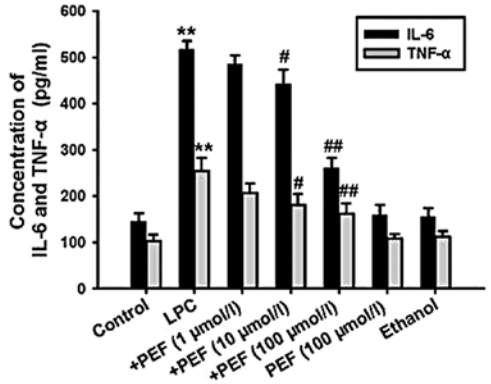
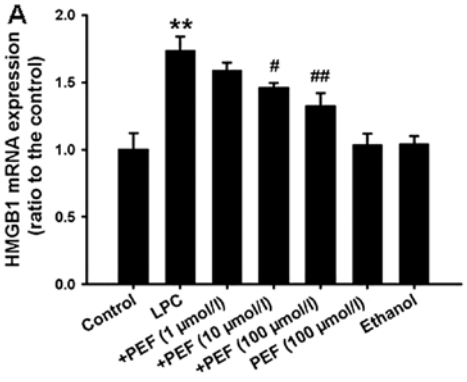


Figure 2. Effect of PEF on LPC-induced inflammatory factor production in HUVECs. Data are expressed as means ± SEM and represent three independent experiments. **P<0.01 vs. control; #P<0.05, ##P<0.01 vs. LPC.

increased the concentration of ICAM-1, MCP-1, IL-6 and TNF-α in cell culture supernatants. This effect of LPC was markedly attenuated by pretreatment with PEF in a concentration-dependent manner. However, PEF alone had no effect on inflammatory factor production (Fig. 2).

Effect of PEF on HMGB1 expression and release induced by LPC. Real-time PCR analysis showed that HMGB1 mRNA expression was significantly upregulated in LPC-treated HUVECs. Consistent with this result, treatment with LPC



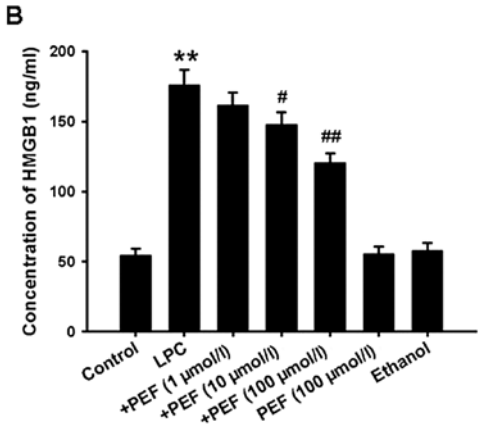


Figure 3. Effect of PEF on LPC-induced HMGB1 mRNA (A) expression and (B) release in HUVECs. Data are expressed as means ± SEM and represent three independent experiments. **P<0.01 vs. control; #P<0.05, ##P<0.01 vs. LPC.

markedly increased the concentration of HMGB1 in cell culture supernatants. However, these effects of LPC were reversed by pretreatment with PEF in a concentration-dependent manner. PEF alone had no effect on HMGB1 mRNA expression and release (Fig. 3).

Effect of PEF on the expression of HMGB1 receptors induced by LPC. The mRNA expression of RAGE, TLR-2 and TLR-4 was significantly upregulated in HUVECs treated with LPC compared with the controls (Fig. 4A). Consistent with the mRNA expression, treatment with LPC significantly increased the protein expression of RAGE, TLR-2 and TLR-4 (Fig. 4B and C). These effects were attenuated by pretreatment with different concentrations of PEF.

Effect of PEF on NF-κB activation induced by LPC. Since activation of NF-κB is essential to inflammatory factor production, we therefore detected NF-κB activity. Treatment of HUVECs with LPC significantly increased NF-κB activity, which was indicated by the increased optical density of NF-κBp65 in the nuclear lysates (Fig. 5). This effect was markedly inhibited by pretreatment with different concentrations of PEF.

Discussion

In the present study, we investigated the inhibitory effect of PEF on LPC-induced inflammatory factor production and

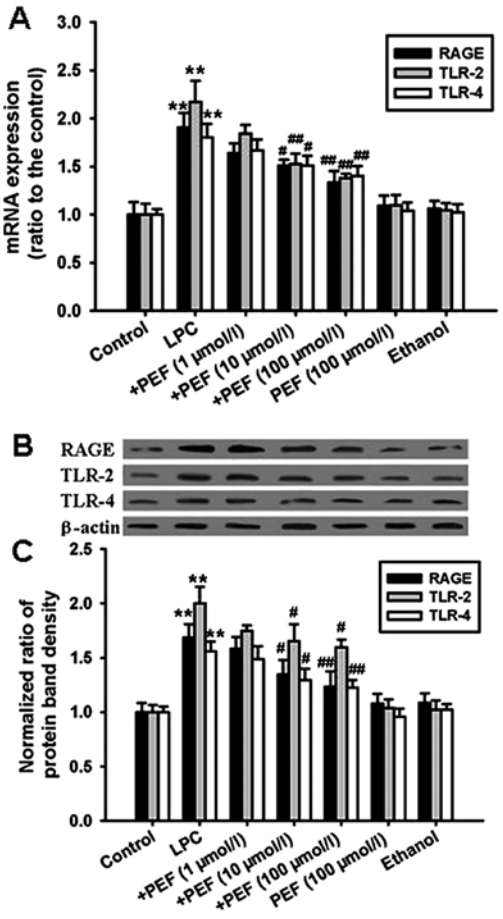


Figure 4. Effect of PEF on LPC-induced RAGE, TLR-2 and TLR-4 expression in HUVECs. (A) mRNA expression of RAGE, TLR-2 and TLR-4 by real-time PCR. (B) Representative image of RAGE, TLR-2 and TLR-4 protein expression by western blotting. (C) Optical density of protein bands. Data are expressed as means ± SEM and represent three independent experiments. **P<0.01 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. LPC.

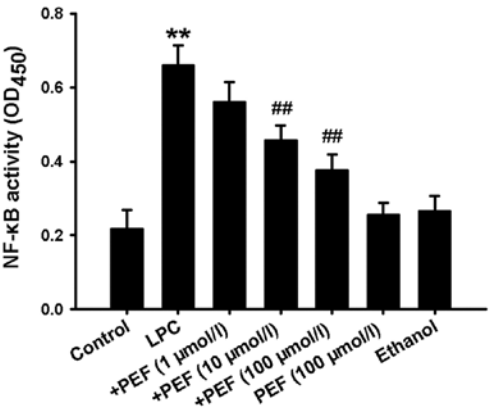


Figure 5. Effect of PEF on LPC-induced NF-κB activation in HUVECs. Data are expressed as the mean optical density (OD) at 450 nm ± SEM and represents three independent experiments. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. LPC.

the underlying mechanisms in HUVECs. The results showed that pretreatment with PEF was able to inhibit LPC-induced inflammatory factor production in a concentration-dependent manner, which was related to inhibition of HMGB1 expression and release, downregulation of RAGE, TLR-2 and TLR-4 expression, and decrease in NF-κB activity.

Vascular inflammatory response is well known to play a crucial role in all stages of atherosclerosis from the nascent lesion to acute coronary syndromes (23). Inhibition of the inflammatory response is a potential strategy to prevent the development of atherosclerosis (8,9,24). LPC is the major bioactive lipid component of ox-LDL and is implicated as an important factor in the atherogenic activity of ox-LDL (25). Many studies have indicated that the atherogenic effect of LPC is closely related to the inflammatory response. Gonçalves *et al* (26) reported that an increased level of LPC plays a key role in human atherosclerotic plaque inflammation. In addition, LPC was found to significantly increase the adherence of monocytes to endothelial cells and upregulate the expression of ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1), which are both biomarkers in inflammatory progression (5). Therefore, inhibition of LPC-induced inflammation may be a promising approach by which to prevent the development of atherosclerosis.

HMGB1, also named amphoterin, is a highly conserved chromatin binding protein that is expressed in multiple cell types including endothelial cells (27). HMGB1 overexpression and release can initiate and amplify the inflammatory response (28). Recently, extracellular HMGB1 was identified as a potent pro-inflammatory cytokine (16) and plays a decisive role in the mediation of inflammatory responses (29). Therefore, HMGB1 is recognized as a potential therapeutic target in inflammatory diseases (28). As previously mentioned, HMGB1 interacts with RAGE, TLR-2 and TLR-4, the cell surface receptors of HMGB1, to amplify inflammatory responses by activating NF-κB and then inducing inflammatory factor expression and release (30). Therefore, the HMGB1-RAGE/TLR-2/TLR-4-NF-κB pathway may be an important inflammatory signaling pathway.

PEF, a monoterpene glucoside isolated from the dry root of *Paeonia*, has been reported to have multiple beneficial effects, such as the lowering of cholesterol levels, anti-platelet agglutination and neuroprotective effects (31-33). Recently, there is growing evidence that PEF exerts an anti-inflammatory effect in animal models of collagen-induced arthritis (34), ischemia/reperfusion-induced cerebral injury (20), lipopolysaccharide-induced acute lung injury (21) and liver inflammatory reactions (35). However, the exact anti-inflammatory mechanisms of PEF are still unclear. Since PEF has anti-inflammatory properties and the HMGB1-RAGE/TLR-2/TLR-4-NF-κB pathway is an important inflammatory signaling pathway, we therefore hypothesized that PEF may be able to inhibit LPC-induced inflammatory factor production in HUVECs, and the mechanism may be associated with inhibition of the HMGB1-RAGE/TLR-2/TLR-4-NF-κB signaling pathway. In the present study, we found that pretreatment of PEF significantly inhibited LPC-induced inflammatory factor production concomitantly with decreased expression and release of HMGB1, downregulated mRNA and protein expression of RAGE, TLR-2 and TLR-4, and decreased NF-κB activity. These results confirmed our hypothesis.

In summary, the present study demonstrated that PEF was able to inhibit LPC-induced inflammatory factor production, which was related to inhibition of the HMGB1-RAGE/TLR-2/TLR-4-NF-κB signaling pathway.

Acknowledgements

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