Danhong Inhibits Oxidized Low-Density Lipoprotein–Induced Immune Maturation of Dendritic Cells via a Peroxisome Proliferator Activated Receptor γ–Mediated Pathway

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Abstract. Danhong injection (DHI), a Chinese Materia Medica standardized product extracted from *Radix Salviae miltiorrhizae* and *Flos Carthami tinctorii*, is effective in the treatment of atherosclerosis (AS)-related diseases. It is widely recognized that AS is a complex inflammatory disease of the arterial wall and the dendritic cells (DCs) is a major player in the pathogenesis of AS via mediating atherosclerotic antigen presenting and T lymphocytes. Here, we determined the effect and possible mechanism of DHI on oxidized low-density lipoprotein (ox-LDL)-induced maturation and immune function of DCs. Human monocyte-derived DCs were incubated with DHI or ciglitazone and were subsequently stimulated with ox-LDL to induce maturation. Similar to ciglitazone, a peroxisome proliferator activated receptor (PPAR) γ agonist, DHI, could significantly reduce ox-LDL-induced expressions of mature markers, enhance the endocytotic function, and inhibit secretions of cytokine on DCs. These effects of DHI could be partly reversed by silencing the PPARγ. In conclusion, DHI could inhibit ox-LDL-induced maturation of DCs partly through activating a PPARγ-mediated signaling pathway.

Keywords: Danhong injection, dendritic cell, peroxisome proliferator activated receptor (PPAR) γ, atherosclerosis

Introduction

Atherosclerosis (AS) is a chronic inflammatory disease of the arterial vessel wall. The recruitment of immune cells such as dendritic cells (DCs), macrophages, and T lymphocytes plays a key role in the initiation and progression of AS (1, 2). Recently, the functional importance of DCs in AS has been highlighted in both experimental and clinical studies (3, 4).

DCs are potent professional antigen-presenting cells required for initiation of innate and adaptive immune responses (5, 6). DCs present in normal arteries are immature and become mature during atherogenesis, and emergence of dendritic cells in rupture-prone regions of vulnerable carotid plaques was documented (7). Proatherogenic factors such as oxidized low-density lipoproteins (ox-LDL) (8, 9), advanced glycation end products (10), and C-reactive protein (CRP) (11) were reported to induce DC maturation and subsequently resulting in T-lymphocyte activation. Therefore, an agent able to inhibit the function of DCs may be beneficial in the treatment of atherosclerotic disease (12).

Danhong injection (DHI), a Chinese Materia Medica standardized product extracted from *Radix Salviae miltiorrhizae* and *Flos Carthami tinctorii*, is effective for treating AS-related diseases. In a randomized clinical trial, Zhao et al. (13) reported that combined conventional therapy with DHI for 2 weeks significantly reduced the plasma levels of endothelin (ET)-1, soluble P-selectin,
and high-sensitivity CRP in patients with acute coronary syndrome (ACS) after percutaneous coronary intervention (PCI). In another randomized clinical trial, Chen et al. (14) showed that DHI intravenous dripping on top of standard therapy was more effective in inhibiting the inflammatory reaction and platelet activation in ACS patients after PCI.

*Salvia miltiorrhiza* BUNGE is one of the main ingredients of DHI, which contains multiple active components such as salvianolic acid (Sal), tanshinone, and tanshinol, that may be responsible for the multiple vasoprotective effects of DHI. Ding et al. (15) showed that the extracts of *Salvia miltiorrhiza* BUNGE and its major ingredients, Sal B and tanshinol, significantly inhibited tumor necrosis factor (TNF)-α-induced endothelial permeability. Fang et al. (16) demonstrated that tanshinone IIA could downregulate the CD40 expression and matrix metalloproteinase (MMP)-2 activity in the high-fat diet–induced rabbit AS model. Kang et al. (17) evidenced that tanshinones significantly inhibited interleukin (IL)-12 production in lipopolysaccharide (LPS)-activated macrophages in a dose-dependent manner. Zhang et al. (18) reported that Sal B could inhibit TNF-α-induced MMP-2 upregulation in human aortic smooth muscle cells via suppression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase–derived reactive oxygen species.

We previously showed that the peroxisome proliferator activated receptor (PPAR) γ agonist ciglitazone could inhibit ox-LDL-induced DC maturation. In this study, we tested the hypothesis that DHI might inhibit DC maturation in a similar way as ciglitazone by analyzing the expression of maturation markers, endocytotic ability, and secretions of cytokines in DCs treated with ox-LDL in the absence or presence of DHI or ciglitazone.

**Materials and Methods**

**Reagents**

The culture medium was RPMI 1640 (Gibco-BRL Life Technologies, Paisley, UK), supplemented with 2 mM L-glutamine, 25 mM HEPES, 10% heat-inactivated FBS (Hyclone, Logan, UT, USA), recombinant human (rh) GM-CSF, and rhIL-4 (R&D Systems Inc., Minneapolis, MN, USA). The following reagents were obtained from the indicated commercial sources: Histopaque 1077, ox-LDL, LPS, ciglitazone, FITC-dextran (40,000 Da), and GW9662 (an irreversible PPARγ antagonist) (Sigma Aldrich Inc., St. Louis, MO, USA); FITC- or PE-conjugated anti-human mAb were used to detect CD1a, CD40, CD86, and HLA-DR (Invitrogen, Inc., Camarillo, CA, USA); IL-12 and TNF-α ELISA kits (eBioscience, Inc., San Diego, CA, USA); annexin V apoptosis detection kit (BD PharMingen, Inc., San Diego, CA, USA); human CD14+ immunomagnetic microbeads (Milteny Biotech, Inc., Bergisch Gladbach, North Rhine-Westphalia, Germany); anti-PPARγ and anti-GAPDH antibodies (Cell Signaling Technologies, Danvers, MA, USA). PPARγ siRNA (sc-29455), control nonsense siRNA (sc-37007), siRNA transfection reagents (sc-29528), and siRNA transfection medium (sc-36868) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). DHI was kindly donated by Shanxi Buchang Pharmaceutical Co. (Xian, Shanxi, China; lot No. 081026).

**Preparation of human monocyte-derived DCs**

Human peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation using Histopaque 1077 as described previously (10). The monocytes (over 98%) were then purified from PBMCs by positive selection with anti-CD14 magnetic beads, seeded into six-well flat-bottom plates with 10⁶ cells per well, cultured in 2 ml RPMI-1640 containing 100 ng/ml rhGM-CSF, 40 ng/ml rhIL-4, and 10% FBS. On culture day 5, cells were pretreated with 100 μg/ml DHI, 25 μg/ml ciglitazone, or 5 μM GW9662 for 24 h and then stimulated by addition of 50 μg/ml ox-LDL or 1 μg/ml LPS for another 48 h, respectively. Cell viability was over 90% as assessed by Trypan blue staining.

**Components and preparation of DHI**

DHI has been approved by state food and drug administration as Chinese herbal patents for CHD patients for more than 5 years and listed in the Chinese Pharmacopoeia (Authorized No. Z20026866). DHI contains 2 medicinal components, *Salvia miltiorrhiza* BUNGE and *Carthamus tinctorius* L.. The herbal drugs were authenticated and standardized on marker compounds according to the Chinese Pharmacopoeia 2005. DHI was prepared as follows: *Salvia miltiorrhiza* BUNGE and *Carthamus tinctorius* L. (3:1) were extracted by boiling distilled water, the aqueous extracts were filtered and concentrated properly to a clear paste, and then the clear paste was placed in ethanol; after the paste was dissolved in ethanol, a pure filtrate was obtained after filtration and evaporation, to which isotonic sodium chloride injection was added in order to adjust pH level to 6 – 7; DHI was then prepared after filtration, potting, and sterilization. To reduce the dose variability of DHI among different batches, the species, origin, harvest time, medicinal parts, and preparation methods for each component were strictly standardized. Moreover, high performance liquid chromatography (HPLC) was applied to quantitate the components of the DHI (Supplementary Methods and Fig. 1: available in the online version only).
Flow cytometric measurement

DCs were washed, resuspended in ice-cold PBS containing 5% FBS and then incubated with FITC- or PE-labeled monoclonal anti-CD1a, anti-CD40, anti-CD86, and anti-HLA-DR antibody for 30 min at 4°C. Cells were washed twice and immunofluorescence analysis was performed using a FACS Calibur (BD Bioscience, San Jose, CA, USA) and analyzed using Cell Quest software.

Determination of endocytosis of DCs

To measure the endocytic activity of DCs, FITC-dextran (1 mg/ml) were incubated with DCs in 100 μl PBS containing 5% FBS at 37°C for 30 min. Thereafter, 2 ml ice-cold PBS containing 1% human serum and 0.02% sodium azide were added to the medium to stop the incubation. The cells were washed twice and analyzed by FACS Calibur.

Measurement of cytokine secretion of DCs

Supernatants of DCs cultures were harvested and stored at −70°C. Human IL-12 and TNF-α levels in culture supernatants were measured using ELISA kits according to the manufacturer’s instructions.

RNA interference

Followed by the protocols provided by Santa Cruz Biotechnology, we first seeded cells into six-well, flat-bottom plates (10⁵ per well), cultured in 2 ml RPMI-1640 containing 100 ng/ml rhGM-CSF, 40 ng/ml rhIL-4, and 10% FBS. On culture day 4, the cells were washed once with 2 ml siRNA transfection medium and then the cells were overlaid with the siRNA duplex solution, which was mixed gently with PPARγ siRNA or control siRNA diluted in siRNA transfection medium for 30 min at room temperature. For each well, 0.8 ml siRNA transfection mixture was added and the cells were incubated at 37°C for 6 h; then the siRNA duplex solution was removed and replaced with RPMI-1640, followed by incubation of the cells for an additional 24 h. After transfection, the cells were stimulated with DHI and then ox-LDL. Whole cell lysates were subjected to western blotting with anti-PPARγ antibody to confirm the effect of siRNA on alteration of PPARγ transcriptional expression.

Western blot analysis

Whole cell extracts (20 μg) were separated by electrophoresis in 12% PAGE and the proteins transferred to PVDF membranes (Millipore Corp., Billerica, MA, USA). Then, the membranes were blocked for 1 h in 0.1% TBS – Tween 20 containing 5% bovine serum albumin, followed by probing with anti-PPARγ (1:1000) or anti-GAPDH (1:1000) antibody at 4°C overnight. The membranes were washed 3 times for 15 min each with TBS-Tween, incubated at room temperature for 2 h with diluted (1:5000) secondary HRP-conjugated antibodies. Immunoreactive proteins were identified using Super Signal West Pico Chemiluminescence Substrate (Thermo, Watham, MA, USA). The ChemiDoc™ XRS gel documentation system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with Quantity One software was used to quantify the immunoreactive proteins. GAPDH was used as the loading control for each lane.

Statistical analyses

Data were presented as means ± S.D. Group mean values were compared with one-way ANOVA followed by post hoc tests using Tukey’s procedure for pairwise comparisons. All statistical analyses were performed with the SPSS 11.5 statistical software, and a P value < 0.05 was considered statistically significant.

Results

DCs morphology

The CD14 microbeads sorted monocytes were symmetrical and the purity was over 98%. Twenty-four hours later, most cells formed different clones, which became larger after 96 h; the dendrites were clearly observed.

DHI suppressed surface molecules of DCs

The expressions of typical surface molecules indicating DCs growth from immature to maturation state were detected. Ox-LDL (50 μg/ml)-treated DCs were exposed to various concentrations of DHI (0, 50, 100, and 200 μg/ml) on the fifth day, and the expressions of costimulatory molecule CD40 were assessed by flow cytometry. The mean fluorescence intensity (MFI) was 103.81 ± 10.97, 87.28 ± 3.85, 40.48 ± 5.88, and 68.13 ± 5.59, respectively. CD40 expression was significantly reduced by DHI at a concentration of 100 μg/ml compared to that in the ox-LDL group (Supplementary Fig. 2: available in the online version only). DHI (100 μg/ml)-induced DCs apoptosis was analyzed by Annexin V/7AAD double staining post 24-h incubation, DHI (100 μg/ml) alone was not toxic to DCs (Supplementary Methods and Fig. 3: available in the online version only). DHI at a concentration of 100 μg/ml was therefore used in the main study. Compared with the control group (culture media alone), the MFI values of CD1a, CD40, CD86, and HLA-DR were significantly increased in the ox-LDL- and LPS-treated groups; these could be significantly attenuated by DHI or ciglitazone (P < 0.05, respectively); however, these could not be inhibited by the PPARγ antagonist GW9662 (P > 0.05, respectively) (Fig. 1).
DHI increased endocytic function of DCs

FITC-dextran was used to identify the endocytotic function of DCs. Compared with the control group, the endocytotic function of DCs was decreased in the ox-

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** Effects of DHI and ciglitazone on the expression of DC surface markers induced by ox-LDL and LPS. PBMCs were separated and purified with anti-CD14 magnetic beads and subsequently cultured with rhGM-CSF (100 ng/ml) and rhIL-4 (40 ng/ml) for 5 days. Then, ox-LDL (50 μg/ml) or LPS (1 μg/ml) was added into the DCs for 48 h in the presence or absence of DHI (100 μl/ml) or ciglitazone (25 μg/ml). The surface molecular markers of DCs (A, CD1a; B, CD40; C, CD86; D, HLA-DR) were analyzed by FACS. The histograms are composite with the surface molecular expressions of DCs treated with culture media alone (control group, CTL, light gray shadow), ox-LDL (dotted line), ox-LDL + ciglitazone (thin continuous line), or ox-LDL + DHI (thick continuous line). The dark gray shadow represents the loading control. The bar graphs show MFI results (mean ± S.D.) of three independent experiments in various groups. *P < 0.05 vs. CTL, †P < 0.05 versus LPS, ‡P < 0.05 vs. ox-LDL. ANT represents the PPARγ antagonist GW9662.
LDL- and LPS-treated groups and were partly reversed by DHI as well as ciglitazone ($P < 0.05$, respectively). However, the decreased endocytotic function of DCs induced by ox-LDL and LPS could not be increased by the PPAR$\gamma$ antagonist GW9662 ($P > 0.05$, respectively) (Fig. 2).

**DHI inhibited cytokine secretion of DCs**

Compared with the control group, the levels of IL-12 and TNF-$\alpha$ were significantly increased in ox-LDL-induced as well as LPS-induced DCs, which were significantly decreased by pretreatment with DHI or ciglitazone, respectively. However, the increased concentrations of IL-12 and TNF-$\alpha$ could not be inhibited by the PPAR$\gamma$ antagonist GW9662 ($P > 0.05$, respectively) (Fig. 3).

**Effect of PPAR$\gamma$ siRNA interference on maturation of DCs**

DCs were transfected with PPAR$\gamma$-specific siRNA and then assayed for PPAR$\gamma$ expression. Western blotting showed that the expression of PPAR$\gamma$ was significantly reduced by the cognate PPAR$\gamma$ siRNA duplex compared with transfection with a control siRNA whose sequence is unrelated to PPAR$\gamma$ (Fig. 4). Silencing the expression of the PPAR$\gamma$ in DCs, the maturation-associated markers CD40, CD1a, and CD86 (Fig. 5A) increased, the endocytotic function (Fig. 5B) decreased, and the levels of IL-12 and TNF-$\alpha$ (Fig. 5C) significantly increased compared with the non-silencing group, respectively. However, the increased expression of HLA-DR exhibited no significant difference compared with no-silencing PPAR$\gamma$ DCs ($P = 0.0535$).

**Discussion**

In the present study, we described an inhibitory effect of DHI, a traditional Chinese herbal medicine, on the...
human DCs maturation induced by ox-LDL. Similar to ciglitazone, preincubation of DCs with DHI could suppress the expressions of maturation-associated markers such as CD40, CD86, CD1a, and HLA-DR, as well as attenuate the secretions of inflammatory cytokines such as IL-12 and TNF-α induced by ox-LDL. The decreased endocytotic function induced by ox-LDL was also significantly increased by DHI. After knocking down the expression of the PPARγ with specific siRNA, these inhibitory effects of DHI were significantly reversed. The present study suggested that DHI mimics the effect of ciglitazone on DCs maturation, which act partly through a PPARγ signaling pathway. To our knowledge, this is the first study to provide evidence that DHI can inhibit ox-LDL-induced DCs maturation.

As a compound of traditional Chinese medicine, DHI was shown to reduce the incidence of coronary events in patients with coronary artery disease. Zhao et al. showed that DHI can significantly reduce the plasma levels of endothelin-1, soluble P-selectin, and high-sensitivity C-reactive protein (hs-CRP) in ACS patients after PCI, suggesting that DHI has certain effects in protecting the endothelial function, inhibiting platelet activation and suppressing the inflammatory reaction (13). Chen et al. showed that DHI can effectively suppress the serum levels of CD62p, glycoprotein IIb/IIIa, fibrinogen C, and hs-CRP, suggesting that DHI can inhibit platelet activation and inflammation in patients of ACS after PCI (14). Multiple actions of DHI including improvement of endothelial function, modulation of lipid level, vasodilatation, anti-oxidation and anti-thrombosis, anti-inflammation, anti-apoptosis, and angiogenesis were reported by previous studies (19 – 23).

The current study included findings showing that one of the vasoactive properties of DHI might be linked with the ability to modulate DC maturation. Pretreatment of ox-LDL-stimulated DCs with DHI could reduce surface expression of costimulatory molecules, such as CD40, CD86, and CD1a, and MHC-II molecule HLA-DR. The presence of such molecules on DCs is required for T-lymphocyte activation and differentiation. In the absence of costimulatory and MHC-II molecules, T lymphocytes interacting with DCs undergo anergy or apoptosis. DHI pretreatment could significantly decrease inflammatory cytokines such as IL-12 and TNF-α production in ox-LDL-induced DCs. A number of cytokines produced by DCs can affect their function at various levels, including their viability, morphology, migration, expression of surface molecules, and binding and processing of antigen peptides. IL-12, a heterodimeric cytokine consisting of p35 and p40 is released mainly by activated macrophages and DCs. IL-12 is a key mediator in inducing the Th1 response and stimulating the production of anti-inflammatory cytokines such as IL-10 and interferon (IFN)-γ from Th1. Inhibition of DC-derived IL-12 production is followed by a down-regulated response of Th1. TNF-α, a pleiotropic cytokine, is also mainly produced by DCs themselves. TNF-α appears to have profound effects on DC function both in vitro and in vivo: it contributes to their activation, maturation (24), and migration to, and accumulation within, draining lymph nodes (25); and it significantly reduces IL-10-mediated inhibition of DC development and function (26). Inhibition of DC-derived TNF-α production is followed by a depressed induction of optimal T-lymphocyte immune responses.

Taken together, we concluded that DHI could effec-
Fig. 5. Effects of PPARγ siRNA transfection on ox-LDL-induced DC maturation in the presence or absence of DHI. PBMCs were separated and purified with anti-CD14 magnetic beads and subsequently cultured with rhGM-CSF (100 ng/ml) and rhIL-4 (40 ng/ml). Then DCs were transfected with PPARγ siRNA, control siRNA, or mock conditions (no siRNA) starting from the 4th day of culture. Subsequently, DCs were stimulated by ox-LDL for 48 h in the absence or presence of DHI. The phenotypic and functional changes of DCs were examined as follows: A, the surface molecular expression (CD40, CD1a, CD86, and HLA-DR) by FACS; B, the endocytosis function by FACS; C, the levels of cytokines (IL-12 and TNF-α) secretion by ELISA. All experiments were performed in triplicate (mean ± S.D.) with similar results. *P < 0.05 vs. CTL, &P < 0.05 vs. ox-LDL, #P < 0.05 vs. ox-LDL + DHI.
tively inhibit ox-LDL-induced DCs maturation. Next, we compared the modulation effect of DHI with that of ciglitazone (a known PPARγ agonist) on ox-LDL-induced DCs maturation and found that the inhibitory effect on DCs maturation was similar between DHI and ciglitazone, suggesting the observed effect of DHI on DCs maturation might be mediated by the PPARγ pathway.

PPAR belongs to the nuclear hormone-receptor superfamily, which consist of three related transcription factors PPARα, PPARβ, and PPARγ. Although PPARγ was initially found to be critical for adipocyte differentiation and function, PPARγ was discovered to play an important role in the cardiovascular system (27, 28). PPARγ appeared to be highly expressed during atherosclerotic lesion formation, particularly in macrophage/foam cells, suggesting that PPARγ might affect atherosclerogenic processes. Ligand-activated PPARγ decreased the inflammatory response in atherosclerogenic cells (29, 30). The previous study from our team also showed that the PPARγ agonist ciglitazone could effectively inhibit ox-LDL-induced DCs maturation (8).

Sung et al. (31) showed that benzo furan lignans extracted from Salvia miltiorrhiza Bunge could activate the PPARγ signaling pathway and its target proteins such as adipocyte protein 2 and stearoyl-CoA desaturase in adipocytes. Our latest study showed that Sal B, a main component of DHI, could effectively suppress ox-LDL-induced DC maturation through PPARγ activation (32). In fact, the effect of DHI on DCs was largely blocked post silencing the expression of PPARγ in DCs. However, the expression on MHC II molecule HLA-DR was not affected by silencing PPARγ. Thus, we speculate that DHI, as a multiple compound medicine, might modulate DCs maturation via multiple ways, and PPARγ is one of the major signaling pathway of DHI.

In conclusion, this study provided insight into the pharmacological functions of DHI, and inhibiting ox-LDL-induced DCs maturation, which was mediated in part via activating the PPARγ pathway, might contribute to DHI’s vasoprotective effects.

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