DBZ Blocks LPS-induced Monocyte Activation and Foam Cell Formation via Inhibiting Nuclear Factor- κ B

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Key Words

Monocyte activation • Inflammation • NF- κ B • Foam cell

Abstract

Background/Aims: It has been widely accepted that chronic inflammation plays important roles in the atherogenesis. Danshensu Bingpian Zhi (DBZ) is a novel synthetic compound derived from the traditional Chinese medicine (TCM) formula Fu Fang Dan Shen (FFDS), which is effective on atherosclerosis clinically. We hypothesized that DBZ possessed the anti-atherosclerosis potentials. Here, we examined the inhibitory effects of DBZ on LPS-induced monocyte activation and foam cell formation. **Methods:** The effects of DBZ were assessed on LPS-induced inflammatory factors expression in monocyte/macrophage. Activation of NF- κ B and AP-1 was analyzed by luciferase reporter assay and signaling pathway of NF- κ B was investigated to elucidate mechanisms underlying DBZ mediated anti-inflammatory activity. Effects of DBZ on macrophage lipid accumulation were evaluated in native LDL and LPS co-incubated macrophages. **Results:** DBZ inhibited LPS-induced inflammatory factors

expression dose dependently in monocytes. DBZ inhibited NF- κ B activation strongly and AP-1 slightly. DBZ suppressed the LPS-induced degradation of I κ B α , thereby decreasing the translocation of p65 to nucleus. Furthermore, DBZ suppressed LPS-activated macrophages lipid accumulation, partly due to inhibiting the expression of LPS-induced aP2 and ADRP in macrophages. **Conclusion:** These results demonstrate that DBZ has potentials on anti-atherosclerosis by suppressing monocyte activation and foam cell formation.

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Introduction

Atherosclerosis is a disease which involves a state of chronic inflammation [1, 2]. Monocyte as the source of macrophage and ultimate foam cell in atherosclerotic lesion, plays important roles in the early stage of the pathogenesis of atherosclerosis [3]. The epidemiological studies have shown that an increased incidence of

coronary artery disease occurs in patients with *Helicobacter pylori* [4], chronic dental [5], and chronic bronchitis infections [6] in which monocytes were activated by microorganisms and accompanied by a chronic inflammation. These studies suggested that monocytes play a very important role in atherogenesis by the way of their proficiency in generating inflammatory mediators, such as cytokines (TNF α , IL-1 β , IL-6), chemokines (MCP1), inducible enzymes (MMP9, iNOS and COX2). These inflammatory mediators have been indicated to mediate all stages of atherosclerosis [7]. LPS, which is a component of the gram negative bacterial wall, could mimic the chronic infection. LPS interacts mainly with monocytes and macrophages via the toll-like receptor 4 (TLR4), and activates a number of intracellular signal pathways which lead to the release of inflammatory mediators. Spontaneous atherosclerosis model apolipoprotein E deficient mice (ApoE^{-/-}), which were subjected to weekly injection of LPS, showed increased atherosclerotic lesion size $\approx$ 100% [8]. These results strongly support that monocyte activation and inflammatory mediators promote the atherogenesis. In addition, anti-inflammatory compounds are proved to be effective on treating atherosclerosis [8, 9]. In this context, we infer that a compound with anti-inflammatory activity would have potentials on the prevention and treatment of atherosclerosis.

The transcription factor NF- κ B is a key player in the development and progression of chronic inflammatory diseases, such as rheumatoid arthritis, asthma and atherosclerosis. Binding sites of NF- κ B have been found in promoter sequences of many inflammatory genes. NF- κ B is activated by various stimuli including TLR agonists and cytokines through activation of the inhibitor of κ B kinase (IKK) which lead to the degradation of inhibitor of κ B α (I κ B α) and translocation of NF- κ B to nucleus [10, 11]. AP-1 is another important transcription factor involved in inflammation activation which is also activated by TLR agonists and cytokines dependent on the activation of c-Jun-NH2-terminal kinase (JNK) [12, 13]. Previous studies have demonstrated that many inflammatory genes are under the control of both NF- κ B and AP-1 [14-16]. So, NF- κ B and AP-1 are regarded as attractive targets to seek for anti-inflammatory agents.

Foam cell formation is a hallmark event during the initial stage of atherosclerosis. It is believed that infection related monocytes activation is involved in the foam cell formation [17-19]. Activation of monocytes by LPS has been shown to increase native LDL uptake and cholesterol accumulation in macrophage [20]. Previous studies have

demonstrated that LPS could influence many aspects associated with macrophage derived foam cell formation, such as promotion of LDL oxidation [21], up-regulation of macrophage fluid phase endocytosis and inhibition of cholesterol efflux [22, 23]. Recently, adipocyte fatty acid-binding protein (aP2/FABP4) and adipocyte differentiation related protein (ADRP/ADFP), which are markers of terminal adipocyte differentiation and facilitate the lipid accumulation during adipocyte maturation, were shown to be induced abundantly during the monocytes activation by TLR agonists and ox-LDL [24-28]. Forced expression of aP2 and ADRP in cells has been shown to enhance uptake of lipid [29-31] and lipid droplet formation [32]. aP2 and ADRP also have been shown to express in macrophages and foam cells at the sites of atherosclerotic lesions [33, 28]. Further studies demonstrated that macrophage-specific aP2/ADRP-deficiency led to a marked protection against atherosclerosis plaque formation in ApoE^{-/-} mice by inhibiting foam cell formation [34, 35]. All these studies suggested that expression of aP2 and ADRP in macrophages is closely related to foam cell formation and atherogenesis. In addition, it's demonstrated that activation of NF- κ B and AP-1 is necessary for inflammatory stimuli induced expression of aP2 and ADRP in monocytes, specific inhibitors of NF- κ B and AP-1 have been indicated to abolish inflammatory stimuli induced expression of aP2 and ADRP [25, 27]. All these studies implicate that aP2 and ADRP are mediators connecting infection stimulated monocytes activation and foam cell formation.

DBZ is a novel synthetic compound that chemically links Danshensu and borneol. The synthesis of this compound was inspired by the 'sovereign and courier' theory of traditional Chinese medicine formula FFDS, which has been indicated to be effective on treating cardiovascular and cerebrovascular diseases for years in China [36, 37]. Danshensu as the primary ingredient from aqueous extract of *Salvia miltiorrhiza* (sovereign in FFDS) has been shown to have pleiotropic activities, such as protection from homocystein induced endothelial dysfunction [38], attenuation of increased endothelial permeability induced by TNF α [39], inhibition of platelet aggregation [40], improvement of microcirculation [41], and protection of myocardium from reperfusion injury of the ischemic heart [42]. Borneol (courier in FFDS) as an adjuvant element in the FFDS formula, is believed to play assisting roles in facilitating the delivery of principal component, increasing bioavailability and promoting active compounds penetrating the biological barriers [43-45]. Based on the understanding of individual functions of

Danshensu and borneol and clinical effect of FFDS on atherosclerosis, we hypothesized that the novel compound DBZ might be effective in inhibiting atherosclerosis. On the other hand, atherosclerosis is widely accepted as an inflammatory disease. We thereby targeted the monocytes/macrophages inflammatory activation to study the activity of DBZ.

Materials and Methods

Cell Culture

The monocytic mouse cell line (Raw264.7) and human cell line (THP-1) were obtained from the American Type Culture Collection (Manassas, VA, USA) and respectively maintained in Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin at 37°C under 5% CO₂ atmosphere. Assays were conducted with cells harvested at 80-90% confluence.

Isolation of Mouse Peritoneal Macrophages

To harvest mouse peritoneal macrophages, the 10-week pathogen-free C57/BL6J male mice were sacrificed, and ice cold RPMI 1640 medium was injected into the peritoneal cavity of each mouse. After 5 minutes, the fluid was carefully collected and centrifuged at 2000 rpm for 10 min. The supernatant was then withdrawn, the cell pellet was washed three times with ice-cold PBS and re-suspended in RPMI 1640 medium containing 10% FBS, allowed to adhere for 3 h, and then washed three times with pre-warmed PBS to remove nonadherent cells. The medium was then replaced with fresh RPMI 1640 medium supplemented with 10% FBS. The cells were treated for the specific times as indicated in the text.

Real time PCR

Raw264.7, THP1 and mouse primary macrophages cells were cultured (5×10^4 cells/ml) in 60 mm tissue culture dish for 24 h, followed by treatment with LPS alone or LPS plus various concentrations of DBZ. At the end of incubation, total RNA were isolated with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Two micrograms of total RNA were reverse transcribed to cDNA using M-MLV transcriptase (Promega, Madison, WI, USA) and oligo (dT)₁₈ primer (Takara, Shuzo, Japan), respectively. cDNA were subsequently subjected to SYBR Green based real time PCR using ABI 7500 real time PCR System (Applied Biosystems, Alameda, CA, USA). Primers used in real time PCR were shown as in Table 1. The gene expression was normalized against the expression of cyclophilin B [46].

Luciferase Reporter Assay

Raw264.7 cells were seeded in 24-well plate and each well was transfected with 1.25 µg of plasmids DNA containing 1.0 µg of NF-κB-luciferase or AP-1-luciferase reporter construct

Gene	Species	Sequence(5' -3')
IL-1β	mouse	Forward:5'-CTGTGTCTTTCCCGTGGACC-3' Reverse:5'-CAGCTCAIATGG GTCCGACA-3'
	human	Forward:5'-ACAGTGGCAATGAGGATGAC-3' Reverse:5'-CCATGGCCACAACAACCTGA-3'
MMP9	mouse	Forward:5'-AAACCAGACCCAGACTCCTCTCT-3' Reverse:5'-GAGGACACAGTCTGACCTGAACCA-3'
	human	Forward:5'-GGACGATGCCTGCAACGT-3' Reverse:5'-CAAATACAGC TGGTCCCAATCT-3'
MCP1	mouse	Forward:5'-GGCTCAGCCAGATGCAGTTAAC-3' Reverse:5'-GCCTACTCATTGGGATCATCTTG-3'
	human	Forward:5'-TGCTCATAGCAGCCACCTTCATTC-3' Reverse:5'-GACACTTGCTGCTGGTGATTCTTC-3'
iNOS	mouse	Forward:5'-CAGATCCCCGAAACGCTTCA-3' Reverse:5'-TGTTGAGGTCTAAAGGCTCCG-3'
	human	Forward:5'-TGGCAGCATCAGAGGGGACC-3' Reverse:5'-GCAGGACAGGGGACCACATCGAA-3'
TNFα	mouse	Forward:5'-CTGAGGTCAATCTGCCCAAGTAC-3' Reverse:5'-CTTCACAGAGCAATGACTCCAAG-3'
	human	Forward:5'-CTCTTCTGCTGTGCTGCACTT-3' Reverse:5'-GGCTACAGGCTGTGCTACTC-3'
COX2	mouse	Forward:5'-CAGAACCGCATTGCCTCTG-3' Reverse:5'-TTGAAGGTGTGCGGCAGC-3'
αP2	mouse	Forward:5'-AGCATCATAACCCTAGATGGCG-3' Reverse:5'-CATAACACATTCCACCACCAGC-3'
ADRP	mouse	Forward:5'-GATTGAATTCGCCAGGAAGA-3' Reverse:5'-TGGCATGTAGTCTGGAGCTG-3'
Cyclophilin B	mouse	Forward:5'-GGAGATGGCACAGGAGGAA-3' Reverse:5'-GCCCGTAGTGTCTCAGCTT-3'
	human	Forward:5'-TTTCATCTGCACTGCCAAGA-3' Reverse:5'-TGCCCAACACCACATGCT-3'

Table 1. Primers used for Real Time PCR.

and 0.25 µg of pRL-TK construct as an internal control (containing *Renilla reniformis* luciferase gene under the thymidine kinase promoter), using VigoFect transfect reagent (Vigorous Biotechnology, Beijing, China). Luciferase activities were determined using the Dual-Luciferase reporter Assay System (Vigorous Biotechnology, Beijing, China) according to the manufacturer's instructions. Luciferase activity was normalized against *Renilla reniformis* luciferase activity.

Nuclear Protein Extraction and Western Blotting

Nuclear proteins were extracted using a commercial kit (KeyGEN Biotechnology, Nanjing, China) according to the manufactory's instruction. Briefly, cells were washed with ice-cold PBS, and allowed to swell after the addition of hypotonic buffer [10 mmol·L⁻¹ HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM DTT and 0.5 mM PMSF] for 10 min. The lysates were centrifuged at 7200 × g for 5 min at 4 °C, the supernatants are cytoplasm proteins. Pellets were re-suspended in hypertonic buffer [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10 mM DTT and 1 mM PMSF] and then incubated further for 30 min on ice. The resultant supernatants from centrifugation at 15 000 × g for 10 min were obtained as nuclear fractions. Concentration of protein in supernatant was measured using Pierce BCA protein assay reagent (Pierce

Chemical Co., Rockford, IL, USA). Protein samples were incubated in $2 \times$ loading buffer for 5 min at 100°C and stored at -20°C . Samples were separated by 8% SDS-PAGE, and subjected to immunoblotting with the appropriate antibodies. Western blot analysis was performed as described previously [46]. All signals were visualized using chemiluminescent reagents (Amersham, Buckinghamshire, UK) and quantified by ImageQuant 5.2 software (Healthcare Bio-Sciences, Philadelphia, PA).

Measurement of IL-6 and NO Contents

IL-6 levels in the culture supernatants were measured using sandwich ELISA kit (Boster Biological Technology, Wuhan, China) according to the manufactory's instruction. The detection limit is between 15.6 pg/ml and 1000 pg/ml for the mouse IL-6. NO_2^- concentrations in culture supernatants was measured to assess NO production. 50 μl of sample aliquots were mixed with 50 μl of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% phosphoric acid) in a 96-well plate and incubated at 25°C in dark for 10 min. The absorbance at 550 nm was measured on a micro-plate reader. NaNO_2 was used as the standard to calculate NO_2^- concentrations.

Immunofluorescence Assay

For the detection of intracellular location of NF- κB p65 subunit, we used Thermo Scientific Cellomics High Content Screening (HCS) system which could analyze the translocation of NF- κB p65 quantitatively based on the statistical amounts of cells. Raw264.7 cells were seeded in 96-well plate for 5×10^3 /well. After incubated overnight, cells were pretreated with different concentrations of DBZ or vehicle for 2 h, and then stimulated with LPS for 30 min or 60 min, respectively. At the end of the stimulation, the cells were fixed with 4% formaldehyde in PBS for 10 min at room temperature and washed with pre-warmed wash buffer for 5 min. The fixed cells were subjected to permeabilization buffer for 10 min and then washed with wash buffer for 5 min. Cells were then incubated in NF- κB p65 primary antibody for 1 h at 37°C . Cells were washed in wash buffer and stained with Dy-light Fluor 488 secondary antibody for 1 h at room temperature. The nuclear were stained with Hoechst Dye. Detailed procedures were referred to Cellomics NF- κB Activation HCS Reagent protocols (Thermo Fisher Scientific). Stained cells were tested on ArrayScan HCS Reader (Thermo Fisher Scientific). The Cytoplasm to Nucleus Translocation BioApplication software was used to calculate the ratio of cytoplasm and nuclear NF- κB intensity [47]. The average intensity of 500 objects (cells) per well was quantified. The ratios were then compared among stimulated, treated, and untreated cells.

Cell Viability Test (MTT Assay)

To determine cell viability, cells (5×10^3 /well) were seeded in sterile 96-well tissue culture plates and treated as indicated. Cell viability was evaluated in each well by the addition of 20 μl of 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT, 2.5 mg/ml in PBS) before 4 h of test, the cell-free

supernatants were removed completely from each well, and 100 μl of DMSO was added. The optical densities of the wells were measured using a spectro photometric multiwell microplate reader (Multiskan MS; Thermo Electron Corporation, Waltham, MA) at wavelength of 570 nm.

LDL Extraction and Purification

LDL was isolated from the fresh plasma of healthy subjects as previously reported [48]. Briefly, after density adjustment to 1.200 g/ml with sodium bromide (NaBr), the plasma was separated by preparative ultracentrifugation at 60,000 rpm for 5.5 h on a Beckman L-80 ultracentrifuge, using a type Ti 90 rotor. The LDL was collected, sterilized, and stored in the dark at 4°C .

Oil Red O Staining

Raw264.7 or primary mouse macrophages were plated at a density of 5×10^4 /ml in 24-well plates overnight prior to infection. Cells were infected with LPS (1 $\mu\text{g}/\text{ml}$) in 500 μl of DMEM with 10 % FBS and LDL (50 $\mu\text{g}/\text{ml}$) in absence or presence of 10 μM DBZ for 18 h. After treatment, cells were washed twice with PBS, fixed for 40 min in 4% formaldehyde (in PBS), and stained for 30 min in 1% Oil Red O (in 60% isopropanol) at 37°C . Cells were then washed with 60% isopropanol quickly and ddH_2O for three times and examined by light microscopy (Olympus, magnification, $\times 40$).

Cellular Lipid Contents Assay

Cells were grown in 6-well plates and treated as in Oil red O staining. At the end of the treatment, cells were washed twice with ice-cold PBS and scraped into 100 μl of ddH_2O . The cell suspension was kept on ice and sonicated for 15 seconds with a sonicator (Sonifier 350; Branson Sonic Power Co., Danbury, CT, USA). Aliquots of 20 μl were assayed separately for total triglyceride and total cholesterol by commercial assay system (Wako chemicals Co., Osaka, Japan). The results were standard by protein concentration determined by the BCA method.

Reagents and Antibodies

DBZ was synthesized by Research and Engineering Center of Modernization of Chinese Herbal Medicine, Northwest University. DBZ was identified by elemental analysis, mass spectrometry, infrared spectroscopy and ^1H - and ^{13}C -nuclear magnetic resonance spectra. Its purity (99.5%) was determined by high performance liquid chromatography-mass spectrometry. Bacterial lipopolysaccharide (LPS) and most of biochemistry reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA); polyclonal antibody to IkB α , p65, iNOS, FABP4/aP2, β -actin and histone H2B were all purchased from Santa Cruz Biotechnology, Inc.(Santa Cruz, CA, USA).

Statistical analysis

Data are expressed as mean $\pm$ standard deviation. ANOVA analysis with a Dunnett's post-hoc test is used to compare the means of normally distributed continuous variables. Statistical significance was set at $P < 0.05$.

Fig. 1. DBZ inhibits LPS-induced inflammatory genes expression in monocytes/macrophages. **A.** Chemical structure of DBZ. **B.** Raw264.7 cytotoxicity was detected using MTT and the cells were treated as presented in C. **C.** Raw264.7 were pretreated with 10, 30 μ M DBZ or vehicle (DMSO) for 2 h and exposed to LPS (1 μ g/ml) stimulation in the presence of DBZ for another 6 h. RNA samples were analyzed by real time PCR with the indicated primers (Table 1). cyclophilin B was used as a internal reference for loading. Ct values from IL-1 β , TNF α , COX-2, iNOS, MCP1, MMP9 measurements were normalized with the values from corresponding cyclophilin B reactions. **D.** Monocytic THP1 cells were treated as same as Raw264.7 and the inflammatory genes expression were also evaluated by real time PCR. **E.** Mouse peritoneal macrophages were isolated and treated as same as Raw264.7, inflammatory genes were detected by real time PCR. Data represent mean ($\pm$ S.D.) from at least 3 independent experiments. *significant difference from LPS treatment $P < 0.05$; ** $P < 0.01$.

Results

DBZ Inhibits LPS-induced Inflammatory Mediators in Monocytes/Macrophages

Inspired from traditional Chinese medicine formula FFDS, we chemically linked DSS and borneol to generate

a novel compound: DBZ (Fig. 1A). We postulated that DBZ would own the activity of the formula FFDS, which is widely applied to prevent and cure atherosclerosis. In order to study the effect of DBZ on atherosclerosis, we targeted to the monocyte inflammation

Fig. 2. DBZ blocks LPS-induced NO production and IL-6 secretion. A. The production of nitric oxide was measured by Griess reaction. Raw264.7 were pretreated with DBZ (10–30 µM) or vehicle (DMSO) for 2 h and followed to LPS (1 µg/ml) stimulation in the presence of DBZ for another 12 h in the 96-well plate. At the end of the treatment, cell supernatants were collected. NO₂⁻ concentration in culture supernatants was measured to assess NO production in Raw264.7 cells by Griess reaction. Data represent mean (± S.D.) from 3 independent experiments*, significant difference from LPS treatment $P < 0.05$; ** $P < 0.01$. B. Expression of iNOS protein was assessed by Western blot analysis. The detection of β-actin was carried out to confirm the equal loading. Protein levels were quantified and data represent mean (± S.D.) from 3 independent experiments. C. IL-6 concentration was measured using sandwich ELISA. Data represent mean (± S.D.) from 3 independent experiments, *significant difference from LPS treatment $P < 0.05$; ** $P < 0.01$.

activation which is considered as an initial event during the atherogenesis. To investigate whether DBZ has anti-inflammatory activity, the murine Raw264.7 and human THP1 monocytic cells were subjected to LPS stimulation to mimic the monocyte activation *in vivo*. Cells were pretreated with DBZ (10, 30 µM) for 2 h followed by treatment with LPS (1 µg/ml) for 6 h. At the end of the stimulation, total RNA were extracted, and the expression of genes encoding cytokines, enzymes and chemokines was measured by real time PCR analysis. In both Raw264.7 (Fig. 1C) and THP1 (Fig. 1D) cells, DBZ effectively inhibited LPS-induced mRNA expression of cytokines, such as IL-1β and TNFα, chemokine MCP1, and inducible enzymes MMP9, iNOS and COX2 in a dose dependent manner. In addition, treatment with DBZ at the concentrations of 10 or 30 µM had little impact on the Raw264.7 viability (Fig. 1B), indicating that DBZ inhibited inflammatory genes expression at these concentrations without affecting the cell viability. 10, 30 µM DBZ also didn't affect THP1 cell viability (data not shown).

Moreover, we questioned whether the suppressive effect of DBZ on inflammatory activation can occur in primary macrophages. The mouse peritoneal macrophages were isolated and subjected to pretreatment with DBZ (10, 30 µM) for 2 h and continuous LPS (1 µg/ml) stimulation for another 6 h. mRNA of primary macrophages were analyzed using real time PCR. DBZ also effectively inhibited LPS-induced mRNA expression of cytokines, chemokines, enzymes in a dose dependent manner in primary macrophages as well (Fig. 1E),

Fig. 3. Effects of DBZ on LPS-induced NF-κB and AP-1 activation. A and B. The effects of DBZ on NF-κB and AP-1 activation were assessed in Luciferase reporter assay. Raw264.7 were transiently transfected with plasmids containing firefly luciferase reporter gene under a promoter containing three tandem repeats of NF-κB (A) or AP-1 (B) binding sites, and *R. reniformis* luciferase expression plasmid as an internal control for 24 h in 48-well plate. Transfected cells were then pretreated for 2 h with DBZ (5–20 µM) or vehicle, continuously activated with LPS (1 µg/ml) in the presence of DBZ for 6 h. Un-stimulated Raw 264.7 cells acted as a negative control. Results were presented as relative luciferase activity (fold difference compared to negative control). All values are means (± S.D.) from 3 independent experiments. *significant difference from LPS treatment $P < 0.05$; ** $p < 0.01$.

indicating that anti-inflammatory effects of DBZ is not restricted to monocytic cell lines. Cell viability results indicated that 10, 30 µM DBZ had no cytotoxic effect on primary peritoneal macrophages (data not shown).

Fig. 4. DBZ blocks translocation of p65 to nucleus induced by LPS in immunofluorescence. A and B. Raw264.7 cells were pretreated with 10, 30 or 50 μ M DBZ or vehicle for 2 h and followed to stimulation with LPS (1 μ g/ml) or not for 30 min or 60 min. Subcellular location of p65 (DyLightTM 488, light green) was sequestered in cytoplasm in cells treated with vehicle or DBZ without LPS stimulation, and the nucleus of cells appears blue (Hoechst). However, p65 translocated to nucleus upon LPS stimulation for 30 min (A) or 60 min (B), so that the distinction between the nucleus (blue) and the cytoplasm (green) blurs after the LPS activation. DBZ pretreatment inhibited translocation of p65 and diminished this blurs obviously in the presence of LPS in a concentration dependent manner. C. The Cytoplasm to Nucleus Translocation BioApplication software was used to calculate the ratio of cytoplasmic and nuclear NF- κ B intensity. The average intensity of 500 objects (cells) per well was quantified. Data represent mean ($\pm$ S.D.) from 3 independent experiments. *significant difference from LPS treatment for 30 min $P < 0.05$; ** $p < 0.01$. # significant difference from LPS treatment for 60 min $P < 0.05$; ## $p < 0.01$.

To confirm the anti-inflammatory activity of DBZ on LPS stimulated monocytes, we detected the protein level of iNOS and its final product NO in culture supernatants. Level of cytokine IL-6 in culture supernatants was also detected to evaluate the effects

of DBZ on synthesis and release of cytokines. Raw264.7 were pretreated with DBZ (10–30 μ M) or vehicle for 2 h, and continuously stimulated with LPS (1 μ g/ml) in the presence or absence of DBZ in the 96-well plate for 12 h. At the end of the treatment, cell supernatants were collected. The levels of NO and IL-6 in the cell culture supernatants were measured by Griess assay and sandwich ELISA, respectively. Total cell lysates were subjected to Western blot to detect the protein level of iNOS. The results indicated that DBZ was able to inhibit the secretion of both NO (Fig. 2A) and IL-6 (Fig. 2C). The suppression of LPS-induced NO secretion was paralleled with the inhibition of expression of iNOS at the protein level (Fig. 2B). These results indicated that LPS-induced inflammatory factors were significantly attenuated by DBZ treatment both at mRNA and protein levels, suggesting a potent anti-inflammatory activity of DBZ.

DBZ Blocks LPS-induced NF- κ B activation

Because DBZ could block LPS-induced inflammatory factors expression at transcriptional level, it is likely that it inhibits transcription factors activation involved in the regulation of inflammatory genes expression. It has been demonstrated that NF- κ B and AP-1 are two important transcription factors involved in the inflammatory genes expression. It also has been demonstrated that LPS induces the expression of inflammatory genes in a NF- κ B and AP-1 dependent manner. To investigate whether the anti-inflammatory effects of DBZ resulted from inhibiting the NF- κ B or AP-1 activation, we detected DBZ's effects on the NF- κ B and AP-1 activation in luciferase reporter assays, respectively. The transfected Raw264.7 cells were pretreated with 5–20 μ M DBZ for 2 h followed by LPS (1 μ g/ml) stimulation for 6 h. Treatment with DBZ ranging from 10–20 μ M significantly inhibited LPS-induced NF- κ B reporter activation (Fig. 3A). However, DBZ just slightly inhibited LPS-induced AP-1 reporter activation at the concentration of 20 μ M (Fig. 3B). These results indicated that DBZ inhibited LPS-induced expression of inflammatory genes primarily by blocking the NF- κ B activation.

DBZ Inhibits LPS-induced Nuclear Translocation of P65 and Degradation of I κ B α

As stated above that DBZ could significantly restrain NF- κ B activation, we continued to wonder whether DBZ could block activation of NF- κ B signaling pathway. It has been reported that NF- κ B (p65/p50) existed as an

Fig. 5. DBZ inhibits LPS stimulated translocation of P65 to nucleus and degradation of IκBα. A and B. Raw264.7 and mouse peritoneal macrophages were pretreated with concentrations of DBZ for 2 h and continuously subjected to stimulation with LPS (1 μg/ml) for 30 min in the presence of DBZ. At the end of the stimulation, nucleic and cytoplasmic proteins were isolated and p65 contents were detected by Western blot. Histone H2B and β-actin were used as the internal controls for nucleic and cytoplasmic proteins loading, respectively. Protein levels were quantified and data represent mean (± S.D.) from 3 independent experiments. *significant difference from LPS treatment $P < 0.05$; ** $p < 0.01$. C and D. Raw264.7 and THP1 were treated as above and total cell lysates were subjected to Western blot to detect IκBα contents, β-actin was carried out to confirm the equal loading. Protein levels were quantified and data represent mean (± S.D.) from 3 independent experiments. *significant difference from LPS treatment $P < 0.05$; ** $p < 0.01$.

inactivated complex with IκB in the cytoplasm in static cells. The activation and nuclear translocation of NF-κB/p65 resulted from IκB phosphorylation and degradation in activated cells. To understand the mechanism by which DBZ inhibits NF-κB activation, we investigated the effects of DBZ on the nuclear translocation of the p65 subunit. Raw264.7 cells were seeded in 96-well plate and pretreated with 10-50 μM of DBZ for 2 h followed by stimulation with LPS (1 μg/ml) for 30 min (Fig. 4A) or 60 min (Fig. 4B) before immunofluorescent examination. The plate was screened and analyzed by the High Content

Screen System. The quantitative NF-κB/p65 intensity translocated to nucleus was analyzed by Cytoplasm to Nucleus Translocation BioApplication software (Fig. 4C). These results showed that DBZ inhibited LPS-induced translocation of p65 to nucleus with a dose dependent manner. To ensure the effect, nuclear extracts of Raw264.7 and mouse primary macrophages were subjected to Western blot analysis. DBZ inhibited LPS-stimulated translocation of p65 to nucleus both in Raw264.7 (Fig. 5A) and primary macrophages (Fig. 5B) in a dose

dependent manner. In concert with the decrease of p65 in nucleus, p65 restrained in cytoplasm were increased both in Raw264.7 (Fig. 5A) and primary macrophages (Fig. 5B) with the pretreatment with DBZ. These data were consistent with the results of immunofluorescence. In summary, these results strongly indicated that DBZ inhibited NF- κ B signaling pathway by blocking the nuclear translocation of p65.

We also determined whether the suppressive effect of DBZ on LPS-induced p65 translocation depended on inhibiting the degradation of I κ B α . In this experiment, Raw264.7 and THP1 cells were treated as presented above. DBZ blocked LPS-mediated I κ B α degradation both in Raw264.7 (Fig. 5C) and THP1 (Fig. 5D) cells in a dose dependent manner. These results suggested that the inhibition of p65 translocation to nucleus mediated by DBZ resulted from the blockade of upstream I κ B α degradation.

DBZ mediated Anti-inflammatory Activity is More Potent than Parent Compounds

In order to elucidate whether DBZ is superior to the parent compounds (DSS and borneol) on anti-inflammatory activity as we postulated, we compared the effects of DBZ with the parent compounds (DSS and borneol) on the NF- κ B activation. We used the NF- κ B luciferase reporter assay to compare the activity of DBZ to the parent compounds. Cells were pretreated with DBZ, DSS, borneol or DSS plus borneol at the concentrations ranging from 10~20 μ M, respectively. It turned out that DSS, borneol and DSS plus borneol at these concentrations had no effect on NF- κ B activation (Fig. 6). In previous studies, it's demonstrated that DSS could inhibit LPS-stimulated inflammatory cytokines secretion in human monocytes at the concentration about 150 μ M [49]. We also detected the activity of DSS at higher concentrations ranging from 50-150 μ M on LPS-induced NF- κ B transactivation and found that DSS only inhibited NF- κ B transactivation at the concentration of 150 μ M significantly in our experiments (data not shown), which is consistent with the previous studies. All these data indicated that chemically linkage of DSS and borneol greatly enhanced the anti-inflammatory activity of parent compounds, suggesting that our initial inspiration from TCM formula FFDS is reasonable.

DBZ Inhibits LPS-induced Expression of aP2 and ADRP

Above data have primarily indicated that DBZ has obvious anti-inflammatory activity in LPS-induced

Fig. 6. DBZ mediated anti-inflammatory activity is more potent than DSS. Raw264.7 were transfected as shown in Fig. 3, cells were pretreated with DBZ, DSS, borneol or DSS plus borneol at the same concentrations ranging from 10-20 μ M, respectively. Luciferase activity was normalized by internal control *R. reniformis* luciferase. Data represent mean ($\pm$ S.D.) from 3 independent experiments. *, significant difference from LPS treatment $P < 0.05$; **, $p < 0.01$.

monocyte activation mainly through suppressing NF- κ B activation. We next detected the effects of DBZ on LPS-induced expression of aP2 and ADRP which are two important mediators responsible for LPS-induced macrophage lipid accumulation. In addition, we detected these two factors considering that NF- κ B was indicated to be key element for inflammation activation associated expression of aP2 and ADRP in macrophages [25, 27]. Raw264.7 cells were pretreated with DBZ for 2 h and continuously stimulation with LPS (1 μ g/ml) for 12 h. DBZ effectively inhibited LPS-induced aP2 and ADRP at transcriptional level (Fig. 7A). The inhibition was paralleled by a decrease in aP2 protein level (Fig. 7B). In primary mouse macrophages, DBZ also inhibited LPS-induced expression of aP2 and ADRP (Fig. 7C). These results demonstrated that DBZ could inhibit LPS-induced expression of aP2 and ADRP, suggesting that DBZ may have effects on LPS induced monocyte lipid accumulation. These results also confirmed the inhibitory effect of DBZ on NF- κ B activation.

DBZ Inhibits LPS-induced Lipid Accumulation in Monocytes/Macrophages

Expression of aP2 and ADRP in macrophages is regarded to contribute to the intracellular lipid accumulation. We detected the effect of DBZ on macrophage lipid accumulation. Raw264.7 cells were co-incubated with LDL (50 μ g/ml) and LPS (1 μ g/ml) for 18

Fig. 7. DBZ inhibits LPS-induced expression of aP2 and ADRP. **A.** Real time PCR analysis of aP2 and ADRP in LPS-stimulated Raw264.7 cells co-treated with DBZ. Ct values from aP₂ and ADRP measurements were normalized with the values from corresponding cyclophilin B reactions. Data represent mean ($\pm$ S.D.) from 3 independent experiments. *significant difference from LPS treatment $P < 0.05$; ** $P < 0.01$. **B.** Western blots detection of aP2 protein level in Raw264.7. β -actin was carried out to confirm the equal loading. Protein levels were quantified and data represent mean ($\pm$ S.D.) from 3 independent experiments. *significant difference from LPS treatment $P < 0.05$; ** $P < 0.01$. **C.** Real time PCR analysis of aP2 and ADRP in LPS-stimulated mouse peritoneal macrophages. Cells were treated as same as Raw264.7.

h in absence or presence of DBZ, the cellular lipid contents were measured. Oil Red O staining showed that the number of lipid droplets in the cytoplasm of DBZ-treated cells was markedly reduced compared to the vehicle treated cells (Fig. 8A). Measurement of

intracellular lipid contents indicated that the level of triglyceride and total cholesterol (Fig. 8B) were significantly reduced by DBZ in a concentration-dependent manner. This inhibitory effect was also confirmed in mouse primary macrophages by the Oil Red O staining (data not shown). DBZ probably affect many aspects related to LPS-induced foam cell formation, in present study, we demonstrated that DBZ ameliorated LPS-induced foam cell formation at least due to its inhibitory effect on induction of aP2 and ADRP.

Discussion

In the present study, we demonstrate that DBZ suppresses LPS-induced monocytes/macrophages activation and foam cell formation mainly through the regulation of NF- κ B activation. The inhibitory effect of DBZ on NF- κ B activation is superior to the parent compounds. These results suggest that DBZ might be a promising therapeutic agent for the prevention and treatment of atherosclerosis by the way of inhibiting monocytes inflammatory activation and foam cell formation.

We targeted on the monocyte activation to explore the anti-atherosclerotic effect of DBZ based on the fact that monocyte inflammatory activation plays important roles during the atherogenesis. Monocyte activation is accompanied by secretion of a series of inflammatory mediators which have been delineated to play roles in atherogenesis. Proinflammatory cytokines IL-1 β , IL-6 and TNF α have been proven to induce the production of a broad spectrum of cytokines and chemokines as well as the expression of adhesion molecules on endothelial cells, thus leading to the recruitment of inflammatory monocytes to the wall of vessel and atherogenesis. In addition, monocytes derived cytokines contribute to the development of vascular damage by stimulating vascular smooth muscle cells proliferation and migration, which lead to form the fibrous cap of atherosclerotic plaque [7]. Monocytes derived chemokines such as MCP1 also has been indicated to lead to monocytes/lymphocyte recruitment and infiltration into the subendothelium, which is regarded as a key step during the initial stage of atherogenesis [50]. Monocyte derived inflammatory mediators have been demonstrated to participate in the all stages of atherogenesis and affect the functions of other types of cells (endothelial cells and smooth muscle cells) in the atherosclerosis plaque. So, it is regarded that anti-inflammatory therapy is a reasonable strategy on

Fig. 8. DBZ suppresses LPS-induced lipid accumulation in Raw264.7. A. Raw264.7 were co-incubated with LPS (1 μ g/ml) and LDL (50 μ g/ml) for 18 h in the presence or absence of DBZ. Oil Red O staining indicated that DBZ obviously inhibited lipid droplets in macrophages. B and C. DBZ blocked LPS induced triglyceride and total cholesterol accumulation in Raw264.7 cells in a dose-dependent manner. Data were presented as means ($\pm$ S.D.) of μ M of triglyceride or cholesterol per milligram protein from 3 independent experiments. *significant difference from LPS treatment $P < 0.05$; ** $P < 0.01$.

treatment of atherosclerosis. DBZ obviously inhibited LPS induced expression of cytokines, chemokines and inducible enzymes during monocyte activation by inhibiting NF- κ B activation, suggesting that DBZ could play anti-atherosclerotic roles in many points during the atherogenesis.

NF- κ B and AP-1 as the pivotal molecules in transcriptional regulation of genes encoding inflammatory mediators play important roles in atherogenesis [51]. Our data showed that DBZ significantly inhibited LPS-induced NF- κ B activation, but had little effect on LPS-induced AP-1 activation, indicating that DBZ mediated anti-inflammatory effect mainly depends on the inhibition of NF- κ B activation. LPS stimulation is capable of activating both NF- κ B and AP-1 signal pathways in macrophages. Initially, LPS interacts with TLR4, which subsequently recruits MyD88/IL-1 receptor-associated kinase and TNF receptor-associated factor 6 (TRAF6). TRAF6 in turn activates downstream kinases responsible for NF- κ B and AP-1 activation. NF- κ B-inducing kinase and mitogen-activated protein kinase/ERK kinase are capable of activating the I κ B kinase (IKK) complex and NF- κ B pathway [52]. In addition, mitogen-activated protein kinase/ERK kinase in association with TRAF6 activates c-Jun-NH2-terminal kinase and leads to the activation of AP-1 pathway [52]. Present findings suggest that DBZ mainly inhibits the signal transduction associated with NF- κ B, indicating that DBZ inhibits LPS-stimulated NF- κ B

signal transduction probably at sites downstream from TRAF6.

We compared the anti-inflammatory effects of DBZ to DSS, borneol and DSS plus borneol. It turned out that DBZ was more potent in inhibiting the NF- κ B activation than the parent compounds, which strongly implicated DBZ is biochemically distinct from the parent compounds and the novel conjugated form is responsible for the inhibitory effect on NF- κ B. One difference between DBZ and DSS in structure is that DBZ is composed of one phenolic ring and a C-6 ring linked by an esteric bond whereas DSS is a phenolic compound only. Caffeic acid is structurally similar to DSS, which are both belong to the phenolic acid compounds. Previous studies demonstrated that caffeic acid phenyl ester which is composed of two phenolic rings linked by esteric bond had higher inhibitory activities against NF- κ B activation [53]. Therefore, we infer that the conjugated form of phenolic ring from DSS with C-6 ring from borneol linked by esteric bond probably plays key roles in DBZ's NF- κ B inhibitory activity. However, the detailed elucidation is still to be unsolved, the relationship between the structure and biological differences of DSS and DBZ needs to be further elucidated.

Foam cell formation is a hallmark event during the initial stage of atherosclerosis. Activation of macrophages by LPS has been shown to increase native LDL uptake and cholesterol accumulation [20]. Since LPS is able to

affect many aspects related to foam cell formation, such as LDL modulation [21], lipid uptake [22], intracellular LDL metabolism [54], fatty acid transport and storage and cholesterol efflux [23]. Here, we just focus on the aP2 and ADRP which are two molecules involved in the uptake of lipid and lipid droplet formation in macrophages and foam cells [24, 31, 32]. We take these two molecules as detecting targets mainly considering that monocyte inflammation activation is closely related to expression of aP2 and ADRP upon pathogen stimulation [24, 26]. Previous studies have demonstrated that selective inhibitors of NF- κ B or AP-1 could prevent the induction of aP2 and ADRP [25, 27]. It's also reported that there are AP-1 binding sites in promoter regions of both aP2 and ADRP [55, 27], indicating that aP2 and ADRP are direct targets of AP-1. However, the specific inhibitor of NF- κ B could inhibit the induction of aP2 and ADRP by an indirect mechanism of the modulation of cytokines whose induction precedes the expression of aP2 and ADRP [27]. Our study demonstrated that DBZ inhibited expression of aP2 and ADRP upon LPS stimulation. Because DBZ has been demonstrated to significantly inhibit NF- κ B activation and slightly inhibit AP-1 activation, we infer that DBZ mediated the inhibition of aP2 and ADRP mainly through the indirect way and dependent on the inhibitory effect on NF- κ B activation.

In conclusion, our study shows that the novel compound DBZ can inhibit LPS-induced NF- κ B activation and macrophage lipid accumulation. We propose that DBZ is likely to be a therapeutic option for atherosclerosis by combining the inhibition of both

monocyte inflammation activation and macrophage derived foam cell formation.

Abbreviations

DBZ (Danshensu Bingpian Zhi); DSS (Danshensu); FFDS (Fu Fang Dan Shen); NF- κ B (nuclear factor κ B); AP-1 (activator protein 1); I κ B α (inhibitor of nuclear factor κ B α); IL-1 β (interleukin 1 β); IL-6 (interleukin 6); iNOS (inducible nitric oxide synthase); COX2 (cyclooxygenase 2); TNF α (tumor necrosis factor α); MCP-1 (monocyte chemoattractant protein-1); MMP9 (matrix metalloproteinase 9); aP2/FABP4 (adipocyte fatty acid-binding protein); ADRP/ADFP (adipocyte differentiation related protein); LDL (low density lipoprotein); LPS (lipopolysaccharide); TLR (Toll like receptor).

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