Salvianolic Acid B Exerts Vasoprotective Effects through the Modulation of Heme Oxygenase-1 and Arginase Activities

Yoonsoo Joe, Min Zheng, Hyo Jeong Kim, Sena Kim, Md. Jamal Uddin, Chul Park, Do Gon Ryu, Shin Sung Kang, Sungwoo Ryoo, Stefan W. Ryter, Ki Churl Chang, and Hun Taeg Chung

School of Biological Sciences (Y.J., H.J.K., S.K., M.J.U., S.S.K., H.T.C.) and Department of Medical Science (M.Z.), University of Ulsan, Ulsan, Korea; Department of Physiology, School of Oriental Medicine, Wonkwang University, Iksan, Korea (C.P., D.G.R.); Department of Biological Sciences, College of Natural Sciences, Kangwon National University, Chuncheon, Korea (S.R.); Division of Pulmonary and Critical Care Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts (S.W.R.); and Department of Pharmacology, Institute of Health Sciences, Gyeongsang National University School of Medicine, Jinju, Korea (K.C.C.)

Received December 5, 2011; accepted March 20, 2012

ABSTRACT

Salvia miltiorrhiza (Danshen), a traditional Chinese herbal medicine, is commonly used for the prevention and treatment of cardiovascular disorders including atherosclerosis. However, the mechanisms responsible for the vasoprotective effects of Danshen remain largely unknown. Salvianolic acid B (Sal B) represents one of the most bioactive compounds that can be extracted from the water-soluble fraction of Danshen. We investigated the effects of Danshen and Sal B on the inflammatory response in murine macrophages. Danshen and Sal B both induced the expression of heme oxygenase-1 (HO-1) and inhibited nitric oxide (NO) production and inducible NO synthase (iNOS) expression in lipopolysaccharide (LPS)-activated RAW 264.7 cells. Inhibition of HO activity using Sn-protoporphyrin-IX (SnPP) abolished the inhibitory effect of Sal B on NO production and iNOS expression. Sal B increased macrophage arginase activity in a dose-dependent manner and diminished LPS-inducible tumor necrosis factor-α production. These effects were also reversed by SnPP. These data suggest that HO-1 expression plays an intermediary role in the anti-inflammatory effects of Sal B. In contrast to the observations in macrophages, Sal B dose-dependently inhibited arginase activity in murine liver, kidney, and vascular tissue. Furthermore, Sal B increased NO production in isolated mouse aortas through the inhibition of arginase activity and reduction of reactive oxygen species production. We conclude that Sal B improves vascular function by inhibiting inflammatory responses and promoting endothelium-dependent vasodilation. Taken together, we suggest that Sal B may represent a potent candidate therapeutic for the treatment of cardiovascular diseases associated with endothelial dysfunction.

Introduction

Danshen, the dried root of Salvia miltiorrhiza (Lamiaceae), is commonly used in traditional Chinese medicine. In Asian and Western countries, Danshen has been widely used for the treatment of various diseases, including cerebrovascular and cardio-

This work was supported by the Korea Research Foundation funded by the Korean Government [Grant BRL-2009-0087350]. Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. http://dx.doi.org/10.1124/jpet.111.190736. [S] The online version of this article (available at http://jpet.aspetjournals.org) contains supplemental material.

ABBRIVIATIONS: Sal B, salvianolic acid B; HO-1, heme oxygenase-1; LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible NO synthase; eNOS, endothelial NOS; SnPP, Sn-protoporphyrin-IX; ROS, reactive oxygen species; L-NAME, N^6-nitro-L-arginine methyl ester; MnTBAP, Mn(III)tetra(4-benzoic acid)porphyrin chloride; MTT, 3-(4,5-Dimethyl-2-yl)-2,5-diphenyltetrazolium bromide; HUVEC, human umbilical vein endothelial cell; RT-PCR, reverse transcription-polymerase chain reaction; TNF-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility-shift assay; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; DHE, dihydroethidium; DAF-FM, 4-amino-5-methylamino-2',7'-difluorofluorescein; CO, carbon monoxide; CORM-2, CO-releasing molecule-2.

vascular diseases (Du et al., 2000; Jiang et al., 2005; Zhou et al., 2005; Cheng, 2006; Fish et al., 2006). Salvianolic acid B (Sal B), a hydrophilic caffeic acid derivative, represents the most abundant and bioactive component of Danshen (Watzke et al., 2006). Sal B can prevent ischemic brain injury in the rat by reducing lipid peroxidation, scavenging free radicals, and improving energy metabolism (Chen et al., 2000). Moreover, Sal B improved regional cerebral blood flow and inhibited platelet aggregation in rats subjected to brain ischemia by cerebral artery occlusion (Tang et al., 2002). To date, the molecular mechanisms underlying the reported beneficial cardiovascular effects of Sal B remain incompletely understood.

http://jpet.aspetjournals.org/content/suppl/2012/03/22/jpet.111.190736.DC1

Supplemental material to this article can be found at:
Sal B regulates vascular homeostasis by exerting a number of vasoprotective effects, including the stimulation of vasodilation, suppression of smooth muscle cell proliferation, and inhibition of inflammatory responses. Many of these effects are mediated by the gaseous second-messenger molecule nitric oxide (NO), the most potent endogenous vasodilator. Endothelial cell-derived NO can act as an important mediator in the cardiovascular, nervous, and immune systems (Moncada et al., 1991; Bretz and Snyder, 1994). NO is synthesized from l-arginine by NO synthase (NOS) enzymes. Several isoforms of NOS have been identified, including the endothelial form of NOS (eNOS), which requires Ca\(^{2+}\)/calmodulin (Fulton et al., 1999), and the inducible Ca\(^{2+}\)/calmodulin-independent form of NOS (iNOS) (Nathan and Xie, 1994; Fleming et al., 1997). iNOS is expressed mainly in macrophages (Stuehr and Marletta, 1985; Nathan, 1997) and other cell types, including endothelial cells, cardiac myocytes, vascular smooth muscle cells, hepatocytes, and mesangial cells (Geller et al., 1993; Shultz et al., 1994; Iwashina et al., 1996; Marcin et al., 1996). The transcriptional and translational regulation of iNOS in various cell types can be induced by cytokines, growth factors, and endotoxins [i.e., lipopolysaccharide (LPS)] (Lamas et al., 1992; Xie et al., 1992; Wang and Marsden, 1995).

The expression of iNOS and the production of NO both are regulated by the inducible stress-protein heme oxygenase-1 (HO-1) (E.C. 1:14:99:3) (Oh et al., 2004, 2006). HO-1 represents a general inducible response to pro-oxidative or proinflammatory states that plays a role in cyto- and tissue protection. Excessive production of NO by iNOS can cause endothelial damage, leading to multiple vascular wall injuries (Nathan, 1997). Arginase, an enzyme that uses l-arginine as a substrate, reciprocally regulates NOS activity (Stephan et al., 2006). Several reports have demonstrated that the up-regulation of arginase functionally inhibits NOS activity and contributes to the pathophysiology of age-related vascular dysfunction (Berkowitz et al., 2003; White et al., 2006; Santhanam et al., 2007). Factors that can selectively regulate the activities of the various NOS isoforms may have therapeutic value for the treatment of cardiovascular disorders (Garvey et al., 1994; Fukuto and Chaudhuri, 1995).

Atherosclerosis involves the formation of arterial lesions that are characterized by inflammation, lipid accumulation, cell death, and fibrosis. Monocyte activation and adhesion to the endothelium play important roles in atherosclerosis and other inflammatory and cardiovascular diseases. In addition to inflammation, endothelial cell dysfunction plays a major role in vascular pathology. To investigate the mechanisms underlying the protective effects of Danshen, we assessed the specific effects of Sal B on inflammatory responses and vasodilatory potential, by examining both inducible and endothelial-dependent NO production.

**Materials and Methods**

**Reagents.** Danshen and salvianolic acid (purity >99%) were obtained from Shanghai Standard Biotech Co. Ltd (Shanghai, China) and freshly prepared in PBS before experiments. Mn(III)tetra(4-benzoic acid)porphyrin chloride (MnTBP) and N\(^{n}\)-nitro-l-arginine methyl ester (l-NNAME) were purchased from Calbiochem (San Diego, CA). 3-(4,5-Dimethyl-2-y1)-2,5-diphenyltetrazolium bromide (MTT) reagent, Griess reagent, and bacterial LPS were purchased from Sigma-Aldrich (St Louis, MO).

**Animals.** All experimental procedures with mice were approved by the Animal Care Committee of the University of Ulsan. Seven-week-old male C57BL/6 wild-type mice were purchased from ORIENT (Pusan, Korea). The mice were maintained under specific pathogen-free conditions at 22°C and given access to food and water ad libitum. Mice were sacrificed by cervical dislocation under anesthesia and used for the collection of liver, kidney, spleen, and aortic tissues. To investigate the effects of Sal B in vivo, mice were randomly assigned to four groups: a sham group (water), an LPS-treated group (1 mg/kg), an LPS + Sal B-treated group (50 mg/kg), and an LPS + Sal B + Sn-protoporphyrin-IX (SnPP) (50 \(\mu\)mol/kg)-treated group. Sal B was given once daily for 5 days by oral administration.

**Cell Culture and Viability.** Cell cultures were maintained at 37°C in humidified incubators containing an atmosphere of 5% CO\(_2\). RAW 264.7 macrophages were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin. Human umbilical vein endothelial cells (HUVECs) were isolated as described previously (de Martin et al., 1993). The cells were grown in 75-cm\(^2\) flasks in endothelial cell basal medium 2 (Lonza Walkersville, Inc., Walkersville, MD) with 20% fetal bovine serum, 0.04% hydrocortisone, 0.04% human fibroblast growth factor, 0.1% vascular endothelial growth factor, 0.1% R3 insulin-like growth factor-1, 0.1% ascorbic acid, 0.1% human epidermal growth factor, 0.1% GA-1000, and 0.1% heparin. Cell viability was determined by using a modified MTT reduction assay as described previously (Pae et al., 2007).

**Arginase Activity Assay.** Arginase activity was assayed in macrophage cell lysates and homogenates prepared from livers and kidneys of anesthetized C57BL/6 mice. In brief, cell or tissue lysates were prepared by incubation or homogenization, respectively, in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and protease inhibitors) at 4°C followed by centrifugation for 20 min at 14,000g at 4°C. The resulting supernatants were used to assay for arginase activity by using the QuantiChrom Arginase assay kit (BioAssay Systems, Hayward, CA).

**NO Production Assay.** As an indicator of NO production, the nitrite concentration in cell culture medium was measured by using the Griess reaction (Kim et al., 2008). In brief, 100 \(\mu\)l of cell culture supernatant was mixed with the same volume of Griess reagent. The absorbance of the mixture at 540 nm was determined by using a microplate reader, and the nitrite concentration was determined by using a dilution of sodium nitrite as the standard. To detect intracellular NO, 4-amino-5-methylamino-2’,7’-difluorofluorescein (DAF-FM) diacetate for 15 min at 4°C followed by centrifugation for 20 min at 14,000g at 4°C. The resulting supernatants were used to assay for arginase activity by using the QuantiChrom Arginase assay kit (BioAssay Systems, Hayward, CA).

**Materials and Methods**

**Reagents.** Danshen and salvianolic acid (purity >99%) were obtained from Shanghai Standard Biotech Co. Ltd (Shanghai, China) and freshly prepared in PBS before experiments. Mn(III)tetra(4-benzoic acid)porphyrin chloride (MnTBP) and N\(^{n}\)-nitro-l-arginine methyl ester (l-NNAME) were purchased from Calbiochem (San Diego, CA). 3-(4,5-Dimethyl-2-y1)-2,5-diphenyltetrazolium bromide (MTT) reagent, Griess reagent, and bacterial LPS were purchased from Sigma-Aldrich (St Louis, MO).
SpectraMax L microplate luminometer (Molecular Devices, Sunnyvale, CA). Firefly luciferase activity was normalized to Renilla luciferase activity in each sample. Each experiment was performed at least three times with triplicate samples.

**Electrophoretic Mobility-Shift Assay.** Nuclear extracts of RAW 264.7 cells were prepared with the NE-PER nuclear extraction reagent (Thermo Fisher Scientific, Waltham, MA). Biotin end-labeled double-stranded oligonucleotides 5′-biotin-(NF-κB-RE-WT, 5′-AGTTGAGGGAAGCTTCCCCAGGC-3′; NF-κB-RE-MUT, 5′-AGTTTAGGCCCCAGGC-3′) were generated by using an oligonucleotide synthesizer. Nuclear extracts were prepared from RAW 264.7 cells by using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific) according to the manufacturer’s instructions. Binding reactions were carried out for 20 min at room temperature in the presence of 50 ng/ml poly(dI-dC), 0.05% Nonidet P-40, 5 mM MgCl₂, 2.5% glycerol in 1× binding buffer (LightShift Chemiluminescent EMSA kit; Thermo Fisher Scientific) using 20 fmol of biotin end-labeled target DNA and 4 µg of nuclear extract. For supershift assays, 2 µg of anti-p65 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or 2 µg of anti-p50 (Santa Cruz Biotechnology, Inc.) was added per 20 µl of binding reaction where indicated. Assays were loaded onto native 5% polyacrylamide gels that were pre-electrophoresed for 60 min in 0.5× Tris borate/EDTA. Samples were then electrophoresed at 100 V before being transferred onto a positively charged nylon membrane (Hybond-N⁺) in 0.5× Tris borate/EDTA at 100 V for 30 min. Transferred DNAs were cross-linked to the membrane at 10 mJ/cm² and detected by using horseradish peroxidase-conjugated streptavidin (Lightshift chemiluminescent EMSA kit) according to the manufacturer’s instructions.

**Western Blot Analysis.** After experimental treatments, cells were harvested, washed twice with ice-cold PBS, lysed with lysis buffer (0.14 M Tris, pH 6.8, 0.21 M SDS, 2.4 M glycerol, and 0.3 mM bromophenol blue) containing protease and phosphatase inhibitors, and boiled for 5 min. Protein concentration was measured with BCA protein assay reagent (Thermo Fisher Scientific). The samples were diluted with lysis buffer containing 1.28 M β-mercaptoethanol, and equal amounts of protein (50 µg) were separated on 8% SDS-polyacrylamide gel electrophoresis followed by transfer to polyvinylidene difluoride membranes (Thermo Fisher Scientific). The membranes were blocked with 5% nonfat milk in PBS containing 0.1% Tween 20 for 1 h and incubated with antibodies against iNOS (Santa Cruz Biotechnology, Inc.) or HO-1 (Enzo Life Sciences, Plymouth Meeting, PA) in PBS containing 0.1% Tween 20 and 3% nonfat milk for 2 h. After washing three times, the membranes were hybridized with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) for 1 h. The blots were detected by using the Amersham ECL system (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The relative signal intensity of bands was determined and standardized by using Scion Image software (Scion Corporation, Frederick, MD).

**Reverse Transcription-Polymerase Chain Reaction.** Total RNA was isolated from RAW 264.7 cells or peritoneal macrophages by using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The forward and reverse primers used in the present study are shown in Supplemental Table 1. In brief, total RNA (0.5 µg) was reverse-transcribed by using an oligo(dT) adaptor as a primer to produce cDNAs. PCRs were conducted by using the following conditions for 30 cycles: denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min, and elongation at 72°C for 1 min. Band intensities of the amplified DNAs were compared after visualization on an UV transilluminator.

**Measurement of NO or Reactive Oxygen Species Generation in Isolated Mice Aorta.** After dissection of mouse aorta, aortic rings were cut open and pinned with the endothelium side up in a Petri dish coated with Sylgard 184 (Dow Corning, Midland, MI). The production of reactive oxygen species (ROS) was quantified with the fluorescent superoxide indicator dihydroethidium (DHE; Invitrogen). The segments were incubated with 5 µM DHE in HEPES buffer at 37°C for 5 min to allow temperature equilibration before data collection. Fluorescence intensity time-lapse images were acquired with an upright microscope (Olympus) equipped with a 10× objective and an intensified camera (Luca 658M-TL) and a custom image acquisition program (Olympus). DHE fluorescence was collected at excitation-emission wavelengths of 470/580 nm. The cell-permeable fluorescent probe DAP-FM diaacetate (Invitrogen) was used to quantify endothelium-derived NO. Fluorescence measurements were acquired, and fluorescence rates were calculated as described previously (Woo et al., 2010).

**Statistical Analyses.** Data were expressed as mean ± S.D. from at least three independent experiments. Statistical significance between experimental and control values was determined by using one-way analysis of variance.

**Results**

Effects of Salvianolic Acid B on HO-1 Expression and NO Production. Sal B (structure in Fig. 1A) has been described as the most potent and effective component of Danshen extracts (Woo et al., 2010). Before studying the effect of Sal B, we evaluated the anti-inflammatory effects of Danshen extract in the RAW 264.7 macrophage cell line by determining HO-1 expression and NO production. Treatment with Danshen extract increased HO-1 expression, whereas it suppressed LPS-inducible NO production and iNOS expres-

**Fig. 1.** Sal B induced HO-1 expression in RAW 264.7 macrophages. A, the chemical structure of Sal B, a pure compound extracted from *S. miltiorrhiza* is shown. B, RAW 264.7 cells were pretreated with various concentrations (100, 200, and 400 µM) of Sal B for 12 h, and then treated with 1 µg/ml of LPS for 18 h. C, cell viability was measured by using the MTT assay. RAW 264.7 cells were incubated for 6 h with the indicated concentrations of Sal B or with 20 µM curcumin as a positive control (left) or incubated for the indicated times with 400 µM Sal B (right). The expression levels of HO-1 were determined by Western immunoblot analysis. α-Actin served as the standard.
sion in a dose-dependent manner (Supplemental Fig. 1). For further analysis of the anti-inflammatory effects of Danshen extract, we used Sal B as a representative bioactive component. First, the cytotoxicity of Sal B was assessed by using the MTT assay after treatment of RAW 264.7 macrophages with various concentrations of Sal B (100–400 μM) in the absence or presence of LPS (1 μg/ml). As shown in Fig. 1B, treatment with Sal B did not affect macrophage cell viability in the absence or presence of LPS. At these concentrations, Sal B dose-dependently increased HO-1 expression in RAW 264.7 cells (Fig. 1C, left). Furthermore, Sal B (400 μM) increased HO-1 expression in a time-dependent manner (Fig. 1C, right). To test the effect of Sal B on LPS-inducible NO production, we measured changes in nitrite levels by using the Griess reaction. LPS-inducible NO production was dramatically decreased by Sal B in a dose-dependent manner (Fig. 2A). Previously, we reported that LPS-inducible NO production is closely correlated with HO-1 expression (Kim et al., 2008). To assess the role of HO-1 in NO production, RAW 264.7 macrophages were treated with 50 μM SnPP, a competitive inhibitor of HO activity, with the addition of Sal B (400 μM) for 12 h before LPS challenge. Inhibition of HO activity by SnPP treatment abolished the inhibitory action of Sal B on NO production (Fig. 2B). To evaluate whether carbon monoxide (CO), a reaction product of HO-1, was involved in the observed down-regulation of iNOS expression by Sal B, Hb was used as a CO scavenger. As shown in Fig. 2C, Hb reversed the inhibitory effect of Sal B on LPS-induced iNOS expression. Moreover, CO gas spontaneously released from the CO-releasing molecule-2 (CORM-2) completely reduced LPS-induced iNOS expression. To confirm the effect of Sal B on NO production, intracellular NO levels were measured by using DAF-FM. As shown in Supplemental Fig. 2, Sal B reduced LPS-induced intracellular NO levels, consistent with the results obtained by the Griess reaction. Our observations suggest that, among the HO-1 products (i.e., CO, iron, and bilirubin), CO is a critical factor that mediates the inhibitory effects of Sal B on iNOS expression and activity. In accordance with the known reciprocal regulatory effect of arginase on NOS activity, Sal B dose-dependently increased arginase

![Fig. 2. Sal B inhibited LPS-induced NO production and increased arginase activity in RAW 264.7 macrophages.](image-url)
activity in RAW 264.7 cells in the presence of LPS (Fig. 2E). This effect of Sal B was also suppressed in the presence of the HO inhibitor (Fig. 2F). Furthermore, we investigated the effect of Sal B on iNOS expression and NO production in peritoneal macrophages (Fig. 3, A and B). Treatment of macrophages for 6 h with Sal B (400 μM) reduced LPS-inducible iNOS expression and NO production. Similar to results in vitro, SnPP, a HO-1 inhibitor, reversed the inhibitory effect of Sal B on LPS-induced TNF-α production. To detect the effect of Sal B on TNF-α levels in peritoneal macrophages, Sal B (400 μM) was administered to macrophages for 6 h in the presence or absence LPS (1 μg/ml). RNA levels and protein production of TNF-α induced by LPS were suppressed by Sal B (Fig. 4C and D). Furthermore, the suppressive effect of Sal B on TNF-α was inhibited by SnPP. The expression of TNF-α is related to NF-κB activation because the TNF-α gene contains four κB motifs in its promoter regions (Collart et al., 1990). Therefore, to study the effect of Sal B on NF-κB activation, p65 levels were detected in the cytoplasmic and nuclear fractions after treatment of RAW 264.7 cells with Sal B (400 μM) followed by treatment with LPS (1 μg/ml). In the cytosol, the LPS-induced decrease in p65 levels was recovered by Sal B treatment (Fig. 5A). On the contrary, the LPS-induced increase in p65 levels in the nucleus was suppressed by Sal B (Fig. 5B). To confirm the role of CO in this response, RAW 264.7 cells were incubated with the CO scavenger Hb for 30 min before treatment with Sal B and LPS. Hb reversed the important role in the inhibition of LPS-induced iNOS and NO production both in vitro and in vivo.

Inhibitory Effect of Salvinorin A on TNF-α Production. To further evaluate the anti-inflammatory effects of Sal B, we tested whether this compound could modulate LPS-inducible TNF-α production in RAW 264.7 macrophages. The production of TNF-α by LPS stimulation was reduced by Sal B in a dose-dependent manner (Fig. 4A). To assess the role of HO-1 in the inhibitory effect of Sal B on TNF-α production, RAW 264.7 cells were treated with the HO inhibitor SnPP (50 μM) and Sal B (400 μM) before treatment with LPS (1 μg/ml). As shown in Fig. 4B, SnPP prevented the inhibitory effect of Sal B on LPS-induced TNF-α production. To detect the effect of Sal B on TNF-α production in RAW 264.7 and murine macrophages, A, RAW 264.7 cells were preincubated with the indicated concentrations of Sal B for 12 h and then treated with LPS (1 μg/ml) for 18 h. B, after incubation with 400 μM Sal B for 12 h in the presence or absence of 50 μM SnPP, RAW 264.7 cells (B) and murine macrophages (C and D) were then stimulated for 18 h with LPS (1 μg/ml). A, B, and D, TNF-α production was measured by ELISA. C, RT-PCR analysis for TNF-α expression was performed. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Fig. 3. Sal B inhibited LPS-induced NO production and increased HO-1 expression in peritoneal macrophages. Cells were preincubated with 400 μM Sal B for 6 h in the presence or absence of 20 μM SnPP, and then stimulated for 18 h with LPS (1 μg/ml). A, the expression levels of iNOS were determined by Western immunoblot analysis. B, NO concentration in the medium was measured using the Griess reaction. C, RT-PCR analysis for HO-1 expression was performed. ***, P < 0.001.

Fig. 4. Sal B inhibited LPS-induced TNF-α production in RAW 264.7 and murine macrophages. A, RAW 264.7 cells were preincubated with the indicated concentrations of Sal B for 12 h and then treated with LPS (1 μg/ml) for 18 h. B to D, after incubation with 400 μM Sal B for 12 h in the presence or absence of 50 μM SnPP, RAW 264.7 cells (B) and murine macrophages (C and D) were then stimulated for 18 h with LPS (1 μg/ml). A, B, and D, TNF-α production was measured by ELISA. C, RT-PCR analysis for TNF-α expression was performed. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
inhibitory effect of Sal B on LPS-dependent NF-κB activation. In an effort to elucidate the transcriptional mechanisms underlying TNF-α expression in the presence of Sal B, luciferase assays and EMSAs for NF-κB were conducted by using nuclear extracts from RAW 264.7 cells treated with LPS (1 μg/ml) only or with LPS (1 μg/ml) and Sal B (400 μM). As shown in Fig. 5C, Sal B reduced LPS-induced activity of an NF-κB response element in RAW 264.7 cells, as determined by luciferase activity. Consistent with reporter assays, we found that Sal B reduced LPS-inducible NF-κB DNA binding activity by using the EMSA (Supplemental Fig. 3). These results indicate that Sal B inhibits TNF-α production by reducing NF-κB activation.

Effects of Salvianolic Acid B on HO-1, NO, and TNF-α Production In Vivo. In this study, we have reported the effects of Sal B on the anti-inflammatory response in vitro. In addition, we investigated whether Sal B had similar effects on HO-1, NO, and TNF-α production in vivo. Mice were randomly assigned to four groups: a sham group (water), an LPS group (1 mg/kg), an LPS + Sal B group (50 mg/kg), and an LPS + Sal B + SnPP (50 μM/kg) group. Sal B was given once daily for 5 days by oral administration. The effect of Sal B on TNF-α production in murine serum was determined by using an ELISA. Administration of Sal B reduced LPS-inducible TNF-α mRNA and protein production (Fig. 6, A and B). This decrease of TNF-α production and TNF-α mRNA by Sal B was reversed by co-oral injection with SnPP. To investigate iNOS expression in mice orally administrated with Sal B, reverse transcriptase-PCR was performed with RNA isolated from the spleen. LPS-induced iNOS expression was decreased with Sal B treatment, and this effect was reversed by cotreatment with SnPP (Fig. 6C). To demonstrate the role of HO-1 in TNF-α and iNOS production, the levels of HO-1 protein and RNA were detected after treatment with LPS or with LPS and Sal B. As shown in Fig. 6, D and E, Sal B increased HO-1 RNA and protein expression in murine spleen. These results indicate that HO-1 activity induced by Sal B attenuates inflammatory responses such as TNF-α and iNOS production in a murine model.

Effects of Salvianolic Acid B on Arginase Activity in Murine Liver and Kidney. To assess the effect of Sal B on the arginase activity of systemic tissues, we performed an arginase activity assay with the lysates of murine livers and kidneys. As shown in Table 1, both arginase I activity in liver lysates and arginase II activity in kidney lysates were significantly decreased by Sal B in a dose-dependent manner. These data indicate that Sal B has an inhibitory effect on both arginase I and II in tissues but exhibits no specificity for arginase isoforms.

Effects of Salvianolic Acid B on NO and ROS Production in Endothelial Cells. Next, we assessed the effect of Sal B on arginase activity in vascular tissue. In the endothelium of aortic tissue isolated from mice, Sal B significantly decreased arginase activity (Fig. 7A). To determine whether
Sal B-dependent arginase inhibition was associated with increases in NO production in the endothelium of murine aorta, we assayed NO production by using DAF, a NO-sensitive fluorescent dye. As shown in Fig. 7B, Sal B significantly increased the average slope of DAF fluorescence in aorta ($P < 0.05$). The NOS inhibitor L-NAME inhibited the Sal B-dependent increases in the slope of DAF fluorescence. Furthermore, we evaluated eNOS levels after Sal B treatment in HUVECs (Fig. 7C). Sal B caused a significant reversal of the LPS-induced decrease in eNOS RNA levels. Treatment with SnPP (a HO-1 inhibitor) and Hb (a CO scavenger) reduced Sal B-induced eNOS levels. This result suggests that the effect of Sal B on eNOS expression is related to HO-1 activity and endogenous CO production. To investigate whether the increases in NO production elicited by Sal B lead to a reduction of vascular ROS production, we measured $O_2^•-$ generation by using the $O_2^•-$-sensitive dye DHE in the endothelium of Sal B-treated aorta. Sal B treatment decreased the slope of DHE fluorescence, which was completely quenched by the superoxide dismutase mimetic MnTBAP (Fig. 8).

**Discussion**

In the current study, we demonstrate that Sal B, the active component of Danshen, protects against endothelial dysfunction by inhibiting inflammatory responses in macrophages and increasing endothelium-dependent NO production. Danshen extracts have been widely used in traditional Chinese medicine for the treatment of cerebrovascular and cardiovascular diseases (Du et al., 2000; Fish et al., 2006). Over the past few years, progress in the pharmacology of Danshen has led to the identification and characterization of the antiatherosclerotic, anti-inflammatory, and antioxidative effects of this complex mixture (Liu et al., 2007). The chemical constituents of Danshen have been studied since the early 1930s (Zhou et al., 2005). Earlier studies focused mainly on the lipophilic compounds (e.g., Tanshinone IIA), whereas more recent studies have focused on hydrophilic compounds. At least 50 components have now been isolated and identified from the aqueous extract of Danshen (Han et al., 2008). Sal B (Fig. 2A) represents the most active water-soluble polyphenolic acid extracted from the root of Danshen (Watzke et al., 2006). The mechanisms responsible for the protective effects of Sal B in cardiovascular diseases remain unclear.

In the milieu of cardiovascular risk factors that disturb vascular homeostasis, inflammation represents a key early event in vascular pathology. Vascular inflammation involves the enhanced production of cytokines and increased expression of cellular adhesion molecules by the endothelium. Monocyte activation and their adhesion to the endothelium are thought to play important pathogenic roles in atherosclerosis (Hansson and Libby, 2006). The endothelium is responsible for maintaining a balance in the production of vasocon-
strictor and vasodilator substances. NO released by the endothelium functions as a potent vasodilator. Defects in the production or activity of NO contribute to endothelial dysfunction, which is an early marker for atherosclerosis.

In humans, atherosclerotic plaques contain blood-borne inflammatory and immune cells, mainly macrophages and T cells, as well as vascular endothelial cells, smooth muscle cells, extracellular matrix, lipids, and cellular lipid-rich debris (Jonasson et al., 1986). Thus, in the arterial intima, activated macrophages secrete more proinflammatory cytokines, such as TNF-α, which promote the development of the disease. We hypothesized that Sal B could improve atherosclerotic plaques through anti-inflammatory effects including the inhibition of iNOS expression, NO production, and TNF-α production.

Here, we found that Danshen extract and its purified derivative Sal B inhibited iNOS expression and NO production in RAW 264.7 macrophages activated with LPS. Our results indicate that the anti-inflammatory effect of Sal B in macrophages with respect to decreased NO production is related to increased expression of the cytoprotective molecule HO-1 and the up-regulation of macrophage arginase activity. The blockage of HO activity by SnPP completely abolished the inhibitory effects of Sal B on iNOS expression, NO production, and TNF-α production. SnPP is well characterized as a potent competitive inhibitor of HO activity, although we cannot completely exclude potential off-target effects. HO activity catalyzes the degradation of heme to biliverdin-IXα, iron, and CO. The mechanism by which HO-1 down-regulates iNOS is incompletely clear, but may involve transcriptional inhibition through CO formation or reduction of heme bioavailability for iNOS synthesis. Previously, HO-1 has been shown to down-regulate proinflammatory cytokine (i.e., TNF-α) production in LPS-stimulated macrophages through the formation of CO (Otterbein et al., 2000). Here, we determined, using the CO scavenging compound Hb, that the regulatory effect of Sal B on TNF-α production may use a similar mechanism.

We also report here that Sal B exerts an important regulatory function on endothelial tissue by promoting NO production. In our experiments, Sal B inhibited arginase activity, which was associated with increased NO production in the endothelium of isolated mouse aorta. These results indicate that arginase competitively inhibits eNOS through the consumption of l-arginine, which is the common substrate for NOS and arginase enzymes. In addition to increasing enzymatic NO production, Sal B decreased ROS production in isolated mouse aorta. Thus, Sal B exerts an antioxidative function that may preserve NO bioavailability by precluding NO consumption through adverse reactions such as peroxynitrite formation. Sal B was also found to act as a general inhibitor of arginase activity in kidney and liver homoge-
nates. Whereas the stimulation of arginase activity by Sal B in macrophages was attributed to secondary effects involving the HO-1 pathway, the mechanism for its inhibitory effect in systemic tissues remains unclear. Our results, taken together, suggest that Sal B can ameliorate endothelial dysfunction, up-regulate anti-inflammatory responses, and promote vasodilation through the increase of HO-1 and decrease of arginase activity.

In conclusion, Sal B exerted anti-inflammatory effects by inhibiting iNOS expression and NO production in LPS-activated RAW 264.7 macrophages. Furthermore, Sal B also played an important role in endothelium-dependent vasodilation by regulating NO production through the inhibition of arginase activity. Therefore, we can conclude that Sal B ameliorates endothelial dysfunction through the up-regulation of anti-inflammatory responses and the promotion of vasodilation. Sal B may provide a new therapeutic approach to the prevention and treatment of various cardiovascular disorders associated with endothelial dysfunction.

**Authorship Contributions**

**Participated in research design:** Joe, Zheng, and Chung

**Conducted experiments:** Joe, Zheng, Park, Ryu, and Ryoo.

**Contributed new reagents or analytic tools:** Ryoo, Chang, and Chung

**Wrote or contributed to the writing of the manuscript:** Joe, Ryter, and Chang

**References**


**Address correspondence to:** Hun Taeq Chung, School of Biological Sciences, University of Ulsan, Ulsan 680-749, Korea. E-mail: chung@ulsan.ac.kr
Salvianolic acid B exerts vasoprotective effects through the modulation of heme oxygenase-1 and arginase activities

Yoonsoo Joe, Min Zheng, Hyo Jeong Kim, Sena Kim, Md. Jamal Uddin, Chul Park, Do Gon Ryu, Shin Sung Kang, Sungwoo Ryoo, Stefan W. Ryter, Ki Churl Chang, and Hun Taeg Chung

Journal of Pharmacology and Experimental Therapeutics (JPET/2011/190736)

Supplemental method

Electrophoretic Mobility Shift Assay (EMSAs)

Nuclear extracts of RAW264.7 cells were prepared with the NE-PER nuclear extraction reagent (Pierce). Biotin end-labeled double-stranded oligonucleotides 5'-biotin- (NF-κB-RE-WT, 5'- AGTTGAGGGGACTTTCCCAGGC -3'; NF-κB-RE-MUT; 5'- AGTTGAGGCC GACTTTCCAGGC -3') was generated using an oligonucleotide synthesizer. Nuclear extracts were prepared form RAW264.7 cells using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacture’s manual. Binding reactions were carried out for 20min at room temperature in the presence of 50 ng/ml poly(dI-dC), 0.05% Nonidet P-40, 5 mM MgCl₂, 10 mM EDTA, and 2.5% glycerol in 1 X binding buffer (LightShift™ Chemiluminescent EMSA kit, Pierce) using 20 fmol of biotin-end-labeled target DNA and 4 μg of nuclear extract. For supershift assay, 2 μg of anti-p65 (sc-8008x, Santa Cruz Biotechnology, Inc.) or 2 μg of anti-p50 (sc-114x, Santa Cruz Biotechnology, Inc.) was added per 20 μl of binding reaction where indicated. Assays were loaded onto native 5% polyacrylamide gels pre-electrophoresed for 60 min in 0.5 X Tris borate/EDTA
and electrophoresed at 100 V before being transferred onto a positively charged nylon membrane (Hybond™-N+) in 0.5 X Tris borate/EDTA at 100 V for 30min. Transferred DNAs were cross-linked to the membrane at 10 mJ/cm² and detected using horseradish peroxidase-conjugated streptavidin (Lightshift™ chemiluminescent EMSA kit) according to the manufacturer’s instructions.

Supplemental Figure 1. Danshen stimulates HO-1, and inhibits LPS-inducible NO production/iNOS expression in RAW 264.7 macrophages. Cells were incubated for 6 h with the indicated concentrations of Danshen extract or with curcumin (Cur, 20 µM) as a positive control (A). Cells were pre-incubated with the indicated concentration of Danshen extracts for 12 h and then treated with 1 µg/ml of LPS for 18 h (B, C). The production of NO was evaluated using the Griess reaction (B). Data are expressed as mean ± SEM from four independent experiments. Results were statistically significant (**P<0.01 compared to control; ***P<0.001 compared to LPS-only treatment) using the one way ANOVA test. Cell lysates were evaluated by Western immunoblot analysis for the expression of iNOS (C). β-actin served as the standard.

Supplemental Figure 2. Sal B inhibited LPS-induced NO production in RAW 264.7 macrophages. Cells were pre-incubated with 400 µM of Sal B (or 50 µM of CORM-2) for 12 h (or 1h) in the presence or absence of 50 µM Hb, and then stimulated for 18 h with LPS (1 µg/mL). Direct visualization of NO production with the fluorescent indicator was performed using a laser-scanning confocal microscope with excitation and emission maxima at 495 and 515 nm, respectively.
Supplemental Figure 3. RAW 264.7 cells were preincubated with 400 μM Sal B in the presence or absence of 50 μM of Hb for 12 h, and then stimulated for 1 h with 1 μg/ml of LPS. Nuclear extracts isolated from RAW 264.7 cells were incubated with 5’-biotin labeled NF-κB-RE-WT probe or NF-κB-RE-MUT probe. The DNA-protein complexes were resolved on a 5% acrylamide gel. To identify p65 or p50 immunoreactivity in the DNA-protein complexes, nuclear extracts were incubated with anti-p65 antibody, anti-p50 antibody, or nonspecific IgG before the addition of the biotinylated probe. Arrows (a) and arrows (b and c) indicate the DNA-protein and DNA-protein-antibody complexes, respectively.

Supplemental Figure 1.
Supplemental Figure 2.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>LPS</th>
<th>LPS+Sal B</th>
<th>LPS+Sal B+Hb</th>
<th>LPS+CO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SalB</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hb</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Supplemental Figure 3.

<table>
<thead>
<tr>
<th>probe</th>
<th>WT</th>
<th>MUT</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LPS</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SalB</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hb</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

References: p55, p50, IgG