

Original Article

Cheonwangbosimdan, a traditional herbal formula, inhibits inflammatory responses through inactivation of NF- κ B and induction of heme oxygenase-1 in RAW264.7 murine macrophages

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Received August 25, 2015; Accepted December 24, 2015; Epub February 15, 2016; Published February 29, 2016

Abstract: Cheonwangbosimdan (CWBSD) is a traditional herbal formula composing 15 medicinal herbs that used for treating mental disorders. In the present study, we investigated inhibitory effects of CWBSD using RAW264.7 murine macrophage cell line. We found that CWBSD induced heme oxygenase-1 (HO-1) expression in a dose-dependent manner with no cytotoxicity. CWBSD significantly reduced production of pro-inflammatory cytokines tumor necrosis factor-alpha (TNF- α), but not interleukin-6 in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. CWBSD also significantly inhibited LPS-induced production of pro-inflammatory mediators such as nitric oxide (NO) and prostaglandin E₂ (PGE₂). In addition, CWBSD suppressed phosphorylation of p38 mitogen-activated protein kinase (MAPK) and c-jun N-terminal kinase (JNK) as well as activation of nuclear factor kappa B (NF- κ B) in LPS-treated macrophages. Overall, our data provide a foundation for further insight into the molecular mechanisms responsible for the anti-inflammatory activity of CWBSD in chronic inflammatory diseases.

Keywords: Cheonwangbosimdan, anti-inflammation, NF- κ B, heme oxygenase-1, macrophages

Introduction

Cheonwangbosimdan (CWBSD, Tian Wang Bu Xin Dan in Chinese) is one of the most widely used traditional herbal formulas in East Asia. CWBSD composes 15 medicinal herbs; *Rehmannia glutinosa*, *Coptis japonica*, *Acorus gramineus*, *Panax ginseng*, *Angelica gigas*, *Schisandra chinensis*, *Asparagus cochinchinensis*, *Liriope platyphylla*, *Thuja orientalis*, *Zizyphus jujuba*, *Scrophularia buergeriana*, *Poria cocos*, *Salvia miltiorrhiza*, *Platycodon grandiflorum*, and *Polygala tenuifolia* [1, 2]. CWBSD has been used for treatment of insomnia, palpitation, mental weakness, forgetfulness, dry throat and mouth, difficulty focusing, nocturnal emissions, constipation, and oral ulceration [3]. Recent studies have reported on the pharmacological activities of CWBSD against central nervous system and Alzheimer's disease in experimental models [4-7]. CWBSD

also showed beneficial effects through anti-oxidative and cytoprotective effect in brain cells such as mouse neuroblastoma 2a cells and BV2 microglial cells [8, 9]. In addition, a recent paper reported that CWBSD inhibited LPS-induced inflammatory responses in microglia, the major immune effector cells in the brain [8, 10].

Inflammation is a biological response caused by a variety of harmful physical or chemical stimuli such as bacterium, irritants or damage cells [11]. Various molecular mediators are involved in inflammatory process to eliminate noxious stimuli and repair damage cells. Pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6) play a critical role to promote systemic inflammation. Previous papers reported successful therapeutic results in inflammatory disease patients by targeting pro-inflammatory cyto-

Table 1. Composition of Cheonwangbosimdan

Latin name	Scientific name	Amount (g)	Origin
Rehmanniae Radix	Rehmannia glutinosa	15.000	Andong, Korea
Coptidis Rhizoma	Coptis japonica	7.500	China
Acori Graminei Rhizoma	Acorus gramineus	3.750	Jeju, Korea
Ginseng Radix Alba	Panax ginseng	1.875	Yeongju, Korea
Angelicae Gigantis Radix	Angelica gigas	1.875	Bonghwa, Korea
Schizandrae Fructus	Schisandra chinensis	1.875	Samcheok, Korea
Asparagi Tuber	Asparagus cochinchinensis	1.875	China
Liriope Tuber	Liriope platyphylla	1.875	Miryang, Korea
Thujae Semen	Thuja orientalis	1.875	China
Zizyphi Semen	Zizyphus jujuba	1.875	China
Scrophulariae Radix	Scrophularia buergeriana	1.875	Uiseong, Korea
Hoelen	Poria cocos	1.875	Pyeongchang, Korea
Salviae Miltiorrhizae Radix	Salvia miltiorrhiza	1.875	China
Platycodi Radix	Platycodon grandiflorum	1.875	Muju, Korea
Polygalae Radix	Polygala tenuifolia	1.875	China
Total		48.750	

kines [12]. Nitric oxide (NO) and prostaglandin E_2 (PGE_2) also involve in the pathogenesis of inflammation and associated with production of inflammatory cytokines [13, 14].

Our present study aimed to investigate whether CWBSD has anti-inflammatory effects by measuring production of pro-inflammatory cytokines and levels of NO and PGE_2 using RAW-264.7 murine macrophage cell line. We also tried to identify the underlying molecular mechanisms on its anti-inflammatory action by analyzing phosphorylation of mitogen-activated protein kinases (MAPKs) and activation of nuclear factor kappa B (NF- κ B).

Material and methods

Plant materials

The 15 crude herbal medicines forming CWBSD were purchased from Kwangmyungdang Medicinal Herbs (Ulsan, Korea). The origin of 15 herbal medicines was confirmed taxonomically by Professor Je-Hyun Lee, Dongguk University, Gyeongju, Republic of Korea. A voucher specimen (2012-KE34-1~KE34-15) has been deposited at K-herb Research Center, Korea Institute of Oriental Medicine.

Preparation of CWBSD decoction

CWBSD was composed of 15 herbs as **Table 1** (total weight = 5.0 kg, about 102.6 times of

composition of single dose) and extracted in distilled water at 100°C for 2 h under pressure (98 kPa) using an electric extractor (COSMOS-660; Kyungseo Machine Co., Incheon, Korea). The extract solution was filtered using a standard sieve (No. 270, 53 mm; Chung Gye Sang Gong Sa, Seoul, Korea) and freeze-dried. The yield of extract was 21.3% (1065.1 g). HPLC analysis was provided in our previous report [15].

Cell culture

The murine macrophage cell line, RAW264.7, was obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco Inc., Grand Island, NY) supplemented with 5.5% heat-inactivated fetal bovine serum (Gibco Inc.), penicillin (100 U/mL), and streptomycin (100 μ g/mL) in a 5% CO_2 incubator at 37°C.

Cytotoxicity assay

Cell viability assay was performed to determine the cytotoxicity of CWBSD using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Cells were plated onto a 96-well microplate at 3×10^3 cells/well and treated with various concentrations of CWBSD for 24 h. After incubation with CCK-8 reagent for 4 h, optical density (OD) at 450 nm was measured by using a Benchmark plus microplate reader (Bio-Rad Laboratories,

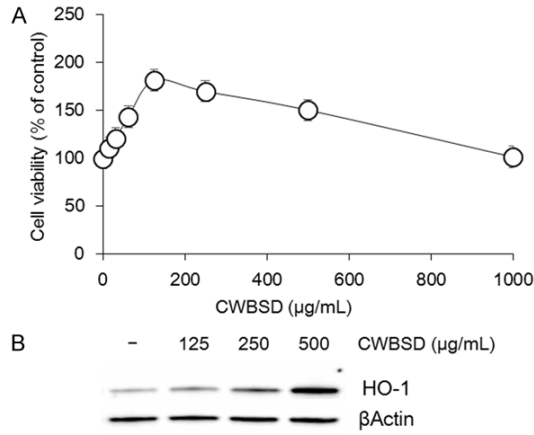


Figure 1. Effect of CWBSD on the cell viability and HO-1 expression in RAW264.7 macrophages. A. Cells were treated with various concentrations of CWBSD (15.625, 31.25, 62.5, 125, 250, 500, or 1000 μg/mL) for 24 h. Cell viability was measured by CCK assay. B. Cells were treated with CWBSD (0, 125, 250, or 500 μg/mL) for 24 h. Cell lysates were prepared and subjected to immunoblotting to detect HO-1 expression. β-actin was used as an internal control.

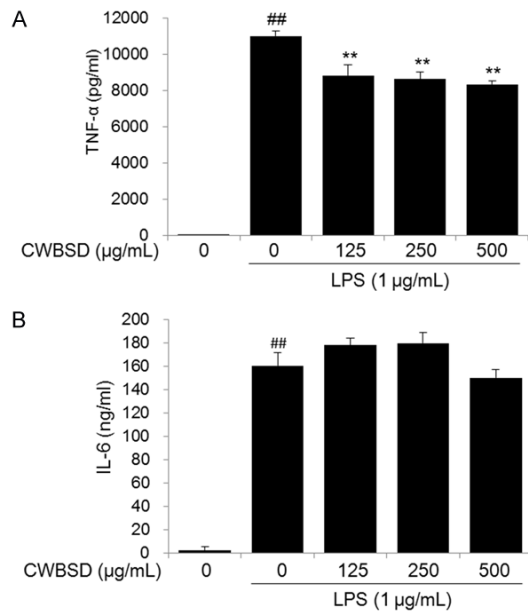


Figure 2. Effect of CWBSD on production of inflammatory cytokines in LPS-stimulated RAW264.7 macrophages. Cells were pretreated with CWBSD (0, 125, 250, or 500 μg/mL) for 4 h and then treated with LPS (1 μg/mL) for 20 h. Supernatants were collected and subjected to ELISAs for (A) TNF-α and (B) IL-6. Bar graphs represent the means from three independent experiments. ##P < 0.01 vs vehicle control cells, and **P < 0.01 vs LPS-treated cells.

Hercules, CA). The cell viability was calculated using the following Equation:

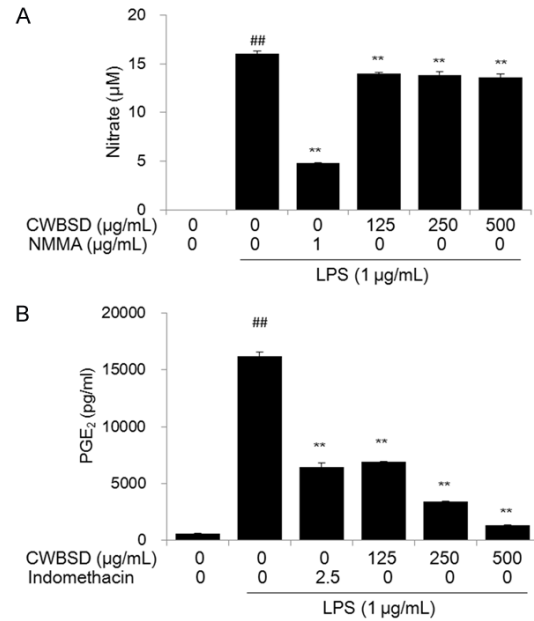


Figure 3. Effect of CWBSD on NO and PGE₂ production in LPS-stimulated RAW264.7 macrophages. Cells were pretreated with CWBSD (0, 125, 250, or 500 μg/mL) for 4 h and then treated with LPS (1 μg/mL) for 20 h. A. Collected supernatants were reacted with Griess reagent and measured absorbance at 540 nm. B. Supernatants were collected and subjected to ELISAs for PGE₂. Bar graphs represent the means from three independent experiments. ##P < 0.01 vs vehicle control cells, and **P < 0.01 vs LPS-treated cells.

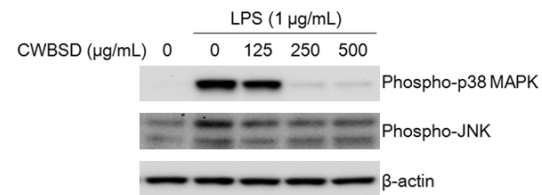


Figure 4. Effect of CWBSD on activation of the MAPKs in LPS-stimulated RAW264.7 macrophages. Cells were pretreated with CWBSD (0, 125, 250, or 500 μg/mL) for 2 h and the treated with LPS (1 μg/mL) for 15 min. Cell lysates were prepared and subjected to immunoblotting for detecting the phosphorylation of p38 MAPK and JNK.

$$\text{Cell viability (\%)} = \frac{\text{Mean OD in CWBSD hyphen treated cells}}{\text{Mean OD in untreated cells}} \times 100$$

Enzyme-linked immunosorbent assays (ELISAs) for TNF-α, IL-6, and PGE₂

Cells were pretreated with various concentrations of CWBSD for 4 h and stimulated with LPS (1 μg/mL; Sigma-Aldrich, St. Louis, MO) for an

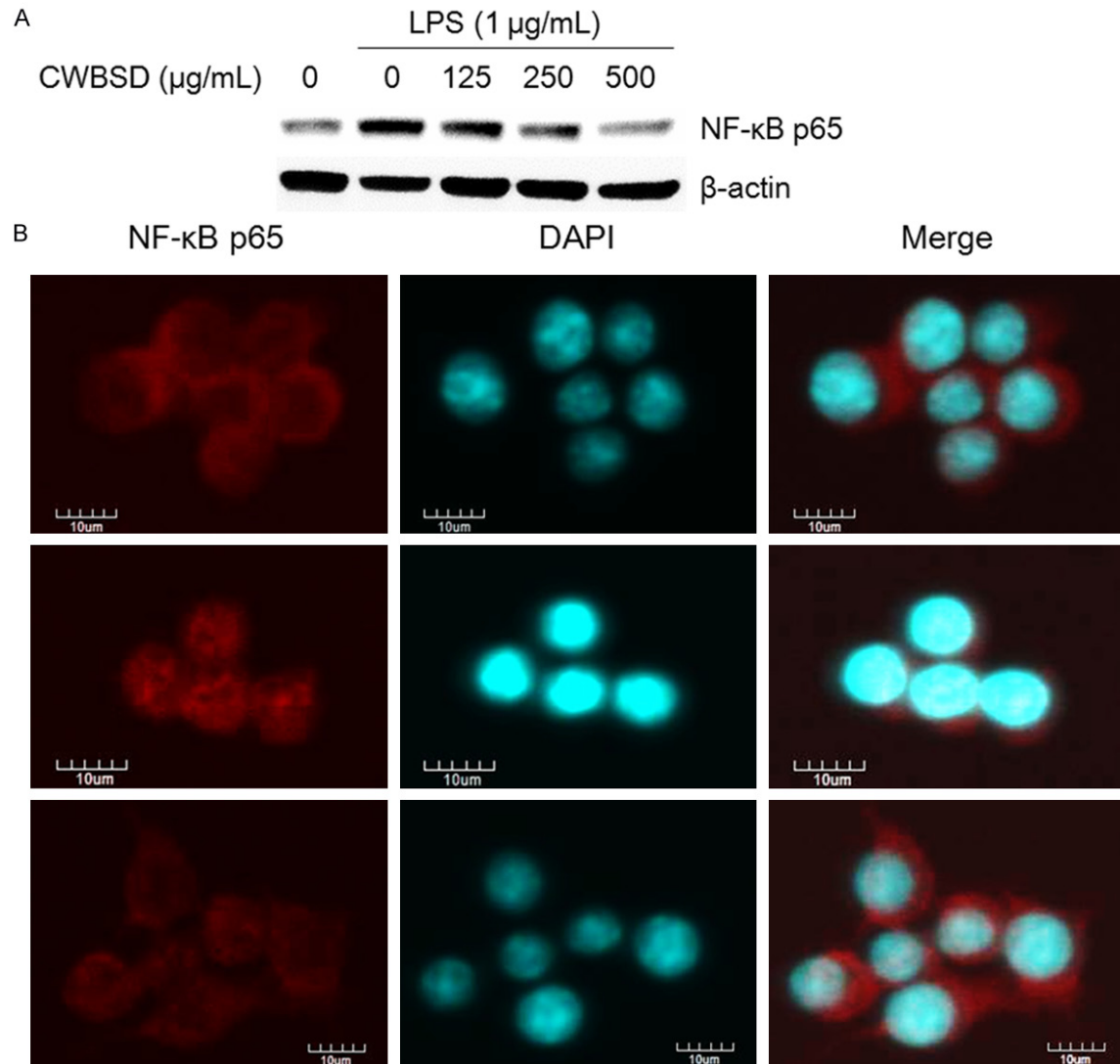


Figure 5. Effect of CWBSD on activation of NF- κ B in LPS-stimulated RAW264.7 macrophages. A. Cells were pretreated with CWBSD (0, 125, 250, or 500 $\mu\text{g/mL}$) for 2 h and the treated with LPS (1 $\mu\text{g/mL}$) for 1 h. Nuclear extracts were prepared and subjected to immunoblotting for NF- κ B p65. B. Cells were pretreated with or without CWBSD (500 $\mu\text{g/mL}$) for 2 h and the treated with LPS (1 $\mu\text{g/mL}$) for 1 h. The cells were fixed in 4% (v/v) methanol free formaldehyde solution (pH 7.4), stained with anti-NF- κ B p65 (Texas Red). The immunostained cells were then mounted with medium containing DAPI and visualized under an Olympus FLUOVIEW FV10i confocal microscopy. Top: Control, middle: LPS, bottom: LPS + CWBSD.

additional 20 h. Production of TNF- α , IL-6, and PGE₂ in the culture supernatants was measured using commercial ELISA kits from R&D systems (Minneapolis, MN), BD Biosciences (Mountain View, CA), and Cayman Chemical Co. (Ann Arbor, MI), respectively.

NO assay

The NO synthesis was analyzed by determining the accumulation of nitrite (NO₂⁻) in culture supernatant using Griess Reagent System (Promega, Madison, WI). Equal volume of supernatant and sulfanilamide solution was mixed

and incubated for 10 min at room temperature, and then added naphthylethylenediamine dihydrochloride solution for additional 5 min. The absorbance was measured at 540 nm by using a Benchmark plus microplate reader (Bio-Rad Laboratories, Hercules, CA). The nitrite concentration was determined from a standard curve generated with sodium nitrite (NaNO₂).

Western blotting

A whole cell extract was prepared by suspending cells in an extraction lysis buffer (Sigma-Aldrich) containing protease inhibitor cocktail

(Roche Applied Science, Indianapolis, IN). A nuclear extract was isolated using the NE-PER Nuclear and Cytoplasmic Extraction reagents (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol. The protein concentration was determined using a Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA). Equal amounts of cell extract (30 µg) were resolved by 4-20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with blocking solution (5% skim milk in Tris-buffered saline containing Tween 20 (TBST)), followed by an overnight incubation at 4°C with the appropriate primary antibody; anti-phospho-p38 MAPK, anti-phospho-JNK (Cell Signaling, Danvers, MA), HO-1 (Abcam, Boston, MA), NF-κB p65, and β-actin (Santa Cruz Biotechnology, Dallas, TX). The membranes were washed three times with TBST, and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. The membranes were again washed three times with TBST, and then developed using an enhanced chemiluminescence (ECL) kit (Thermo Scientific, Rockford, IL). Image capture was performed using ChemiDoc (Bio-Rad).

Immunofluorescence staining

Cells were plated onto poly-L-lysine coated slide glass and fixed in 4% (v/v) methanol free formaldehyde solution (pH 7.4) at 4°C for 25 min. The cells were permeabilized in 0.2% (w/v) Triton X-100, blocked in 5% (w/v) bovine serum albumin (BSA) in humidified chamber, followed by immunostaining with NF-κB p65 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) and Texas Red-conjugated secondary antibody. The slides were covered with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA) and visualized using a Fluoview FV10i confocal microscope (Olympus, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using GraphPad InStat 3.05 software (GraphPad Software Inc., La Jolla, CA). Results are expressed as the mean ± standard error of the mean (SEM). Statistical significance was calcu-

lated using one-way analysis of variance (ANOVA) with a post Dunnett's multiple comparisons being used to assess the differences between the groups. $P < 0.05$ was considered significant.

Results

CWBSD enhances HO-1 expression in RAW264.7 macrophages

Cytotoxicity of CWBSD extract against RAW-264.7 cells was measured by CCK assay. Cells were treated with various concentrations of CWBSD extract (15.625, 31.25, 62.5, 125, 250, 500, or 1000 µg/mL) for 24 h. As shown in **Figure 1A**, cell viability was maintained over 100% up to 1000 µg/mL.

To examine anti-inflammatory effect of CWBSD, protein expression of HO-1, one of target of anti-inflammatory agents [16], was analyzed by immunoblotting. CWBSD extract clearly increased HO-1 expression in a dose-dependent manner (**Figure 1B**).

CWBSD inhibits LPS-mediated production of TNF-α in RAW264.7 macrophages

To assess whether CWBSD influences production of pro-inflammatory cytokines, cells were treated with CWBSD in LPS-stimulated RAW-264.7 cells. Levels of TNF-α and IL-6 in the culture supernatant were analyzed by ELISAs. As expected, LPS significantly increased the productions of TNF-α and IL-6 in the cells (**Figure 2**). By contrast, CWBSD extract significantly reduced LPS-induced TNF-α production in a dose-dependent manner (**Figure 2A**). However, CWBSD had no significant effect on IL-6 production (**Figure 2B**).

CWBSD prevents LPS-induced production of NO and PGE₂ in RAW264.7 macrophages

To evaluate the effect of CWBSD extract on NO production, the amount of nitrite secreted into the supernatant was measured in LPS-treated RAW264.7 macrophages. As shown in **Figure 3A**, CWBSD extract significantly reduced LPS-stimulated NO production. We also investigated the inhibitory effect of CWBSD on PGE₂ generation in LPS-treated RAW264.7 cells. LPS significantly increased level of PGE₂ whereas CWBSD extract significantly decreased LPS-induced PGE₂ production (**Figure 3B**).

CWBSD suppresses phosphorylation of MAPKs in LPS-stimulated RAW264.7 macrophages

To examine the molecular mechanisms responsible for anti-inflammatory action of CWBSD, we analyzed the phosphorylation of p38 MAPK and JNK in LPS-treated RAW264.7 cells with or without CWBSD. LPS treatment markedly increased phospho-levels of p38 MAPK and JNK compared with untreated control. Treatment with CWBSD extract dramatically suppressed phosphorylation of p38 MAPK at 250 or 500 µg/mL, but no effect at 125 µg/mL. However, CWBSD treatment weakly reduced LPS-induced phosphorylation of JNK (**Figure 4**), and has no effect on phosphorylation of extra extracellular signal-regulated kinase (ERK) (data not shown).

CWBSD abrogates LPS-mediated NF-κB activation in RAW264.7 macrophages

We also investigated the effect of CWBSD on activation of NF-κB, a major inflammatory factor [17], by LPS stimulation. In our study, LPS stimulation increased expression of NF-κB p65 in the nucleus. By contrast, CWBSD reduced the nuclear level of NF-κB p65 in a dose-dependent manner in LPS-stimulated RAW264.7 cells (**Figure 5A**). Consistently, immunocytochemistry results revealed that CWBSD treatment blocked the LPS-mediated nuclear translocation of NF-κB p65 in RAW264.7 macrophages compared with LPS treatment alone (**Figure 5B**).

Discussion

Although traditional herbal formulas have been utilized to prevent or treat various diseases, the scientific evidences are still rare to support their biological and pharmacological effects. We here report that traditional herbal formula CWBSD exerts anti-inflammatory effects via inducing HO-1 expression in RAW264.7 macrophages. CWBSD inhibited LPS-mediated production of pro-inflammatory mediators TNF-α, NO, and PGE₂, as well as suppressed phosphorylation of MAPKs and inactivation of NF-κB.

Inflammation is a complex immune reaction against biological or chemical stimuli [11]. Inflammatory process can be progressed by increasing TNF-α, NO and/or PGE₂ [18, 19]. Especially, TNF-α is a major inflammatory factor and considered an attractive candidate mole-

cule for anti-inflammatory agents as anti-TNF-α therapy [20]. In the present study, inflammatory reaction was induced by stimulation with LPS according to the MacMicking's report [21]. We found that CWBSD significantly decreased levels of TNF-α in a dose-dependent manner in LPS-stimulated RAW264.7 cells. In contrast, no effect was observed on IL-6 production. We also detected suppression of NO and PGE₂ by CWBSD in LPS-treated macrophages, suggesting anti-inflammatory action of CWBSD.

HO-1 plays an important role in the regulation of oxidative stress and inflammatory response. HO-1 activation is associated with inhibition of pro-inflammatory cytokine release and suppression of pro-inflammatory enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) [22]. Because our data revealed inhibitory effects of CWBSD on TNF-α, iNOS-derived NO and COX-2 derived PGE₂, we examined whether CWBSD influence HO-1 expression. As expected, CWBSD enhanced level of HO-1 protein expression in a dose-dependent manner in RAW264.7 cells.

Inflammation is regulated at the molecular levels by the intracellular signaling pathways such as MAPKs and NF-κB [23, 24]. Many papers reported that activation of MAPK and/or NF-κB is correlated with production of pro-inflammatory mediators, such as TNF-α, NO, and PGE₂. To determine the molecular mechanisms responsible for inhibition of inflammatory response by CWBSD, alteration of MAPK phosphorylation and NF-κB activation in LPS-treated RAW264.7 cells with or without CWBSD. LPS stimulation markedly increased phospho-levels of MAPK p38 and JNK whereas CWBSD suppressed LPS-induced phosphorylation of MAPK p38 and JNK. In contrast, CWBSD had no effect on ERK phosphorylation (data not shown). In addition, CWBSD reduced nuclear expression of NF-κB p65 in LPS-treated cells. Consistently, CWBSD treatment clearly blocked cellular localization of NF-κB p65 in the nucleus of LPS-stimulated cells. These results imply the crucial role of JNK and NF-κB pathways in CWBSD-induced anti-inflammatory activity.

Among 15 herbal composition of CWBSD, most of them revealed anti-inflammatory effects *in vitro* or *in vivo* [25-38]. Thus, inhibition of inflammatory reaction by CWBSD may be results from coordination of the herbal compo-

sition. In our previous report, we established the simultaneous analysis of 7 marker compounds 5-hydroxymethyl-2-furaldehyde, coptisine, berberine, nodakenin, harpagoside, cinnamic acid, and β -asarone by using high-performance liquid chromatography (HPLC) method [15]. We will be necessary to identify a bioactive compound to express anti-inflammatory activity of CWBSD in the same experimental condition.

In summary, our present study suggests the scientific evidence that CWBSD exerts anti-inflammatory effects, which was shown to involve in the production of TNF- α , NO, and PGE₂ by inhibiting NF- κ B activation in macrophages. We also showed CWBSD induces HO-1 expression, indicating anti-oxidative effect of CWBSD, and will be required additional assays to support anti-oxidation of CWBSD.

Acknowledgements

This work was supported by a grant from the Korea Institute of Oriental Medicine (No. K15250).

Disclosure of conflict of interest

None.

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