Dendrobium moniliforme Stem Extract Inhibits Lipoteichoic Acid-Induced Inflammatory Responses by Upregulation of Heme Oxygenase-1

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The stems of *Dendrobium moniliforme* have been used in traditional herbal medicine for the treatment of fever and lack of body fluid in Korea. In this study, we investigated anti-inflammatory effects of the aqueous extract of *D. moniliforme* stems (DM) in response to lipoteichoic acid (LTA), a major constituent of the cell wall of Gram-positive bacteria. DM inhibited LTA-induced expression of a pro-inflammatory mediator inducible nitric oxide synthase (iNOS) in the murine macrophages. And DM induced expression of heme oxygenase-1 (HO-1) at the transcriptional level. Conversely, the knockdown of HO-1 expression by siRNA markedly reversed the inhibitory effects of DM on LTA-induced iNOS expression. We also demonstrated that nuclear translocation of Nrf2 was increased following treatment with DM. In addition, DM-mediated Nrf2 activation and HO-1 expression were suppressed by PI3K/Akt and p38 inhibitors; treatment with DM also resulted in phosphorylation of Akt and p38. These results suggest that DM inhibits the expression of iNOS in LTA-stimulated macrophages, and that these effects are mediated by the upregulation of HO-1 expression via PI3K/Akt/p38-Nrf2 signaling.

Keywords: *Dendrobium moniliforme*, heme oxygenase-1 (HO-1), inducible nitric oxide synthase (iNOS), lipoteichoic acid, NF-E2-related factor 2 (Nrf2)

Introduction

Lipoteichoic acid (LTA) is a major component of the cell wall of Gram-positive bacteria including *Staphylococcus aureus*, a common cause of abscesses, sinusitis, food poisoning, and wound infections following surgery. LTA plays an important role in a number of infectious diseases including skin infections [1], pneumonia [2], meningitis [3], and sepsis [4], and has led to high mortality [5]. LTA is a pathogen-associated molecular pattern (PAMP) molecule in Gram-positive pathogenic bacteria and may bind to Toll-like receptors-2 (TLR-2) [6, 7]. Signal transduction via TLR-2 activates nuclear factor-kappaB (NF-κB), leading to the expression of pro-inflammatory molecules such as inducible nitric oxide synthase (iNOS), cytokines, and chemokines. A large amount of nitric oxide (NO) synthesized by iNOS has been implicated in the pathogenesis of various inflammatory disease [8]. Thus inhibition of NO production has been suggested as an effective strategy for the treatment of

inflammatory diseases.

Heme oxygenase-1 (HO-1) is an inducible enzyme that mediates the degradation of cellular heme into carbon monoxide (CO), free iron, and biliverdin, which is further converted to bilirubin by biliverdin reductase [9]. HO-1 end-products CO and biliverdin are thought to have anti-apoptotic and anti-inflammatory functions, and increased intracellular levels of iron up-regulate ferritin, an iron-storing protein that participates in the cytoprotective machinery [9, 10]. Growing evidences have demonstrated that many compounds induce HO-1 expression and exhibit their anti-inflammatory effects via HO-1 up-regulation [10–16]. Furthermore, human and murine HO-1 deficiency exhibit increased oxidative stress, persistent vascular injury, and chronic inflammation [17, 18].

NF-E2-related factor 2 (Nrf2) is known to be major transcription factor which regulates HO-1 expression [19]. In basal conditions, Nrf2 is present in the cytoplasm bound to Kelch-like ECH-associated protein 1 (Keap1), and degraded

by the ubiquitin-dependent 26S proteasome system [20]. During activation, Nrf2 is released from inhibition by Keap1, translocates to the nucleus, heterodimerizes with Maf, and binds to antioxidant response elements (AREs) located in the promoter regions of many detoxifying/antioxidant genes, including HO-1 [20, 21].

The stems of *Dendrobium moniliforme* (L.) Sw have been used in traditional Chinese and Korean medicine as a Yin tonic to nourish the stomach, promote the production of body fluid, and reduce fever [22, 23]. *D. moniliforme* is known to produce a variety of secondary metabolites, such as alkaloids [24], sesquiterpenes [25, 26], and phenanthrenes [27, 28]. Several biological activities have been reported for *D. moniliforme*, such as free radical scavenging [23, 29], immunoregulatory [25], anti-inflammatory [27, 30], and anti-tumor [28] activities, as well as reno-protective effects in high fat diet-treated mice [31]. We previously examined that *D. moniliforme* inhibits lipopolysaccharide-stimulated production of NO and IL-1 β in macrophages [30]. However, the mechanism responsible for its anti-inflammatory actions has not been fully understood. In this study, we investigated the effects of the aqueous extract of *D. moniliforme* (DM) stem on the LTA-induced inflammatory response in macrophages. Our finding is the first report clarifying *D. moniliforme* exhibits anti-inflammatory effects via upregulation of HO-1.

Materials and Methods

Materials and Preparation of Extract

Cobalt protoporphyrin (CoPP) was purchased from Enzo Life Sciences, Inc. (USA). Actinomycin D (AD), cycloheximide (CHX), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and LTA from *Staphylococcus aureus* were purchased from Sigma-Aldrich Co. (USA). LY294002, PD98059, SP600125 and SB203580 were obtained from A.G. Scientific (USA). The antibodies for iNOS, HO-1, Nrf2, Akt, p38, TATA-box binding protein (TBP) and histone deacetylases (HDAC) were purchased from Santa Cruz Biotechnology (USA), the antibodies for phospho-Akt and phospho-p-38 were purchased from Cell Signaling Technology (USA), and the anti-tubulin antibody was purchased from Bio Genex (USA). The dry shoot systems of *D. moniliforme* Sw. were obtained and extracted as described previously [30]. In brief, the dry shoot systems (300 g) were extracted with distilled water at 100°C for 4 h. The extract was filtered through a 0.45- μ m filter, freeze-dried (yield: 8 g), and stored at 4°C. The dried extract was dissolved in phosphate-buffered saline (PBS) and filtered through a 0.22 μ m-filter before use.

Cell Culture

Murine macrophage RAW 264.7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with glutamine

(1 mM) and 10% FBS at 37°C in an atmosphere of 5% CO₂.

Measurement of Nitrite Concentration

NO production in cell culture was measured by a microplate assay method as described previously [15].

Cell Viability Assay

The cytotoxicity of DM was estimated using a microculture MTT-based colorimetric assay as described previously [15].

Western Blot Analysis

Cytosolic and nuclear proteins were extracted as described previously [32]. Western blotting was performed as described previously [16]. Tubulin or TATA binding protein (TBP) were used as protein-loading controls for each lane. Quantitative image analysis was performed using image analysis software, ImageJ (<http://rsb.info.nih.gov/ij>) and data were presented as fold of control.

Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

The total cellular RNA was isolated using RNA-spin mini RNA isolation kits (GE Healthcare, USA) in accordance with the manufacturer's instructions. Total RNA (1 μ g) was reverse-transcribed using Maxime RT PreMix (Intron Biotechnology, Korea) with oligo-dT15-primers. PCR was performed in a TaKaRa PCR Thermal Cycler Dice (TaKaRa Bio Inc., Japan). The primer sequences were as follows: HO-1-sense (5'-GTTGACGGACCC CAAAAGAT-3'), HO-1-antisense (5'-CCTCATCCTGGAAGGTCC AC-3'), GAPDH-sense (5'-AGGTGGTCTCCTCTGACTTC-3'), and GAPDH-antisense (5'-TACCAGGAAATGAGCTTGAC-3').

Immunofluorescence Confocal Microscopy

Immunofluorescence confocal microscopy was performed as described previously [16].

Interference of HO-1

The siRNAs for HO-1 (GenBank Accession No. NM 010442.1) were synthesized by Bioneer (Korea). The cells were transfected with HO-1 siRNA or negative control siRNA using INTERFERin (Polyplus transfection, Illkirch, France) and then incubated for 48 h until the protein expression was detected.

Statistical Analysis

All results were expressed as the mean $\pm$ SE (standard error). Each experiment was repeated at least three times. Statistical analysis was performed by using SPSS software to determine significant differences. We used one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for comparison of three or more groups. A value of $p < 0.05$ was considered statistically significant.

Results

DM Inhibits iNOS Expression in Macrophages

To investigate the anti-inflammatory effect of DM, we

Fig. 1. Effect of DM on the production of NO in macrophages. (A) RAW 264.7 cells were incubated with various concentrations of DM for 1 h and then stimulated with LTA (10 μg/ml) for 20 h. The production of NO was measured via Griess test. (B) Cells were treated as mentioned above, and then expression of iNOS was analyzed via Western blot and analyzed according to the Materials and methods. (C) Cell viability was measured via MTT assay. **p* < 0.05 vs. LTA-treated group.

first examined the effect of DM on NO synthesis in murine macrophage RAW 264.7 cells. Pretreatment of DM suppressed LTA-induced NO release in a dose-dependent manner (Fig. 1A). Additionally, the expression level of iNOS was significantly decreased by DM in a dose-dependent manner

in Western blot analysis (Fig. 1B). On the other hand, DM did not influence the cell viability as measured via MTT assay (Fig. 1C). These results suggest that DM inhibits iNOS expression and NO production without affecting cell viability.

Fig. 2. Inhibitory effect of HO-1 on iNOS expression. (A) Cells were incubated with DM (500 μg/ml) or CoPP (10 μM) for 1 h and then stimulated with LTA (10 μg/ml) for 20 h. Protein levels of iNOS and HO-1 were analyzed via Western blot. (B) Protein expression was relatively quantified according to the Materials and methods. **p* < 0.05 vs. LTA-treated group. (C) Cells were transfected with HO-1 siRNA or control siRNA, pretreated with DM (500 μg/ml) for 1 h and then stimulated with LTA (10 μg/ml) for 20 h. Protein levels of iNOS and HO-1 were analyzed via Western blot. (D) Protein expression was relatively quantified. si-Con and si-HO-1 represents control siRNA and HO-1 siRNA, respectively. **p* < 0.05 for each group (both of iNOS and HO-1).

DM Exhibits Anti-Inflammatory Effects Via Induction of HO-1

To examine whether HO-1 suppresses the expression of iNOS, cells were pretreated with CoPP, an inducer of HO-1, and incubated with LTA for 20 h. As shown in Figs. 2A and 2B, CoPP treatment significantly inhibited the expression of iNOS in parallel with HO-1 induction, and DM treatment also showed similar effects. To confirm DM exhibits its anti-inflammatory effect via the induction of HO-1 expression, we used an HO-1 small interfering (si) RNA system. Decreased HO-1 expression by HO-1 si-RNA blocked DM-mediated inhibition of LTA-stimulated iNOS expression, whereas control siRNA showed no effect (Figs. 2C and 2D).

DM Induces HO-1 Expression in Murine Macrophages

Next, we examined the expression of HO-1 by DM in macrophages. DM dramatically increased HO-1 protein and mRNA levels in a dose-dependent manner (Figs. 3A–3C). CoPP was used as a positive control. To verify that DM-induced HO-1 expression is mediated by transcription and translation, we used AD to inhibit DNA-dependent RNA polymerase, and CHX to inhibit ribosomal protein

synthesis. AD or CHX significantly decreased DM-induced HO-1 expression (Figs. 3D and 3E). Taken together, these results indicate that DM induces HO-1 expression at the transcriptional level.

DM-Induced HO-1 Expression Is Mediated by Nrf2

Since the expression of HO-1 is known to be regulated by Nrf2 [19], we investigated whether DM activates Nrf2 in RAW 264.7 cells. Nuclear levels of Nrf2, which are critical to its transcriptional activity, were examined via Western blotting and immunofluorescence confocal microscopy. As shown in Fig. 4, DM significantly increased nuclear level of Nrf2 in a dose-dependent manner. These results suggest that DM activates Nrf2, which in turn induces HO-1 expression.

DM Mediates HO-1 Expression through PI3K/Akt and p38 Kinase

To investigate what signaling pathway is involved in DM-mediated Nrf2 activation and HO-1 expression, we used pharmacological inhibitors of PI3K and MAPKs (PI3K/Akt: LY294002, ERK: PD98059, JNK: SP600125, and p38: SB203580). Pre-treatment of RAW 264.7 cells with

Fig. 3. Induction of HO-1 by DM.

(A) Cells were incubated with various concentrations of DM or CoPP (10 µM) for 8 h, and HO-1 protein levels were analyzed via Western blot. (B) Protein expression was relatively quantified. * $p < 0.05$ vs. control group. (C) Cells were treated as mentioned above and mRNA levels were analyzed via RT-PCR. (D) Cells were treated with DM (500 µg/ml) for 8 h in the absence or presence of AD (1 µg/ml) or CHX (1 µg/ml). Protein levels of HO-1 were analyzed via Western blot. (E) Protein expression was relatively quantified. * $p < 0.05$ vs. DM-treated group.

Fig. 4. Effect of DM on Nrf2 activation.

Cells were incubated with DM (500 µg/ml) for indicated times (A), or indicated concentrations of DM for 1 h (C). Nuclear Nrf2 and TBP (Nuc-Nrf2 and Nuc-TBP) and cytosolic Nrf2 and tubulin (Cyt-Nrf2 and Cyt-Tubul) were assayed via Western blot, and relative expression of Nrf2 was analyzed (B and D). * $p < 0.05$ vs. control group. (E) Cells were incubated with DM (500 µg/ml) for 1 h on coverslips in 35 mm dishes, and nuclear accumulation of Nrf2 was confirmed via confocal microscopy.

LY294002 or SB203580 inhibited DM-induced HO-1 expression and Nrf2 nuclear accumulation (Figs. 5A and 5B). In addition, treatment of RAW 264.7 cells with DM increased phosphorylation of Akt and p38 kinase (Figs. 5C and 5D). These results suggest that DM increases Nrf2 activation and HO-1 expression via PI3K/Akt and p38 kinase signaling pathways.

Discussion

In the present study, we examined the effects of DM on

LTA-induced inflammation in vitro, and the relationship between HO-1 expression and the inhibition of LTA-induced inflammation. We found that DM inhibited the production of NO and suppressed the expression of iNOS in the murine macrophages (Fig. 1). Excessive production of NO by iNOS in macrophages is believed to play a critical role in the pathogenesis of various inflammation-related diseases. Thus suppression of iNOS expression in macrophages could represent an attractive target to treat various inflammatory diseases [33–37]. Thus our results suggest that DM could be a useful remedial agent for Gram-

Fig. 5. Involvement of Akt and p38 kinase in DM-mediated expression of HO-1.

(A) Cells were incubated with LY294002 (LY, 10 μ M), SP600125 (SP, 20 μ M), PD98059 (PD, 20 μ M), and SB203580 (SB, 20 μ M) for 30 min and then treated with DM (500 μ g/ml) for 8 h for HO-1 or 1 h for Nrf2. Equal amounts of cytosolic (for HO-1) or nuclear (for Nrf2) extract were analyzed via Western blot. (B) Protein expression was relatively quantified. * $p < 0.05$ vs. DM-treated group. (C) Cells were incubated with various concentrations of DM for 30 min and equal amounts of cytosolic extract were analyzed via Western blot. (D) Protein expression was relatively quantified. * $p < 0.05$ vs. control group.

positive bacteria-mediated inflammatory diseases such as skin infections, pneumonia, meningitis, and sepsis.

HO-1 is known to exhibit anti-inflammatory activities by inhibiting production of pro-inflammatory mediators [11, 12, 38], suggesting a potential therapeutic strategy for treating inflammatory diseases. We found that DM markedly induced HO-1 expression at the transcriptional level (Fig. 3). Because HO-1 is known to be induced by chemical-mediated oxidative stress [39], we examined whether DM increased HO-1 expression by inducing cell damage. A cell viability assay (MTT assay) revealed that DM does not affect cell viability (Fig. 1C), suggesting that HO-1 is specifically induced by DM, not by cell damage. Moreover, the reduction of HO-1 expression via siRNA significantly repealed the inhibitory effects of DM on LTA-induced iNOS expression, while treatment with CoPP suppressed iNOS expression (Fig. 2). These data suggest that DM inhibit iNOS expression via HO-1 induction. Although the effect of HO-1 end-products was not examined in this study, several studies indicate CO and biliverdin could play a role to ameliorate inflammation [40, 41].

This study showed that DM increased nuclear accumulation

of Nrf2 in a dose-dependent manner (Fig. 4), suggesting that DM activates Nrf2 translocation into the nucleus, which gives rise to induction of HO-1 expression. Because Nrf2 regulates HO-1 as well as other detoxifying and antioxidant proteins including NAD(P)H quinone oxidoreductase 1 (NQO-1), thioredoxin-1 (Trx1) and glutathione S-transferase (GST) [42], it is possible that DM may upregulate NQO-1, Trx1 and/or GST, although this was not examined in our study. Several studies demonstrated that PI3K and MAPKs such as ERK, JNK, and p38 are involved in Nrf2 and HO-1 signaling in response to various stimuli [14, 43]. However, these signaling pathways depend on the type of cells and stimuli in terms of their contribution to HO-1 expression. This study showed that DM-mediated Nrf2 activation and HO-1 expression were regulated by PI3K/Akt and p38 (Fig. 5). Recently, Sun *et al.* [44] described that Nrf2 is phosphorylated at multiple sites by MAPK to facilitate its nuclear translocation. Thus it is possible that DM-induced PI3K/Akt and p38 activities directly phosphorylate Nrf2 and promote nuclear translocation of Nrf2, even if phosphorylation of Nrf2 was not examined in this study. Naturally, the possibility that PI3K/Akt and p38 regulate

the Nrf2 activity through indirect mechanisms should not rule out.

In conclusion, we demonstrated that DM inhibits the expression of iNOS in LTA-stimulated macrophages in vitro, and that this effect is mediated by upregulation of HO-1 expression via PI3K/Akt/p38-Nrf2 signaling. Our findings could help us understand the molecular mechanism of the anti-inflammatory actions of DM.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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