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Tribulus terrestris Suppresses the Lipopolysaccharide-Induced Inflammatory Reaction in RAW264.7 Macrophages through Heme Oxygenase-1 Expressions

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The fruit of *Tribulus terrestris* L. (Zygophyllaceae) is an important source of traditional Korean and Chinese medicines. In this study, NNMBS223, consisting of the ethanol extract of T. terrestris, showed potent anti-inflammatory activities in RAW264.7 macrophages. We investigated the effect of NNMBS223 in suppressing the protein expression of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2 and production of iNOS-derived nitric oxide (NO), COX-2-derived prostaglandin E2 (PGE2) in lipopolysaccharide (LPS)-stimulated macrophages. In addition, NNMBS223 induced expression of heme oxygenase (HO)-1 through nuclear translocation of nuclear factor E2-related factor 2 (Nrf2) in macrophages. The effects of NNMBS223 on LPS-induced production of NO and PGE2 were partially reversed by the HO activity inhibitor tin protoporphyrin (SnPP). These findings suggest that Nrf2-dependent increases in expression of HO-1 induced by NNMBS223 conferred anti-inflammatory activities in LPS stimulated RAW264.7 macrophages.

Key words : *Tribulus terrestris* L., Nitric oxide synthase, Cyclooxygenase, Heme Oxygenase-1, Nuclear factor E2-related factor 2

Introduction

The fruit of *Tribulus terrestris* L., which belonging to the Zygophyllaceae family, is used as a folk medicine for treatment of sexual impotence, rheumatism, edema, abdominal distention, hypertension and kidney stones[1-3]. Previous phytochemical studies of *T. terrestris* have reported the isolation of several bioactive compounds like alkaloids and saponins[4,5]. Beneficial effects of this plant have been investigated, however, its direct molecular targets and the mechanism of action against inflammation are not known.

Recently emerging evidence has shown that excessive inflammatory response causes disturbance of tissue functions and can lead to disease pathogenesis. The inflammatory response is a complex biological process mediated by activation of immune cells such as neutrophils, eosinophils, mononuclear phagocytes, and macrophages[6,7]. Among them, macrophages play a key role in management of the immune response to invading pathogens through phagocytosis and cytokine secretion. Upon activation, macrophages contribute to the inflammatory process by enhancing signal transduction, which induces pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, nitric oxide (NO), and prostaglandin (PG) E_2[8]. Under inflammatory conditions, these proinflammatory mediators and cytokines activate cells involved in chronic hepatitis, atherosclerosis, and rheumatoid arthritis[9,10].

Heme oxygenase (HO)-1, an enzyme essential for heme degradation, catalyzes the first and rate-limiting step in the oxidative degradation of free heme into carbon monoxide (CO), biliverdin/bilirubin, and ferrous iron[11]. HO-1 and the subsequent metabolites of heme catabolism have recently been recognized as having major immunomodulatory and anti-inflammatory properties in various inflammation models. In particular, HO-1 expression or CO administration has potent anti-inflammatory effects in activated macrophages through attenuation of the expression of pro-inflammatory enzymes and cytokines, and bilirubin/biliverdin suppresses iNOS expression and NO production following stimulation of macrophages with lipopolysaccharide (LPS)[12-15]. Nuclear transcription factor-E2-related factor 2 (Nrf2) is a basic leucine zipper transcription factor that interacts with its cognate DNA binding domains in the promoter to regulate the gene

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expression of phase II detoxifying enzymes, including heme oxygenase-1 (HO-1), NADPH, quinine oxioreductase-1 (NQO1), and glutathione S-transferase[16]. Recent studies have suggested that natural products can activate Nrf2 by promoting dissociation of the Nrf2-Keap1 complex, resulting in induction of anti-inflammatory proteins, including HO-1. Therefore, regulating the production of proinflammatory mediators via the Nrf2-dependent upregulation of HO-1 would be effective for prevention and treatment of a variety of inflammatory diseases.

T. terrestris has recently been known to exert anti-oxidative and anti-inflammatory activities. However, the underlying mechanisms of action against inflammation have not yet been demonstrated. In this study, The aim of this study was to examine the role of NNMBS223, consisting of the ethanol extract of *T. terrestris*, as an anti-inflammatory HO-1 inducer and its regulation in RAW264.7 murine macrophages.

Materials and Methods

1. Plant material and preparation of NNMBS223

The ripe fruits of *T. terrestris* (Zygophyllaceae) were purchased from the University Oriental Herbal Drugstore, Iksan, Korea, in August 2010, and a voucher specimen was deposited at the Herbarium of the College of Pharmacy at Wonkwang University, Korea. Ripe fruits of T. terrestris (50 g) were extracted twice with hot 70% ethanol (1 l) for 2 h at room temperature, and filtered with filter paper. The filtrate was evaporated in vacuo to produce a 70% ethanol extract (3.21 g, 6.42 w/w%). The 70% ethanol extract was suspended in distilled water (100 ml), followed by filtration. The residue derived from the filtration was dissolved in hot ethanol and filtered again. The filtrate was then evaporated in vacuo to obtain a standardized fraction of *T. terrestris* (NNMBS223, 247 mg, 0.5 w/w%). NNMBS223 was deposited at the Standardized Material Bank for New Botanical Drugs, Wonkwang University. For each experiment, NNMBS223 was dissolved in dimethylsulfoxide (final culture concentration, 0.05%). Serum-free medium was used as a vehicle control. Preliminary studies have indicated that the solvent had no effect on cell viability at the concentration used.

2. Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). Primary antibodies, including mouse/goat/rabbit anti-HO-1, anti-iNOS, anti-COX-2, anti-Nrf2 and secondary antibodies, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An enzyme-linked immunosorbent assay (ELISA) kit for PGE2 was purchased from R & D Systems, Inc. (Minneapolis, MN, USA). Tin protoporphyrin IX (SnPP IX; inhibitor of HO activity) and cobalt protoporphyrin (CoPP; HO-1 inducer) were obtained from Porphyrin Products (Logan, UT, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

3. Cell culture and viability assay

RAW264.7 macrophages were obtained from American Type Culture Collection (ATCC; Manassas, VA). The cells were maintained at 5×10^5 cells/ml in DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G and incubated at 37℃ in a humidified atmosphere containing 5% CO_2 and 95% air. For determination of cell viability, 50 mg/ml of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to 1 ml of cell suspension (1×10^5 cells per 1 ml in 96-well plates) and incubated for 4 h. The formazan formed was dissolved in acidic 2-propanol, and optical density was measured at 590 nm.

4. Nitrite assay

The method used for determination of NO production follows the protocol published by Lee et al.[17]. The nitrite concentration in the medium was measured as an indicator of NO production using the Griess reaction; 100 μl of each supernatant was mixed with the same volume of Griess reagent, and the absorbance of the mixture was determined at 525 nm using an ELISA plate reader. The nitrite concentration was determined using a standard curve of sodium nitrite prepared in Dulbecco's Modified Eagle Medium (DMEM) that was free of phenol red.

5. PGE2 assay

RAW264.7 macrophages were cultured in 24-well plates, pre-incubated for 12 h with various concentrations of sample, and then treated with LPS for 18 h. Culture medium was collected and the concentration of PGE2 was determined using ELISA kits (R&D Systems) as per the manufacturer's instructions.

6. Western blot analysis

Cell homogenates (30 μg of protein) were separated on 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. Blots were then washed with H_2O, blocked with 5% skimmed milk powder in Tris-Buffered Saline Tween-20 (TBST) (10 mM Tris-HCl, pH 7.6, 150 mM NaCl,

0.05% Tween-20) for 1 h, and incubated with the appropriate primary antibody at dilutions recommended by the supplier. The membrane was then washed and primary antibodies were detected with goat anti-rabbit-IgG or rabbit anti-mouse-IgG conjugated to horseradish peroxidase, and the bands were visualized with enhanced chemiluminescence (Amersham Bioscience, Buckinghamshire, UK). Protein expression levels were determined by analysis of signals captured on nitrocellulose membranes using the Fusion FX7 chemiluminescence imaging system (Vilber Lourmat, Marne-la-vallée, France).

7. Preparation of nuclear and cytoplasmic fractions

Nuclear and cytoplasmic extracts of RAW264.7 macrophages (3×10^6 cells per 3 ml in 60 mm dishes) were isolated and assayed using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific) as per the manufacturer's instructions. The supernatant was separated and stored at -70℃ until further use. The nucleus and cytoplasmic extracts were then analyzed for protein content using the Bradford assay.

8. Statistical analysis

Data are expressed as a mean ± S.D., and the data were analyzed using one-way ANOVA followed by a Dunnett's test or Student's t test for determination of any significant differences. P value < 0.05 was considered significant.

Results

Initially, the cytotoxic potential of NNMBS223 in RAW264.7 macrophages was measured.

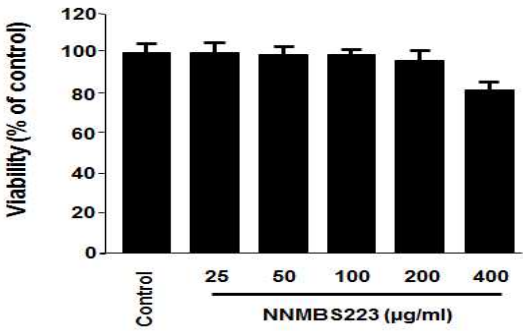

Fig. 1. Effects of NNMBS223 on cell viability. RAW264.7 macrophages were incubated for 36 h with various concentrations of NNMBS223 (25 - 400 μg/ml). Cell viability was determined as described in materials and methods. Bar represents the mean ± S.D. of 3 independent experiments.

As shown in Fig. 1, results of the MTT assay performed at 200 μg/ml NNMBS223 showed no cytotoxic effects in macrophages. Therefore, for further experiments, the macrophages were treated with NNMBS223 in the concentration range of 25-200 μg/ml. At non-cytotoxic concentrations (25-200 μg/ml), RAW264.7 macrophages were challenged with LPS (1 μg/ml) in the presence or absence of NNMBS223 and the levels of iNOS and COX-2 expression were measured by western blot analysis.

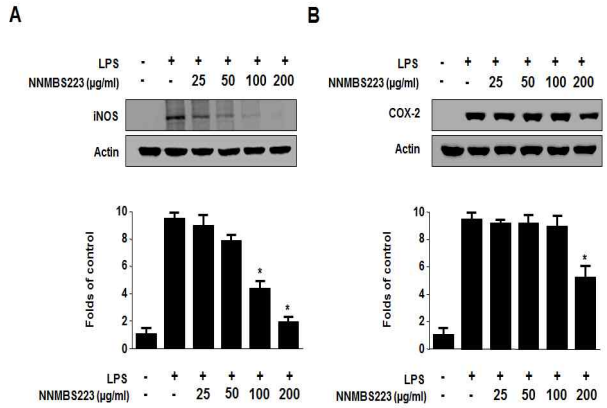

Fig. 2. Effects of NNMBS223 on LPS-induced iNOS and COX-2 protein expression in RAW264.7 macrophages. Macrophages were pre-treated for 12 h with indicated concentrations of NNMBS223 and stimulated for 18 h with LPS (1 μg/ml). Western blot analyses for iNOS and COX-2 expression (A, B) were performed as described in materials and methods. Representative blots of 3 independent experiments are shown. *p < 0.05 compared to the group treated with LPS.

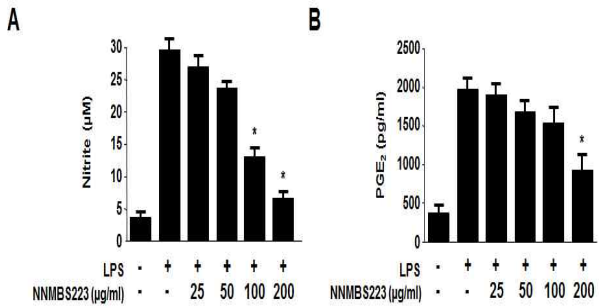

Fig. 3. Effects of NNMBS223 on LPS-induced NO and PGE2 production in RAW264.7 macrophages. Macrophages were pre-treated for 12 h with indicated concentrations of NNMBS223 and stimulated for 18 h with LPS (1 μg/ml). The concentrations of NO, PGE2 were determined as described under materials and methods. Data represent mean values of 3 experiments ± S.D. *p < 0.05 compared to the group treated with LPS.

As shown in Fig. 2, pre-treatment of macrophages with NNMBS223 for 12 h resulted in suppressed expression of iNOS and COX-2, thereby suppressing production of iNOS-derived NO and COX-2-derived PGE2 (Fig. 3A and B). In addition, I attempted to determine whether NNMBS223 affected HO-1 protein expression in RAW 264.7 cells. As shown in Fig. 4A, NNMBS223 treatment upregulated the expression of HO-1 protein in a dose-dependent manner in RAW 264.7 cells. As a positive control, the HO-1 inducer, cobalt protophorphyrin

(CoPP), increased HO-1 expression at 10 μM. Induction of HO-1 by NNMBS223 reached a peak at 200 μg/ml. At a concentration of 200 μg/ml, HO-1 expression was evident after 6 h, and the maximum increase was observed at around 18 h in macrophages(Fig. 4B).

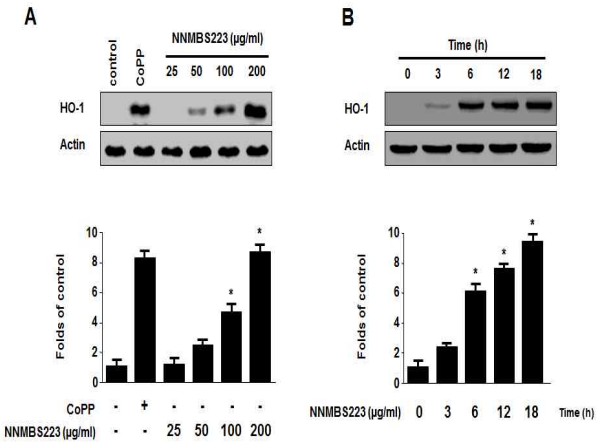

Fig. 4. Effects of NNMBS223 on HO-1 expression in RAW264.7 macrophages. Macrophages were incubated for 18 h with indicated concentrations of NNMBS223 (A) and periods with 200 μg/ml of NNMBS223 (B). Western blot analysis for HO-1 expression was performed as described in materials and methods, and representative blots of 3 independent experiments are shown. Data represent the mean values of 3 experiments ± S.D. *p < 0.05 compared to the control group.

Nuclear translocation of activated Nrf2 is known as an important event upstream of HO-1 expression in a wide variety of cells, including macrophages. Therefore, I next examined the effects of NNMBS223 on Nrf2 nuclear translocation in macrophages. The cells were incubated with NNMBS223 for 0.5, 1, 1.5 and 3 h at a concentration of 200 μg/ml. Western blot analysis showed that NNMBS223 increased the expression of Nrf2 in nuclear fraction, with a concomitant decrease in cytoplasmic fractions(Fig. 5).

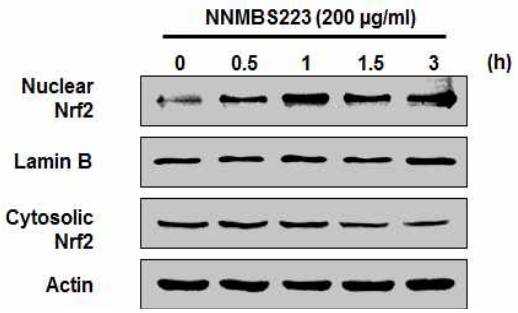

Fig. 5. Effects of NNMBS223 on nuclear translocation of Nrf2 in RAW264.7 macrophages. Quiescent macrophages were incubated for the indicated periods with 200 μg/ml of NNMBS223. Nrf2 protein was detected by western blot as described in the materials and methods, and representative images or blots of 3 independent experiments are shown.

To confirm that the suppressive effect of NNMBS223 on pro-inflammatory mediators is correlated with NNMBS223-mediated expression of HO-1, I attempted to determine whether the effect of NNMBS223-mediated expression of HO-1 was reversed by pre-treatment with SnPP, an inhibitor of HO activity. Macrophages were pre-treated with 200 μg/ml NNMBS223 for 12 h in the absence or presence of SnPP, followed by stimulation with LPS. As shown in Fig. 6, SnPP partially abolished the inhibition of LPS-induced production of NO and PGE2 by NNMBS223.

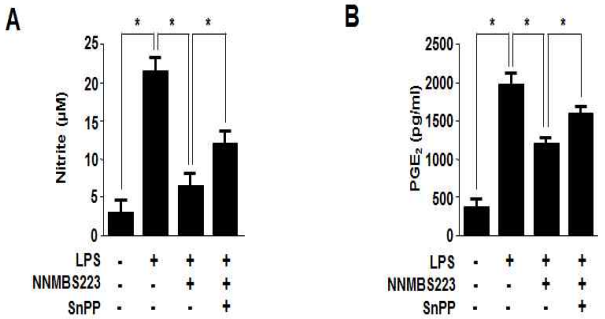

Fig. 6. HO-1 mediates the suppressive effect of NNMBS223 on LPS-stimulated pro-inflammatory mediator production. Macrophages were pre-treated for 12 h with NNMBS223 (200 μg/ml) in the presence or absence of SnPP (50 μM), and stimulated for 18 h with LPS (1 μg/ml). The concentrations of NO and PGE2 were determined as described in materials and methods. SnPP was pretreated with macrophages for 3 h in this experiment. Data represent mean values of 3 experiments ± S.D. *p< 0.05.

Discussion

Recent reports, have suggested that regulation of pro-inflammatory mediators by HO-1 is an important mechanism for the cellular pathophysiological condition of inflammation. To confirm that the suppressive effect of NNMBS223 on pro-inflammatory mediators is correlated with NNMBS223-mediated expression of HO-1, I attempted to determine whether the effect of NNMBS223-mediated expression of HO-1 was reversed by pre-treatment with SnPP, an inhibitor of HO activity. The result in Fig. 6 indicates that SnPP partially abolished the inhibition of LPS-induced production of NO and PGE2 by NNMBS223. As shown in this study, because NNMBS223 actively induces expression of HO-1 in macrophages, it is possible that the anti-inflammatory effects of NNMBS223 are mediated in part by the products of the HO-1 enzyme reaction, namely, CO, bilirubin/ biliverdin.

Previous studies have reported that *T. terrestris* possesses a variety of beneficial properties, including anti-oxidant, anti-inflammatory, and analgesic activities in diverse *in vivo* or *in vitro* models[18,19]. Ehrman et al.[20] reported that alkaloids from *T. terrestris* show anti-inflammatory activity through inhibition of the c-Jun terminal-NH2 kinase (JNK) pathway. In

addition, the methanolic extract of this plant showed potent inhibition of COX-2 activity on LPS stimulated macrophages[21]. However, the relevant mechanisms that could substantially explain the anti-inflammatory effect of this plant remain to be elucidated. The inflammatory response is a complex reaction of the immune system, which is regulated by several inflammatory mediators. Expression of pro-inflammatory enzymes, including iNOS and COX-2, plays an essential role in immune-activated macrophages by production of iNOS-derived NO and COX-2-derived PGE2. Several previous studies have reported that HO-1 and its enzymatic by-products play a crucial role in inflammatory response targeted against macrophages[13,22]. In the current study, I indicate that inhibition of HO activity by the HO inhibitor SnPP resulted in partial reversal of the inhibitory effects of NNMBS223 on production of NO and PGE2 in LPS-stimulated macrophages. These results suggested that NNMBS223-induced expression of HO-1 plays a vital role in the inhibition of inflammatory responses in LPS-stimulated macrophages.

In conclusion, NNMBS223, the ethanol extract of *T. terrestris*, actively induced expression of HO-1 through the Nrf2 pathway in RAW264.7 macrophages leading to suppression of LPS-induced production of NO and PGE2 and mitigation of the inflammatory process. These results suggest that NNMBS223 might be a promising therapeutic agent for further development in treatment of a variety of inflammatory diseases. Further detailed studies investigating the anti-inflammatory effects of NNMBS223 *in vitro* and *in vivo* models would help to clarify its therapeutic potential.

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