Full Paper

Asperlin From the Marine-Derived Fungus *Aspergillus* sp. SF-5044 Exerts Anti-inflammatory Effects Through Heme Oxygenase-1 Expression in Murine MacrophagesDong-Sung Lee^{1,†}, Gil-Saeng Jeong^{2,†}, Bin Li¹, Sang Un Lee³, Hyuncheol Oh³, and Youn-Chul Kim^{1,*}¹Standardized Material Bank for New Botanical Drugs, College of Pharmacy, Wonkwang University, Iksan 570-749, Republic of Korea²College of Pharmacy, Keimyung University, Dae-gu 704-701, Republic of Korea³College of Medical and Life Sciences, Silla University, Busan 617-736, Republic of Korea

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Abstract. Asperlin is a fungal metabolite isolated from *Aspergillus* sp. SF-5044. In the present study, we isolated asperlin from the marine-derived fungus *Aspergillus* sp. SF-5044 and demonstrated that it inhibited inducible nitric oxide synthase (iNOS) expression, reduced iNOS-derived NO, suppressed cyclooxygenase (COX)-2 expression, and reduced COX-derived prostaglandin (PG) E₂ production in lipopolysaccharide (LPS)-stimulated RAW264.7 and murine peritoneal macrophages. Similarly, asperlin reduced the production of tumor necrosis factor (TNF)- α and interleukin (IL)-1 β . In addition, asperlin inhibited the phosphorylation and degradation of I κ B- α , as well as the nuclear translocation of p65 caused by the stimulation of LPS in RAW264.7 macrophages. Furthermore, asperlin induced heme oxygenase (HO)-1 expression through nuclear translocation of nuclear factor E2-related factor 2 and increased HO activity in RAW264.7 macrophages. The effects of asperlin on the LPS-induced expression of iNOS and COX-2 and production of NO, PGE₂, TNF- α , and IL-1 β were partially reversed by a HO-1 inhibitor, tin protoporphyrin. These findings suggest that asperlin-induced HO-1 expression plays a role in the anti-inflammatory effects of asperlin in macrophages.

Keywords: asperlin, heme oxygenase (HO)-1, anti-inflammatory effect, RAW264.7 macrophage, murine peritoneal macrophage

Introduction

Chronic or acute inflammation is a complex biological process mediated by activating immune cells such as neutrophils, eosinophils, mononuclear phagocytes, and macrophages (1, 2). During the process of inflammation, these immune cells including macrophages play a key role in managing the immune response to invading pathogens through phagocytosis and cytokine secretion. In addition, macrophages orchestrate many different physiological immune responses, including the overproduction of proinflammatory cytokines and overexpres-

sion of proinflammatory enzymes such as inducible nitric oxide synthase (iNOS), nitric oxide (NO), cyclooxygenase (COX)-2, prostaglandin (PG) E₂, tumor necrosis factor (TNF)- α , and interleukin (IL)-1 β (3). Under inflammatory conditions, these proinflammatory cytokines and enzymes not only induce cell and tissue injury but also activate cells involved in chronic hepatitis, atherosclerosis, and rheumatoid arthritis (4, 5).

Heme oxygenase (HO)-1 is a stress-responsive protein that can be induced by stimulants such as inflammatory cytokines, heat shock, heavy metals, and oxidants. In particular, HO-1 and the subsequent metabolites of heme catabolism appear to play key roles in regulating various important biological responses, including oxidative damage, cell injury, and inflammation (6). Degradation of free heme by HO-1 leads to the generation of bilirubin/biliverdin, free iron (Fe²⁺), and carbon monoxide (CO)

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(7). Recent studies have shown that the gaseous CO molecule and the natural antioxidant bilirubin/biliverdin appear to be responsible for most of the anti-inflammatory effects of HO-1 (8, 9). In particular, HO-1 and CO can inhibit the overproduction of proinflammatory cytokines in activated macrophages (10–12), and bilirubin/biliverdin suppresses iNOS expression and NO production following lipopolysaccharide (LPS) stimulation of macrophage cell lines (13). Therefore, the upregulation of HO-1 expression inhibits the expression of proinflammatory enzymes, including iNOS and COX-2, and also suppresses the production of proinflammatory cytokines such as iNOS-derived NO, COX-2-driven PGE₂, TNF- α , and IL-1 β (14, 15). It has also been reported that the expression of HO-1 suppresses iNOS expression and NO production via the inactivation of nuclear factor (NF)- κ B (16, 17). In other reports, it has been suggested that various transcription factors, such as nuclear factor E2-related factor 2 (Nrf2), interact with their cognate DNA binding domains in the HO-1 promoter to upregulate *ho-1* gene transcription, leading to expression of HO-1 (18). In addition, phytochemicals can activate Nrf2 by directly binding to Keap1 through a covalent linkage, resulting in the induction of cytoprotective proteins, including HO-1 (19). Nrf2 is required for the expression of some inducible proteins such as glutathione S-transferase, quinone reductase, and HO-1 (20).

Asperlin is a crystalline metabolite isolated from the marine-derived fungus *Aspergillus* sp. SF-5044. It has been shown to exhibit antitumor and antibacterial activity (21, 22). However, its anti-inflammatory activity and molecular mechanism have not yet been demonstrated. In the present study, we first investigated whether asperlin could downregulate the expression of proinflammatory enzymes and the production of proinflammatory mediators in RAW264.7 macrophages and murine macrophages obtained from C57BL/6 mice. We further provide evidence to support a vital role for HO-1 expression in mediating the anti-inflammatory effects of asperlin.

Materials and Methods

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL Co. (Carlsbad, CA, USA). Tin protoporphyrin IX (SnPP IX), an inhibitor of HO activity (23, 24), was obtained from Porphyrin Products (Logan, UT, USA). Thioglycollate was purchased from BD Pharmingen (San Diego, CA, USA). Trizol reagent and the polymerase chain reaction kit were purchased from Invitrogen (Carlsbad, CA, USA). RAW264.7 macrophages were obtained from the American Type

Culture Collection (Manassas, VA, USA). C57BL/6 mice were purchased from Orient Bio Co. (Sungnam, KyungKiDo, Republic of Korea). Primary antibodies including HO-1, COX-2, iNOS, I κ B- α , p-I κ B- α , p65, β -actin, proliferating cell nuclear antigen (PCNA), lamin B, and secondary antibodies used for western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). NS-398 (*N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide), a COX-2-selective inhibitor (25, 26), was purchased from Calbiochem (Darmstadt, Germany). Enzyme-linked immunosorbent assay (ELISA) kits for PGE₂, TNF- α , and IL-1 β were purchased from R&D Systems (Minneapolis, MN, USA). All other chemicals including the HO-1 inducer cobalt protoporphyrin (CoPP) (24, 27) and L-NAME (L-N^G-nitro arginine methyl ester), a standard NOS inhibitor (28, 29), were obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless indicated otherwise. All of the asperlin used in this paper was the same preparation and isolated from *Aspergillus* sp. SF-5044.

General experimental procedures

The structure of asperlin from *Aspergillus* sp. SF-5044 was confirmed by its spectral data. Optical rotation was recorded on a Perkin Elmer 341 digital polarimeter (Perkin Elmer, Waltham, MA, USA). NMR spectra (1D and 2D) were recorded in CDCl₃ using a JNM ECP-400 spectrometer (400 MHz for ¹H and 100 MHz for ¹³C; JEOL, Tokyo). Chemical shifts were referenced relative to tetramethylsilane ($\delta_H/\delta_C = 0$). ESIMS data were obtained using an API-2000 ESI-MS instrument from Applied Biosystems (Foster City, CA, USA) at Pukyong National University, Korea. Solvents for extractions and flash column chromatography were reagent grade and used without further purification. Solvents used for HPLC were analytical grade. Flash column chromatography was carried out using LiChroprep RP-18 (40–63 μ m; Merck, Darmstadt, Germany). HPLC separations were performed on a Capcell Pak C₁₈ column (10 $\times$ 250 mm; 5- μ m particle size; Shiseido, Tokyo) with a flow rate of 2 mL/min. Compounds were detected by UV absorption at 210 nm.

Fermentation, extraction, and isolation

Details of isolation and identification of the marine-derived fungus *Aspergillus* sp. SF-5044 have been described elsewhere (30). The fungal strain was cultured on 110 petri-dish plates (90 mm), each containing 20 mL of potato dextrose agar media with 3% NaCl. Plates were individually inoculated with 2 mL seed cultures of the fungal strain. Plate cultures were incubated at 25°C for a period of 10 days. Extraction of the agar media with EtOAc (2 L) provided an organic phase, which was then

concentrated *in vacuo* to yield 2.0 g of an extract. The EtOAc extract was subjected to C₁₈ flash column chromatography (5 × 26 cm), eluting with a stepwise gradient of 20%, 40%, 60%, 80%, and 100% (v/v) MeOH in H₂O (500 mL each). The fractions eluted at 40% MeOH (452.7 mg) was re-applied to C₁₈ flash column chromatography (5 × 22 cm), eluting with a stepwise gradient of 10%–100% (v/v) MeOH in H₂O (500 mL each, 10% increment for each fraction). The fraction eluted with 40% MeOH (167.7 mg) was further purified by semi-preparative reversed-phase HPLC, eluting with a gradient from 7 to 30% MeOH in H₂O (0.1% formic acid) over 90 min to yield 1 (61 mg, *t_R* = 83 min). The structure of 1 was determined as (+)-asperlin by comparison of MS and NMR data with those in the literature (31), along with its [α]_D value.

Cell culture and viability assay

RAW264.7 macrophages were maintained at 5×10^5 cells/mL in DMEM medium supplemented with 10% heat-inactivated FBS, penicillin G (100 units/mL), streptomycin (100 mg/mL), and L-glutamine (2 mM) and incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. The effects of various experimental modulations on cell viability were evaluated by determining mitochondrial reductase function, as measured by the ability to reduce tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), into formazan crystals (32). The formation of formazan is proportional to the number of functional mitochondria in living cells. For determination of cell viability, 50 mg/mL of MTT was added to 1 mL of cell suspension (1×10^5 cells/mL in 96-well plates) for 4 h, and the resulting formazan was dissolved in acidic 2-propanol; optical density was measured at 590 nm. The optical density of the formazan formed in control (untreated) cells was taken as 100% viability.

Peritoneal macrophages culture

Thioglycollate-elicited peritoneal macrophages were harvested from C57BL/6 mice 4 days after i.p. injection of 3 mL thioglycollate (33). Peritoneal lavage was washed using 8 mL of Hanks' Balanced Salt Solution containing 10 U/mL heparin. The cells were distributed in RPMI-1640 supplemented with 10% heat-inactivated FBS in 6-well tissue culture plates (5×10^6 cells/mL). After incubation for 3 h, nonadherent cells were removed, and adherent cells were treated with LPS in the absence or presence of asperlin.

Nitrite determination

Production of nitrite, a stable end product of NO oxidation, was used as a measure of NOS activity. Nitrite

present in the conditioned media was determined spectrophotometrically, based on the Griess reaction (34). An aliquot of each supernatant (100 μ L) was mixed with the same volume of Griess reagent (0.1% [w/v] *N*-(1-naphthyl)-ethylenediamine and 1% [w/v] sulfanilamide in 5% [v/v] phosphoric acid) for 10 min at room temperature. The absorbance of the final reactant at 525 nm was measured using an ELISA plate reader, and the nitrite concentration was determined by using a standard curve of sodium nitrite made up in DMEM free of phenol red.

PGE₂, TNF- α , and IL-1 β assays

The levels of PGE₂, TNF- α , and IL-1 β present in each sample were determined with the use of commercially available kits from R&D Systems (Abingdon, UK) (35). The assay was performed according to the manufacturer's instructions. Briefly, RAW264.7 macrophages were cultured in 24-well plates (5×10^5 cells/mL), preincubated for 12 h with different concentrations of asperlin and then stimulated for 18 h with LPS. Cell culture supernatants were collected immediately after treatment and centrifuged at $13,000 \times g$ for 2 min to remove any particulates. The medium was added to a 96-well plate precoated with affinity-purified polyclonal antibodies specific for PGE₂, mouse TNF- α , or IL-1 β . An enzyme-linked polyclonal antibody specific for PGE₂, mouse TNF- α , or IL-1 β was added to the wells and left to react for 20 or 2 h, followed by a wash to remove any unbound antibody-enzyme reagent. The intensity of the color detected at 450 nm (correction wavelength set at 540 or 570 nm) was measured after the addition of a substrate solution and was proportional to the amount of PGE₂, TNF- α , and IL-1 β produced.

Preparation of cytosolic and nuclear fractions

RAW264.7 macrophages were homogenized (1:20, w:v) in PER-Mammalian Protein Extraction buffer (Pierce Biotechnology, Rockford, IL, USA) containing freshly added protease inhibitor cocktail I (EMD Biosciences, San Diego, CA, USA) and 1 mM phenylmethylsulfonyl fluoride. The cytosolic fraction of the cell was prepared by centrifugation at $15,000 \times g$ for 10 min at 4°C. Nuclear and cytoplasmic extracts of the RAW264.7 macrophages were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology). After treatment, RAW264.7 macrophages (3×10^6 cells/3 mL in a 60-mm dish) were collected and washed with phosphate-buffered saline. After centrifugation, cell lysis was performed at 4°C by vigorous shaking for 15 min in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl [pH 7.4], 50 mM glycerophosphate, 20 mM NaF, 20 mM ethylene glycol tetraacetic acid, 1 mM dithiothreitol, 1 mM

Na₃VO₄, and protease inhibitors). After centrifugation at 15,000 × *g* for 15 min, the supernatant was separated and stored at -70°C until further use. Protein content was determined by a bicinchoninic acid protein assay kit (Pierce Biotechnology).

Western blot analysis

Western blot analysis was performed by lysing cells in 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethylsulfonyl fluoride, 5 mg/mL aprotinin, 5 mg/mL pepstatin A, and 1 mg/mL chymostatin). Protein concentration was determined with the Lowry protein assay kit (P5626, Sigma). An equal amount of protein for each sample was resolved using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred onto a Hybond enhanced chemiluminescence nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% skim milk and sequentially incubated with primary antibody (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence detection (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Measurement of heme oxygenase activity

The method used for the determination of HO activity followed a protocol published by Motterlini et al. (36). Briefly, after the incubation process, the cells were washed twice with PBS, gently scraped off the dish, and centrifuged (1,000 × *g* for 10 min at 4°C). The cell pellet was resuspended in MgCl₂ (2 mM) phosphate (100 mM) buffer (pH 7.4), frozen at -70°C, thawed 3 times, and finally sonicated on ice before centrifugation at 18,000 × *g* for 10 min at 4°C. The supernatant (400 μL) was added to an NADPH-generating system containing 0.8 mM NADPH, 2 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate-L-dehydrogenase, 2 mg of protein from rat liver cytosol (prepared from the 15,000 × *g* supernatant fraction as a source of biliverdin reductase), potassium phosphate buffer (100 mM, pH 7.4), and hemin (10 mM) in a final volume of 200 μL. The reaction was conducted for 1 h at 37°C in the dark and terminated by the addition of 1 mL chloroform. The amount of extracted bilirubin was calculated by the difference in absorption between 464 and 530 nm using a quartz cuvette (extinction coefficient, 40 mM⁻¹ cm⁻¹ for bilirubin).

DNA binding activity of NF-κB

RAW264.7 macrophages were pretreated for 12 h with the indicated concentrations of asperlin and stimulated for 1 h with LPS (1 μg/mL). The DNA-binding activity of NF-κB in nuclear extracts was measured (TransAM

kit; Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Sample preparation: The indicated concentrations of asperlin were added to the RAW264.7 macrophages and then they were stimulated with LPS for 1 h. After treatment, 20 μg of nuclear fraction was extracted from each macrophage sample and each was then diluted in complete lysis buffer to a total volume of 20 μL, followed by addition of 30-μL aliquot of complete binding buffer (DTT, herring sperm DNA, and Binding Buffer AM3). The samples were then pipetted into the individual wells of a 96-well plate and incubated for 1 h at room temperature with mild agitation (100 rpm on a rocking platform). After washing each well with wash buffer, 100 μL of diluted NF-κB antibody (1:1000 dilution in 1 × Antibody Binding Buffer) was added to each well and then incubated for 1 h with mild agitation (100 rpm on a rocking platform). After washing each well with wash buffer, 100 μL of diluted HRP-conjugated antibody (1:1000 dilution in 1 × Antibody Binding Buffer) was added to each well and then incubated for 1 h with mild agitation (100 rpm on a rocking platform). Developing solution was added to each well and left to react for 5 min followed by a wash to remove the supernatant. The 450-nm absorbance of each sample was then read on a spectrophotometer within 5 min.

Statistical analyses

Data are expressed as the mean ± S.D. of at least 3 independent experiments. To compare 3 or more groups, one-way analysis of variance followed by a Newman-Keuls post hoc test was used. *P* < 0.05 was considered statistically significant. Statistical analysis was performed with GraphPad Prism software, version 3.03 (GraphPad Software Inc., San Diego, CA, USA).

Results

Cytotoxic effects of asperlin on cell viability in RAW264.7 macrophages and murine peritoneal macrophages

To investigate the cytotoxic potential of asperlin (Fig. 1A), its effects on the viability of murine peritoneal macrophages (Fig. 1B) and RAW264.7 macrophages (Fig. 1C) were first measured in an MTT assay. As shown in Fig. 1, B and C, cell viability was not significantly altered by asperlin concentrations up to 40 μM. These results suggest that the concentrations of asperlin tested (below 40 μM) were nontoxic to murine peritoneal macrophages and RAW264.7 macrophages. Thus, for further experiments, the cells were treated with asperlin concentrations of 5 – 40 μM. A higher concentration of asperlin reduced the viability of these macrophages (Fig. 1: B and C).

Fig. 1. Chemical structure of asperlin (A) and the effects of asperlin on cell viability (B, C). Macrophages were incubated for 36 h with various concentrations of asperlin (1–60 μ M). Data represent the mean values of 3 experiments ($\pm$ S.D.).

Effects of asperlin on expression of proinflammatory enzymes and production of proinflammatory cytokines in murine peritoneal macrophages and RAW264.7 macrophages stimulated with LPS

We investigated the effect of asperlin on LPS-induced iNOS and COX-2 expression in murine peritoneal macrophages (Fig. 2) and RAW264.7 macrophages (Fig. 3). Thioglycollate-elicited macrophages from C57BL/6 mice were cultured either in medium alone or in medium containing LPS (1 μ g/mL). Cells were challenged with LPS (1 μ g/mL) in the presence or absence of asperlin at noncytotoxic concentrations ranging from 5 to 40 μ M, and the levels of iNOS and COX-2 expression were measured in murine peritoneal macrophages (Fig. 2: A and B) and RAW264.7 macrophages (Fig. 3: A and B). Western blot analysis revealed that asperlin suppressed LPS-induced iNOS and COX-2 expression in a dose-dependent manner in both murine peritoneal macrophages (Fig. 2: A and B) and RAW264.7 macrophages (Fig. 3: A and B). Pretreatment of the cells with asperlin for 12 h resulted in decreased iNOS expression and reduced iNOS-derived NO production in both murine peritoneal macrophages (Fig. 2: A and C) and RAW264.7 macrophages (Fig. 3: A and C). L-NAME, a standard NOS inhibitor (28, 29), caused a significant inhibition of iNOS expression (Figs. 2A and 3A) and NO production (Figs. 2C and 3C) at a concentration of 50 μ M. Under the same conditions, asperlin also suppressed COX-2 expression

and reduced COX-2-derived PGE₂ production in both murine peritoneal macrophages (Fig. 2: B and D) and RAW264.7 macrophages (Fig. 3: B and D). NS-398, a COX-2 selective inhibitor (25, 26), significantly inhibited the expression of COX-2 (Figs. 2B and 3B) and production of PGE₂ (Figs. 2D and 3D) at a concentration of 5 μ M. Because our results showed that asperlin suppressed the LPS-induced proinflammatory mediators iNOS, NO, COX-2, and PGE₂, we further tested the effects of asperlin on the LPS-induced production of TNF- α and IL-1 β in an enzyme immunoassay. Both cells were pre-incubated with asperlin for 12 h, followed by LPS stimulation for 18 h. The asperlin decreased TNF- α and IL-1 β production in a concentration-dependent manner in both murine peritoneal macrophages (Fig. 2: E and F) and RAW264.7 macrophages (Fig. 3: E and F).

Effects of asperlin on protein expression levels of p-I κ B- α and I κ B- α , NF- κ B activation, and NF- κ B-DNA binding activity in LPS-stimulated RAW264.7 macrophages

We investigated the effects of asperlin on the protein expression levels of I κ B- α and the phosphorylation of I κ B- α , an inhibitor associated with NF- κ B in the cytoplasm. Asperlin suppressed the LPS-induced phosphorylation and degradation of I κ B- α in the cytoplasm, as shown by western blotting (Fig. 4A). As shown in Fig. 4A, I κ B- α was degraded after treatment with LPS in RAW264.7 macrophages for 1 h, and this degradation

Fig. 2. Effects of asperlin on iNOS (A) and COX-2 (B) expression and iNOS-derived NO (C) and COX-2-derived PGE₂ (D) production in murine peritoneal macrophages stimulated with LPS. Cells were pretreated for 12 h with the indicated concentrations of asperlin in the presence or absence of L-NAME (50 μM) and NS-398 (5 μM) for 0.5 h and stimulated for 18 h with LPS (1 μg/mL). Representative western blot analyses of 3 independent experiments are shown. Data represent the mean values of 3 experiments (± S.D.). **P* < 0.05, compared with the group treated with LPS alone.

was markedly suppressed by pretreatment of the cells with asperlin (5–40 μM) for 12 h. These results suggest that asperlin inhibited the nuclear translocation of NF-κB (p65) by the stimulation of LPS through prevention of IκB-α degradation. We also observed the appearance of NF-κB-DNA binding activity (Fig. 4B) in nuclear extracts of RAW 264.7 macrophages stimulated with LPS for 1 h. As shown in Fig. 4B, treatment with LPS alone increased the levels of NF-κB-DNA binding activity. However, asperlin suppressed this increase in a dose-dependent manner (Fig. 4B). In addition, our data showed that the translocation of nuclear p65 protein in RAW 264.7 macrophages was increased after cells were treated with LPS for 1 h, whereas the level of p65 declined in response to LPS after pretreatment with asperlin (5–40 μM) for 12 h (Fig. 4C). It is clear that the density ratio of LPS-induced p65 was significantly decreased in a dose-

dependent manner by asperlin (Fig. 4C). On the other hand, the total amount of RelA (p65) contents were not changed in pretreatment with LPS in the presence or absence of asperlin, as shown in Fig. 4C.

Effects of asperlin on HO-1 expression through nuclear translocation of Nrf2 and HO activity in RAW264.7 macrophages

Furthermore, we examined whether asperlin induces HO-1 expression and HO activity in RAW264.7 macrophages. RAW264.7 macrophages were treated with various noncytotoxic concentrations of asperlin (5–40 μM) for 12 h. Asperlin increased HO-1 expression (Fig. 5A) and HO activity (Fig. 5B) in a dose-dependent manner. The HO-1 inducer CoPP (24, 27) increased HO-1 expression and resulted in significant HO activity at a concentration of 20 μM (Fig. 5: A and B). We also exam-

Fig. 3. Effects of asperlin on iNOS (A) and COX-2 (B) expression and on the production of iNOS-derived NO (C) and COX-2-derived PGE₂ (D) in RAW264.7 macrophages stimulated with LPS. Cells were pretreated for 12 h with the indicated concentrations of asperlin in the presence or absence of L-NAME (50 μM) and NS-398 (5 μM) for 0.5 h and stimulated for 18 h with LPS (1 μg/mL). Representative western blot analyses of 3 independent experiments are shown. Data represent the mean values of 3 experiments (± S.D.). **P* < 0.05, compared with the group treated with LPS alone.

ined the effects of SnPP, a competitive inhibitor of HO activity (24, 27), in the presence of asperlin at a concentration of 40 μM (Fig. 5B). SnPP treatment inhibited the induction of HO activity by asperlin (40 μM) (Fig. 5B). The induction of HO-1 by asperlin reached a peak at an asperlin concentration of 40 μM. HO-1 induction was obvious after 6 h and reached a maximum after 18 h of treatment (Fig. 5C). In accordance with the concentration-dependent increase in HO-1 expression, asperlin also increased HO activity (Fig. 5D). Therefore, we investigated whether treatment of RAW264.7 macrophages with asperlin induced the translocation of Nrf2 to the nucleus. The macrophages were incubated with asperlin for 0.5, 1, and 1.5 h at a concentration of 40 μM. The nuclear fractions of asperlin-treated macrophages showed a gradual increase in Nrf2 levels, whereas Nrf2 levels decreased concomitantly in the cytosolic fractions (Fig.

6). On the other hand, asperlin did not affect the change of total Nrf2 contents in whole cell lysis protein (Fig. 6).

Effects of SnPP on inhibition of iNOS and COX-2 expression and nitrite, PGE₂, TNF-α, and IL-1β production by asperlin pretreatment of LPS-stimulated macrophages

We also examined whether HO-1 mediated the suppressive effect of asperlin on LPS-stimulated expression of proinflammatory enzymes, including iNOS and COX-2, and production of proinflammatory mediators such as NO, PGE₂, TNF-α, and IL-1β. Macrophages were pretreated with 40 μM asperlin for 12 h in the presence or absence of SnPP, a competitive inhibitor of HO-1 activity, followed by LPS stimulation. As shown in Fig. 7, SnPP treatment partly attenuated the inhibitory activity of asperlin on LPS-stimulated expression of iNOS and

Fig. 4. Effects of asperlin on the protein expression level of I κ B- α phosphorylation, degradation of I κ B- α (A), NF- κ B-DNA binding activity (B), and NF- κ B activation (C) in RAW264.7 macrophages. Cells were pretreated for 12 h with the indicated concentrations of asperlin and stimulated for 1 h with LPS (1 μ g/mL). Representative western blot analyses of 3 independent experiments are shown. Data represent the mean values of 3 experiments ($\pm$ S.D.). * P < 0.05, compared with the group treated with LPS alone.

COX-2 and production of NO, PGE₂, TNF- α , and IL-1 β .

Discussion

Macrophages are widely used as a cellular model to screen potential anti-inflammatory compounds. They also play significant roles in the host defense mechanism. Inflammatory mediators and proinflammatory cytokines are also involved in host defense, but their overproduction contributes to the pathogenesis of various diseases such as rheumatoid arthritis, periodontitis, chronic inflammation, otitis media, autoimmune diseases, hearing loss, and bacterial sepsis (37, 38). Natural products provide new avenues for drug development and have therefore played a key role in the discovery and development of drugs for the treatment of some inflammatory diseases. Recently, many studies have focused on finding a potent anti-inflammatory agent in natural products.

Asperlin is a crystalline metabolite isolated from the

marine-derived fungus *Aspergillus* sp. SF-5044. Although asperlin has been shown to exhibit antitumor and antibacterial activity (21, 22), its anti-inflammatory activity and molecular mechanism have not yet been reported. In the present study, we first investigated whether asperlin isolated from the marine-derived fungus *Aspergillus* sp. SF-5044 could down-regulate the expression of proinflammatory enzymes and the production of proinflammatory mediators through HO-1 expression in murine macrophages. We also investigated the potential involvement of HO-1 expression, through nuclear translocation of Nrf2, in the anti-inflammatory activity elicited by asperlin. Initially, we showed that pretreatment of LPS-stimulated RAW264.7 and murine macrophages obtained from C57BL/6 mice with asperlin (5–40 μ M) inhibited iNOS and COX-2 expression, thereby suppressing production of COX-2-derived PGE₂ and iNOS-derived NO production (Figs. 2 and 3). Furthermore, we also examined that the inhibitory effects of asperlin on iNOS and COX-2 expression, and PGE₂ and NO produc-

Fig. 5. Effects of asperlin on HO-1 expression and HO activity in RAW264.7 macrophages. Cells were incubated for 12 h with the indicated concentrations of asperlin (A) in the presence or absence of SnPP (50 μM) and stimulated for 18 h with LPS (1 μg/mL) (B) and periods with 40 μM of asperlin (C, D). Representative western blot analyses of 3 independent experiments are shown. Data represent the mean values of 3 experiments ($\pm$ S.D.). * P < 0.05 (Newman-Keuls post hoc test).

Fig. 6. Effects of asperlin on the nuclear translocation of Nrf2 in RAW264.7 macrophages. Macrophages were treated with 40 μM of asperlin for 0.5, 1, and 1.5 h. The nuclei were fractionated from the cytosol using PER-Mammalian Protein Extraction buffer as described in "Materials and Methods." Nrf2 protein was detected by western blot analysis, and representative blots of 3 independent experiments are shown.

tion was compared with NS-398 or L-NAME. NS-398 is well known to inhibit COX-2 activity and expression, selectively. It has been reported that NS-398 blocks COX-2 mRNA expression and enzyme activity as well as PGE₂ production in RAW264.7 macrophages (25, 26).

L-Arginine analogs including L-NAME are non-specific NOS inhibitors that attenuate NO production induced by LPS and cytokines (28, 29). The expression of proinflammatory enzymes, including iNOS and COX-2, plays a crucial role in immune-activated macrophages by producing iNOS-derived NO and COX-2-derived PGE₂ (15, 16). Nitric oxide, a free oxygen radical, can act as a cytotoxic agent in pathological processes, especially in many inflammatory diseases (39). After exposure to stimulants, including LPS, iNOS can be induced to trigger several detrimental cellular responses, including inflammation (40). The overproduction of iNOS-derived NO is involved in the pathology of various inflammatory disorders and tissue damage conditions (41). Changes in NO level caused by the inhibition of iNOS enzyme activity or iNOS induction provide a means of assessing the effect of these agents on the inflammatory process.

Asperlin also inhibited LPS-induced TNF- α and IL-1 β production in activated macrophages (Figs. 2 and 3). The proinflammatory activities of TNF- α and IL-1 β are well known, and these inflammatory cytokines play a major role in regulating inflammation and tumor progression (42). NF- κ B (a heterodimer of p65 and p50) is located in the cytoplasm as an inactive complex bound to I κ B- α , which is phosphorylated and subsequently degraded, and then dissociates to produce activated NF- κ B (43, 44). It is also well known that NF- κ B is a significant factor regu-

Fig. 7. Effects of SnPP on inhibition of iNOS (A) and COX-2 (B) expression and nitrite (C), PGE₂ (D), TNF- α (E), and IL-1 β (F) production by asperlin pretreatment of LPS-stimulated macrophages. Cells were pretreated for 12 h with asperlin (40 μ M) in the presence or absence of SnPP (50 μ M) and stimulated for 18 h with LPS (1 μ g/mL). Representative western blot analyses of 3 independent experiments are shown. Data represent the mean values of 3 experiments ($\pm$ S.D.). * P < 0.05 (Newman-Keuls post hoc test).

lating the expression of inflammation-associated enzymes and cytokine genes, such as iNOS, COX-2, TNF- α , and IL-1 β , which contain NF- κ B binding motifs within their respective promoters (43). Many anti-inflammatory agents exhibit their potency by suppressing NF- κ B signaling (44–46). Thus, we also investigated whether NF- κ B activation and DNA-binding activity, a key signaling pathway leading to iNOS expression, could be an upstream target for the inhibitory effects of asperlin. We examined the effects of asperlin on the phosphorylation of I κ B α and the nuclear translocation of a NF- κ B subunit, p65, in RAW264.7 macrophages. Our results clearly showed that phosphorylation and degradation of I κ B α and the nuclear translocation of p65 induced by LPS were significantly reduced after pretreatment with 5–40 μ M of asperlin for 12 h (Fig. 4A). We also observed the appearance of NF- κ B-DNA binding activity (Fig. 4B) of NF- κ B in nuclear extracts of RAW 264.7 macrophages stimulated with LPS for 1 h. As shown in Fig. 4B, asperlin dose-dependently inhibited the increase of NF- κ B-DNA binding activity in RAW 264.7 macrophages stimu-

lated with LPS (Fig. 4B). These results suggest that asperlin, at least in LPS-stimulated macrophages, exerted its anti-inflammatory effects by suppressing the expression of the proinflammatory enzymes iNOS and COX-2, as well as the production of the proinflammatory cytokines NO, PGE₂, TNF- α , and IL-1 β .

The induction of HO-1 expression and activity plays a role in mediating the anti-inflammatory effects in LPS-stimulated macrophages (47, 48). Although the exact mechanisms for the anti-inflammatory effects of HO-1 have not yet been completely elucidated, one or more of the enzymatic by-products of HO-1 have been evaluated as possible factors. The beneficial effects of HO-1 induction have been attributed to several factors, including the degradation of the pro-oxidant heme, formation of biliverdin/bilirubin, and the release of CO (49–51). In particular, CO has been reported to suppress PGE₂, NO, TNF- α , and IL-1 β production in LPS-stimulated macrophages through its interference in NF- κ B activation (10–12, 14, 15); also, bilirubin/biliverdin suppresses iNOS expression and NO production following LPS

stimulation of the macrophage cell lines (13). Previous studies have suggested that a series of plant-derived compounds, including curcumin and resveratrol, can induce HO-1 and exert anti-inflammatory effects in different types of cells (52, 53). Also in our previous studies, we demonstrated that the anti-inflammatory effects of plant-isolated compounds, including sulfuretin from *Rhus verniciflua* and cudraticusxanthone A from *Cudrania tricuspidata*, might result from the induction of HO-1 in macrophages (23, 24). In addition, recent reports have suggested that phytochemicals can activate Nrf2 by directly binding to Keap1 through covalent linkage, resulting in the induction of cytoprotective proteins, including HO-1 expression (19, 20). Cobalt protoporphyrin (CoPP) is known to be an effective inducer of HO-1 expression in many different cells (54–57). HO-1 is induced by CoPP through nuclear translocation of Nrf2, and the enzymatic function of HO-1 reduces the overproduction of NO in macrophages (54). In addition, HO-1 expression by CoPP exerts anti-inflammatory effects, including down-regulated proinflammatory cytokines and cytoprotective functions in acute lung injury followed by LPS challenge (55). Up-regulation of HO-1 by CoPP significantly inhibited LPS-induced iNOS and COX-2 expression and NO and PGE₂ production in RAW264.7 macrophages (56) and murine bEnd.3 cells (57).

In this study, we tested the effects of asperlin on HO-1 expression and HO activity (Fig. 5). In addition, it is well known that nuclear translocation of activated Nrf2 is an important upstream contributor in the mechanism of HO-1 expression (18). We showed here that asperlin treatment led to an increase in the levels of Nrf2 in the nucleus in RAW264.7 macrophages (Fig. 6). This experiment suggests that Nrf2 may play a role in asperlin-induced HO-1 expression. In addition, we determined if SnPP treatment inhibited the induction of HO activity by asperlin (Fig. 5B). There were no effects of SnPP in the absence of asperlin (data not shown). We also examined whether asperlin-mediated HO-1 expression was correlated with the inhibition of LPS-induced proinflammatory enzymes, including iNOS and COX-2, and proinflammatory mediators such as NO, PGE₂, TNF- α , and IL-1 β . Our results indicate that the inhibition of HO activity by the HO inhibitor SnPP partially reversed the inhibitory effects of asperlin on NO, PGE₂, TNF- α , and IL-1 β production in LPS-stimulated macrophages (Fig. 7). Because SnPP blocks HO enzymatic activity, these data confirmed that asperlin could partially inhibit NO, PGE₂, TNF- α , and IL-1 β production via modulation of HO-1 expression. Thus, these data indicate that asperlin-induced HO-1 expression and HO activity were at least partly responsible for the anti-inflammatory effects of

this compound.

In conclusion, we isolated asperlin from the marine-derived fungus *Aspergillus* sp. SF-5044 and demonstrated that asperlin increased HO-1 expression, which inhibited NO, PGE₂, TNF- α , and IL-1 β production, in LPS-induced macrophages. On the basis of these findings, we suggest that asperlin might be a beneficial therapeutic agent that can be considered for further development in the treatment of a variety of inflammatory diseases.

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