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Whiskey Congeners Suppress LPS/IFNγ-Induced NO Production in Murine Macrophage RAW 264 Cells by Inducing Heme Oxygenase-1 Expression

Tomohiro Itoh,*[,†] Masashi Ando,[†] Yasuyuki Tsukamasa,[†] Toshiyuki Wakimoto,[§] and Haruo Nukaya[#]

[†]Laboratory of Aquatic Food Science, Department of Fisheries, Faculty of Agriculture, Kinki University, 3327-204 Nakamachi, Nara 631-8505, Japan

[§]Graduate School of Pharmaceutical Sciences, Faculty of Pharmaceutical sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-Ku, Tokyo 113-0033, Japan

[#]University of Shizuoka, 52-1 Yada, Suruga-Ku, Shizuoka 422-8526, Japan

Supporting Information

ABSTRACT: Whiskey includes many nonvolatile substances (whiskey congeners; Whc) that seep from the oak cask during the maturation process. To date, many functions of Whc have reported, such as antiallergy and antimelanogenesis. This study examined the effect of Whc on LPS/IFNγ-induced nitric oxide (NO) production in murine macrophage RAW 264 cells. Whc suppressed LPS/IFNγ-induced NO production in a concentration-dependent manner. To determine the active compounds in Whc, the effect of 10 major compounds isolated from Whc on LPS/IFNγ-induced NO production was examined. Coniferylaldehyde (CA) and sinapylaldehyde (SiA) strongly suppressed LPS/IFNγ-induced NO production. Pretreatment with Whc, CA, and SiA induced heme oxygenase-1 (HO-1) expression. The expression of HO-1 by Whc, CA, and SiA pretreatment was due to activation of Nrf2/ARE signaling via the elevation of intracellular reactive oxygen species. To investigate the in vivo effects of Whc, Whc was administered to mice with antitype II collagen antibody-induced arthritis, and we the arthritis score and hind paw volume were measured. Administration of Whc remarkably suppressed the arthritis score and hind paw volume. Taken together, these findings suggest that Whc is beneficial for the treatment of inflammatory disease.

KEYWORDS: *whiskey congeners, nitric oxide, heme oxygenase-1, Nrf2/ARE signaling, intracellular ROS*

INTRODUCTION

Nitric oxide (NO) is produced by the inducible isoform of NO synthase (iNOS), which catalyzes the oxidation of the equivalent guanidine nitrogen of L-arginine.[1−4] NO is involved in several important biological functions, such as host defense against pathogens, inhibition of tumor cells, and neurotransmission.[5−8] However, excessive NO production is detrimental and can trigger rheumatoid arthritis, gastritis, bowel inflammation, neuronal cell death, and bronchitis.[9,10] Because macrophages express CD14, CD11b/18, Toll-like receptors (TLRs), and type I and type II interferon (IFN) receptors on their cell surfaces, the cells produce large amounts of NO after stimulation with lipopolysaccharide (LPS), IFNs, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β).[11−13] LPS binds to CD14/TLR4, which activates NF-κB, PI3K/Akt, and mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal protein kinase, and p38 MAPK (p38). The activation of these downstream molecules leads to the expression of iNOS, cyclooxygenase-2 (COX-2), TNF-α, and IFNβ.[14,15] IFNβ and INFγ also bind to type I and type II IFN receptors, activate Janus kinase (JAK)-signal transducers and activators of transcription (Stat) signaling, and consequently up-regulate IFN regulatory factor 1 (IRF1).[16] Both IRF1 and Stat1 bind to the 5′-flanking region of the iNOS promoter and enhance NO production. Thus, modulation of LPS/IFNγ signaling can alter iNOS expression and NO production.

Whiskey is matured in oak casks during the manufacturing process. The color, aroma, and flavor of matured whiskey are influenced by nonvolatile substances (whiskey congeners; Whc) exuded from the oak cask. Whc include hundreds of flavor components, such as ellagitannin, vanillin, protocatechuic acid, *p*-hydroxybenzoic acid, sinapylaldehyde (SiA), syringaldeyde, and coniferylaldeyde (CA).[17] We recently reported that Whc and their major components (syringaldeyde, lyoniresinol, and ellagic acid) attenuated IgE-mediated degranulation in rat basophilic leukemia RBL-2H3 cells.[17] Whc have been shown to interfere with FcεRI−IgE binding, inhibit melanogenesis, and protect against ethanol-induced gastric mucosal damage.[18−20]

In this study, we demonstrated the inhibitory effect of Whc on LPS/IFNγ-costimulated NO production in murine macrophage RAW 264 cells and identified the anti-inflammatory compounds of Whc. Furthermore, we have described the mechanism by which Whc inhibit NO production, and we found that Whc intake ameliorated erythema and paw swelling in a rheumatoid arthritis mouse model.

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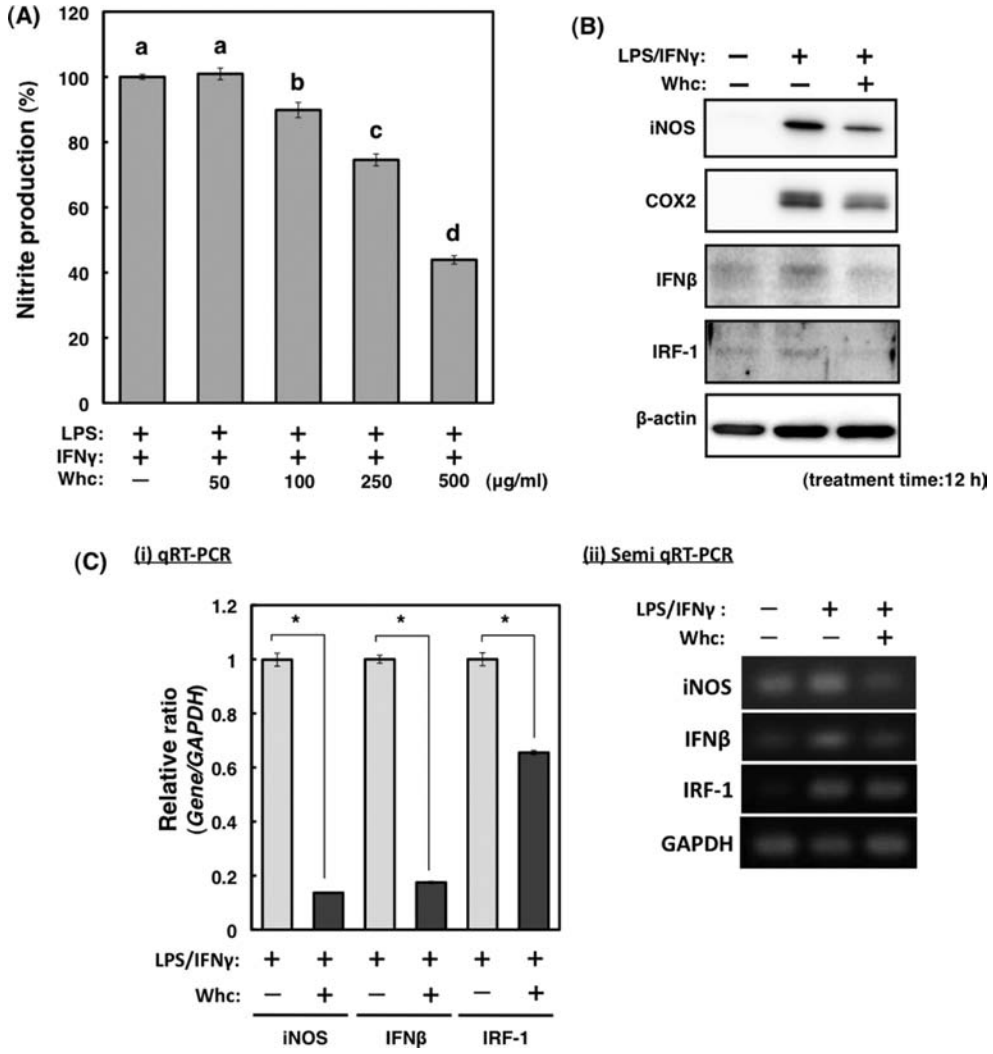

Figure 1. Whiskey congeners (Whc) inhibit nitric oxide production and inflammatory-related gene expressions in LPS/IFNγ-induced RAW 264 cells. (A) RAW 264 cells were pretreated with or without whiskey congeners (50−500 μg/mL) for 2 h. The cells were then stimulated with or without LPS (200 ng/mL)/IFNγ (25 ng/mL) for 24 h. At 24 h later, cell culture media were harvested for measurement of nitrite, a stable metabolite of NO (mean ± SE, n = 9). Statistical significance is considered at $p < 0.01$, as determined by Fisher's multiple-range test. (B) The cell lysates were harvested and subjected to Western blot analysis for iNOS, COX2, IFNβ, IRF-1, and β-actin. A representative blot from three independent experiments is shown. (C) The total RNA of Whc-treated RAW 264 cells was harvested and subjected to quantitative RT-PCR (qRT-PCR, i) and semi-qRT-PCR (ii) for *iNOS*, *IFNβ*, and *IRF-1* (mean ± SD, n = 3).

MATERIALS AND METHODS

Preparation of Whiskey Congeners (Whc) and Isolation of Major Compounds from Whc. In previous study, we described the preparation of Whc and the isolation of major compounds from them.[17] Thus, the Whc (7.91 g) were prepared from whiskey at the age of 12 years using freeze dehydration equipment. The powdered Whc was subjected to column chromatography (Amberlite XAD-2) and eluted with a stepwise gradient with water and methanol. The methanol fraction was further separated by a Sephadex LH-20 column and eluted with methanol, to give fraction 1 (2436 mg), fraction 2 (846 mg), fraction 3 (110 mg), fraction 4 (148 mg), and fraction 5 (41 mg). These fractions were purified by preparative high-performance liquid chromatography, and the chemical structures were identified by ^{1}H and ^{13}C NMR and mass spectrometric analysis.

Reagents. Antibodies against p44/42 MAPK (ERK), phospho-p44/42 MAPK (Thr202/Tyr204) (p-ERK), p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182) (p-p38), SAPK/JNK (JNK), phospho-SAPK/JNK (Thr183/Tyr185) (p-JNK), iNOS, COX2, Stat1, phospho-Stat1, IκBα, NF-κB (p65), and HO-1 were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies to IFNβ, IRF-1, Nrf2, Rac, p22^{phox}, p47^{phox}, and gp91^{phox} were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against β-actin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture and Treatment. Murine macrophage RAW 264 cells were purchased from RIKEN Bio Resource Center (Tsukuba, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 100 U/mL of penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. RAW 264 cells (2×10^5 cells/well) were seeded into a 24-well multiplate and cultured for 12 h. After 12 h of incubation, cells were pretreated with Whc or each isolated compound from Whc for 2 h. The treated cells were then costimulated with or without LPS (final concentration = 200 ng/mL) (Sigma-Aldrich) and IFNγ (final concentration = 25 ng/mL) (Millipore, Bedford, MA, USA).

Measurement of NO Production. Cell culture media were collected and then centrifuged at 1500g and 4 °C for 5 min. A Griess reagent kit (Promega, Madison, WI, USA) was used to measure the amount of nitrite, a stable metabolite of NO, in the supernatants. Briefly, 50 μL of each culture medium was added to a 96-well plate in triplicate, and then the same volume of sulfanilamide solution was dispensed. After incubation at room temperature for 10 min, 50 μL of

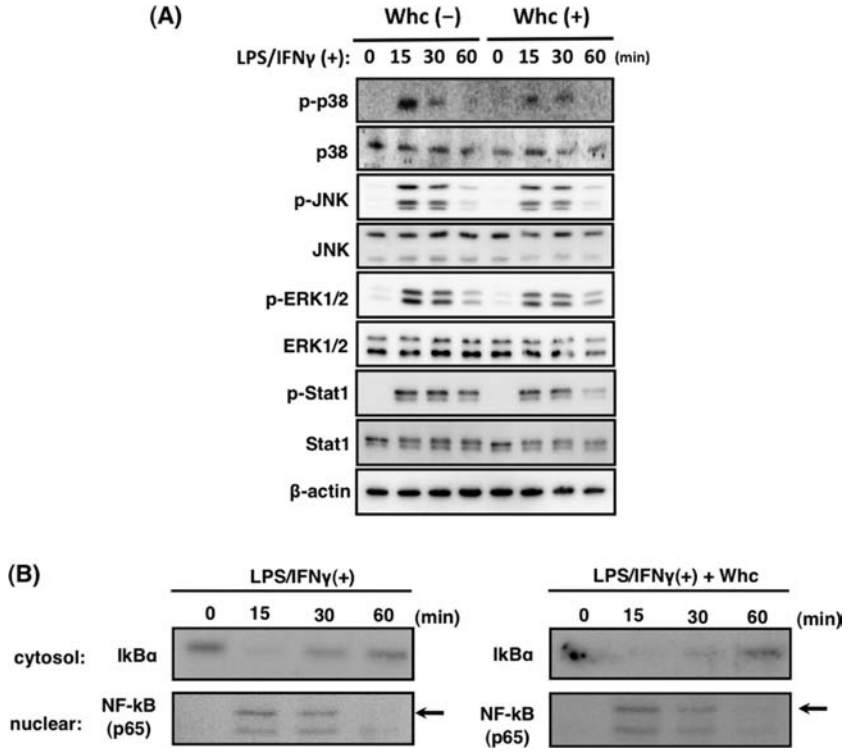

Figure 2. Effect of Whc on LPS/IFNγ-mediated signal transduction in RAW 264 macrophage cells. RAW 264 macrophage cells were incubated for 2 h in the presence or absence of Whc and then treated with or without LPS and IFNγ. (A) The cell lysates were harvested at the indicated time after LPS/IFNγ stimulation and subjected to Western blot analysis for indicated proteins. A representative blot from three independent experiments is shown. (B) After incubation in the presence or absence of Whc for indicated time periods, the cytosolic and nuclear fractions were separated and subjected to Western blot analysis for indicated proteins. A representative blot from three independent experiments is shown.

N-1-naphthylethylenediamine dihydrochloride solution was added to all wells. After incubation at room temperature for 10 min, absorbance was measured at 540 nm using a colorimetric microplate reader (Corona Grating Microplate Reader SH-9000, Tokyo, Japan).

Western Blotting. Whole cell extracts were prepared by lysing cells in RIPA buffer containing a complete protease inhibitor cocktail and a phosphatase inhibitor cocktail (Roche, Penzberg, Germany). Samples were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene difluoride (PVDF) membranes. These PVDF membranes were incubated with a primary antibody, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Immunolabeled proteins were detected using an ECL chemiluminescence kit (GE Healthcare, Piscataway, NJ, USA) and an LAS-4000 luminoimage analyzer (Fuji Film, Tokyo, Japan).

Quantitative Real Time-PCR. Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) followed by DNase I treatment. cDNA was synthesized from 0.25 mg of total RNA using a PrimeScript reagent kit (Takara Bio, Ohtsu, Japan). cDNA was subjected to quantitative RT-PCR using a Thermal Cycler Dice Real-Time PCR system (TP800; Takara Bio). Primers for *iNOS*, *IFNβ*, *IRF-1*, and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) were purchased from Takara Bio. The expression level of each gene was determined using the comparative Ct method and normalized to that of *GAPDH*, which was used as an internal control. The PCR reaction consisted of 45 cycles (95 °C for 10 s and 60 °C for 40 s) after an initial denaturation step (95 °C for 10 min).

Measurement of Intracellular Reactive Oxygen Species (ROS) Levels. Intracellular levels of ROS were determined using a cell-permeable fluorescent probe, CM-H_2DCF-DA (Invitrogen). Cells were incubated with 10 μM CM-H_2DCF-DA for 1 h at 37 °C. After treatment, cells were washed twice with PBS and lysed in RIPA buffer. The absorbance of the lysates was measured with an excitation wavelength of 490 nm and an emission wavelength of 530 nm using the MTP-600 fluorometric imaging plate reader (Corona Electric, Ibaraki, Japan).

Transfection and Luciferase Assay. The antioxidant response element (ARE) reporter gene contained two copies of the ARE enhancer sequences found in the promoter region of glutathione *S*-transferase Ya subunit gene upstream of thymidine kinase minimal promoter that was fused to a firefly luciferase gene (pGL3-ARE-TK-luc, Promega). The firefly luciferase gene *ARE* (2 μg) and pRLSV40 (Promega) were cotransfected into cells plated on 24-well plates by using Lipofectamine2000 (Invitrogen). Luciferase activities were measured 24 h after treatment with Whc, CA, or SiA at the indicated concentrations using a Dual-Glo Luciferase Assay System (Promega).

Antitype II Collagen Antibody-Induced Arthritis in Mice. Inflammatory arthritis was induced using the arthritogenic mouse monoclonal anti-type II collagen 5-clone antibody cocktail (Iwai Chemical, Tokyo, Japan). Seven-week-old mice, which were fed Whc (500 or 1000 mg/kg body weight) for 2 weeks, were injected intravenously with 1 mg of the arthritogenic cocktail. Treatment with Whc continued after the injection. Three days later, 25 μg of LPS (*Escherichia coli* O111:B4) was injected intraperitoneally. Two weeks after the antibody injection, we photographed the hind and front paws, evaluated the arthritis scores. and measured the foot volume of hind paws using a plethysmometer (MK550M, Muromachi-kikai, Tokyo, Japan). The arthritis score was determined by examination of ankles (0, unaffected; 1, erythema; 2, swelling of one hind limb; 3, severe swelling of both hind limbs).

Statistical Analysis. All data were analyzed by one-way analysis of variance (ANOVA) and subsequently by Fisher's multiple-range test among many groups or Student's *t* test between two groups, at a significance level of $p < 0.01$.

RESULTS

Whc Inhibit NO Production and Inflammatory-Related Gene Expression in LPS/IFNγ-Induced RAW

264 Cells. Whc suppressed LPS/IFNγ-induced NO production in a concentration-dependent manner (Figure 1A). We next determined the protein and mRNA expression levels of iNOS, IFNβ, and IRF-1. Protein and mRNA expression levels of all molecules were significantly suppressed by Whc treatment (Figure 1B,C), suggesting that the reduced LPS/IFNγ-stimulated NO production was due to the suppression of iNOS expression.

Whc Inhibit LPS/IFNγ-Mediated Signal Transduction. We further examined the molecular mechanisms underlying the inhibitory effect of Whc on LPS/IFNγ-induced NO production in murine macrophage RAW 264 cells. We initially examined the activation of MAPK (p38, SAPK/JNK, and ERK) and Stat1 following LPS/IFNγ stimulation. The level of p38 MAPK at 15 min after LPS/IFNγ stimulation in RAW 264 cells treated with Whc was dramatically suppressed compared with that in RAW 264 cells without Whc treatment (Figure 2A). Other molecules, such as SAPK/JNK, ERK, and Stat1, were also slightly suppressed by Whc treatment. We then examined the effect of Whc treatment on the translocation of NF-κB to the nucleus. As shown in Figure 2B, the translocation NF-κB in Whc-treated RAW 264 cells was slightly suppressed. These results suggested that the suppression of LPS/IFNγ-induced NO production by Whc was mainly due to the inactivation of stress-related kinases.

Whc Suppress LPS/IFNγ-Induced Intracellular ROS Levels. Upon LPS/IFNγ costimulation in macrophages, ROS are produced via the NADPH oxidase system.[21] Because p38 MAPK and SAPK/JNK are activated by ROS, we measured the levels of intracellular ROS using a ROS-specific fluorescence probe. Whc significantly suppressed LPS/IFNγ-induced ROS production (Figure 3). This finding suggested that the inhibition of LPS/IFNγ-induced NO production by Whc is due to the suppression of oxidative stress-related pathway.

CA and (SiA in Whc Strongly Suppress LPS/IFNγ-Induced NO Production. To determine the active compounds in Whc, we examined the inhibitory effects of 10 major compounds isolated from Whc on LPS/IFNγ-induced NO production in RAW 264 cells. Interestingly, CA and SiA strongly suppressed LPS/IFNγ-induced NO production. Additionally, ellagic acid (Ella) also decreased NO production by 60% (Figure 4A,B). Suzuki et al. reported that CA and SA, isolated from Whc, induce heme oxygenase-1 (HO-1), which is one of the phase II enzymes in human endothelial cells.[22] Similar results were obtained in our study. Several natural

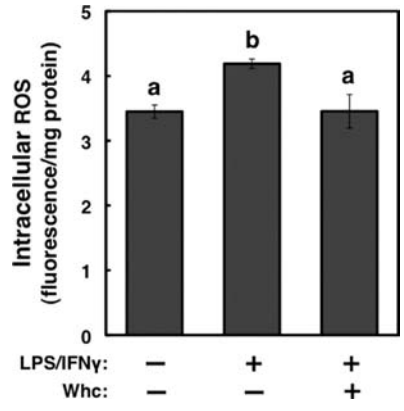

Figure 3. Effect of Whc on the elevation of intracellular ROS levels in LPS/IFNγ-induced RAW 264 cells. The RAW 264 cells pretreated with Whc for 2 h. After pretreatment, the cells were further treated with CM-H_2DCF-DA and incubated for 30 min. Cells were then stimulated with or without LPS/IFNγ, and ROS-mediated DCF oxidation was measured. Values are given as the mean ± SEM (n = 12). Statistical significance is considered at $p < 0.01$, as determined by one-way ANOVA and Fisher's multiple-range test.

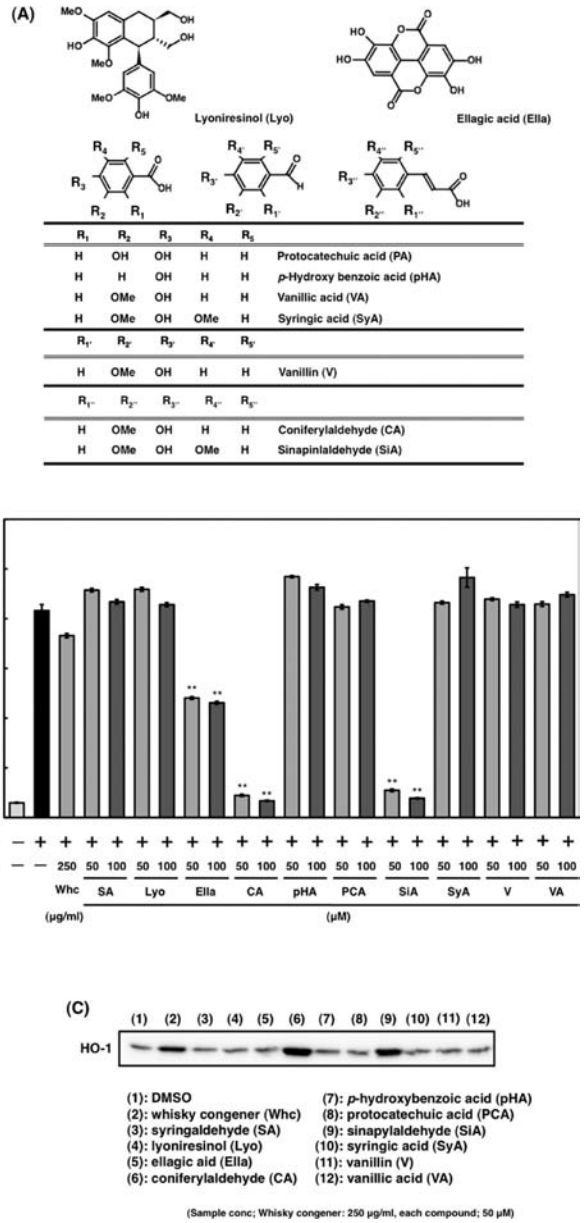

Figure 4. Effects of Whc or Whc major components on LPS/IFNγ-induced NO production and expressions of heme oxygenase-1 (HO-1) in Whc- or Whc major components-pretreated RAW 264 cells. (A) Chemical structures of major substances isolated from Whc. (B) RAW 264 cells were pretreated with or without Whc (250 μg/mL) or Whc major substances (50 or 100 μM) for 2 h. After LPS/IFNγ stimulation for 24 h, cell culture media were harvested for measurement of nitrite, a stable metabolite of NO (mean ± SE, n = 9). Asterisks indicate statistical significance as determined by Student's t test (**, $p < 0.01$, vs only LPS/IFNγ-stimulated RAW 264 cells). (C) After incubation in the presence or absence of Whc or Whc major substances for 2 h, the cell lysates were harvested and subjected to Western blot analysis for HO-1. A representative blot from three independent experiments is shown.

extracts suppress LPS-induced NO production in RAW 264 cells through HO-1 induction.[23−25] We also examined HO-1 expression in RAW 264 cells treated with each compound for 2 h and found that Whc, CA, and SiA remarkably induced HO-1 expression (Figure 4C). HO-1 protein expression in Whc-, CA-, or SiA-treated RAW 264 cells dramatically increased at 2 h before incubation (Supporting Information). On the other hand, pretreatment with Ella did not induce HO-1 expression in RAW 264 cells. By using Zn-protoporphyrin (ZnPP), a specific inhibitor of HO-1, we further examined whether HO-1 expression was involved in the inhibitory effect of Whc, CA, or SiA on LPS/IFNγ-induced NO production. NO production in ZnPP+CA- and ZnPP+SiA-treated RAW 264 cells was higher than that in CA- or SiA-treated RAW 264 cells (Figure 5).

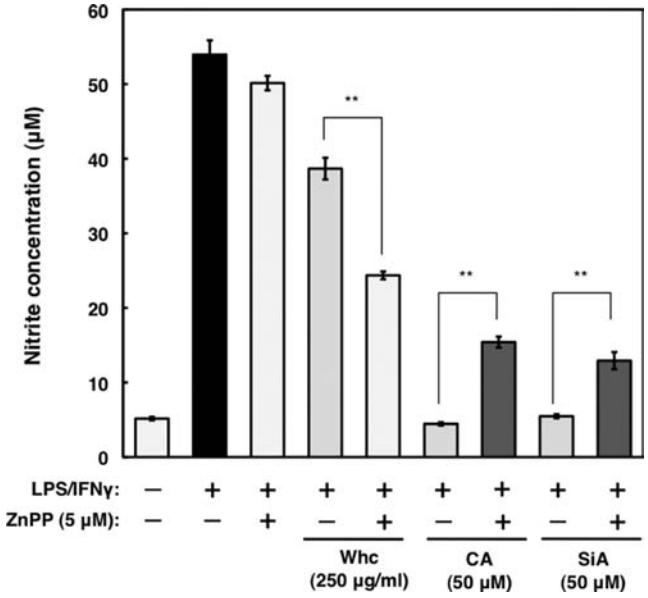

Figure 5. Effect of HO-1 inhibitor on LPS/IFNγ-induced NO production in Whc-, CA-, or SiA-treated RAW 264 cells. RAW 264 macrophage cells were incubated for 2 h in the presence or absence of ZnPP (10 μM). After incubation, the cells were further pretreated with or without Whc (250 μg/mL) or CA or SiA (50 μM) for 2 h. After each treatment, the cells were stimulated with LPS/IFNγ and then incubated for 24 h. The cell culture media were harvested for measurement of nitrite, a stable metabolite of NO (mean ± SE, $n = 9$). Asterisks indicate statistical significance as determined by Student's t test (**, $p < 0.01$).

However, ZnPP treatment did not restore completely the inhibitory effect of CA or SiA on LPS-stimulated NO production. The levels of NO production in 10 or 20 μM CA and SiA treatment in the presence of ZnPP increased (data not shown). It was thus presumed that the inhibitory effects of CA or SiA on LPS/IFNγ-induced NO production were very strong at the examined concentration (50 μM). Conversely, LPS/IFNγ-induced NO production in Whc+ZnPP-treated RAW 264 cells was not elevated. Probably, the result occurred through non-HO-1-inducing mechanisms, such as the inhibitory effect of Ella in Whc.

Pretreatment with Whc, CA, or SiA Activates the Keap1/Nfr2/ARE Pathway. HO-1 expression regulates the Nrf2/ARE pathway;[26−30] hence, we next examined ARE enhancer activation by Whc, CA, or SiA using an ARE reporter gene. As shown in Figure 6A, Whc, CA, and SiA significantly increased ARE enhancer activity. Moreover, the translocation of

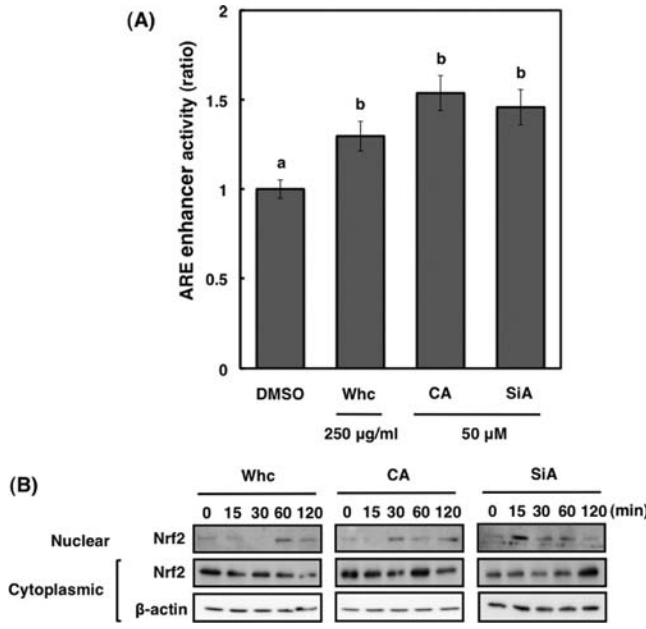

Figure 6. Effects of Whc, CA, and SA pretreatment on Nrf2/ARE activation and nuclear translocation of Nrf2 in RAW 267 cells. (A) RAW 264 cells transfected with the ARE firefly luciferase reporter vector and Renilla luciferase control vector were treated with Whc (250 μg/mL) or CA or SiA (50 μM) for 24 h, followed by luciferase assay. The firefly luciferase activity was normalized to the Renilla luciferase activity. The ARE enhancer activity is shown as the ratio to the DMSO control. Data are expressed as the mean ± SEM ($n = 6$). Statistical significance is considered at $p < 0.01$, as determined by one-way ANOVA and Fisher's multiple-range test. (B) At indicated times after treatment with Whc, CA, or SiA (50 μM), nuclear and cytoplasmic fractions were separated from each RAW 264 cell by using the NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Waltman, MA, USA). These fractions were subjected to Nrf2. Western blot analysis was used for HO-1 and β-actin. A representative blot from three independent experiments is shown.

Nrf2 to the nucleus was also observed after pretreatment with Whc, CA, or SiA (Figure 6B). Then, we measured the levels of intracellular ROS in Whc-, CA-, or SiA-pretreated RAW 264 cells. All treatments facilitated intracellular ROS production (Figure 7A). NADPH oxidase is an enzyme complex composed of membrane-bound subunits ($gp91^{phox}$ and $p22^{phox}$), cytosolic subunits ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$), and a monomeric GTP-binding protein of the Rho family (Rac2).[31,32] To activate phagocytic NADPH oxidase, the cytosolic subunits are translocated to the membrane, thus initiating the production of superoxide.[31,32] As shown in Figure 7B, Whc, CA, and SiA pretreatment enhanced the translocation of the cytosolic subunits ($p47^{phox}$ and Rac2) of NADPH oxidase to the plasma membrane. The reduction of intracellular ROS by *N*-acetylcysteine (NAC) in Whc-, CA-, or SiA-pretreated RAW 264 cells was significantly suppressed by HO-1 expression (Figure 7C). From these results, we concluded that HO-1 expression in Whc-, CA-, or SiA-pretreated RAW 264 cells regulates intracellular ROS.

Pretreatment with Whc, CA, or SiA Activates MAPKs and PI3K/Akt. Intracellular ROS is one of the molecules upstream of MAP kinases and PI3K/Akt.[33,34] Phosphorylated p38 MAPK and JNK were detected 15 min after Whc and SiA treatment (Figure 8A). In Whc-pretreated cells, Akt was more

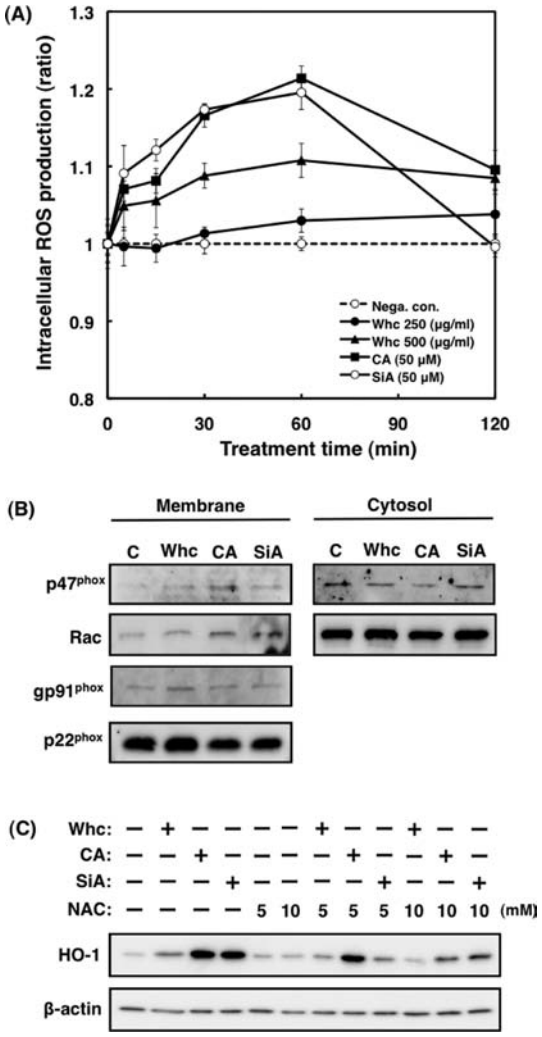

Figure 7. Effects of Whc, CA, or SiA pretreatment on the intracellular ROS production in RAW 264 cells. (A) At indicated times after treatment with Whc (250 and 500 μg/mL) or CA or SiA (50 μM), RAW 264 cells were subjected to measurement for intracellular ROS levels using the fluorescent probe CH−H_2DCFDA. The intracellular ROS levels are shown as a ratio to the initiation level. Data are expressed as the mean ± SEM (n = 10). (B) The cytosolic and membrane fractions were separated by using a ProteoExtract subcellular proteome extraction kit (Merck KGaA, Darmstdt, Germany). Whc, CA, SiA pretreament enhanced the translocation of cytosolic subunits of NADPH oxidase (p47^{phox} and Rac) to the membrane. A representative blot from three independent experiments is shown. (C) RAW 264 cells were treated for 1 h with or without NAC (5 or 10 mM) and then treated for 2 h with Whc (250 μg/mL) or CA or SiA (50 μM). Cell lysates were prepared and subjected to Western blot analysis for HO-1 and β-actin. A representative blot from three independent experiments is shown.

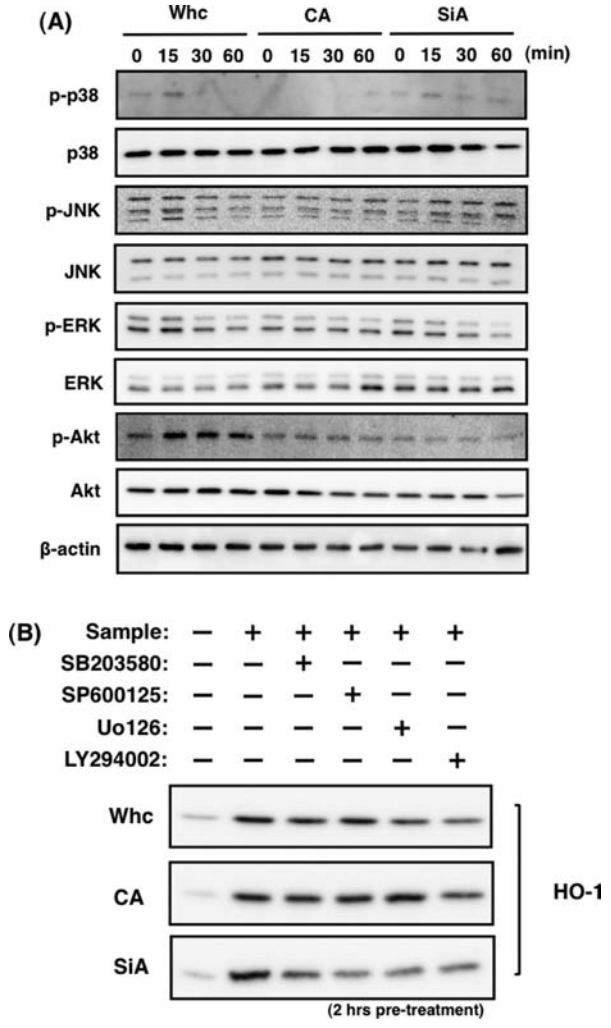

Figure 8. Effects of Whc, CA, or SiA pretreatment on signal transuduction in RAW 264 cells. (A) At indicated times after pretreatment with Whc (250 and 500 μg/mL) or CA or SiA (50 μM) cell lysates were prepared from RAW 264 cells and subjected to Western blot analysis for phosphorylated and total p38, JNK, and ERK proteins. A representative blot from three independent experiments is shown. (B) RAW 264 cells were pretreated for 1 h with either SB203580 (5 μM), SP600125 (10 μM), Uo126 (1 μM), or PD98059 (10 μM) and then treated with Whc, CA, or SiA for 2 h. Cell lysate was prepared and subjected to Western blot analysis for HO-1 and β-actin. A representative blot from three independent experiments is shown.

strongly phosphorylated than in CA- or SiA-pretreated cells. Changes in phosphorylated ERK after each treatment were not clearly detectable. The activation of MAPKs and Akt was abolished by NAC treatment (data not shown). Furthermore, inhibitors of MAPKs and Akt down-regulated Whc-, CA-, or SiA-induced HO-1 expression (Figure 8B). Thus, intracellular ROS acts upstream of HO-1 expression through the activation of MAPKs and Akt.

Oral Intake of Whc Attenuates Anti-type II Collagen Antibody-Induced Arthritis in Mice. The arthritis score gradually decreased upon Whc intake in a dose-dependent manner (Figure 9A). A shown in Figure 9B, hind and front paws swelled after LPS injection in arthritogenic cocktail-treated mice. However, swelling and erythema were ameliorated by Whc feeding. The volume of swelling was also measured using a plethysmometer. Whc feeding significantly decreased hind paw volume compared with that of control mice (Figure 9C).

■ DISCUSSION

In the present study, Nrf2 distinctly translocated to the nucleus after Whc, CA, and SiA pretreatment, thereby promoting ARE enhancer activation and HO-1 expression. The suppression of LPS/IFNγ-stimulated NO production by Whc, CA, or SiA was also clearly abolished by ZnPP treatment. This result suggested

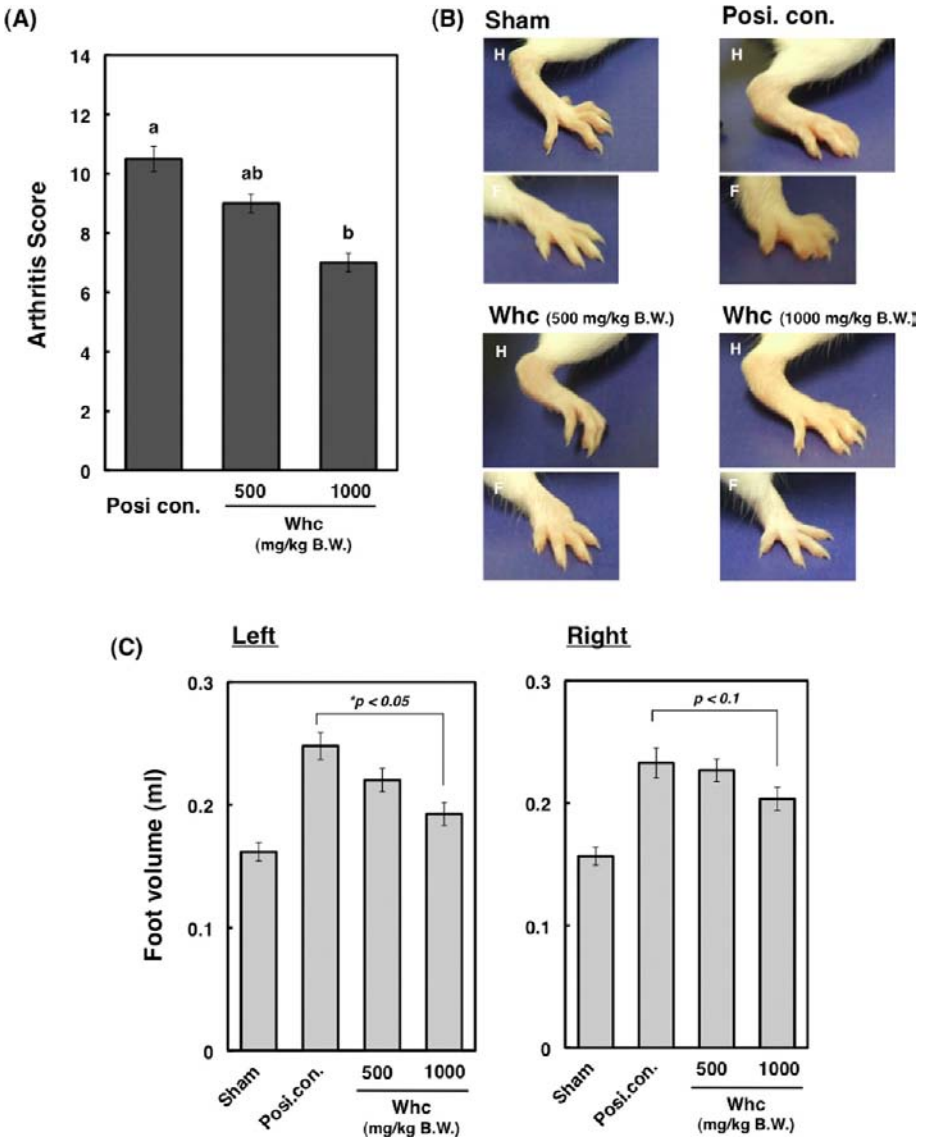

Figure 9. Effects of oral intake of Whc on antitype II collagen antibody-induced arthritis in mice. BALB/c Cr Slc female mice were fed with or without Whc (500 and 1000 mg/kg body weight) throughout the experiments. After treatment with or without Whc for 2 weeks, mice were injected intravenously with 1 mg of the arthritogenic mouse monoclonal antitype II collagen antibody cocktail. Three days later, 25 μg of LPS was injected intraperitoneally. (A, B) Two weeks after the antibody injection, photographs of the hind and front paws were taken, and the arthritis score was evaluated. Statistical significance is considered at $p < 0.01$, as determined by one-way ANOVA and Fisher's multiple-range test. (C) The foot volume of hind paws was measured using a plethysmometer. Values are expressed as the mean ± SD ($n = 5$). Asterisks indicate statistical significance as determined by Student's t test ($*$, $p < 0.05$).

that Whc, CA, and SiA inhibit LPS/IFNγ-induced inflammation through activation of the Nrf2/ARE pathway (Figure 10). ROS modifies sulfhydryl groups of kelch-like ECH-associated protein 1 (Keap1), thereby activating Nrf2/ARE signaling.[35,36] Under normal conditions, Nrf2 binds with Keap1 and then undergoes proteasome-dependent degradation. Keap1 has cystein thiol groups that work as sensors for electrophiles or ROS. When cystein thiol groups in Keap1 are modified by electrophiles or ROS, Keap1 alters its conformation and releases Nrf2, which is then translocated to the nucleus. Nrf2 binds to and activates the ARE enhancer present within the promoter region of genes encoding for phase II enzymes, such as glutathione peroxidase, γ-glutamylcysteine synthetase, HO-1, NADPH quinone oxidoreductase, and glutathione *S*-transferase. Because intracellular ROS levels were transiently elevated by Whc, CA, and SiA pretreatments in our experiment, the release and translocation of Nrf2 to the nucleus were presumably triggered by ROS. To determine whether ROS production by pretreatment with Whc, CA, and SiA plays a critical role in Nrf2/ARE signaling, we examined the effect of HO-1 expression by using an antioxidant, NAC. NAC treatment in Whc-, CA-, or SiA-pretreated RAW 264 cells significantly down-regulated the expression of HO-1 protein and the activation of MAPKs and PI3K/Akt. Moreover, the expression of HO-1 protein in Whc-, CA-, or SiA-pretreated RAW 264 cells was also abolished by MAPKs or PI3K/Akt inhibitors. Many reports show that protein kinases, such as MAPKs and PI3K/Akt phosphorylate ,released Nrf2 and consequently showed an anti-inflammatory effect. These results suggested that elevation of intracellular ROS is an upstream signal of the modification of Keap1 and MAPKs and PI3K activation for the activation of Nrf2/ARE signaling.

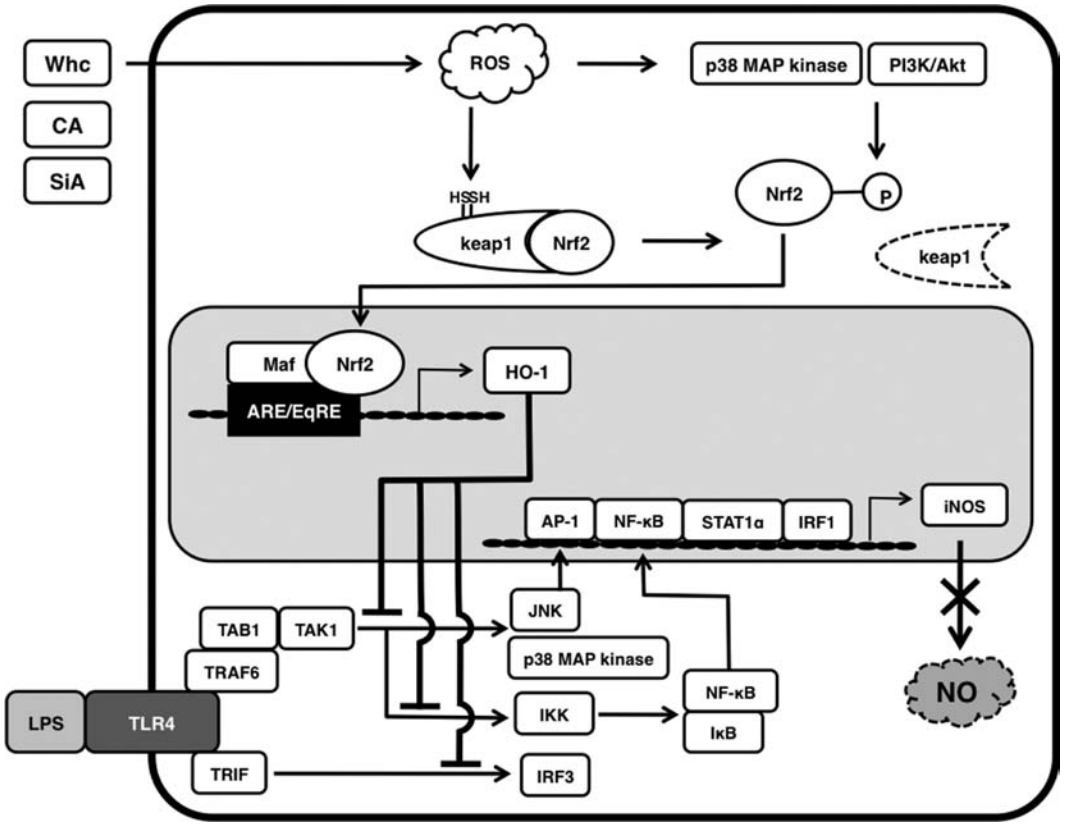

Figure 10. Schematic representation of mechanisms underlying anti-inflammatory effects by Whc, CA, and SiA through Nrf2/ARE activation. Whc, CA, and SiA up-regulate intracellular ROS production and the activation of p38 MAPkinase and PI3K/Akt. The resultant Nrf2/ARE activation induces expression of HO-1 and augments the antioxidant ability in the cells, resulting in suppression to LPS/IFNγ-induced inflammatory responses.

HO-1 is a rate-limiting catabolic enzyme involved in heme degradation, in which cabon monoxide (CO), ferrous iron, biliverdin, and bilirubin are produced as byproducts.[37−39] These byproducts could contribute to anti-inflammatory effects as described here. Wang et al. reported that CO attenuates TLR4 signaling by increasing the interaction of caveolin-1 with TLR4.[40] Sarady-Andrews et al. showed that biliverdin administration to LPS-macrophage inhibits the expression of iNOS.[41] Additionally, bilirubin has an inhibitory effect on iNOS expression.[42] Biliverdin is rapidly converted to bilirubin by biliverdin reductase; therefore, the inhibitory effect of biliverdin on iNOS expression may be due to converted bilirubin. Furthermore, Taille et al. reported that HO-1 induction inhibited NADPH oxidase activity.[43] Upon LPS/IFNγ stimulation in RAW 264 cells, NADPH oxidase produces intracellular ROS, which in turn promote iNOS expression.[21] In the present study, the elevation of intracellular ROS after LPS/IFNγ stimulation in Whc-treated RAW 264 cells significantly reduced compared with that after LPS/IFNγ-stimulated RAW 264 cells not treated with Whc. It was thus presumed that the reduction of intracellular ROS level in Whc-treated RAW 264 cells after LPS/IFNγ stimulation might be due to an augment of antioxidant contents in cells through the induction of HO-1. Moreover, Ella, a component of Whc, also decreased NO production by 60%. Ella has antioxidative activity;[44] hence, it also contributes to the reduction of intracellular ROS level in Whc-treated RAW 264 cells after LPS/IFNγ stimulation. The fact that the levels of intracellular ROS production and HO-1 expression in Whc-pretreated RAW 264 cells were lower than those in CA- or SiA-pretreated RAW 264 cells clearly reflects that Ella acts as an antioxidant in Whc-pretreated RAW 264 cells. These results indicate that Ella reduces intracellular ROS production after LPS/IFNγ stimulation that is inflammatory inducing molecules rather than the activation of Nrf2/ARE signaling.

In conclusion, the activation of Nrf2/ARE signaling and the induction of HO-1 expression by pretreatment with Whc, CA, and SiA in RAW 264 cells were regulated by two distinct mechanisms via the elevation of intracellular ROS. First, Whc, CA, and SiA pretreatment in RAW 264 cells modified sulfhydryl groups of Keap1 through NADPH oxidase-mediated ROS production. Second, Whc, CA, and SiA pretreatment increased the phosphorylation of p38 MAPK and PI3K/Akt. This result strongly suggests that ROS are important molecules for the activation of Nrf2/ARE signaling in Whc-, CA-, and SiA-pretreated RAW 264 cells. Preconditioning by Whc, CA, and SiA led to the production of antioxidant components through Nrf2/ARE signaling, enabling cells to adapt to inflammatory signaling after LPS/IFNγ costimulation. Although only approximately 2 ppm CA and SiA are contained in Whc, our in vitro and in vivo data suggest that Whc, CA, and SiA could be effective agents for inflammatory diseases.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figure. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: 81-742-43-6708. Fax: 81-742-43-1316. E-mail: titoh@nara.kindai.ac.jp.

Notes

The authors declare no competing financial interest.

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