JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Anti-inflammatory Activities of 6β-Acetoxy-7α-hydroxyroyleanone from *Taiwania cryptomerioides* Hayata ex Vivo and in Vivo

Guan-Jhong Huang,[†] Jeng-Shyan Deng,[‡] Shyh-Shyun Huang,[†] Chi-I Chang,[▽] Tien-Ning Chang,[†] Pei-Hsin Shie,[†] and Yueh-Hsiung Kuo*[,§,⊥,⊗]

[†]School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Pharmacy, China Medical University, Taichung, Taiwan

[‡]Department of Health and Nutrition Biotechnology, Asia University, Taichung 413, Taiwan

[▽]Graduate Institute of Biotechnology, National Pingtung University of Science and Technology, Pingtung 912, Taiwan

[§]Department of Chemistry, National Taiwan University, Taipei, Taiwan

[⊥]Agricultural Biotechnology Research Center, Academic Sinica, Taipei, Taiwan

[⊗]Tsuzuki Institute for Traditional Medicine, China Medical University, Taichung, Taiwan

ABSTRACT: Excess production of nitric oxide (NO) by inducible NO synthase (iNOS) in activated macrophages is linked to acute and chronic inflammation. Thus, it would be valuable to develop inhibitors of NO production and/or iNOS for potential therapeutic use. This study investigated the anti-inflammatory effects of 6β-acetoxy-7α-hydroxyroyleanone (AHR), a compound isolated from the bark of *Taiwania cryptomerioides* Hayata, using lipopolysaccharide (LPS)-stimulated mouse macrophage (RAW 264.7) ex vivo and carrageenan (Carr)-induced mouse paw edema model in vivo. When RAW 264.7 macrophages were treated with different concentrations of AHR (0, 0.31, 0.62, 1.25, and 2.50 μg/mL) together with LPS (100 ng/mL), a significant concentration-dependent inhibition of NO production was detected. Western blotting revealed that AHR blocked protein expression of iNOS and cyclooxygenase-2 (COX-2) in LPS-stimulated RAW 264.7 macrophages, significantly. In the anti-inflammatory test, AHR (1.25 and 2.50 mg/kg) decreased paw edema at 4 and 5 h after λ-carrageenan (Carr) administration and increased the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in the liver tissue. It was also demonstrated that AHR significantly attenuated the malondialdehyde (MDA) level in the edema paw at 5 h after Carr injection. AHR (0.62, 1.25, and 2.50 mg/kg) decreased the NO levels on both edema paw and serum at 5 h after Carr injection. Also, AHR diminished the serum tumor necrosis factor (TNF-α) at 5 h after Carr injection. Western blotting revealed that AHR (2.50 mg/kg) decreased Carr-induced iNOS and COX-2 expressions at 5 h in the edema paw. An intraperitoneal (ip) injection treatment with AHR also diminished neutrophil infiltration into sites of inflammation, as did indomethacin (Indo). The anti-inflammatory activities of AHR might be related to the decrease in the levels of MDA, iNOS, and COX-2 in the edema paw and to the increase in the activities of CAT, SOD, and GPx in the liver through the suppression of TNF-α and NO.

KEYWORDS: 6β-acetoxy-7α-hydroxyroyleanone, anti-inflammation, NO, TNF-α

INTRODUCTION

Taiwania cryptomerioides Hayata (Taxodiaceae) is an endemic plant and also an important building material. It grows at elevations from 1800 to 2600 m in the central mountains in Taiwan. The heartwood of *T. cryptomerioides* is yellowish-red with distinct purplish-pink streaks. In our previous investigation, various sesquiterpenes, lignans, abietane-type diterpenes, and bisflavones were analyzed from the heartwood,[1] bark,[2] and leaves[3] of this plant. 6β-Acetoxy-7α-hydroxyroyleanone (AHR), a diterpenoid compound, was isolated from the bark of *T. cryptomerioides*.[4] AHR processing of abietane-type diterpenes showed antioxidant, antibacterial, and anti-inflammatory activities.[5] AHR also shows free radical scavenging activity[6] and blocks Kv1.2 channels on C-type inactivation.[7] However, little information is available on the anti-inflammatory effects of AHR.

Inflammation has been shown to be associated with a number of chronic diseases, including asthma, rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, and Alzheimer's disease, and it plays a role in various human cancers. One of the major factors involved in the inflammation response is inducible nitric oxide synthase (iNOS), which is induced by lipopolysaccharide (LPS) and various inflammatory mediator cytokines such as interferons, interleukins, and tumor necrosis factor (TNF)-α.[8] Papers also have reported that the inflammatory effect induced by λ-carrageenan (Carr) could be associated with free radical formation. Free radicals, prostaglandin, and NO will be released when Carr is administered for 1−5 h. The edema effect was raised to maximum at the third hour, and its malondialdehyde (MDA) production was due to free radical attack on the plasma membrane.[9] Thus, the inflammatory effect would result in the accumulation of MDA. Therefore, in this paper we examined the anti-inflammatory effects of AHR on LPS-induced RAW 264.7 cells and Carr-induced paw edema in mice. We detected the

Received: February 9, 2011
Revised: August 9, 2011
Accepted: August 10, 2011
Published: August 10, 2011

levels of iNOS and COX-2 in either RAW 264.7 cells or paw edema. Also, the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in the liver at 5 h after Carr injection were investigated to understand the relationship between the anti-inflammatory mechanism of the AHR and antioxidant enzymes.

MATERIALS AND METHODS

Chemicals. LPS (endotoxin from *Escherichia coli*, serotype 0127:B8), Carr, indomethacin (Indo), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). TNF-α was purchased from Biosource International Inc. (Camarillo, CA). Anti-iNOS, anti-COX-2, and anti-β-actin antibodies (Santa Cruz, CA) and a protein assay kit (Bio-Rad Laboratories Ltd., Watford, Herts, U.K.) were obtained as indicated. Poly(vinylidene fluoride) membrane (Immobilon-P) was obtained from Millipore Corp. (Bedford, MA).

Plant Material. The bark of *T. cryptomerioides* was collected in Tai Chung, Taiwan. The plant material was identified by Muh-Tsuen Gun, formerly a technician of the Department of Botany, National Taiwan University. A voucher specimen has been deposited at the Herbarium of the Department of Botany, National Taiwan University, Taipei, Taiwan.

Isolation and Determination of the Active Compound. Air-dried pieces of *T. cryptomerioides* bark (12 kg) were extracted three times with acetone (12 L) at room temperature (7 days each time). The acetone extract was evaporated in vacuo to leave a black residue, which was suspended in H_2O and then partitioned (three times) with 11 L of ethyl acetate (EtOAc). The EtOAc fraction (360 g) was chromatographed on silica gel using *n*-hexane and EtOAc of increasing polarity as eluent and further purified by high-performance liquid chromatography eluting with *n*-hexane/EtOAc (25:75). AHR (Figure 1A) was eluted with 10% EtOAc in hexane and recrystalled with EtOH.[4]

AHR: yellow solid; mp 182−183 °C; $[\alpha]^{22}_{D} = -51.5°$ (*c* 0.8, $CHCl_3$); IR, ν_{max} 3395, 1745, 1725, 1650, 1620, 1600, 1250 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz), δ 1.01, 1.03 (each 3H, s, H-18, 19), 1.22 (6H, d, *J* = 7.2 Hz, H-16, 17), 1.56 (3H, s, H-20), 2.01 (3H, s, $-OCOCH_3$), 2.62 (1H, br d, *J* = 12.8 Hz, H-1$_\beta$), 3.15 (1H, sept, *J* = 7.2 Hz, H-15), 4.48 (1H, br s, H-6), 5.50 (1H, br s, H-7), 7.25 (1H, s, 12-OH); EIMS (70 eV), *m*/*z* (rel intensity %) 390 $[M]^+$ (6), 330 (100), 315 (63), 261 (38), 248 (56), 232 (30).

Animals. Imprinting control region (ICR; 6−8-week-old males) mice were obtained from the BioLASCO Taiwan Co., Ltd. The animals were kept in plexiglass cages at a constant temperature of 22 ± 1 °C and a relative humidity of 55 ± 5% with a 12 h dark−light cycle for at least 2 weeks before the experiment. They were given food and water ad libitum. All experimental procedures were performed according to the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*. In addition, all tests were conducted under the guidelines of the International Association for the Study of Pain.[10]

After a 2 week adaptation period, male ICR mice (18−25 g) were randomly assigned to four groups (*n* = 6) of animals in the study. The control group received normal saline (intraperitoneal, ip). The other three groups include a Carr-treated, a positive control (Carr + Indo), and an AHR-administered group (Carr + AHR).

Cell Culture. A murine macrophage cell line RAW 264.7 (BCRC 60001) was purchased from the Bioresources Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in plastic dishes containing Dulbecco's Modified Eagle Medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma) in a CO_2 incubator (5% CO_2 in air) at 37 °C and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin−0.02% EDTA in Ca^{2+},Mg^{2+}-free phosphate-buffered saline (DPBS).

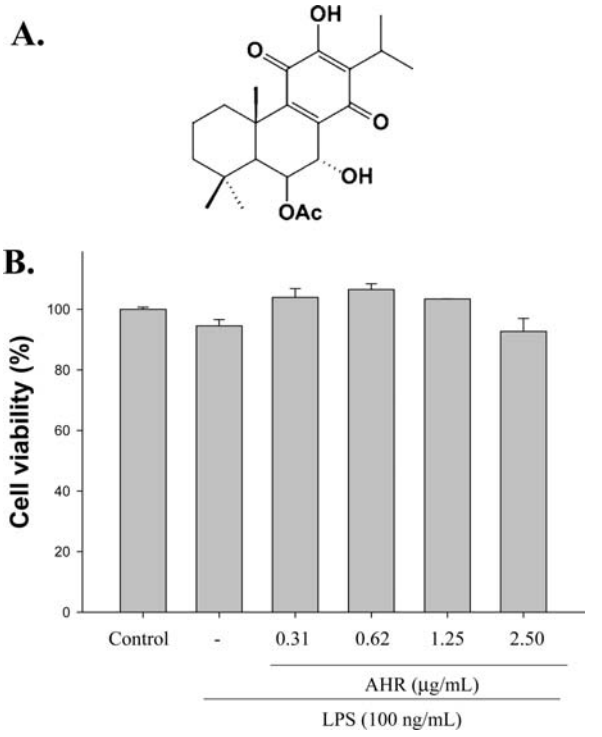

Figure 1. Chemical structure of 6β-acetoxy-7α-hydroxyroyleanone (AHR) (A) and cytotoxic effects of AHR in RAW 264.7 cells (B). Cells were incubated for 24 h with 100 ng/mL of lipopolysaccharide (LPS) in the absence or presence of AHR (0, 0.31, 0.62, 1.25, and 2.50 μg/mL). AHR was added 1 h before incubation with LPS. Cell viability was evaluated using the MTT assay. The data are presented as the mean ± SD for three different experiments performed in triplicate.

Cell Viability. Cells (2×10^5) were cultured in a 96-well plate containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultured with AHR in the presence of 100 ng/mL LPS for 24 h. After that, the cells were washed twice with DPBS and incubated with 100 μL of 0.5 mg/mL MTT for 2 h at 37 °C, testing for cell viability. The medium was then discarded, and 100 μL of dimethyl sulfoxide (DMSO) was added. After 30 min of incubation, absorbance at 570 nm was read using a microplate reader (Molecular Devices, Sunnyvale, CA).

Measurement of Nitric Oxide/Nitrite. NO production was indirectly assessed by measuring the nitrite levels in the cultured media and serum determined by a colorimetric method based on the Griess reaction.[11] The cells were incubated with AHR (0, 0.31, 0.62, 1.25, and 2.50 μg/mL) in the presence of LPS (100 ng/mL) at 37 °C for 24 h. Then, cells were dispensed into 96-well plates, and 100 μL of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 5% phosphoric acid) and incubated at room temperature for 10 min; the absorbance was measured at 540 nm with a Micro-Reader (Molecular Devices). Serum samples were diluted four times with distilled water and deproteinized by adding $^1/_{20}$ volume of zinc sulfate (300 g/L) to a final concentration of 15 g/L. After centrifugation at 10000*g* for 5 min at room temperature, 100 μL of supernatant was applied to a microtiter plate well, followed by 100 μL of Griess reagent. After 10 min of color development at room temperature, the absorbance was measured at 540 nm with a Micro-Reader. By using sodium nitrite to generate a standard curve, the concentration of nitrite was measured from the absorbance at 540 nm.

Carr-Induced Edema. The Carr-induced hind paw edema model was used for determination of anti-inflammatory activity.[11] Animals were

ip-treated with AHR (0.62, 1.25, and 2.50 mg/kg), Indo, or normal saline, 30 min prior to injection of 1% Carr (50 μL) in the plantar side of the right hind paws of the mice. The paw volume was measured immediately after Carr injection and at 1, 2, 3, 4, and 5 h intervals after the administration of the edematogenic agent using a plethysmometer (model 7159, Ugo Basile, Varese, Italy). The degree of swelling induced was evaluated by the ratio a/b, where a is the volume of the right hind paw after Carr treatment and b is the volume of the right hind paw before Carr treatment. Indo was used as a positive control. After 5 h, the animals were sacrificed and the Carr-induced edema feet were dissected and stored at −80 °C. Also, blood was withdrawn and kept at −80 °C.

In the secondary experiment, the right hind paw tissue and liver tissue were taken at 5 h. The right hind paw tissue was rinsed in ice-cold normal saline and immediately placed in cold normal saline 4 times its volume and homogenized at 4 °C. Then the homogenate was centrifuged at 12000g for 5 min. The supernatant was obtained and stored at −20 °C for MDA assays. The whole liver tissue was rinsed in ice-cold normal saline and immediately placed in cold normal saline 1 times its volume and homogenized at 4 °C. Then the homogenate was centrifuged at 12000g for 5 min. The supernatant was obtained and stored at −20 °C for the antioxidant enzymes (CAT, SOD, and GPx) activity assays.

Total Protein Assay. The protein concentration of the sample was determined according to the Bradford dye-binding assay (Bio-Rad, Hercules, CA).

MDA Assay. MDA from the Carr-induced edema foot was evaluated by using the thiobarbituric acid reacting substances (TBARS) method.[11] Briefly, MDA reacted with thiobarbituric acid in the acidic high temperature and formed a red TBARS complex. The absorbance of TBARS was determined at 532 nm.

Measurement of Serum TNF-α by an Enzyme-Linked Immunosorbent Assay (ELISA). Serum levels of TNF-α were determined using a commercially available ELISA kit (Biosource International Inc., Camarillo, CA) according to the manufacturer's instruction. TNF-α was determined from a standard curve. The concentrations were expressed as picograms per milliliter.

Antioxidant Enzyme Activity Measurements. The following biochemical parameters were analyzed to check the hepatoprotective activity of AHR by the methods given below.

Total SOD activity was determined by the inhibition of cytochrome c reduction.[12] The reduction of cytochrome c was mediated by superoxide anions generated by the xanthine/xanthine oxidase system and monitored at 550 nm. One unit of SOD was defined as the amount of enzyme required to inhibit the rate of cytochrome c reduction by 50%. Total CAT activity was based on that of Aebi.[13] In brief, the reduction of 10 mM H_2O_2 in 20 mM phosphate buffer (pH 7) was monitored by measuring the absorbance at 240 nm. The activity was calculated using a molar absorption coefficient, and the enzyme activity was defined as nanomoles of dissipating hydrogen peroxide per milligram of protein per minute. Total GPx activity in cytosol was determined according to Paglia and Valentine's method.[14] The enzyme solution was added to a mixture containing hydrogen peroxide and glutathione in 0.1 mM Tris buffer (pH 7.2), and the absorbance at 340 nm was measured. The activity was calculated by using a calibration curve of GPx established from bovine whole blood. A linear relationship between the activity (units per milliliter) of GPx and the reduction of NADPH absorbance at 340 nm was found, and the enzyme activity was defined as nanomoles of NADPH oxidized per milligram of protein per minute.

Protein Lysate Preparation and Western Blot Analysis of iNOS and COX-2. Stimulated murine macrophage cell line RAW 264.7 cells were washed with PBS and lysed in an ice-cold lysis buffer [10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM EGTA, 10 mM NaF, 1 mM $Na_4P_2O_7$, 20 mM Tris buffer (pH 7.9), 100 mM β-glycerophosphate, 137 mM NaCl, 5 mM EDTA, and one protease inhibitor cocktail tablet (Roche, Indianapolis, IN)] on ice for 1 h, followed by centrifugation at 12000g for 30 min at 4 °C. Soft tissues were removed from individual mouse paws and homogenized in a solution containing 10 mM CHAPS, 1 mM phenylmethanesulfonyl fluoride (PMSF), 5 μg/mL, aprotinin, 1 μM pepstatin, and 10 μM leupeptin. The homogenates were centrifuged at 12000g for 20 min, and 30 μg of protein from the supernatants was then separated on 10% sodium dodecyl sulfate−polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. After transfer, the membrane was blocked for 2 h at room temperature with 5% skim milk in Tris-buffered saline−Tween (TBST; 20 mM Tris, 500 mM NaCl, pH 7.5, 0.1% Tween 20). The membranes were then incubated with mouse monoclonal anti-iNOS or anti-COX-2 antibody in 5% skim milk in TBST for 2 h at room temperature. The membranes were washed three times with TBST at room temperature and then incubated with a 1:2000 dilution of anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (Sigma, St. Louis, MO) in 2.5% skim milk in TBST for 1 h at room temperature. The membranes were washed three times, and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) using hyperfilm and ECL reagent (Amersham International plc., Buckinghamshire, U.K.). The results of Western blot analysis were quantified by measuring the relative intensity compared to the control using Kodak Molecular Imaging Software (version 4.0.5, Eastman Kodak Co., Rochester, NY) and represented in relative intensities.

Histological Examination. For histological examination, biopsies of paws were taken 5 h following the interplanetary injection of Carr. The tissue slices were fixed in 1.8% formaldehyde and 1.0% acetic acid for 1 week at room temperature, dehydrated by graded ethanol, and embedded in paraffin (Sherwood Medical). Sections (thickness = 5 μm) were deparaffinized with xylene and stained with hematoxylin and eosin (H&E) stain. All samples were observed and photographed with a BH-2 Olympus microscope. Every three to five tissue slices were randomly chosen from Carr-, Indo-, and AHR-treated (2.5 mg/kg) groups. Histological examination of these tissue slices revealed an excessive inflammatory response with massive infiltration of neutrophils [polymorphonuclear leukocytes (PMNs)] by microscopy. The numbers of neutrophils were counted in each scope (400×) and thereafter their average count obtained from five scopes of every tissue slice.[15]

Statistical Analysis. Experimental results are presented as the mean ± standard deviation (SD) of three parallel measurements. Data (Figures 4−6; Table 1) are expressed as the mean ± standard error of the mean (SEM). Statistical evaluation was carried out by one-way analysis of variance (ANOVA followed by Scheffe's multiple-range tests). Statistical significance is expressed as (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$.

Table 1. Effects of AHR and Indo on the Liver CAT, SOD, and GPx Activities in Mice[a]

group	catalase (U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)
control	5.28 ± 0.22	24.38 ± 0.31	3.42 ± 0.13
Carr	3.58 ± 0.31	15.64 ± 0.28	1.86 ± 0.19
Carr + Indo	4.73 ± 0.23**	22.53 ± 0.58**	2.82 ± 0.21**
Carr + AHR (0.62 mg/kg)	3.83 ± 0.28*	17.23 ± 0.45*	1.98 ± 0.26
Carr + AHR (1.25 mg/kg)	4.37 ± 0.24**	19.82 ± 0.35**	2.57 ± 0.39*
Carr + AHR (2.50 mg/kg)	4.98 ± 0.48***	22.93 ± 0.63**	2.83 ± 0.24**

[a] Each value represents the mean ± SEM. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ as compared with the Carr group (one-way ANOVA followed by Scheffe's multiple-range test).

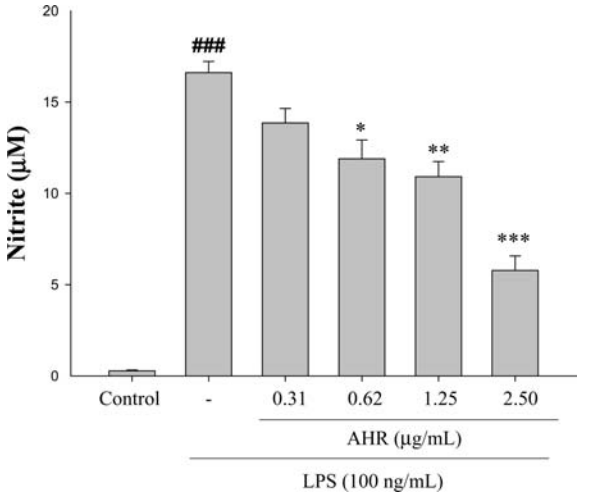

Figure 2. Effects of AHR on LPS-induced NO production of RAW 264.7 macrophages. Cells were incubated for 24 h with 100 ng/mL of LPS in the absence or presence of AHR (0, 0.31, 0.62, 1.25, and 2.50 μg/mL). AHR was added 1 h before incubation with LPS. Nitrite concentration in the medium was determined using Griess reagent. The data are presented as the mean ± SD for three different experiments performed in triplicate. (###) as compared with sample of control group; (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ as compared with LPS-alone group.

RESULTS

Cell Viability and Effect of AHR on LPS-Induced NO Production in Macrophages. The effect of AHR on RAW 264.7 cell viability was determined by a MTT assay. Cells cultured with AHR at concentrations of 0, 0.31, 0.62, 1.25, and 2.50 μg/mL used in the presence of 100 ng/mL LPS for 24 h did not change cell viability (Figure 1B).

NO plays a role as a neurotransmitter, vasodilator, and immune regulator in a variety of tissues at physiological concentration. High levels of NO produced by iNOS have been defined as cytotoxic molecules in inflammation.[16] AHR did not interfere with the reaction between nitrite and Griess reagents at 2.50 μg/mL (data not shown). Unstimulated macrophages, after 24 h of incubation in culture medium, produced background levels of nitrite. When RAW 264.7 macrophages were treated with different concentrations of AHR (0.31, 0.62, 1.25, and 2.50 μg/mL) together with LPS (100 ng/mL) for 24 h, a significant concentration-dependent inhibition of nitrite production was detected. There was either a significant decrease in the nitrite production of group treated with 0.62 μg/mL AHR ($p < 0.05$) or a highly significant decrease of groups treated, respectively, with 1.25 and 2.50 μg/mL of AHR when compared with the LPS-alone group ($p < 0.01$ or $p < 0.001$). The IC_{50} value for inhibition of nitrite production of AHR was about 1.92 ± 0.26 μg/mL (Figure 2).

Inhibition of LPS-Induced iNOS and COX-2 Protein by AHR. To investigate whether the inhibition of NO production was due to decreased iNOS and COX-2 protein levels, the effect of AHR on iNOS and COX-2 protein expression was studied by immunoblot. The results showed that incubation with AHR (0, 0.62, 1.25, and 2.50 μg/mL) in the presence of LPS (100 ng/mL) for 24 h inhibited iNOS and COX-2 protein expression in mouse macrophage RAW 264.7 cells in a dose-dependent manner (Figure 3A). The detection of β-actin was also performed in the same blot as an internal control. The intensity of protein bands was analyzed using Kodak Quantity software in three independent

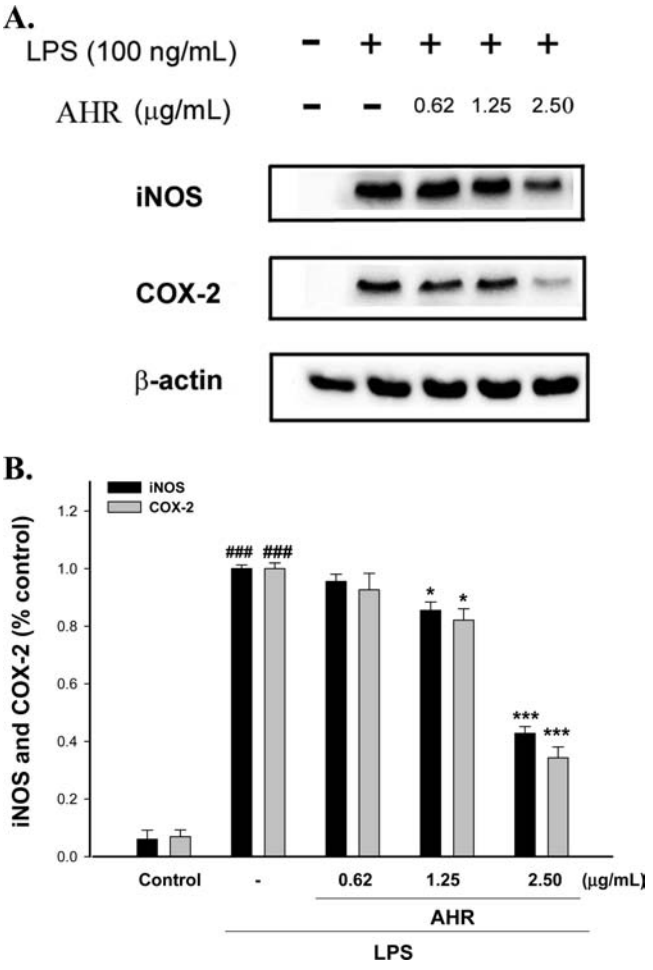

Figure 3. Inhibition of iNOS and COX-2 protein expression by AHR in LPS-stimulated RAW 264.7 cells. Cells were incubated for 24 h with 100 ng/mL LPS in the absence or presence of AHR (0, 0.62, 1.25, and 2.50 μg/mL). AHR was added 1 h before incubation with LPS. Lysed cells were then prepared and subjected to Western blotting using an antibody specific for iNOS and COX-2. β-Actin was used as an internal control. (A) A representative Western blot from two separate experiments is shown. (B) Relative iNOS and COX-2 protein levels were calculated with reference to a LPS-stimulated culture. (###) as compared with sample of control group. The data are presented as the mean ± SD for three different experiments performed in triplicate. (*) $p < 0.05$ and (***) $p < 0.001$ as compared with the LPS-alone group.

experiments and showed averages of 57.2 and 65.7% down-regulation of iNOS and COX-2 proteins, respectively, after treatment with AHR at 2.50 μg/mL compared with LPS alone (Figure 3B).

Effects of AHR on Carr-Induced Mice Paw Edema. Because AHR effectively inhibited iNOS and COX-2 inductions in macrophages, studies were extended to determine whether AHR affected acute phase inflammation in animal models. In this study, we used Carr-induced edema because this model is widely employed for screening the effects of anti-inflammatory drugs. Carr-induced paw edema is shown in Figure 4. AHR (2.5 mg/kg) inhibited ($p < 0.001$) the development of paw edema induced by Carr at 4 and 5 h after treatment, significantly. Indo (10 mg/kg) significantly decreased the Carr-induced paw edema at 4 and 5 h after treatment ($p < 0.001$).

Effects of AHR on the MDA Level. The MDA level increased significantly in the edema paw at 5 h after Carr injection ($p < 0.001$).

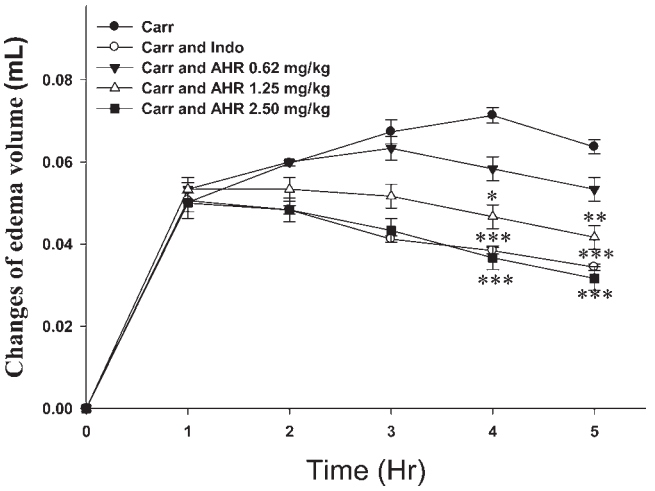

Figure 4. Effects of AHR and Indo on hind paw edema induced by Carr in mice. Each value represents the mean ± SEM. (∗) $p < 0.05$, (∗∗) $p < 0.01$, and (∗∗∗)$p < 0.001$ as compared with the Carr group (one-way ANOVA followed by Scheffe's multiple-range test).

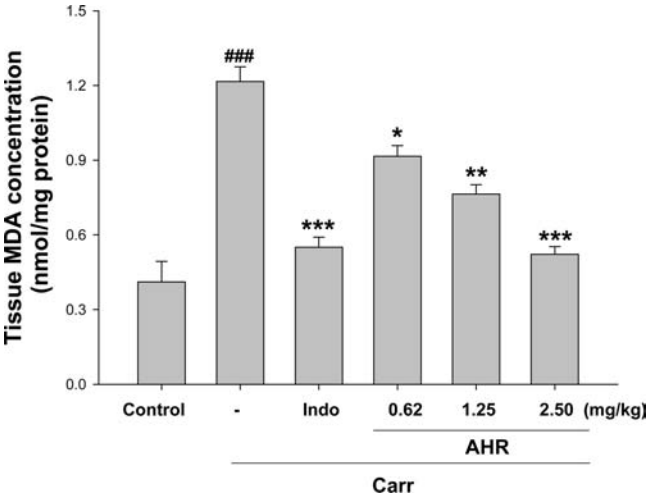

Figure 5. Effects of AHR and Indo on tissue MDA concentration in mice. Each value represents the mean ± SEM. (###) $p < 0.001$ as compared with the control group; (∗) $p < 0.05$, (∗∗) $p < 0.01$, and (∗∗∗) $p < 0.001$ as compared with the Carr group (one-way ANOVA followed by Scheffe's multiple-range test).

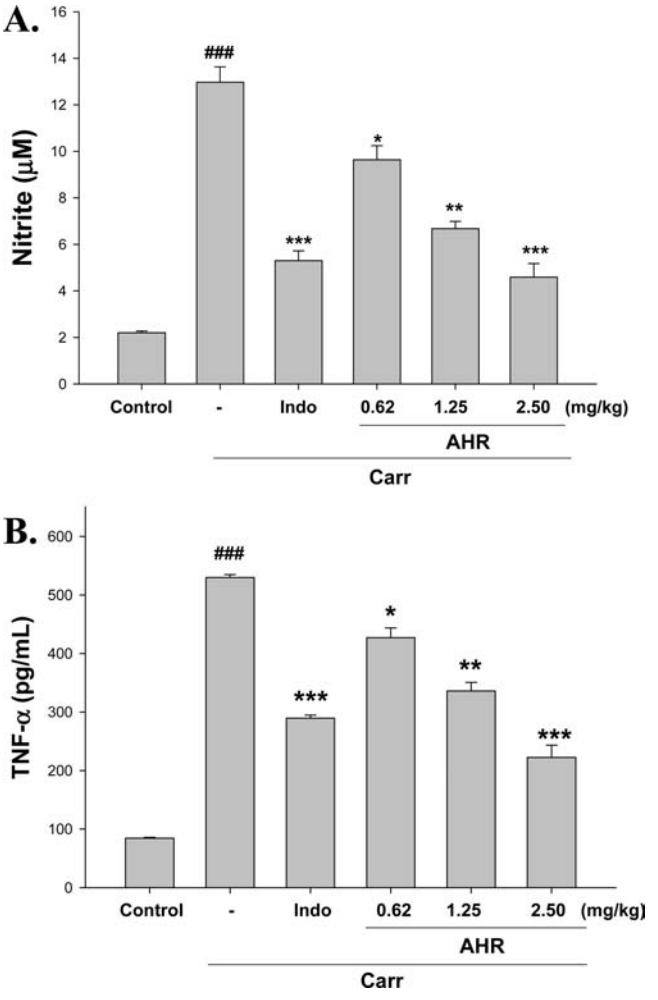

Figure 6. Effects of AHR and Indo on Carr-induced NO and TNF-α concentrations of serum at 5 h in mice. Each value represents the mean ± SEM. (###) $p < 0.001$ as compared with the control group; (∗) $p < 0.05$, (∗∗) $p < 0.01$, and (∗∗∗) $p < 0.001$ as compared with the Carr group (one-way ANOVA followed by Scheffe's multiple-range test).

However, the MDA level was decreased significantly by treatment with AHR (2.5 mg/kg) ($p < 0.001$), as well as 10 mg/kg Indo (Figure 5).

Effects of AHR on NO Level. In Figure 6A, the NO level increased significantly in the edema serum at 5 h after Carr injection ($p < 0.001$). AHR (2.5 mg/kg) significantly decreased the serum NO level ($p < 0.001$). The inhibitory potency was similar to that of Indo (10 mg/kg) at 5 h after induction.

Effects of AHR on TNF-α Level. The TNF-α level increased significantly in serum at 5 h after Carr injection ($p < 0.001$). However, AHR (1.25 or 2.50 mg/kg) decreased the TNF-α level in serum at 5 h after Carr injection ($p < 0.01$ or $p < 0.001$), as well as 10 mg/kg Indo (Figure 6B).

Effects of AHR on Activities of Antioxidant Enzymes. At 5 h after the intrapaw injection of Carr, liver tissues were also analyzed for the biochemical parameters such as CAT, SOD, and GPx activities. CAT, SOD, and GPx activities in liver tissue were decreased significantly by Carr administration. CAT, SOD, and GPx activities were increased significantly after treatment with 2.5 mg/kg AHR and 10 mg/kg Indo ($p < 0.01$ or $p < 0.001$) (Table 1).

Effects of AHR on Carr-Induced iNOS and COX-2 Protein Expressions in Mouse Paw Edema. To investigate whether the inhibition of NO production was due to decreased iNOS and COX-2 protein levels, the effect of AHR on iNOS and COX-2 protein expression was studied by Western blot. The results showed that injection of AHR (2.5 mg/kg) on Carr-induced edema at 5 h inhibited iNOS and COX-2 protein expression in mouse paw edema (Figure 7A). The intensity of protein bands was analyzed using Kodak Quantity software in three independent experiments and showed averages of 65.8 and 73.2% down-regulation of iNOS and COX-2 protein, respectively, after treatment with AHR at 2.5 mg/kg compared with Carr-induced alone (Figure 7B). In addition, the protein expression showed averages of 52.4 and 63.1% down-regulation of iNOS and COX-2 protein after treatment with Indo at 10.0 mg/kg compared with Carr-induced alone (Figure 7B). The down-regulation of iNOS

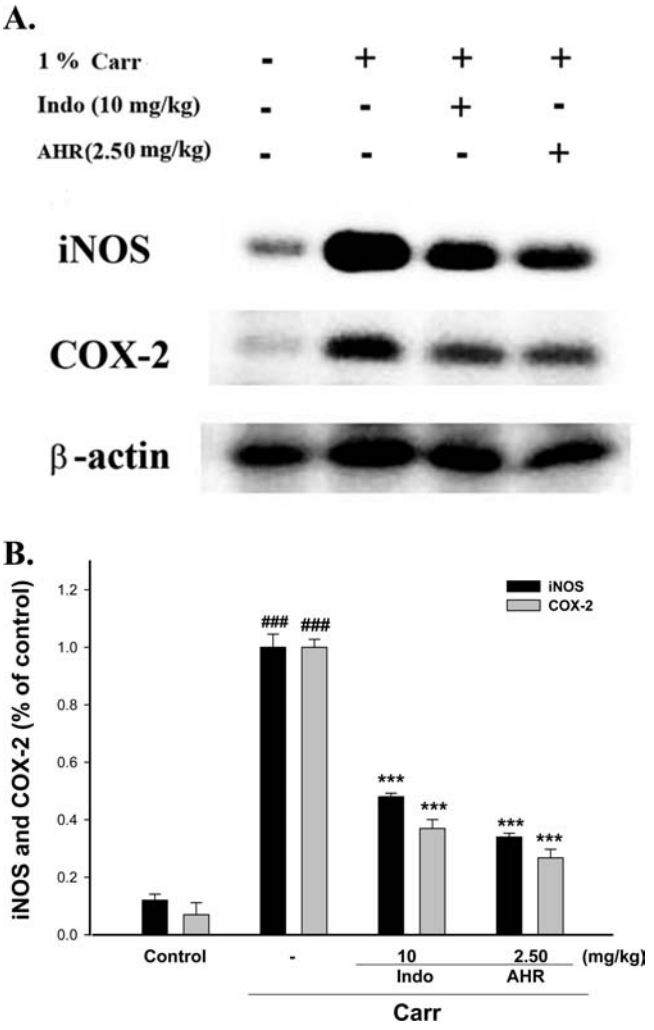

Figure 7. Inhibition of iNOS and COX-2 protein expression by AHR induced by Carr at 5 h in mice. Tissue suspension was then prepared and subjected to Western blotting using an antibody specific for iNOS and COX-2. β-Actin was used as an internal control. (A) A representative Western blot from two separate experiments is shown. (B) Relative iNOS and COX-2 protein levels were calculated with reference to a Carr-injected mouse. (###) compared with sample of control group. The data are presented as the mean ± SD for three different experiments performed in triplicate. (***) $p < 0.001$ as compared with Carr-alone group.

and COX-2 activities of the AHR (2.5 mg/kg) was better than that of Indo (10.0 mg/kg).

Histological Examination. Paw biopsies of Carr model animals showed marked cellular infiltration in the connective tissue. The infiltrates accumulated between collagen fibers and into intercellular spaces. Paw biopsies of animals treated with AHR (2.5 mg/kg) showed a reduction in Carr-induced inflammatory response. Actually, inflammatory cells were reduced in numbers and were confined to near the vascular areas. Intercellular spaces did not show any cellular infiltrations. Collagen fibers were regular in shape and showed a reduction of intercellular spaces. Moreover, the hypodermal connective tissue was not damaged (Figure 8). Neutrophil levels were significantly increased with Carr treatment ($p < 0.01$). Indo (10.0 mg/kg) and AHR (2.5 mg/kg) could decrease the neutrophil numbers as compared to the Carr-treated group ($p < 0.01$ or $p < 0.001$) (Figure 8E), significantly.

DISCUSSION

In the present study, we demonstrated anti-inflammatory activities of AHR in both ex vivo and in vivo experimental systems, using LPS-stimulated RAW 264.7 macrophages and a mouse model of topical inflammation, respectively. Dual inhibitory activities against iNOS as shown in in vitro assays appear to confer on AHR a potent in vivo efficacy in mouse, suggesting its potential therapeutic usage as a novel topical anti-inflammatory source of health food. The pathology of inflammation is initiated by complex processes triggered by microbial pathogens such as LPS, which is a prototypical endotoxin. LPS can directly activate macrophages, which trigger the production of inflammatory mediators, such as NO, PGE_2, TNF-α, and leukotrienes.[9] However, no report has been issued on the anti-inflammatory effect of AHR and the mode of action involved. Thus, this study was aimed to evaluate the anti-inflammatory effect of AHR by screening the effects of AHR on LPS-induced pro-inflammatory molecules ex vivo and on acute phase inflammation in vivo.

Although iNOS plays a pivotal role in immunity against infectious agents by producing an excess amount of NO, this enzyme has come into the spotlight for its detrimental roles in inflammation-related diseases. Excessive production of NO plays a critical role in the aggravation of circulatory shock and chronic inflammatory diseases, such as septic shock, inflammatory hepatic dysfunctions, inflammatory lung disease, and colitis.[16] As many of these conditions exhibit rapid onset and development, often resulting in the failure of conventional anti-inflammatory therapies and extremely high mortality rates, a simultaneous suppression of NO production pathways, as shown by AHR, may satisfy the so far unmet need for control of the rapid progression of the inflammatory process. In vitro models such as macrophage cells or other cell lines are useful materials with a steady high-level production of NO. The mechanisms by which AHR inhibits macrophage functions have not been elucidated. Results in vitro showed that AHR suppressed LPS-induced production of NO (Figure 2) and the expression of inflammatory protein products such as iNOS and COX-2 (Figure 3). Examination of the cytotoxicity of AHR in RAW 264.7 macrophages using the MTT assay has indicated that AHR even at 2.50 μg/mL did not affect the viability of RAW 264.7 cells. Therefore, inhibition of LPS-induced nitrite production by AHR was not the result of a possible cytotoxic effect on these cells.

Carr-induced rat paw edema is a suitable test for evaluating anti-inflammatory drugs and has frequently been used to assess the antiedematous effect of natural products.[17] The degree of swelling of the Carr-injected paws was maximal 3 h after injection. Statistical analysis revealed that AHR and Indo significantly inhibited the development of edema 4 h after treatment ($p < 0.05$ or $p < 0.001$) (Figure 4). The third phase of the edema-induced by Carr, when the edema reaches its highest volume, is characterized by the presence of prostaglandins and other compounds of slow reaction.[18] It was found that the injection of Carr into the rat paw induces the liberation of bradykinin, which later induces the biosynthesis of prostaglandin and other autacoids, which are responsible for the formation of the inflammatory exudates.[19]

In studies of the mechanism of inflammation, the L-arginine−NO pathway has been proposed to play an important role in the Carr-induced inflammatory response.[20] Our present results also confirm that the Carr-induced paw edema model results in the production of NO. The expression of iNOS has been

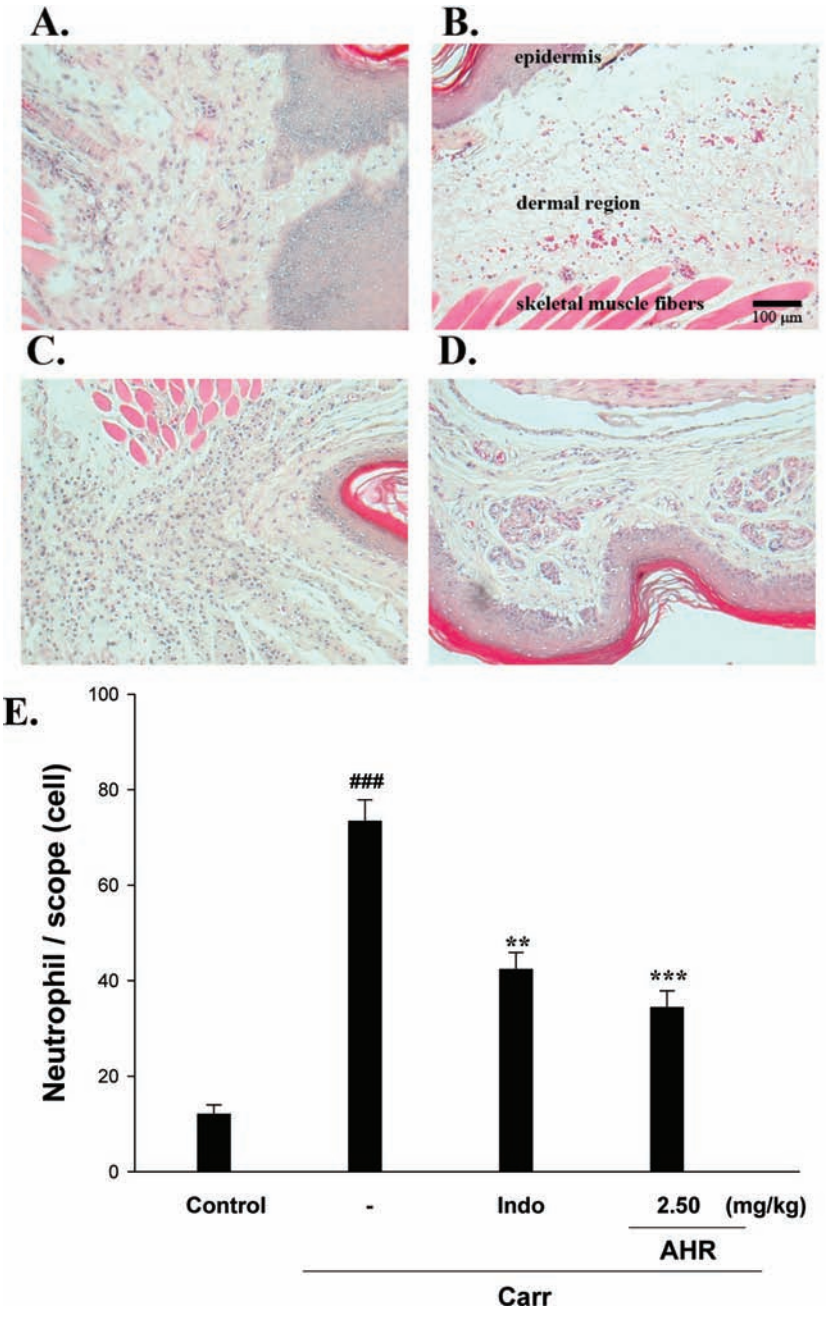

Figure 8. Representative light micrographs of mouse hind footpad H&E stained to reveal hemorrhage, edema, and inflammatory cell infiltration in control mice (A), Carr-treated mice demonstrating hemorrhage with moderately extravascular red blood cell and large amount of inflammatory leukocyte, mainly neutrophils, infiltration in the subdermis interstitial tissue of mice (B), and mice given indomethacin (Indo) (10 mg/kg) before Carr (C). AHR showed significant morphological alterations (100×) (D) and numbers of neutrophils in each scope (400×) (E) compared to subcutaneous injection of Carr only. (###) $p < 0.001$ as compared with the control group. The data are presented as the mean ± SD for three different experiments performed in triplicate. (**) $p < 0.01$ and (***) $p < 0.001$ as compared with the Carr group. Scale bar = 100 μm.

proposed as an important mediator of inflammation.[21] In our study, the level of NO was decreased significantly by treatment with 0.62, 1.25, and 2.5 mg/kg AHR. We suggest the anti-inflammatory mechanism of AHR may be through the L-arginine−NO pathway because AHR significantly inhibits NO production.

The pro-inflammatory cytokines such as TNF-α and IL-1β are small secreted proteins, which mediate and regulate immunity and inflammation. The production of TNF-α is crucial for the synergistic induction of NO synthesis in IFN-γ- and/or LPS-stimulated macrophages. TNF-α induces a number of physiological effects including septic shock, inflammation, and cytotoxicity.[22] Also, TNF-α is a mediator of Carr-induced inflammatory incapacitation and is able to induce the further release of kinins and leukotrienes, which is suggested to have an important role in the maintenance of long-lasting nociceptive response.[23] In this study, we found that AHR decreased the TNF-α level in serum after Carr injection by treatment with 1.25 and 2.50 mg/kg AHR, significantly.

The Carr-induced inflammatory response has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such as hydrogen peroxide, superoxide, and hydroxyl radicals, as well as the release of other neutrophil-derived mediators.[23] Researchers demonstrated that the inflammatory effect induced by Carr is associated with free radicals. Free radicals, prostaglandin, and NO will be released when Carr is administered for 1−6 h. The edema effect was raised to the maximum at 3 h. MDA production is due to free radical attack on the plasma membrane. Thus, the inflammatory effect would result in the accumulation of MDA.[11] Glutathione (GSH) acts as an oxy radical scavenger by scavenging NO and other oxidants. The increased GSH level may favor reduced MDA production. GSH plays an important role against Carr-induced local inflammation.[24] In this study, there were significantly increases in CAT, SOD, and GPx activities with AHR treatment (Table 1). Furthermore, there were significantly decreases in MDA level with AHR treatment (Figure 5). We assume the suppression of MDA production is probably due to the increases of CAT, SOD, and GPx activities.

In conclusion, these results suggested that AHR possessed anti-inflammatory effects. The anti-inflammatory mechanism of AHR may be related to iNOS, and it is associated with the increase in the activities of antioxidant enzymes (CAT, SOD, and GPx). AHR may be used as a pharmacological agent in the prevention or treatment of disease in which free radical formation is a pathogenic factor.

AUTHOR INFORMATION

Corresponding Author

*Phone: +886 4 2207-1693. Fax: +886 4 2207-1693. E-mail: kuoyh@mail.cmu.edu.tw.

Funding Sources

We appreciate the financial support from the National Science Council (NSC100-2313-B-039-004- and NSC 100-2320-B-039-033-), China Medical University (CMU) (CMU99-S-29, CCM-P99-RD-042, and CMU99-COL-10), and Taiwan Department of Heath Clinical Trial and Research Center of Excellence (DOH100-TD-B-111-004).

ACKNOWLEDGMENT

We thank Dr. Jeffrey Conrad for critically reading the manuscript.

REFERENCES

(1) Kuo, Y. H.; Cheng, Y. S.; Lin, Y. T. Extractive components from the wood of *Taiwania cryptomerioides* Hayata: three new sequiterpene alcohols, muurolane-3-ene-9α-ol-2-one, muurolane-2α,9β-diol-3-ene and muurolane-2β,9β-diol-3-ene. *Tetrahedron Lett.* **1969**, 2375–2377.

(2) Chien, S. C.; Chen, C. C.; Chiu, H. L.; Chang, C. I.; Tseng, M. H.; Kuo, Y. H. 18-*nor*-Podocarpanes and podocarpanes from the bark of *Taiwania cryptomerioides*. *Phytochemistry* **2008**, *69*, 2336–2340.

(3) Lin, W. H.; Fang, J. M.; Cheng, Y. S. Diterpenoids and steroids from *Taiwania cryptomerioides*. *Phytochemistry* **1998**, *48*, 1391–1397.

(4) Chang, C. I.; Tseng, M. H.; Kuo, Y. H. Five new diterpenoids from the bark of *Taiwania cryptomerioides*. *Chem. Pharm. Bull.* **2005**, *53*, 286–289.

(5) Pferschy-Wenzig, E. M.; Kunert, O.; Presser, A.; Bauer, R. In vitro anti-inflammatory activity of larch (*Larix decidua* L.) sawdust. *J. Agric. Food Chem.* **2008**, *56*, 11688–11693.

(6) Wang, S. Y.; Wu, J. H.; Shyur, L. F.; Kuo, Y. H.; Chang, S. T. Antioxidant activity of abietane-type diterpenes from heartwood of *Taiwania cryptomerioides* Hayata. *Holzforschung* **2002**, *56*, 487–492.

(7) Leung, Y. M.; Wong, K. L.; Lin, C. H.; Chao, C. C.; Chou, C. H.; Chang, L. Y.; Chen, S. W.; Cheng, T. H.; Kuo, Y. H. Dependence of 6 β-acetoxy-7α-hydroxyroyleanone block of Kv1.2 channels on C-type inactivation. *Cell. Mol. Life Sci.* **2010**, *67*, 147–156.

(8) Yamamoto, Y.; Gaynor, R. B. Therapeutic potential of the NF-κB pathway in the treatment of inflammation and cancer. *J. Clin. Invest.* **2001**, *107*, 135–142.

(9) Lai, C. S.; Lee, J. H.; Ho, C. T.; Liu, C. B.; Wang, J. M.; Wang, Y. J.; Pan, M. H. Rosmanol potently inhibits lipopolysaccharide-induced iNOS and COX-2 expression through downregulating MAPK, NF-κB, STAT3 and C/EBP signaling pathways. *J. Agric. Food Chem.* **2009**, *57*, 10990–10998.

(10) Zimmermann, M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* **1983**, *16*, 109–110.

(11) Chang, H. Y.; Sheu, M. J.; Yang, C. H.; Leu, Z. C.; Chang, Y. S.; Peng, W. H.; Huang, S. S.; Huang, G. J. Analgesic effects and the mechanisms of anti-inflammation of hispolon in mice. *Evidence-Based Complement. Altern. Med.* **2009**, doi: 10.1093/ecam/nep027.

(12) Flohe, L.; Otting, F. Superoxide dismutase assays. *Methods Enzymol.* **1984**, *105*, 93–104.

(13) Aebi, H. Catalase *in vitro*. *Methods Enzymol.* **1984**, *105*, 121–126.

(14) Paglia, E. D.; Valentine, W. N. Studies on the quantitative and qualitative characterization of erythrocytes glutathione peroxidase. *J. Lab. Clin. Med.* **1967**, *70*, 158–169.

(15) Huang, G. J.; Huang, S. S.; Lin, S. S.; Shao, Y. Y.; Chen, C. C.; Hou, W. C.; Kuo, Y. H. Analgesic effects and the mechanisms of anti-inflammation of ergostatrien-3β-ol from *Antrodia camphorata* submerged whole broth in mice. *J. Agric. Food Chem.* **2010**, *58*, 7445–7452.

(16) Pan, M. H.; Lai, C. S.; Dushenkov, S.; Ho, C. T. Modulation of inflammatory genes by natural dietary bioactive compounds. *J. Agric. Food Chem.* **2009**, *57*, 4467–4477.

(17) Spector, W. G.; Willoughb, D. A. The inflammatory response. *Bacteriol. Rev.* **1963**, *27*, 117–154.

(18) Tohda, C.; Nakayama, N.; Hatanaka, F.; Komatsu, K. Comparison of anti-inflammatory activities of six *Curcuma* rhizomes: a possible curcuminoid-independent pathway mediated by *Curcuma phaeocaulis* extract. *Evidence-Based Complement. Altern. Med.* **2006**, *3*, 255–260.

(19) Salvemini, D.; Wang, Z.; Bourdon, D. M.; Stern, M. K.; Curne, M. G.; Manning, P. T. Evidence of peroxynitrite involvement in the carrageenan induced rat paw edema. *Eur. J. Clin. Pharmacol.* **1996**, *303*, 217–220.

(20) Cuzzocrea, S.; Zingarelli, B; Calapai, G.; Nava, F.; Caputi, A. P. Zymosan activated plasma induces paw oedema by nitric oxide and prostaglandin production. *Life Sci.* **1997**, *60*, 215–220.

(21) Pan, M. H.; Lai, C. S.; Dushenkov, S.; Ho, C. T. Modulation of inflammatory genes by natural dietary bioactive compounds. *J. Agric. Food Chem.* **2009**, *57*, 4467–4477.

(22) Yun, K. J.; Koh, D. J.; Kim, S. H.; Park, S. J.; Ryu, J. H.; Kim, D. G.; Lee, J. Y.; Lee, K. T. Anti-inflammatory effects of sinapic acid through the suppression of inducible nitric oxide synthase, cyclooxygase-2, and proinflammatory cytokines expressions via nuclear factor-κB inactivation. *J. Agric. Food Chem.* **2008**, *56*, 10265–10272.

(23) Dawson, J.; Sedgwick, A. D.; Edwards, J. C.; Lees, P. A comparative study of the cellular, exudative and histological responses to carrageenan, dextran and zymosan in the mouse. *Int. J. Tissue React.* **1991**, *13*, 171–185.

(24) Chaturvedi, P. Inhibitory response of *Raphanus sativus* on lipid peroxidation in albino rats. *Evidence-Based Complement. Altern. Med.* **2008**, *5*, 55–59.