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ARTICLE

Anti-inflammatory Activity of Flavokawain B from *Alpinia pricei* Hayata

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Alpinia pricei (Zingiberaceae) is a spicy herb indigenous to Taiwan. A potent anti-inflammatory compound, flavokawain B (FKB), was obtained from *A. pricei*. FKB significantly inhibited production of NO and PGE_2 in LPS-induced RAW 264.7 cells. Moreover, it also notably decreased the secretion of TNF-α. Expression of iNOS and COX-2 proteins was also inhibited by FKB in a dose-dependent manner. FKB blocked the nuclear translocation of NF-κB induced by LPS, which was associated with prevention IκB degradation, and subsequently decreased NF-κB protein levels in the nucleus. Similar anti-inflammatory activities of FKB were observed in an animal assay. NO concentrations in mouse serum rose dramatically from 3.2 to 28.8 μM after mice were challenged with LPS; however, preadministration of 200 mg/kg FKB reduced the NO concentration to 3.8 μM after challenge with LPS. Moreover, FKB strongly suppressed LPS-induced iNOS, COX-2, and NF-κB proteins expression in mouse liver.

KEYWORDS: *Alpinia pricei*; flavokawain B; anti-inflammatory activity; NF-κB

INTRODUCTION

The rhizomes of Zingiberaceae plants are widely used as spices or traditional medicine in Asian countries, eaten raw, or cooked as vegetables and as flavoring. Leaves of several Zingiberaceae have also been used for food flavoring and in traditional medicine (*1*). Many *Alpinia* species are well-known medicinal herbs that have been shown by several previous studies to have various effects, namely, anti-inflammatory (*2*,*3*), antioxidant, antimicrobial (*4*,*5*), antidermatophytic (*6*), antinociceptive (*7*), hepatoprotective (*8*), immunostimulatory (*9*), and anticancer (*10*, *11*) activities.

Alpinia pricei Hayata is a perennial rhizomatous plant indigenous to Taiwan. It has various traditional and commercial uses, such as use of the leaves to make traditional zongzi (a glutinous rice dumpling) in Taiwan and use of the aromatic rhizomes as a folk medicine for dispelling abdominal distension and enhancing stomach secretion and peristalsis. However, to the best of our knowledge, the phytochemistry and bioactivity of *A. pricei* have not yet been investigated.

In the present study, four chalcone flavonoids, namely, cardamonin (**1**), flavokawain B (FKB) (**2**), 2′,4′,6′-trimethoxychalcone (**3**), and pinostrobin chalcone (**4**), were isolated from *A. pricei* (**Figure 1**), and their effects on inflammation were investigated. Among them, FKB showed potent anti-inflammatory activity. The anti-inflammatory mechanism of FKB was further elucidated by in vitro and in vivo assays.

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MATERIALS AND METHODS

Plant Materials and Chemicals. Rhizomes of *A. pricei* were collected from Ping-tung County in southern Taiwan in March 2007 and identified by Dr. Yen-Hsueh Tseng (National Chung Hsing University, Taiwan). A voucher specimen was deposited in the herbarium of the same university. Dulbecco's modified Eagle's medium (DMEM) and other cell culture reagents including fetal bovine serum (FBS) were obtained from Gibco BRL (Invitrogen), Grand Island, NY. Dimethyl sulfoxide (DMSO), penicillin, tryspin−EDTA, Tris-HCl, sodium dodecyl sulfate (SDS), lipopolysaccharide (LPS; *Escherichia coli* 0127:138), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Griess reagent were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents used in this study were of reagent or HPLC grade.

Animals. Male ICR mice (6 weeks old, 25−28 g) were purchased from BioLasco Co. (Taipei, Taiwan) and housed at a temperature of 25 ± 2 °C and 55 ± 5% relative humidity; lighting was provided from 6:00 a.m. to 6:00 p.m. with food and water ad libitum. Mice were allowed to adapt to their laboratory condition for at least 1 week prior to the experiment. All animal experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and Taiwan laws relating to the protection of animals and were approved by the local ethics committee.

Extract Preparation, Isolation, and Compound Identification. Air-dried rhizome of *A. pricei* (2.0 kg) was extracted exhaustively with 70% ethanol (EtOH) at ambient temperature. Total crude EtOH extract was concentrated under vacuum to yield a residue (166 g) and then partitioned in ethyl acetate (EtOAc)/H_2O to give an EtOAc-soluble fraction (17.7 g) and an H_2O-soluble fraction. The EtOAc-soluble fraction was further separated by chromatography over silica gel (60−80 mesh) and eluted with an *n*-hexane/EtOAc gradient (*n*-hex/EtOAc, 95:5; 90:10; 85:15; 80:20; 70:30; 60:40; 50:50; 40:60, 0:100, each 1 L) to produce 23 fractions. The process was continued through the last active fraction, which was

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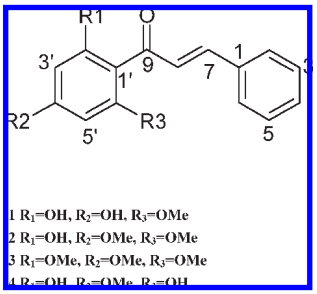

Figure 1. Structures of cardamonin (**1**), flavokawain B (**2**), 2′,4′,6′-trimethoxychalcone (**3**), and pinostrobin chalcone (**4**).

resolved into individual compounds by high-performance liquid chromatography (HPLC) using a Luna silica column (250×10 mm; Phenomenex, Torrance CA) eluted with an *n*-hex/dichloromethane/EtOAc solvent system to obtain four major compounds, cardamonin (**1**, 73.9 mg) (*12*), flavokawain B (**2**, 1538.1 mg) (*13*), 2′,4′,6′-trimethoxychalcone (**3**, 231.1 mg) (*12*), and pinostrobin chalcone (**4**, 39.8 mg) (*12*, *14*) (**Figure 1**). The structures of these compounds were elucidated and confirmed by spectroscopic analyses. UV spectra were recorded on a Jasco V-550 and IR spectra on a Bio-Rad FTS-40 spectrometer. Electron-impact mass spectrometry (EIMS) and high-resolution electron-impact mass spectrometry (HREIMS) data were collected with a Finnigan MAT-958 mass spectrometer, and NMR spectra were recorded with Bruker Avance 500 and 300 MHz FT-NMR spectrometers, at 500 MHz (^{1}H) and 75 MHz (^{13}C).

Cell Viability Assay. Cell viability was evaluated by an MTT assay (*15*). In the MTT assay, the cell suspension (2×10^5 cells/well) was plated in a 96-well microculture plate. After seeding, various concentrations of the test compound were added to the plate and incubated for 24 h. After 24 h, the culture supernatant were removed, and 50 μL of MTT mixed with fresh medium was added to the each well and incubated for 1 h. The absorbance of formazan at 490 nm was measured directly from the 96-well assay plates without additional processing.

Nitric Oxide Inhibitory Assay. Effects of compounds **1**, **2**, **3**, and **4** on NO production were measured indirectly by analysis of nitrite levels using the Greiss reaction (*16*, *17*). Briefly, murine macrophage cell line RAW 264.7 grown in a 75 cm^2 culture dish was seeded in 96-well plates at a density of 2×10^5 cells/well. Cells were cultured at 37 °C in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 incubator as recommended by the American Type Culture Collection (ATCC). Adherent cells were then incubated with or without 1 μg/mL of LPS for 24 h, in the absence or presence of test compounds. Nitrite concentration (as an estimate of NO production) was measured using the culture supernatant from the RAW 264.7 cells by the Griess reaction (*18*). The amount of nitrite in the samples was obtained by means of the $NaNO_2$ serial dilution standard curve, and nitrite production was quantified by a microplate reader (μQuant, Bio-Tek Instruments, Inc., Winooski, VT).

Determination of Prostaglandin E_2 Production. RAW 264.7 cells (2×10^5 cells/well) seeded on a 96-well plate were pretreated with 500 μM aspirin for 3 h to inactivate endogenous COX-1 (*19*), washed twice with PBS, and then treated with FKB (5, 10, 20, and 40 μM) and curcumin 20 μM) for 1 h. The cells were then incubated for 16 h in fresh DMEM medium with or without 1 μg/mL of LPS. The concentration of PGE_2 produced from endogenous arachidonic acid was measured in cell culture supernatant by an enzyme-linked immunosorbent assay (ELISA) kit according to the supplier's instructions (EIA; Cayman Chemical, Ann Arbor, MI).

Determination of TNF-α Production. The level of TNF-α in cell culture media was measured using an ELISA kit (Biosource, Camarillo, CA). Briefly, RAW 264.7 cells were seeded in a 96-well plate at a density of 2×10^5 cells/well and incubated with FKB (5, 10, 20, and 40 μM) or curcumin 20 μM in the presence or absence of LPS (1 μg/mL) for 18 h. TNF-α levels in the culture medium were assayed using the ELISA kit following the supplier's instructions. The medium was diluted with working reagent (1:2); 100 μL of each diluted sample was used for ELISA, and TNF-α levels were estimated by absorbance at 450 nm using a microplate reader (μQuant, Bio-Tek Instruments, Inc.).

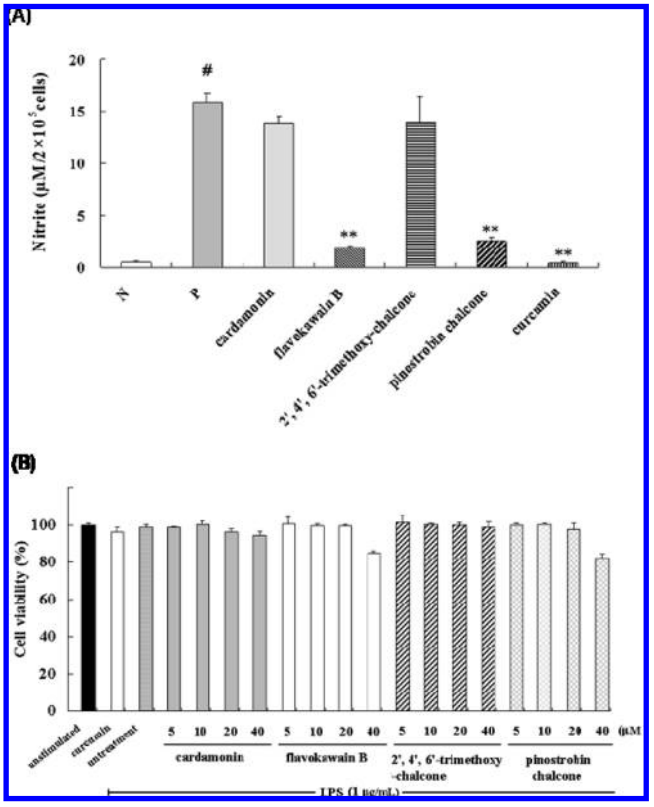

Figure 2. Effects of 20 μM cardamonin, flavokawain B, 2′,4′,6′-trimethoxychalcone, and pinostrobin chalcone from *Alpinia pricei* and curcumin on LPS-induced NO production and cell viability in RAW 264.7 cells. (**A**) NO production was measured indirectly by analysis of nitrite levels: LPS challenge alone, P; no LPS or drug, N. (**B**) Cell viability was evaluated using the MTT assay. Cells were incubated with indicated concentrations of cardamonin, flavokawain B, 2′,4′,6′-trimethoxychalcone, and pinostrobin chalcone for 24 h. The results are displayed as percentage of control samples (mean ± SE; $n = 3$; ∗, $P < 0.05$; ∗∗, $P < 0.01$, by Dunnett's test vs P).

Preparation of Whole Cell, Cytosolic, and Nuclear Extracts. The preparation of whole cell extract was previously described (*20*). Briefly, RAW 264.7 cells (5×10^6 cells/well) seeded on 6 cm dishes were treated with different concentrations of FKB (5, 10, 20, and 40 μM), stimulated with LPS (1 μg/mL), and incubated at 37 °C with 5% CO_2 for 16 h before COX-2 and iNOS assay. For the measurement of NF-κB (p50 and p65), IκBα, and IKK, cells were treated for only 1 h. Curcumin (20 μM) was used as a reference compound. For whole cell extracts, cells were lysed in Mammalian Protein Extraction Reagent (Cayman Chemicals), lysates were centrifuged at 4 °C for 10 min, and the supernatant was collected. The cytosolic and nuclear extracts were prepared according to protocol 78833 using a Nuclear and Cytoplasmic Extraction Reagents (NE-PER) kit (Pierce Biotechnology Inc., Rockford, IL). The protein content was quantified by absorbance at 595 nm according to the Bradford method (*21*) using bovine serum albumin as a standard.

In Vivo Experiments. Mice were divided into four groups consisting of six mice each. FKB and curcumin were dissolved in 1% DMSO and administered to mice by intraperitoneal injection with or without various concentrations of FKB (50, 100, and 200 mg/kg). Curcumin (100 mg/kg) was given 4 h before LPS injection (5 μg/kg) as described by Chen et al. (*22*). Control mice received vehicle (1% DMSO) only. Mice were sacrificed by decapitation during anesthesia with ethyl ether 12 h after LPS injection. Blood was collected by eye bleeding or cardiac puncture in EDTA tubes and centrifuged at 500*g* for 10 min at 4 °C, and NO concentrations in serum were measured indirectly using the Griess reaction (*18*) for determination of nitrite from blood serum; reduction of nitrate to nitrite was performed under alkaline conditions prior to Griess assay (*23*). Whole cell, cytosolic, and nuclear proteins were collected from

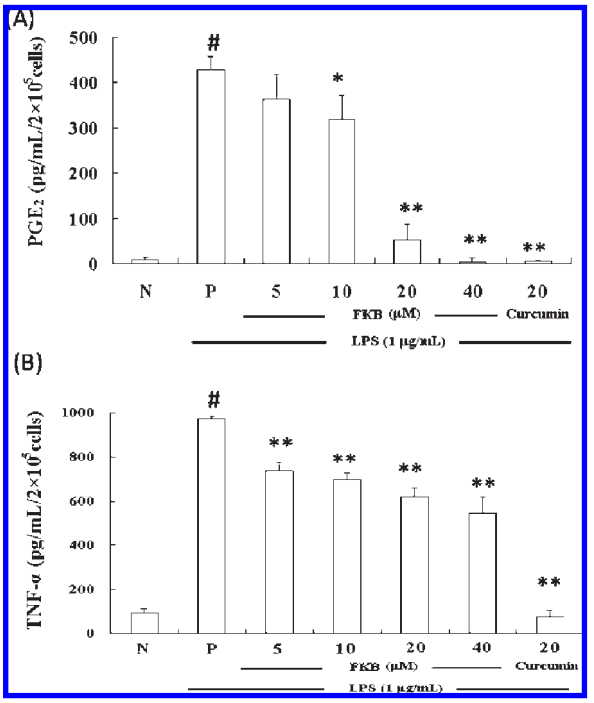

Figure 3. Effects of flavokawain B and curcumin on LPS-induced PGE_2 (**A**) and TNF-α (**B**) production in RAW 264.7 cells. The cells were pretreated for 1 h with different concentrations (5, 10, 20, 40 μM) of flavokawain B or curcumin (20 μM), and then LPS (1 μg/mL) was added and incubated for 16 h; LPS challenge alone, P; no LPS or drug, N (mean ± SE; $n = 3$; ∗, $P < 0.05$; ∗∗, $P < 0.01$, by Dunnett's test vs P).

liver tissues 12 h after LPS injection. The liver was quickly removed, frozen in liquid nitrogen, and milled. Whole proteins were isolated using Mammalian Protein Extraction Reagent (Cayman). The cytosolic and nuclear extracts were prepared as above using a kit (Pierce Biotechnology Inc., Rockford, IL), and protein content was quantified according to the Bradford method by absorbance at 595 nm (*21*).

Western Blot Analysis. RAW 264.7 cells were incubated with or without various concentrations of FKB, curcumin, and 1 μg/mL of LPS to measure the expressions of iNOS, COX-2, IKK, IκBα, and NF-κB proteins. Whole cell (iNOS and COX-2), cytoplasmic (IκB and IKK), and nuclear (p50/p65 NF-κB) protein extracts were collected from treated and untreated cells. Equal amounts of protein (20 μg) were loaded and resolved by 7−12% SDS-PAGE. The size-separated proteins were electroblotted onto polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, Bedford, MA) at 300 mA for 90 min. The membranes were incubated in blocking buffer (10% w/v skim milk in TBST buffer) for 1 h and then incubated with anti-iNOS (1:1000) or anti-COX-2 (1:1000) (Cayman), anti-NF-κB (1:500) (Abcam, Cambridge, U.K.), anti-IKK (1:1000) and anti-Iκ-Bα (1:1000) polyclonal antibody (Cell Signaling Technology, Danvers, MA), and antiactin (1:500) monoclonal antibody (Sigma). After two washes with 0.1% TBST (TBS containing 0.1% Tween 20), the membranes were incubated with anti-rabbit secondary antibodies conjugated with horseradish peroxidase and detected by the enhanced chemiluminescence reagents (ECL, Pierce). Actin levels were measured as a loading control.

Statistical Analysis. Data are expressed as means ± SE. The significance of differences between group means was analyzed by analysis of variance (ANOVA) using Dunnett's test. Differences were declared to be significant if P values were < 0.05 (indicated by ∗) or < 0.01 (∗∗).

RESULTS

Effects of Chalcones on NO Production and Cell Viability in LPS-Induced Macrophage Cells. In the present study, four flavonoids based on the chalcone skeleton, namely, cardamonin (**1**), FKB (**2**), 2′,4′,6′-trimethoxychalcone (**3**), and pinostrobin chalcone

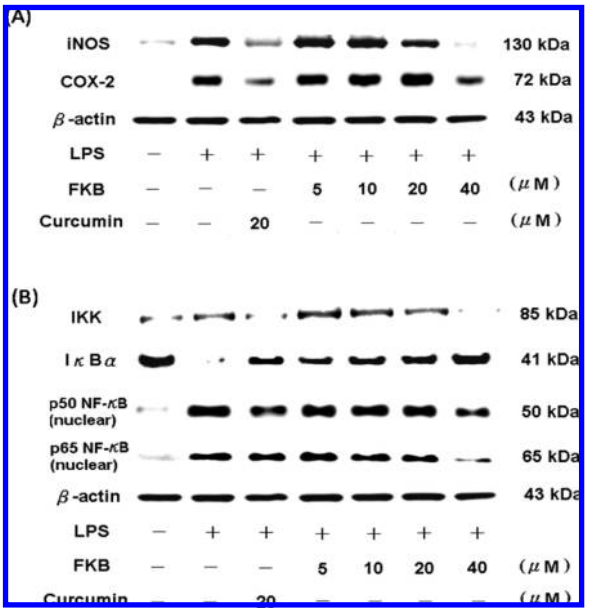

Figure 4. (**A**) Effects of flavokawain B and curcumin on the expression of iNOS and COX-2 protein in LPS-stimulated RAW 264.7 cells. Cells were incubated with or without LPS (1 μg/mL) for 18 h. iNOS and COX-2 were determined by immunoblotting using specific antibodies. (**B**) Effects of flavokawain B and curcumin on the expression of IKK, IκBα, and NF-κB protein in LPS-stimulated RAW 264.7 cells. Cells were incubated with or without LPS (1 μg/mL) for 1 h. IKK, IκBα, and NF-κB were detected by immunoblotting using specific antibodies.

(**4**), were isolated from the rhizome of *A. pricei*, and their effects on NO, PGE_2, and TNF-α production were studied in LPS-induced RAW 264.7 cells at 20 μM (**Figure 2A**). Compounds **2** and **4** were the most efficacious of the four chalcones, with effects similar to those of the 20 μM curcumin used as a positive control (*27*). The concentration of chalcones required to inhibit 50% of NO production (IC_{50}) was estimated from the percent inhibition versus concentration plot using a linear regression algorithm (data not shown). The IC_{50} values for cardamonin (**1**), FKB (**2**), 2′,4′,6′-trimethoxychalcone (**3**), and pinostrobin chalcone (**4**) were determined to be 60.6, 9.8, 79.0, and 12.0 μM, respectively. To examine whether chalcones were cytotoxic to macrophage cells, RAW 264.7 cells were incubated with indicated concentrations of chalcones for 24 h. An MTT assay showed that cultured cells were healthy and viable at the tested concentrations of chalcones and LPS (**Figure 2B**).

Effects of FKB on PGE_2 and TNF-α Production in LPS-Induced Macrophage Cells. Next, we determined the effects of FKB on PGE_2 production and TNF-α activity. As shown in **Figure 3A**, FKB inhibits PGE_2 production in LPS-stimulated RAW 264.7 cells in a concentration-dependent manner. PGE_2 production fell significantly from 428.4 to 52.1 pg/mL in the supernatant with a concentration of 20 μM and to 2.9 pg/mL with 40 μM. A similar observation was found for the effect of FKB on the production of the inflammatory cytokine TNF-α (**Figure 3B**).

Inhibitory Effects of FKB on LPS-Induced iNOS and COX-2 Expressions in Macrophage Cells. Western blot was performed to determine whether the inhibitory effects of FKB on the pro-inflammatory mediators (NO, PGE_2, and TNF-α) are related to the modulation of iNOS and COX-2 protein expression. In unstimulated RAW 264.7 cells, iNOS (130 kDa) and COX-2 (72 kDa) proteins were almost undetectable (**Figure 4A**, LPS: −; FKB: −). However, in response to LPS at 1 μg/mL, iNOS and COX-2 expressions were markedly augmented (**Figure 4A**,

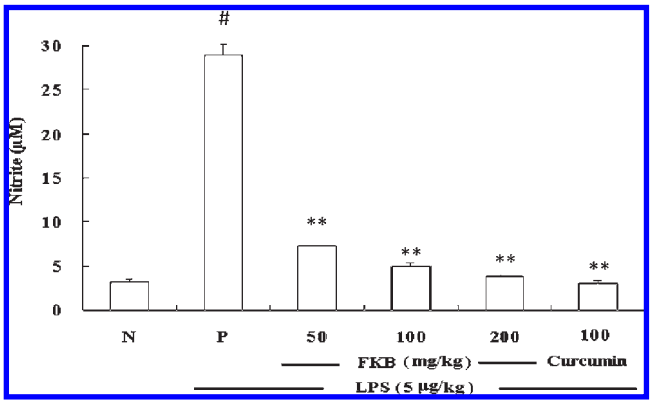

Figure 5. Effect of flavokawain B and curcumin on LPS-induced NO production in liver tissues; LPS challenge alone, P; no LPS or drug, N (mean ± SE; $n = 3$; **, $P < 0.01$, by Dunnett's test vs LPS alone).

LPS: +; FKB: −). FKB markedly suppressed LPS-induced iNOS and COX-2 protein level in a dose-dependent manner (**Figure 4A**), whereas β-actin was used as a loading control.

Effects of FKB on NF-κB, IκBα, and IKK Protein Expression in LPS-Induced Macrophage Cells. To further investigate the mechanism of FKB-mediated inhibition of iNOS and COX-2 expression, we focused on the well-known NF-κB-mediated inflammatory pathway. Western blot analysis confirmed that FKB prevents the LPS-induced nuclear translocation of p50/p65 NF-κB subunits (**Figure 4B**). In unstimulated cells, NF-κB is localized in the cytosol by binding with IκB; when stimulated by LPS, IκB rapidly degraded via 20S proteosomal degradation required with the IκB kinase (IKK) activation. Here, we further investigated whether FKB prevents degradation of IκBα and activation of IKK. We observed FKB pretreated cells inhibit the degradation of the inhibitory subunit IκBα and activation of IKK (**Figure 4B**), whereas it does not attenuate β-actin used as a loading control. In summary, these results demonstrate that FKB extracted from *A. pricei* is a potent inhibitor of LPS-induced NO, PGE_2, and TNF-α production at the gene expression level by blocking NF-κB activation.

Effects of FKB on LPS-Induced NO Production and COX-2 and iNOS Protein Expression in LPS-Induced Mice. As shown in **Figure 5**, pretreatment with FKB inhibits LPS-induced NO production. When mice were treated with LPS alone, the nitrite concentration in mouse serum increased markedly from 3.2 to 28.8 μM; however, when the mice was pretreated with 50, 100, or 200 mg/kg of FKB, the nitrite concentrations were reduced to 7.3, 5.1, and 3.8 μM, respectively. This result confirmed that FKB also strongly inhibits NO production in vivo. We further investigated the iNOS and COX-2 tissue protein level in mouse liver tissues. Injection of LPS (5 μg/kg) increased tissue protein level of iNOS and COX-2. However, pretreatment with FKB clearly attenuated the LPS-induced increase of iNOS, COX-2, and NF-κB (p65/p50) protein levels in the liver (**Figure 6**), whereas it does not attenuate β-actin used as a loading control. In comparison with curcumin (100 mg/kg), FKB was a more effective chemopreventive agent in vivo.

DISCUSSION

NO is a free radical generated through the conversion of L-arginine to citrulline, catalyzed by three isoforms of nitric oxide synthase (NOS), namely, the neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) isoforms. iNOS is induced in various cell types by inflammatory inducers (*24*). High NO levels cause a variety of pathophysiological processes including inflammation (*25*) and carcinogenesis (*26*). In the present study, four chalcone flavonoids, namely, cardamonin (**1**), flavokawain B (FKB) (**2**), 2′,4′,6′-trimethoxychalcone (**3**), and pinostrobin chalcone (**4**), were isolated from *A. pricei* (**Figure 1**), and their effects on inflammation were investigated. According to these results, the hydroxyl group at the C2′ position is an important substitution in chalcone analogues for the inhibition of inflammatory mediators such as NO; compound **3** had the lowest activity due to a methoxyl group at C2′ (see **Figure 1**). Next, bu comparison of the structures of compounds **1**, **2**, and **4**, compound **1** was less active than **2** and **4**, suggesting that the methoxyl group at C4′ is also an important characteristic for NO inhibition. That is to say, the activities of **2** and **4** were stronger than those of **1** and **3**, indicating that the hydroxyl at C2′ and the methoxyl at C4′ are important substitutions. Overall, FKB (**2**) is the major compound having the higher activity of the tested chalcones.

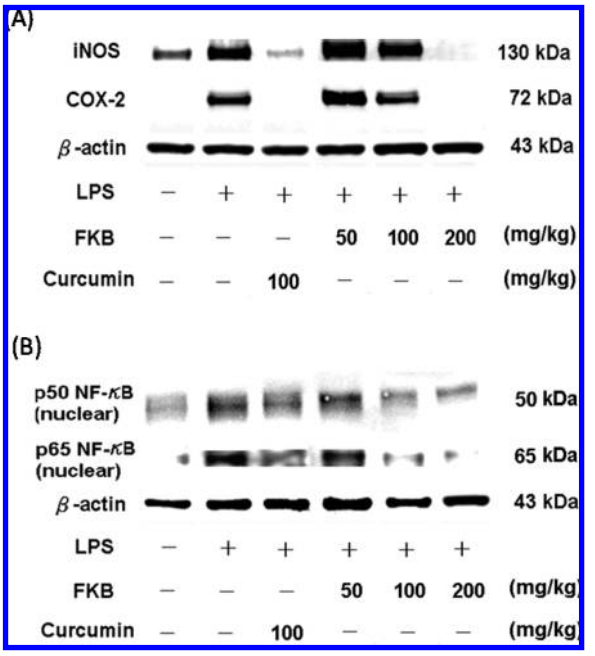

Figure 6. Effect of flavokawain B and curcumin on the expression of iNOS, COX-2 (**A**), and NF-κB (p65/p50) (**B**) protein in liver. Mouse liver tissue was incubated with or without LPS (5 μg/kg) for 12 h. iNOS, COX-2, and NF-κB (p65/p50) were detected by immunoblotting using specific antibodies; β-actin levels served as a loading control.

NO produced by iNOS and prostaglandins, autacoids of which biosynthesis originates from the enzyme COX-2, have been implicated as important mediators in the processes of inflammation (*28*). Inhibition of NO and PGE_2 production from COX-2 and iNOS can effectively treat inflammatory disease (*29*, *30*). As FKB significantly inhibited LPS-induced NO, TNF-α, and PGE_2 production, we reasoned that this inhibition could be due to suppression at the transcriptional level of iNOS and COX-2, leading to inhibition of iNOS and COX-2 protein expression.

To further examine the mechanism of the FKB-mediated inhibition of iNOS and COX-2 transcription, we looked at effects on the NF-κB pathway, one of the major signaling pathways leading to the activation of iNOS and COX-2 genes (*31*). NF-κB is composed of p50 and p65 subunits and is normally sequestered in the cytosol by binding with IκB. When stimulated by inflammatory mediators such as LPS and TNF-α, IκB is phosphorylated by IKK, the IκB kinase, ubiquitinated, and rapidly degraded via 26S proteosomal degradation, thus releasing NF-κB to travel to the nucleus and induce gene expression (*32*).

To follow up on our in vitro observations, we tested the anti-inflammatory activity of FKB in vivo. Mice were pretreated with FKB (50, 100, and 200 mg/kg) or curcumin (100 mg/kg) for 4 h and then with LPS (5 μg/kg) for 12 h followed by analysis of serum nitrite levels. Our results indicated that FKB reduced the LPS-induced serum nitrite level. Moreover, FKB also suppressed iNOS and COX-2 levels in LPS-challenged mice liver tissues. NF-κB reductions in nuclear fractions were also evidence that FKB strongly inhibited nuclear translocation of NF-κB subunits.

In summary, we confirm that FKB inhibits the production of pro-inflammatory mediators such as NO, TNF-α, and PGE_2 in LPS-stimulated macrophages. We also show here that this anti-inflammatory effect occurs by down-regulation of iNOS and COX-2 expression via suppression of the transcription factor NF-κB, the activation of which is a key feature in the pathophysiology of inflammatory disease. FKB or an extract of *A. pricei* could be used as a nutraceutical or to provide leads for better pharmaceutical treatment of inflammatory and pain diseases.

ABBREVIATIONS USED

COX-2, cyclooxygenase-2; DMEM, Dulbecco's modified Eagle's medium; FKB, flavokawain B; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IKK, Iκ-B kinase; iNOS, inducible nitric oxide synthase; Iκ-B, inhibitor of nuclear factor kappa-B; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor kappa-B; NO, nitric oxide; PGE_2, prostaglandin-E_2; TNF-α, tumor necrosis factor alpha.

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