JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of Immunological Activities of Peanut Stilbenoids, Arachidin-1, Piceatannol, and Resveratrol on Lipopolysaccharide-Induced Inflammation of RAW 264.7 Macrophages

BAMBANG DJOKO,† ROBIN Y.-Y. CHIOU,† JIA-JEN SHEE,‡ AND YI-WEN LIU*,§

Department of Food Science, College of Life Sciences, National Chiayi University, Chiayi, Taiwan, Division of Urology, Department of Surgery, Chang Gung Memorial Hospital, Chiayi, Taiwan, and Graduate Institute of Biopharmaceutics, College of Life Sciences, National Chiayi University, Chiayi, Taiwan

Biological activities of peanut stilbenoids, mainly resveratrol and its derivatives, have attracted increased attention and interest because of peanut being a potent producer and a dietary channel to convey these polyphenols to the human body. As arachidin-1 and piceatannol are structurally close to resveratrol, it is worthy to investigate their immunological activities on inhibition of lipopolysaccharide (LPS)-induced production of PGE_2 and NO and mediation of the related transcription factors (NF-κB and C/EBP) of RAW 264.7 macrophage cells. Productions of PGE_2 and NO were inhibited by all the test stilbenoids in a dose-dependent manner while gene and protein expressions of COX-2 and iNOS were not inhibited. As shown by NF-κB-driven luciferase assay, LPS-induced NF-κB activities were also reduced by the stilbenoids. In further, when these stilbenoids were subjected to monitoring their inhibitory effectiveness on LPS-induced transcription factor expressions of C/EBPδ and C/EBPβ, only C/EBPδ expressions were reduced. Thus, these stilbenoids were effective in inhibition of PGE_2- or NO-mediated inflammation and NF-κB- or C/EBPδ-mediated inflammatory gene expression. In comparison, the highest inhibitory activity on LPS-induced PGE_2/NO production, C/EBPδ gene expression, and NF-κB activation was piceatannol which was followed in order by arachidin-1 and resveratrol. The observed anti-inflammatory activities of these peanut stilbenoids are of merit in further consideration for nutraceutical applications.

KEYWORDS: Arachidin-1; piceatannol; resveratrol; inflammation; transcription factors; macrophages

INTRODUCTION

In recent years, resveratrol has been demonstrated as a health-enhancing phytochemical mainly because of its observed various potent bioactivities, namely, antioxidant, anti-inflammatory, anti-angiogenesis, estrogenic, and antitumor activities (*1*, *2*). Piceatannol is an anti-inflammatory, immunomodulatory, and anti-proliferative stilbene that has attracted attention on its biomedical functions (*3*, *4*). Piceatannol was biosynthesized and detected in peanut callus (*5*). In comparison, bioactivities of arachidin-1, a peanut secondary metabolite containing an additional hydroxyl, and an isopentenyl moiety to resveratrol and only an additional isopentenyl moiety to piceatannol (**Figure 1**) have been meagerly studied. Arachidin-1 could be biosynthesized and isolated from germinated peanut sprouts (*5*−*8*) to a substantial amount in our laboratory (*9*). In this study, it is worthy to investigate immunological activities of arachidin-1 along with piceatannol and reveratrol on inhibition of lipopolysaccharide (LPS)-induced production of PGE_2 and NO and mediation of the related transcription factors (NF-κB and C/EBP) of RAW 264.7 macrophage cells.

Cells of the monocytic/macrophage lineage play an important role in response to inflammation, infection, and the associated innate and adaptive immune responses. LPS, a component of the Gram-negative bacteria cell wall, is well-known as an effective stimulus in activation of macrophages to secrete proinflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), and secondary mediators, such as nitric oxide (NO), leukotrienes, and prostaglandins (PGs). Cyclooxygenase (COX), the key enzyme in the production of PGs, exists in two isoforms, for example, COX-1 is expressed constitutively in most cell types and COX-2 is an inducible enzyme expressed in activated macrophages, fibroblasts, and several other cell types. In vivo, expression of COX-2 was observed in chronic inflammatory conditions such as arthritis (*10*) and human colon cancer tissue (*11*). In vitro, COX-2

* To whom correspondence should be addressed. Phone: 886-5-2717809. Fax: 886-5-2717778. E-mail: ywlss@mail.ncyu.edu.tw.
† Department of Food Science, National Chiayi University.
‡ Chang Gung Memorial Hospital.
§ Graduate Institute of Biopharmaceutics, National Chiayi University.

10.1021/jf062741a CCC: $37.00
Published on Web 02/23/2007

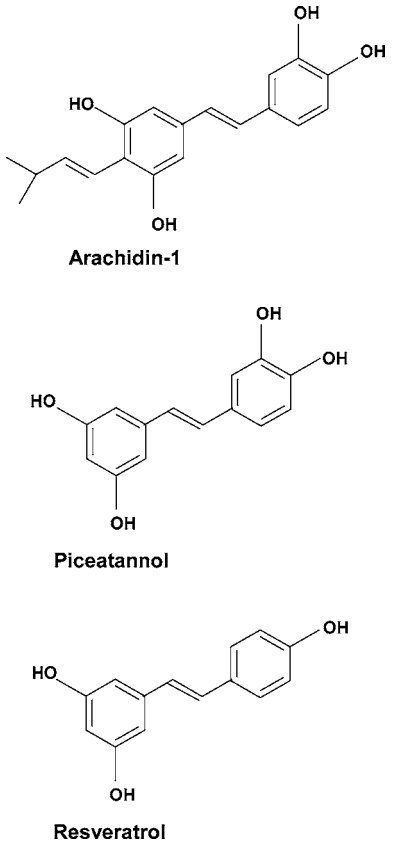

Figure 1. Structures of arachidin-1, piceatannol, and resveratrol.

expression was induced in response to stimuli such as LPS and growth factors (*12*, *13*). After release of arachidonic acid from cell membrane by phospholipase A_2, arachidonic acid was converted into PGH_2 by COX and then was further metabolized to PGE_2 in most cells. Because the prostaglandin products of COX-2 pathway, especially PGE_2, are very effective in proliferation enhancement of normal and cancer cells, biologically active compound screening for anti-inflammatory and anticancer is eventually focused on inhibition of COX-2 expression and its enzymatic activity.

Besides COX-2, inducible nitric oxide synthase (iNOS) is another enzyme contributing to inflammation. Like COX-2, iNOS is generally not present in the resting cells but is induced by various stimuli, including LPS and TNF-α (*14*). Although large production of NO by iNOS may promote host-defensive potency (*15*, *16*), NO also contributes to septic and hemorrhagic shock, rheumatoid arthritis, and chronic infections (*17*). Recently, NO was found to be reactive to activate COX-2 to synthesize prostaglandin and eventually to enhance inflammation. Therefore, the anti-inflammatory compound screening criteria need to include the inhibitory activity on NO production.

LPS stimulation of macrophages activates several nuclear transcription factors including NF-κB (p65/p50) and CCAAT/enhancer-binding proteins (C/EBPs) to mediate induction of many genes encoding inflammatory mediators (*18*, *19*). In this study, mouse macrophage cell line RAW 264.7 stimulated with LPS was used as an in-vitro model to investigate the inhibitory characteristics of arachidin-1, piceatannol, and resveratrol on LPS-induced production of PGE_2 and NO and mediation of the related transcription factors of NF-κB and C/EBP.

MATERIALS AND METHODS

Materials and Cell Culture. RAW 264.7 cells were cultured at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Carlsbad, CA) supplemented with 10% FBS (Biowest, France). LPS (*Salmonella typhosa*), anti-β-actin antibody, resveratrol (*trans*-3,4′,5-trihydroxystilbene), and piceatannol (*trans*-3,3′,4′,5-tetrahydroxystilbene) were from Sigma-Aldrich (St. Louis, MO). Arachidin-1 (*trans*-4-(3-methyl-but-1-enyl)-3,3′,4′,5-tetrahydroxystilbene) was prepared from peanut (*7*, *8*) isolated by high-performance liquid chromatography (HPLC) and was identified by NMR spectroscopic analyses in our laboratory (*9*). SuperSignal West Pico Chemiluminescent Substrate was from Pierce (Rockford, IL). Antibodies against Sp1 and COX-2 were purchased from Santa Cruz (Santa Cruz, CA). Antibodies against iNOS/NOS II were from Upstate (Lake Placid, NY). The pNFκB-Luc vectors were from BD Clontech (Palo Alto, CA). The jetPEI was from Polyplus transfection (Illkirch, France). PGE_2 enzyme immunoassay kit was from Cayman (Ann Arbor, MI). PRO-PREP protein extraction solution was from iNtRON Biotechnology (Kyungki-Do, Korea). Ro106-9920 was from TOCRIS (Ellisville, MO). Bio-Rad protein assay reagent was purchased from Bio-Rad (Hercules, CA). TRIzol reagent and SuperScript II were from Invitrogen (Carlsbad, CA).

RNA Extraction and RT−PCR. RAW 264.7 cells were cultured in 3.5-cm dish the day before LPS treatment. The cells were treated with each stilbenoids 1 h prior to introduction of 100 ng/mL LPS. The RNA was extracted with TRIzol reagent and was detected by RT−PCR technique. Reverse transcription was performed on 2 μg of total RNA by random primers (9 mers) and SuperScript II, and then $^1/_{20}$ volume of reaction mixture was pooled, followed by PCR with mouse COX-2 specific primers (5′-CAGCAAATCCTTGCTGTTCC-3′ and 5′-TGGGCAAAGAATGCAAACATC-3′), mouse iNOS specific primers (5′-GTCAACTGCAAGAGAACGGAGAAC-3′ and 5′-GAGCTCCTC-CAGAGGGTAGGCT-3′), mouse C/EBP δ specific primers (5′-ATCGCTGCAGCTTCCTATGT-3′ and 5′-GGTTAAGCCCGCAAA-CATTA-3′), or β-actin specific primers (5′-CCTAAGGCCAACCGTGAAAA-3′ and 5′-TCTTCATGGTGCTAGGAGCCA-3′). Cycle numbers of PCR were 25 cycles for COX-2, C/EBPδ, iNOS, and β-actin. The RT−PCR products were separated on 1% agarose gel and analyzed.

Protein Extraction. RAW 264.7 cells were precultured in 6-cm dish for nuclear extracts and in 3.5-cm dish for cell lysates extraction 1 day before LPS treatment. The cells were treated with each compound 1 h prior to introduction of 100 ng/mL LPS. Nuclear extracts were obtained by lysing the cells in buffer A (10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM EDTA, 0.5 mM PMSF, 10 mM NaF, 1 mM orthovanadate, 0.5 mM DTT, 2 μg/mL leupeptin, 2 μg/mL pepstatin A, and 10 mM β-glycerolphosphate) at 4 °C for 10 min and then were centrifuged at 7500*g* for 30 s at 4 °C. The nuclear pellets were then resuspended in buffer C (20 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM EDTA, 0.5 mM PMSF, 10 mM NaF, 1 mM orthovanadate, 0.5 mM DTT, 2 μg/mL leupeptin, 2 μg/mL pepstatin A, and 10 mM β-glycerolphosphate) at 4 °C for 20 min and were centrifuged at 7500*g* for 2 min at 4 °C. Total cell lysates were prepared by lysing the cells in buffer containing PRO-PREP Protein Extraction Solution containing 10 mM NaF and 1 mM orthovanadate at 4 °C for 15 min and were centrifuged at 7500*g* at 4 °C for 30 s. The supernatants containing protein extracts were stored at −80 °C for stabilization.

Western Blotting. Protein concentration was determined by using Bio-Rad protein assay reagent. Extracted protein (30 μg) was separated in 8% SDS-PAGE and was transferred to PVDF membrane. After blotting, the membrane was incubated with specific primary antibodies overnight at 4 °C and then was further incubated for 1 h with HRP-conjugated secondary antibody and eventually was incubated with SuperSignal West Pico Chemiluminescent Substrate for 1−2 min. The bounded antibodies were detected by Kodak digital science (Image Station 440CF).

Prostaglandin E_2 (PGE_2) Determination. RAW 264.7 cells were cultured in 24-well plate 1 day before LPS treatment. The cells were treated with each inhibitor 1 h prior to introduction of 100 ng/mL LPS. The extracellular medium containing synthesized PGE_2 was determined by Cayman PGE_2 enzyme immunoassay kit 23 h after LPS treatment. PGE_2 production was measured by an OD reading at 420 nm.

Nitric Oxide (NO) Determination. RAW 264.7 cells were cultured in 24-well plate 1 day before LPS treatment. The cells were treated

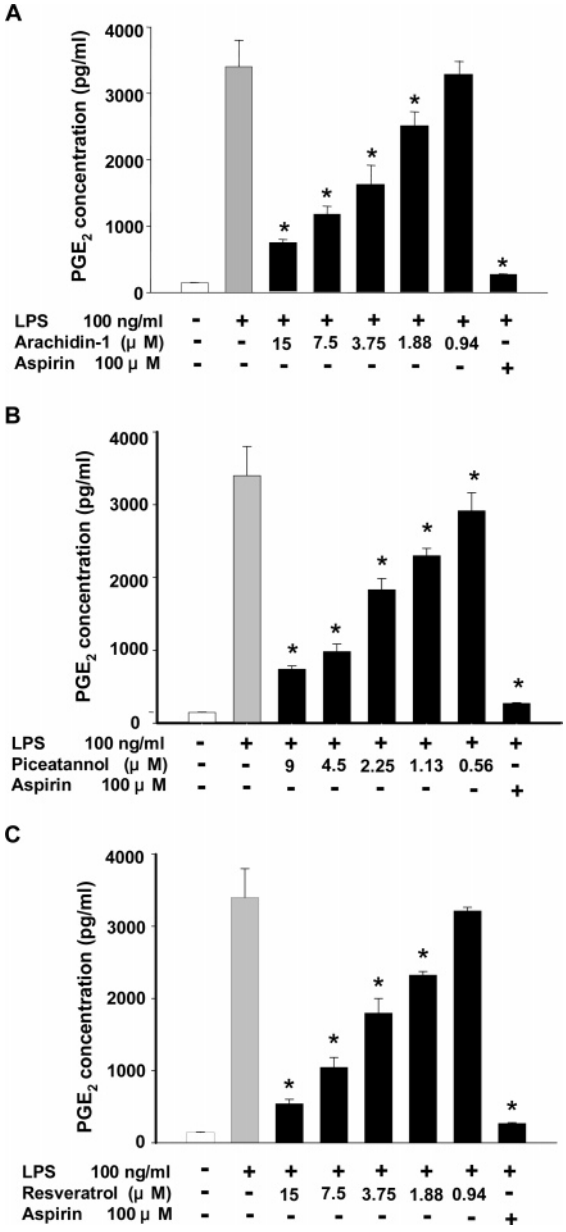

Figure 2. Dose-dependent inhibition of stilbenoids on LPS-induced PGE_2 synthesis in RAW 264.7 cells. Aliquots of 5×10^5 macrophage cell line RAW 264.7 were cultured in 24-well plate with DMEM supplemented with 10% FBS 1 day before LPS treatment. The stilbenoids (**A**, arachidin-1; **B**, piceatannol; **C**, resveratrol) were added 1 h prior to introduction of LPS. Aspirin (100 μM) was used as a positive inhibition control. The COX-2 metabolite, PGE_2, was determined by Cayman PGE_2 enzyme immunoassay 23 h after addition of LPS. Measurements were performed in triplicate and were presented as mean ± SD. *p*-Values are calculated by comparing with the LPS-treated group.

with each stilbenoids 1 h prior to introduction of 100 ng/mL LPS. The extracellular medium containing NO was determined by Griess Reagent System. The isolated supernatants were incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and were incubated at room temperature for 10 min. $NaNO_2$ was used to generate a standard curve, and nitrite production was measured by an OD reading at 550 nm.

Determination of NF-κB Activity by Luciferase Reporter Assay. Cells were transfected with the firefly luciferase gene construct containing NF-κB-binding element (pNFκB-Luc vector) by using jetPEI according to the manufacturer's instruction with a slight modification. Cells were replated 16 h before transfection at a density of 2.8×10^5 cells in 1 mL of fresh culture medium in a 12-well plastic dish. For use in transfection, jetPEI was incubated with pNFκB-Luc vector (1 μL of jetPEI/0.5 μg of total plasmid) in 0.1 mL of serum-free medium for 15 min at room temperature. A total of 0.5 mL of culture medium (with 10% FBS) was added to the DNA/jetPEI mix, then was added dropwise to the cells, and then was incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 5 h. After the change of DNA/jetPEI medium to 1 mL of fresh culture medium, cells were treated with the stilbenoids for 1 h and with added LPS for another 2 h. The cell lysates were prepared for luciferase and protein concentration assays. Luciferase activity was measured by the luciferase assay system in a Berthold Lumat LB 9507 luminometer. All the relative luciferase activities were normalized to the same protein concentration.

Statistical Analysis. Differences among the data of LPS-treated control and further treatments with various chemicals were analyzed by one-way ANOVA with SigmaStat 3.10 (Systat Software, Inc.) followed by Dunnett posthoc test. The minimum level of significance used throughout was $p < 0.05$.

RESULTS

Cytotoxicity of Arachidin-1, Piceatannol, and Resveratrol in Mouse Macrophages. To characterize the biological activities of arachidin-1, piceatannol, and resveratrol under their cytotoxic doses, MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide) assay was employed to detect the cytotoxicity of the peanut stilbenoids. After introduction of the stilbenoids to mouse macrophages RAW 264.7 cells for 24 h, piceatannol showed highest cytotoxicity in mouse macrophages ($IC_{50} = 19.0 \pm 1.0$ μM) and was followed in order by that of arachidin-1 ($IC_{50} = 31.7 \pm 1.5$ μM) and resveratrol ($IC_{50} = 73.0 \pm 7.8$ μM).

Effects of Arachidin-1, Piceatannol, and Resveratrol on LPS-Induced PGE_2 Synthesis in Mouse Macrophages. Because resveratrol inhibits phorbol ester-induced COX-2 transcription (*20*) and cyclooxygenase activity (*2*), it is interesting to compare the effects of arachidin-1, resveratrol, and piceatannol on inhibition of LPS-induced PGE_2 production. After the cells were treated with the peanut stilbenoids and then were stimulated with LPS for 23 h, PGE_2 productions were determined. As shown in **Figure 2**, PGE_2 production was suppressed by arachidin-1, piceatannol, and resveratrol in a dose-dependent manner. These stilbenoids exhibited obvious inhibitory effects on the production of PGE_2 even at the concentration lower than 5 μM, in which piceatannol showed a higher inhibition potency ($IC_{50} = 2.53$ μM), and a similar potency was observed between arachidin-1 and resveratrol ($IC_{50} = 3.51$ μM).

Effects of Arachidin-1, Piceatannol, and Resveratrol on LPS-Induced Protein and mRNA Expression of COX-2. Since these stilbenoids inhibited LPS-induced PGE_2 synthesis, the effects of arachidin-1, piceatannol, and resveratrol on the expression of COX-2 protein and mRNA were further investigated. As shown in **Figure 3**, it reveals that 30 μM arachidin-1, 15 μM piceatannol, or 30 μM resveratrol did not suppress both LPS-induced COX-2 protein and mRNA expression. These results suggest that the inhibition of arachidin-1, piceatannol, and resveratrol on PGE_2 synthesis is mainly through post-translational inhibition of LPS-induced COX-2 gene expression.

Effects of Arachidin-1, Piceatannol, and Resveratrol on Inhibition of LPS-Induced NO Production. On the basis of some research to indicate that LPS-induced NO production in macrophages is inhibited by resveratrol (*21*, *22*), arachidin-1, piceatannol, and resveratrol were subjected to determine their effectiveness to inhibit LPS-induced NO production in this study. As the results shown in **Figure 4**, piceatannol exhibited the strongest inhibition on LPS-induced NO production

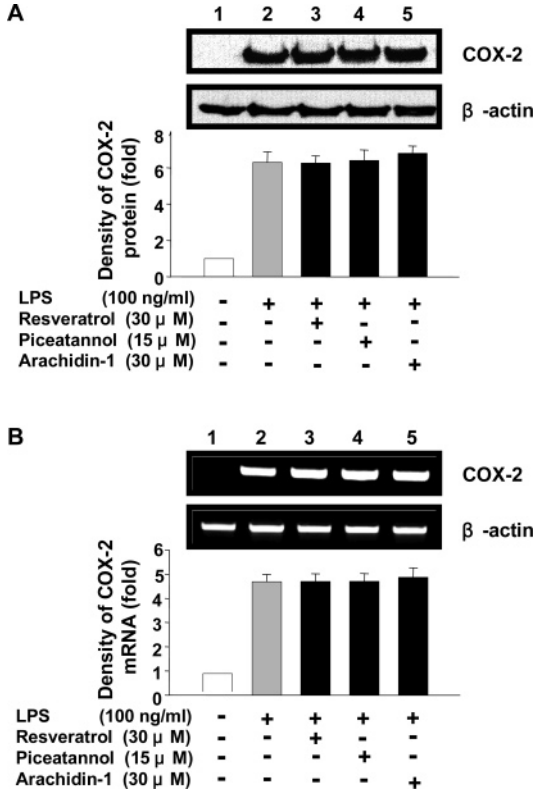

Figure 3. Effects of stilbenoids on LPS-induced COX-2 expression in RAW 264.7 cells. (**A**) Aliquots of 4×10^6 macrophage cell line RAW 264.7 were cultured in 6-cm dish with DMEM supplemented with 10% FBS 1 day before LPS treatment. The stilbenoids were added 1 h prior to introduction of LPS. The total cell lysates were extracted 5 h later and were analyzed by Western blotting. (**B**) Aliquots of 2.8×10^6 macrophage cell line RAW 264.7 were cultured in 3.5-cm dish with 10% FBS supplemented DMEM medium 1 day before the inhibitors and LPS were added. The stilbenoids were added 1 h prior to introduction of LPS. The total RNA was extracted 3 h later. Total RNA (2 μg) was applied to detect COX-2 mRNA by RT–PCR technique as described in Materials and Methods. The RT–PCR products were separated on 1% agarose gel and were digitally imaged after staining with ethidium bromide. The traces represent the means from three independent experiments, which are presented as mean ± SD.

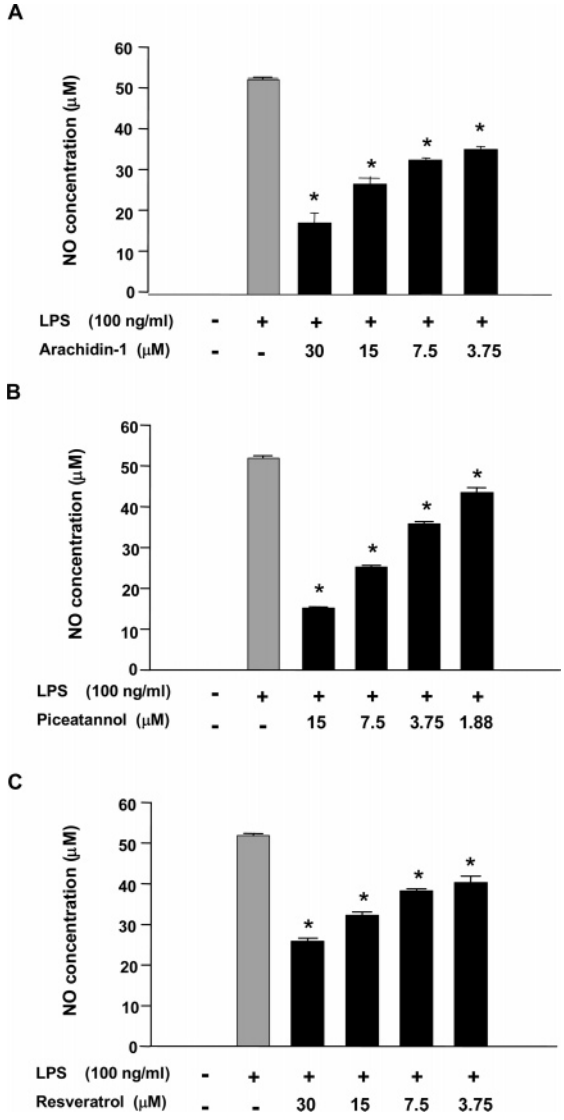

Figure 4. Dose-dependent inhibition of stilbenoids on LPS-induced NO production in RAW 264.7 cells. Aliquots of 5×10^5 macrophage cell line RAW 264.7 were cultured in 24-well plate with 10% FBS supplemented DMEM medium 1 day before addition of stilbenoids and LPS. Arachidin-1, piceatannol, or resveratrol was added 1 h prior to introduction of LPS for 18 h. The extracellular medium containing nitrite was determined by Griess reagent system. Measurements were performed in triplicate and are presented as mean ± SD. *p*-Values are calculated by comparing with the LPS-treated group.

(IC_{50} = 8.96 μM) and was followed in order by arachidin-1 (IC_{50} = 17.2 μM) and resveratrol (IC_{50} = 29.9 μM).

Effects of Arachidin-1, Piceatannol, and Resveratrol on LPS-Induced Protein and mRNA Expression of iNOS. As these stilbenoids were observed to effectively inhibit LPS-induced NO production, their effects on LPS-induced iNOS protein and mRNA expression were investigated. As shown in **Figure 5**, 30 μM of arachidin-1, 15 μM piceatannol, or 30 μM resveratrol had minimal effect on suppressing both protein and mRNA expression of iNOS. Since these stilbenoids exhibited higher inhibitory effect on LPS-induced NO production (**Figure 4**) than on LPS-induced iNOS mRNA and protein expression (**Figure 5**) at the same doses, it suggests that the inhibition of arachidin-1, piceatannol, and resveratrol on NO production are mainly through post-translational inhibition mechanism at the test doses.

Effects of Arachidin-1, Piceatannol, and Resveratrol on LPS-Induced NF-κB Activation. Even these stilbenoids did not inhibit LPS-induced COX-2 and iNOS gene expression at the test doses (**Figures 3** and **5**); LPS-mediated NF-κB activation accounts for a part of LPS-induced activation of many other inflammatory genes (*18*), and so it is necessary to identify their effects on the transcriptional activity of NF-κB. The NF-κB activity was monitored by a NF-κB-driven luciferase plasmid pNFκB-Luc which contains four NF-κB binding sites and a luciferase reporter gene. As shown in **Figure 6**, after plasmid transfection and LPS stimulation, the luciferase activity was increased about 5 folds, and this increase was decreased about 40% by 30 μM arachidin-1, 15 μM piceatannol, and 30 μM resveratrol and was completely inhibited by Ro106-9920, an IκB degradation inhibitor. In comparison, piceatannol also showed the greatest inhibitory potency on LPS-induced NF-κB activity and was followed by about the same levels of arachidin-1 and resveratrol.

Effects of Arachidin-1, Piceatannol, and Resveratrol on LPS-Induced Nuclear Expression of Transcription Factor C/EBPβ and C/EBPδ. Besides the NF-κB signal pathways,

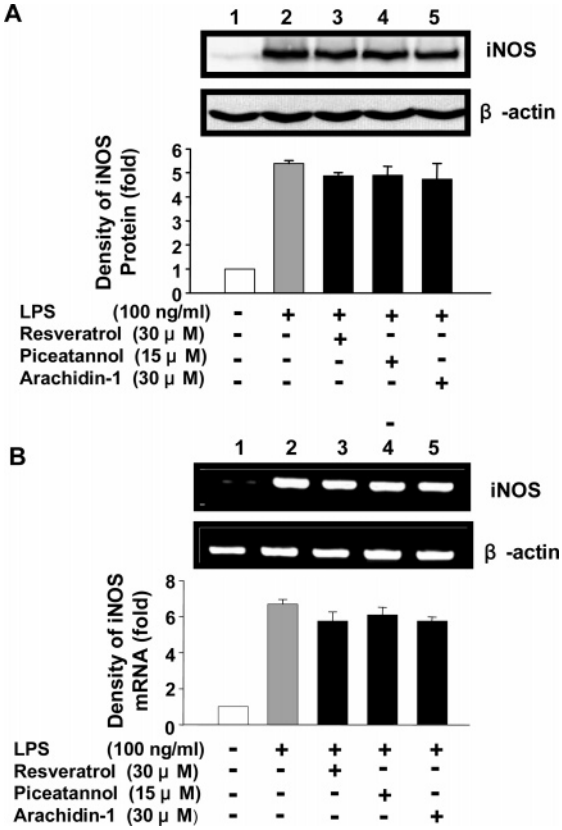

Figure 5. Effects of stilbenoids on LPS-induced iNOS expression in RAW 264.7 cells. (**A**) Aliquots of 4×10^6 macrophage cell line RAW 264.7 were cultured in 6-cm dish with DMEM supplemented with 10% FBS 1 day before LPS treatment. The stilbenoids were added 1 h prior to introduction of LPS. The total cell lysates were extracted 15 h later and were analyzed by Western blotting. (**B**) Aliquots of 2.8×10^6 macrophage cell line RAW 264.7 were cultured in 3.5-cm dish with 10% FBS supplemented DMEM medium 1 day before addition of stilbenoids and LPS. The stilbenoids were added 1 h prior to introduction of LPS. Total RNA was extracted 9 h later. Total RNA (2 μg) was applied to detect iNOS mRNA by RT–PCR technique as described in Materials and Methods. The RT–PCR products were separated on 1% agarose gel and were digitally imaged after staining with ethidium bromide. The traces represent the means from three independent experiments, which are presented as mean ± SD.

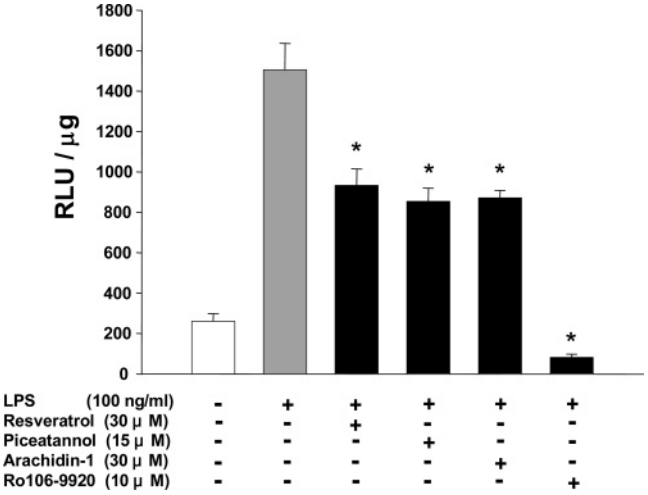

Figure 6. Effects of stilbenoids on LPS-induced NF-κB activation in RAW 264.7 cells. Cells were replated 16 h before transfection at a density of 2.8×10^5 cells in 1 mL of fresh culture medium in a 12-well plastic dish. jetPEI was incubated with pNFκB-Luc plasmid in 0.1 mL of serum-free medium for 20 min at room temperature. A total 0.5 mL of culture medium (with 10% FBS) was added to the DNA/jetPEI mix, then was added dropwise to the cells, and then was incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 5 h. After the change of DNA/jetPEI medium to 1 mL of fresh culture medium, cells were treated with stilbenoids for 1 h and added LPS for another 2 h. The cell lysates were prepared for luciferase and protein concentration assays. All the relative luciferase activities were normalized to the same protein concentration. Results are shown as mean ± SD of three independent experiments. *p*-Values are calculated by comparing with the LPS-treated group.

transcription factor C/EBPβ and C/EBPδ protein contents are increased by LPS stimulation in RAW 264.7 cells (*23*−*25*). When the effects of arachidin-1, piceatannol, and resveratrol on LPS-induced gene expression of C/EBPβ and C/EBPδ were monitored, all test stilbenoids increased LPS-induced C/EBPβ nuclear protein expression in mouse macrophages (**Figure 7A**) but inhibited LPS-induced C/EBPδ nuclear protein and mRNA expressions (**Figure 7B** and **7C**). Piceatannol also showed the highest potency in inhibiting C/EBPδ expression and was followed in order by arachidin-1 and resveratrol.

DISCUSSION

The immunological activities of arachidin-1, piceatannol, and reveratrol on inhibition of LPS-induced production of PGE_2 and NO and mediation of the related transcription factors (NF-κB and C/EBP) of RAW 264.7 macrophage cells have been intensively investigated in this study. The achieved data have shown that arachidin-1, piceatannol, and resveratrol did not exhibit inhibitory effect on the gene and protein expression of COX-2 (**Figure 3**) but inhibited PGE_2 production in a dose-dependent manner (**Figure 2**). This mode of a post-translational inhibition was similar to an in-vivo study on LPS-induced rat airway inflammation. That study suggests resveratrol reduces lung tissue neutrophilia and prostanoid levels but does not inhibit COX-2 gene expression (*26*). Resveratrol has also been demonstrated to have a direct suppression on PG synthesis by inhibiting the enzyme activity of COX-1 and COX-2 in a dose-dependent manner (*2*, *20*). Moreover, resveratrol in suppression of arachidonic acid catalysis through inhibiting PLA_2 enzyme activity has been reported (*27*). Therefore, arachidin-1 and piceatannol might follow the same mechanisms in inhibiting PGE_2 production.

Similar to COX-2 and PGE_2, arachidin-1, piceatannol, and resveratrol exhibited a marked inhibitory effect on LPS-induced NO production but only a minimal effect on the protein and mRNA expression of iNOS (**Figures 4** and **5**). It is obvious that the NO inhibition is mediated mainly by post-translation mechanism at the test doses. This phenomenon has been reported (*21*), and 60 μM resveratrol exhibits only a slight inhibition on LPS-induced iNOS mRNA expression (*22*). However, gene activation of iNOS being inhibited by resveratrol has also been reported (*28*, *29*). These contrary results of resveratrol on LPS-induced iNOS gene expression might be due to LPS source and laboratory difference, such as bacterial strain of LPS, cell sources, and applied drug concentration. In this study, it is clear that these stilbenoids at the test doses do not inhibit LPS-induced iNOS expression but reduce NO production. It was known that 100 μM of piceatannol and resveratrol shows only slightly inhibition on iNOS enzyme activities (*29*). Another study demonstrated that resveratrol and oxyresveratrol exhibit a scavenger for free nitric oxide in a spectrofluorimetric cell-free

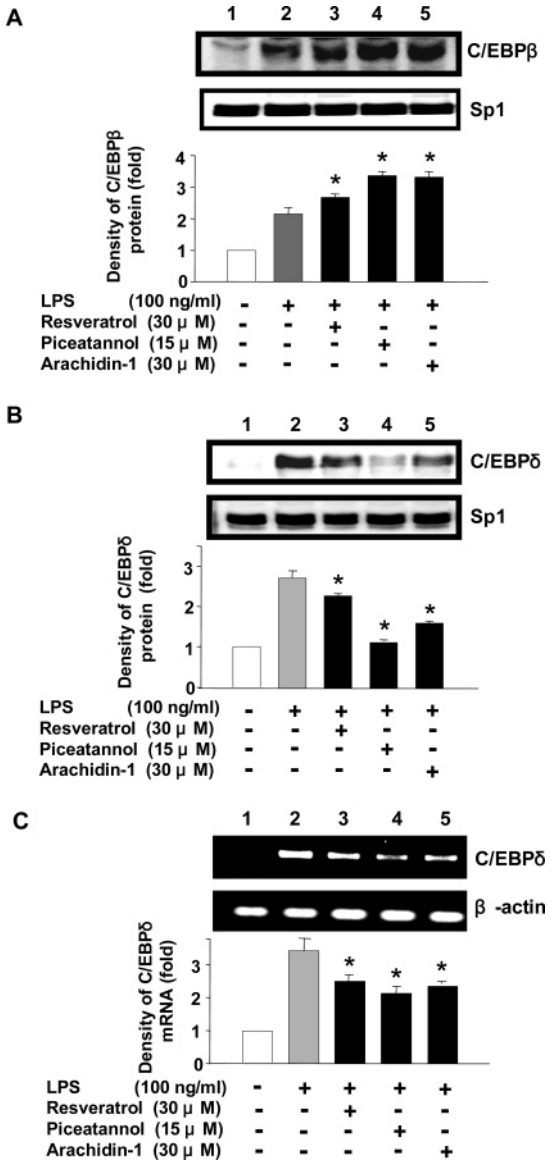

Figure 7. Effects of stilbenoids on C/EBPβ and C/EBPδ expression in LPS-activated RAW 264.7 cells. (**A** and **B**) Aliquots of 4×10^6 macrophage cell line RAW 264.7 were cultured in 6-cm dish with DMEM supplemented with 10% FBS 1 day before the LPS treatment. The stilbenoids were added 1 h prior to introduction of LPS. The nuclear extracts were extracted 5 h after LPS stimulation and were analyzed by Western blotting. (**C**) Aliquots of 2.8×10^6 macrophage cell line RAW 264.7 were cultured in 3.5-cm dish with 10% FBS supplemented DMEM medium 1 day before addition of stilbenoids and LPS. The stilbenoids were added 1 h prior to introduction of LPS. Total RNA was extracted 3 h later. Total RNA (2 μg) was applied to detect C/EBPδ mRNA by RT–PCR technique as described in Materials and Methods. The RT–PCR products were separated on 1% agarose gel and were digitally imaged after staining with ethidium bromide. The traces represent the means from three independent experiments and are presented as mean ± SD. *p*-Values are calculated by comparing with the LPS-treated group.

assay (*30*). In the present study, the inhibitory effects on LPS-induced NO production of these stilbenoids at their test doses may be partly through the direct scavenge activity of intracellular NO.

LPS-mediated NF-κB activation accounts for a part of LPS-induced iNOS gene activation (*31*). NF-κB is also involved in transcriptional control of IL-1β-induced COX-2 gene (*32*). In this study, it showed that all these stilbenoids compounds decreased the transcription activity of NF-κB about 40% at the test doses (**Figure 6**). However, they still did not exhibit marked inhibitory effect on LPS-induced COX-2 and iNOS expression (**Figures 3** and **5**). It suggests that NF-κB is not the only transcription factor in LPS-induced COX-2 and iNOS gene expression in the mouse macrophages. A correspondent result had been reported that NF-κB site is not required for LPS-induced COX-2 gene expression in RAW 264.7 cells (*33*).

In mouse macrophages, LPS-activated COX-2 and iNOS mRNA expression requires both C/EBPβ and C/EBPδ (*24*, *33*, *34*). When the roles of arachidin-1, piceatannol, and resveratrol on the C/EBPβ and C/EBPδ expression were investigated, the effects of these stilbenoids on LPS-induced protein expression of C/EBPβ are distinguished from C/EBPδ (**Figure 7**). These stilbenoids had minimal effect to increase the nuclear protein expression of C/EBPβ but decreased LPS-induced C/EBPδ expression. C/EBPβ might be more essential than C/EBPδ in LPS-induced COX-2 expression because the C/EBPβ-/- macrophages are defective in LPS-induced COX-2 expression (*24*). The inability of these stilbenoids on the inhibition of LPS-induced C/EBPβ expression might also account for a part of their inability in inhibition of LPS-induced COX-2 and iNOS expression.

Among those peanut stilbenoids, they mainly differentiate in number of hydroxyl groups, with or without isopentenyl moiety. The 4′-hydroxyl group of resveratrol was reported to be more active than the 3′- and 5′-hydroxyl groups because of resonance effects, and in conjunction with the *trans*-olefin structure of the parent stilbene skeleton, the hydroxyl groups were the most important determinants of antioxidant and antiproliferation bioactivity (*35*). In addition to the hydroxyl group, antifungal activities of those compounds are also affected by their molecular lipophilicity (*36*). In this study, piceatannol (tetrahydroxystilbene) was observed to have the strongest inhibitory potency on LPS-induced PGE_2/NO production, C/EBPδ gene expression, and NF-κB activation. The tested bioactive potency of arachidin-1, containing an additional isopentenyl group to piceatannol, is slightly lower than piceatannol and higher than resveratrol in NO and C/EBPδ inhibition or similar to resveratrol in PGE_2 and NF-κB inhibition. In general, all three stilbenoids have performed effective anti-inflammatory activities following an identical mechanism but varied with different potency among molecules. This suggests that arachidin-1 along with piceatannol and resveratrol might be of importance in further development for nutraceutical or chemopreventive applications.

ABBREVIATIONS

COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2; iNOS, inducible nitric oxide synthase; NO, nitric oxide; C/EBP, CCAAT/enhancer-binding protein; NF-κB, nuclear factor-κB; FBS, fetal bovine serum.

ACKNOWLEDGMENT

We are greatly indebted to Dr. Ching-Hsein Chen for his critical reading of the manuscript.

LITERATURE CITED

(1) Aggarwal, B. B.; Bhardwaj, A.; Aggarwal, R. S.; Seeram, N. P.; Shishodia, S.; Takada, Y. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. *Anticancer Res.* **2004**, *24*, 2783−2840.

(2) Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W.; Fong, H. H.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **1997**, *275*, 218−220.

(3) Ashikawa, K.; Majumdar, S.; Banerjee, S.; Bharti, A. C.; Shishodia, S.; Aggarwal, B. B. Piceatannol inhibits TNF-induced NF-kappaB activation and NF-kappaB-mediated gene expression through suppression of IkappaBalpha kinase and p65 phosphorylation. *J. Immunol.* **2002**, *169*, 6490−6497.

(4) Wieder, T.; Prokop, A.; Bagci, B.; Essmann, F.; Bernicke, D.; Schulze-Osthoff, K.; Dorken, B.; Schmalz, H. G.; Daniel, P. T.; Henze, G. Piceatannol, a hydroxylated analog of the chemopreventive agent resveratrol, is a potent inducer of apoptosis in the lymphoma cell line BJAB and in primary, leukemic lymphoblasts. *Leukemia* **2001**, *15*, 1735−1742.

(5) Ku, K. L.; Chang, P. S.; Cheng, Y. C.; Lien, C. Y. Production of stilbenoids from the callus of *Arachis hypogaea:* a novel source of the anticancer compound piceatannol. *J. Agric. Food Chem.* **2005**, *53*, 3877−3881.

(6) Wang, K. H.; Lai, Y. H.; Chang, J. C.; Ko, T. F.; Shyu, S. L.; Chiou, R. Y. Germination of peanut kernels to enhance resveratrol biosynthesis and prepare sprouts as a functional vegetable. *J. Agric. Food Chem.* **2005**, *53*, 242−246.

(7) Wotton, H. R.; Strange, R. N. Circumstantial evidence for phytoalexin involvement in the resistance of peanuts to *Aspergillus flavus*. *J. Gen. Microbiol.* **1985**, *131*, 487−494.

(8) Sobolev, V. S.; Cole, R. J.; Dorner, J. W. Isolation, purification, and liquid chromatographic determination of stilbene phytoalexins in peanuts. *J. AOAC Int.* **1995**, *78*, 1177−1182.

(9) Chang, J. C.; Lai, Y. H.; Djoko, B.; Wu, P. L.; Liu, C. D.; Liu, Y. W.; Chiou, R. Y. Y. Biosynthesis enhancement and antioxidant and anti-inflammatory activities of peanut (*Arachis hypogaea* L.) arachidin-1, arachidin-3, and isopentadienylresveratrol. *J. Agric. Food Chem.* **2006**, *54*, 10281−10287.

(10) Sano, H.; Hla, T.; Maier, J. A.; Crofford, L. J.; Case, J. P.; Maciag, T.; Wilder, R. L. In vivo cyclooxygenase expression in synovial tissues of patients with rheumatoid arthritis and osteoarthritis and rats with adjuvant and streptococcal cell wall arthritis. *J. Clin. Invest.* **1992**, *89*, 97−108.

(11) Kargman, S. L.; O'Neill, G. P.; Vickers, P. J.; Evans, J. F.; Mancini, J. A.; Jothy, S. Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. *Cancer Res.* **1995**, *55*, 2556−2559.

(12) O'Sullivan, M. G.; Chilton, F. H.; Huggins, E. M., Jr.; McCall, C. E. Lipopolysaccharide priming of alveolar macrophages for enhanced synthesis of prostanoids involves induction of a novel prostaglandin H synthase. *J. Biol. Chem.* **1992**, *267*, 14547−14550.

(13) Chen, L. C.; Chen, B. K.; Chang, J. M.; Chang, W. C. Essential role of c-Jun induction and coactivator p300 in epidermal growth factor-induced gene expression of cyclooxygenase-2 in human epidermoid carcinoma A431 cells. *Biochim. Biophys. Acta* **2004**, *1683*, 38−48.

(14) Ding, A. H.; Nathan, C. F.; Stuehr, D. J. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immunol.* **1988**, *141*, 2407−2412.

(15) Green, S. J.; Nacy, C. A.; Meltzer, M. S. Cytokine-induced synthesis of nitrogen oxides in macrophages: a protective host response to *Leishmania* and other intracellular pathogens. *J. Leukocyte Biol.* **1991**, *50*, 93−103.

(16) Lorsbach, R. B.; Murphy, W. J.; Lowenstein, C. J.; Snyder, S. H.; Russell, S. W. Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. Molecular basis for the synergy between interferon-gamma and lipopolysaccharide. *J. Biol. Chem.* **1993**, *268*, 1908−1913.9.

(17) Nussler, A. K.; Billiar, T. R. Inflammation immunoregulation, and inducible nitric oxide synthase. *J. Leukocyte Biol.* **1993**, *54*, 171−178.

(18) Guha, M.; Mackman, N. LPS induction of gene expression in human monocytes. *Cell. Signalling* **2001**, *13*, 85−94.

(19) Wu, K. K.; Liou, J. Y.; Cieslik, K. Transcriptional control of COX-2 via C/EBPbeta. *Arterioscler., Thromb., Vasc. Biol.* **2005**, *25*, 679−685.

(20) Subbaramaiah, K.; Chung, W. J.; Michaluart, P.; Telang, N.; Tanabe, T.; Inoue, H.; Jang, M.; Pezzuto, J. M.; Dannenberg, A. J. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. *J. Biol. Chem.* **1998**, *273*, 21875−21882.

(21) Wadsworth, T. L.; Koop, D. R. Effects of the wine polyphenolics quercetin and resveratrol on pro-inflammatory cytokine expression in RAW 264.7 macrophages. *Biochem. Pharmacol.* **1999**, *57*, 941−949.

(22) Chan, M. M.; Mattiacci, J. A.; Hwang, H. S.; Shah, A.; Fong, D. Synergy between ethanol and grape polyphenols, quercetin, and resveratrol, in the inhibition of the inducible nitric oxide synthase pathway. *Biochem. Pharmacol.* **2000**, *60*, 1539−1548.

(23) Liu, Y. W.; Chen, C. C.; Tseng, H. P.; Chang, W. C. Lipopolysaccharide-induced transcriptional activation of interleukin-10 is mediated by MAPK- and NF-kappaB-induced CCAAT/enhancer-binding protein delta in mouse macrophages. *Cell. Signalling* **2006**, *18*, 1492−1500.

(24) Caivano, M.; Gorgoni, B.; Cohen, P.; Poli, V. The induction of cyclooxygenase-2 mRNA in macrophages is biphasic and requires both CCAAT enhancer-binding protein beta (C/EBP beta) and C/EBP delta transcription factors. *J. Biol. Chem.* **2001**, *276*, 48693−48701.

(25) Liu, Y. W.; Tseng, H. P.; Chen, L. C.; Chen, B. K.; Chang, W. C. Functional cooperation of simian virus 40 promoter factor 1 and CCAAT/enhancer-binding protein beta and delta in lipopolysaccharide-induced gene activation of IL-10 in mouse macrophages. *J. Immunol.* **2003**, *171*, 821−828.

(26) Birrell, M. A.; McCluskie, K.; Wong, S.; Donnelly, L. E.; Barnes, P. J.; Belvisi, M. G. Resveratrol, an extract of red wine, inhibits lipopolysaccharide induced airway neutrophilia and inflammatory mediators through an NF-kappaB-independent mechanism. *FASEB. J.* **2005**, *19*, 840−841.

(27) Moreno, J. J. Resveratrol modulates arachidonic acid release, prostaglandin synthesis, and 3T6 fibroblast growth. *J. Pharmacol. Exp. Ther.* **2000**, *294*, 333−338.

(28) Tsai, S. H.; Lin-Shiau, S. Y.; Lin, J. K. Suppression of nitric oxide synthase and the down-regulation of the activation of NFkappaB in macrophages by resveratrol. *Br. J. Pharmacol.* **1999**, *126*, 673−680.

(29) Kageura, T.; Matsuda, H.; Morikawa, T.; Toguchida, I.; Harima, S.; Oda, M.; Yoshikawa, M. Inhibitors from rhubarb on lipopolysaccharide-induced nitric oxide production in macrophages: structural requirements of stilbenes for the activity. *Bioorg. Med. Chem.* **2001**, *9*, 1887−1893.

(30) Lorenz, P.; Roychowdhury, S.; Engelmann, M.; Wolf, G.; Horn, T. F. Oxyresveratrol and resveratrol are potent antioxidants and free radical scavengers: Effect on nitrosative and oxidative stress derived from microglial cells. *Nitric Oxide* **2003**, *9*, 64−76.

(31) Lowenstein, C. J.; Alley, E. W.; Raval, P.; Snowman, A. M.; Snyder, S. H.; Russell, S. W.; Murphy, W. J. Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 9730−9734.

(32) Newton R.; Kuitert, L. M.; Bergmann, M.; Adcock, I. M.; Barnes, P. J. Evidence for involvement of NF-kappaB in the transcriptional control of COX-2 gene expression by IL-1beta. *Biochem. Biophys. Res. Commun.* **1997**, *237*, 28−32.

(33) Wadleigh, D. J.; Reddy, S. T.; Kopp, E.; Ghosh, S.; Herschman, H. R. Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. *J. Biol. Chem.* **2000**, *275*, 6259−6266.

(34) Cieslik, K.; Zhu, Y.; Wu, K. K. Salicylate suppresses macrophage

nitric-oxide synthase-2 and cyclo-oxygenase-2 expression by inhibiting CCAAT/enhancer-binding protein-beta binding via a common signaling pathway. *J. Biol. Chem.* **2002**, *277*, 49304−49310.

(35) Stivala, L. A.; Savio, M.; Carafoli, F.; Perucca, P.; Bianchi, L.; Maga, G.; Forti, L.; Pagnoni, U. M.; Albini, A.; Prosperi, E.; Vannini, V. Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol. *J. Biol. Chem.* **2001**, *276*, 22586−22594.

(36) Arnoldi, A.; Merlini, L. Lipophilicity-antifungal activity relationships for some isoflavonoid phytoalexins. *J. Agric. Food Chem.* **1990**, *38*, 834−838.

Received September 25, 2006. Revised December 26, 2006. Accepted January 4, 2007. This work was supported by grants NSC 93-2320-B-415-002 and NSC 94-2313-B-415-011 from National Science Council of the Republic of China.

JF062741A