Biochemical Pharmacology, Vol. 59, pp. 1445–1457, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00255-0

Oroxylin A Inhibition of Lipopolysaccharide-Induced iNOS and COX-2 Gene Expression via Suppression of Nuclear Factor-κB Activation

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ABSTRACT. Polyphenols are major components of many traditional herbal remedies, which exhibit several beneficial effects including anti-inflammation. The exact mechanism of the anti-inflammatory action of polyphenols, however, has not been determined. In the present study, we examined the effects of eight different polyphenols isolated from Chinese herbs, including two flavonoids (myricitrin and oroxylin A), four ellagitannins (penta-*O*-galloyl-β-glucopyranose, woodfordin C, oenothein B, and cuphiin D1), and two anthraquinones (emodin and physcion), on lipopolysaccharide (LPS)-induced nitric oxide (NO) production, and inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) gene expression in RAW264.7 macrophages. The results indicated that only oroxylin A and emodin concentration-dependently inhibited LPS-induced NO production. The remaining compounds slightly inhibited LPS-induced NO production only at the highest concentration examined. Furthermore, oroxylin A inhibited the expression of LPS-induced iNOS and COX-2 proteins and mRNAs without an appreciable cytotoxic effect on RAW264.7 cells. Emodin also inhibited LPS-induced iNOS protein as potently as oroxylin A, but it inhibited LPS-induced iNOS mRNA expression only slightly and did not affect COX-2 mRNA and proteins. This was consistent with the findings that oroxylin A but not emodin or physcion inhibited prostaglandin E_2 synthesis induced by LPS. The inhibitory effects of oroxylin A on LPS-induced iNOS and COX-2 gene expression were also demonstrated in Bcl-2-overexpressing RAW264.7 macrophages, suggesting that oroxylin A inhibition of iNOS and COX-2 expression was not due to its antioxidant effect. Furthermore, oroxylin A but not emodin blocked nuclear factor-κB (NF-κB) binding and transcriptional activation associated with decreased p65 proteins in the nucleus induced by LPS. These results indicated that oroxylin A, an active component in *Huang Qin*, inhibited LPS-induced iNOS and COX-2 gene expression by blocking NF-κB activation, whereas emodin inhibition of LPS-induced iNOS expression may be mediated by a different transcription factor. BIOCHEM PHARMACOL **59**;11: 1445–1457, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. lipopolysaccharide; inducible nitric oxide synthase; cyclooxygenase 2; nuclear factor-κB; oroxylin A; polyphenolic compounds; macrophages

NO‖ has been shown to be an important regulatory molecule in diverse physiological functions such as vasodilation, neural communication, and host defense [1, 2]. Molecular cloning and sequencing analysis have revealed the existence of at lease three main types of NOS isoforms [3–5]. NOS present in the vascular endothelium (eNOS) and that in central and peripheral neurons (nNOS) are constitutive (cNOS), and their activation is Ca^{2+}-dependent. Continuous release of NO by cNOS plays a role in keeping the vasculature in an active state of vasodilation. Various agonists such as bradykinin and acetylcholine have been shown to trigger cNOS-mediated NO production through increasing intracellular Ca^{2+}. NOS in macrophages and hepatocytes, on the other hand, is inducible (iNOS), and its activation is Ca^{2+}-independent [6, 7]. After exposure to endotoxin and/or cytokines, iNOS can be induced in various cells such as macrophages, Kupffer cells, smooth muscle cells, and hepatocytes. The induced iNOS catalyzes the formation and release of a large amount of NO, which

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‖ *Abbreviations:* NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; IκB, inhibitor κB; IFN-γ, interferon-γ; Bcl-2, B-cell lymphoma protein-2; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; DTT, dithiothreitol; NP-40, Nonidet P-40; PGE_2, prostaglandin E_2; EMSA, electrophoretic mobility shift assay; and MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

Received 20 April 1999; accepted 25 October 1999.

plays a key role in the pathophysiology of a variety of diseases including septic shock [8–11]. NO production catalyzed by iNOS, therefore, may reflect the degree of inflammation and provides a measure by which effects of drugs on the inflammatory process can be assessed. Expression of COX-2 in various tissue preparations following LPS treatment also has been reported [12–14]. This enzyme is considered to play a major role in the inflammatory process by catalyzing the production of prostaglandins.

A portion of the 5′-flanking region of the murine iNOS gene has been cloned [15]. The promoter of the murine iNOS gene contains a TATA box and consensus sequences for the binding of transcription factors associated with stimuli that induce iNOS expression [16]. Two regulatory regions, a promoter proximal region containing the NF-κB site and a more distal enhancer region, are known to mediate expression of the iNOS gene. It has been reported that binding of NF-κB to the upstream NF-κB site of the iNOS promoter plays an important role in maximal expression of the iNOS gene induced by LPS and IFN-γ [17]. A number of traditional plant-derived medicines have been found to be rich in polyphenolic compounds [18]. These include flavonoids, tannins, and anthraquinones and have been shown to exhibit anti-inflammatory activities [19–21]. The exact mechanism of anti-inflammatory action of these compounds is not established. The possibility that these compounds exhibit their biological effects by blocking iNOS and COX-2 expression, therefore, was examined in the present study. Specifically, we studied the effects of two flavonoids (myricitrin, isolated from *Cuphea hyssopifolia* [22], and oroxylin A, isolated from *Scutellaria baicalensis*), four ellagitannins (penta-*O*-galloyl-β-glucopyranose, woodfordin C, oenothein B, and cuphiin D1; all isolated from *C. hyssopifolia* [22]), and two anthraquinones (emodin and physcion; both extracted from *Rheum palmatum*) on LPS-induced NO and PGE_2 production and expression of iNOS and COX-2 in RAW264.7 macrophages. The data demonstrated that oroxylin A was the most potent among the compounds tested in blocking LPS-induced iNOS and COX-2 gene expression. These inhibitory effects of oroxylin A were also demonstrated in Bcl-2-overexpressing RAW264.7 cells. The blocking effect of oroxylin A occurred via inhibition of binding of the transcription factor NF-κB to the iNOS promoter. Emodin, which also was a potent inhibitor of LPS-induced NO production, appeared to affect different transcription factors.

MATERIALS AND METHODS

Cells

RAW264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection. Cells were cultured in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics (100 U/mL of penicillin A and 100 U/mL of streptomycin) and 10% heat-inactivated fetal bovine serum (Gibco/BRL) and maintained at 37° in a humidified incubator containing 5% CO_2.

Agents

Eight different polyphenolic compounds (chemical structures shown in Fig. 1) were isolated from Taiwanese and Chinese herbal plants. These are classified into three types: (1) flavonoids: myricitrin (N1) isolated from the leaves of *C. hyssopifolia* (Lythraceae) and oroxylin A (N2) from the root of *S. baicalensis* (Labiatae); (2) ellagitannins: penta-*O*-galloyl-β-glucopyranose (N3), woodfordin C (N4), oenothein B (N5), and cuphiin D1 (N6), all isolated from the leaves of *C. hyssopifolia* (Lythraceae); and (3) anthraquinones: emodin (N7) and physcion (N8) were isolated from *R. palmatum* (Polygonaceae).

Nitrite Assay

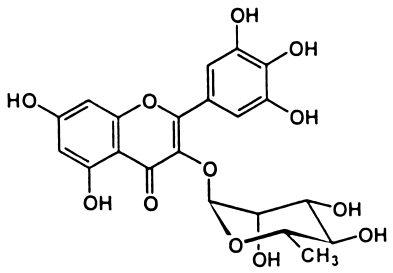
The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction [23]. One hundred microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water); absorbance of the mixture at 550 nm was determined with an ELISA plate reader (Dynatech MR-7000; Dynatech Laboratories).

Western Blots

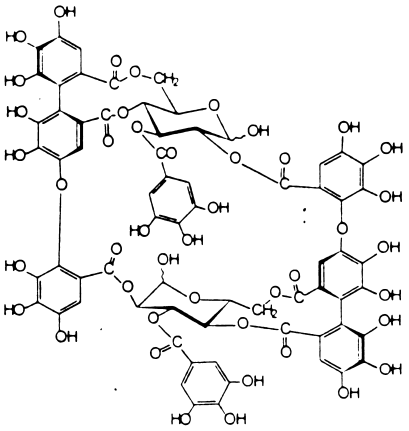
Total cellular extract, cytosolic fractions (for IκB), and nuclear fraction (for p65) were prepared according to Müller *et al.* [24], separated on SDS–polyacrylamide minigels (8% for iNOS or COX-2, and 10% for IκB or p65), and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). The membrane was incubated overnight at 4° with 1% bovine serum albumin and then incubated with anti-iNOS, anti-COX-2, or anti-α-tubulin monoclonal antibodies (Transduction Laboratories), or with anti-IκB or anti-p65 polyclonal antibodies (Santa Cruz Biochemicals). iNOS, IκB, p65, COX-2, and α-tubulin were detected by NBT and BCIP staining (Sigma Chemical Co.).

Northern Blot Analysis

Total RNA (20 μg/lane) was separated by electrophoresis on 1.2% agarose gel containing 6.7% formaldehyde and transferred to a Hybond-N nylon membrane (Amersham Life Science) in 20x standard saline citrate (3 M sodium chloride and 0.3 M sodium citrate, pH 7.0). After being heated at 80° for 2 hr and subjected to prehybridization for 4 hr, the filters were hybridized with ^{32}P-labeled murine iNOS cDNA probes at a concentration of 3×10^6 cpm/mL for 16–18 hr at 42°. The probe was labeled with [α-^{32}P]dCTP by using a Random Primer labeling kit (Stratagene). Then the filters were washed, dried, and autoradiographed with Kodak X-Omat XAR-film using intensifying screens at −80°.



Myricitrin (N1)



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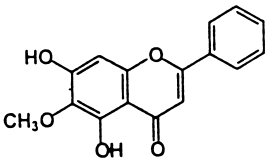
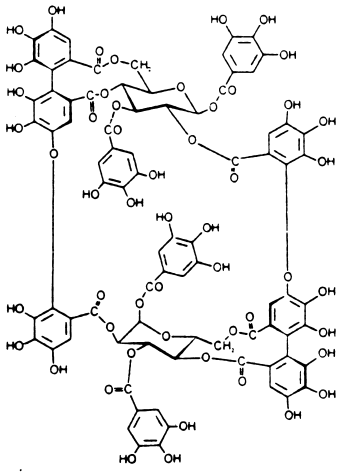
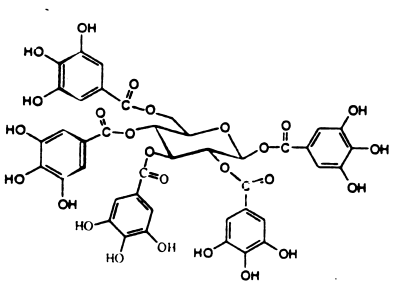


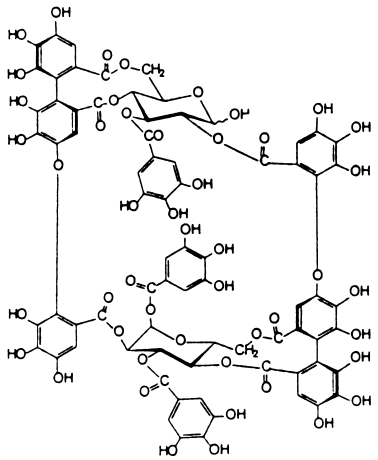
Oroxylin A (N2)



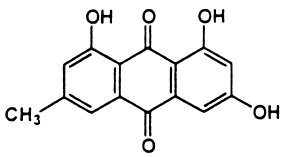
Cuphiin D1 (N6)



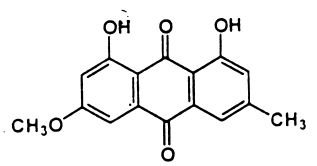
penta-O-galloyl-β-glucopyranose (N3)



Woodfordin C (N4)



Emodin (N7)



Physcion (N8)

FIG. 1. Chemical structures of polyphenolic compounds examined in the present study. For simplicity, each chemical is represented by the letter N with a number (N1–N8).

Transient Transfections and Luciferase Activity Assay

All transfectants were carried out using a standard calcium phosphate precipitation procedure. For luciferase activity assays, RAW264.7 macrophages were transfected with 2 μg of reporter plasmid containing 5 NF-κB sites in its enhancer element (STRATAGENE). After 48–72 hr, cells were treated with LPS alone or LPS plus each indicated compound for 4 hr, and then cells were lysed with lysis buffer (0.5 M HEPES, pH. 7.4; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 1% Triton X-100). Analysis of luciferase activity was performed using a Luciferase reporter gene assay kit (Packard BioScience Co.).

Establishment of Bcl-2/RAW264.7 Macrophage Cells

RAW264.7 cells expressing Bcl-2 were prepared by electroporation (model T800; BTx) of RAW264.7 cells with the Bcl-2 expression vector pC-Δj-bcl-2 (a gift from Dr. S-F. Yang of the Institute of Molecular Biology, Academia Sinica) or with a neo-controlled vector. pC-Δj-bcl-2, the expression vector that carries the human Bcl-2 cDNA under control of the SV40 promoter/enhancer sequence, was developed according to our previous report [25]. Briefly, cells were suspended in 1 mL of HEPES-buffered saline containing plasmid DNA, and then received electric treatment as follows: electric amplitude, 350 V; pulse width, 99 μsec. Stable transfectants resistant to G418 (Gibco BRL) were obtained. The levels of Bcl-2 expression of each clone were examined by western blotting.

Measurement of PGE_2 Production

RAW264.7 cells were subcultured in six-well plates and were incubated with selected compounds (oroxylin A, emodin, and physcion) for 12 hr. One hundred microliters of supernatant of culture medium was collected for the determination of PGE_2 concentrations by ELISA (Cayman Enzyme Immunoassay kit).

EMSA

Nuclear and cytoplasmic extracts were prepared according to a modified method of Chen *et al.* [25]. At the end of culture, cells were suspended in hypotonic buffer A (10 mM HEPES, pH 7.6; 10 mM KCl; 0.1 mM EDTA; 1 mM DTT; 0.5 mM phenylmethylsulfonyl fluoride) for 10 min on ice and vortexed for 10 sec. Nuclei were pelleted by centrifugation at 12,000 *g* for 20 sec. The supernatants containing cytosolic proteins were collected. The pellet containing nuclei was suspended in buffer C (20 mM HEPES, pH 7.6; 25% glycerol; 0.4 M NaCl; 1 mM EDTA; 1 mM DTT; 0.5 mM phenylmethylsulfonyl fluoride) for 30 min on ice. The supernatants containing the nuclear proteins were collected by centrifugation at 12,000 *g* for 10 min and stored at -70°. For the electrophoretic mobility assay, 10-μg aliquots of nuclear proteins were mixed with the labeled double-stranded NF-κB oligonucleotide, 5′-AGTTGAGGG-GACTTTCCCAGGC-3′, and incubated at room temperature for 20 min (underlining indicates κB consensus sequence or binding site for NF-κB/c-Rel homodimeric and heterodimeric complexes). The incubation mixture included 1 μg of poly(dI-dC) in a binding buffer (25 mM HEPES, pH 7.9; 0.5 mM EDTA; 0.5 mM DTT; 1% NP-40; 5% glycerol; 50 mM NaCl). The DNA/protein complex was electrophoresed on 4.5% nondenaturing polyacrylamide gels in 0.5x Tris/borate/EDTA buffer (0.0445 M Tris; 0.0445 M borate; 0.001 M EDTA). A double-stranded mutated oligonucleotide, 5′-AGTTGAGGCGACTTTC-CCAGGC-3′, was used to examine the specificity of binding of NF-κB to DNA (the underlined sequence is identical to the κB consensus sequence except for a G-to-C substitution in the NF-κB/Rel DNA binding motif). The specificity of binding was also examined by competition with the unlabeled oligonucleotide.

RESULTS

Effects of Flavonoids, Ellagitannins, and Anthraquinones on LPS-Induced NO Production in RAW264.7 Macrophages

The chemical structures of the two flavonoids (myricitrin, N1; oroxylin A, N2), four ellagitannins (penta-*O*-galloyl-β-glucopyranose, N3; woodfordin C, N4; oenothein B, N5; cuphiin D1, N6), and two anthraquinones (emodin, N7; physcion, N8) that were used in the present study are shown in Fig. 1. The effects of these eight compounds on NO production in RAW264.7 macrophages were investigated. The accumulated nitrite, estimated by the Griess method, in the culture medium was used as an index for NO synthesis from these cells. Each of these eight compounds, at a concentration of 20 μg/mL, did not interfere with the reaction between nitrite and Griess reagent (data not shown). Unstimulated macrophages, after 24 hr of incubation in culture, produced background levels of nitrite (Fig. 2). When the resting cells were incubated with each indicated compound alone, the concentration of nitrite in the medium was maintained at a background level similar to that in the unstimulated samples. After treatment with LPS (100 ng/mL) for 24 hr, nitrite concentration was increased markedly about 20-fold (~35 μM). When macrophages were incubated with various concentrations of each compound (5, 10, or 20 μg/mL) together with 100 ng/mL of LPS for 24 hr, significant concentration-dependent inhibition of nitrite production was found in the presence of oroxylin A (N2) and emodin (N7). The remaining compounds showed slight inhibition of LPS-induced nitrite production only at the highest concentration (20 μg/mL) (Fig. 2). Examination of the effects of these eight compounds at 20 μg/mL on RAW264.7 cell viability as determined by the MTT assay indicated that only emodin at this high concentration caused slight cytotoxicity (~30%), whereas other compounds did not affect cell viability (data not shown).

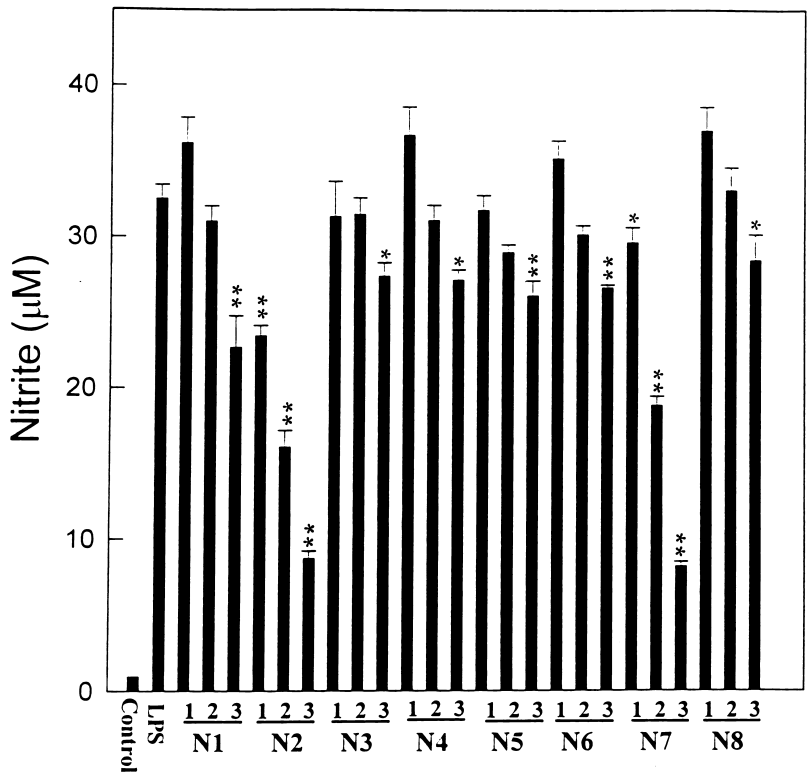


FIG. 2. Effects of various polyphenols (N1–N8) on LPS-induced nitrite production in RAW264.7 macrophages. The cells were treated with 100 ng/mL of LPS only (LPS) or LPS plus 5 μg/mL (1), 10 μg/mL (2), or 20 μg/mL (3) of each indicated compound (N1–N8) at 37° for 24 hr. At the end of each incubation, 100 μL of the medium was removed for measuring nitrite production. Control values were obtained in the absence of LPS or polyphenols. Data were obtained from three independent experiments and expressed as means ± SD. Key: (*) $P < 0.05$ and (**) $P < 0.01$ indicate significant differences from the LPS-treated group.

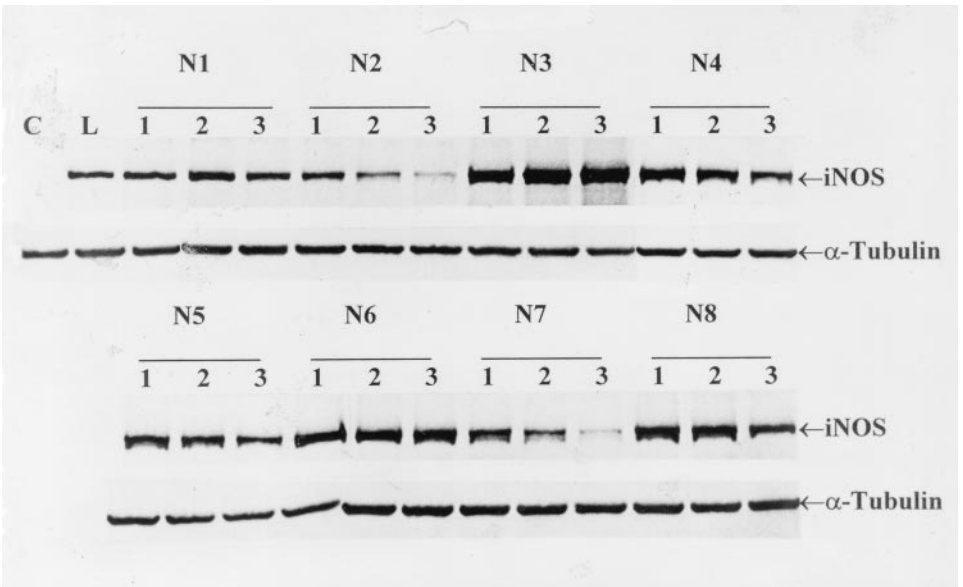


FIG. 3. Inhibition of LPS-induced iNOS protein in RAW264.7 macrophages by various polyphenolic compounds (N1–N8). The cells were treated as described in Fig. 2. Equal amounts of total proteins (50 μg/lane) were subjected to 10% SDS–PAGE, and expression of iNOS and α-tubulin protein was detected by western blotting using specific anti-iNOS and anti-α-tubulin antibodies. α-Tubulin protein was used as an internal control. C = control; L = LPS-treated.

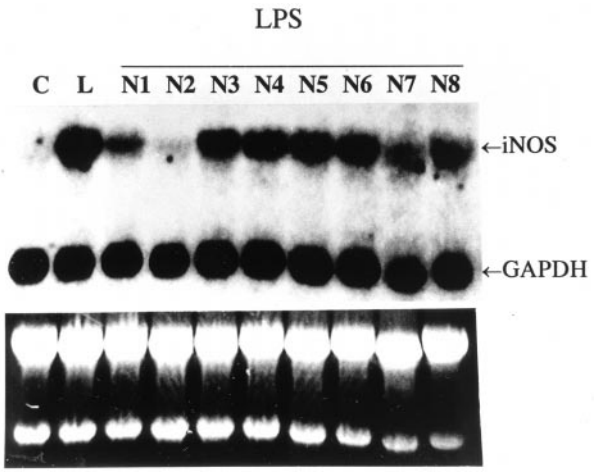


FIG. 4. Effects of various polyphenolic compounds (N1–N8) on LPS-induced iNOS mRNA in RAW264.7 macrophages. The cells were treated with 100 ng/mL of LPS only (L) or LPS plus 20 μg/mL of each indicated compound (N1–N8), and incubated at 37° for 7 hr. Detection of iNOS mRNA was performed by northern blotting using mouse iNOS cDNA as a probe, and GAPDH was used as an internal control. Ethidium bromide staining of 28S and 18S rRNA (lower panel) was performed to confirm that an equal amount of total RNA was loaded in each lane.

Effects of Various Polyphenolic Compounds on the Expression of iNOS and COX-2 Genes in RAW264.7 Macrophages

RAW264.7 macrophages did not express detectable iNOS protein (Fig. 3) or iNOS mRNA (Fig. 4) when incubated in the medium alone for 24 or 7 hr, respectively. The basal level of iNOS in RAW264.7 cells was not affected when incubated with each of the eight compounds alone (data not shown), whereas 100 ng/mL of LPS induced a dramatic increase in iNOS protein (Fig. 3) and mRNA (Fig. 4) in these cells. Among these eight compounds, only oroxylin A and emodin inhibited LPS-induced iNOS protein in a concentration-dependent manner, while N3, N4, N6, and N8 appeared to increase iNOS protein induced by LPS. The amount of α-tubulin protein as an internal control remained unchanged (Fig. 3). Among these compounds, oroxylin A was the most potent in inhibiting iNOS mRNA, followed by myricitrin. Emodin inhibited LPS-induced iNOS mRNA only slightly, while N3, N4, N6, and N8 did not seem to affect LPS-induced iNOS mRNA (Fig. 4).

Since NO may directly activate expression of COX isoforms, and induction of COX gene expression has been shown to be involved in LPS-mediated response [12–14], we investigated the effects of these eight compounds on LPS-induced COX-2 gene expression. The results indicated that only oroxylin A inhibited LPS-induced COX-2 gene expression at both mRNA and protein levels (Fig. 5, A and B). Furthermore, the marked increase in PGE_2, a product of the COX-2 enzyme, induced by LPS was inhibited significantly by oroxylin A (5–20 μg/mL). On the other hand, emodin and physcion at similar concentrations did not inhibit LPS-induced PGE_2 production (Fig. 5C).

Effect of Oroxylin A and Emodin on LPS-Induced iNOS and COX-2 Gene Expression in Bcl2-Overexpressing RAW264.7 Macrophages

RAW264.7 macrophages were transfected with the plasmid pC-Δj-bcl-2, also carrying a neomycin resistance gene. Stable Bcl-2 protein expression was assessed by western blot analysis with an anti-human Bcl-2 specific antibody. Two independent clones, Bcl2/RAW-1 and Bcl2/RAW-2, showed substantial Bcl-2 overexpression (Fig. 6A). Both clones expressed higher levels of Bcl-2 protein, and neomycin-vector transfected RAW264.7 cells (neo/RAW) lacked any human Bcl-2 protein. The endogenous iNOS protein in Bcl-2/RAW-1 and Bcl-2/RAW-2 cells was higher than that in neo/RAW cells, and the levels of iNOS protein were in the order Bcl-2/RAW-2 > Bcl-2/RAW-1 > neo/RAW. Upon treatment with 100 ng/mL of LPS, significant induction of iNOS protein (Fig. 6A) and nitrite production (Fig. 6B) was detected in Bcl-2/RAW-1 and neo/RAW cells; however, Bcl-2/RAW-2 cells were less sensitive to LPS treatment. To determine whether oroxylin A and emodin inhibited LPS-induced iNOS and COX-2 gene expression in Bcl-2-overexpressing RAW264.7 cells, both Bcl-2/RAW-1 and Bcl-2/RAW-2 were incubated with oroxylin A or emodin (20 μg/mL) followed by activation with LPS (100 ng/mL). Analysis of iNOS and COX-2 gene expression performed by northern and western blots indicated that oroxylin A (N2) and emodin (N7) inhibited LPS-induced nitrite production and iNOS gene expression in both Bcl-2-transfected cells (Figs. 7 and 8A). Upon analysis of COX-2 mRNA, oroxylin A but not emodin inhibited LPS-induced COX-2 mRNA in Bcl-2-overexpressing RAW264.7 macrophages (Fig. 8B). These results were in accordance with those derived from parental RAW264.7 macrophages.

Inhibition of LPS-Induced NF-κB Activation by Various Compounds

NF-κB is a transcription factor that is activated in response to stimulation by LPS, and activation of NF-κB is an essential step in inducing iNOS gene expression in macrophages [17]. To assess the effect of these compounds (20 μg/mL) on early stages of iNOS gene expression, the activation of NF-κB in RAW264.7 macrophages was examined using EMSA (Fig. 9, A and B). One hour following incubation with LPS, the binding of NF-κB was increased markedly in the nuclear fraction of macrophages. This inductive NF-κB binding activity was inhibited markedly by myricitrin, oroxylin A, and oenothein B (Fig. 9, A and B). In contrast, emodin did not affect the activation of NF-κB by LPS, although it inhibited nitrite production and iNOS gene expression. The NF-κB complex formation was blocked specifically by the addition of an excessive amount

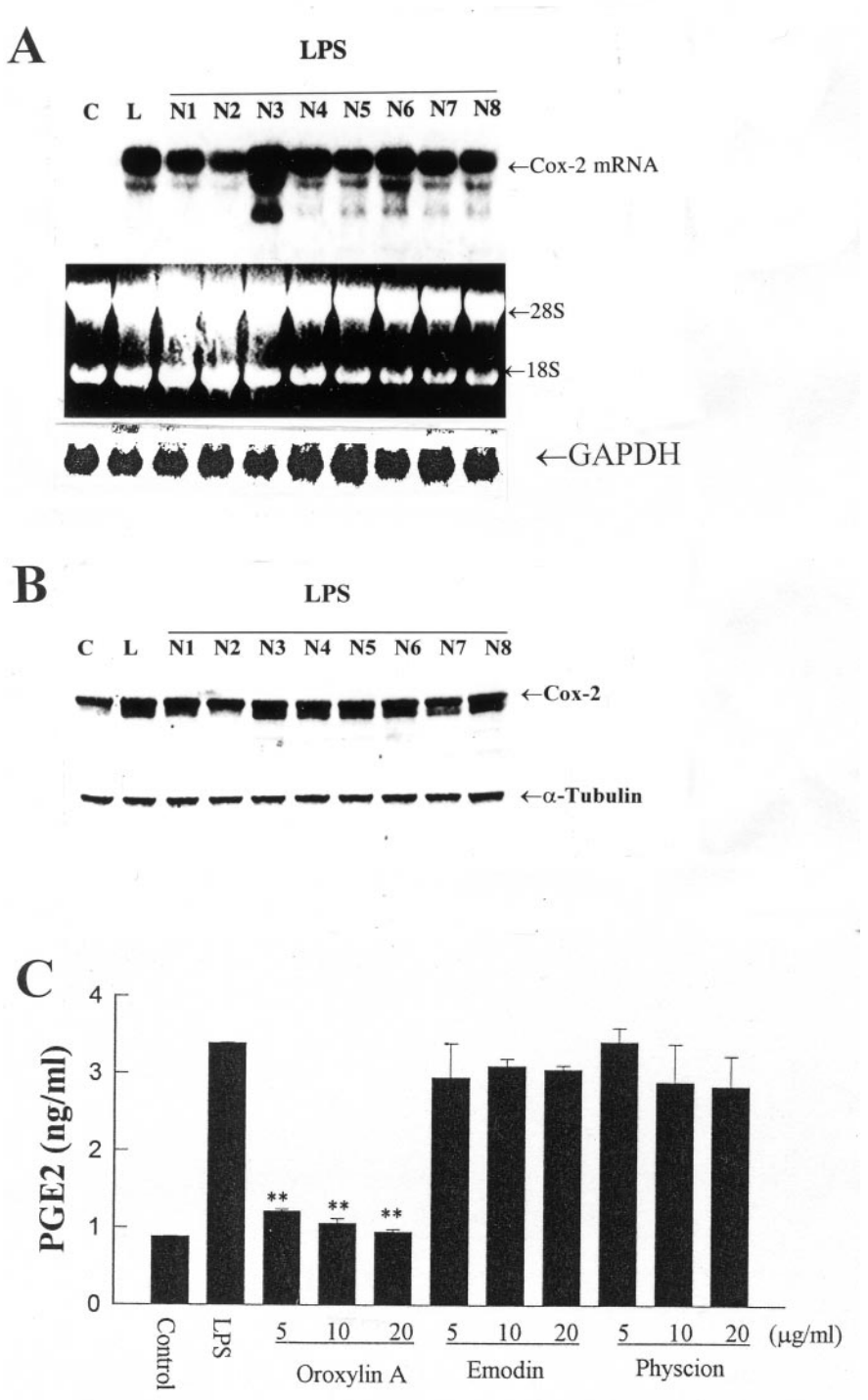


FIG. 5. Effects of various polyphenolic compounds (N1–N8) on expression of COX-2 mRNA and protein in RAW264.7 macrophages. Cells were treated with LPS (100 ng/mL) only (L), or LPS plus each indicated compound (20 μg/mL) for 24 hr for expression of COX-2 protein and 7 hr for COX-2 mRNA. (A) Expression of COX-2 mRNA was detected by northern blotting using mouse COX-2 cDNA as a probe. Ethidium bromide staining of total RNA and the amount of GAPDH were used to confirm that an equal amount of total RNA was loaded in each lane as indicated in the lower panel in A. (B) Detection of COX-2 protein was performed by western blotting using a specific anti-COX-2 antibody. α-Tubulin protein was used as an internal control among these eight compounds. (C) Effects of different polyphenolic compounds on LPS-induced PGE_2 synthesis in cultured smooth muscle cells. PGE_2 concentrations in the culture medium were measured by ELISA. Key: (**) significantly different ($P < 0.01$) from LPS-treated samples.

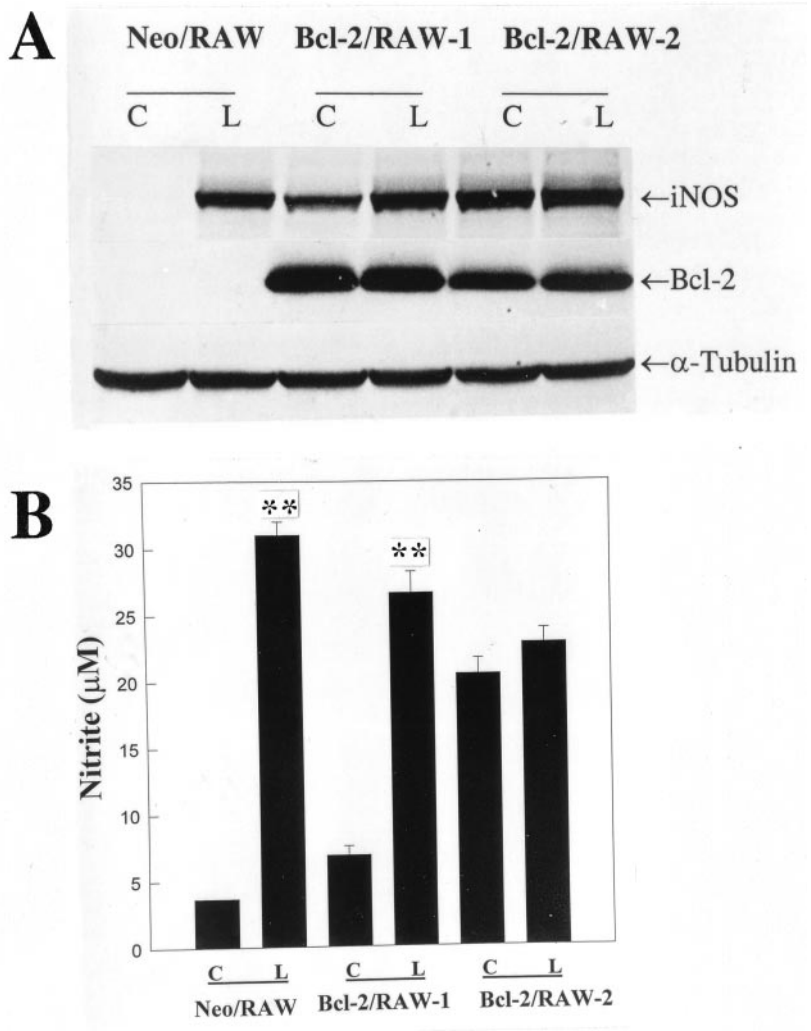


FIG. 6. Detection of iNOS protein and nitrite production in LPS-treated parental RAW264.7 and Bcl-2-overexpressing RAW 264.7 cells (Bcl-2/RAW-1 and Bcl-2/RAW-2). Bcl-2/RAW-1 and Bcl-2/RAW-2 cells were derived from two independent transfection experiments and grew well in G418-containing medium. The cells were treated with (L) or without (C) LPS (100 ng/mL) for 24 hr. iNOS and Bcl-2 proteins were detected by western blotting using specific antibodies (panel A). Measurement of nitrite production in control and LPS-treated RAW264.7 (Neo/RAW), Bcl-2/RAW-1, and Bcl-2/RAW-2 cells is shown in panel B. Data were obtained from three independent experiments and expressed as means ± SD. Key: (**) significantly different ($P < 0.01$) from the respective control.

of specific unlabeled consensus oligomer, but was not inhibited by the mutated unlabeled oligomer (data not shown). To further demonstrate that inhibition of NF-κB activation by oroxylin A, a Luc-reporter plasmid containing 5 NF-κB binding sites in the enhancer element was transfected transiently into RAW264.7 cells, and analysis of luciferase activity was performed to identify the levels of NF-κB activation. The results indicated that oroxylin A efficiently decreased the LPS-induced luciferase activity by 4-fold (Fig. 9C). These data were consistent with the results of analysis of NF-κB binding by EMSA. The heteromeric NF-κB complex is sequestered in the cytoplasm as an inactive precursor complexed with an inhibitory protein, an IκB-like protein, and LPS induced NF-κB activation through increasing nuclear p65 protein associated with a decreased cytosolic IκB protein. Incubation of RAW264.7 macrophages with 100 ng/mL of LPS for 30 min resulted in increased NF-κB (p65) in the nuclear fraction and decreased IκB protein in the cytosol (Fig. 9D). This phenomenon was inhibited significantly by 20 μg/mL of myricitrin (N1), oroxylin A (N2), penta-*O*-galloyl-β-glucopyranose (N3), and oenothein B (N5) (Fig. 9C). Emodin (N7) and cuphiin D1 (N6) treatment did not block the increased p65 in the nucleus or degradation of IκB in the cytosol induced by LPS.

DISCUSSION

Results of the present study indicated that several naturally occurring polyphenolic compounds such as flavonoids, ellagitannins, and anthraquinones differentially inhibited LPS-induced NO production and iNOS gene expression in RAW264.7 macrophages. Among eight polyphenolic compounds examined, oroxylin A and emodin were the most

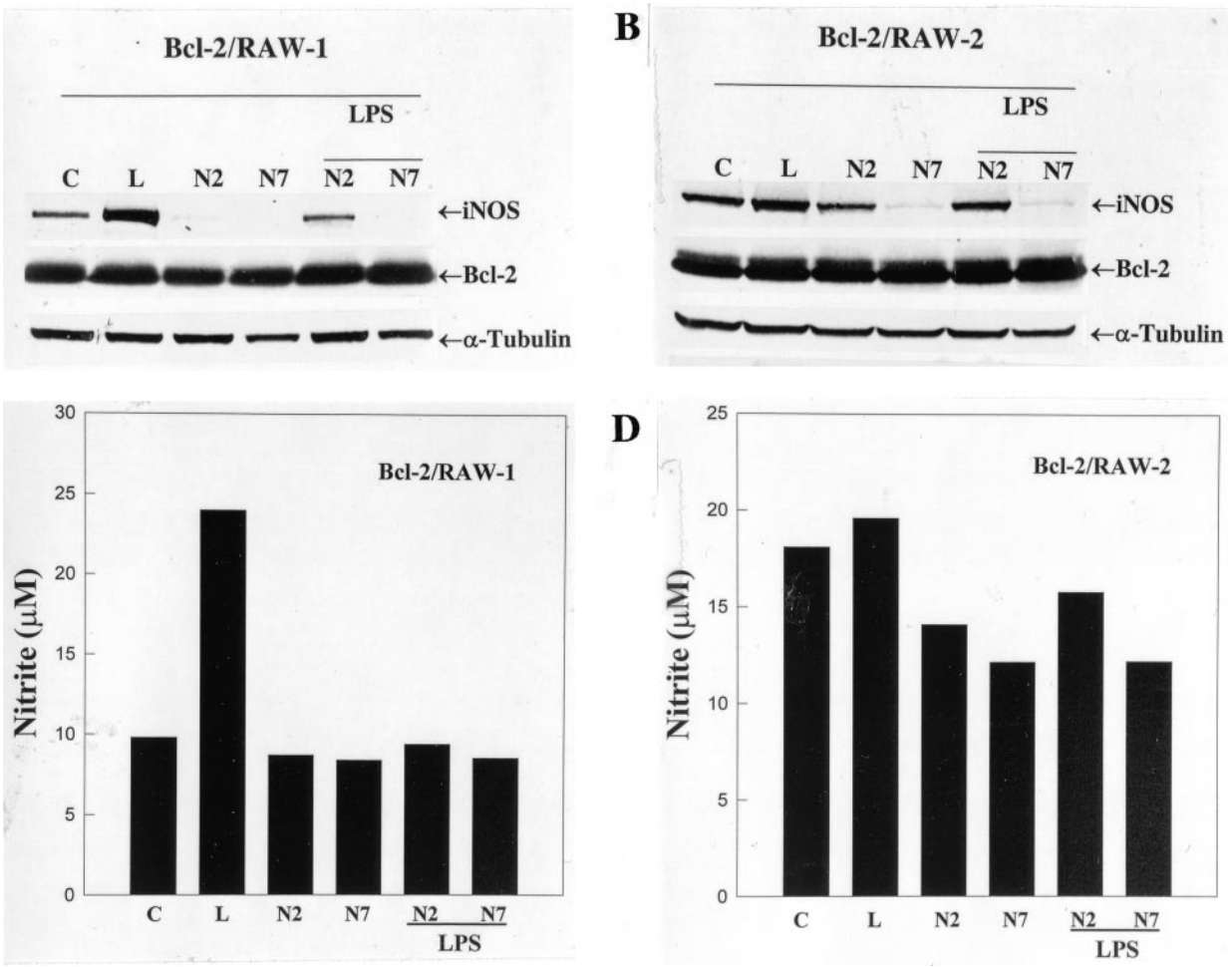


FIG. 7. Effects of oroxylin A (N2) and emodin (N7) on LPS-induced iNOS protein and nitrite production in Bcl-2/RAW-1 and Bcl-2/RAW-2 cells. The cells were treated with 100 ng/mL of LPS (L), 20 μg/mL of oroxylin A (N2), or 20 μg/mL of emodin (N7) only, or LPS plus 20 μg/mL of oroxylin A or LPS plus 20 μg/mL of emodin. Panels A and B show detection of iNOS, Bcl-2, and α-tubulin proteins. Panels C and D show nitrite production in treated cells as described in Fig. 2. Each value is the mean of three independent experiments.

potent inhibitors of LPS-induced NO production and iNOS gene expression. The remaining compounds (myricitrin, penta-*O*-galloyl-β-glucopyranose, woodfordin, oenothein B, cuphiin D1, and physcion) slightly but significantly inhibited LPS-induced NO production only at a high concentration (20 μg/mL). The mechanisms of inhibition of iNOS by oroxylin A and emodin, however, were different. The former but not the latter inhibited NF-κB activation. In addition, oroxylin A but not emodin or other phenolic compounds inhibited COX-2 gene expression. The latter finding is consistent with reports by others that the immunosuppressive effect of emodin in human mononuclear cells is not affected by indomethacin (a nonspecific COX-2 inhibitor) [26].

Oroxylin A used in the present study was isolated from Scutellariae radix (*Huang Qin*), the root of *S. baicalensis* Georgi (Labiatae). *Huang Qin* has been used in Chinese medicine as a remedy for treating inflammation, suppurative dermatitis, allergic diseases, hyperlipemia, and arteriosclerosis [27]. The active principle in *Huang Qin* that exhibits these beneficial effects, however, has not been determined fully. Results of the present study indicated that oroxylin A inhibited the LPS induction of iNOS and COX-2 gene expression in macrophages without appreciable cytotoxic effects. These findings were consistent with a decrease caused by oroxylin A in LPS-induced NO and PGE_2 production. These results suggest that oroxylin A is an important active principle in *Huang Qin*, which may play an important role in inhibiting inflammatory processes.

It should be noted that other phenolic compounds such as penta-*O*-galloyl-β-glucopyranose (N3), woodfordin C (N4), cuphiin D1 (N6), and physcion (N8) increased iNOS proteins induced by LPS. These four compounds, however, only slightly affected or did not affect LPS-induced iNOS mRNA expression, and they also did not increase LPS-induced NO production. At higher concentrations (20 μg/mL), however, these compounds slightly but significantly inhibited NO production induced by LPS. The reason for the discrepancy between iNOS proteins and NO production in the presence of these compounds (in particular, penta-*O*-galloyl-β-glucopyranose and cuphiin

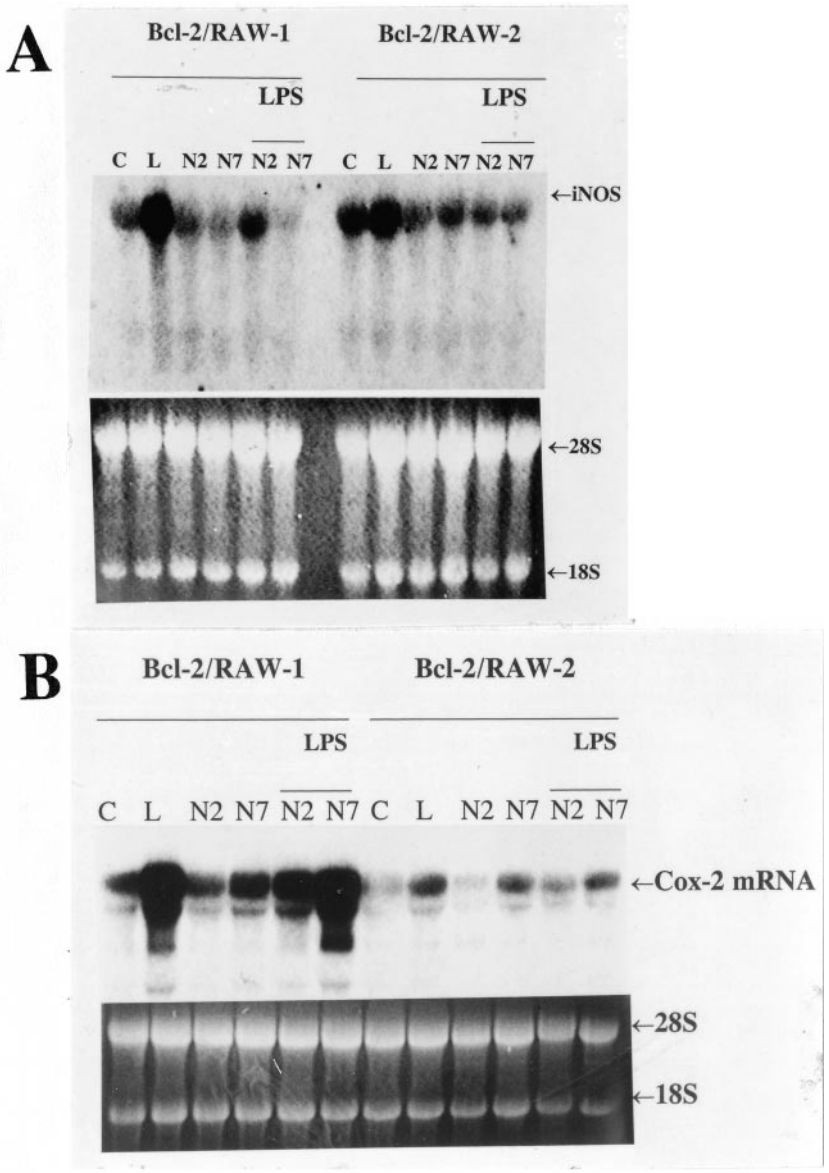


FIG. 8. Effects of oroxylin A (N2) and emodin (N7) on LPS-induced iNOS (A) and COX-2 mRNA (B) in Bcl-2-overexpressing RAW264.7 cells. Bcl-2/RAW-1 and Bcl-2/RAW-2 cells were treated with 100 ng/mL of LPS only (L), LPS plus oroxylin A (N2), or LPS plus emodin (N7). iNOS and COX-2 mRNA were detected by northern blotting using mouse iNOS and COX-2 cDNA as a probe. Ethidium bromide staining of total RNA was shown in the lower blots of panels A and B to confirm that a similar amount of total RNA was loaded in each lane.

D1) is not known. The reason for the increase in iNOS proteins was not examined in the present study. It is possible that these compounds may scavenge NO production through their antioxidant effects [28]. Accordingly, minor net changes in NO concentrations were not detected.

On the other hand, myricitrin at a high concentration (20 μg/mL) inhibited LPS-induced iNOS mRNA significantly, but inhibited LPS-induced iNOS protein and NO production only slightly. At much higher concentrations (40 or 80 μg/mL), myricitrin did inhibit LPS-induced iNOS expression in mRNA, protein levels, and NO production (our preliminary data). It appears that myricitrin also is an inhibitor of iNOS expression, but it is less potent than oroxylin A in inhibiting expression of this enzyme.

Emodin (N7), an anthraquinone derivative isolated from *R. palmatum*, which also has been used in Chinese medicine to treat various diseases including inflammation [26], is another potent inhibitor of LPS-induced NO production and iNOS protein synthesis. At 20 μg/mL, it almost abolished LPS-induced iNOS protein synthesis and NO production. However, it reduced LPS-induced iNOS mRNA expression only slightly. Among the eight compounds examined in the present study, emodin was the only compound exhibiting cytotoxicity in about 30% of the cells at 24 hr post-treatment, whereas the other compounds did not affect cell viability at the concentrations examined. This result suggests that the decrease in iNOS proteins and NO production 24 hr after treatment with emodin may be due, in part, to its cytotoxic effect on cell viability.

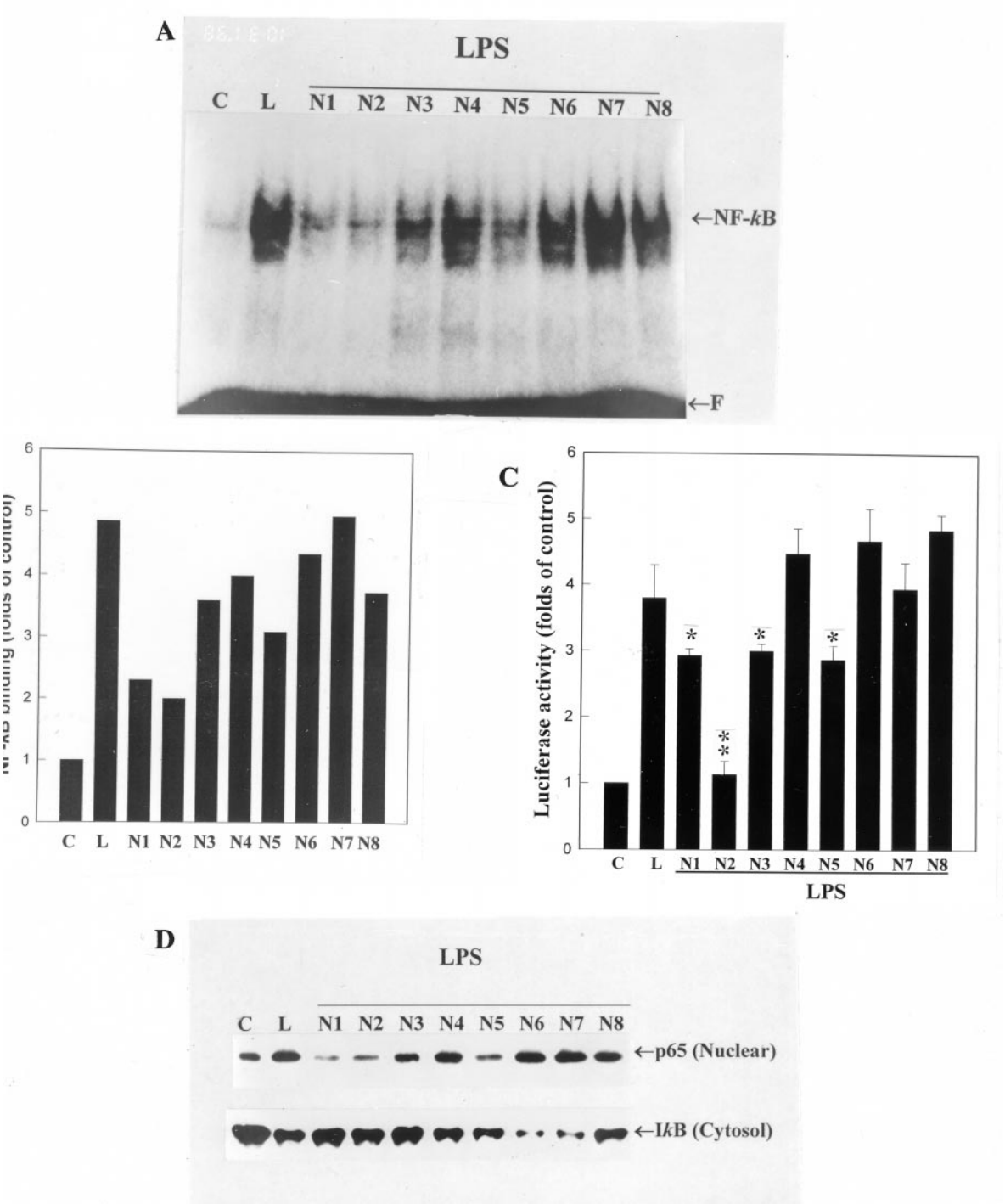


FIG. 9. Effects of various polyphenolic compounds (N1–N8) on LPS-induced NF-κB binding in RAW264.7 macrophages. (A) The cells were treated with 100 ng/mL of LPS only (L) or LPS plus 20 μg/mL of each indicated compound for 1 hr. Detection of NF-κB binding activities was performed by EMSA. (B) The intensity of each band was quantitated by densitometry analysis (IS-1000), and the intensity of the control group (C) was defined as 1. Panel C indicates effects of various polyphenols on LPS-induced NF-κB transcriptional activation. A reporter plasmid containing 5 NF-κB sites in its enhancer element was transiently transfected into RAW264.7 cells for 48 hr and further treated with LPS only, or LPS plus each indicated compound (20 μg/mL) for 1 hr. The luciferase activity was measured by LUCILITE, and the control group value was defined as 1. Data were derived from three independent experiments and expressed as means ± SD. Key: (*) $P < 0.05$ and (**) $P < 0.01$ indicate a significant difference from the LPS-only treated group. (D) Effects of various polyphenols on LPS-induced p65 protein in the nucleus and IκB degradation in cytosol. The cells were treated as described in panel A, and analysis of p65 and IκB proteins was performed by western blotting using specific anti-p65 or anti-IκB antibodies.

It has been established that RAW264.7 macrophages are highly susceptible to endogenously generated or exogenously supplied NO [29]. Treatment of RAW264.7 macrophages with LPS and IFN-γ has been shown to result in NOS induction and apoptosis. Both nitrite accumulation and apoptosis were blocked by the NOS inhibitor N^G-monomethyl-L-arginine. Meβmer *et al.* [30] reported that Bcl-2-overexpressing RAW264.7 macrophages appeared highly resistant to LPS/IFN-γ-induced apoptosis, although inducible NO synthase levels increased with concomitant nitrite production similar to parental cells [30]. In the present study, Bcl-2-overexpressing RAW264.7 cells showed higher endogenous iNOS proteins and nitrite production under unstimulated conditions than did neotransfected cells. The inhibitory effects of oroxylin A on LPS-induced iNOS and COX-2 in Bcl-2 transfectants were similar to those derived from parental RAW264.7 macrophages, indicating that Bcl-2 proteins did not affect the inhibitory effect of oroxylin A on LPS-induced iNOS and COX-2 gene expression. Bcl-2 proteins have been shown to function in an antioxidant pathway and to protect various types of cells by inhibiting formation or action of reactive oxygen species [31, 32], and inducing endogenous cellular antioxidants [33, 34]. Accordingly, comparable inhibition by oroxylin A of LPS-induced iNOS and COX-2 expression in both parental and Bcl-2-overexpressing RAW264.7 macrophages suggests that the inhibitory effect of oroxylin A on LPS-induced iNOS and COX-2 expression was unlikely due to its antioxidant property.

The expression of iNOS in murine macrophages has been shown to be dependent on NF-κB activation [35]. The possibility that oroxylin A may inhibit the activity of NF-κB was examined. The results indicated that oroxylin A inhibition of expression of both iNOS and COX-2 mRNA and proteins was most likely due to oroxylin A suppression of NF-κB activation. This is consistent with the reports that NF-κB response elements are present on the promoters for both iNOS and COX-2 genes [35–40]. NF-κB is composed mainly of two proteins, p50 and p65 [35]. Under unstimulated conditions, NF-κB is present in the cytosol and is bound to the inhibitory protein IκB. After induction by a variety of agents such as LPS, IκB is phosphorylated to trigger proteolytic degradation of IκB. NF-κB is then released from IκB and is translocated into the nucleus. In the present study, we found an inhibition of LPS-induced IκB degradation in cytosol with an inhibition of p65 protein increase in the nucleus by oroxylin A but not emodin, indicating that oroxylin A blocked LPS-induced activation of NF-κB. These results suggest that oroxylin A inhibition of LPS-induced expression of the iNOS and COX-2 genes occurs through blocking NF-κB activation, although inhibition of other factors such as AP-1, IFN-γ response elements (IRF-1), and γ-activated site (GAS) may be involved [41–44]. There are several transcription factor binding sites located on the promoter of the murine iNOS gene such as IFN-γ response elements (IRF), GAS, OCT-1, and NF-κB sites. In the present study, inhibition of LPS-induced iNOS gene expression by oroxylin A was found to correlate well with the inhibition of NF-κB binding and *trans*-activating activities as seen by EMSA and NF-κB-Luc transient transfection assays. The binding activities of AP-1, IRF-1, and OCT-1, however, did not change significantly in oroxylin A-treated macrophages (our unpublished data). These results again suggest that inhibition of LPS-induced iNOS expression by oroxylin A most likely occurs through blocking NF-κB. The failure of emodin to block LPS-induced NF-κB activation suggests that emodin inhibits LPS-activated iNOS gene expression by blocking binding activities of different transcription factors [41, 42].

In summary, results of the present study indicated that oroxylin A was an effective inhibitor of LPS-induced iNOS and COX-2 gene expression by blocking NF-κB activation in RAW264.7 macrophages. Emodin, which also inhibited LPS-induced nitrite production and iNOS protein without blocking COX-2 gene expression, appeared to act through a different transcription pathway. Other polyphenols, including myricitrin, penta-*O*-galloyl-β-glucopyranose, woodfordin C, oenothein B, cuphiin D1, and physcion, slightly inhibited LPS-induced NO production only at the highest concentration examined (20 μg/mL). Oroxylin A, an active component of the Chinese herb *Huang Qin*, appears to be a potential therapeutic agent for treating LPS-induced sepsis syndrome.

The authors gratefully acknowledge the financial support of this work from the Juridical Person of Yen's Foundation in Taiwan, and grants from the National Institutes of Health HL 27763 and HL47574, and SIU/EAM & CRC.

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