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Wogonin, baicalin, and baicalein inhibition of inducible nitric oxide synthase and cyclooxygenase-2 gene expressions induced by nitric oxide synthase inhibitors and lipopolysaccharide

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Abstract

We previously reported that oroxylin A, a polyphenolic compound, was a potent inhibitor of lipopolysaccharide (LPS)-induced expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). In the present study, three oroxylin A structurally related polyphenols isolated from the Chinese herb Huang Qui, namely baicalin, baicalein, and wogonin, were examined for their effects on LPS-induced nitric oxide (NO) production and iNOS and COX-2 gene expressions in RAW 264.7 macrophages. The results indicated that these three polyphenolic compounds inhibited LPS-induced NO production in a concentration-dependent manner without a notable cytotoxic effect on these cells. The decrease in NO production was in parallel with the inhibition by these polyphenolic compounds of LPS-induced *iNOS* gene expression. However, these three compounds did not directly affect iNOS enzyme activity. In addition, wogonin, but not baicalin or baicalein, inhibited LPS-induced prostaglandin E_2 (PGE_2) production and *COX-2* gene expression without affecting COX-2 enzyme activity. Furthermore, *N*-nitro-L-arginine (NLA) and *N*-nitro-L-arginine methyl ester (L-NAME) pretreatment enhanced LPS-induced iNOS (but not COX-2) protein expression, which was inhibited by these three polyphenolic compounds. Wogonin, but not baicalin or baicalein, similarly inhibited PGE_2 production and COX-2 protein expression in NLA/LPS or L-NAME/LPS-co-treated RAW 264.7 cells. These results indicated that co-treatment with NOS inhibitors and polyphenolic compounds such as wogonin effectively blocks acute production of NO and, at the same time, inhibits expression of *iNOS* and *COX-2* genes.

Keywords: Baicalin; Baicalein; Wogonin; Lipopolysaccharide; Inducible nitric oxide synthase; Cyclooxygenase-2

1. Introduction

Macrophages play an important role in the host defense mechanism against bacterial as well as viral infections [1,2]. When activated by bacterial toxins such as LPS or lipoteicholic acid (LTA), macrophages inhibit the growth of a wide variety of tumor cells and invade microorganisms through releasing factors such as NO, cytokines, tumor necrosis factor-α, and eicosanoid mediators of the immune response [3]. NO has been shown to be a significant regulatory molecule in diverse physiological functions including vasodilation, neural communication, and host defense [4,5]. Molecule cloning and sequencing analysis have revealed at least three types of NOS isoforms existing in cells [6–8]. NOS isozymes that appear in the vascular endothelium (eNOS) and central and peripheral neurons (nNOS) are constitutive (cNOS). Release of NO catalyzed by cNOS plays a role in maintaining active vasodilation through a Ca^{2+}-dependent pathway. On the other hand, NOS in macrophages and hepatocytes is inducible, and its activation is Ca^{2+}-independent. Following exposure to LPS or cytokines, iNOS can be induced in various cells such as mac-

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Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, LPS, lipopolysaccharide; NLA, *N*-nitro-L-arginine; and L-NAME, *N*-nitro-L-arginine methyl ester.

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rophages, Kupffer cells, smooth muscle cells, and hepatocytes. iNOS activation catalyzes the formation of a large amount of NO, which plays a key role in the pathogenesis of a variety of diseases including septic shock [9–11]. Therefore, NO production by iNOS may reflect the degree of inflammation and provides a measure to assess the effect of drugs on the inflammatory process.

Cyclooxygenase is the enzyme catalyzing the conversion of arachidonic acid to prostaglandin H_2, the precursor of a variety of biological active mediators such as PGE_2, prostacyclin, and thromboxane A_2 [12–14]. Two forms of this enzyme have been identified: COX-1, a constitutive cyclooxygenase, and COX-2, an isoform induced in response to many stimulants and activated at the inflammatory site to give rise to pain, swelling, and stiffness [15–17]. Recent findings have suggested that COX-2 may play important roles in the pathogenesis of diseases such as colon carcinoma, Alzheimer's disease, heart failure, and hypertension [18–20]. Therefore, there is an increasing interest in the usefulness of COX-2 inhibitors.

Medicinal plants have been used as traditional remedies for hundreds of years. *Scutellaria baicalensis* Georgi (Huang Qui) is one of the important medicinal herbs widely used for the treatment of various inflammatory diseases, hepatitis, tumors, and diarrhea in East Asian countries such as China, Korea, Taiwan, and Japan [21]. The plant has been reported to contain a large number of flavonoids, frequently found as glucosides and other constituents, including phenethyl alcohols, sterols, and essential oils and amino acids. In our previous study, oroxylin A (a polyphenolic compound) isolated from Huang Qui was found to be a potent inhibitor of LPS-induced NO and PGE_2 productions by blocking *iNOS* and *COX-2* gene activation [22]. In the present study, three oroxylin A structurally related polyphenolic compounds, i.e. baicalin, baicalein, and wogonin, were examined for their effects on LPS-induced *iNOS* and *COX-2* gene expression. The results demonstrated that baicalin, baicalein, and wogonin significantly inhibited LPS-induced NO production and *iNOS* gene expression in a concentration-dependent manner, but did not inhibit iNOS enzyme activity. Furthermore, wogonin, but not baicalin or baicalein, inhibited LPS-induced PGE_2 production and *COX-2* gene expression. Similar results were obtained in NLA or L-NAME plus LPS-treated RAW 264.7 macrophages.

2. Materials and methods

2.1. Cells

RAW 264.7, 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 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.

2.2. Agents

Three structurally related polyphenolic compounds (baicalin, baicalein, and wogonin) were isolated from the Chinese herbal plant Huang Qui. Sulfanilamide, naphthylethylenediamine dihydrochloride, NLA, L-NAME, indomethacin, and LPS were obtained from Sigma Chemical Co.

2.3. Cell viability assay

RAW 264.7 cells were plated at a density of 10^4 cells/well into 96-well plates. After overnight growth, cells were treated with a different concentration of flavonoids for 24 hr. At the end of treatment, 20 μL of combined solution of the tetrazolium compound MTT and an electron coupling reagent, phenazine methosulfate, were added to each well. After incubation for 1 hr at 37° in a humidified 5% CO_2 atmosphere, absorbance at 600 nm was recorded using an ELISA plate reader.

2.4. Extraction and isolation

Dried *S. baicalensis* roots (5 kg) were cut into small pieces, immersed, and extracted with acetone (50 L $\times$ 2) at room temperature for two weeks. After filtration, the residues were then reflux-extracted with 50% aqueous ethanol (20 L $\times$ 2). Acetone and 50% aqueous ethanol extracts were concentrated under reduced pressure to 1 and 5 L, respectively. Ethanol was added into the concentrated 50% aqueous ethanol extracts and produced a large amount of yellow precipitate. A portion of the precipitate (5.0 g) was recrystallized with aqueous ethanol to obtain baicalin (3.6 g). The acetone extracts were subjected to column chromatography on silica gel (10 cm i.d. $\times$ 30 cm) eluted with $CHCl_3$ and $CHCl_3$–MeOH (10:1 to 1:1 gradient) to yield a total ten fraction. $CHCl_3$ elute was coated with Celite 545 (Merck) and rechromatographed on silica gel (2 cm i.d. $\times$ 30 cm) eluted with hexane–acetone (10:1 to 2:1 gradient) to yield oroxylin A (740 mg) and wogonin (1.5 g). A portion of the $CHCl_3$–MeOH (10:1) elute (4 g) was subjected to gel permeation chromatography on Sephadex LH-20 (2 cm i.d. $\times$ 45 cm) eluted with MeOH to yield baicalein (2.5 g). Each compound was identified by direct comparison of its spectroscopic data with authentic samples. Purity tests of baicalin, baicalein, oroxylin A, and wogonin were performed by HPLC equipped with a 280-nm detector and LiChrospher 100 RP-18e column (4 mm i.d. $\times$ 125 mm). The mobile phase was composed of CH_3CN–0.1 M H_3PO_4 (28:72) and the flow rate was 1.0 mL/min. The purity of all compounds was more than 99.5%.

2.5. 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 dihydrocholide in water); absorbance of the mixture at 550 nm was determined with an ELISA plate reader (Dynatech MR-7000; Dynatech Laboratories Inc.).

2.6. Measurement of iNOS enzyme activity

For the assay in intact cells, RAW 264.7 cells were plated in 100-mm tissue culture dishes (4×10^6 cells) and incubated with LPS (100 ng/mL) for 12 hr. Then, the cells were washed twice with PBS. Cells were harvested and plated into a 96-well plate (2×10^5 cells/well) and incubated in the absence or presence of tested compounds for a further 12 hr without LPS in medium. The supernatants were removed, and the Griess reaction was performed as above. For the assay in cell lysates, RAW 264.7 cells were washed three times with PBS, scraped into cold PBS, and centrifuged at $500 \times g$ for 10 min at 4°. The cell pellet was resuspended in 0.5 mL 40 mM Tris buffer (pH 8.0) containing 5 mg/mL of pepstatin A, 1 μg/mL of chymostatin, 5 μg/mL of aprotinin, and 100 μM phenylmethylsulfonyl fluoride and lysed by three freeze–thaw cycles. Aliquots of the lysate were used for Bradford protein assay. iNOS enzyme activity was measured as described [24]. Briefly, 50 mg of cell lysate protein was incubated in 20 mM Tris–HCl (pH 7.9) containing 4 μM FAD, 4 μM tetrahydrobiopterin, 3 mM dithiothreitol (DTT), and 2 mM each of L-arginine and NADPH. The reaction was carried out in duplicate for 180 min at 37° in 96-well plates. Residual NADPH was oxidized enzymatically and the Griess reaction was performed as above.

2.7. Western blot analysis

Total cellular extracts were prepared according to our previous papers [25,26], separated on 8% SDS–polyacrylamide minigels, and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). The membrane was incubated overnight at 4° with 1% BSA and then incubated with anti-iNOS, anti-Cox-2, or anti-α-tubulin monoclonal antibodies (Transduction Laboratories). Expression of protein was detected by staining with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma Chemical Co.).

2.8. Measurement of COX-2 enzyme activity

RAW 264.7 cells were plated at 1×10^5 cells/well in a 12-well plate and incubated for 6 hr with LPS. The cell supernatants were removed, and cells in each well were washed twice with fresh culture medium and allowed to equilibrate in the absence of tested compounds for 30 min. The cells were further incubated with 100 μM arachidonic acid for 15 min without LPS in medium. The supernatants were removed and assayed for PGE_2.

2.9. Measurement of PGE_2 production

RAW 264.7 cells were subcultured in 6-well plates and incubated with indicated compounds for 12 hr. One hundred microliters of supernatant of culture medium was collected for the determination of PGE_2 concentration by ELISA (Cayman Enzyme Immunoassay kit).

2.10. Statistics

The values are expressed as means ± SE. The significance of difference from the respective controls for each experimental test condition was assayed by using Student's *t*-test for each paired experiment. *P* values <0.05 were regarded as indicating significant differences.

3. Results

3.1. Inhibition of LPS-induced NO production by baicalin, baicalein, and wogonin in RAW 264.7 macrophages

The chemical structures of baicalin, baicalein, and wogonin are shown in Fig. 1. These polyphenolic compounds are flavonoids. The extraction and isolation of each compound from the Chinese herb Huang Qui (*S. baicalensis*) was described in Materials and Methods, and the purity of each compound was more than 99.5%. The effects of baicalin, baicalein, and wogonin on LPS-induced NO production in RAW 264.7 macrophages were investigated by measuring the accumulated nitrite, as estimated by the Griess reaction, in the culture medium. Baicalin, baicalein, and wogonin at 40 μM did not interfere with the reaction between nitrite and Griess reagents (data not shown). Unstimulated macrophages, after 24 hr of incubation in culture medium, produced background levels of nitrite (Fig. 2). When the cells were incubated with the indicated compounds 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 concentrations in the medium increased remarkably by about 20-fold ($\sim$30 μM). When RAW 264.7 macrophages were treated with different concentrations of the indicated compounds together with LPS (100 μg/mL) for 24 hr, a significant concentration–dependent inhibition of nitrite production was detected in the presence of baicalin, baicalein, and wogonin. The IC_{50} values of the three polyphenols in inhibiting LPS-induced NO production were 15 ± 1.4, 19.4 ± 1.0, and 9.5 ± 0.8 μM (N = 3), respectively. The rank of potencies in inhibiting LPS-induced NO production

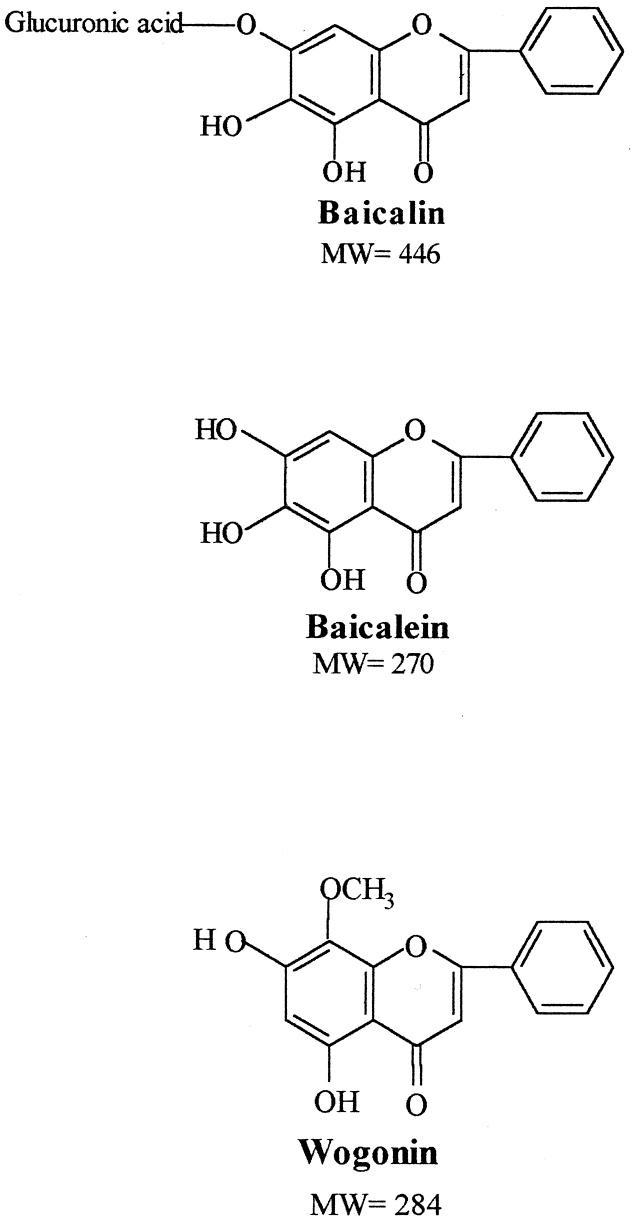


Fig. 1. Chemical structures of baicalin, baicalein, and wogonin.

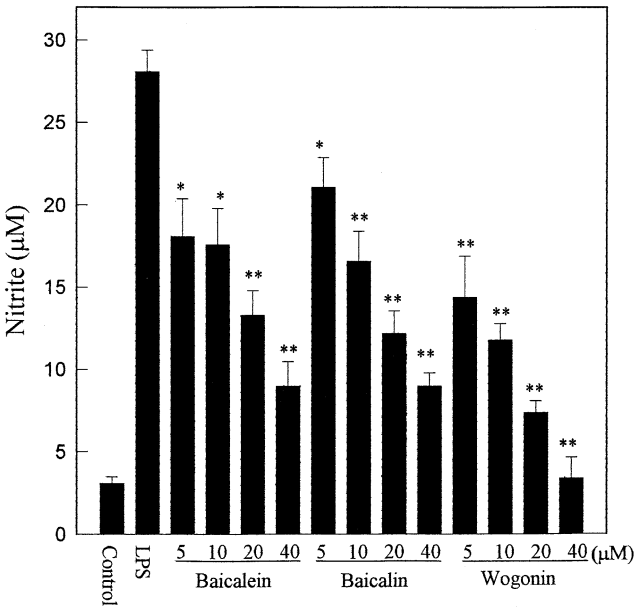


Fig. 2. Effects of baicalin, baicalein, and wogonin on LPS-induced nitrite production in RAW 264.7 macrophages. The cells were treated with 100 ng/mL of LPS only (LPS) or LPS plus different concentrations (5, 10, 20, and 40 μM) of baicalin, baicalein, or wogonin at 37° for 24 hr. At the end of incubation, 100 μL of the medium was removed to measure nitrite production. Control values were obtained in the absence of LPS or polyphenols. Data were derived from three independent experiments and expressed as means ± SEM. *$P < 0.05$ and **$P < 0.01$ indicate statistically significant differences from the LPS-treated group.

was wogonin > baicalin > baicalein ($P < 0.01$, N = 3) (Fig. 2). Examination of the cytotoxicity of baicalin, baicalein, and wogonin in RAW 264.7 macrophages by MTT assay indicated that all three compounds, even at 40 μM, did not affect the viability of RAW 264.7 cells (data not shown). Therefore, inhibition of LPS-induced nitrite production by baicalin, baicalein, and wogonin was not the result of a possible cytotoxic effect on these cells.

3.2. Baicalin, baicalein, and wogonin inhibition of LPS-induced iNOS gene expression by Western blot analysis

RAW 264.7 cells did not express detectable iNOS protein when incubated in the medium without LPS for 24 hr, and the basal level of iNOS protein was not affected when incubated with baicalin, baicalein, or wogonin (data not shown). Upon LPS (100 ng/mL) treatment for 24 hr, iNOS protein drastically increased in these cells, and co-treatment of cells with LPS (100 ng/mL) and different concentrations (20 and 40 μM) of the indicated compounds for 24 hr significantly inhibited iNOS protein induction in RAW 264.7 macrophages (Fig. 3). The amount of α-tubulin protein as an internal control remained unchanged.

3.3. Wogonin inhibition of LPS-induced PGE_2 production and COX-2 gene expression

Unstimulated RAW 264.7 macrophages in culture medium for 24 hr produced a basal amount of PGE_2 (1.5 ± 0.2 ng/mL) in the medium. After treatment with LPS (100 ng/mL) for 24 hr, the medium concentration of PGE_2 elevated significantly to 7 ± 0.7 ng/mL. This increase was inhibited by co-treatment of cells with different concentrations of wogonin (Fig. 4). However, LPS-induced PGE_2 production was not inhibited by baicalin or baicalein except at 40 μM, the highest concentration examined. Western blot analysis demonstrated that unstimulated RAW 264.7 macrophages expressed only a small amount of COX-2 proteins. Baicalin, baicalein, and wogonin treatment alone did not affect the basal COX-2 expression. Upon LPS (100 ng/mL) treatment for 24 hr, COX-2 protein drastically increased in these cells. The increase was significantly inhibited by co-

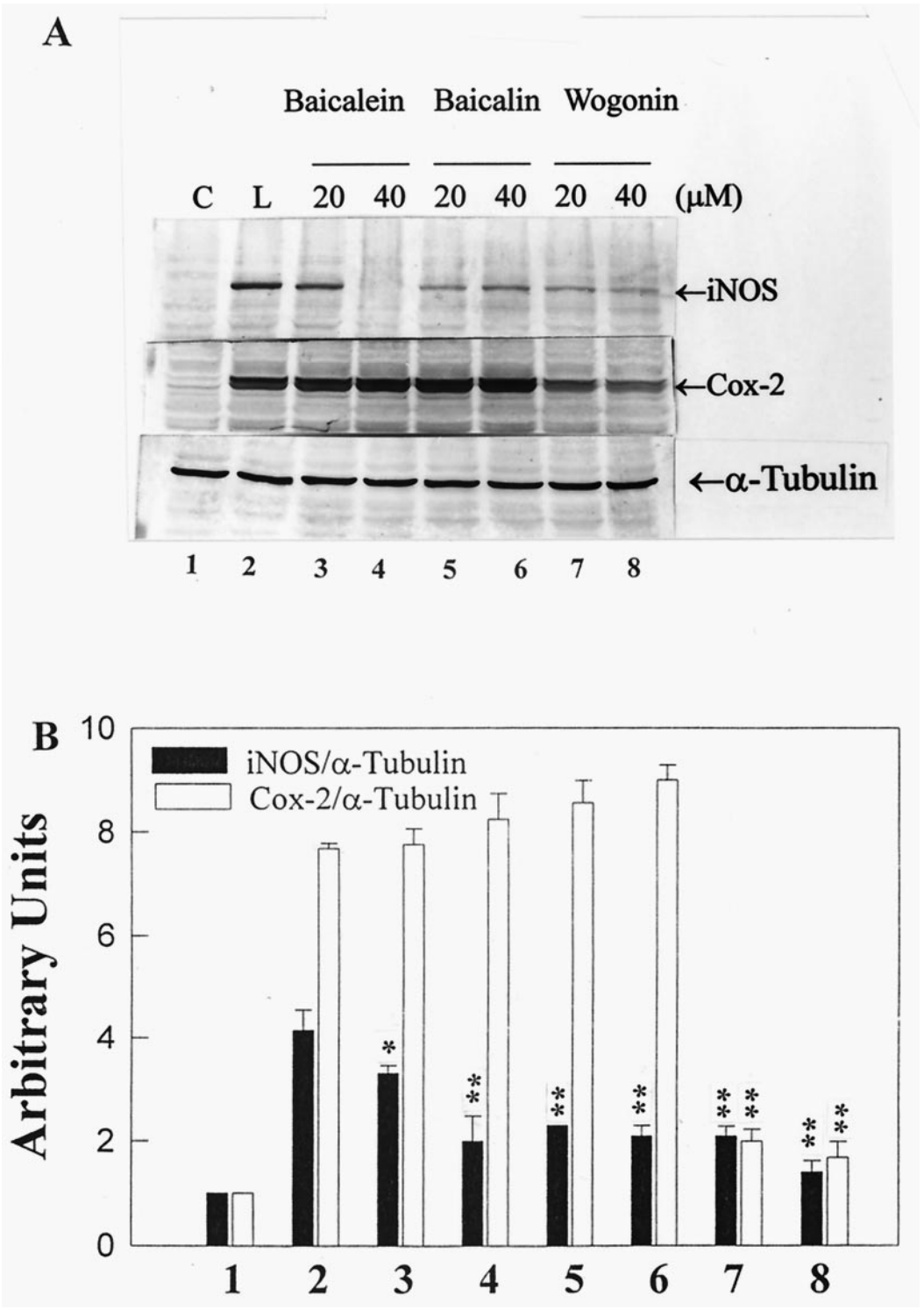


Fig. 3. Western blot analysis of the inhibition of LPS-induced iNOS and COX-2 protein expression by polyphenols. (A) The cells were treated as described in Fig. 2. Equal amounts of total proteins (50 μg/lane) were subjected to 10% SDS–PAGE, and expression of iNOS, COX-2, and α-tubulin protein was detected by Western blotting using specific antibodies. α-Tubulin protein was used here as an internal control. C: control; L: LPS alone. (B) Quantification of band intensities in (A) from three independent experimental results by densitometry (IS-1000 Digital Imaging System). Data were described as means ± SE of iNOS/α-tubulin or Cox-2/α-tubulin. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant differences from the LPS-treated group.

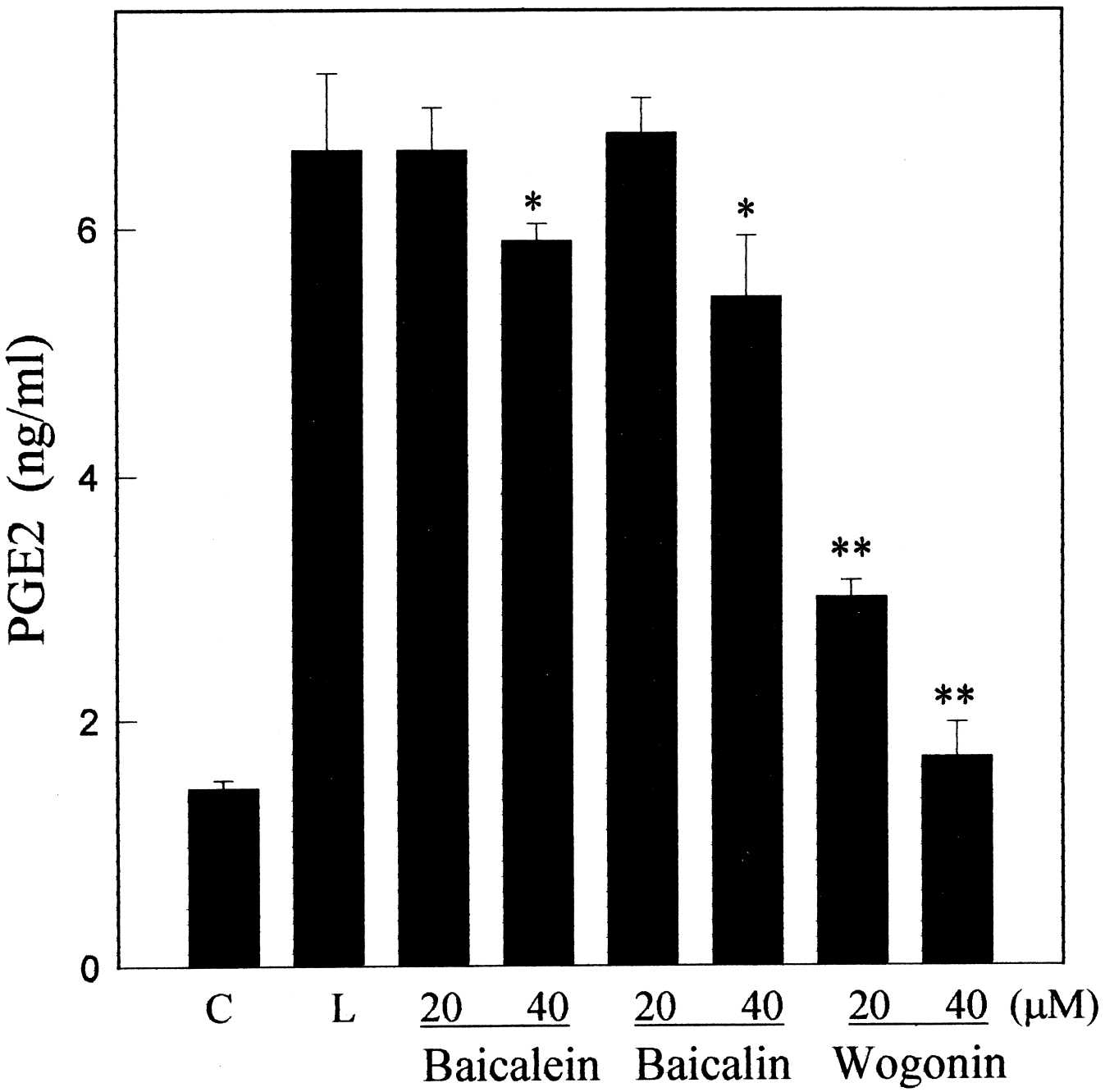


Fig. 4. Inhibition of LPS-induced PGE_2 production by baicalin, baicalein, and wogonin. RAW 264.7 macrophages were treated with LPS (100 ng/mL) followed by treatment with polyphenols at the indicated concentrations and incubated at 37° for 24 hr. The amount of PGE_2 in the medium was measured as described in section 2. C: control; L: LPS alone. Data were derived from three independent experiments and expressed as means ± SE. $*P < 0.05$ and $**P < 0.01$ indicate statistically significant differences from the LPS alone group.

treatment of cells with different concentrations (20 and 40 μM) of wogonin for 24 hr. Baicalin and baicalein at similar concentrations, however, did not inhibit LPS-induced COX-2 protein synthesis (Fig. 3).

3.4. Effects of baicalin, baicalein, and wogonin on iNOS and cyclooxygenase enzyme activities in RAW 264.7 macrophages

Table 1 indicates that the addition of different concentrations (20 or 40 μM) of baicalin, baicalein, and wogonin to RAW 264.7 macrophages, which had been pretreated with LPS to induce NOS, did not affect NOS enzyme activity in intact cells when the amount of nitrite production in the medium was measured. In parallel experiments, the NOS inhibitors NLA and L-NAME significantly decreased nitrite production in the medium, but did not alter iNOS enzyme activity in the cell lysates by direct NOS enzyme activity assays *in vitro*. The lack of direct enzyme inhibition by baicalin, baiclein, and wogonin was

Table 1
Effects of baicalin, baicalein, and wogonin on LPS-induced NO synthesis and iNOS enzyme activity in RAW 264.7 macrophages

LPS pretreatment of cells	Addition to LPS-treated RAW 264.7 cells	NO in medium (μM/6 $\times$ 10^5 cells)	iNOS specific activity: NO formation (μM/200 μg protein)
None	DMSO control	0.0 ± 0.0	1.5 ± 0.8
LPS (100 ng/mL), 12 hr	Control	16.9 ± 0.5	8.9 ± 1.2
	Baicalein		
	20 μM	15.4 ± 0.3	9.8 ± 2.3
	40 μM	13.1 ± 0.9	8.1 ± 1.9
	Baicalin		
	20 μM	14.5 ± 0.5	8.7 ± 1.3
	40 μM	14.8 ± 1.5	9.4 ± 2.1
	Wogonin		
	20 μM	16.5 ± 0.3	8.7 ± 3.1
	40 μM	16.3 ± 0.3	8.9 ± 2.7
	NLA		
	2 mM	0.3 ± 0.1**	9.4 ± 2.1
	L-NAME		
	2 mM	0.4 ± 0.2**	9.7 ± 1.5

RAW 264.7 macrophages were stimulated with LPS (100 ng/mL) for 12 hr, and cells were washed twice with PBS to remove LPS. RAW cells were then scraped and placed in a 24-well plate, and the indicated compounds were added and incubated at 37° incubator for an additional 12 hr. The amount of NO accumulated in the medium and the alternation of iNOS enzyme activity in cell lysates were detected by indirect and direct NOS enzyme assays as described in Materials and Methods. Data are means ± SE from three independent experiments.

** $P < 0.01$ indicates significantly different from LPS alone.

further supported by findings that different concentrations (20 and 40 μM) of baicalin, baicalein, and wogonin did not inhibit LPS-induced NO production, whereas both NLA and L-NAME treatment significantly inhibited such production (Table 2). When different concentrations (20 or 40 μM) of baicalin, baicalein, and wogonin were added to RAW 264.7 macrophages in which COX-2 proteins had already been induced by LPS, there was no decrease in PGE_2 production using added arachidonic acid as a substrate (Table 3). Neither NLA nor L-NAME affected PGE_2 production which, however, was significantly inhibited by the cyclooxygenase enzyme inhibitor indomethacin.

Table 2
Effects of baicalin, baicalein, and wogonin on iNOS activity by direct enzyme activity assay in RAW 264.7 cell lysates

Pretreatment of cells before lysis	Addition to lysate	iNOS specific activity: NO formation (μM/200 μg total protein)[a]
None	DMSO	1.1 ± 0.5
LPS (100 ng/mL), 12 hr	DMSO	8.6 ± 0.4
	Baicalin	
	20 μM	8.7 ± 1.0
	40 μM	9.0 ± 1.1
	Baicalein	
	20 μM	7.2 ± 0.1
	40 μM	9.2 ± 1.8
	Wogonin	
	20 μM	8.1 ± 0.2
	40 μM	7.5 ± 0.1
	NLA	
	4 mM	4.2 ± 0.4**
	L-NAME	
	4 mM	3.9 ± 0.6**

[a] The values were obtained from three separate experiments and described as means ± SE. Lysate preparation and iNOS activity assay were described in section 2. Each indicated compound was added to lysates (200 μg) from LPS-treated RAW 264.7 macrophages, and iNOS activity was measured.

** $P < 0.01$ indicates significantly different from LPS alone.

3.5. Effects of baicalin, baicalein, and wogonin on NLA- or L-NAME-stimulated LPS-induced iNOS and COX-2 gene expression

It has been suggested that NO is a key factor in terminating inflammation through an autoregulatory feedback inhibition of iNOS synthesis in LPS- or cytokine-treated cells [27]. Accordingly, NOS enzyme inhibitors such as NLA and L-NAME significantly inhibit NO production while stimulating *iNOS* gene expression. In the present study, NLA and L-NAME significantly inhibited LPS-induced NO (but not PGE_2) production (Table 4). NLA and L-NAME enhanced LPS (100 ng/mL)-induced *iNOS* (but not *COX-2*) gene expression by about 3-fold ($P < 0.01$, compared with the LPS-treated group) (Fig. 5A). The increased expression of iNOS was inhibited by baicalin, baicalein, or wogonin in a concentration-dependent manner. Wogonin, but not baicalin or baicalein, inhibited expression of COX-2 proteins in NLA (or L-NAME) plus LPS-cotreated RAW 264.7 macrophages (Fig. 5B).

4. Discussion

NO has been recognized to be an important mediator of cellular communication in several preparations (in addition

Table 3
Effects of added baicalein, baicalin, wogonin, indomethacin, NLA, and L-NAME on LPS-induced COX-2 enzyme in RAW 264.7 cells

LPS treatment of cells[a]	Addition to LPS-treated RAW 264.7 cells	PGE_2 (ng/mL)[b]
None	DMSO	0.2 ± 0.0
LPS	DMSO	6.5 ± 0.2
	Baicalin	
	20 μM	6.1 ± 0.6
	40 μM	5.9 ± 0.4
	Baicalein	
	20 μM	6.1 ± 0.2
	40 μM	6.2 ± 0.5
	Wogonin	
	20 μM	5.9 ± 0.7
	40 μM	5.7 ± 0.4
	Indomethacin	
	20 μM	2.0 ± 0.7**
	NLA	
	2 mM	5.6 ± 0.9
	L-NAME	
	2 mM	6.2 ± 0.6

[a] RAW 264.7 cells were stimulated with LPS (100 ng/mL) for 6 hr, and cells were washed twice with fresh medium. Baicalin, baicalein, or other indicated compounds were then added and incubated at 37° for 30 min. The cells were further incubated with arachidonic acid (100 μM) for 15 min.

[b] The amount of PGE_2 in the supernatant was assayed as described in Materials and Methods. Data are means ± SE of three samples from two independent experiments. Duplicate determinations were made for each experiment.

** $P < 0.01$ indicates significantly different from LPS alone.

Fig. 5. Inhibition of NLA/LPS- or L-NAME/LPS-induced iNOS and COX-2 proteins by baicalin, baicalein, and wogonin in RAW 264.7 macrophages. (A) The cells were treated with LPS (100 ng/mL) plus NLA or L-NAME at 37° for 24 hr. The expression of iNOS, COX-2, and α-tubulin was determined by Western blotting. (B) Effects of pretreatment of baicalin, baicalein, and wogonin (40 μM) in LPS/NLA- or LPS/L-NAME-stimulated RAW 264.7 macrophages (37° for 24 hr) on the expression of iNOS, COX-2, and α-tubulin protein was detected by Western blotting using specific antibodies as described in Materials and Methods. (C) Quantification of band intensities in (B) from three independent experiments by densitometry (IS-1000 Digital Imaging System). Data were described as means ± SE of iNOS/α-tubulin or Cox-2/α-tubulin. ** $P < 0.01$ indicates statistically significant differences from the LPS + NOS inhibitor (NLA or L-NAME)-treated group.

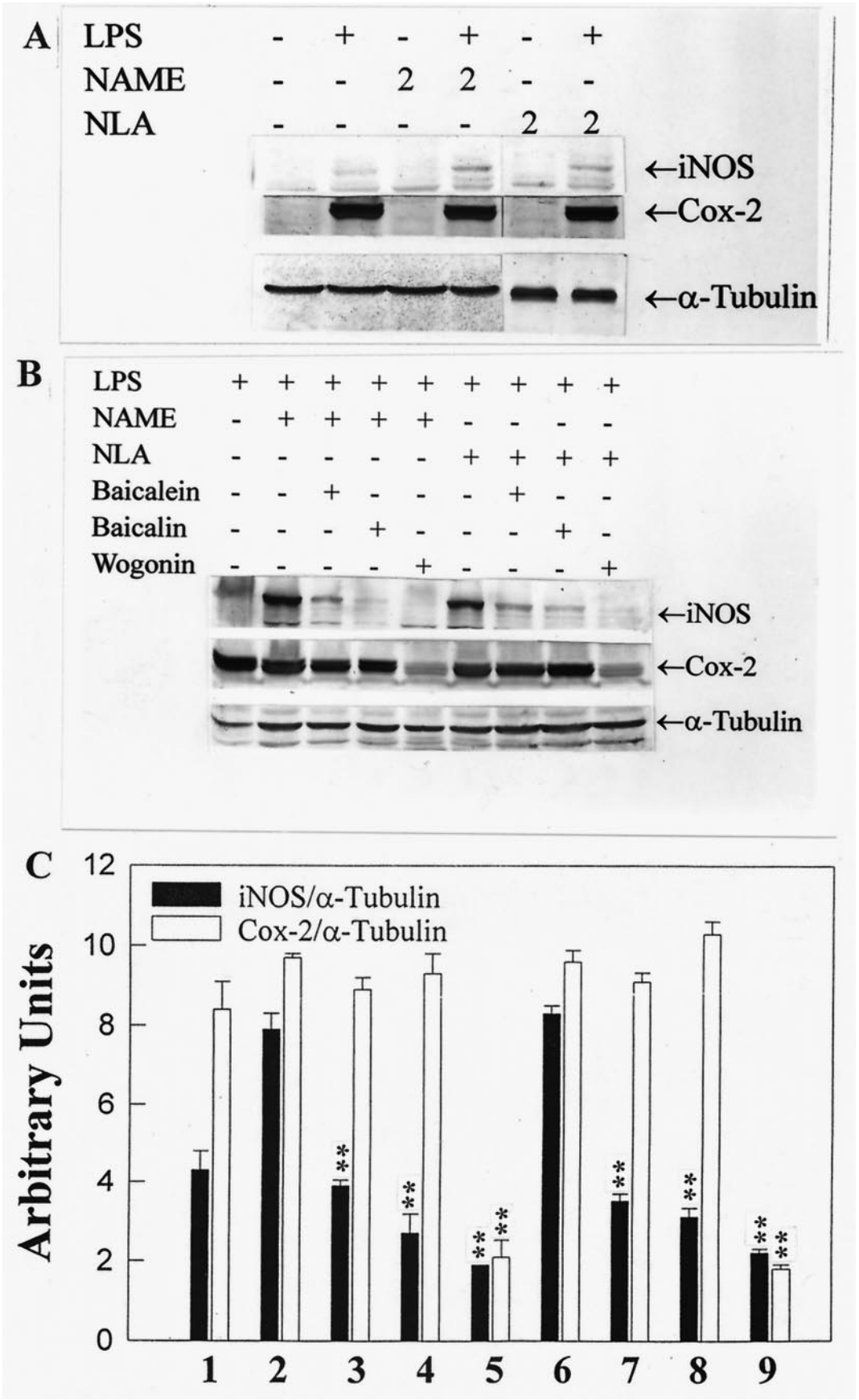
to endothelial cells) such as macrophages, neutrophils, smooth muscle, autonomic nervous system, and central nervous system [28–33]. Several studies have demonstrated that induction of iNOS produces a large amount of NO during endotoxemia and under inflammatory conditions. Therefore, drugs that inhibit iNOS expression and/or enzyme activity resulting in decreased NO generation may have beneficial therapeutic effects in the treatment of diseases due to overproduction of NO [34,35]. In this study, exposure of RAW 264.7 macrophages to LPS for several hours was associated with an accumulation of nitrite in the medium, suggesting an enhanced NO production. This LPS-induced NO production was inhibited by baicalin, baicalein, and wogonin, which were co-incubated with LPS in a time- and concentration-dependent manner without notable cytotoxicity. The present study also demonstrated that the inhibitory effect of these compounds on LPS-induced NO production was not observed when iNOS was already expressed by preactivation with LPS. This was supported by the findings that these three polyphenolic compounds did not directly inhibit NOS enzyme activity. Results from Western blotting analysis further indicated that expression of iNOS proteins in RAW 264.7 macrophages treated with LPS was prevented by baicalin, baicalein, and wogonin in a concentration-dependent manner. These results suggest that inhibition of LPS-induced NO production by baicalin, baicalein, and wogonin occurs through inhibition of *iNOS* gene expression, but not the activity of NOS. This mechanism of action of these three compounds appears to be similar to that of glucocorticoids and oroxylin A, the latter also a polyphenolic compound [4,22,36,37].

Table 4
Effects of baicalin, baicalein, and wogonin on NLA- or L-NAME-stimulated LPS-induced NO and PGE_2 productions in RAW 264.7 macrophages[a]

Treatment of cells	NO (μmol/4 × 10^5)	PGE_2 (ng/mL)
Control	3.7 ± 0.7	1.8 ± 0.4
LPS	30.1 ± 0.6	7.1 ± 0.8
NLA (2 mM)	3.7 ± 0.3	1.5 ± 0.5
LPS + NLA (2 mM)	9.7 ± 0.2**	7.2 ± 0.6
LPS + NLA (2 mM) + baicalin (40 μM)	7.1 ± 0.3**	5.8 ± 1.1*
LPS + NLA (2 mM) + baicalein (40 μM)	8.3 ± 1.3**	5.5 ± 0.8*
LPS + NLA (2 mM) + wogonin (40 μM)	6.7 ± 0.7**	2.1 ± 0.4**
L-NAME (2 mM)	5.1 ± 0.4	1.3 ± 0.6
LPS + L-NAME (2 mM)	7.3 ± 1.2**	6.9 ± 0.9
LPS + L-NAME (2 mM) + baicalin (40 μM)	8.5 ± 0.9**	6.1 ± 0.3
LPS + L-NAME (2 mM) + baicalein (40 μM)	7.9 ± 1.2**	6.2 ± 0.5
LPS + L-NAME (2 mM) + wogonin (40 μM)	6.8 ± 0.5**	2.3 ± 0.7**

[a] RAW 264.7 cells were co-treated with LPS (100 ng/mL) and indicated compounds for 24 hr. The amount of NO and PGE_2 accumulated in the supernatant was detected by Griess assay and the PGE_2 assay kit as described in Materials and Methods. Data are means ± SE from three independent experiments.

* $P < 0.05$ and ** $P < 0.01$ indicate significant difference from LPS alone.


A
LPS - + - + - +
NAME - - 2 2 - -
NLA - - - - 2 2
←iNOS
←Cox-2
←α-Tubulin
B
LPS + + + + + + + + +
NAME - + + + + - - - -
NLA - - - - - + + + +
Baicalein - - + - - - + - -
Baicalin - - - + - - - + -
Wogonin - - - - + - - - +
←iNOS
←Cox-2
←α-Tubulin
C
Arbitrary Units
iNOS/α-Tubulin
Cox-2/α-Tubulin
0 2 4 6 8 10 12
1 2 3 4 5 6 7 8 9

L-arginine analogs such as NLA and L-NAME are non-specific NOS inhibitors that attenuate NO production induced by LPS and cytokines. However, several *in vitro* and *in vivo* studies have demonstrated that NO may function as an anti-inflammatory mediator. For example, NO released by NO donors can decrease cytokine-induced endothelial cell activation [38,39], inhibit leukocyte–endothelium interaction [40–42], and attenuate vascular inflammation [43]. Peng *et al*. [27] recently reported that treatment with L-NAME decreased LPS- and interferon-γ (IFN-γ)-stimulated NO production, whereas it augmented LPS- and IFN-γ-induced iNOS expression by 2.5-fold. A similar result was found in the present study. NLA and L-NAME equally and significantly decreased LPS-induced NO production by $93 \pm 3.5\%$ while increasing LPS-induced iNOS protein by 3.9-fold in RAW 264.7 macrophages. Pretreatment with baicalin, baicalein, and wogonin decreased NO production and iNOS protein expression in RAW 264.7 cells pretreated with LPS plus NLA or LPS plus L-NAME. Wogonin appears to be most potent in inhibiting NO production and iNOS expression. In addition, wogonin, but not baicalin or baicalein, significantly inhibited PGE_2 production and *COX-2* gene expression in NLA/LPS- or NAME/LPS-treated RAW 264.7 macrophages.

Polyphenols are common components existing in natural plants. Several studies have demonstrated that polyphenolic compounds derived from herbs possess several biological activities, including free radical scavenging [44,45] and anti-inflammatory [46] and anticarcinogenic activities [47–49]. Huang Qui has been used in Chinese medicine as a remedy for treating inflammation, suppurative dermatitis, allergic diseases, hyperlipemia, and arteriosclerosis [21]. The active principles in Huang Qui have not been determined fully. Our previous report indicated that oroxylin A, also derived from Huang Qui, was a potent inhibitor of LPS-induced *iNOS* and *COX-2* gene expressions. Inhibition of LPS-induced *iNOS* and *COX-2* gene expression by oroxylin A was mediated by blocking the activation of nuclear factor-κB [22]. Results of the present and our previous studies indicated that baicalin, baicalein, wogonin, and oroxylin A inhibited LPS-induced *iNOS* gene expression in macrophages without appreciable cytotoxic effects. These findings are consistent with reports by others [50,51]. Among these flavonoids, only wogonin and oroxylin A showed significant inhibition on LPS-induced PGE_2 production and COX-2 expression. It is interesting to note that only oroxylin A and wogonin contain a methoxyl (CH_3O) group on the A ring.

In summary, the results of the present study indicated that baicalin, baicalein, and wogonin pretreatment inhibited LPS-induced *iNOS* gene expression and NO production. Wogonin, but not baicalin or baicalein, inhibited LPS-induced COX-2 expression. These compounds did not affect iNOS and COX-2 activity. In combination with NOS inhibitors, wogonin, like oroxylin A [22], may be useful in the prevention and treatment of diseases due to increased expression of iNOS and COX-2 such as in endotoxemia [10–12,17].

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