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International Immunopharmacology 7 (2007) 1054–1063

International Immunopharmacology

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Wogonin but not Nor-wogonin inhibits lipopolysaccharide and lipoteichoic acid-induced iNOS gene expression and NO production in macrophages

Guan-Cheng Huang [a], Jyh-Ming Chow [b], Shing-Chuan Shen [c], Liang-Yo Yang [d], Cheng-Wei Lin [e], Yen-Chou Chen [e,f,*]

[a] *Department of Internal Medicine, Chi-Mei Medical Center, Tainan, Taiwan*
[b] *Section of Hematology-Oncology, Department of Internal Medicine, Taipei Municipal Wan-Fang Hospital, Taipei Medical University, Taiwan*
[c] *Department of Dermatology, Taipei Municipal Wan-Fang Hospital, Taipei, Taiwan*
[d] *Department of Physiology and Graduate Institute of Neuroscience, Taipei Medical University, Taipei, Taiwan*
[e] *Graduate Institute of Pharmacognosy, School of Pharmacy, Taipei Medical University, Taipei, Taiwan*
[f] *Topnotch Stroke Research Center, Taipei Medial University, Taipei, Taiwan*

Received 19 February 2007; received in revised form 28 March 2007; accepted 2 April 2007

Abstract

Wogonin (Wog; 5,7-dihydroxy-8-methoxy flavone) has been shown to effectively inhibit lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) gene expression and nitric oxide production in our previous study. In the present study, we found that Nor-wogonin (N-Wog; 5,7,8-trihydroxyl flavone), a structural analogue of Wog with an OH substitution at C8, performed different effect on LPS- or lipoteichoic acid (LTA)-induced iNOS gene expression and nitric oxide (NO) production in macrophages. Wog, but not N-Wog, significantly inhibits LPS- or LTA-induced NO production through suppressing iNOS gene expression at both protein and mRNA without affecting NO donor sodium nitroprusside-induced NO production, NOS enzyme activity, and cells viability. Activation of JNKs (not ERKs) via phosphorylation induction, and an increase in c-Jun (not c-Fos) protein expression were involved in LPS- and LTA-treated RAW264.7 cells, and those events were blocked by Wog, but not N-Wog, addition. Furthermore, 5,7-diOH flavone, but not 5-OH flavone, 7-OH flavone, 5-OH-7-OCH_3 flavone, significantly inhibits LPS-induced iNOS protein expression and NO production, and 7,8-diOCH_3 flavone performs more effective inhibitory activity on LPS-induced NO production and iNOS protein expression than 7-OCH_3-8-OH flavone. These data suggest that OHs at both C5 and C7 are essential for NO inhibition of flavonoids, and OCH_3 at C8 may contribute to this activity, and suppression of JNKs–c-Jun activation is involved.

Keywords: Wogonin; Nor-wogonin; LPS; LTA; JNKs; iNOS; NO

Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; LTA, lipoteichoic acid; SNP, sodium nitroprusside; ERKs, extracellular regulated protein kinases; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; JNKs, c-Jun N-terminal kinases; PCR, polymerase chain reaction; Wog or W, wogonin; N-Wog or NW, Nor-wogonin

* Corresponding author. Graduate Institute of Pharmacognosy, School of Pharmacy, Taipei Medical University, Taipei, Taiwan. Tel.: +886 2 27361661x6152; fax: +886 2 23787139.
E-mail address: yc3270@tmu.edu.tw (Y.-C. Chen).

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doi:10.1016/j.intimp.2007.04.001

1. Introduction

Flavonoids, a group of polyphenolic compound, are widely found in plants, vegetables, and fruits. Flavonoids possess various pharmacological effects including anti-tumor, anti-inflammatory, antioxidant, and antiviral activities [1]. Among them, anti-inflammatory effect of flavonoids has been extensively applied in Chinese herbal medicine to inhibit chronic inflammation. However, the structural activity relationship of flavonoids in anti-inflammation is still unclear.

Nitric oxide synthase (NOS, E.C. 1.14.13.39) is an enzyme responsible of the biosynthesis of nitric oxide (NO), a free radical, via the conversion of L-arginine to L-citrulline. At least three NOS isoforms, usually designated as inducible (iNOS), endothelial (eNOS), and

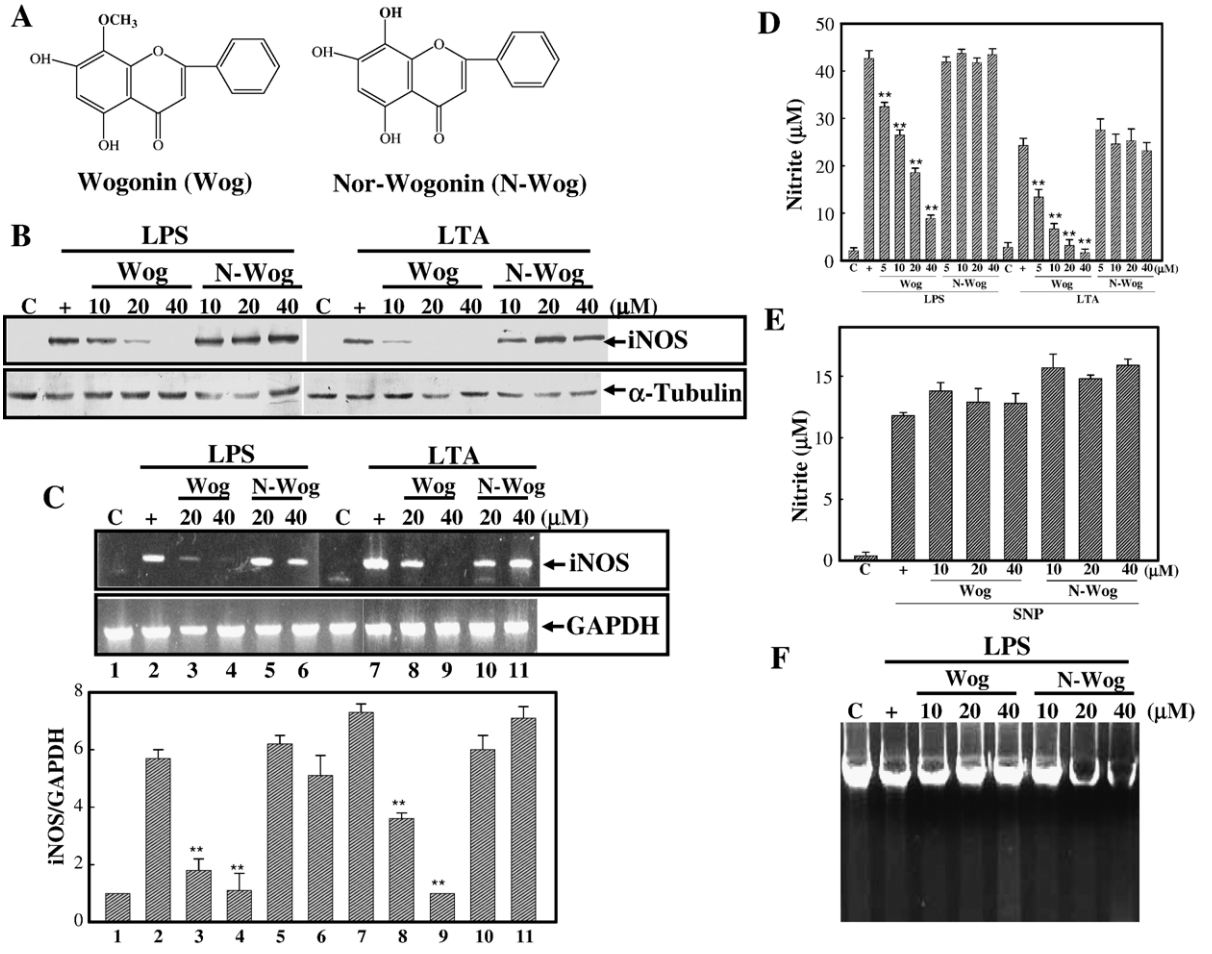

Fig. 1. Wogonin (Wog) but not Nor-wogonin (N-Wog) inhibited lipopolysaccharide (LPS)- or lipoteichoic acid (LTA)-induced NO production and iNOS gene expression at both the protein and mRNA levels in RAW264.7 cells. (A) The chemical structures of wogonin (Wog) and Nor-wogonin (N-Wog). (B) RAW264.7 cells were treated with different doses (10, 20, and 40 μM) of Wog or N-Wog for 30 min followed by the addition of LPS (100 ng/mL) or LTA (1 μg/mL) for an additional 12 h. The expressions of iNOS and α-tubulin protein were detected by Western blotting using specific antibodies. (C) Wog but not N-Wog inhibited LPS- or LTA-induced iNOS mRNA levels in RAW264.7 cells. Cells were treated with different doses (20 and 40 μM) of Wog or N-Wog for 30 min, followed by the addition of LPS or LTA for a further 6 h. (Upper panel) The expressions of iNOS and GAPDH mRNA in each sample were detected by RT-PCR using specific primers. (Lower panel) The intensities of iNOS and GAPDH mRNA were examined by a densitometric analysis, and data derived from three independent experiments are expressed as mean±SE. (D) Wog but not N-Wog inhibited LPS- or LTA-induced NO production. As described in (A), the amount of NO in the medium was detected by the Griess reaction as detailed in "Materials and methods". (E) Neither Wog nor N-Wog directly scavenged NO molecules elicited by SNP in vitro. Different doses (10, 20, and 40 μM) of Wog and N-Wog were mixed with sodium nitroprusside (SNP; 100 μM) in the condition with 10 mM Tris (pH 7.5) for 12 h, and the amount of NO in the medium was detected by the Griess reaction. (F) The viability of RAW264.7 cells was not affected by adding Wog or N-Wog. Cells were treated under the conditions described in (A), and the DNA integrity of cells in each group was analyzed by agarose electrophoresis. $^{**}P<0.01$ significantly differed from the LPS- or LTA-treated group as analyzed by Student's *t*-test.

Table 1
Effects of wogonin and Nor-wogonin on NOS enzyme activity and cellular viability in RAW264.7 cells

LPS pretreatment of cells	Addition to LPS-treated cells	NO in medium[a] (6×10^5 cells; μM)	Cytotoxicity[b] (folds of control)
RAW264.7 cells			
	Control	21.5±1.2	1.00
	Wogonin (μM)		
	10	18.9±2.1	1.08±0.03
	20	18.2±1.9	1.02±0.03
	40	19.1±1.1	1.00±0.09
	Nor-wogonin (μM)		
	10	22.1±2.3	1.10±0.04
	20	22.5±1.4	1.07±0.03
	40	22.8±2.2	1.11±0.05
	NLA (mM)		
	2	2.4±0.5**	1.02±0.04

[a] Cells were stimulated with LPS (100 ng/mL) for 12 h, and washed twice with sterilized PBS to remove LPS, followed by incubating with Wog and N-Wog (10, 20, and 40 μM) or NOS inhibitor *N*-nitro-L-arginine (NLA, 2 mM) a further for 12 h. The amount of nitrite in the medium was detected as described in the section of Materials and methods. **$P<0.01$ indicates significantly different from LPS alone.
[b] Cells were treated with Wog and N-Wog (10, 20, and 40 μM) or NOS inhibitor *N*-nitro-L-arginine (NLA, 2 mM) for 12 h, and the viability of cells under different treatments was detected by MTT assay.

neuronal (nNOS) had been identified. Both eNOS and nNOS isoforms are constitutively expressed, and the releasing of NO by eNOS through a Ca^{+2}-dependent pathway could act as a neurotransmitter in neuron signal transmission, which play a key factor for maintaining the normal vasoactivity. NOS in macrophages and hepatocytes is iNOS, and its activation is Ca^{+2}-independent. After exposure to endogenous and exogenous stimulators, overproduction of NO by iNOS can be induced quantitatively in macrophages, smooth muscle cells and hepatocytes to trigger several disadvantage cellular responses and caused some diseases including inflammation, sepsis, stroke, and with the development of atherosclerosis [2–4]. Lipopolysaccharide (LPS) and lipoteichoic acid (LTA) are components of Gram-negative and Gram-positive bacteria, respectively, and the induction of NO overproduction and iNOS gene expression by LPS and LTA has been demonstrated [5,6]. Therefore, the development of agents capable of blocking LPS-and LTA-induced NO overproduction, as well as iNOS gene expression, can be regarded as a potential therapeutic target for the treatment of inflammation.

Wogonin (5,7-diOH-8-OCH_3 flavone; Wog), one of the active constituents found in the roots of *Scutellaria baicalensis* Georgi, possesses several beneficial effects, as previously reported. Zao et al. indicated that Wog was able to inhibit hemin-nitrite-H_2O_2-induced liver injury by blocking the oxidation of proteins [7]. Bonham et al. indicated that Wog inhibits the proliferation of prostate cancer cells and inhibition of the androgen receptor signaling pathway is involved [8]. Park et al. found that Wog significantly reduced alcohol-induced gastropathy through anti-inflammation and apoptosis induction in vivo [9]. Our previous study indicated that Wog effectively inhibited LPS-induced NO production in macrophages [10], and in vivo anti-inflammatory effect of Wog against LPS-induced NO was identified [11]. In addition, we also found that Wog addition effectively reduces LPS plus TPA-induced transformation in glioma C6 cells through inhibiting MMP-9 activation and iNOS gene expression [12]. Although several biological effects of Wog have been shown, the importance of structural substitutions including OH at C5 and C7, and OCH_3 at C8 on the action of Wog is still unclear. In the present study, we examined the importance of OH substitution at C5 and C7, and OCH_3 at C8, by studying the effects of structurally related compounds, Wog and Nor-Wog. In addition, Wog-induced inhibition on NO production was elucidated.

2. Materials and methods

2.1. Cells

RAW264.7 is a mouse macrophage cell line, and was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco/BRL, Gaithersburg, MD, USA) supplemented with antibiotics (100 U/mL of penicillin A and 100 U/mL of streptomycin), and 10% heat-inactivated fetal bovine serum (Gibco/BRL, Gaithersburg, MD, USA) and maintained at 37 °C in 5% CO_2 humidified air.

2.2. Agents

The structurally related flavonoids of wogonin, Nor-wogonin, 5-OH flavone, 7-OH flavone, 5,7-diOH flavone, 7,8-diOCH_3 flavone, and 7-OCH_3-8-OH flavone were obtained from Sigma Chemical (St. Louis, MO, USA). Lipopolysaccharide (LPS) (*Escherichia coli*, serotype 055:B5), lipoteichoic acid (LTA) (*Bacillus subtilis*), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), nitroblue tetrazolium (NBT), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from Sigma Chemical (St. Louis, MO, USA). The antibodies for detecting the expression of iNOS, total and phosphorylated JNKs, total and phosphorylated ERKs, c-Jun, phosphorylated c-Jun, and α-tubulin protein were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All chemical solvents were purchased from Merck KGaA (Darmstadt, Germany).

2.3. Cell viability assay

MTT was used as an indicator of cell viability as determined by its mitochondrial-dependent reduction to formazone. Cells were plated at a density of 10^5 cells/well into 96-well plates for 12 h, followed by treatment with different concentrations of each compound for a further 16 h. Cells were washed with PBS twice, and MTT (50 mg/mL) was added to the medium for 4 h. Furthermore, the supernatant was removed, and the formazone crystals were dissolved using 0.04 N HCl in isopropanol. The absorbance was read at 600 nm with an ELISA analyzer (Dynatech MR-7000; Dynatech Laboratories; Chantilly, VA, USA).

2.4. Nitrite assay

Cells were plated at a density of 5×10^5 cells/mL in 24-well plates for 12 h, followed by treatment with LPS or LTA and different concentrations of the indicated compounds for a further 12 h. NO_2^- accumulation was used as an indicator of NO production in the cell culture medium by the Griess reaction. One hundred microliters of each supernatant were mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylendiamine dihydrochloride in water). The absorbance of each sample after Griess reaction was determined by ELISA plate reader (Dynatech MR-7000; Dynatech Laboratories) at 530 nm [13]. The nitrite concentration is evaluated by means of a calibration curve (2.5 to 40 μM), using sodium nitrite as a standard.

2.5. Western blotting

Total cellular extracts were prepared according to our previous paper [14], separated on 8%–12% SDS-polyacrylamide minigels, and transferred to immobilon polyvinylidenedifluoride membranes (Millipore; Billerica, MA, USA). The membrane was blocked with 1% bovine serum albumin at room temperature for

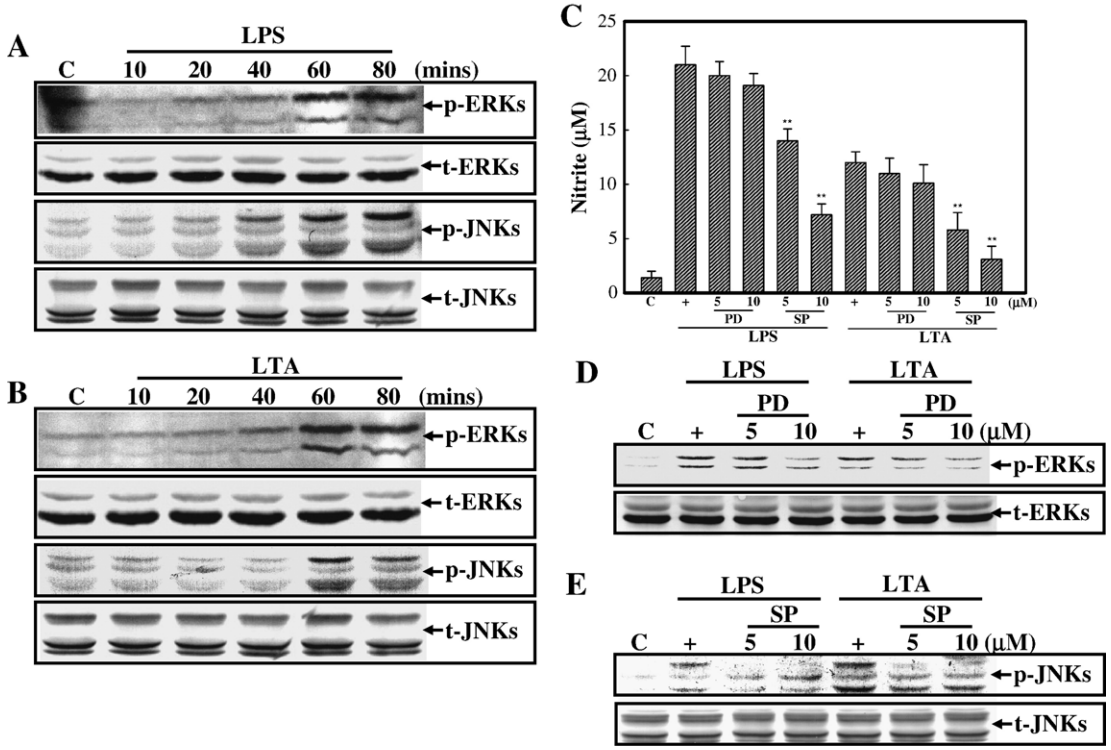

Fig. 2. Activation of JNKs but not ERKs involves in LPS- and LTA-induced NO production and iNOS protein expression. (A, B) Time-dependent induction of ERKs and JNKs protein phosphorylation is detected in LPS- and LTA-treated RAW264.7 cells. Cells were treated with LPS or LTA for different times (10, 20, 40, 60, and 80 min). The expression of phosphorylated and total ERKs and JNKs protein was detected by Western blotting using specific antibodies. (C) SP600125, but not PD98059, addition inhibits LPS- and LTA-induced NO production. Cells were treated with PD98059 and SP600125 for 30 min followed by LPS and LTA treatment for 12 h. The amount of NO in medium was detected by Griess reaction. $^{**}P<0.01$ significantly different from LPS- or LTA-treated group as analyzed by Student's *t*-test. (D, E) ERKs inhibitor PD98059 and JNKs inhibitor SP600125 addition inhibit LPS- and LTA-induced ERKs and JNKs protein phosphorylation respectively. Cells were treated with PD98059 or SP600125 (5 and 10 μM) for 30 min followed by adding LPS or LTA for a further 60 min. The expression of phosphorylated and total ERKs and JNKs protein was examined by Western blotting.

1 h and then incubated with antibodies for detecting iNOS, α-tubulin, c-Jun, phospho-c-Jun, JNKs, phospho-JNKs, ERKs, and phospho-ERKs protein overnight at 4 °C. Expression of the protein was detected by staining with NBT and BCIP.

2.6. RT-PCR

RAW264.7 cells were treated with indicated compounds and LPS for 4 h and washed out with ice-cold PBS. Total RNA was isolated by total RNA extraction kit (Amersham Biosciences, Buckinghamshire, UK), and the total RNA concentration was detected at 260 nm using a spectrophotometer (Shimadzu UV1601). Total RNA (1 μg) was converted to cDNA with oligo d(T). PCR was performed on the cDNA using the following sense and antisense primers, respectively, for iNOS: CCCTTCCGAAGTTTCTGGCAGCAGC and GGCTGTCAGAGAGCCTCGTGGCTTTGG; and GAPDH: TGAAGGTCGGTGTGAACGGATTTGGC and CATGTAGGCCATGAGGTCCA CCAC. PCR of the cDNA was performed in a final volume of 50 μL containing PCR primers, oligo (d)T, total RNA, and DEPC H_2O by RT-PCR Beads Kit (Amersham Biosciences, Buckinghamshire, UK). The amplification sequence protocol was 95 °C for 30 s, 56 °C for 30 s, 72 °C for 45 s for 30 cycles. The PCR products were separated by electrophoresis on 1.2% agarose gels and visualized by staining with ethidium bromide.

2.7. DPPH radical scavenging activity assay

The scavenging activities of tested compounds against DPPH radical were measured. In brief, different concentrations of tested compounds were added to 0.1 mL of 1 M Tris–HCl buffer (pH 7.9) and then mixed with 1.2 mL of 500 μM DPPH in methanol for 20 min under light protection. The absorbance at 517 nm was determined. Deionized water was used as a control group. The decrease of absorbance at 517 nm was calculated for scavenging activity.

2.8. Measurement of iNOS enzyme activity

In order to assay the iNOS enzyme activity in intact cells, RAW264.7 cells were planted in 100-mm tissue dishes (4×10^6) cells and incubated with LPS (100 ng/mL) for 12 h. Then the cells were washed twice with PBS, and cells were harvested and planted in a 96-well plate (2×10^5 cells/well), and incubated in the presence or absence of different doses of Wog and N-Wog for a further 12 h. The amount of NO in the supernatant was detected by Griess reaction as described above.

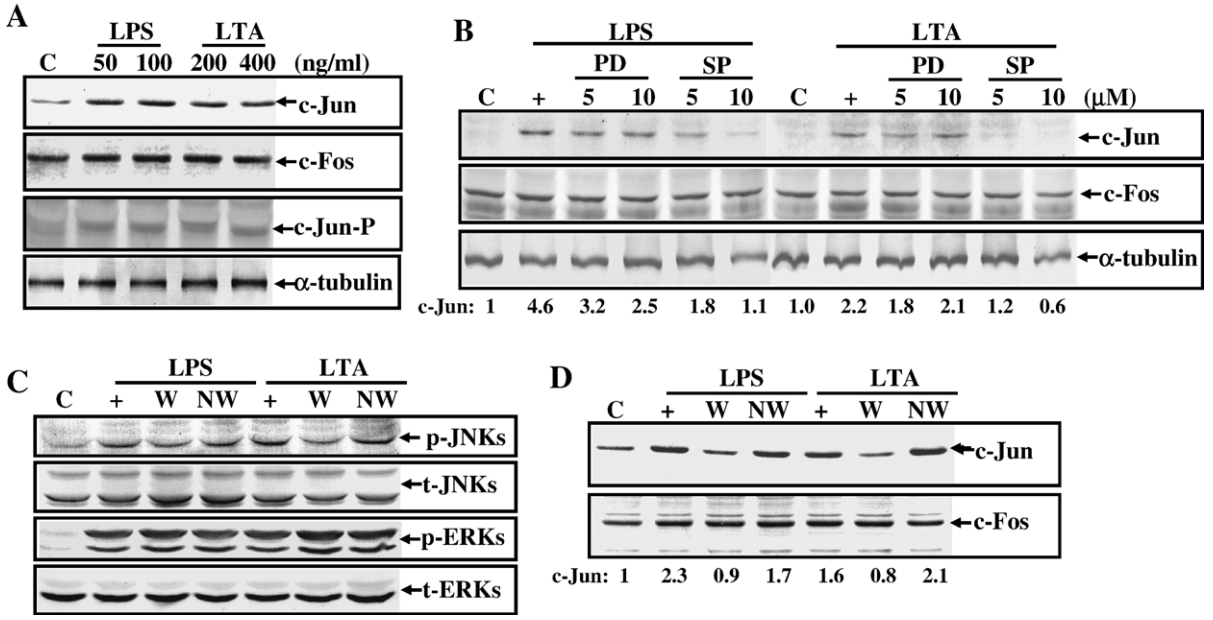

Fig. 3. Wog, but not N-Wog, inhibition of LPS- and LTA-induced JNKs and c-Jun protein expression in RAW264.7 cells. (A) LPS and LTA addition induce c-Jun, but not c-Fos, protein expression. Cells were treated with LPS or LTA for 6 h, and the expression of c-Jun, c-Fos, α-tubulin, and phosphorylated c-Jun (c-Jun-P) protein was examined by Western blotting. (B) SP600125, but not PD98059, addition reduces LPS- and LTA-induced c-Jun, but not c-Fos, protein expression. Cells were treated with PD98059 or SP600125 for 30 min, followed by adding LPS or LTA for an additional 6 h. The expression of c-Jun, c-Fos and α-tubulin protein was examined by Western blotting. (C) Wog (W), but not N-Wog (NW) inhibits LPS- and LTA-induced JNKs, but not ERKs, protein phosphorylation in RAW264.7 macrophages. Cells were treated with Wog or N-Wog (40 μM) for 30 min, followed by adding LPS or LTA for further 60 min. The expression of phosphorylated and total ERKs and JNKs protein was detected by Western blotting. (D) Wog (W), but not N-Wog (NW) inhibits LPS- and LTA-induced c-Jun protein expression in RAW264.7 cells. Cells were treated with Wog or N-Wog (40 μM) for 30 min, followed by adding LPS or LTA for further 6 h, and the expression of c-Jun and c-Fos protein was detected by Western blotting.

2.9. Statistical analysis

Values are expressed as mean ± S.E. The significance of the difference from the respective controls for each experimental test condition was assayed by using Student's *t*-test for each paired experiment. A P value $<.05$ or 0.01 was considered as statistically significant difference.

3. Results

3.1. Wog but not N-Wog inhibits LPS- or LTA-induced NO production and iNOS gene expression in RAW264.7 cells

The chemical structures of wogonin (W; Wog; 5,7-diOH-8-COH_3 flavone) and Nor-wogonin (NW; N-Wog; 5,7,8-triOH flavone) are shown in Fig. 1A. The difference between Wog and N-Wog is a methoxyl (OCH_3) group at C8 of Wog and a hydroxyl (OH) group at C8 of N-Wog. We further examined the effects of Wog and N-Wog on LPS- or LTA-induced NO production and iNOS gene expression. RAW264.7 cells were treated with different doses of Wog and N-Wog (5, 10, 20 and 40 μM) for 30 min followed by incubation with LPS (100 ng/mL) or LTA (1 μg/mL) for 12 h, and the amount of nitrite in the medium under different treatments was measured with the Griess reaction. Data in Fig. 1B show that Wog but not N-Wog dose-dependently inhibited iNOS protein expression induced by LPS and LTA. Data from the RT-PCR revealed that an increase in iNOS mRNA expression was detected in LPS- or LTA-treated RAW264.7 cells, and that was attenuated by the addition of Wog but not N-Wog (Fig. 1C). An equal amount of

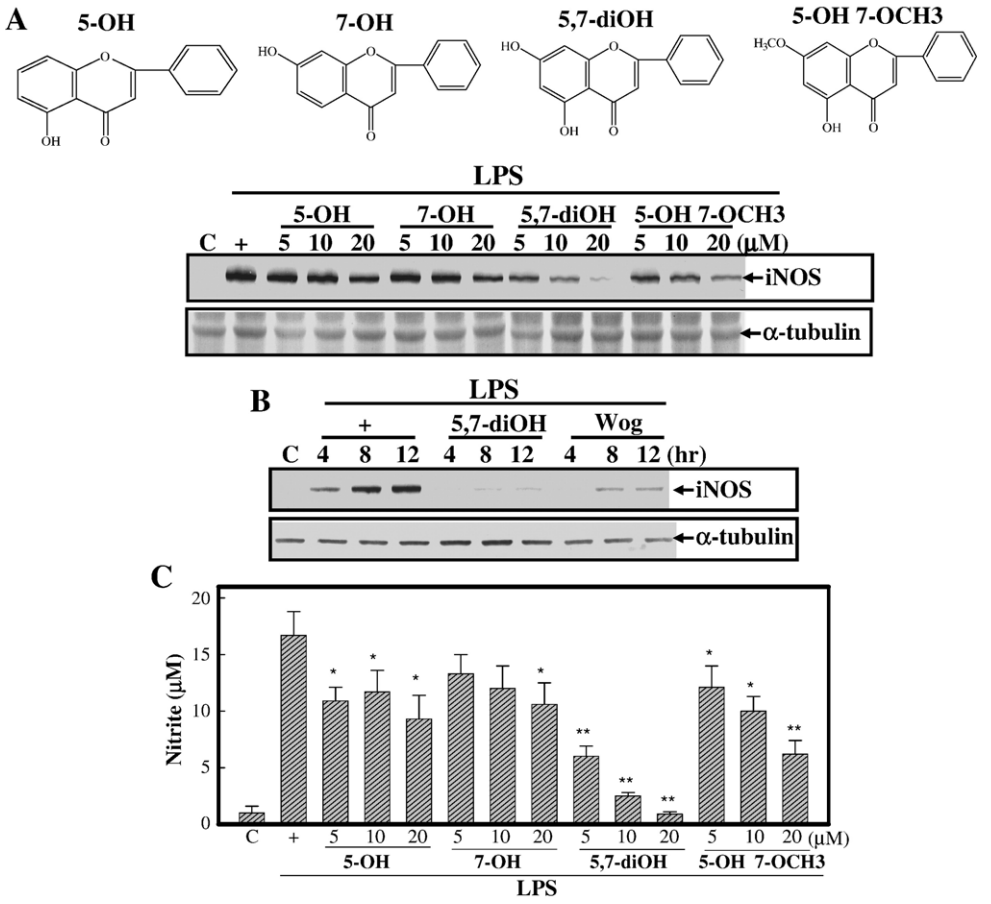

Fig. 4. Differential inhibitory effects of 5-OH, 7-OH, 5,7-diOH, 5-OH-7-OCH_3 flavone on LPS-induced NO production and iNOS protein expression in RAW264.7 cells. (A) (Upper panel) Chemical structure of tested compounds was described. (Lower panel) 5,7-diOH flavone performed significant dose-dependent inhibitory effect on LPS-induced iNOS protein expression. Cells were treated with different doses (5, 10, and 20 μM) of indicated compounds for 30 min, followed by incubating with LPS for a further 12 h. The expression of iNOS and α-tubulin protein was detected by Western blotting using specific antibodies. (B) 5,7-diOH flavone and Wog time-dependently inhibit LPS-induced iNOS protein expression. Cells were treated with LPS in the presence or absence of a 30 min prior 5,7-diOH flavone and Wog (20 μM) treatment for 4 to12 h, and the expression of iNOS and α-tubulin protein was detected. (C) 5,7-diOH flavone expresses the most potent inhibitory effect among tested compounds on LPS-induced NO production. As described in (B), the amount of NO in the medium was measured by Griess reaction. *$P<0.05$, **$P<0.01$ significantly different from LPS-treated group as analyzed by Student's *t*-test.

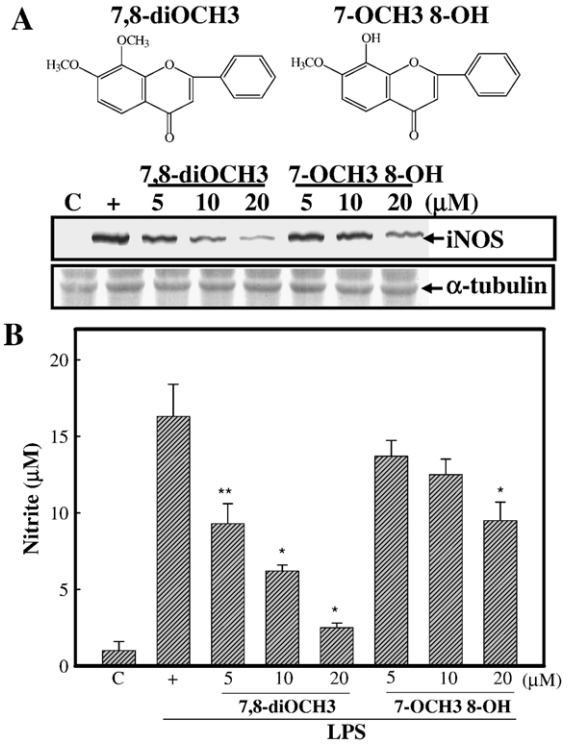

Fig. 5. 7,8-diOCH$_3$ flavone performed more potent inhibition on LPS-induced NO production and iNOS protein expression than 7-OCH$_3$-8-OH flavone. (A) (Upper panel) Chemical structure of both compounds was described. (Lower panel). Cells were treated with different doses (5, 10, and 20 μM) of both compounds for 30 min, followed by incubating with LPS for a further 12 h. The expression of iNOS and α-tubulin protein was detected by Western blotting using specific antibodies. (B) 7,8-diOCH$_3$ flavone expresses more potent inhibitory effect among tested compounds on LPS-induced NO production. As described in (A), the amount of NO in the medium was measured by Griess reaction. *$P<0.05$, **$P<0.01$ significantly different from LPS-treated group as analyzed by Student's *t*-test.

GAPDH mRNA was used as an internal standard. In the lower panel of Fig. 1C, the quantitative data of the iNOS/GAPDH mRNA ratio from three independent experiments are provided. In the same part of the experiment, Wog but not N-Wog dose-dependently inhibited LPS- or LTA-induced NO production in RAW264.7 cells (Fig. 1D). We further examined if Wog's inhibition of NO production occurred by the direct scavenging of NO molecules. Sodium nitroprusside (SNP) is considered as a direct NO donor, and an increase of NO production via dissociation of SNP in aqueous solution has been identified. As illustrated in Fig. 1E, an increase in NO production was detected in the medium of SNP-treated RAW264.7 cells, and neither Wog nor N-Wog affected NO production induced by SNP. Data of an indirect NOS enzyme activity assay showed that Wog and N-Wog were unable to reduce NO production in this assay, and reduction of NO production by an L-arginine analogue (NLA) as a positive control was described (Table 1). Data of MTT assay show that neither Wog nor N-Wog expresses any cytotoxic effect in RAW264.7 cells (Table 1). Similarly, Wog and N-Wog addition did not affect the integrity of DNA of RAW264.7 cells (Fig. 1F). These data indicated that Wog but not N-Wog possesses an inhibitory ability to block LPS-or LTA-induced NO production through suppressing iNOS gene expression at both the mRNA and protein levels.

3.2. Activation of JNKs but not ERKs involves in LPS- and LTA-induced NO production

We further examined the role of JNKs and ERKs in LPS- and LTA-induced NO production. As illustrated in Fig. 2A and B, both LPS and LTA time-dependently induced the expressions of phosphorylated JNK and ERK proteins, however the total forms of both proteins remained constant. Application of the JNK inhibitor, SP600125, but not the ERK inhibitor, PD98059, significantly inhibited LPS- and LTA-induced NO production (Fig. 2C). PD98059 and SP600125 addition respectively attenuated LPS- and LTA-induced ERKs and JNKs protein phosphorylation in cells (Fig. 2D and E). It indicates that activation of JNKs but not ERKs via phosphorylation induction participate in LPS- and LTA-induced iNOS protein expression and NO production in macrophages.

3.3. Wog inhibits LPS- and LTA-induced phosphorylated JNKs and c-Jun protein expression in RAW264.7 macrophages

Expression of c-Jun and c-Fos protein has been shown at the downstream of JNKs activation. We therefore investigate if induction of c-Jun and c-Fos protein is involved in LPS- and LTA-treated RAW264.7 macrophages. As shown in Fig. 3A, an

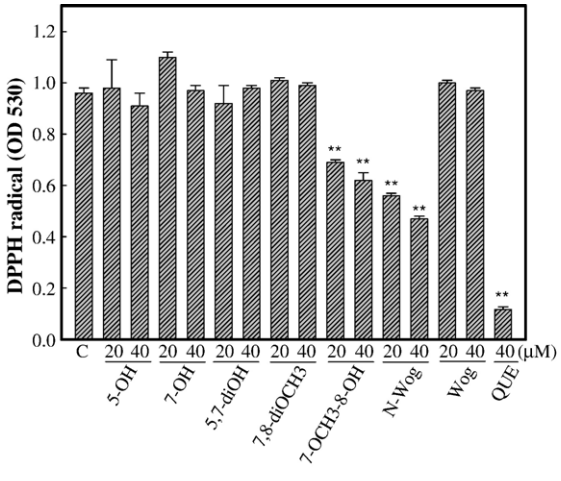

Fig. 6. Differential DPPH radical scavenging effects of flavonoids. As described in the Materials and methods, different doses (20 and 40 μM) of tested compounds were mixed with DPPH solution for 20 min. The absorbance at 517 nm was measured. The decrease in the value of OD517 was calculated for scavenging activity. QUE, quercetin.

increase in c-Jun and phosphorylated c-Jun, but not c-Fos protein, is detected in LPS- and LTA-treated RAW264.7 cells by Western blotting. Incubation of cells with SP600125, but not PD98059, significantly inhibits LPS- and LTA-induced c-Jun protein expression, however no change in the expression of c-Fos and α-tubulin protein was observed (Fig. 3B). Wog, but not N-Wog, treatment attenuates LPS- and LTA-induced phosphorylated JNKs, but not ERKs, protein expression (Fig. 3C). A decrease in c-Jun, but not c-Fos, protein was detected in Wog, but not N-Wog,-treated macrophages. These data suggest that inhibition of iNOS gene expression and NO production by Wog is mediated by blocking JNKs/c-Jun activation in macrophages.

3.4. Hydroxylation at C5 and C7 is essential for flavone inhibition of NO production and iNOS protein expression

Both Wog and N-Wog contain OH groups at C5 and C7, therefore we investigate the differential effect of 5-OH, 7-OH, 5,7-diOH, and 5-OH-7-OCH_3 flavone on LPS-induced iNOS protein expression and NO production. Structure of 5-OH, 7-OH, 5,7-diOH, and 5-OH-7-OCH_3 flavone were described in the upper panel of Fig. 4A. Data of Western blotting show that 5,7-diOH flavone expresses significant inhibitory effect on LPS-induced iNOS protein expression in a dose-dependent manner (Fig. 4A; lower panel). As the same part of experiment, 5,7-diOH flavone and Wog time-dependently inhibit LPS-induced iNOS protein expression (Fig. 4B). Data of the Griess reaction in Fig. 4C supported that 5,7-diOH flavone performed the most potent inhibition against LPS-induced NO production among test compounds in RAW264.7 cells (Fig. 4C). The IC_{50} values of 5,7-diOH flavone and 5-OH-7-OCH_3 flavone on LPS-induced NO production are 7.4±0.8 and 17.3±0.4 μM, respectively.

3.5. OH at C8 attenuates the NO inhibition of flavone

Previous data indicated that Wog, but not N-Wog, inhibited LPS and LTA-induced NO production. The structural difference between Wog and N-Wog is an OCH_3 and OH at C8 of Wog and N-Wog, respectively. In order to provide more evidences to indicate the role of OH and OCH_3 at C8 of flavone on LPS-induced NO production, analysis of 7,8-diOCH_3 and 7-OCH_3-8-OH flavone on LPS-induced iNOS protein expression, as well as NO production, was performed. The chemical structure of 7,8-diOCH_3 and 7-OCH_3-8-OH flavone has been shown in the upper panel of Fig. 5A. In the presence of LPS treatment, both 7,8-diOCH_3 and 7-OCH_3-8-OH flavone expresses differential inhibitory activity on LPS-induced iNOS protein expression, and the inhibitory activity of 7,8-diOCH_3 flavone is more potent than that of 7-OCH_3-8-OH flavone (Fig. 5A; lower panel). Data of Griess reaction show that 7,8-diOCH_3 significantly inhibits LPS-induced NO production, and the IC_{50} values of 7,8-diOCH_3 and 7-OCH_3-8-OH flavone on LPS-induced NO production are 12.6±0.9 and >20 μM, respectively (Fig. 5B). It suggests that OCH_3 but not OH substitution at C8 may contribute to the NO inhibition of flavonoids.

3.6. Analyzing differential anti-DPPH radical activity of 5-OH, 7-OH, 5,7-diOH, 7,8-diOCH3, 7-OCH3-8OH flavone, N-Wog, Wog, and quercetin

In order to examine if NO inhibition of flavonoids attributes to their differential free radical scavenging activity, in vitro anti-DPPH radical was performed. As illustrated in Fig. 6, 7-OCH_3-8-OH flavone and N-Wog significantly reduced DPPH radical production in the present assay. Neither 5-OH, 7-OH, 5,7-diOH, 7,8-diOCH_3 flavone nor Nor-Wog expresses any inhibitory effect against DPPH radical production. A significant inhibition of DPPH radical production by quercetin (QUE) is described as a positive control. It suggests that anti-radical activity is not parallel with NO inhibition of flavonoids.

4. Discussion

We provide evidence to support the roles of OH at C5 and C7, and OCH_3 at C8 on the NO inhibition of Wog. Data of the present study indicate that OH substitutions at C5 and C7 are essential for NO inhibition of flavonoids in LPS- and LTA-induced macrophages. However, OH substitution at C8 may perform inhibitory effect against NO production. The importance of OH or OCH_3 substitution at C8 in the NO inhibition of flavonoids is proposed, and suppression of JNKs–c-Jun signal events is identified.

Activation of intracellular kinase cascades has been reported in LPS- or LTA-induced inflammatory responses. Chang et al. indicated that LTA-induced NO production was mediated by activation of protein kinase A and p38 MAPK in RAW264.7 cells [5]. Wu et al. found that LTA-induced COX-2/PGE2 was mediated by activation of PKC and ERKs in rat cortical neuron cells [15]. Han et al. indicated that activation platelet-activating factor receptor and Jak2 was involved in LTA-induced nitric oxide production [16]. More evidences supported that activation of MAPKs participated in LPS-induced NO production [17,18]. Data of the present study show that activation of ERKs and JNKs, via inducing protein phosphorylation, was detected in LPS- and LTA-treated macrophages, and addition of the JNKs inhibitor SP600125, but not ERKs inhibitor PD98059, reduced the NO production induced by LPS and LTA. It suggests that activation of JNKs, but not ERKs, may participate in LPS- and LTA-induced NO production and iNOS protein expression. Moreover, induction of c-Jun, but not c-Fos, protein is observed in LPS and LTA-treated RAW264.7 cells, which is blocked by SP600125, but not PD98059. The role of JNKs/c-Jun activation in LPS- and LTA-induced NO production is identified in the present study. The effects of Wog and

N-Wog on LPS- and LTA-induced JNKs/c-Jun are still unclear. Data of the present study show that addition of Wog, but not N-Wog, effectively inhibits LPS- and LTA-induced phosphorylated JNKs and c-Jun protein. It suggests that Wog inhibition of iNOS gene expression induced by LPS and LTA is mediated by blocking JNKs/c-Jun activation in macrophages.

The antioxidative properties of flavonoids may be partly related to their anti-inflammatory actions; therefore, it is interesting to investigate the relationship between antioxidant and anti-inflammation of flavonoids. Noroozi et al. indicated that increasing OH numbers might enhance the ROS-scavenging activity of flavonoids [19]. Park et al. reported that flavonoids with three free hydroxyl (OH) groups seemed to produce the optimal effects in increasing ocular blood flow [9]. Silva et al. indicated that the presence of catechol group in the B ring, OH at C3, and the C2–C3 double bond are important for anti-oxidant effect of flavonoids [20]. Rosenkranz et al. suggested that OH at C4′ with a free C5′-position is sufficient for COX-2-inhibiting activity of flavonoids [21]. Our previous studies have shown that OH at C3 is critical for flavone inhibition of epidermal growth factor-induced proliferation [22]. In addition, hydroxylation at C4′ or C6 is essential for apoptosis-inducing activity of flavanone via production of reactive oxygen species [23]. Data of the present study show that Wog, 5,7-diOH flavone, and 7,8-diOCH_3 flavone are effective in inhibiting LPS- or LTA-induced iNOS protein and NO production, however these compounds express no scavenging effect against the production of DPPH radical. Thus, we suggest that antioxidative properties probably do not play a critical role in flavonoids inhibition of iNOS/NO production.

Wog is an active compound in Chinese Herb *S. baicalensis* Georgi, and several biological activities of Wog have been identified. Yu et al. has reported that Wog inhibited N-acetyltransferase activity and gene expression in human leukemia cells HL-60 [24]. Lin et al. indicated that Wog attenuated LPS-induced angiogenesis via inhibition of VEGF/VEGFR-2 pathway [25]. Treatment of malignant cells with Wog sensitizes them to TNF-α and TRAIL-induced apoptosis [26]. Our series studies have shown that Wog effectively inhibited LPS-induced NO production in vitro and in vivo [10,11], and Wog addition suppressed LPS plus TPA-induced malignant transformation through blocking iNOS gene expression and NO production in glioma C6 [12,27]. Although NO inhibition by Wog has been investigated, the roles of structural substitutions including OH at C5 and C7, and OCH_3 at C8 on Wog inhibition of NO production are still undefined. Data of the present study show that (a) Wog, but not N-Wog is effective in inhibiting NO production elicited by LPS and LTA; (b) 5,7-diOH flavone addition significantly inhibited LPS-induced NO production; (c) 7,8-diOCH_3 flavone is more potent than 7-OCH_3-8-OH flavone in NO inhibition stimulated by LPS. It suggests that OH substitutions at C5 and C7 may contribute to the NO inhibition of flavonoids, and the inhibitory role of OH at C8 is identified. These data provide SAR evidences for further development of anti-inflammatory drugs from flavonoids.

NO has been shown in the cause of several chronic human diseases such as heart disease, stroke, cancer, and atherosclerosis [28]. Miyoshi et al. reported that deficiency of iNOS reduces advances atherosclerosis in apolipoprotein E-deficient mice [29]. Data of the present study show that flavonoids such as Wog and 5,7-diOH flavone posses ability to inhibit LPS- and LTA-induced iNOS gene expression and NO production. It indicates that these flavonoids not only can be used for anti-inflammation, but also can be applied to treat chronic human diseases. The therapeutic effect of flavonoids and its SAR against NO-mediated chronic diseases may reserve importance for further investigation.

Acknowledgements

This study was supported by the National Science Council of Taiwan (NSC94-2320-B-038-049 and 95-2320-B-038-029-MY2), Chi Mei Medical Center (96CM-TMU-03), and Topnotch Stroke Research Center Grant, Ministry of Education.

References

[1] Middleton Jr E, Kandaswami C, Theoharis C. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 2000;52:673–751.
[2] Duval DL, Miller DR, Collier J, Billings RE. Characterization of hepatic nitric oxide synthase: identification as the cytokine-inducible form primarily regulated by oxidants. Mol Pharmacol 1996;50:277–84.
[3] Marletta MA. Nitric oxide synthase structure and mechanism. J Biol Chem 1993;268:12231–4.
[4] Nathan C. Nitric oxide as a secretory product of mammalian cells. FASEB J 1992;6:3051–64.
[5] Chang YC, Li PC, Chen BC, Chang MS, Wang JL, Chiu WT, et al. Lipoteichoic acid-induced nitric oxide synthase expression in RAW 264.7 macrophages is mediated by cyclooxygenase-2, prostaglandin E2, protein kinase A, p38 MAPK, and nuclear factor-kappaB pathways. Cell Signal 2006;18:1235–43.
[6] Jiang W, Pisetsky DS. The role of IFN-alpha and nitric oxide in the release of HMGB1 by RAW 264.7 cells stimulated with polyinosinic–polycytidylic acid or lipopolysaccharide. J Immunol 2006;177:3337–43.
[7] Zhao Y, Li H, Gao Z, Gong Y, Xu H. Effects of flavonoids extracted from *Scutellaria baicalensis* Georgi on hemin-nitrite-H_2O_2 induced liver injury. Eur J Pharmacol 2006;536:192–9.

[8] Bonham M, Posakony J, Coleman I, Montgomery B, Simon J, Nelson PS. Characterization of chemical constituents in *Scutellaria baicalensis* with antiandrogenic and growth-inhibitory activities toward prostate carcinoma. Clin Cancer Res 2005;11:3905–14.
[9] Park S, Hahm KB, Oh TY, Jin JH, Choue R. Preventive effect of the flavonoid, wogonin, against ethanol-induced gastric mucosal damage in rats. Dig Dis Sci 2004;49:384–94.
[10] Chen YC, Shen SC, Chen LG, Lee TJF, Yang LL. Wogonin, baicalin, and baicalein inhibition of inducible nitric oxide synthase and cyclooxygenase 2 gene expressions induced by NOS inhibitors and lipopolysaccharide. Biochem Pharmacol 2001;61:1417–27.
[11] Shen SC, Lee WR, Lin HY, Huang HC, Ko CH, Yang LL, et al. In vitro and in vivo inhibitory activities of rutin, wogonin, and quercetin on lipopolysaccharide-induced nitric oxide and prostaglandin E_2 productions. Eur J Pharmacol 2002;446:187–94.
[12] Shen SC, Lin CW, Lee HM, Chien LL, Chen YC. LPS plus TPA induction of migration and invasion of glioma cells in vitro and in vivo: differential inhibitory effects of flavonoids. Neuroscience 2006;140:477–89.
[13] Lin HY, Shen SC, Chen YC. Anti-inflammatory effect of heme oxygenase 1: glycosylation and nitric oxide inhibition in macrophages. J Cell Physiol 2005;202:579–90.
[14] Ko CH, Shen SC, Lee TJF, Chen YC. Myricetin inhibits matrix metalloproteinase 2 protein expression and enzyme activity in colorectal carcinoma cells. Mol Cancer Ther 2005;4:281–90.
[15] Wu HH, Hsieh WS, Yang YY, Tsai MC. Lipoteichoic acid induces prostaglandin E(2) release and cyclooxygenase-2 synthesis in rat cortical neuronal cells: involvement of PKCepsilon and ERK activation. Life Sci 2006;79:272–80.
[16] Han SH, Kim JH, Seo HS, Martin MH, Chung GH, Michalek SM, et al. Lipoteichoic acid-induced nitric oxide production depends on the activation of platelet-activating factor receptor and Jak2. J Immunol 2006;176:573–9.
[17] Jung SW, Lee MH, Kim YC, Paik SG, Choi YH, Kim YS, et al. Modulation of the transactivation function of nuclear factor-kappaB by lipopolysaccharide in RAW264.7 macrophages. Int J Oncol 2004;25:1081–7.
[18] Kim JH, Kim DH, Baek SH, Lee HJ, Kim MR, Kwon HJ, et al. Rengyolone inhibits inducible nitric oxide synthase expression and nitric oxide production by down-regulation of NF-kappaB and p38 MAP kinase activity in LPS-stimulated RAW 264.7 cells. Biochem Pharmacol 2006;71:1198–205.
[19] Noroozi M, Angerson WJ, Lean ME. Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. Am J Clin Nutr 1998;67:1210–8.
[20] Silva MM, Santos MR, Caroco G, Rocha R, Justino G, Mira L. Structure–antioxidant activity relationships of flavonoids: a re-examination. Free Radic Res 2002;36:1219–27.
[21] Rosenkranz HS, Thampatty BP. SAR: flavonoids and COX-2 inhibition. Oncol Res 2003;13:529–35.
[22] Shen SC, Ko CH, Hsu KC, Chen YC. 3-OH flavone inhibition of epidermal growth factor-induced proliferation through blocking prostaglandin E_2 production. Int J Cancer 2004;108:502–10.
[23] Ko CH, Shen SC, Chen YC. Hydroxylation at c4′ or c6 is essential for apoptosis-inducing activity of flavanone through activation of the caspase 3 cascade and production of reactive oxygen species. Free Radic Biol Med 2004;36:897–910.
[24] Yu CS, Yu FS, Chuang YC, Lu HF, Lin SY, Chiu TH, et al. Wogonin inhibits N-acetyltransferase activity and gene expression in human leukemia HL-60 cells. Anticancer Res 2005;25:127–32.
[25] Lin CM, Chang H, Chen YH, Li SY, Wu IH, Chiu JH. Protective role of wogonin against lipopolysaccharide-induced angiogenesis via VEGFR-2, not VEGFR-1. Int Immunopharmacol 2006;6:1690–8.
[26] Fas SC, Baumann S, Zhu JY, Giaisi M, Treiber MK, Mahlknecht U, et al. Wogonin sensitizes resistant malignant cells to TNFalpha- and TRAIL-induced apoptosis. Blood 2006;108:3700–6.
[27] Chen TJ, Shen SC, Lin HY, Chien LL, Chen YC. Lipopolysaccharide enhancement of 12-*i*-tetradecanoylphorbol 13-acetate-mediated transformation in rat glioma C6, accompanied by induction of inducible nitric oxide synthase. Toxicol Lett 2004;147:1–13.
[28] Radi R. Nitric oxide, oxidants, and protein tyrosine nitration. Proc Natl Acad Sci U S A 2004;101:4003–8.
[29] Miyoshi T, Li Y, Shih DM, Wang X, Laubach VE, Matsumoto AH, et al. Deficiency of inducible NO synthase reduces advanced but not early atherosclerosis in apolipoprotein E-deficient mice. Life Sci 2006;79:525–31.