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Biochemical Pharmacology 66 (2003) 1821–1832
Biochemical Pharmacology
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# Inhibition of lipopolysaccharide-induced nitric oxide production by flavonoids in RAW264.7 macrophages involves heme oxygenase-1

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Received 22 January 2003; accepted 12 May 2003

**Abstract**

The role of heme oxygenase-1 (HO-1) played in the inhibitory mechanism of flavonoids in lipopolysaccharide (LPS)-induced responses remained unresolved. In the present study, flavonoids, including 3-OH flavone, baicalein, kaempferol, and quercetin, induced HO-1 gene expression at the protein and mRNA levels in the presence or absence of LPS in RAW264.7 macrophages. This effect was associated with suppression of LPS-induced nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) protein expression. Hemin induced HO-1 protein expression and this was associated with the suppression of LPS-induced NO production and iNOS protein expression in a dose-dependent manner. In addition, an increase in bilirubin production was found in flavonoid- and hemin-treated cells. Hemin, at the doses of 10, 20, and 50 μM, dose-dependently stimulated the flavonoid (50 μM)-induced HO-1 protein expression, and enhanced their inhibitory effects on LPS-induced NO production and iNOS protein expression. Pretreatment of the HO-1 inhibitor, tin protoporphyrin (10 μM), attenuated the inhibitory activities of the indicated flavonoids on LPS-induced NO production. Morphologic analysis showed that 3-OH flavone, baicalein, kaempferol, quercetin, hemin, and tin protoporphyrin did not cause any change in cell viability in the presence or absence of LPS. In contrast, only 3-OH flavone showed a significant inhibition of cell growth using the MTT assay. Transfection of an HO-1 vector in macrophages (HO-1/RAW264.7) resulted in a 3-fold increase in HO-1 protein compared with that the parental RAW264.7 cells. NO production mediated by LPS in HO-1 over-expressed RAW264.7 cells (HO-1/RAW264.7) was significant less than that in parental RAW264.7 cells. 3-OH Flavone, baicalein, kaempferol, and quercetin showed a more significant inhibition on LPS-induced NO production in HO-1/RAW264.7 cells than in parental RAW264.7 cells. These results provide evidence on the role of HO-1 in the inhibition of LPS-induced NO production by flavonoids. A combination of HO-1 inducers (i.e. hemin) and flavonoids might be an effective strategy for the suppression of LPS-induced NO production.


*Keywords:* Flavonoid; Lipopolysaccharides; Nitric oxide; Inducible nitric oxide; Heme oxygenase-1

## 1. Introduction

Heme oxygenase (HO) is the rate-limiting enzyme in the oxidative degradation of heme into bilirubin, iron, and CO, whereas HO-2 and HO-3 are constitutively expressed and HO-1 is the inducible form that provides protection against oxidative stress [1,2]. HO-1-null mice and HO-1-deficient humans showed that HO-1 is an important molecule in the host's defense against oxidative stress and that it has potent anti-inflammatory properties because both HO-1-deficient

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*Abbreviations:* HO-1, heme oxygenase-1; NO, nitric oxide; LPS, lipopolysaccharide; SnPP, tin protoporphyrin; iNOS, inducible nitric oxide synthase; CO, carbon monoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; RT–PCR, reverse transcriptase–polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; GAPDH, glutaldehyde-3-phosphate dehydrogenase.

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doi:10.1016/S0006-2952(03)00422-2

mice and humans have a phenotype of an increased inflammatory state [3,4]. Enhancement of HO-1 protein through the use of agonists prevented the development of hypoxic pulmonary hypertension and indicated that CO might be involved in the vasodilating and antiproliferative action [5–7]. Exogenous administration of HO-1 by gene transfer protected lung cells from free radical-induced lethal effects and showed potent anti-inflammatory effects in the lung [8,9]. Willis *et al*. reported that induction of HO activity and HO-1 expression in macrophages inhibits a carragenin-induced pleural model of acute inflammation in rats [10]. Inhibition of HO activity may result in increased inflammatory responses, and prior induction of HO enzyme activity causes a dramatic decrease in inflammatory parameters [10–12]. These previous data suggested that induction of HO is beneficial in response to stressful situations, such as inflammation; however, the action of HO in the anti-inflammatory process remains unclear.

Production of NO is modulated by NOS, which converts L-arginine to L-citrulline, accompanied by NO production. At least three NOS have been identified, including endothelial NOS (eNOS), iNOS, and neural NOS (nNOS). The small amount of NO produced by constitutive NOS, including eNOS and nNOS, is an important regulator of physical homeostasis, whereas the large amount of NO produced by iNOS has been closely correlated with the pathophysiology in a variety of diseases and inflammation. After exposure to inducers, such as LPS from Gram-negative bacteria or lipoteichoic acid (LTA) from Gram-positive bacteria, iNOS can be induced quantitatively in various cells, such as macrophages, smooth muscle cells, and hepatocytes, to trigger several disadvantageous cellular responses and caused responses similar to inflammation, sepsis, and stroke [13,14]. Therefore, NO production induced by LPS or LTA through iNOS induction may reflect the degree of inflammation and may provide a measure to assess the effect of drugs on the inflammatory process.

Flavonoids have been identified as either simple or complex glycosides in plants, and humans have been estimated to consume approximately 1 g of flavonoids/day [15]. Several beneficial biological effects of flavonoids have been identified, including antioxidant, free radical-scavenging, anticancer, and anti-inflammatory activities [16–20]. Although flavonoids with multiple beneficial biological functions, application of flavonoids in treatment of human diseases is not popular due to their poor absorptive efficacy and higher effective doses. Therefore, trying to understand their action mechanism and interaction with other compounds is an important issue. Our recent studies have demonstrated that flavonoids, such as oroxylin A, quercetin, and wogonin, show potent inhibitory activities on LPS-induced NO and $PGE_2$ production through suppression of iNOS and COX-2 protein expression [18–20]. In the present study, we show scientific evidences that 3-OH flavone, baicalein, kaempferol, and quercetin, are potent HO-1 inducers in RAW264.7 macrophages, and the relationship of HO-1 and inhibitory activities of flavonoids on LPS-induced NO production will be demonstrated in the present study.

## 2. Materials and methods

### 2.1. Cells

RAW264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM glutamine, antibiotics (100 units/mL penicillin A and 100 units/mL streptomycin), and 10% heat-inactivated fetal bovine serum (FBS; Gibco/BRL) and maintained in a 37° humidified incubator containing 5% $CO_2$.

### 2.2. Agents

The four structurally related flavonoids, including 3-OH flavone, baicalein, kaempferol, and quercetin, were obtained from Sigma Chemical Co. LPS (*Escherichia coli*, serotype 055:B5), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), $BaCl_2{\cdot}2H_2O$, hemin, and tin protoporphyrin (SnPP) were purchased from Sigma. Benzene and Giemsa solution were purchased from Merck. The antibodies of anti-HO-1, anti-iNOS, anti-COX-2, and anti-α-tubulin were obtained from Santa Cruz Biotechnology.

### 2.3. Morphological assay

RAW264.7 cells were plated at a density of $5 \times 10^5$ cells/well into 24-well plates for 12 hr and treated with indicated compounds for a further 12 hr. The supernatant was removed and cells were washed twice with PBS. Giemsa solution was added into the cells, and extra-Giemsa solution was removed by PBS. The alternative morphology of cells was detected under microscopy observation.

### 2.4. Cell viability assay

MTT was used as an indicator of cell viability as determined by the mitochrondrial-dependent reduction to formazone. RAW264.7 cells were plated at a density of $10^5$ cells/well into 96-well plates for 12 hr, followed by treatment with different concentrations of each compound for a further 16 hr. Cells were washed with PBS three times and MTT (50 mg/mL) was added to the medium for 4 hr. 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) [20].

### 2.5. Determination of bilirubin in culture medium

RAW264.7 cells were plated a density of $5 \times 10^5$ cells/well into 24-well plates for 12 hr and treated indicated compounds for 12 hr. The 0.5 mL supernatant was collected and 250 mg $BaCl_2 \cdot 2H_2O$ was added. After vortexing, 0.75 mL benzene was added and mixed well again. The benzene phase containing the bilirubin was separated by centrifugation for 30 min at 13,000 *g*. The extraction of bilirubin was determined as a difference in absorbance between 450 and 600 nm ($\varepsilon_{450}$: 27.3 $mM^{-1}$, $cm^{-1}$) [21,22].

### 2.6. Nitrite assay

RAW264.7 cells were plated at a density of $5 \times 10^5$ cells/mL in 24-well plates for 12 hr, followed by treatment with LPS (50 ng/mL) and different concentrations of the indicated compounds, such as flavonoids, hemin, and SnPP, for a further 12 hr. The amount of NO production in the medium was detected with the Griess reaction. 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). The absorbance of the mixture at 530 nm was determined with an ELISA plate reader (Dynatech MR-7000; Dynatech Laboratories), and nitrite concentration was determined using a dilution of sodium nitrite as a standard [19].

### 2.7. Western blotting

Total cellular extracts were prepared according to our previous papers [18–20], separated on 8–12% SDS–polyacrylamide minigels, and transferred to immobilon polyvinylidenedifluoride membranes (Millipore). Membranes were incubated with 1% BSA and then incubated with anti-iNOS, anti-COX-2, or anti-α-tubulin antibodies (Santa Cruz Biotechnology) overnight at 4°. Expression of protein was detected by staining with NBT and BCIP (Sigma) [18].

### 2.8. Reverse transcriptase–polymerase chain reaction (RT–PCR)

RAW264.7 cells were treated with each of the flavonoid compounds (100 μM) for 6 hr and then washed with ice-cold PBS. Total RNA was isolated by RNAzol B (Amersham), and the total RNA concentration was detected using a spectrophotometer. Total RNA (2 μg) was converted to cDNA with oligo d(T). PCR was performed on the cDNA using the following sense and antisense primers, respectively, for HO-1: CTGTGTAACCTCTGCTGTTCC and CCACACTACCTGAGTCTACC; and glutaldehyde-3-phosphate dehydrogenase (GAPDH): TGAAGGTCGGTGTGAACGGATTTGGC and CATGTAGGCCATGAGGTCCACCAC. PCR of the cDNA was performed in a final volume of 50 μL containing PCR primers, oligo d(T), total RNA, and DEPC·$H_2O$. The amplification sequence protocol was 95° for 30 s, 54° for 30 s, 72° for 45 s for 35 cycles. The PCR products were separated by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining [21].

### 2.9. Establishment of HO-1 transfectants

The pCMV-HO-1, a constitutive expression vector, carries full-length human HO-1 cDNA under control of the CMV promoter/enhancer sequence. We transfected pCMV-HO-1 or pCMV into RAW264.7 cells using the Transfast™ transfection reagent (Promega Co). After 48 hr, cells were trypsinized and replated in DMEM with 10% FBS and 400 μg/mL G418. G418 resistant cells were selected and expanded. The level of HO-1 was analyzed by Western blotting.

### 2.10. Statistical analysis

Values are expressed as the mean ± SE. 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 <0.05, or 0.01, was regarded as indicating a significant difference.

## 3. Results

### 3.1. Induction of HO-1 gene expression and bilirubin production by 3-OH flavone, baicalein, kaempferol, and quercetin in RAW264.7 macrophages

The chemical structures of 3-OH flavone, baicalein, kaempferol, and quercetin used in the present study are shown in Fig. 1. In 3-OH flavone-, baicalein-, kaempferol-, and quercetin- (100 μM) treated RAW264.7 cells, HO-1 protein was induced in a time-dependent manner, and the largest induction of HO-1 protein by the indicated flavonoids appeared at 12 hr (Fig. 2A). In the same part of the experiment, RAW264.7 cells were treated with different doses (50, 100, and 200 μM) of the indicated flavonoids for 12 hr, and the expression of HO-1 protein was analyzed by Western blotting. Results in Fig. 2B show that a dose-dependent induction of HO-1 protein was detected in 3-OH flavone-, baicalein-, kaempferol-, and quercetin-treated macrophages. In order to determine if induction of HO-1 gene expression by 3-OH flavone, baicalein, kaempferol, and quercetin appeared at the transcriptional level, RT–PCR using specific primers for HO-1 and GAPDH was performed. Results in Fig. 2C show that 3-OH flavone, baicalein, kaempferol, and quercetin (100 μM) induced HO-1 gene expression at the mRNA level. The mRNA level of GAPDH in

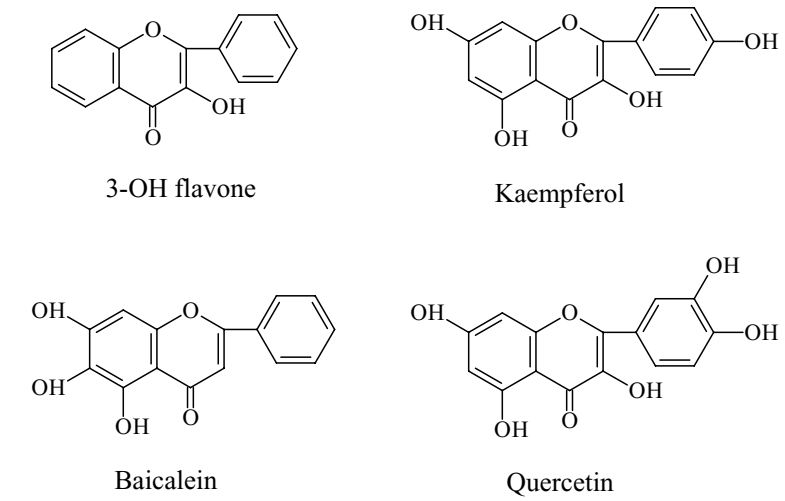


Fig. 1. Chemical structures of 3-OH flavone, baicalein, kaempferol, and quercetin.

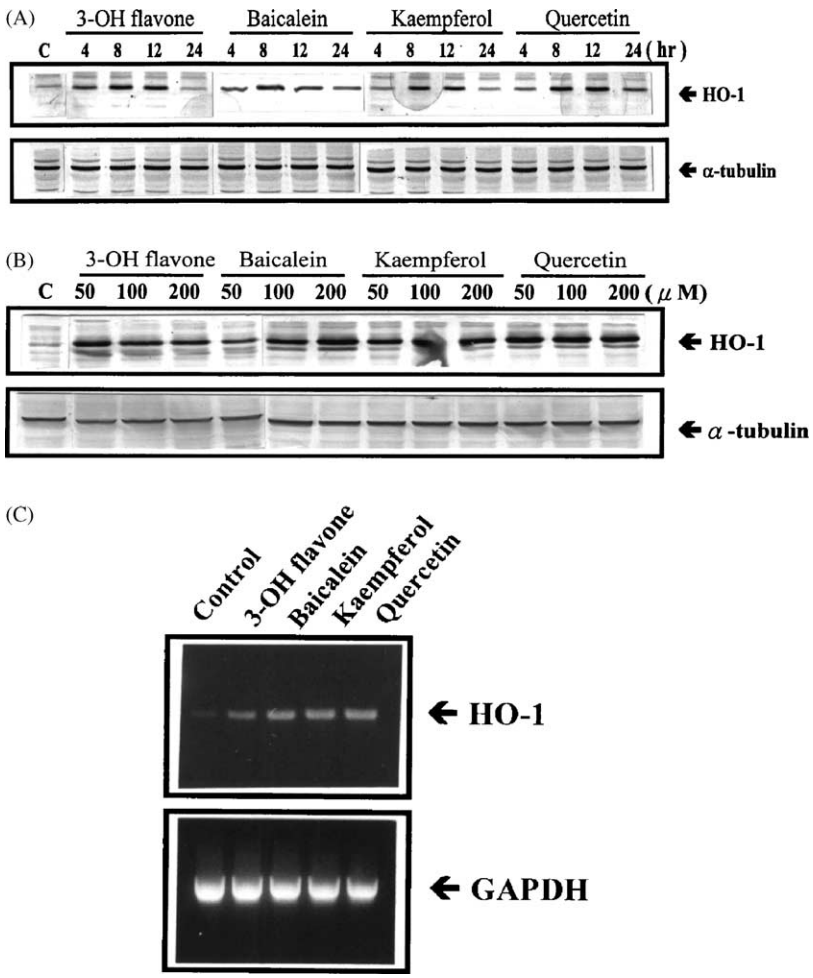


Fig. 2. Induction of HO-1 gene expression by 3-OH flavone, baicalein, kaempferol, and quercetin in RAW264.7 macrophage cells. (A) Time-dependent induction of HO-1 protein by flavonoids. RAW264.7 cells were treated with 3-OH flavone, baicalein, kaempferol, and quercetin (100 μM) for 4, 8, 12, and 24 hr and expression of HO-1 protein in cells was analyzed by Western blotting. (B) Dose-dependent induction of HO-1 protein by flavonoids. Cells were treated with different concentrations (50, 100, and 200 μM) of the indicated compounds for 12 hr, and the expression of HO-1 protein was analyzed. α-Tubulin was used as an internal control. C: control. (C) Induction of HO-1 mRNA levels by flavonoids. RAW264.7 cells were treated with 3-OH flavone, baicalein, kaempferol, and quercetin (100 μM) for 6 hr and total cellular RNA was extracted. Expression of HO-1 mRNA was examined by RT–PCR using specific primers for the HO-1 gene; and GAPDH was used as an internal control.

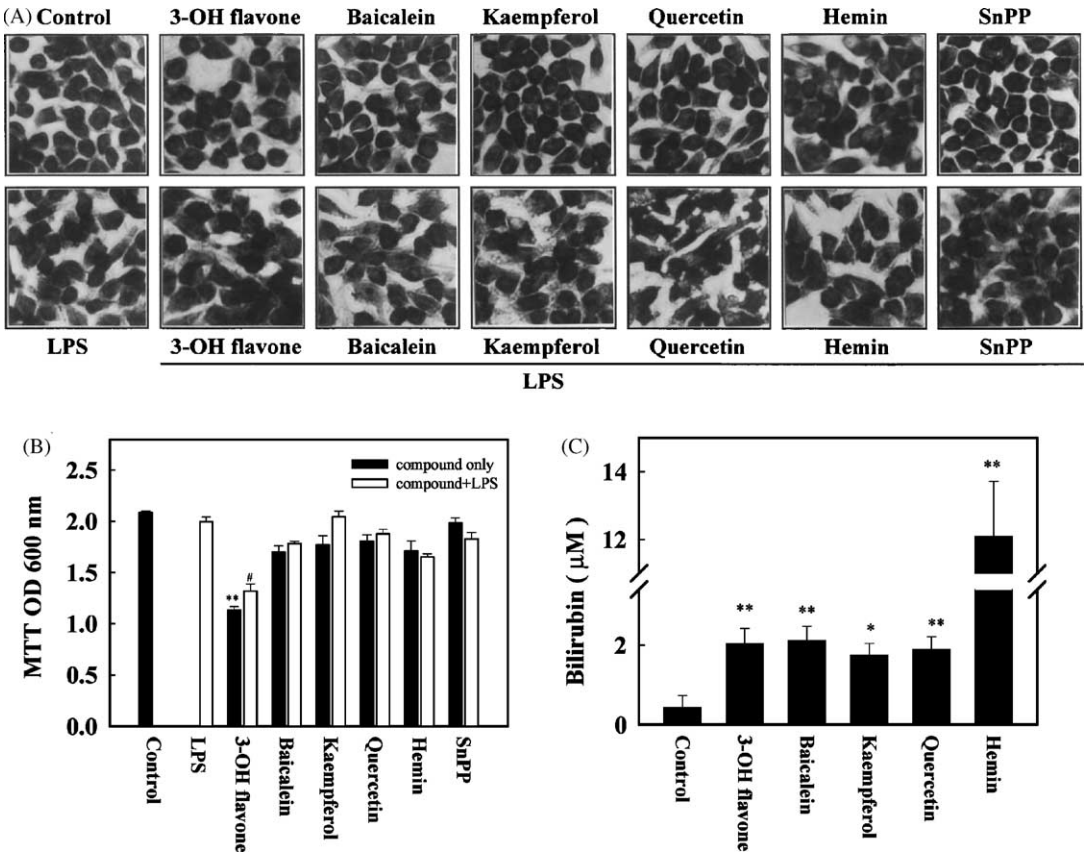


Fig. 3. Effects of flavonoids in RAW264.7 cells by morphological observation, MTT assay, and bilirubin production assay. (A) RAW264.7 cells were treated indicated compounds (100 μM) or SnPP (10 μM) for 12 hr in the presence or absence of LPS, and the morphology of cells was detected by Giemsa staining, followed by a microscopic observation. (B) RAW264.7 cells were treated indicated compounds (100 μM) and SnPP (10 μM) with or without LPS (50 ng/mL) for 12 hr, and the growth condition of cells was determined by MTT assay. (C) RAW264.7 cells were treated indicated compounds (100 μM) for 12 hr, and the amount of bilirubin in the medium was measured as described in Section 2. Data were obtained from six independent experiments, and expressed as the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant difference from the control-treated group, and $^{\#}P < 0.05$ indicates significant difference from the LPS-treated group as analyzed by Student's *t*-test.

cells under different treatments remained unchanged in the RT–PCR assay as an internal control. Furthermore, MTT assay and morphological observation were performed to examine the direct effects of flavonoids on RAW264.7 cells. Results of morphological study showed that 3-OH flavone, baicalein, kaempferol, and quercetin did not cause morphological change in cells under microscopic observation. In the MTT assay, 3-OH flavone but not others showed the significant growth inhibition in cells, compared with control (Fig. 3A and B). Activation of HO-1 enzyme converts heme to bilirubin, an indicative of HO activity. In order to identify if activation of HO-1 enzyme activity in flavonoid-treated cells, production of bilirubin in medium was measured by a $BaCl_2$ extraction method as described in Section 2. Results of Fig. 3C showed that 3-OH flavone, baicalein, kaempferol, and quercetin significantly induced bilirubin production in RAW264.7 cells. HO-1 inducer hemin induction of bilirubin production was described as a positive control. These results demonstrate that 3-OH flavone, baicalein, kaempferol, and quercetin are potent HO-1 inducers, and induction of HO-1 gene expression by these flavonoids were examined at both the transcriptional and translational levels.

### *3.2. Inhibition of LPS-induced NO production and iNOS protein by 3-OH flavone, baicalein, kaempferol, and quercetin associated with an increase of HO-1 protein in RAW264.7 cells*

In the present study, effects of 3-OH flavone, baicalein, kaempferol, and quercetin on LPS-induced NO production in RAW264.7 macrophages were investigated. Nitrite, which had accumulated in the culture medium, was estimated using the Griess reaction as an index for NO synthesis from cells. None of the compounds, at a concentration of 200 μM, interfered with the reaction between nitrite and the Griess reagents (data not shown). After a 12 hr incubation, unstimulated macrophages produced a background level of nitrite of about 2 μM in the culture medium. When cells were incubated with 3-OH flavone, baicalein, kaempferol, or quercetin alone, the amount of nitrite in the medium was maintained at a background level similar to that in the unstimulated

samples (data not shown). After treatment with LPS (50 ng/mL) for 12 hr, nitrite concentration in the medium increased remarkably by about 40 μM. When RAW264.7 macrophages were treated with different concentrations of indicated compounds together with LPS (50 ng/mL) for 12 hr, a concentration-dependent inhibition of nitrite production was detected in the presence of 3-OH flavone, baicalein, kaempferol, and quercetin (Fig. 4B). The $IC_{50}$ values for 3-OH flavone, baicalein, kaempferol, and quercetin on LPS-induced NO production were 32.37, 55.75, 37.84, and 16.52 μM, respectively. Furthermore, the effects of the indicated flavonoids on LPS-induced iNOS and COX-2 protein expression were examined by Western blotting. HO-1 protein was increased slightly in LPS-treated cells, and 3-OH flavone, baicalein, kaempferol, and quercetin all inhibited LPS-induced iNOS protein and induced HO-1 protein in dose-dependent manners; however, the expression of COX-2 protein induced by LPS did not change under treatment with the indicated flavonoids (Fig. 4A).

### *3.3. The HO-1 inducer, hemin, inhibited LPS-induced NO production and iNOS protein, associated with increasing HO-1 protein*

In order to study the relationship between HO-1 protein and LPS-induced NO production, a well-known HO-1 inducer hemin was used in the study. In the absence of LPS, hemin showed a concentration-dependent induction of HO-1 protein in RAW264.7 macrophages (Fig. 5A). In the presence of LPS (50 ng/mL), HO-1 inducer hemin exhibited concentration-dependent inhibition of LPS-induced NO production, accompanied by a concentration-dependent induction of HO-1 protein (Fig. 5B and C). Analysis of iNOS protein expression by Western blotting showed that hemin concentration-dependently inhibited LPS-induced iNOS protein expression (Fig. 5B). MTT assays indicated that hemin at the highest test dose showed no significant cytotoxicity in RAW264.7 macrophages (Fig. 3A and B). It is suggested that induction of HO-1 gene expression was able to inhibit LPS-induced NO production.

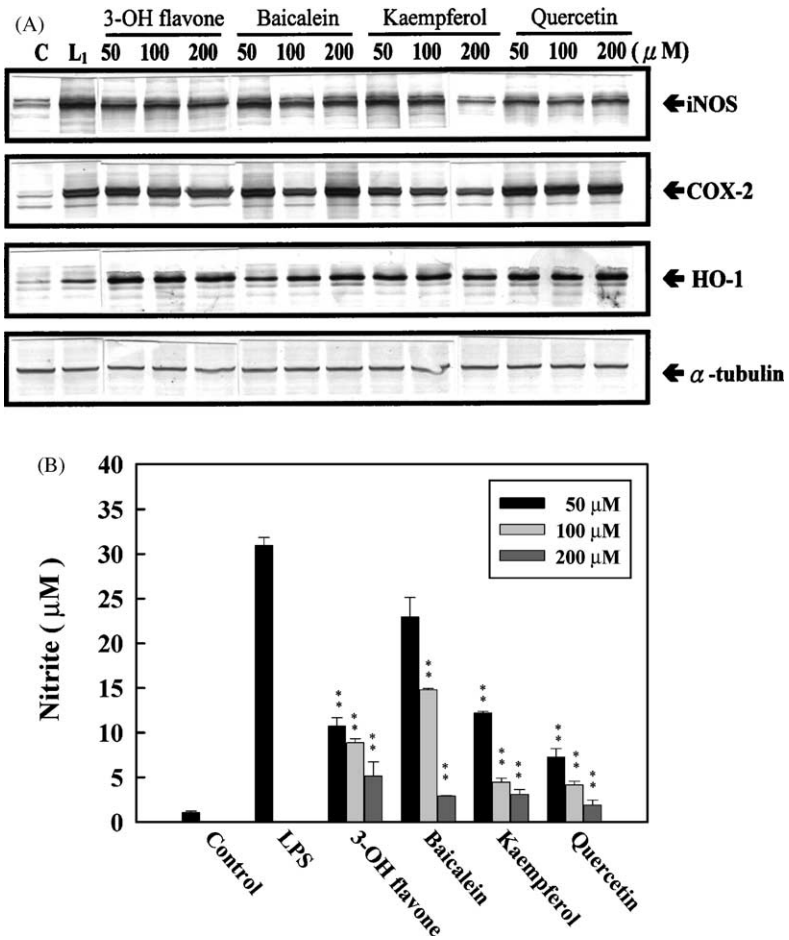


Fig. 4. 3-OH Flavone, baicalein, kaempferol, and quercetin inhibition of LPS-induced iNOS and NO production, associated with increasing HO-1 protein expression. (A) Cells were treated with LPS (50 ng/mL) in the presence of different concentrations (50, 100, and 200 μM) of 3-OH flavone, baicalein, kaempferol, and quercetin for 12 hr, and expressions of iNOS, COX-2, HO-1, and α-tubulin protein were detected by Western blotting. C: control. $L_1$: LPS. (B) NO production in the medium under different treatments was measured by the Griess reaction. The amount of NO production was quantitatively assessed using $NaNO_2$ as a standard. Data were obtained from three independent experiments and are expressed as the mean ± SE. $^{**}P < 0.01$ indicates a significant difference from the LPS-treated group, as analyzed by Student's *t*-test.

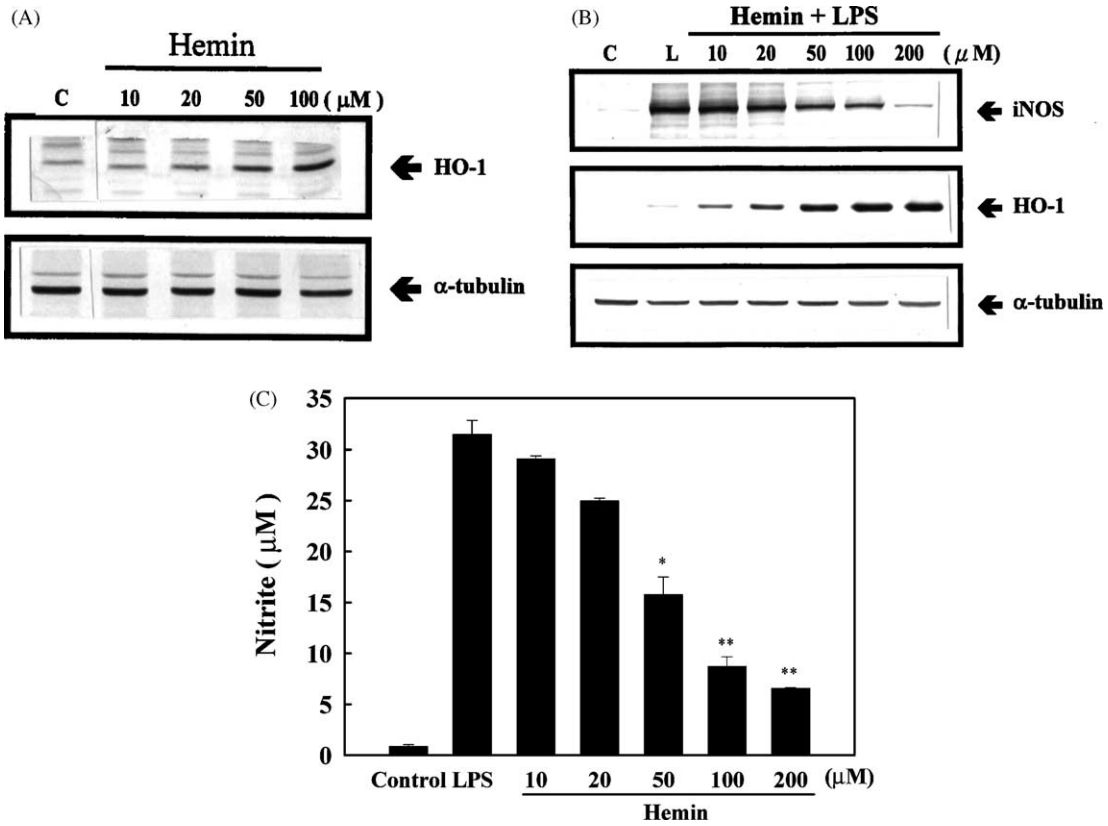


Fig. 5. HO-1 inducer hemin inhibition of LPS-induced iNOS protein and NO production, with increasing HO-1 protein. (A) Dose-dependent induction of HO-1 protein by the HO-1 inducer, hemin. Cells were treated with hemin (10, 20, 50, and 100 μM) for 12 hr, and expressions of HO-1 and α-tubulin protein were analyzed as described in Fig. 2. (B) Dose-dependent inhibition of iNOS protein expression by hemin. Cells were treated with different concentrations of hemin in the presence of LPS (50 ng/mL) for 12 hr; expressions of iNOS, HO-1, and α-tubulin protein were examined by Western blotting. α-Tubulin was used as an internal control. C: control. L: LPS. (C) The amount of NO produced in the medium under different treatments as measured by the Griess reaction, with $NaNO_2$ used as a standard. Data were obtained from three independent experiments and are expressed as the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant difference from the LPS-treated group, as analyzed by Student's *t*-test.

### *3.4. Pretreatment of macrophages with the flavonoids or hemin inhibits following LPS-induced NO production and iNOS gene expression*

Previous data indicated the simultaneous treatments of compounds with LPS suppress NO production and iNOS gene expression, accompanied by inducing HO-1 protein. It is interesting to identify if HO-1present induced by flavonoids or hemin prior to LPS treatment also showed the inhibitory effect on LPS-induced NO production. Results in Fig. 3 indicated that flavonoids time-dependently induced HO-1 protein expression. Therefore, macrophages were pretreated with indicated flavonoids or hemin for 8 hr prior to LPS treatment, and NO production and iNOS gene expression were analyzed. Results of Fig. 6 showed that pretreatment of cells with 3-OH flavone, baicalein, kaempferol, and quercetin (50 and 100 μM) for 8 hr inhibits LPS-induced iNOS gene expression and NO production (Fig. 6). Similarly, hemin (50 and 100 μM) pretreatment inhibits LPS-induced iNOS gene expression and NO production in RAW264.7 cells (Fig. 6). These data suggested that HO-1 induced by flavonoids or hemin prior to LPS treatment inhibits NO production and iNOS gene expression.

### *3.5. Co-inhibitory effects of hemin and indicated flavonoids on LPS-induced NO production and iNOS protein expression*

Results described above indicated that induction of the HO-1 protein might be involved in the inhibition by flavonoids of LPS-induced NO production. In order to provide evidence to identify if induction of HO-1 participated in flavonoid-inhibited NO production, macrophages were treated with the indicated flavonoids (50 μM) with or without low doses of hemin (10, 20, and 50 μM) in the presence of LPS (50 ng/mL) for 12 hr, and NO production, iNOS and HO-1 proteins expression were analyzed by Griess reaction and Western blotting, respectively. Hemin at the doses of 10, 20, and 50 μM showed a slight inductive effect on HO-1 protein expression and exhibited a slight inhibition on LPS-induced NO production as shown in Fig. 5B and C. Results in Fig. 7 show that hemin dose-dependently enhanced the inhibitory activities of 3-OH flavone, baicalein, kaempferol, and quercetin on LPS-induced NO production and iNOS protein expression, associated with stimulating 3-OH flavone-, baicalein-, kaempferol-, and quercetin-induced

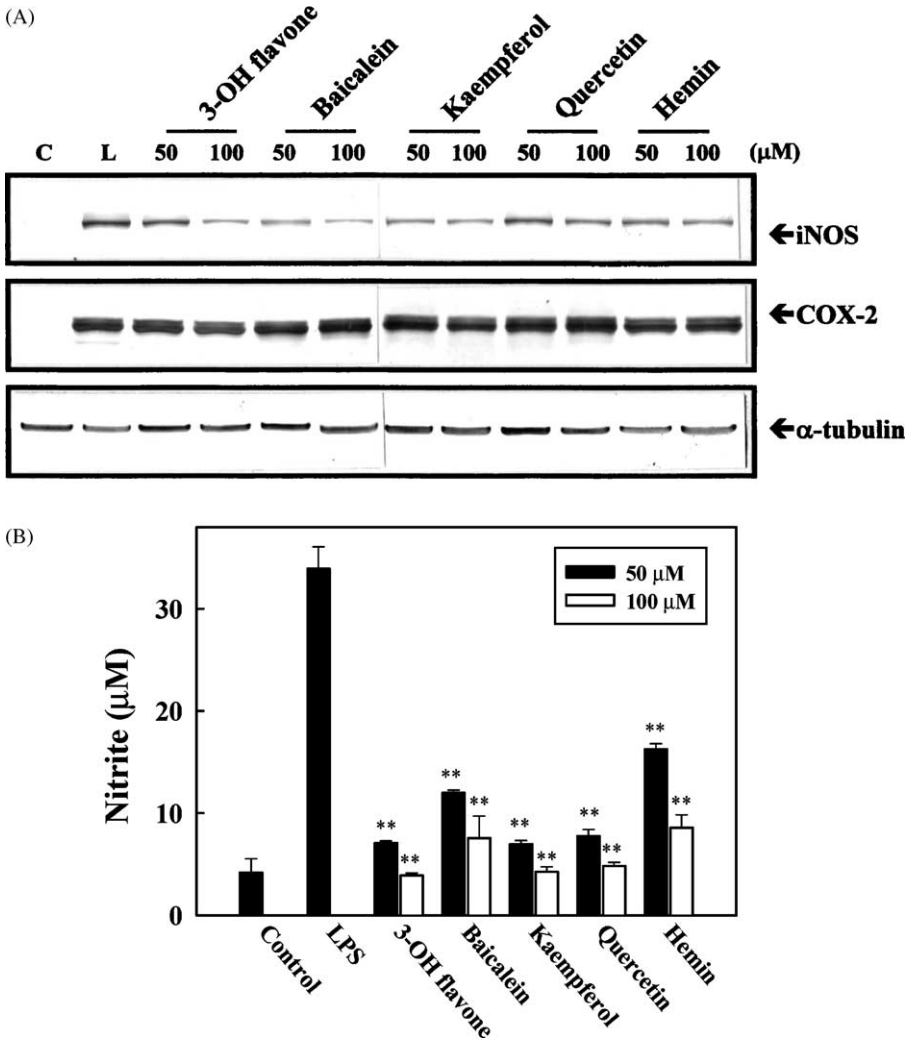


Fig. 6. Pretreatment of 3-OH flavone, baicalein, kaempferol, quercetin, or hemin inhibits LPS-induced iNOS gene expression and NO production. Macrophages pretreated with 3-OH flavone, baicalein, kaempferol, quercetin or hemin (50 and 100 μM) for 8 hr followed by LPS treatment (50 ng/mL) for a further 12 hr. The expression of iNOS protein expression (A) and NO production (B) were examined by Western blotting and Griess reaction, respectively. Data were obtained from three independent experiments and are expressed as the mean ± SE. $^{**}P < 0.01$ indicates significant difference from the LPS-treated group, as analyzed by Student's *t*-test.

HO-1 protein expression. These data demonstrate that induction of HO-1 protein expression by the HO-1 inducer hemin was able to potentiate the inhibitory effects of the indicated flavonoids on LPS-induced NO production, and an additive effect of hemin and the indicated flavonoids on LPS-induced NO production was observed.

### *3.6. The HO inhibitor, SnPP, attenuated the inhibitory activities of 3-OH flavone, baicalein, kaempferol, and quercetin on LPS-induced NO production*

SnPP has been described as an inhibitor of HO activity [23,24]. Therefore, co-treatment of macrophages with 3-OH flavone, baicalein, kaempferol, and quercetin (50 μM) with or without SnPP in the presence of LPS (50 ng/mL) was performed, and the amount of NO production was measured with the Griess reaction. The amount of NO in the medium increased in LPS-treated cells, while 3-OH flavone, baicalein, kaempferol, and quercetin inhibited its induction. Co-treatment of cells with SnPP and the indicated flavonoids in the presence of LPS significantly attenuated their inhibitory activities of flavonoids on LPS-induced NO production (Fig. 8). These data suggest that activation of HO-1 participated in the inhibitory mechanism of flavonoids 3-OH flavone, baicalein, kaempferol, and quercetin on LPS-induced NO production.

### *3.7. Over-expression of HO-1 protein potentiated the inhibitory activity of flavonoids on LPS-induced NO production*

In order to provide more direct evidence to support HO-1 could be involved in flavonoid-inhibited NO production, HO-1 over-expressed RAW264.7 cells were established in our study. RAW264.7 macrophages cells were transfected with HO-1 expression vector, and a stable clone of HO-1

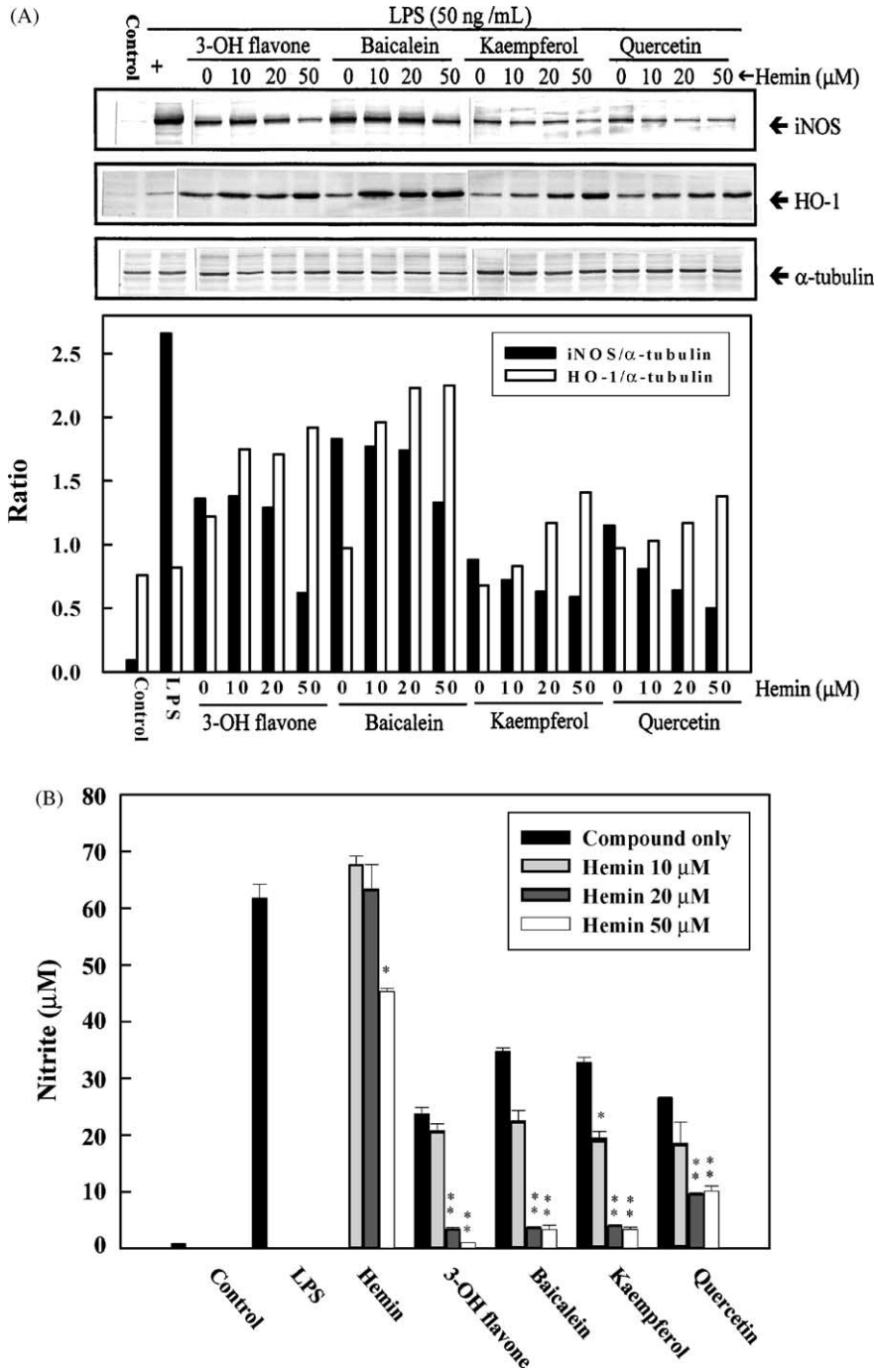


Fig. 7. Stimulation by low doses of hemin of the inhibitory activities of flavonoids on LPS-induced NO production. Cells were treated with low doses of hemin (10, 20, and 50 μM) and the indicated flavonoids, including 3-OH flavone, baicalein, kaempferol, and quercetin (50 μM), in the presence of LPS (50 ng/mL) for 12 hr. (A) Expressions of iNOS, HO-1, and α-tubulin protein were examined by Western blotting using specific antibodies. Band intensities of iNOS and HO-1 were quantified by densitometry analysis, and the data were expressed as mean ratio of iNOS/α-tubulin and HO-1/α-tubulin derived from three independent experiments. (B) The amount of NO produced in the medium under different treatments was measured by the Griess reaction. Data were obtained from three independent experiments and are expressed as the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant differences from the indicated flavonoid-treated group, as analyzed by Student's *t*-test. The hemin-treated group showed a significant difference from the LPS-treated group.

over-expressed RAW264.7 macrophages was established by G418 selection method (HO-1/RAW264.7). The control vector (pCMV) transfected RAW264.7 cells show the same HO-1 protein expression and NO production induced by LPS as in parental RAW264.7 cells (data not shown). Western blotting analysis was performed to identify the inductive level of HO-1 protein in HO-1/RAW264.7 cells. Results of Fig. 9A showed that expression of HO-1 protein was increased about 3-fold in HO-1/RAW264.7 cells, compared with parental RAW264.7 macrophages. In the presence of different doses of LPS, NO production induced by LPS in HO-1/RAW264.7 is significant less than that in

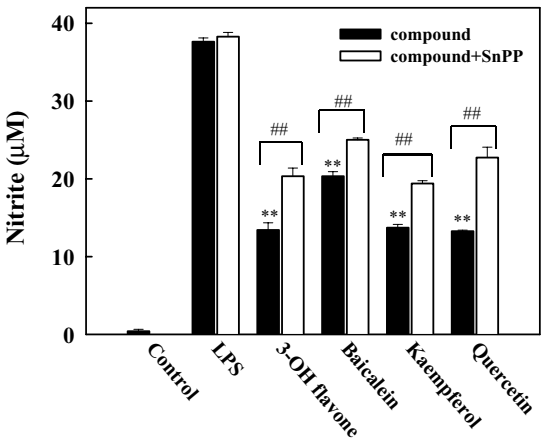


Fig. 8. Tin protoporphyrin (SnPP) attenuation the inhibitory effects of flavonoids on LPS-induced NO production in RAW264.7 cells. Cells were treated with the indicated flavonoids (50 μM) and LPS (50 ng/mL) in the presence or absence of SnPP (10 μM) for 12 hr, and the amount of NO produced in the medium was determined by the Griess reaction. Data were obtained from six independent experiments and are expressed as the mean ± SE. $^{**}P < 0.01$ indicates significant differences from the indicated flavonoid-treated group; $^{\#\#}P < 0.01$ indicates significant difference from respective group, as analyzed by Student's *t*-test.

parental RAW264.7 cells (Fig. 9B). It is indicated that HO-1 over-expression attenuated the responses of cells to LPS. Accordingly, 3-OH flavone, baicalein, kaempferol, and quercetin showed more significant inhibition on LPS-induced NO production in HO-1/RAW264.7 cells than that in parental RAW264.7 cells (Fig. 9C). These data show that HO-1 may participate in flavonoids inhibition of LPS-induced NO production, and increasing intracellular HO-1 protein potentiates the inhibitory activity of flavonoids on LPS-induced NO production.

## 4. Discussion

Flavonoids exist extensively in the plants and multiple biological functions in several previous studies [25–27]. However, the role that HO-1 plays in the inhibitory effects of flavonoids on LPS-induced NO production has remained unclear. In the present study, we demonstrate that 3-OH flavone, baicalein, kaempferol, and quercetin were able to inhibit LPS-induced NO production, accompanied by the induction of HO-1 gene expression. The inhibitory effects of 3-OH flavone, baicalein, kaempferol, and quercetin on LPS-induced NO production were attenuated by the HO inhibitor, SnPP, and potentiated by the HO-1 inducer, hemin. In addition, HO-1 over-expressed RAW264.7 cells exhibited less sensitive to NO production induced by LPS than parental RAW264.7 cells, and flavonoids showed the more potent inhibitory activity to LPS-induced NO production in HO-1/RAW264.7 cells than those in parental RAW264.7 cells. Results of the present study provide the first evidence demonstrating that HO-1 involves in the anti-inflammatory effects of flavonoids, and a combination of an HO-1 inducer, such as hemin and flavonoids, might be a promising strategy for the inhibition of LPS-induced NO production.

HO-1 (HSP32) is one member of the heat shock protein (HSP) group which responds to a number of cellular injuries, including thermal or oxidant stress, and also plays an essential role in the cell's adaptive responses to protect cells against thermal and oxidative stresses [28]. Hemin is an effective inducer of HO-1 mRNA expression and activated HO-1 enzyme activity in human hepatoma cells. Previous studies demonstrated that HO-1 activity increased following treatment with hemin, and the metabolites heme to biliverdin and bilirubin that have been shown to possess potent antioxidant properties [29,30]. Scapagnini *et al*. demonstrated that plant-derived phenolic compounds of caffeic acid phenethyl ester (CAPE) and curcumin increased heme oxygenase activity and HO-1 protein in astrocytes with elevating intracellular GSH level [31]. These data suggested that induction of HO-1 involved in the antioxidative process. In this study, hemin significantly induced HO-1 gene expression associated with inhibition of LPS-induced NO production. The inhibitory effects of flavonoids on LPS-induced NO production were potentiated significantly by lower doses of hemin. These data suggest that the inhibition of flavonoids on LPS-induced NO production parallels the inhibitory action of hemin, and the increase in HO-1 protein was able to potentiate the inhibitory effects of flavonoids on NO production induced by LPS. SnPP is a common HO inhibitor, and the inhibition of HO activity by SnPP has been suggested in several previous studies [23,24]. In this study, SnPP attenuated the inhibitory activities of the indicated flavonoids on LPS-induced NO production. These data suggest that activation of HO activity is involved in flavonoid inhibition of LPS-induced NO production. Two other HO inhibitors, ZnPP and CoPP, were also used in the present study; however, attenuation of flavonoid-inhibited NO production by ZnPP and CoPP was not observed because ZnPP and CoPP showed potent inductive effects on HO-1 gene expression in cells (data not shown). Similar results of HO-1 induction by ZnPP and CoPP have been reported in previous studies [32–34].

In order to examine if the inhibitory effects of flavonoids on LPS-induced NO production through their cytotoxic effect, microscopic observation and MTT assay were performed in the present study. Baicalein, kaempferol, and quercetin showed no significant cytotoxic effect in cells. However, only 3-OH flavone decreases the number of viable cells by MTT assay. 3-OH Flavone is an interesting compound, and a decrease in the number of viable cells was found by MTT and trypan blue exclusion assays. However, neither morphological change by microscopic observation nor DNA fragmentation by agarose electrophoresis was found (data not shown). Our unpublished data showed that 3-OH flavone was able to suppress cell growth through modulation of cell cycle progression, and more detailed action of 3-OH flavone in cell cycle regulation is under investigating.

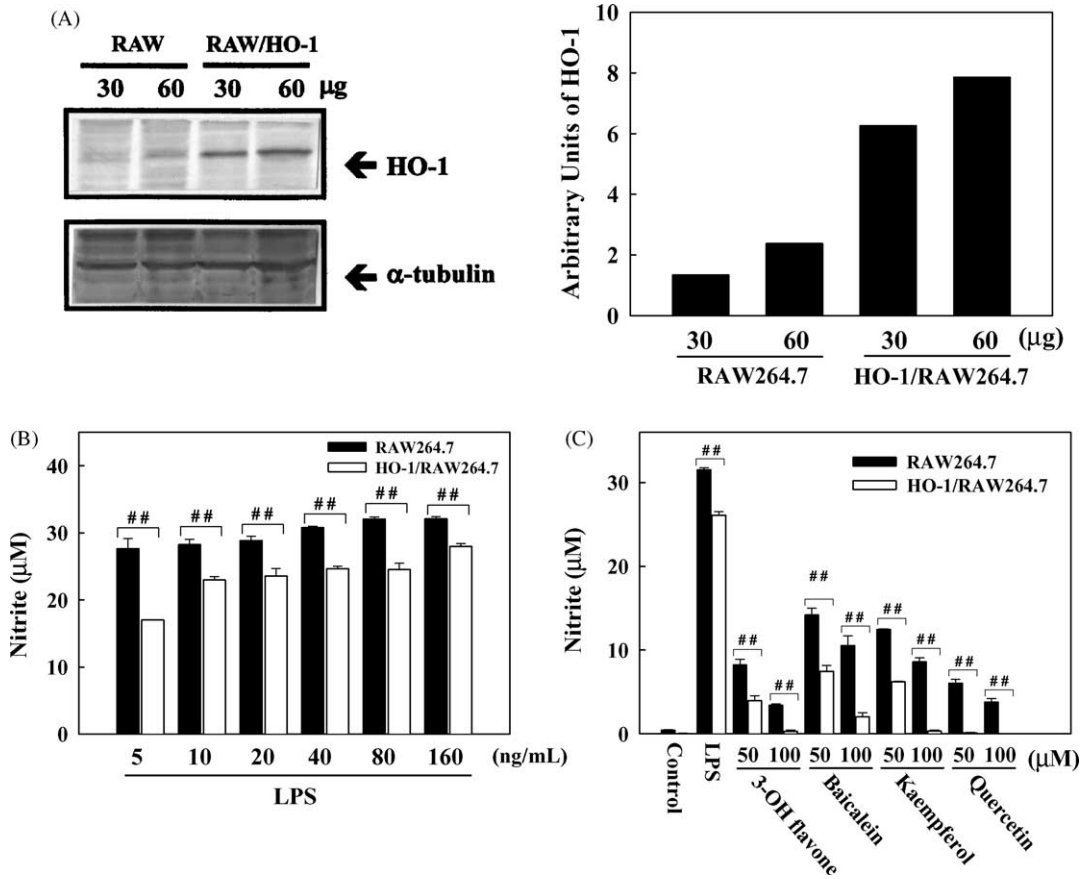


Fig. 9. 3-OH Flavone, baicalein, kaempferol, and quercetin showed more significant NO inhibition in HO-1/RAW264.7 cells than that in parental RAW264.7 cells. (A) The different amount of total protein (30 and 60 μg) extracted from RAW264.7 cells and HO-1/RAW264.7 cells were applied to SDS–PAGE electrophoresis, and the level of HO-1 protein expression was detected by Western blotting. α-Tubulin was described as an internal control. Right panel: Band intensity of HO-1 protein was quantified by a densitometry analysis, and expressed as a mean value derived from three independent experiments. (B) The amount of NO production induced by LPS in RAW264.7 cells and HO-1/RAW264.7 was measured by Griess reaction. Both cells were treated with different doses of LPS (5, 10, 20, 40, 80, and 160 ng/mL), and the amount of nitrite in the medium was measured as described previously. (C) 3-OH Flavone, baicalein, kaempferol, and quercetin showed the more significant inhibition in HO-1/RAW264.7 cells than that in parental RAW264.7 cells. Both RAW264.7 cells and HO-1/RAW264.7 cells were treated with 3-OH flavone, baicalein, kaempferol, and quercetin (50 and 100 μM) in the presence of LPS (50 ng/mL) for 12 hr, and NO produced in medium was detected by Griess reaction. NO production was determined using dilution of $NaNO_2$ as a standard and the absorbance at O.D. = 530 nm. Data were obtained from six independent experiments and expressed as mean ± SE. $^{\#\#}P < 0.01$ indicates significant difference from respective group.

The relationship between HO-1 induction and NO inhibition by flavonoids was first suggested in the present study. Results of direct and indirect NOS activity assays showed that flavonoids and the HO-1 inducer, hemin, caused slight but significant inhibition of NO production in an indirect NOS enzyme activity assay; however, decreasing NO production by these compounds was not examined in a direct NOS activity assay (data not shown). In the indirect NOS activity, HO-1 protein increased in cells which were pretreated with LPS for 12 hr followed by indicated HO-1 inductive compounds treatment. It is indicated that differential inhibitory effects of flavonoids and hemin on NO production between indirect and direct NOS activity assays might be due to HO-1 protein expression in the indirect NOS activity assay.

In conclusion, results of our present study demonstrate that induction of HO-1 gene expression is involved in the inhibitory effects of flavonoids on LPS-induced NO production, and increases in HO-1 protein potentiate their inhibitory activities. We suggest that a combination of HO-1 inducers, such as hemin, and functional flavonoids, such as 3-OH flavone, baicalein, kaempferol, and quercetin, would be very effective in preventing the NO production induced by LPS in cells. In the study, inhibition of NO production occurred simultaneously with a decrease of iNOS protein in flavonoid- and/or HO-1 inducer hemin-treated macrophages under LPS treatment. This suggests that induction of HO-1 is able to inhibit NO production through suppression of iNOS gene expression. Otterbein and *et al*. reported that metabolites of hemin, including biliverdin and bilirubin, were able to inhibit NOS activity through their antioxidant activities [35]. It is suggested that induction of HO-1 was able to decrease NO production induced by LPS through directly inhibited NOS activity or suppressed iNOS gene expression. The relationship between HO-1 induction and iNOS protein inhibition is still undefined, and more necessary evidence will be provided in further studies.

## Acknowledgments

This study was supported by the National Science Council of Taiwan (NSC 89-2320-B-038-054, NSC 90-2320-B-038-027, and NSC 91-2320-B-038-040).

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