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Effects of Naturally Occurring Flavonoids on Nitric Oxide Production in the Macrophage Cell Line RAW 264.7 and Their Structure–Activity Relationships

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ABSTRACT. Flavonoids affect the inflammatory process of the mammalian system and possess anti-inflammatory as well as immunomodulatory activities *in vitro* and *in vivo*. Since nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) is one of the inflammatory mediators, the effects of various naturally occurring flavonoids on NO production in LPS-activated RAW 264.7 cells were evaluated *in vitro*. Flavonoids such as apigenin, wogonin, luteolin, tectorigenin, and quercetin inhibited NO production, as measured by nitrite formation at 10–100 μM. The most active among 26 flavonoid derivatives tested were apigenin, wogonin, and luteolin, having IC_{50} values of 23, 17, and 27 μM, respectively, while AMT, a synthetic selective iNOS inhibitor, had an IC_{50} value of 0.09 μM. In contrast, flavanones, such as naringenin, and flavonoid glycosides, such as apiin, did not demonstrate significant inhibition up to 100 μM. These results clearly indicated that a C-2,3 double bond might be important, and that the potency of inhibition depended upon the substitution patterns of the flavonoid molecules. The inhibitory activity of flavonoids was not due to direct inhibition of iNOS enzyme activity because they did not reasonably inhibit iNOS activity, as measured by [^{3}H]citrulline formation from [^{3}H]arginine, up to 100 μM. In contrast, wogonin and luteolin concentration-dependently reduced iNOS enzyme expression, when measured by western blotting, at 10–100 μM. All these results clearly demonstrated that certain flavonoids inhibit NO production in lipopolysaccharide-activated RAW 264.7 cells, and their inhibitory activity might be due to reduction of iNOS enzyme expression. BIOCHEM PHARMACOL **58**;5:759–765, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. flavonoid; wogonin; luteolin; RAW 264.7; nitric oxide; inducible nitric oxide synthase; western blotting; inflammation

NO† is synthesized from L-arginine by NOS in various animal cells and tissues. Among the isoforms identified thus far, iNOS is an inducible one, newly synthesized by activating signals such as LPS and/or cytokines [1]. iNOS produces a micromolar amount of NO for a long period when induced. NO produced in this way shows cytotoxicity and tissue damage, whereas nanomolar concentrations of NO produced by constitutive forms of NOS are essential to maintain normal cellular functions [1, 2]. NO is involved in various biological processes including inflammation and immunoregulation [3, 4]. Therefore, inhibition of NO production by iNOS may have potential therapeutic value when related to inflammation and septic shock. In addition to many synthetic inhibitors of iNOS, natural products inhibiting NO production also have been found [5–7]. And there have been several studies to investigate the inhibitory activity of flavonoids, one of the large families of plant constituents. Genistein, an isoflavone known as a tyrosine kinase inhibitor, shows mild inhibitory activity of NO production from LPS-activated RAW 264.7 cells [8]. Flavone and amino-substituted flavones have been reported to inhibit NO production from murine activated peritoneal macrophages [9]. Epigallocatechin gallate from tea, one of the flavonoids (polyphenol), has been demonstrated to inhibit NO production and iNOS enzyme expression from mouse peritoneal cells [10, 11]. And recently, several flavonoids including quercetin, morin, and apigenin have been reported to inhibit NO production from C6-astrocytes [12]. Although these previous results demonstrated that certain flavonoids/polyphenols might have the potential to affect NO production and iNOS enzyme activity, the effects of flavonoids based on their chemical structures have not been determined. Therefore, in this investigation, we evaluated the effects of structurally diverse flavonoid derivatives on NO production from LPS-activated RAW 264.7 cells, on iNOS enzyme activity, and on iNOS enzyme expression. We found that certain flavonoids having a C-2,3 double bond, such as wogonin and luteolin, strongly

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† *Abbreviations*: NO, nitric oxide; NOS, nitric oxide synthase; LPS, lipopolysaccharide; AMT, 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine hydrochloride; 7-NINA, 7-nitroindazole sodium; BH_4, (6*R*)-5,6,7,8-tetrahydrobiopterin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; and MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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inhibited NO production, mainly by reducing iNOS enzyme expression, at 10–100 μM.

MATERIALS AND METHODS

Chemicals

AMT and 7-NINA were purchased from Tocris Cookson. LPS (*Escherichia coli* 0127:B8), NADPH, FAD, and BH_4 were obtained from the Sigma Chemical Co. DMEM and other cell culture reagents including FBS were products of Gibco Laboratories. Dowex-50W X8 cation exchanger and a protein assay kit were purchased from Bio-Rad Laboratories. L-[2,3,4,5-^{3}H]Arginine monohydrochloride (58 Ci/mmol) was obtained from Amersham Life Sciences.

Flavonoids

Flavanone, naringenin, (+)-catechin, flavone, apigenin, luteolin, biochanin A, galangin, quercetin, morin, and myricetin were purchased from the Aldrich Chemical Co. Flavonol was obtained from TCI. Chrysin, wogonin, and skullcapflavone II were isolated from Scutellaria radix, and isoflavones such as tectorigenin, iristectorigenin A, and irisflorentin were isolated from Bellamcanda rhizome as described previously [13]. Daidzein, formononetin, genistein, and puerarin were isolated from Pueraria radix according to a previously published method [14]. Vitexin and isovitexin were isolated from mung bean as has been described [15]. Apiin and icariin were isolated from celery and Epimedii herba, respectively, and identified using authentic standards provided by Dr. Sam Sik Kang (Natural Products Research Institute, Seoul National University).

NO Production from RAW 264.7 Cells

RAW 264.7, a murine monocyte/macrophage cell line, was obtained from the American Type Culture Collection. Cells were cultured in Falcon plates (100 mm) with DMEM supplemented with 10% FBS and 1% antibiotics under 5% CO_2 at 37°. To study the effects of flavonoids on NO production, cells were plated in 96-well plates (2×10^5 cells/well) using fresh medium. After preincubation for 2 hr, LPS (1 μg/mL) and flavonoids were added and incubated for another 24 hr. Flavonoids or reference compounds were dissolved in DMSO on the day of experiment and diluted with serum-free DMEM into appropriate concentrations. The final concentration of DMSO was adjusted to 0.1% (v/v). Control wells also received the same amount of DMSO. At this concentration of DMSO, no significant change in cytotoxicity and NO productivity was observed in preliminary experiments. For testing cell viability, the MTT assay was used in the presence or absence of LPS and/or testing compounds based on the method previously described [16]. MTT solution (0.5 mg/mL) was added to each well and incubated for 4 hr. The formazan crystals were dissolved by addition of DMSO (200 μL), and absorbance was measured (570 nm). Sometimes a dye exclusion test using trypan blue also was employed.

Measurement of Nitrite Concentration

To measure the concentration of NO produced during a 24-hr incubation period, unless otherwise specified, the stable conversion product of NO, nitrite (NO_2^-), was measured. A standard procedure using Griess reagent [1:1 mixture (v/v) of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 5% H_3PO_4] was employed. Medium (100 μL) and Griess reagent (100 μL) were mixed in the 96-well plates and left for 10 min at room temperature. Optical density was measured with a microplate reader (Spectra Max, Molecular Devices) at 550 nm.

iNOS Activity Assay

The enzyme preparation was obtained from RAW 264.7 cells cultured in a 100-mm plate after activation with LPS (1 μg/mL) for 20 hr. The cells were collected and washed twice with serum-free DMEM. Then they were homogenized in a Teflon homogenizer in 50 mM Tris–HCl buffer, pH 7.4, containing 0.1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 2 μM leupeptin, and 0.1% 2-mercaptoethanol. The lysate was centrifuged at 15,000 *g* for 30 min at 4°, and the supernatant was used as the iNOS source. iNOS enzyme activity was measured by [^{3}H]citrulline formation from [^{3}H]arginine, essentially following the procedure of Kobuchi *et al.* [7] with minor modifications. Briefly, the reaction mixture (170 μL) consisted of 1 mM NADPH, 10 μM FAD, 1 mM dithiothreitol, 100 μM BH_4, 0.2 mM EGTA, [^{3}H]arginine (0.5 μCi/mL of reaction medium), and 50 μL of homogenate in 50 mM HEPES buffer (pH 7.5). After incubation for 1 hr at 37°, the reaction was terminated by the addition of 200 μL of cold stop buffer (50 mM morpholinoethanesulfonic acid, pH 5.5, and 5 mM EDTA). Then the reaction mixture was applied to a 0.6 mL column of Dowex 50W X8 resin (Na^+ form), prewashed with 0.5 N NaOH and distilled water, and the column was eluted with 1 mL of stop buffer. Radioactivity of the combined eluate was measured using a liquid scintillation counter (LKB model 1209).

Western Blotting of iNOS Enzyme

RAW 264.7 cells were cultured in a 100-mm plate in the presence or absence of LPS (1 μg/mL) and/or flavonoids for 20 hr. Cells were washed, harvested, and homogenized. The lysate was centrifuged at 15,000 *g* for 30 min, and the supernatant was freeze-dried. Using Tris-glycine gel (4–20%), electrophoresis was carried out with a Novex Mini-cell-II apparatus. Bands were blotted to a PVDF membrane. iNOS antibody (N32030, Transduction Laboratory) was incubated, and iNOS bands were visualized by treatment of secondary antibody and DAB reagent (Vector Laboratory).

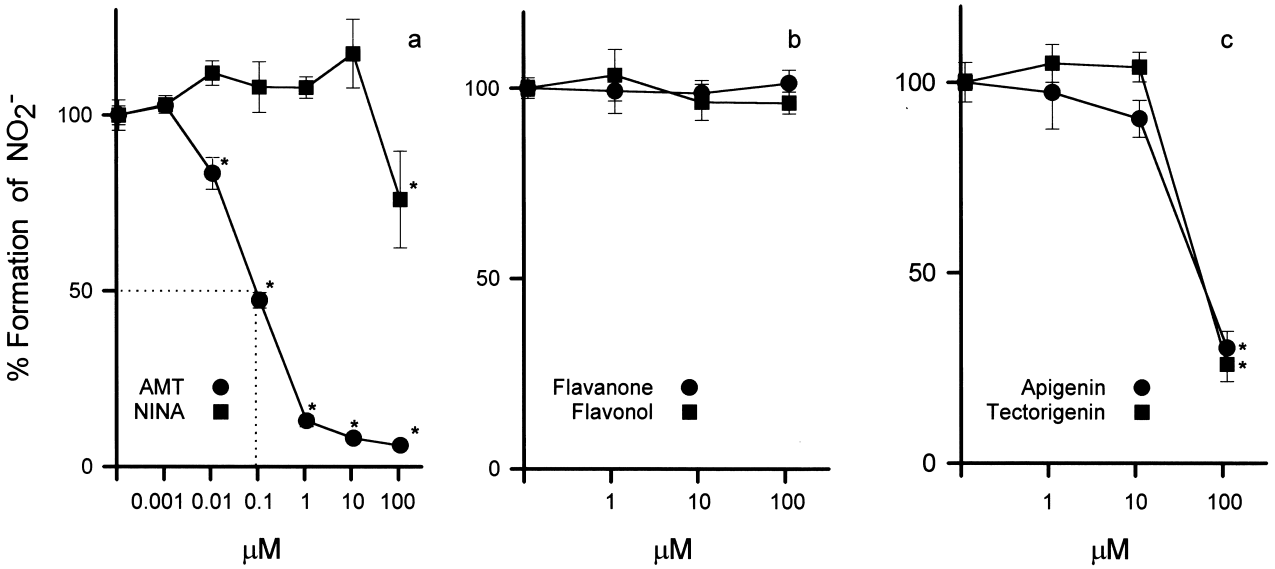


FIG. 1. Effects of various flavonoids on nitrite production of LPS-activated RAW 264.7 cells. LPS (1 μg/mL) with or without compounds was added to RAW 264.7 cells, and nitrite concentration was measured. Percent formation of nitrite was calculated after subtracting the nitrite concentration of the control group only with vehicle (0.1% DMSO) for 24 hr. Nitrite concentrations of LPS-activated controls for AMT, NINA, flavanone, flavonol, apigenin, and tectorigenin were 53.6 ± 1.5, 55.2 ± 2.4, 52.4 ± 1.1, 52.4 ± 1.0, 54.8 ± 2.9, and 53.3 ± 0.9 μM, respectively. Data points and bar represent arithmetic means ± SD (N = 3). Key: (*) $P < 0.001$, significantly different from the LPS-activated group.

Statistical Analysis

All values are the arithmetic mean ± SD. Student's unpaired *t*-test was used to determine statistical significance. All experiments were performed at least twice and showed similar results.

RESULTS

Effects of Flavonoids on NO Production from RAW 264.7 Cells

When LPS (1 μg/mL) was added to RAW 264.7 cells, NO production, measured as nitrite, was increased dramatically up to 58.0 ± 4.5 μM (35.4 ± 1.1 nmol/well/2 × 10^5 cells in a 96-well plate) for 24 hr from the basal level of 2.8 ± 0.1 μM without LPS (N = 7). NO production from LPS-activated RAW 264.7 cells was inhibited almost completely by AMT, a selective iNOS synthase inhibitor (IC_{50} = 90 nM), whereas 7-NINA, a relatively selective cNOS synthase inhibitor, inhibited NO production (24.0%) only at a high concentration (100 μM), as shown in Fig. 1a. To determine the effects of various flavonoid derivatives on NO production, different concentrations of flavonoids (1, 10, and 100 μM) were incubated. Flavonoid aglycones such as flavanone, naringenin, (+)-catechin, skullcapflavone II, daidzein, irisflorentin, flavonol, galangin, and morin did not inhibit NO production significantly up to 100 μM (Fig. 1b). In addition, all flavonoid glycosides tested, including vitexin, isovitexin, apiin, puerarin, and icariin, were inac-

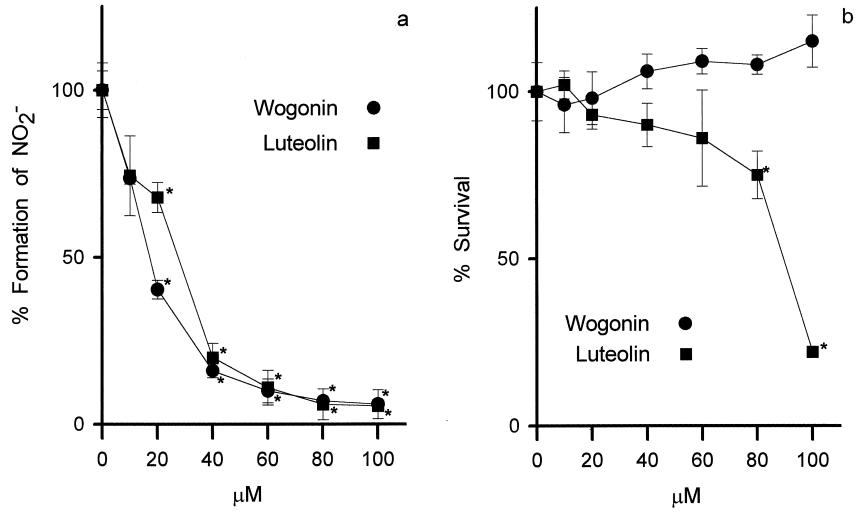
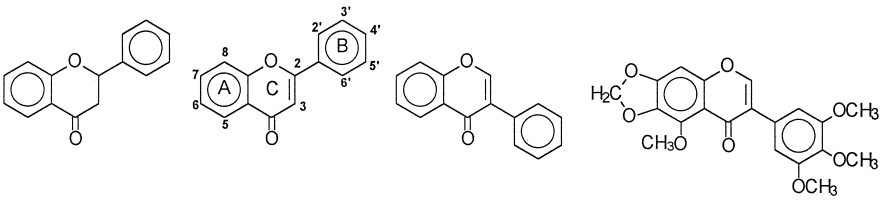


FIG. 2. Effects of wogonin and luteolin on nitrite production of LPS-activated RAW 264.7 cells. Panel a represents the concentration-dependent inhibition of nitrite production, and panel b indicates the cytotoxicity of these flavonoids on RAW cells in the presence of LPS, measured by the MTT assay. Nitrite concentrations of LPS-activated controls for wogonin and luteolin were 55.8 ± 3.3 and 54.7 ± 4.5 μM, respectively. Percent survival of LPS-activated controls for both groups was 100.0 ± 8.7%. Data points and bar represent arithmetic means ± SD (N = 3). Key: (*) $P < 0.001$, significantly different from the LPS-activated group.

TABLE 1. Effects of various flavonoids on NO production from RAW 264.7 cells



Class (derivatives)	Substitutions	Nitrite produced (μM)	IC_{50} (μM)
LPS-treated		54.7 ± 3.5*	
AMT†		8.5 ± 1.2 (84.4)‡	0.09
7-Nitroindazole		44.8 ± 3.2	>100
Flavanones			
Flavanone		55.4 ± 1.9	
Naringenin	4′,5,7-(OH)	49.8 ± 2.6	
(+)-Catechin	(+)-3,3′,4′,5,7-(OH)	57.8 ± 1.2	
Flavones			
Flavone		36.3 ± 2.1 (33.6)	
Chrysin	5,7-(OH)	33.7 ± 1.1 (38.4)	
Apigenin	4′,5,7-(OH)	16.5 ± 2.4 (69.8)	23
Wogonin	5,7-(OH)-8-(OCH_3)	5.1 ± 2.4 (90.6)	17
Luteolin	3′,4′,5,7-(OH)	5.9 ± 0.1 (89.2)	27
Skullcapflavone II	2′,5,5′,7-(OH)-6′,8-(OCH_3)	49.6 ± 4.0	
Isoflavones			
Daidzein	4′,7-(OH)	59.1 ± 10.4	
Formononetin	7-(OH)-4′-(OCH_3)	43.6 ± 3.0 (20.3)	
Genistein	4′,5,7-(OH)	31.5 ± 1.9 (42.4)	
Biochanin A	5,7-(OH)-4′-(OCH_3)	34.0 ± 2.6 (37.8)	
Tectorigenin	4′,5,7-(OH)-6-(OCH_3)	14.2 ± 2.5 (74.1)	73
Iristectorigenin A	3′,5,7-(OH)-4′,6-(OCH_3)	24.3 ± 3.0 (55.6)	100
Irisflorentin		71.2 ± 6.6	
Flavonols			
Flavonol		52.6 ± 1.6	
Galangin	3,5,7-(OH)	46.7 ± 9.5	
Quercetin	3,3′,4′,5,7-(OH)	27.3 ± 2.2 (50.1)	107
Morin	2′,3,4′,5,7-(OH)	53.3 ± 1.5	
Myricetin	3,3′,4′,5,5′,7-(OH)	25.7 ± 3.0 (53.1)	110
Flavonoid glycosides			
Vitexin	4′,5,7-(OH)-8-C-Glc	55.3 ± 6.3	
Isovitexin	4′,5,7-(OH)-6-C-Glc	53.2 ± 1.9	
Apiin	4′,5-(OH)-7-O-Apio-Glc	56.3 ± 0.4	
Puerarin	4′,7-(OH)-8-C-Glc	54.5 ± 3.5	
Icariin	5-(OH)-4′-(OCH_3)-8-prenyl-3-O-rham-7-O-Glc	52.0 ± 3.0	

*Arithmetic mean ± SD (N = 3).
†All compounds were incubated at 100 μM.
‡Values in parentheses represented percent inhibition of NO production (only flavonoids showing more than 20% inhibition are shown).

tive. Weakly active ones (less than 45% inhibition at 100 μM) were flavone, chrysin, formononetin, genistein, and biochanin A. Flavones/isoflavones/flavonols including apigenin, wogonin, luteolin, tectorigenin, iristectorigenin A, quercetin, and myricetin showed strong inhibitory activity on NO production at > 10 μM (Fig. 1c). The concentration-dependent inhibition of the most active flavonoids, wogonin and luteolin, is shown in Fig. 2. All results including IC_{50} values are summarized in Table 1. The cytotoxic effects of flavonoids were measured using the MTT assay. When luteolin was added to LPS-activated RAW 264.7 cells and incubated for 24 hr, it clearly reduced the viability of RAW 264.7 cells at > 60 μM (Fig. 2b). Luteolin (1–100 μM), however, did not show cytotoxicity when incubated without LPS treatment. All other flavonoids tested did not demonstrate any significant cytotoxic effects on RAW 264.7 cells up to 100 μM with or without LPS treatment.

Effects of Flavonoids on iNOS Enzyme Activity

To determine the inhibitory mechanism of NO production from LPS-activated RAW 264.7 cells, iNOS enzyme activity was measured, using cell homogenate, based on the

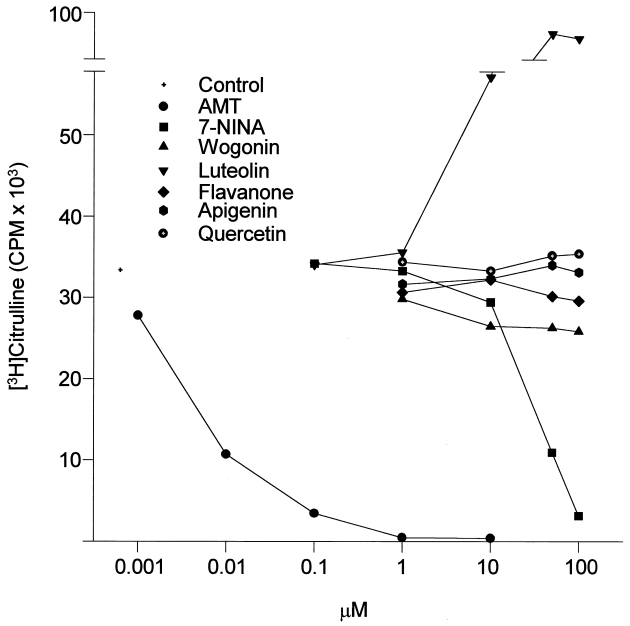


FIG. 3. Inhibition of iNOS enzyme activity by flavonoids. Homogenate of LPS-activated RAW 264.7 cells for 20 hr was used for the enzyme assay. [^{3}H]Citrulline formation from [^{3}H]arginine by the homogenate without LPS treatment was negligible. Control indicates the activity of the homogenate from LPS-activated RAW 264.7 cells. Data points represent arithmetic means of two separate assays.

conversion of [^{3}H]arginine to [^{3}H]citrulline. Under our assay conditions, iNOS enzyme activity was increased linearly depending on protein concentration (up to 100 μg/tube) and incubation time (up to 1 hr). The cell homogenate obtained without LPS activation showed negligible activity (data not shown). AMT strongly inhibited iNOS enzyme activity (IC_{50} = 8 nM), while 7-NINA inhibited enzyme activity only at a high concentration (100 μM), as expected. When flavonoids were examined, wogonin was found to inhibit iNOS enzyme activity slightly (less than 20% at 100 μM), as shown in Fig. 3. Other flavonoids such as flavanone, apigenin, and quercetin were inactive. Interestingly, luteolin increased iNOS enzyme activity in a concentration-dependent manner (an approximately 4-fold increase at 100 μM).

Effects of Flavonoids on iNOS Enzyme Expression

The effects of flavonoids on the induction of iNOS enzyme expression were checked using a western blotting technique. As shown in Fig. 4, wogonin and luteolin concentration-dependently reduced the induction of iNOS enzyme expression at 10–100 μM. AMT did not reduce iNOS enzyme expression.

DISCUSSION

Flavonoids possess anti-inflammatory and immunomodulatory activities *in vitro* and *in vivo*. The cellular action mechanisms of flavonoids for these pharmacological activities have been reported partly as involving inhibition of cyclooxygenase/lipoxygenase and their antioxidative nature [17, 18].

In this investigation, our study clearly showed that certain flavonoids inhibit NO production from LPS-activated RAW 264.7 cells. The strongly active flavonoids possessed a C-2,3 double bond and 5,7-dihydroxyl groups in the A-ring. The 8-methoxyl group in the A-ring and 4′- or 3′,4′-vicinal substitutions in the B-ring may have affected inhibitory activity favorably, while 2′,4′-(meta)-hydroxyl substitution in the B-ring (morin) abolished inhibitory activity. In addition, the 3-hydroxyl moiety in the C-ring was revealed to reduce the activity, since flavone derivatives such as flavone, chrysin, apigenin, and luteolin inhibited more strongly than did flavonol derivatives such as flavonol, galangin, and quercetin. Flavanone derivatives, which do not have a C-2,3 double bond, were inactive up to 100 μM. These results indicated that a planar ring system in the flavonoid molecule is important to exhibit inhibitory activity of NO production. Flavonoid glycosides were not active up to 100 μM regardless of the types of aglycones (flavone, isoflavone, flavonol) and types of linkage (C- or O-glycosides). At present, we do not know the

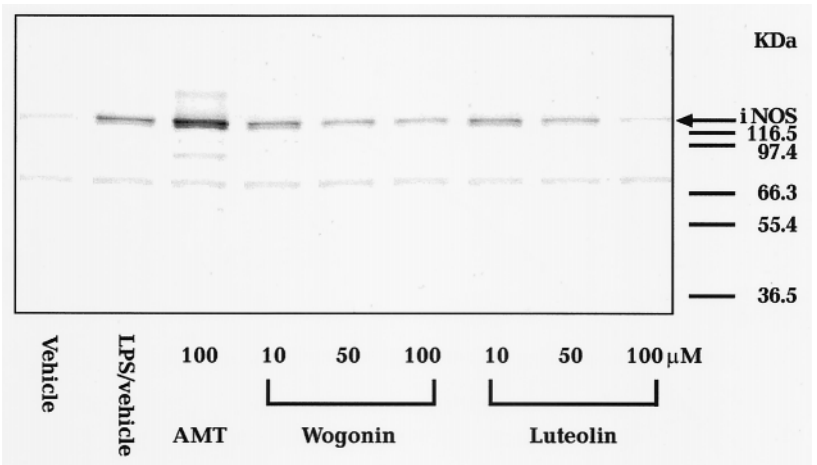


FIG. 4. Inhibition of iNOS enzyme induction by flavonoids (western blot). The same amount of protein (30 μg) was used in each lane.

reason why glycoside derivatives were not active. It is possible that flavonoid glycosides may not penetrate the cell membrane due to their hydrophilicity, or there might be steric hindrance due to their bulky glycosyl residues, as suggested previously [19].

To obtain clear knowledge of the inhibitory mechanism of NO production, the effects of the selected flavonoids on iNOS enzyme activity were examined. As shown in Fig. 3, flavonoids that strongly inhibited NO production at 10–100 μM did not strongly inhibit iNOS enzyme activity up to 100 μM. Rather, luteolin enhanced iNOS enzyme activity. Among the flavonoids tested, wogonin showed weak inhibition only at 100 μM, but this is not likely to be the main mechanism of inhibitory activity of NO production. This speculation also was supported by evidence that flavonoids including flavanone, apigenin, wogonin, luteolin, and quercetin added 24 hr after the addition of LPS did not significantly inhibit NO production from RAW 264.7 cells, in which iNOS was already fully induced (data not shown). In contrast, wogonin and luteolin did reduce iNOS enzyme expression in a concentration-dependent manner, as revealed by western blotting. Therefore, it is strongly suggested that inhibition of NO production by these flavonoids may be due to their reduction of iNOS enzyme expression, at least in part. It should be noted, however, that we could not distinguish, based on the results obtained in this study, whether the inhibitory activity of NO production by luteolin was due to a selective reduction of iNOS enzyme expression or to cytotoxicity to RAW cells.

The effect of flavonoids on other forms of NOS is not certain at present. Tamura *et al.* [20] have shown that quercetin and apigenin inhibit NADPH diaphorase in mouse brain, probably the neuronal form of NOS (nNOS), whereas flavone does not. These results indicated that some flavonoids inhibit nNOS activity directly at 10–100 μM, whereas our results demonstrated that flavonoids did not strongly inhibit iNOS enzyme activity at the same concentration range. Although, being relatively hydrophilic, flavonoids might not penetrate the blood–brain barrier so that they could not affect the levels of NO concentration in the brain, flavonoids seem to have differential effects on NOS isoforms. Further study is needed to clarify this point.

Our study demonstrated that certain flavonoids reduced iNOS enzyme expression. However, the cellular mechanism of flavonoids for down-regulating iNOS enzyme in RAW 264.7 cells is not understood. There may be several possibilities. Flavonoids may affect the translational level of iNOS enzyme expression, or they may have effects upstream of signal transduction such as activation/translocation of transcription factors including nuclear factor kappa B (NF-κB). Alternatively, flavonoids may down-regulate iNOS enzyme by modulation of enzyme activities related to signal transduction. Protein kinase C and tyrosine kinase are known to be involved in LPS-induced induction of iNOS in RAW 264.7 cells [21]. Certain flavonoids were reported previously to inhibit protein kinase C, tyrosine kinase, and phospholipases A_2 and C [22]. Another possible mechanism includes a down-regulation of iNOS indirectly by inhibition of eicosanoid production through the cyclooxygenase/lipoxygenase pathway. However, the precise mechanism will be unveiled by further study.

In conclusion, certain flavonoids, especially flavones including apigenin, wogonin, and luteolin, inhibited NO production from LPS-activated RAW 264.7 cells *in vitro*. The main inhibitory mechanism of these flavonoids may be the reduction of iNOS enzyme expression. Inhibition of NO production may contribute to the anti-inflammatory and immunoregulatory activities of flavonoids.

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