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Flavonoids Differentially Modulate Nitric Oxide Production Pathways in Lipopolysaccharide-Activated RAW264.7 Cells

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Naturally occurring flavonoids are known to modulate various inflammatory and immune processes. Based on structural property, in this study, molecular mechanism of flavonoids in modulating nitric oxide (NO) production and its signaling pathway were investigated using lipopolysaccharide (LPS)-activated RAW264.7 cells. Although flavonol-typed flavonoids (kaempferol and quercetin) more potently scavenged reactivity of nitric oxide (·NO) as well as peroxynitrite ($ONOO^-$) than isoflavones (genistein and genistin), kaempferol, quercetin and genistein showed a little difference in inhibition of both inducible NO synthase expression and NO production, with IC_{50} values of 13.9, 20.1 and 26.8 μM. However, there was a striking pattern related to structural feature in modulation of LPS-mediated signaling pathways. Thus, flavonols only inhibited transcription factor AP-1 activation, whereas isoflavones suppressed the DNA binding activation of NF-κB and C/EBPβ. Therefore, these data suggest that structural feature may be linked to decide drugs target molecule in LPS-mediated signaling pathways, rather than its potency.

Key words: Flavonoids, Flavone, Isoflavone, Nitric oxide (NO), NO signaling pathway

INTRODUCTION

Oxidative responses are mainly mediated by nitric oxide (·NO) and superoxide ($\cdot O_2^-$) that are produced from several activated cells such as endothelial cells, Kupffer cells, neutrophils, and macrophages (Huie and Padmaja, 1993). In particular, NO is a notable reactive component generated from L-arginine by at least three different isoforms of NO synthase (NOS) (Stuehr, 1999). Although NO plays important physiological roles in many different cellular processes, excessive production of NO mediated by inducible NOS (iNOS) in inflammatory cells such as macrophages has been reported to mediate acute and chronic inflammatory diseases, such as rheumatoid arthritis, sepsis and pancreatic cancer (Kroncke *et al.*, 1998; Wheeler and Bernard, 1999). Therefore, protection against excessive NO production is attributed to important tool in controlling such relevant diseases.

Flavonoids are naturally occurring polyphenolic compounds which are known to be involved in many different biological activities, such as anti-inflammatory, anti-viral, anti-fungal and anti-cancer effects (Kim *et al.*, 1999; Cho *et al.*, 2000; Lopez-Lazaro, 2002; Shen *et al.*, 2002). Because various sources including tea, wine, vegetables, fruits and medicinal plants, are being familiar with human daily life, it is importantly regarded that an understanding pharmacological mechanism of flavonoids may contribute to develop non-toxic and more promising therapeutic drugs. To date, the molecular mechanism of flavonoids in modulating many different biological effects was not clearly elucidated yet, but anti-oxidative properties are considered as major pharmacological action (Haenen *et al.*, 1997; Heijnen *et al.*, 2001). Indeed, structural features (positions and numbers of hydroxyl groups, and existence of catechol group and double bond) required for scavenging effects of flavonoids against peroxynitrite and superoxide anion have been reported to be in agreement with inhibitory effects on the production of tumor necrosis factor-a and NO (Haenen *et al.*, 1997; Kim *et al.*, 1999; Heijnen *et al.*, 2001; Choi *et al.*, 2002; Olszanecki *et al.*, 2002; Klotz and Sies, 2003; Matsuda *et al.*, 2003). Having found that flavonoids directly act as inhibitors of protein

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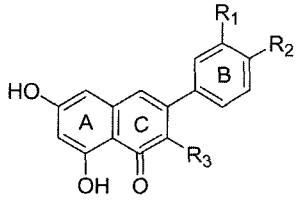
kinase C (PKC), protein tyrosine kinase (PTK) and phosphoinositoid 3-kinase (PI-3K), however, it is noted that these compounds could directly modulate cellular event as an inhibitory compound rather than simple antioxidants. Indeed, some groups have reported inhibition of mitogen-activated protein kinases (MAPK) and transcription factors involved in lipopolysaccharide (LPS)-activated intracellular signaling by flavonoids (Liang *et al.*, 1999), although there was no report to explain the relationship between flavonoid structure and its biochemical targets.

In the present study, therefore, we explored molecular basis of inhibitory effect by flavonoids on NO production in terms of its signaling pathway, based on structural property. Our results suggest that structural features of flavonoids may be linked to deciding intracellular target molecules involved in NO production pathway.

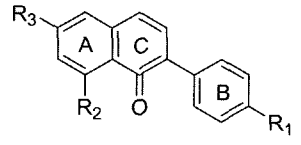
MATERIALS AND METHODS

Materials

Flavonoids [genistein, genistin, quercetin and kaempferol (Fig. 1) were purified from several *Prunus serrulata* var. spontanea leaves (Jung *et al.*, 2002). Bovine serum albumin type V, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, lipopolysaccharide (LPS), sodium nitroprusside (SNP), standard flavonoids (genistein, genistin, quercetin and kaempferol) and nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma Chemical Co. (St. Louis, MO). Polyvinylidene fluoride (PVDF) membrane was obtained from Millipore Corp. (Bedford, MA). Antibody to nitrotyrosine was from Upstate Biotechnology (Lake Placid, NY). Antibodies to iNOS, phospho-specific antibodies to extracellular signal-related kinases (ERK) 1/2, p38 and JNK were from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) western blotting detection reagents were from Amersham Life Science (Buckinghamshire, England). Nuclear factor (NF)-κB (5'-GAGAGGCAAGGGGATTCCCTTAGTTAGGA-3'), activator protein (AP)-1 (5'-CTAGTGATGAGTCAG-CCGGATC-3) and CCAAT/enhancer-binding protein β (C/EBPβ) (5'-CTGCCGCTGCGGTTCTTGCGCAACTCACT-3') consensus oligonucleotides were from Bioneer (Daejeon, Korea). All other chemicals were of the highest purity available from either Sigma Chemical Co. or Junsei Chemical Co. (Tokyo, Japan).



$R_1 = R_2 = R_3 = OH$ Quercetin
$R_1 = H$, $R_2 = R_3 = OH$ Kaempferol



$R_1 = R_2 = R_3 = OH$ Genistein
$R_1 = R_2 = OH$, R_3 = O-Glucose Genistin

Fig. 1. Chemical structures of flavonoids

Measurement of $ONOO^-$ and $^{\cdot}NO$ scavenging activities by flavonoids

$ONOO^-$ and $^{\cdot}NO$ scavenging activities by flavonoids (quercetin, kaempferol, genistein and genistin) and other standard compounds were measured by monitoring the oxidation of DHR 123 and DAF-2 induced by the generating agents for $ONOO^-$ and $^{\cdot}NO$ [$ONOO^-$ (10 mM) and sodium nitroprusside (2 mM)], as reported previously (Kooy *et al.*, 1994; Nagata *et al.*, 1999). The oxidation of DHR 123 and DAF-2 was measured using a microplate fluorescence spectrophotometer FL 500 (Bio-Tek Instruments, USA) with excitation and emission wavelengths of 485 nm and 530 nm, respectively, at room temperature.

GSH reductase activity with $ONOO^-$ and flavonoids

Enzymatic activity of GSH reductase was determined by the NADPH reduction method (Mavis and Stellwagen, 1968). The samples with 0.6 U/mL of GSH reductase were incubated with shaking at 37°C for 5 min. Then $ONOO^-$ was added, this was followed by another 10min of incubation with shaking at 37°C. The test mixture was added to GSH reductase solution containing 1 mM GSSG, 0.09 mM β-NADPH, 0.13% (w/v) BSA in 75 mM potassium phosphate buffer with 2.6 mM EDTA (pH 7.4). GSH reductase activity was followed spectrophotometrically at 340 nm. Each assay was performed three times with similar results.

Cell culture

RAW 264.7 cells obtained from American Type Culture Collection (Rockville, MD) were cultured with DMEM medium (Nissui Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), glutamine (233.6 mg/mL) and antibiotics (0.25 mg/mL amphotericin B and 72 mg/mL gentamicin) at 37°C with 5% CO_2.

Quantification of NOx level (nitrite and nitrate)

RAW 264.7 cells were preincubated with or without flavonoids or L-NAME for 3 h and continuously activated with 100 ng/mL LPS for 24 h. NO metabolites [NO_2^- and

NO_3^- (NOx)] were measured from culture supernatant after deproteining with an equal volume of methanol. The assay was carried out using NO-analyzing system (ENO-20, Eicom Corp., Kyoto, Japan) (Kimura et al., 1999) The absorbance of the product dye at 540 nm was measured by a flow-through spectrophotometer.

Cell viability

The viability of cells treated with flavonoids was assessed by MTT assay, as previously described by Tada *et al.* (1986). RAW 264.7 cells in a 24-well plate were pre-incubated for overnight. Cells were pretreated with or without flavonoids for 3 hrs and then treated with 100 ng/ml LPS for 24 hrs. After addition of MTT solution, optical density was determined at 560 nm on a microplate reader (SpectraCount, Packard, Meriden, CT, USA).

Preparation of cytosolic fraction and nuclear extracts

The cytosolic fraction and nuclear extracts were prepared from the cells, as reported previously (Kim *et al.*, 2002). Flavonoid-treated cells in the presence or absence of LPS were washed with ice-cold PBS, and harvested to get the cell pellet by centrifugation (3000 rpm, 5 min) at 4°C. The pellets were suspended in buffer A [10 mM Tris buffer (pH 8.0) containing $MgCl_2$ (1.5 mM), dithiothreitol (DTT, 1 mM), 0.1% Nonidet P-40, 5 μg/mL each of pepstatin and aprotinin] and incubated on ice for 15 min. Cytosolic and nuclear fractions were separated by centrifugation (12,000 rpm, 15 min) and the nuclei were further resuspended in buffer B [10 mM Tris (pH 8.0), with 50 mM KCl, 100 mM NaCl, 1 mM DTT, 5 mg/mL each of pepstatin and aprotinin] and incubated on ice for 30 min to obtain nuclear extracts. Protein concentrations were determined by bicinchoninic acid method (Smith *et al.*, 1985).

Western blot analysis

Total protein equivalents were separated on 8~12% SDS-polyacrylamide mini-gel using a Laemmli buffer system at 100 V and were transferred to PVDF membrane at 100 V for 1.5 h in a wet transfer system (Bio-Rad, Hercules, CA). The membrane was immediately placed in a blocking solution [5~10% non-fat dry milk in TBS-T buffer (pH 7.5) containing 10 mM Tris, 100 mM NaCl, and 0.1% Tween 20] at 4°C overnight. The membrane was then washed in TBS-T buffer for 30 min and further incubated with antibodies to nitrotyrosine (1: 2000), iNOS (1: 1000), phospho-ERK1/2 (1: 500) and phospho-p38 (1: 500) at room temperature for 1~2 h. After washing, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody at room temperature. Immunoreactive bands were visualized by the enhanced chemiluninescence system.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed by the method reported previously (Kim *et al.*, 2002). Fifteen mg of nuclear protein extract was incubated in binding buffer containing 5% glycerol, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM ethylenediamine tetraacetic acid (EDTA), 2 mM DTT, 1% Nonidet P-40, 10 mM Tris (pH 7.5), and 1 mg of poly(dI-dC)• poly(dI-dC) for 20 min at 4°C. Radiolabeled NF-κB consensus oligonucleotide (20,000 cpm of ^{32}P) or AP-1 consensus oligonucleotide (20,000 cpm of ^{32}P) was then added and further incubated for 20 min at room temperature. The DNA-binding complexes were separated by 7% native polyacrylamide gel in 0.5 × TBE buffer (0.045 M Tris-borate/0.001 M EDTA). Specific binding was confirmed by competition reaction with a 50-fold excess of unlabeled, identical oligonucleotides.

Statistic analysis

The Student's *t*-test and oneway ANOVA were used to determine the statistical significance of differences between values for various experimental and control groups. Data are expressed as means ± standard errors (SEM) of at least 3 independent experiments performed with triplicates.

RESULTS

Antioxidant activity of flavonoids

As antioxidant property of flavonoids is one of important biological activities of the compounds, we first tested scavenging activities against $ONOO^-$ and $^{\cdot}NO$ to confirm whether these compounds are truly working in our assay system. As Table I and Fig. 2(A and B) show, flavonoids tested significantly inhibited both the oxidation of DHR by

Table I. IC_{50} (micromolar) values in radical-mediated oxidation and reduction of NOx of flavonoids

Flavonoids	IC_{50} (μM)		
	$^{\cdot}NO$	$ONOO^-$	NOx
Quercetin	5.9 ± 0.3	0.9 ± 0.1	13.9 ± 0.3
Kaempferol	8.2 ± 0.8	3.0 ± 0.1	20.1 ± 0.7
Genistein	>100	24.4 ± 1.2	26.8 ± 0.6
Genistin	>100	44.5 ± 6.1	70.3 ± 1.4
Carboxy-PTIO*	1.5 ± 0.1	-	-
Penicillamine*	-	3.2 ± 0.4	-
L-NAME			195.4 ± 3.7

*$^{\cdot}NO$ scavenging activity was measured by generation of fluorescent DAF-2. $ONOO^-$ scavenging activity was measured by monitoring the oxidation of DHR 123. NOx concentration was determined by analyzing nitrite (NO_2) and nitrate (NO_3) levels using Griess assay as described in Material and Methods. Each experiment was performed by triplicate observations. Data are mean ± S.E. of three different experiments.
*Used as positive control.

$ONOO^-$ and the generation of fluorescent DAF-2 by NO from SNP. The neutralizing effects (IC_{50} values) of quercetin, kaempferol, genistein and genistin against $ONOO^-$ were 0.9, 3.0, 24.4, and 44.5 μM, respectively. These compounds displayed relatively weak scavenging activities against NO reactivity, compared to $ONOO^-$. Thus, only flavonols (quercetin and kaempferol) but not isoflavones (genistein and genistin) neutralized NO reactivity, with IC_{50} values of 5.9 and 8.2 μM, respectively. Positive control drugs, penicillamine (for $ONOO^-$ assay) and carboxy PTIO (for NO assay) showed strong neutralizing effects with IC_{50} values of 3.2 and 1.5 μM, respectively (Table I).

In vitro cytotoxicity

Although numerous articles showed that flavonoids did not have any cytotoxicity up to 100 μM, we firstly checked the effects of flavonoids (quercetin, kaempferol, genistein and genistin) on RAW264.7 cell viability in the absence of LPS using MTT assay. Pretreatment of unstimulated cells with testing compounds did not significantly block cell

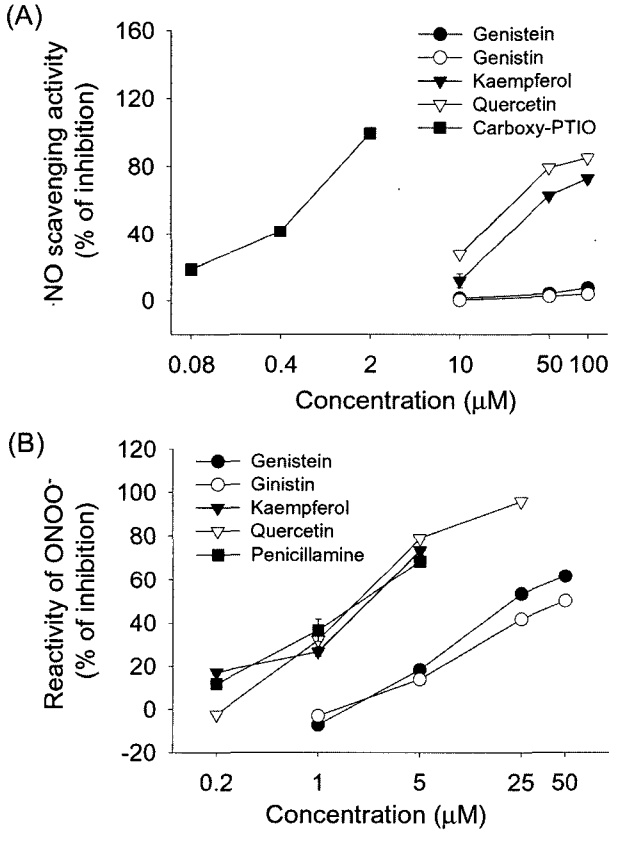


Fig. 2. Radical scavenging activities of flavonoids (genistein, genistin, kaempferol and quercetin). (A) NO scavenging activity was measured by generation of fluorescent DAF-2. (B) $ONOO^-$ scavenging activity was measured by monitoring the oxidation of DHR 123 as described in Materials and Methods. Each experiment was performed by triplicate observations. Data are mean ± S.E. of three different experiments.

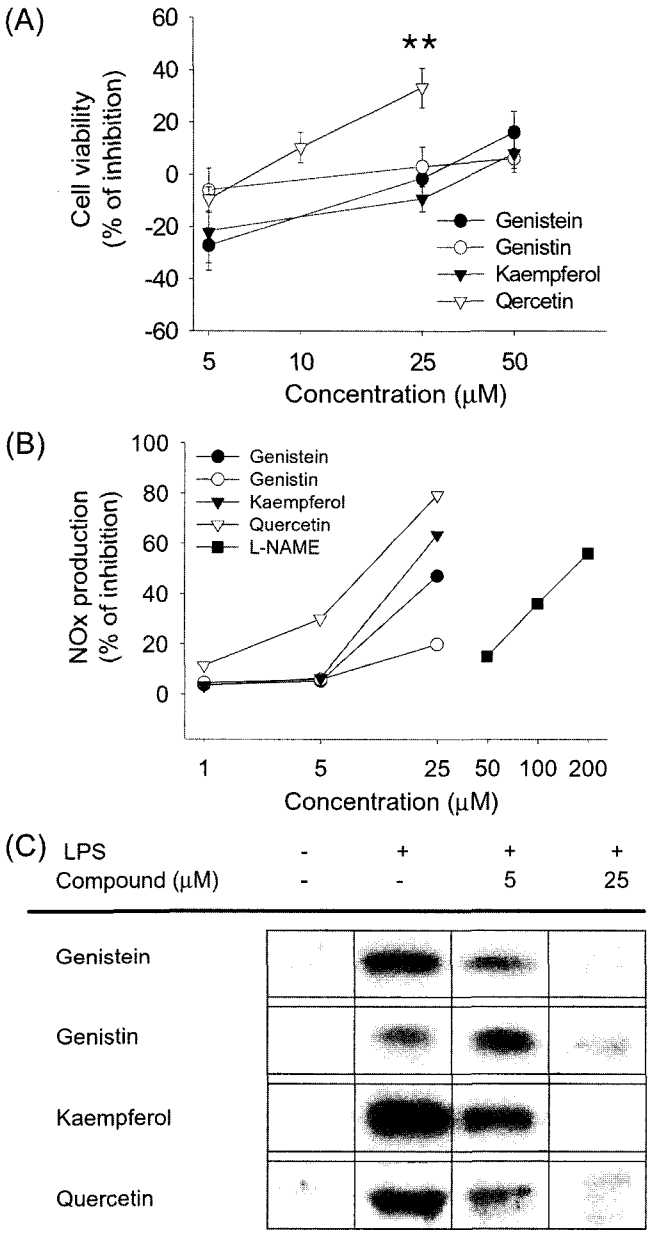


Fig. 3. Effects of flavonoids on RAW 264.7 cell viability (A), NOx production (B) and iNOS expression (C) upon LPS exposure. (A) RAW 264.7 cells were treated with different concentrations of flavonoids (genistein, genistin, kaempferol and quercetin) for 24 h and the viability of cells was determined by tryphan blue dye exclusion as described in Materials and Methods. (B) RAW 264.7 cells were pre-treated with different concentrations of flavonoids (genistein, genistin, kaempferol and quercetin) for 3 h and further incubated with LPS (100 ng/mL) for 24 h. The levels of NO_2 and NO_3 were determined by Griess reaction as described in Materials and Methods. Each experiment was performed by triplicate observations. Data are mean ± S.E. of three different experiments. (C) RAW 264.7 cells were pre-treated with different concentrations of flavonoids (genistein, genistin, kaempferol and quercetin) for 3 h and further incubated with LPS (100 ng/mL) for 12 h. iNOS levels were detected using an ECL kit as described in Materials and Methods. Data are a representative of three independent experiments.**: $p<0.01$.

viability up to 50 μM, except quercetin (Fig. 3A).

Flavonoids suppress NO production and iNOS expression

The inhibitory effect of flavonoids on NO production was assessed by determining NOx level including nitrite (NO_2) and nitrate (NO_3) from LPS-activated RAW264.7 cells. As reported previously, that the tested flavonoids diminished NO production in a dose dependent manner, with IC_{50} values of 13.6, 20.1, 26.81, and 70.3 μM, respectively (Fig. 3B). To confirm the inhibitory effect of flavonoids on NO production, iNOS expression was examined from LPS-stimulated macrophages. As shown in Fig. 3C, LPS induced highly iNOS expression and flavonoids suppressed its expression in a dose-dependent manner. Indeed, non-cytotoxic concentration (5 μM) of flavonoids except genistin significantly inhibited iNOS expression and at 50 μM all flavonoids showed completely or significantly suppressed iNOS expression.

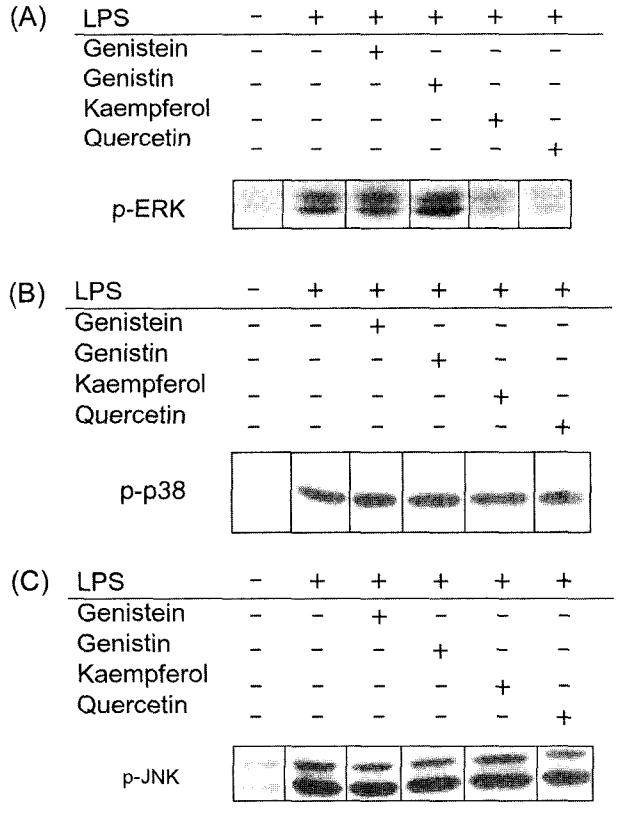


Fig. 4. *Effects of flavonoids on the phosphorylation of ERK (A), p38 (B) and JNK (C) in LPS-stimulated RAW 264.7 cells. The cells were pretreated with genistein (50 mM), genistin (50 mM), kaempferol (50 mM), and quercetin (50 mM) and further incubated for 1 hour in the presence or absence of LPS (100 ng/mL). Total cellular proteins were separated on SDS-polyacrylamide gels and blotted with phospho-specific antibodies to ERK1/2 (A), p38 (B) and JNK (C). Data are a representative of three independent experiments.*

Standard compound, L-NAME, also attenuated NO production with IC_{50} value of 195.4 μM.

Flavonoids differentially inhibit LPS-induced signaling for NO production

To access molecular basis of NO inhibitory effect by flavonoids, the regulation of LPS-induced signaling cascade by flavonoids was investigated. To do this, the activation of MAP kinases (ERK, p38 and JNK) and transcription factors (NF-κB, AP-1 and C/EBPβ) induced by LPS (Carter *et al.*, 1999a, 1999b; Cobb and Godsmith, 1999; Guha and Mackman, 2001) was evaluated.

The MAPK pathway is known to play an important role in the transcriptional regulation of LPS-induced iNOS expression *via* activation of transcription factors. Of the tested flavonoids, flavonol-typed compounds, quercetin and kaempferol strongly blocked LPS-induced ERK (p44/42) phosphorylation, a hallmark for activation (Cobb and Godsmith, 1999), while isoflavones did not (Fig. 4A). Unfortunately, however, we could not obtain significant inhibitory effects of flavonoids on the activation of p38 and JNK, even though LPS significantly induced the phosphorylation of the MAPKs (Fig. 4B and C). It may be due to different experimental conditions such as low LPS concentration (100 ng/mL) affecting weak activation of p38 and JNK.

Flavonoids, however, clearly exhibited distinct regulatory effects on transcript factor activation, judged by DNA binding assay, in a structure-dependent manner. Fig. 5 illustrates that DNA-binding activities of NF-κB, AP-1 and C/EBPβ were increased at 60 min upon LPS exposure. In particular, only pretreatment with isoflavones, genistein and genistin, potently diminished the DNA-binding of NF-κB (p50/50 and p50/65) and C/EBPβ (Fig. 5A and B). In contrast, two favonols only suppressed AP-1 binding activity (Fig. 5C).

DISCUSSION

Flavonoids are naturally occurring plant polyphenols abundant in our daily diets. Indeed, it has been reported that total consumption is likely to be as high as 100 mg per day (Hertog *et al.*, 1995). With this respect, many reports were tried to address their biological and pharmacological activities in anti-inflammatory and anti-cancer effects, although molecular mechanisms underlying these effects are still unclear. In this study, we have demonstrated that flavonoids differentially modulate NO production pathway in a structure-dependent manner. Consequently, the tested flavonoids (quercetin, kaempferol, genistein and genistin) blocked NO production, with IC_{50} values of 13.9, 20.1, 26.8, and 70.3 μM, respectively. These effects were not due to non-specific cytotoxicity

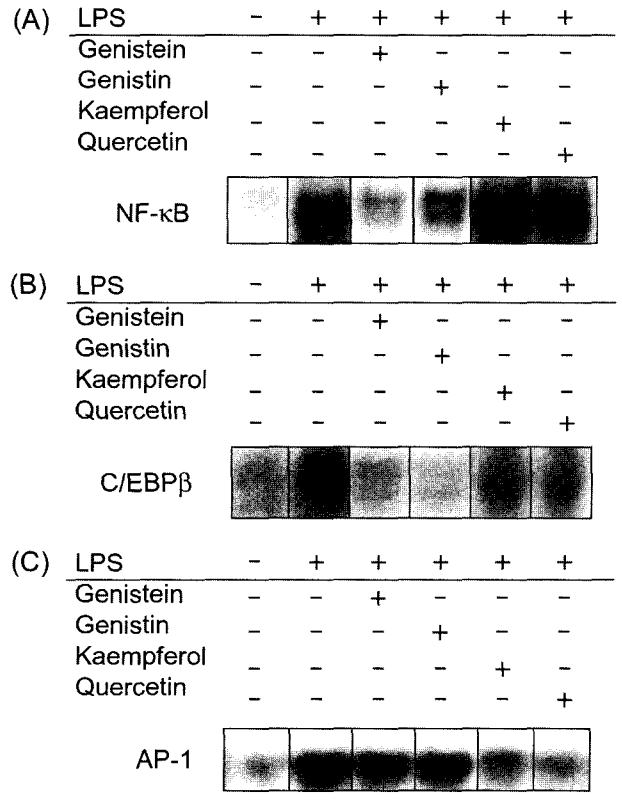


Fig. 5. Effects of flavonoids on the DNA binding activities of NF-κB (A), C/EBPb (B) and AP-1 (C) in LPS-stimulated RAW 264.7 cells. The cells were pre-treated with genistein (50 mM), genistin (50 mM), kaempferol (50 mM), and quercetin (50 mM) and further incubated for 1 hour in the presence or absence of LPS (100 ng/mL). Nuclear extracts were prepared and assayed for DNA binding of NF-κB (A) C/EBPb (B) and AP-1 (C) by EMSA as described in Materials and Methods. Data are a representative of three independent experiments.

and direct scavenging activities against reactive •NO, since there were no cytotoxic compounds on the RAW264.7 cell viability and none of isoflavone neutralized the reactivity of •NO (Table I). The inhibitory activities of these compounds were similar to the results from Yoshikawa group with IC_{50} of 29, 36, and 26 μM, respectively, but quite different from other reports in which IC_{50} values of kaempferol and quercetin from Olszanecki group were reported as 5.7 and 92.1 μM, respectively (Olszanecki *et al.,* 2002) and IC_{50} values of quercetin from Kim group were 107 μM (Kim *et al.,* 1999). Probably, the discrepancy may be due to different experimental conditions such as different cell type, and LPS doses. Otherwise, position of ring B in the presence of the 2,3-double bond with the 4-oxo functionality in the C ring (Rice-Evans *et al.,* 1996) may be not critical point to decide to their inhibitory potency, unlike scavenging effects against peroxynitrite and superoxide anion. Rather, it may be due to maintaining a planar form of three-dimensional structure (Olszanecki *et al.,* 2002). However, glycosylation seems to be still crucial factor to diminish NO production inhibitory activity by flavonoids.

How flavonoids inhibit induction of iNOS and NO production is not clearly understood yet, but several explanations are argued. The first possibility may be derived from the antioxidant property of flavonoids by which these compounds scavenge reactive free radicals. Indeed, LPS enhances iNOS biosynthesis *via* generation of reactive oxygen species (Mathy-Hartert *et al.,* 2002). However, some evidence supports that the possibility can be denied. Thus, firstly, membrane permeable scavenger such as Mn(III)TBAP did not suppress iNOS induction (Faulkner *et al.,* 1994). Secondly, inhibitory potency of flavonoids on scavenging effect was not in agreement with that on NO production (Haenen *et al.,* 1997; Kim *et al.,* 1999; Heijnen *et al.,* 2001; Matsuda *et al.,* 2003). Thirdly, the inhibitory effects of flavonoids were more distinct in inhibition of peroxynitrite formation than NO production (Table I). Finally, flavonoids are reported to be generator of free radicals in a certain conditions (Ohshima *et al.,* 1998). Therefore, many research groups and we favor the second possibility that flavonoids may act as a LPS-signaling molecule inhibitor. Indeed, certain flavonoids are known to inhibit some signaling enzymes such as protein kinase C, protein tyrosine kinase, phospholipase A2, phospholipase C and phosphatidylinositol 3-kinase (Akiyama *et al.,* 1987; Agullo *et al.,* 1997; Middleton *et al.,* 2000). Although the exact mechanism is not clearly elucidated, ATP-binding domain of the kinases is thought to be the common target site of flavonoids (Geahlen *et al.,* 1989; Cushman *et al.,* 1991). Recent articles, however, suggested that MAPK and transcription factors are much more considerable molecular targets of flavonoid. Thus, importantly, many papers reported that some flavonoids are potent inhibitor of ERK activation or NF-κB binding, although the papers did not compare biochemical targets of flavonoids in a structural feature. In this study, however, we clearly showed that flavonoids have different biochemical property in modulating LPS-induced signaling. Thus, flavonol-type flavonoids (kaempferol and quercetin) more potently suppressed the phosphorylation of ERK and activation of AP-1, whereas isoflavone-type flavonoids (genistein and genistin) strongly diminished the activation of NF-κB and C/EBPβ). Although the exact target molecules for flavonoids are not elucidated, these patterns may suggest that the target molecules can be recognized by flavonoids in a structural feature-dependent manner and a fraction composed of many different kinds of flavonoids may be able to have various biochemical targets in the same cells. Because of the fact, our data seem to also explain one important meaning regarding why some medicinal plant extracts sometimes lose their biological activities when these are activity-guided

fractionated (Cho *et al.*, 1999).

In conclusion, we have shown that flavonoids inhibit iNOS expression and NO production *via* negative modulation of signaling components activated by LPS exposure. The target signaling enzyme was decided by structural feature of flavonoids. Our data may help to understand how flavonoids modulate LPS-induced signaling in terms of molecular mechanism.

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