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Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages

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Prostaglandins biosynthesis and nitric oxide production have been implicated in the process of carcinogenesis and inflammation. In this study, we investigated the effect of various flavonoids and (–)-epigallocatechin-3-gallate on the activities of inducible cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages. Apigenin, genistein and kaempferol were markedly active inhibitors of transcriptional activation of COX-2, with IC_{50} < 15 μM. In addition, apigenin and kaempferol were also markedly active inhibitors of transcriptional activation of iNOS, with IC_{50} < 15 μM. Of those compounds tested, apigenin was the most potent inhibitor of transcriptional activation of both COX-2 and iNOS. Western and northern blot analyses demonstrated that apigenin significantly blocked protein and mRNA expression of COX-2 and iNOS in LPS-activated macrophages. Transient transfection experiments showed that LPS caused an ~4-fold increase in both COX-2 and iNOS promoter activities, these increments were suppressed by apigenin. Moreover, electrophoretic mobility shift assay (EMSA) experiments indicated that apigenin blocked the LPS-induced activation of nuclear factor-kB (NF-kB). The inhibition of NF-kB activation occurs through the prevention of inhibitor kB (IkB) degradation. Transient transfection experiments also showed that apigenin inhibited NF-kB-dependent transcriptional activity. Finally, we showed that apigenin could inhibit the IkB kinase activity induced by LPS or interferon-γ. The results of further studies suggest that suppression of transcriptional activation of COX-2 and iNOS by apigenin might mainly be mediated through inhibition of IkB kinase activity. This study suggests that modulation of COX-2 and iNOS by apigenin and related flavonoids may be important in the prevention of carcinogenesis and inflammation.

Introduction

Flavonoids are plant polyphenolic compounds whose main dietary sources are fruit and vegetables and comprise several classes including flavonols, flavanones, flavanols and flavans. Epidemiological studies have shown that the consumption of vegetables, fruits and tea is associated with a decreased risk of cancer and flavonoids are believed to play an important role in delaying or preventing carcinogenesis (1,2). In the Western diet, the average daily intake of mixed flavonoids amounts to ~1 g (3). Consumption of a traditional diet rich in flavonoids profoundly decreases the risk of some diseases in Asia (4). For example, breast, prostate, endometrial and colon cancer and coronary heart disease have a lower incidence in Asia than in Western countries (5). Some flavonoids have been found to possess anticarcinogenic (2,6,7), anti-inflammatory (8), antiviral (9), cytotoxic (10), cytostatic (11) and antioxidant (12) properties. A plant-derived diet might contain certain flavonoids that exert these healthy effects, thereby protecting us from some diseases. Identification and characterization of such flavonoids might provide us with additional new agents for cancer chemoprevention.

Increased levels of prostaglandins and cyclooxygenase (COX) activity have been detected in multiple epithelial cancers (13,14). Prostaglandins, especially prostaglandin E_2 (PGE_2), affect cell proliferation, tumor growth and suppress the immune response to malignant cells. Therefore, high levels of prostaglandins could favor malignant growth (15,16). Two isozymes, designated COX-1 and COX-2, have been identified which are encoded by separate genes. The COX-1 isozyme is believed to be a housekeeping protein in most tissues and seems to catalyze the synthesis of protaglandins for normal physiological functions. COX-1 is expressed at essentially constant levels and does not fluctuate in response to various stimuli. In contrast, COX-2 is rapidly induced by tumor promoters, growth factors, cytokines and mitogens in various cell types (17,18). Many cell types associated with inflammation, such as macrophages, endothelial cells and fibroblasts, express the COX-2 gene upon induction (19).

Nitric oxide synthase (NOS) plays a major role in regulating vascular tone, neurotransmission, killing of microorganisms and tumor cells and other homeostatic mechanisms (20). High levels of nitric oxide (NO) have been described in a variety of pathophysiological processes including various forms of circulatory shock (21), inflammation (22) and carcinogenesis (23). Molecular cloning and sequencing analyses revealed the existence of at least three main types of NOS isoforms. Both neuronal NOS and endothelial NOS are constitutively expressed (24), whereas inducible NOS (iNOS) is inducible in response to interferon-γ (IFN-γ), lipopolysaccharide (LPS) and a variety of pro-inflammatory cytokines (21). The inducible isoform of iNOS is responsible for the overproduction of NO in inflammation (21). Overexpression of either COX-2 or iNOS has been implicated in the pathogenesis of many disease processes. Prostaglandin production by COX-2 and NO production by iNOS is mainly regulated at the transcriptional level (25,26). In macrophages, LPS activates the transcription factor nuclear factor-kB (NF-kB), which leads to

Abbreviations: COX, cyclooxygenase; EGF, epidermal growth factor; EGCG, (–)-epigallocatechin-3-gallate; EIA, enzyme immunoassay; EMSA, electrophoretic mobility shift assay; iNOS, inducible nitric oxide synthase; IkB, inhibitor kB; IKK, IkB kinase; IFN-γ, interferon-γ; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LPS, lipopolysaccharide; NF-kB, nuclear factor-kB; NO, nitric oxide; NOS, nitric oxide synthase; PGE_2, prostaglandin E_2.

induction of expression of many immediate early genes (27). The presence of the *cis*-acting NF-kB element has been demonstrated in the 5′-flanking regions of both COX-2 and iNOS genes (26,28). Activation of NF-kB by LPS is induced by a cascade of events leading to the activation of inhibitor kB (IkB) kinase (IKK), which phosphorylates IkB, leading to its degradation and translocation of NF-kB to the nucleus (29). Thus, NF-kB translocates to the nucleus and induces gene transcription through the *cis*-acting kB element.

Although the anti-inflammatory and anticarcinogenic characteristics of flavonoids have been studied, their mechanism(s) of action is not fully understood. We previously reported that the tea flavonoid (–)-epigallocatechin-3-gallate (EGCG) could reduce the autophosphorylation levels of epidermal growth factor (EGF) receptor induced by EGF and block EGF binding to its receptor (30). Another study from our laboratory used LPS-activated peritoneal macrophages to demonstrate that the tea flavonoid EGCG could reduce NO radical production through preventing binding of NF-kB to the iNOS promoter (31). In this study, we examined the effects of selected flavonoids on the production of PGE_2 and NO from mouse macrophages and employed both mouse iNOS and COX-2 reporter plasmids and an IKK activity assay to seek possible reaction mechanisms.

Materials and methods

Materials

LPS (*Escherichia coli* 0127:B8), apigenin, genistein, kaempferol, myricetin, quercetin, sufanilamide and naphthylethylenediamine dihydrochloride were purchased from Sigma Chemical Co. (St Louis, MO). Mouse IFN-γ was purchased from Rand D Systems Inc. (Minneapolis, MN). Isotopes were obtained from Amersham (Arlington Heights, IL). EGCG was purified as previously described (32).

Cell culture

The mouse macrophage cell line RAW 264.7 (ATCC, T1B71) was cultured in RPMI containing 10% heat-inactivated fetal bovine serum (Gibco BRL Life Technologies Inc., Grand Island, NY), 100 U/ml penicillin and 100 μg/ml streptomycin. For all assays except the luciferase assay, cells were plated in 60 mm dishes at 5×10^6 cells/dish and allowed to grow for ~18–24 h. Treatment with vehicle (0.01% DMSO), test compounds and/or LPS was carried out under serum-free conditions.

Determination of PGE_2 and nitrite

The culture medium of control and treated cells was collected, centrifuged and stored at –70°C until tested. The level of PGE_2 released into culture medium was quantified using a specific enzyme immunoassay (EIA) according to the manufacturer's instructions (Amersham). The nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction (33).

LPS-induced COX-2 and iNOS enzyme activities

The cells were plated in a 24-well plate and treated with LPS (50 ng/ml) for 6 h. The cells were washed three times with fresh medium and treated with one of the six flavonoids for 30 min. The cells were further incubated with 100 μM arachidonic acid for 15 min, then the supernatants were removed and assayed for PGE_2 (34) as described above. For the iNOS enzyme activity assay, the cells were cultured in 100 mm tissue culture dishes and incubated with LPS (50 ng/ml) for 12 h. The cells were harvested and plated in a 24-well plate and treated with one of the six flavonoids for a further 12 h. The supernatants were removed and assayed for nitrite (34) as described above.

Western blotting

Equal amounts of total cellular protein (50 μg) were resolved by SDS–PAGE and transferred onto Immobilon-P membrane (Millipore, Bedford, MA) as described previously (30). The membrane was then incubated with an anti-COX-2 antiserum (Transduction Laboratories, Lexington, KY), anti-COX-1 antiserum, anti-IkB antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-macrophage NOS antiserum (Transduction Laboratories). The membranes were subsequently probed with anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and visualized using enhanced chemiluminescence kits (ECL; Amersham). The densities of the bands were quantitated with a computer densitometer (IS-100 Digital Imaging System).

IKK assay

Equal amounts of total cellular protein (200 μg) were immunoprecipitated with IKK-α specific antibody (Santa Cruz Biotechnology) and protein A/G-PLUS agarose for 12 h at 4°C. The kinase assay was carried out in 45 μl of kinase buffer (40 mM Tris–NaOH, pH 7.5, 500 mM NaCl, 0.1% NP-40, 6 mM EDTA, 6 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM *p*-nitrophenyl phosphate, 300 μM sodium orthovanadate, 1 mM benzamidine, 2 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM dithiothreitol) containing 5 μM cold ATP, 10 μCi [γ-^{32}P]ATP (5000 Ci/mmol; Amersham) and 1 μg GST–IkBα fusion protein (Santa Cruz Biotechnology) as substrate and incubated for 20 min at 25°C. Each sample was mixed with 8 μl of 5× Laemmli's loading buffer to stop the reaction, heated for 10 min at 100°C and subjected to 8% SDS–PAGE. The gels were dried, visualized by autoradiography and quantitated by densitometry (IS-1000 Digital Imaging System) (35).

Probe

PCR primers for mouse iNOS and COX-2 cDNA probes were synthesized according to the following oligonucleotide sequences: iNOS, forward primer 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ (2944–2968), reverse primer 5′-GGCTGTCAGAGAGCCTCGTGGCTTTGG-3′ (3416–3440); COX-2, forward primer 5′-GGAGAGACTATCAAGATAGTGATC-3′ (1094–1117), reverse primer 5′-ATGGTCAGTAGACTTTTACAGCTC-3′ (1931–1954). Both probes were prepared as detailed in our previous report (31).

Northern blotting

Total RNA was isolated as described by Chomczynski and Sacchi (36). For northern blots, 25 μg of total RNA were separated by electrophoresis on a 1.2% agarose gel containing 6.7% formaldehyde and transferred to a Hybond-N nylon membrane (Amershan). After baking at 80°C for 2 h, membranes were prehybrized for 12–16 h at 42°C in hybridization buffer (50% formamide, 6× SCC, 10× Denhart's solution, 10 mM EDTA, 0.1% SDS and 100 μg/ml single-stranded salmon sperm DNA). Hybridization was carried out for 16 h at 42°C with iNOS or COX-2 cDNA probe (3×10^6 c.p.m./ml). The probe was labeled with [α-^{32}P]dCTP using a Rediprime II labeling system (Amersham). Then the membranes were washed and autoradiographed on X-ray film (Kodak XAR-5) using an intensifying screen at –80°C. After autoradiography, the blot was stripped and reprobed with a probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (37) as a control.

Plasmids

The mouse iNOS promoter plasmid was generously provided by Dr Charles J. Lowenstein (Johns Hopkins University) (28). The construct contains a 1.75 kb *Hin*cIII restriction fragment upstream of the macrophage NOS gene (28). The pNFkB-Luc plasmid was purchased from Stratagene Corp. (La Jolla, CA). The mouse COX-2 promoter plasmid containing a 1035 bp fragment, –996 to +70 relative to the transcription start, was amplified from mouse genomic DNA using the primers 5′-TGGCCAACACAAACACAGTAG-3′ (sense) and 5′-CAGTGCTGAGATTCTTCGTGA-3′ (antisense). Each 5′ amplimer contained a *Xho*I site and each 3′ amplimer contained a *Hin*dIII site, such that the resulting PCR product, upon digestion with *Xho*I and *Hin*dIII, could be ligated in-frame into the unique *Xho*I/*Hin*dIII site present within the pGL2Enhance plasmid (Promega Corp., Madison, WI). pGL2Enhance was linearized with *Xho*I and *Hin*dIII (BioLab, Beverly, MA), and ligated to the above *Xho*I/*Hin*dIII-digested PCR products using standard techniques. Sequence identities were confirmed with an ABI PRISM 377 DNA Analysis System (Perkin-Elmer Corp., Taipei, Taiwan).

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins and ^{32}P-labeled double-stranded oligonucleotide probe were prepared as described previously (31). For the EMSA, 2 μg of each nuclear extract was mixed with the labeled double-stranded NF-kB oligonucleotide 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and incubated at room temperature for 20 min (underlining indicates a kB consensus sequence or a binding site for NF-kB/c-Rel homodimeric and heterodimeric complexes). The specificity of binding was examined by competition with the unlabeled oligonucleotide. The DNA–protein complex was separated on 6% non-denaturing acrylamide gels before vacuum drying and autoradiography.

Transient transfection and luciferase assay

RAW 264.7 cells were seeded in a 60 mm dish. When the cells were confluent, the medium was replaced with serum-free Opti-MEM (Gibco BRL). Then the cells were transfected with the pNFkB-Luc, pGL2-iNOS or pGL2-COX2 plasmid reporter gene using LipofectAMINE™ (Gibco BRL). After 24 h incubation, the medium was replaced with complete medium. After 24 h, the cells were trypsinized and equal numbers of cells were plated in 12-well

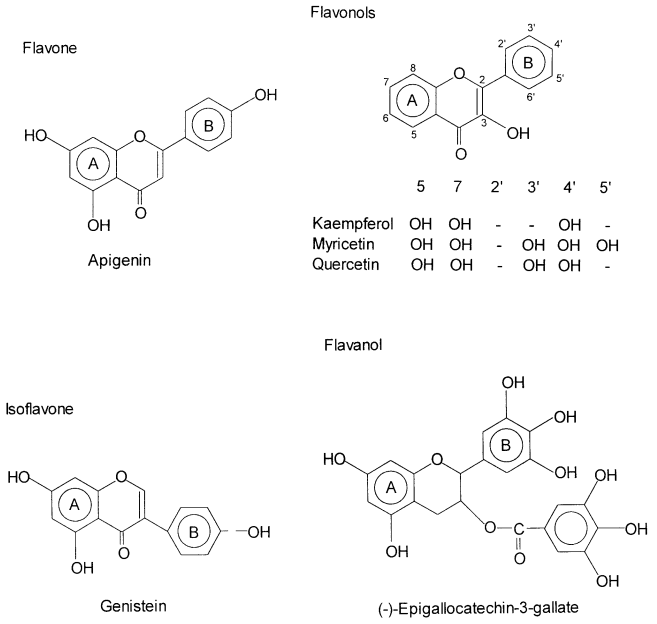


Fig. 1. Structures of the different flavonoids.

tissue culture plates for 12 h. Cells were then treated with LPS (100 ng/ml) and/or test compound for 3 h. Each well was washed twice with cold phosphate-buffered saline and harvested in 150 µl of lysis buffer (0.5 M HEPES, pH 7.8, 1% Triton N-101, 1 mM $CaCl_2$ and 1 mM $MgCl_2$). Aliquots of 100 µl of cell lysate were used to assay luciferase activity with the LucLite™ luciferase reporter gene assay kit (Packard Instrument Co., Meriden, CT). Luminescence was measured in a TopCount Microplate Scintillation and Luminescence Counter (Packard 9912V1; Meriden, CT) in single photon counting mode for 0.05 min/well, following 5 min adaptation in the dark. Luciferase activities were normalized to protein concentrations.

Statistical analysis

Data are presented as means ± SE for the indicated number of independently performed experiments. Statistical analysis was done by one way Student's *t*-test.

Results

Several flavonoids inhibit prostaglandin and/or nitrite production in LPS-activated macrophages

To investigate the anti-inflammatory effects of flavonoids, six flavonoids (Figure 1) were tested with regard to their effect on prostaglandin and nitrite production in LPS-activated macrophages. As shown in Figure 2A, apigenin, genistein and kaempferol inhibited PGE_2 production by >50% at 15 µM. The data indicated that apigenin was markedly more active than genistein and kaempferol. In contrast, EGCG and myricetin slightly enhanced PGE_2 production. Quercetin inhibited PGE_2 production to a lesser extent. With the exception of myricetin, the tested flavonoids also inhibited nitrite production at a concentration of 25 µM ($P < 0.05$), as shown in Figure 2B. At 15 µM the flavonoids could be ranked according to their inhibitory potency as apigenin > quercetin > kaempferol > EGCG > genistein. These flavonoids inhibit LPS-induced PGE_2 and nitrite production in a dose-dependent manner (Figure 2). Among them, apigenin was the most potent inhibitor for both PGE_2 (IC_{50} 8.04 µM) and nitrite (IC_{50} 9.86 µM) production in macrophages. Inhibition of PGE_2 and nitrite production was not due to toxicity, as determined by the trypan blue exclusion assay.

Several flavonoids inhibit COX-2 and/or iNOS protein expression in LPS-activated macrophages. We wanted to understand whether the inhibitory effects on inducible PGE_2 and

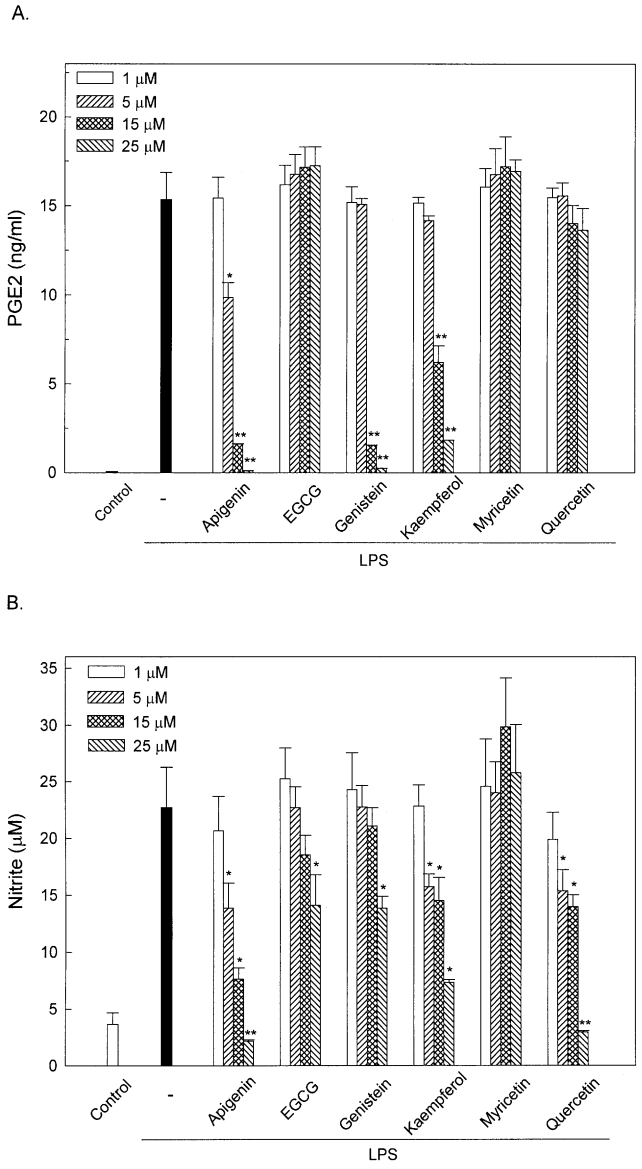


Fig. 2. Effects of the six flavonoids on LPS-induced (**A**) PGE_2 and (**B**) nitrite production in RAW 264.7 cells. The cells were treated with different concentrations of flavonoids and LPS (50 ng/ml) for 24 h. Production of PGE_2 and nitrite were determined by EIA and Griess reaction, respectively, as described in Materials and methods. The values are expressed as means ± SE of triplicate tests. *$P < 0.05$; **$P < 0.001$ versus LPS treatment.

nitrite production were due to a direct effect of these flavonoids on intrinsic enzyme activities of COX-2 and iNOS, respectively. The experimental data indicated that the main inhibitory effect of these flavonoids (at <25 µM) was not on the intrinsic activities of the COX and NOS enzymes ($P > 0.05$, no significant difference from LPS alone; Tables I and II). Only EGCG weakly inhibited the enzyme activity of COX ($0.01 < P < 0.05$). We next investigated whether these flavonoids might affect levels of COX-2 and iNOS protein. As shown in Figure 3, apigenin strongly suppressed the protein levels of both COX-2 and iNOS, whereas EGCG and quercetin slightly enhanced COX-2 protein level, but inhibited iNOS protein level, as measured by densitometer scans (Figure 2, bottom). Genistein and kaempferol markedly inhibited the protein levels of both COX-2 and iNOS. However, myricetin did not affect these protein levels. These data suggest that transcriptional

Table I. Effect of the six flavonoids on the activity of COX-2 enzyme in RAW264.7 cells

LPS induction of cells[a]	Treatment with flavonoid	PGE_2 (ng/ml)[b]
None	DMSO (control)	0.82 ± 0.02
LPS (50 ng/ml)	DMSO (control)	10.05 ± 0.37
	Apigenin	8.69 ± 0.47[c]
	EGCG	8.71 ± 0.18[d]
	Genistein	9.04 ± 0.26[c]
	Kaempferol	11.45 ± 0.23[d]
	Myricetin	9.60 ± 0.25[c]
	Quercetin	11.99 ± 0.22[d]

[a]RAW 264.7 cells were stimulated with LPS (50 ng/ml) for 6 h and the cells were washed with fresh medium. One of the six flavonoids (25 μM) was added and incubated for 30 min, then arachidonic acid (100 μM) was added for a further 15 min.
[b]The amount of PGE_2 in the supernatant was assayed by EIA as described in Materials and methods. The values are expressed as means ± SE of triplicate tests.
[c]$P > 0.05$, no significant difference from LPS alone.
[d]$P < 0.05$.

Table II. Effect of the six flavonoids on the activity of iNOS enzyme in RAW264.7 cells

LPS induction of cells[a]	Treatment with flavonoids	Nitrite (μM)[b]
None	DMSO (control)	2.02 ± 0.02
LPS (50 ng/ml)	DMSO (control)	3.40 ± 0.11
	Apigenin	3.08 ± 0.23[c]
	EGCG	3.21 ± 0.18[c]
	Genistein	3.32 ± 0.26[c]
	Kaempferol	3.42 ± 0.09[c]
	Myricetin	3.66 ± 0.31[c]
	Quercetin	3.11 ± 0.24[c]

[a]RAW 264.7 cells were stimulated with LPS (50 ng/ml) for 12 h and the cells were washed with fresh medium. The cells were harvested and plated into a 24-well plate and incubated with one of the six flavonoids (25 μM) for 12 h.
[b]The amount of nitrite in the supernatant was assayed by the Griess reaction as described in Materials and methods. The values are expressed as means ± SE of triplicate tests.
[c]$P > 0.05$, no significant difference from LPS alone.

events are involved in inhibition of LPS-induced COX-2 and iNOS expression by these flavonoids.

Apigenin decreases COX-2 and iNOS expression in LPS-activated macrophages

Among the flavonoid compounds tested, apigenin was the most potent inhibitor of COX-2 and iNOS expression in LPS-activated macrophages. Therefore, we studied the inhibitory effect of apigenin on COX-2 and iNOS activities in LPS-activated macrophages in detail. Western blotting analysis indicated that the levels of both COX-2 and iNOS proteins were gradually decreased with increasing concentrations of apigenin (Figures 4 and 5). Apigenin at a concentration of 5 μM inhibited protein expression of COX-2 and iNOS by ~50%. The constitutive COX-1 protein level was not affected by apigenin treatment (Figure 4A). The amounts of PGE_2 and nitrite, as a measure of COX-2 and iNOS activity, in the supernatants from each treatment of the RAW cells were determined and corresponded with the protein data (Figures 4 and 5). Changes in amounts of COX-2 and iNOS enzyme could reflect altered protein synthesis or degradation. Northern blotting was done to investigate whether apigenin suppressed

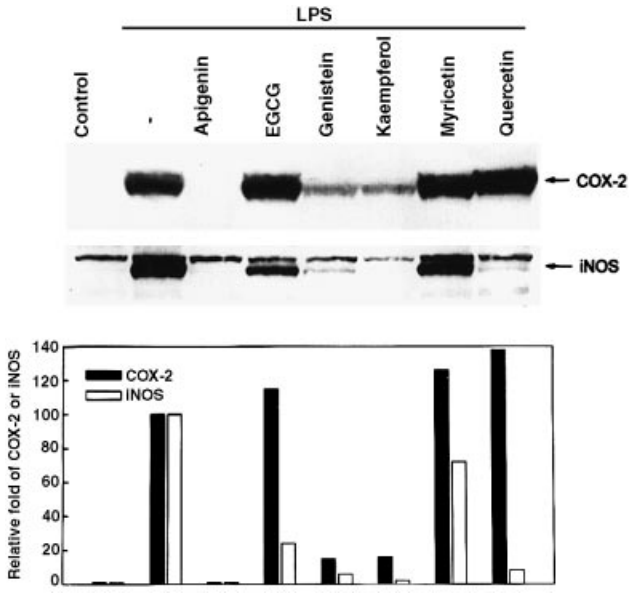


Fig. 3. Effects of the six flavonoids on LPS-induced COX-2 and iNOS protein levels in RAW 264.7 cells. The cells were treated with various flavonoids (12.5 μM) and LPS (50 ng/ml) for 24 h. Total cellular protein (50 μg/lane) was separated on 10% SDS–polyacrylamide gels and blotted with antibodies specific for COX-2 and iNOS as described in Materials and methods.

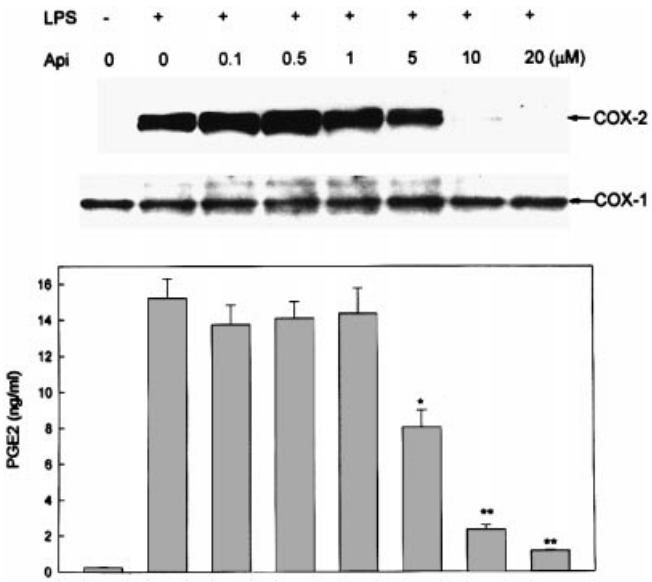


Fig. 4. Effect of apigenin on LPS-induced COX-1 and COX-2 protein levels and PGE_2 production in RAW 264.7 cells. The cells were treated with different concentrations of apigenin and LPS (50 ng/ml) for 24 h. Total cellular protein (50 μg/lane) was separated on 10% SDS–polyacrylamide gels and blotted with antibodies specific for COX-1 and COX-2 as described in Materials and methods. Production of PGE_2 was determined by EIA as described in Materials and methods. The values are expressed as means ± SE of triplicate tests. *$P < 0.005$; **$P < 0.001$ versus LPS treatment.

LPS-mediated induction of COX-2 and iNOS via a pre-translational mechanism. As shown in Figure 6, LPS (50 ng/ml) induced the COX-2 and iNOS mRNA transcript in RAW cells. Both COX-2 and iNOS mRNA induction by LPS was markedly attenuated by apigenin in a dose-dependent manner. These data suggest that apigenin inhibited COX-2 and iNOS expression at the transcription levels.

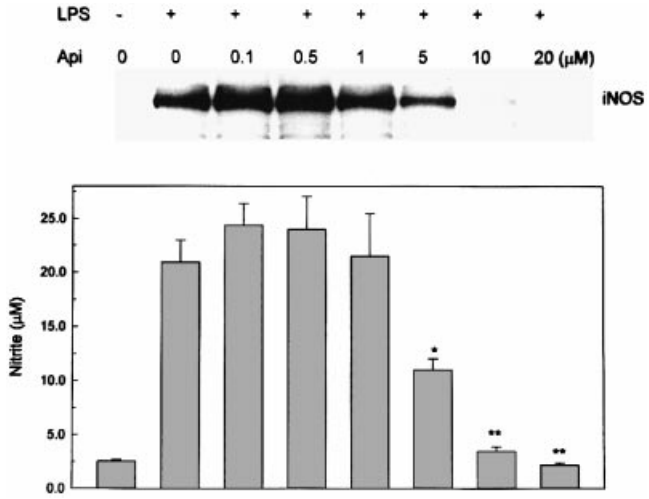


Fig. 5. Effect of apigenin on LPS-induced iNOS protein levels and nitrite production in RAW 264.7 cells. The cells were treated with different concentrations of apigenin and LPS (50 ng/ml) for 24 h. Total cellular protein (50 μg/lane) was separated on 10% SDS–polyacrylamide gels and blotted with antibodies specific for iNOS as described in Materials and methods. Production of nitrite was determined by the Griess reaction as described in Materials and methods. The values are expressed as means ± SE of triplicate tests. *$P < 0.005$; **$P < 0.001$ versus LPS treatment.

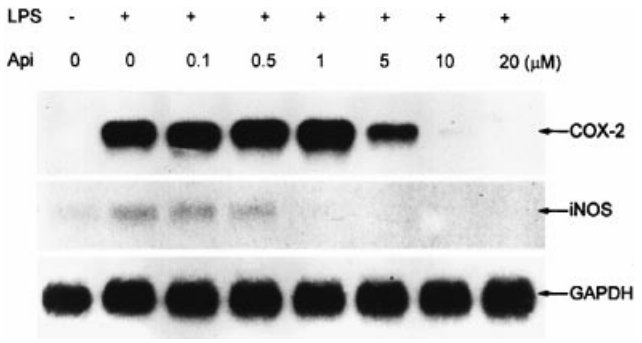


Fig. 6. Effect of apigenin on LPS-induced COX-2 and iNOS mRNA levels in RAW cells. The cells were treated with different concentrations of apigenin and LPS (50 ng/ml) for 18 h. Total RNA (25 μg) was successively hybridized to ^{32}P-labeled COX-2, iNOS and GAPDH probes as described in Materials and methods.

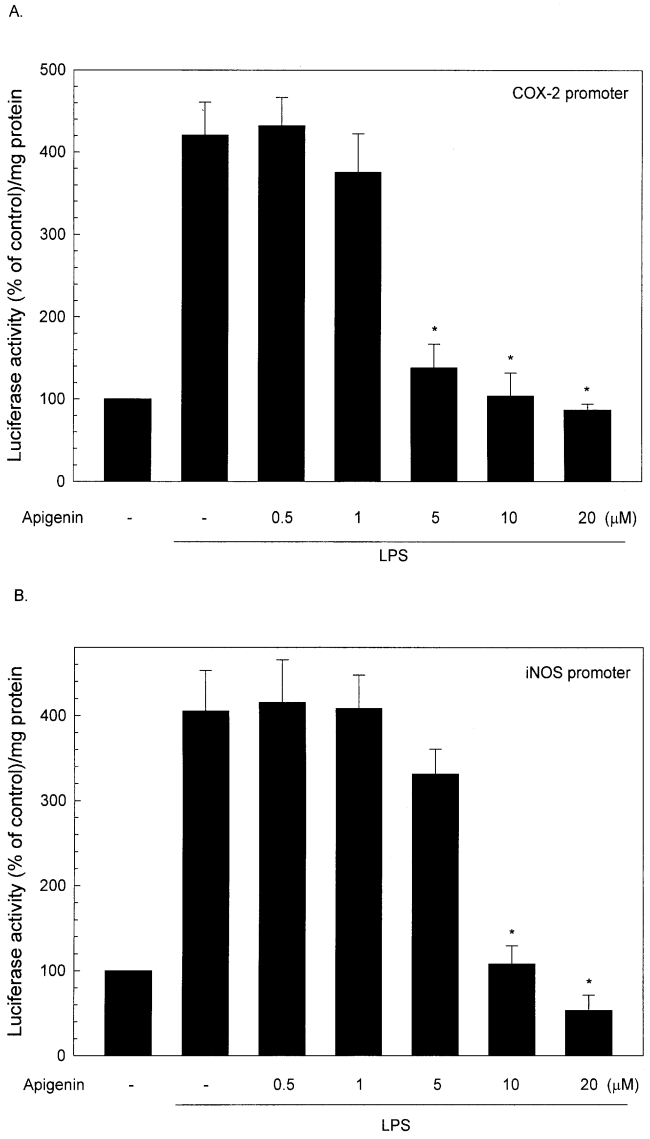


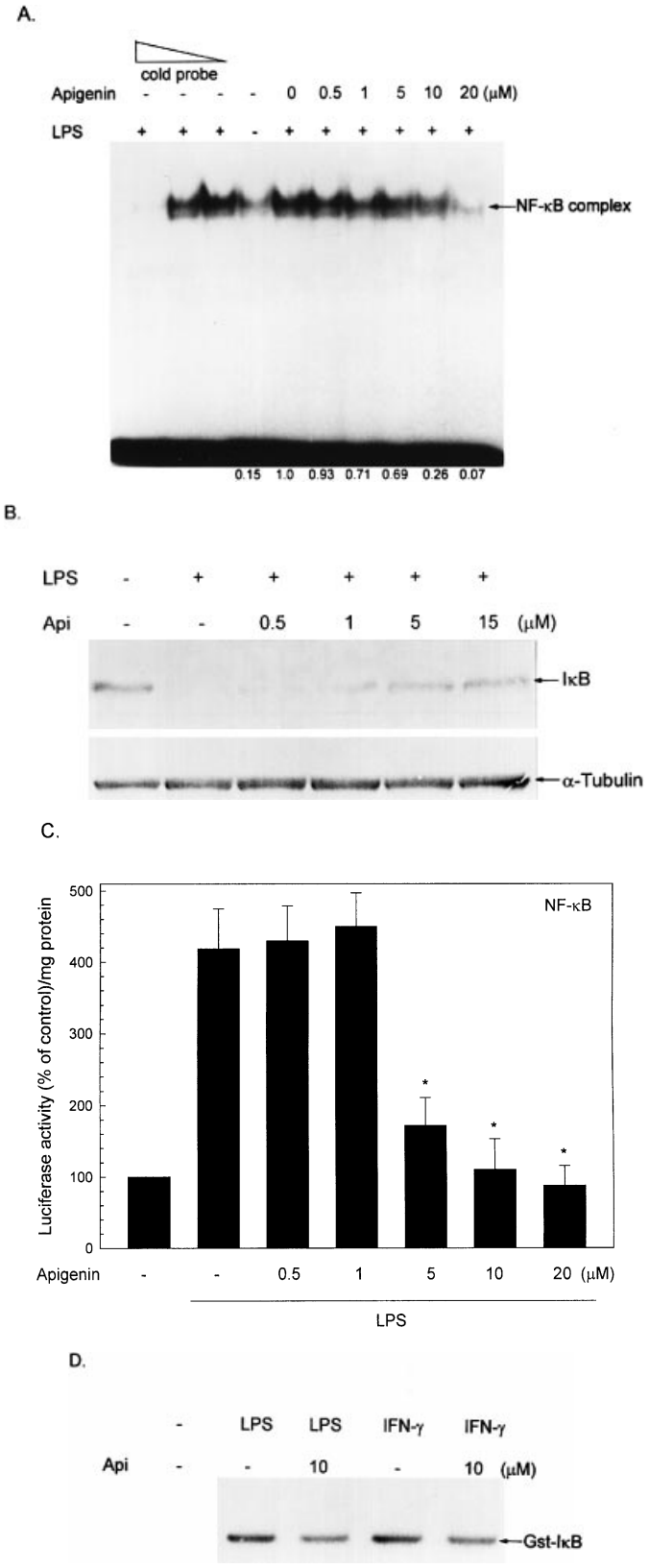
Fig. 7. Effect of apigenin on LPS-induced (**A**) COX-2 and (**B**) iNOS promoter activities in RAW 264.7 cells. The cells were transfected with 2.5 μg of mouse COX-2 promoter constructs (–996 to +70) or mouse iNOS promoter (–1592 to +160). After transfection, cells were subcultured in 12-well plates, then treated with different concentrations of apigenin and LPS (100 ng/ml) for 3 h. Cells were harvested and the levels of luciferase activities were determined as described in Materials and methods. The values are expressed as means ± SE of triplicate tests. *$P < 0.001$ versus LPS treatment.

To further investigate the importance of LPS and apigenin in modulating expression of COX-2 and iNOS, transient transfections were performed using mouse COX-2 and iNOS luciferase promoter constructs. Treatment with 100 ng/ml LPS led to an ~4-fold increase in COX-2 and iNOS promoter activities that was inhibited by apigenin in a dose-dependent manner (Figure 7A and B). These results could potentially be explained if apigenin blocked LPS-mediated signal transduction.

Apigenin suppresses the activation of NF-kB in LPS-activated macrophages

Because activation of NF-kB is critical for induction of both COX-2 and iNOS by LPS or other inflammatory cytokines (38,39), we determined whether apigenin might suppress NF-kB activation in LPS-activated macrophages. As shown in Figure 8A by EMSA, incubation of RAW cells with 100 ng/ml LPS for 3 h increased NF-kB binding activity ~7-fold. Induction of NF-kB binding activity by LPS was markedly inhibited by apigenin in a dose-dependent manner (Figure 8A). The addition of excess unlabeled consensus oligonucleotide completely prevented the band shifts, demonstrating the specificity of the protein–DNA interaction. Since it has been well documented that activation of NF-kB correlates with rapid proteolytic degradation of IkB, prevention of IkB degradation was also studied as an indication of inhibition of NF-kB activation by apigenin. As shown in Figure 8B, LPS induced transient degradation of IkB in RAW cells, whereas apigenin prevented degradation of IkB in a dose-dependent manner. In an additional study, transient transfection with a NF-kB-dependent luciferase reporter plasmid was done to confirm whether apigenin inhibited NF-kB binding activity in LPS-induced macrophages. As shown in Figure 8C, apigenin inhibited LPS-induced NF-kB transcriptional activity in a dose-dependent manner. Finally, we investigated whether apigenin could inhibit IKK activity. As shown in Figure 8D, 10 μM

apigenin significantly inhibited the IKK activity induced by LPS or IFN-γ. There is no effect of apigenin on the level of IKK protein (data not shown). These results suggest that inhibition of COX-2 and iNOS expression by apigenin occurred via suppression of IKK activity resulting in prevention of NF-kB activation.



Discussion

Flavonoids are naturally occurring plant polyphenols found in abundance in diets rich in fruit, vegetables and plant-derived beverages such as tea. Several flavonoids are biochemically active compounds with known anti-inflammatory, anticarcinogenic and free radical scavenging properties. However, the anti-inflammatory functions of some flavonoids are not well established. In a previous study, it was shown that tea flavonoids like EGCG suppress NOS expression in LPS-activated peritoneal macrophages (31). In this study, we indicate that several flavonoids exhibit inhibitory effects on LPS-induced COX-2 or iNOS activity in RAW macrophages. These findings provide a significant molecular basis as to how dietary flavonoids are active in preventing cancer and inflammation.

COX enzyme possesses both cyclooxygenase and peroxidase functions. Prostaglandins formed by COX impair immune surveillance and modulate proliferation in a variety of cell types (16). The peroxidase function contributes to the activation of procarcinogens (40). During infection and inflammation, high production of NO has been shown to cause DNA damage as well as mutations *in vivo* (41). The formation of carcinogenic *N*-nitrosamines resulting from elevated NO formation has also been demonstrated in cell cultures and *in vivo* (42). Recently, it was indicated that overexpression of either COX-2 or iNOS may be intimately involved in the pathogenesis of many diseases, such as colon cancer (43), multiple sclerosis (42), neurodegerative diseases and heart infarction (45,46). Although numerous agents have been synthesized that are effective inhibitors by acting as substrate analogs for COX-2 or iNOS, an alternative approach might be taken to determine whether combining agents that suppress transcription of these two enzymes is more effective than either alone.

Phosphorylation of proteins seems to play a significant role in LPS signaling pathways, including protein tyrosine kinase, mitogen-activated protein kinase, protein kinases C and A, G protein and ceramide-activated protein kinase (47). LPS activates protein kinase C, which in turn induces NADPH oxidase activity (48). Activated NADPH oxidase rapidly generates a series of free radicals and activates protein tyrosine kinase.

Fig. 8. Effect of apigenin on (**A**) LPS-induced NF-kB activity determined by EMSA, (**B**) IkB degradation, (**C**) pNFkB-Luc reporter plasmid and (**D**) LPS- or IFN-γ-induced IkB kinase activity. (A) The cells were treated with different concentrations of apigenin and LPS (100 ng/ml) for 1 h. Nuclear extracts were prepared and EMSAs were carried out as described in Materials and methods. Specificity of NF-kB complex formation was verified in a LPS-only sample by displacement with a 25- (lane 1), 2.5- (lane 2) or 0-fold (lane 3) excess of the unlabeled consensus oligonucleotide. Band intensities were quantified by densitometry and the relative amounts of radioactivity are presented at the bottom of the figure. (B) The cells were treated with different concentrations of apigenin and LPS (50 ng/ml) for 30 min. Total cellular protein (50 μg/lane) was separated on 10% SDS–polyacrylamide gels and blotted with antibodies specific for IkB as described in Materials and methods. (C) The cells were transfected with 2.5 μg of pNFkB-Luc reporter plasmid. After transfection, cells were subcultured in 12-well plates, then treated with different concentrations of apigenin and LPS (100 ng/ml) for 3 h. Cells were harvested and the levels of luciferase activity were determined as described in Materials and methods. The values are expressed as means ± SE of triplicate tests. *$P < 0.001$ versus LPS treatment. (D) The cells were treated with 10 μM apigenin and LPS (50 ng/ml) or IFN-γ (20 ng/ml) for 15 min. Total cell lysates (200 μg) were used for immunoprecipitation. IkB kinase activities were assayed with GST–IkB as substrate. The experiment was performed as described in Materials and methods and ^{32}P-labeled GST–IkB is shown.

Protein tyrosine kinase has been implicated in NF-kB activation by LPS, UV light and hypoxia (48). Apigenin is a novel chemopreventive agent and the effects have been investigated both *in vitro* and *in vivo* (49,50). It is relatively non-toxic and non-mutagenic in comparison with other flavonoids such as quercetin (51). Our previous studies demonstrated that apigenin and other flavonoids inhibit protein kinase C activity in 12-*O*-tetradecanoylphorbol-13-acetate-induced NIH 3T3 fibroblast cells (52,53). Another report demonstrated that apigenin-induced reversion of v-H-*ras*-transformed NIH 3T3 cells by inhibiting mitogen-activated protein kinase and its downstream oncogenes (54). Apigenin is also an antioxidant, preventing oxidative DNA damage in human lymphocytes (55). All of these properties of apigenin might contribute to inhibition of LPS-induced phosphorylation and degradation of IkB. Therefore, IkB still binds to NF-kB and prevents NF-kB translocation to the nucleus. Recently, activation of p38 or ERK-1 and -2 has been shown to be involved in stimulation of NF-kB activity and subsequent expression of iNOS and COX-2 in murine macrophages (56–58). Apigenin might also inhibit these kinases activities, leading to NF-kB activation at or before the phosphorylation step of IkB. Suppression of activation of NF-kB by apigenin may partially account for this, because there are known response elements on the promoters of both the COX-2 and iNOS genes (59). However, apigenin might also inhibit other transcription factors induced by LPS and suppress the gene transcription of COX-2 and iNOS. Recently, the anti-inflammatory properties of aspirin have been linked to inhibition of IKK, thereby preventing activation of NF-kB (60). Our laboratories have also indicated that some flavonoids have the potential to inhibit the activity of IKK (35). In this study we further demonstrate that the anti-inflammatory properties of apigenin might be correlated with suppression of IKK activity. These results suggest that inactivation of IKK is important for prevention of inflammation.

Previous studies have indicated that several flavonoids modulate arachidonic acid metabolism in platelet and peritoneal leukocytes (61,62). Cellular mechanisms underlying these effects are still unclear but are thought to be linked to their antioxidant properties. In this study we demonstrate that apigenin, genistein and kaempferol inhibit both COX-2 and iNOS in a dose-dependent manner. Quercetin and EGCG inhibit iNOS activity, but slightly enhance COX-2 activity. Myricetin is without inhibitory effect on both COX-2 and iNOS. These phenomena might involve the inhibitory mechanism of flavonoids and might depend on their structures. The inclusion of more than two OH groups on the B ring decreases the inhibitory potency of flavonoids on COX-2 activity. The polyhydroxylated B ring of EGCG and myricetin seems to be insufficient to inhibit iNOS activity. Considering the biological functions of flavonoids presented in this and previous studies, it is quite possible that flavonoids might contribute to the preventive effects of a vegetarian diet on inflammation and carcinogenesis. The possible relationships between the structural properties of flavonoids and their anti-inflammatory and anticarcinogenic activities deserves further investigation.

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