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Production of Cytokine and NO by RAW 264.7 Macrophages and PBMC *In Vitro* Incubation with Flavonoids

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Flavonoids, a group of low molecular weight phenylbenzopyrones, have various pharmacological properties including antioxidant activity, anticancer, and immunomodulatory effects. In the present study, lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate/phytohemagglutinin (PMA/PHA) were used as stimulants for RAW 264.7 macrophages and human peripheral blood mononuclear cell (hPBMC), and tumor necrosis factor (TNF)-α and interleukin (IL)-2 productions were measured. In addition, flavonoids were examined for their effects on LPS-induced NO production in RAW 264.7 macrophages. The results showed that all compounds were not strongly cytotoxic at the tested concentrations on hPBMC and RAW 264.7 macrophages. On immunomodulatory properties, catechin, epigallocatechin (EGC), naringenin, and fisetin repressed NO production and TNF-α secretion. Furthermore, catechin, epigallocatechin gallate (EGCG), epicatechin (EC), luteolin, chrysin, quercetin, and galangin increased IL-2 secretion while EGC, apigenin, and fisetin inhibited the secretion. These results indicated that flavonoids have the capacity to modulate the immune response and have a potential anti-inflammatory activity. There was no obvious structure-activity relationship regard to the chemical composition of the flavonoids and their cell biological effects.

Key words: Flavonoids, Tumor necrosis factor-α (TNF-α), Nitric oxide (NO), Interleukin-2 (IL-2), RAW 264.7, PBMC

INTRODUCTION

Flavonoids are found in seeds, citrus fruits, olive oil, tea and red wine and are commonly consumed with the human diet (Middleton Jr. *et al.*, 2001), approximately 1 g flavonoids/day. The basic structure of flavonoids consists of an o-heterocyclic ring fused to an aromatic ring with a third ring system attached at either C3 or C4 of the hetero cyclic ring (Table I). The 6 most common chemical classes are the anthocyanidins, catechins, flavones, flavonols, flavanones, and isoflavonoids (Wiseman, 1996). Various pharmacological roles including antioxidant, bactericidal, anti-malarial, anti-inflammatory, and anticancer effects have been demonstrated.

There is a good evidence to suggest that the systemic immune response is at least partially controlled by a group of cytokines that allow the cellular components of this defense mechanism to communicate with each other, and which are also capable of directing the nature of the response. Some of these molecules such as tumor necrosis factor (TNF)-α is predominantly synthesized and secreted by activated macrophages. On the other hand, interleukin (IL)-2, IL-4, IL-5, IL-10, and interferon (INF)-γ are produced by activated T lymphocytes. This second group of cytokines has now been classified into two subsets (Th1-type and Th2-type cells) based on the types of cytokines they produce. Th1 cells secrete mostly IL-2 and INF-γ, and Th2 cells secrete IL-3, IL-4, IL-5, IL-6, and IL-10 (Radford-Smith and Jewell, 1996).

TNF-α is a potent cytokine produced by many cell types including macrophages, monocytes, and lymphocytes in response to inflammation, infection, and injury. The increasing understanding of the role of cytokines in autoimmunity, and the observation that TNF-α is central to the inflammatory and destructive process common to several human autoimmune diseases, has led to a new generation of therapeutics, the TNF-α blocking agents (Andreakos *et al.*, 2002).

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Table I. Classification, structure, and substitution patterns of flavonoids

Compound	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$
(+/-) Catechin hydrate	OH	OH	H	OH	H	H	OH	OH	OH
EGC	OH	OH	H	OH	H	H	OH	OH	H
EGCG	OH	OH	H	OH	H	H	OH	OH	OH
EC	OH	OH	H	OH	H	H	OH	OH	H
Naringenin	H	OH	H	OH	H	H	H	OH	H
Naringin	H	OH	H	Rhamnoglucoside	H	H	H	OH	H
Apigenin	H	OH	H	OH	H	H	H	OH	H
Luteolin	H	OH	H	OH	H	H	OH	OH	H
Chrysin	H	OH	H	OH	H	H	H	H	H
Rutin	manno/gluco-pyranosyl	OH	H	OH	H	H	H	OH	OH
Fisetin	OH	H	H	H	H	H	OH	OH	H
Myricetin	OH	OH	H	OH	H	H	OH	OH	OH
Quercetin	OH	OH	H	OH	H	H	OH	OH	H
Galangin	OH	OH	H	OH	H	H	H	H	H

Th1 cells are responsible for cell-mediated immunity: phagocyte-dependent host response, cytotoxicity, and macrophage activation (Kovacs, 2000). IL-2 has effect on several other immune cells, including natural killer (NK) cells, B cells, monocyte/macrophages, and neutrophils (Mingari *et al.*, 1984; Waldmann *et al.*, 1984; Robertson and Ritz, 1990).

Cytokines such as interleukin (IL)-2, and other inflammatory stimuli such as bacterial lipopolysaccharide (LPS) regulate the activity of inducible NO synthase (iNOS) in macrophages. It has also been shown that the production of TNF-α is crucial for the synergistic induction of NO synthesis in IFN-γ and/or LPS-stimulated macrophages (Park *et al.*, 2000). NO is involved in various biological processes including inflammation and immunoregulation (Ialenti *et al.*, 1992; Stichtenoth and Frolich, 1998). 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 (Chiou *et al.*, 1997; Kobuchi *et al.*, 1997; Ryu *et al.*, 1998) and there have been several studies to investigate the inhibitory activity of flavonoids.

In the present study, the possible anti-inflammatory effects of flavonoids have been investigated *in vitro* in order to evaluate whether they have potential as anti-inflammatory agents in chronic inflammatory diseases. The production of the pro-inflammatory cytokine TNF-α by LPS-stimulated human peripheral blood mononuclear cell (hPBMC) and RAW 264.7 macrophages after incubation with flavonoids was determined. Also, the effect of flavonoids on Th-1 derived cytokine IL-2 protein secretion in hPBMC was investigated. In addition, we evaluated the effects of structurally diverse flavonoid derivatives on NO production on LPS-activated RAW 264.7 cells.

MATERIALS AND METHODS

Reagents

Flavanol; (+/-)-catechin hydrate, epigallocatechin (EGC), epigallocatechin gallate (EGCG), epicatechin (EC), flavanone; naringenin, naringin, flavone; apigenin, luteolin, chrysin, rutin, and flavonol; fisetin, myricetin, quercetin, galangin were purchased from Sigma (St. Louis, MO, U.S.A.). LPS (*Escherichia coli* 0127:B8), phorbol 12-myristate 13-acetate (PMA) and phytohemagglutinin (PHA) were obtained from Aldrich Chemical Co. (St. Louis, MO). Flavonoids were dissolved in DMSO on the day of experiment and diluted with phosphate-buffered saline (PBS) into appropriate concentrations. Fetal bovine serum (FBS), penicillin-streptomycin, and RPMI 1640 and Minimum Essential Medium (MEM) were from Gibco BRL (Grand Island, NY, U.S.A.). RAW 264.7, a mouse macrophage cell line was obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea).

Isolation of human PBMC

Blood specimens were obtained with consent from healthy human volunteers (aged between 20 and 50 years). Heparinised whole blood were diluted with RPMI 1640 medium and hPBMC were isolated by buoyant density centrifugation over Histopaque 1077 (Sigma, Poole, UK) at 450 g for 16 min. PBMC harvested from the 'buffy' layers were washed twice by centrifugation at 600 g for 6 min with RPMI 1640 medium. The cells were counted and resuspended in complete cell culture medium (CTCM)

consisting of RPMI 1640 medium with 5% FBS, 2 mM L-glutamine, and 5×10^{-5} M 2-mercaptoethanol.

Cell culture

RAW 264.7 macrophages were maintained in MEM and hPBMC were maintained in RPMI 1640 cultured in 75 cm^2 plastic flasks (Falcon-Becton Dickinson Labwares, Franklin Lakes, NJ, U.S.A.) and were supplemented with 10% (v/v) heat-inactivated FBS, 100U/mL penicillin and 100 μg/mL streptomycin at a temperature of 37°C in a 5% CO_2 humidified incubator (Sanyo, Japan). For cytokine production, RAW 264.7 macrophages were detached by vigorous pipetting. After centrifugation, the cells were incubated (5×10^5 cells/mL) with fresh medium in 24-well flat-bottomed tissue culture plates (Corning, NY, U.S.A.) at 37°C in a 5% CO_2 humidified incubator. Cells were supplemented in the absence or presence of flavonoids and stimulants (1 μg/mL LPS for TNF-α and NO, 50 ng/mL PMA and 1 μg/mL PHA for IL-2) for various time intervals. Cultures were centrifuged at 300×g for 10 min to separate supernatant from cells.

Cell density determination

Cell numbers and viability were assessed by trypan blue (Sigma, Poole, U.K.) dye exclusion. The dye exclusion test is based on the concept that viable cells do not take up trypan blue dye, whereas dead cells are permeable to this dye. Twenty microliters of trypan blue solution was mixed with 20 μL of cell suspension in a microtube to obtain a final density about $0.3\text{-}2\times10^6$ cells/mL, and was loaded onto a hemocytometer. The cells that excluded the dye were counted in the standard manner within 1-5 min after mixing of the dye and cell suspension.

Cytotoxicity assay

In order to discern whether inhibition of proliferation was due to cytostatic or cytotoxic effect, the viability of RAW 264.7 cells and hPBMC were measured 48 h post-treatment. The *in vitro* cytotoxic effect was measured by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, Sigma Chemical Co.) assay colorimetric dye reduction method (Freshney, 2000). Cells (1×10^5 cells/mL) were seeded in 96 well flat-bottomed tissue culture plates (Corning) in the absence or presence of flavonoid for 24 h at 37°C in a 5% CO_2 humidified incubator. At the end of the incubation, 50 μL of filter sterilized MTT stock solution was added and the plate was incubated for a further 4 h. Dimethylsulfoxide (DMSO, DUCHEFA Biochemie, Netherlands) and Sörensen's buffer (0.1 M glycine, 0.1 M NaCl adjusted to pH 10.5 with 1 M NaOH) was added. The absorbance was detected using an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular devices, Menlo Park, CA, U.S.A.) at 570 nm. The cytotoxicity was measured as IC_{50} values and each assay was done in triplicate.

TNF-α production from LPS stimulated hPBMC and RAW 264.7 macrophages

The TNF-α secretion in culture supernatants were assayed using a commercially available ELISA kits obtained from Assay Designs, Inc. (Ann Arbor, MI, USA). Briefly, supernatants were added to the 96-well plates coated with monoclonal Ab with specificity for human or murine TNF-α. Between subsequent steps in the assay, coated plates were washed 4 times with PBS containing 0.05% (v/v) Tween-20. After exposure to medium, assay plates were sequentially exposed to biotin-conjugated anti-cytokine antibodies and detected by streptavidin-peroxidase anti-rabbit antibody. The plate was developed using 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide solution and optical density readings at 450 nM were taken using a microplate reader (Dynex). The concentration of TNF-α in the culture supernatants were determined by extrapolation from the reference standard TNF-α curve.

IL-2 production from PMA/PHA stimulated hPBMC

Flavonoids were initially diluted into CTCM and then tested in triplicate wells at concentrations ranging from 1 nM to 10 mM in 200 μL of CTCM, containing 100,000 hPBMC which were separately challenged with 50 ng/mL PMA and 1 μg/mL PHA. Cell stimulations of hPBMC were incubated for 24 h at 37°C in 5% CO_2-air and 100 μL of culture supernatants was removed for the determination of IL-2. The levels of cytokine produced in the culture supernatants were determined in a 'sandwich' ELISA. Briefly, 96-well Nunc MaxiSorp (Life Technologies, Paisley, UK) plates were coated overnight at 4°C with 'capture' mouse anti-human IL-2 monoclonal antibody (BD Pharmingen, U.K.). After the plates were blocked with 1% (wt/vol) bovine serum albumin (BSA) (Sigma, Poole, U.K.) 100 μL of cell culture supernatants were added and incubated overnight at 4°C; standard human IL-2 concentrations (BD Pharmingen, U.K.) ranging from 8 to 500 pg/mL were included for each plate. The 'captured' IL-2 was sandwiched with a biotinylated mouse anti-human IL-2 monoclonal antibody (BD Pharmingen, U.K.). The presence of biotinylated antibodies was detected by the addition of streptavidin-peroxidase (BD Pharmingen, U.K.). After thorough washes the assay was developed using tetramethyl benzidine substrate (Sigma, Poole, U.K.). The enzyme reaction was stopped with 2.5 M H_2SO_4 and the colorimetric development was read at 450 nm with a spectrophotometric 96-well plate reader (Dynex).

Measurement of nitrite and nitrate concentration

To study the effects of flavonoid on NO production, a

commercially available ELISA kits obtained from Assay Designs, Inc. (Ann Arbor, MI, U.S.A.) was used. Nitric oxide was measured as nitrite released from mouse macrophage cells, RAW 264.7. To measure nitrite (NO_2^-), cells were suspended in CTCM consisting of RPMI 1640 medium with 5% FBS, 2 mM L-glutamine, and 5×10^{-5} M 2-mercaptoethanol, and plated in 96-well culture plate (2.5×10^5 cells/well). The cells were incubated for 48 h at 37°C in an atmosphere of 5% CO_2 and 95% humidity. Thereafter, 100 μL of medium was aspirated from each well and replenished with the same amount of fresh medium. Further incubation for 24 h was done with desired concentration of different flavonoids in the presence or absence of LPS. NO production in supernatant was measured in terms of amount of nitrite, its stable product in a microplate assay. To measure nitrite, 100 μL of macrophage culture supernatant was collected, mixed with an equal volume of Griess reagent (A solution of sulfanilamide and *N*-(1-naphthyl)-ethylenediamine in 2M hydrochloric acid) and incubated for 10 min at room temperature. Nitrite concentration was determined by measuring the absorbance at 540 nm with a microplate reader. $NaNO_2$ was used for external calibration. Nitrate (NO_3^-) was measured by reducing nitrate to nitrite with nitrate reductase, then measuring nitrite by using Griess reagent. There was no interference of the test compounds either with the ELISA or with Griess reagent.

Statistical analysis

All values expressed as mean ± S.E.M. were obtained from at least five observations and were compared using analysis of variance (ANOVA). Differences were significant; probability values of <0.001, <0.01 or <0.05 were considered significant with 99.9%, 99% or 95% of confidence, respectively.

RESULTS

Cytotoxic effect of flavonoids

The cytotoxicity effects of flavonoids on RAW 264.7 macrophage cell line and hPBMC were investigated. When the cells were treated with flavonoids at various concentrations of 1 nM-100 μM for 48 h, all compounds were not strongly cytotoxic at the tested concentration as shown in Table II. Thus, it seems unlikely that the induction or inhibition of cytokine production (shown later) was due to any cytotoxic effects of flavonoids.

Effects of flavonoids on TNF-α secretion in LPS-stimulated hPBMC and RAW 264.7 macrophages

We also studied the effects of flavonoids on TNF-α secretion in hPBMC and RAW 264.7 macrophages (Fig. 1, 2). In unstimulated macrophages, only small amounts of TNF-α were secreted into the medium. The stimulation of PBMC and macrophages with only LPS (positive control cells) resulted in a significant increase in TNF-α secretion monitored 24 h after administration. Pretreatment of unstimulated cells with flavonoids over 24 h did not result in any change in the secretion of TNF-α into the medium. When the cells were treated with flavonoids and LPS, all flavanols tested ((+/-)-catechin hydrate, EC, EGC, and EGCG) (Fig. 1A) and most flavonols tested (myricetin, quercetin, and galangin) slightly inhibited LPS-induced TNF-α secretion, but fisetin which is also a flavonol significantly decreased the TNF-α level (Fig. 1D). Also, all flavones tested (apigenin, luteolin, and chrysin) decreased the secretion of TNF-α almost down to the baseline levels (Fig. 1C). We had similar results in RAW 264.7 cells on TNF-α production (Fig. 2) and the only difference was that naringenin which is a flavanone significantly inhibited the TNF-α production in macrophages (Fig. 2B).

Table II. Flavonoid classes and IC_{50} for RAW 264.7 macrophages and hPBMC

Compound	Class	IC_{50} value (μM)	
		RAW 264.7	hPBMC
(+/-) Catechin hydrate	Flavanol	>100	>100
EGC	Flavanol	>100	>100
EGCG	Flavanol	>100	>100
EC	Flavanol	>100	>100
Naringenin	Flavanone	>100	>100
Naringin	Flavanone	>100	>100
Apigenin	Flavone	23.8 ± 1.7	51.2 ± 3.4
Luteolin	Flavone	35.2 ± 0.2	>100
Chrysin	Flavone	>100	>100
Rutin	Flavone	>100	>100
Fisetin	Flavonol	26.7 ± 4.4	67.9 ± 1.1
Myricetin	Flavonol	62.2 ± 1.2	>100
Quercetin	Flavonol	44.0 ± 0.4	>100
Galangin	Flavonol	>100	>100

For basic structure flavonoid, see Table I. >100: no inhibition observed below 100 μM. Data shown are the average IC_{50} of five experiments ± S.D.

Effects of flavonoids on IL-2 secretion in PMA/PHA-stimulated hPBMC

The stimulation of hPBMC with some flavonoids shifted the PMA/PHA-induced cytokine secretion toward a more immunostimulating response. In the presence of flavonoids (5-80 μg/mL), an increased release of IL-2 ($p<0.05$) was observed compared with PMA/PHA control. Among flavonols, galangin, and myricetin increased PMA/PHA-induced IL-2 secretion (Fig. 3D), and among flavanols, (+/-) catechin hydrate, EC, and EGCG increased IL-2

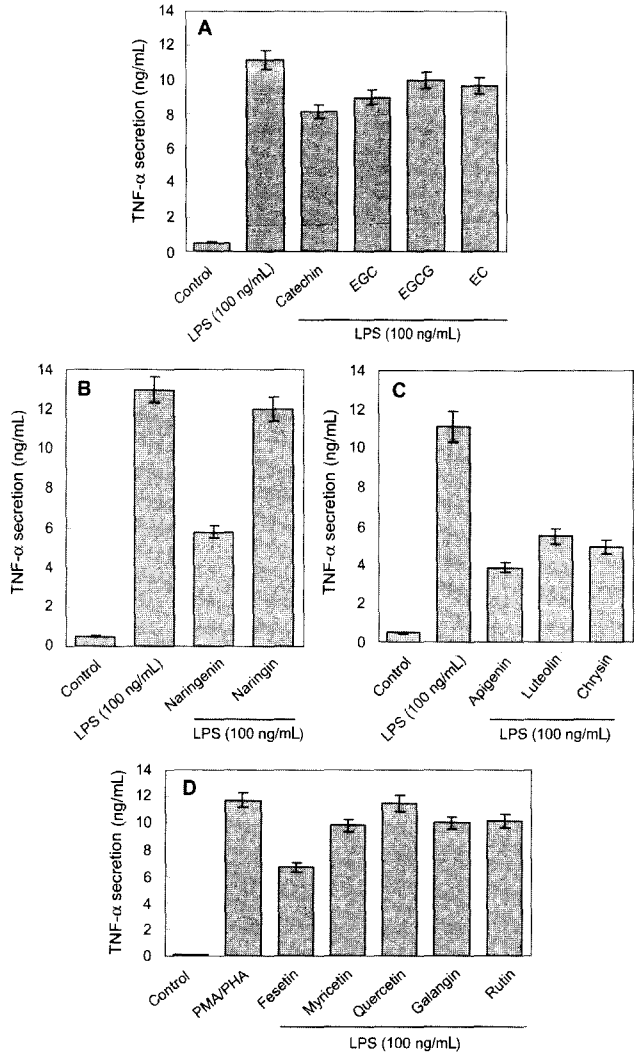

Fig. 1. Effects of flavonoids (A: flavanol, B: flavanone, C: flavone, D: flavonol) on TNF-α secretion in unstimulated and LPS (100 ng/mL) stimulated hPBMC. The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 h in the presence or absence of flavonoids and LPS. The TNF-α measurement in the culture supernatant were performed by an ELISA TNF-α set. Flavonoids tested in this were supplemented at the following concentrations: cathechin, EC, naringenin, naringin, rutin = 100 μM; EGCG, myricetin = 10 μM; chrysin = 50 μM; EGC, apigenin, luteolin, fisetin, quercetin, galangin = 5 μM. The results are expressed as nanograms per milliliter ± S.D. in triplicate. Pretreatment of unstimulated cells with flavonoids over 24 h did not result in any change in the secretion of TNF-α into the medium.

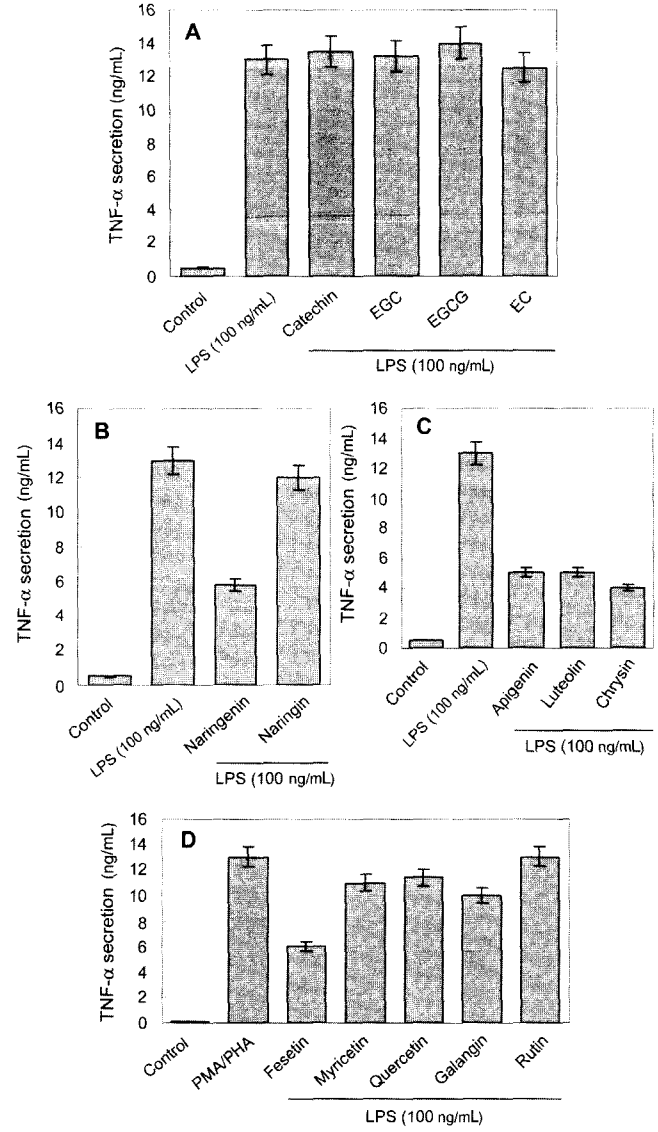

Fig. 2. Effects of flavonoids (A: flavanol, B: flavanone, C: flavone, D: flavonol) on TNF-α secretion in unstimulated and LPS (100 ng/mL) stimulated RAW 264.7 macrophages. The same conditions were used as Fig. 1 to culture the cells and treat them with flavonoids. Pretreatment of unstimulated cells with flavonoids over 24 h did not result in any change in the secretion of TNF-α into the medium.

secretion (Fig. 3A). Also, among flavones, luteolin and chrysin increased the secretion (Fig. 3C). In contrast, the levels of IL-2 released from hPBMC in the presence of other flavonoids including EGC, apigenin, and fisetin revealed a significant decrease (Fig. 3). The control (CTCM) did not exert any effect on the PMA/PHA-stimulated release of IL-2. Pretreatment of unstimulated cells with flavonoids over 24 h did not result in any change in the secretion of IL-2 into the medium. Overall, the levels of IL-2 released from PMA/PHA stimulated hPBMC in the presence of flavonoids showed a certain immunomodulatory effect.

Effects of flavonoids on NO production in LPS-stimulated RAW 264.7 macrophages

The effects of flavonoids on LPS-induced NO production in RAW 264.7 macrophages were investigated by measuring the accumulated nitrite and nitrate, as estimated by the Griess reaction in the culture medium. At the used concentrations, flavonoids did not interfere with the reaction between nitrite, nitrate and Griess reagents (data

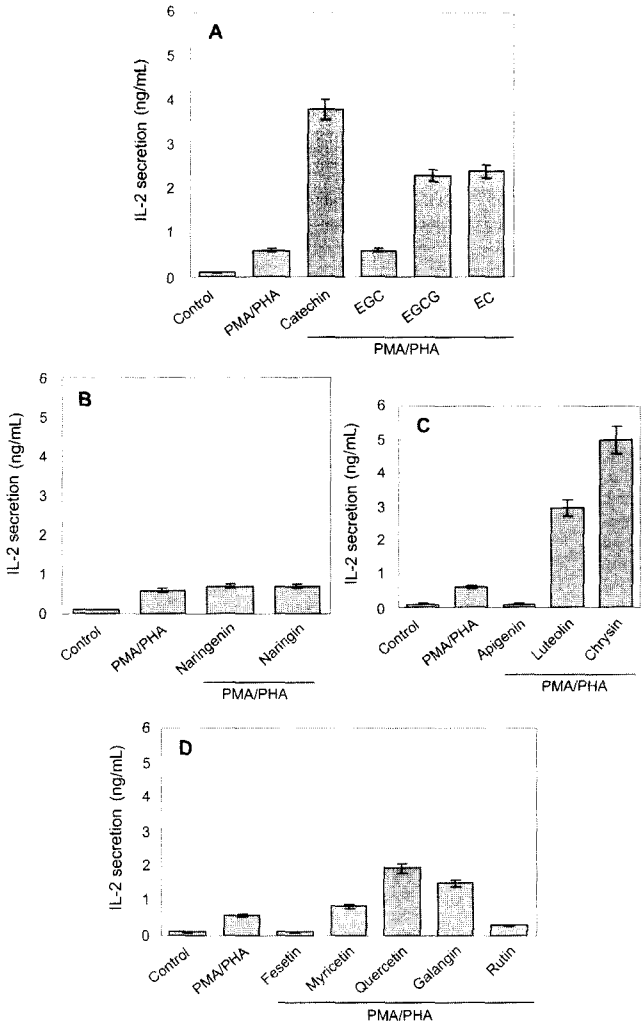

Fig. 3. Effects of flavonoids (A: flavanol, B: flavanone, C: flavone, D: flavonol) on IL-2 secretion in unstimulated and 50 ng/mL PMA and 1 μg/mL PHA stimulated human PBMC. The cells were incubated with or without flavonoids and PMA/PHA at 37°C in a humidified 5% CO_2 atmosphere for 24 h. Then the supernatants were collected, and the measurement of IL-2 was performed by specific ELISA. Flavonoids tested in this were supplemented at the following concentrations: EC, quercetin = 80 μM; cathechin, chrysin, galangin = 40 μM; EGC, EGCG, fisetin, myricetin, rutin, naringenin, naringin = 20 μM; apigenin, luteolin = 5 μM. The results are expressed as picograms per milliliter ± S.D. in triplicate. Pretreatment of unstimulated cells with flavonoids over 24 h did not result in any change in the secretion of IL-2 into the medium.

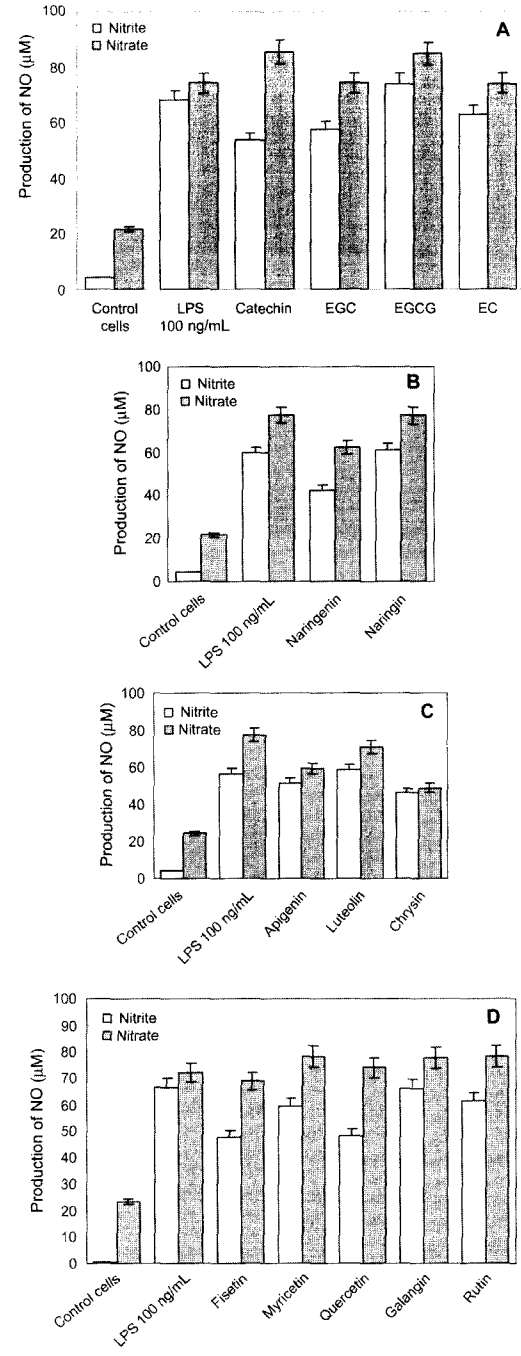

Fig. 4. Effects of flavonoids (A: flavanol, B: flavanone, C: flavone, D: flavonol) on NO production in unstimulated and LPS (100 ng/mL) stimulated RAW 264.7 macrophages. The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 h in the presence or absence of flavonoids and LPS. The amounts of nitrite and nitrate released by macrophages were measured by the method of Griess. Flavonoids tested in this were supplemented at the following concentrations: cathechin, EC, naringenin, naringin, rutin = 100 μM; EGCG, myricetin = 10 μM; chrysin = 50 μM; EGC, apigenin, luteolin, fisetin, quercetin, galangin = 5 μM. The results are expressed as IM ± S.D. in triplicate. Pretreatment of unstimulated cells with flavonoids over 24 h did not result in any change in the production of NO into the medium.

not shown). Unstimulated control cells, after 24 h of incubation in culture medium, produced a negligible amount of background levels of NO (<5 μM). When the cells were incubated with the indicated compounds alone, the concentration of NO in the medium was maintained at a background level similar to that in the unstimulated samples (Fig. 4). However, in LPS-stimulated cells, a substantial increase in the production of NO was evident. Pretreatment of macrophages with flavones (apigenin,

chrysin, and rutin,) slightly decreased LPS-induced NO production (Fig. 4C), as compared to positive control cells. Additionally, Flavanols ((+/-) catechin hydrate and EGC) (Fig. 4A) and flavonols (myricetin and quercetin) (Fig. 4D) showed a mild inhibitory effect on NO production. In contrast to other flavonols, fisetin significantly inhibited secretion of NO (Fig. 4D). Naringenin, which is a flavanone, also strongly decreased NO production, while another flavanone, naringin, had little effect on NO (Fig. 4C).

DISCUSSION

Flavonoids, a group of low molecular weight phenylbenzopyrones, are common in vascular plants and are found in particular spices, vegetables, fruits, nuts, seeds as well as tea and wine. Several properties have been ascribed to flavonoids. The ability of certain flavonoids to inhibit pro-inflammatory mediators like TNF-α could be useful in the treatment of several chronic inflammatory diseases. TNF-α is a pro-inflammatory cytokine which is important in the inflammatory stages of several chronic inflammatory diseases. In the present study, the production of TNF-α by LPS-stimulated hPBMC and macrophage cell line RAW 264.7 after incubation with flavonoids were determined. In negative control cells, TNF-α was produced in vanishingly small quantities but when stimulated with LPS, a large increase in the secretion of TNF-α into the medium was observed in the cells. When the cells were treated with flavonoids and LPS, most of the flavonoids that were tested inhibited LPS-induced TNF-α secretion. In particular, fisetin, apigenin, luteolin, chrysin, and naringenin significantly decreased the TNF-α level by 50% or more (Fig. 1, 2).

Flavonoids are considered to be antioxidants as they participate in an antioxidant network and likely spare endogenous cellular vitamin E and glutathione (Rimbach *et al.*, 1999). It has been described that flavonoids with 4-6 OH groups act as strong antioxidants in an aqueous milieu, where as those with more or fewer OH groups show low or no antioxidant activities (Rice-Evans *et al.*, 1995). In addition, it was found that OH groups in the ortho-position at ring B as well as the double bond between C2 and C3 together with the carbonyl function in ring C are important structural determinants for the antioxidant effects (Bors *et al.*, 1990). However, there were flavonoids carrying only two or three OH groups that showed high antioxidant potencies. Moreover, ortho-OH groups in ring B or double bond between C2 and C3 did not show significant difference between other flavonoids in NO production (Fig. 4). Therefore, there was no obvious structure-activity relationship with regard to the chemical composition (number of the OH group or the position) of the flavonoids and their cell biological effects. However both rutin and naringin had no effect on TNF-α, IL-2, and NO production, and this may be due to the fact that these are flavonoid glycoside molecules (Table I) and had little or no activity.

Pro-inflammatory cytokines such as TNF-α are strong stimulators and/or co-stimulators for inducible nitric oxide synthase (iNOS) gene expression in certain cell types (Xie *et al.*, 1993). Flavonoids such as (+/-) catechin hydrate, EGC, naringenin, and fisetin which suppressed TNF-α also effectively inhibited LPS-induced NO production in macrophages (Fig. 4) suggesting a linked pathway. The primary mechanism of anti-inflammatory actions of several plant extracts and their role in immunomodulation is through inhibition of TNF-α production and scavenging of free radicals (Sandoval *et al.*, 2000). Thus, the ability of flavonoids to inhibit NO production and TNF-α may have therapeutic implications for conditions where TNF-α plays a major role in pathogenesis. TNF-α also has a profound stimulatory influence on mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB (Williams *et al.*, 1994). It has been reported that some flavonoids decrease NF-κB-dependent gene expression in LPS-stimulated RAW 264.7 macrophages (Park *et al.*, 2000) and inhibits various tyrosine and serine/threonine kinases including mitogen-activated MAPK (Hagiwara *et al.*, 1988). Further studies are necessary to determine the exact mechanism by which flavonoids inhibit TNF-α and NO production.

Since cytokines play an important role in the development of immune responses, it was decided to study the effect of flavonoids on the production of Th1 secreted cytokine.

One of the important T-cell growth factor is IL-2, which is produced by activated CD4+ T cells, and plays an important role in the immune response, T-cell and B-cell activation of proliferation. The data presented here prove that flavonoids modulate PBMC production of Th1 cytokine (IL-2). The levels of IL-2 released from PMA/PHA stimulated hPBMC in the presence of certain flavonoids including EGC, apigenin, and fisetin revealed a significant decrease (Fig. 3). Since IL-2 is an essential component in the activation and maintenance of cell proliferation, the antiproliferative effect of these flavonoids may be due, at least in part, to the decrease of IL-2 production. Also, as mentioned above, there has been a report showing that some flavonoids decrease NF-κB-dependent gene expression (Park *et al.*, 2000). NF-κB regulates many important functions in lymphocytes and play a crucial role in their proliferation and expression of cytokines and their receptors. In the nucleus, binds directly to the IL-2 gene enhancer region and combines with nuclear factor for activated T cell (NF-ATc) to form active form of NF-ATn. This binding to IL-2 promoter is crucial for the expression of the IL-2 and IL-2R gene (Li and Verma, 1992). Therefore, the above flavonoids probably act at one or more of the steps

involved in the activation pathways of NF-κB. On the other hand, other flavonoids including (+/-) catechin hydrate, EGCG, EC, luteolin, chrysin, quercetin, and galangin induced the production of IL-2 (Fig. 3). IL-2 can increase immunoglobulin synthesis and J-chain transcription in B cells, potently augment the cytolytic activity of nature killer (NK) cells, and induce the cytolytic activity of lymphokine-activated killer (LAK) cells (Leonard, 1999). The above flavonoids appear to enhance lymphocyte functions and can thus potentially initiate acquired immune responses.

Overall, these results indicate that flavonoids have the capacity to modulate the immune response and have a potential anti-inflammatory activity. Also, the capability of flavonoids for modulating the immune system can not be predicted on the basis of their chemical composition and structure.

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