Polymethoxylated Flavones Derived from Citrus Suppress Tumor Necrosis Factor-α Expression by Human Monocytes

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Received October 2, 1998

Flavonoids isolated from citrus were evaluated for their ability to affect the inflammation response through suppression of cytokine expression by human monocytes. Several polymethoxylated flavones inhibited lipopolysaccharide-induced monocyte expression of tumor necrosis factor (TNFα). Subsequent studies centered on the compound 3,5,6,7,8,3′,4′-heptamethoxyflavone (HMF) which produced the highest inhibition ($IC_{50} = 5\ \mu M$). HMF was also a potent inhibitor of macrophage inflammatory protein-1α (MIP-1α) and interleukin-10 (IL-10) production, but not of IL-1β, IL-6, or IL-8 production. Suppression of TNFα production was at the level of mRNA induction as determined by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). HMF was also a potent inhibitor of human phosphodiesterase activity and was shown to induce a substantial elevation of cAMP levels in monocytes. The similarity of these results to the inhibition profile of the known phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, suggests that the polymethoxylated flavones inhibit cytokine production in part by suppression of phosphodiesterase activity. The ability of HMF to also inhibit IL-10 production suggests the additional existence of a phosphodiesterase-independent mechanism for this compound.

Flavonoids are widely distributed in the plant kingdom, and as many as 4000 flavonoid-related compounds have been described.[1] Ingested at hundreds of mg per day in the Western diet, flavonoids appear to have several beneficial effects on human health. Of particular interest are the flavonoids derived from citrus, many of which exhibit anticancer, anticarcinogenic, antiviral, antioxidant, antithrombogenic, and antiatherogenic properties.[2] Polymethoxylated flavones have been shown to reduce the invasiveness of tumors in animal models[3] and to induce the differentiation of myeloid leukemic cells and suppress proliferation while promoting apoptosis.[4,5] Polymethoxylated flavones have further been shown to reduce lymphocyte proliferation[6] and platelet aggregation[7] and to suppress ethanol-induced gastric hemorrhagic lesions[8] while promoting chloride secretion by human colonic epithelial cells.[9] Recently, the antiinflammatory properties of these dietary flavonoids and others have received considerable attention. Select hydroxylated flavones block adhesion molecule biosynthesis by cytokine-induced endothelial cells.[10,11] In other studies, flavonoids have been shown to block activation-induced degranulation of neutrophils and mast cells.[12] Significantly, a formulation of citrus flavonoids has been shown to block capillary leakage and leukocyte infiltration in animal models.[13,14]

Central to many aspects of inflammation are the cytokines that behave as autocrine or paracrine protein factors. In particular, the cytokine, tumor necrosis factor-α (TNFα), drives many critical components of inflammatory processes.[15,16] Monocytes are a major cellular source of TNFα and other cytokines. To further characterize the beneficial pharmacological properties of dietary flavonoids, we undertook an evaluation of citrus flavonoids for the ability to modulate cytokine expression by human monocytes. In this study, several polymethoxylated flavones were found to strongly inhibit bacterial lipopolysaccharide-induced expression of TNFα, whereas flavonoid glycosides were inactive. Hence, this study focuses on the activities of the polymethoxylated flavones and characterizes aspects of their activities pertaining to possible modes of action.

Results and Discussion

An extensive collection of flavonoids from citrus was evaluated for the suppression of TNFα production in cultures of human monocytes. Of these compounds, the polymethoxylated flavones consistently showed the highest inhibition of TNFα production (Table 1). The IC_{50} values for these compounds occurred in a narrow range of 5−30 μM. With the exception of tetra-*O*-methylscutellarein, negligible cytotoxicity was detected with these compounds. The citrus flavanone- and flavone-*O*- and *C*-glycosides, naringin, hesperidin, diosmin, isosakuranetin rutinoside, narirutin, margaretin, isomargaretin, and isovitexin, were inactive as inhibitors ($IC_{50} > 200\ \mu M$) and showed no cytotoxicity (data not shown). A number of polyhydroxylated flavone aglycons were inhibitory toward TNFα production but were also significantly cytotoxic toward cultured human monocytes as measured by depletion of cellular LDH activity (Table 1). Significantly, other polyhydroxylated flavone aglycons, including myricetin, hesperetin, fisetin, chrysin, epicatechin, baicalein, galagin, 7-hydroxyflavone, 3-hydroxyflavone, catechin, flavanone, 4,5,6-trihydroxyflavone, 3,4,7-trihydroxyflavone, eriodictyol, gossypetin, and robinin, lacked activity and any associated cytotoxicity (data not shown).

The inhibition of cytokine production by the polymethoxylated flavones was further characterized by determining the ability of 3,5,6,7,8,3′,4′-heptamethoxyflavone (HMF) to inhibit the production of the other main monocyte-derived cytokines, including IL-1β, IL-6, IL-8, IL-10, and MIP-1α. The ability of HMF ($IC_{50,TNF\alpha} = 5\ \mu M$) to modulate the production of these other cytokines was evaluated in monocytes from three independent human

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10.1021/np980431j CCC: $18.00
Published on Web 03/04/1999

Table 1. Results of Flavonoid-Mediated Inhibition of TNFα Production

compound	IC_{50},[a] μM
polymethoxylated flavones	
5,6,7,4′-tetramethoxyflavone (tetra-*O*-methylscutellarein)	22[b]
5,6,7,8,4′-pentamethoxyflavone (tangeretin)	30
3,5,6,7,8,3′,4′-heptamethoxyflavone	5
5,6,7,8,3′,4′-hexamethoxyflavone (nobiletin)	10
5,6,7,3′,4′ -pentamethoxyflavone (sinensetin)	8
5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone	5[c]
5,7,8,3′,4′-pentamethoxyflavone	16
7-hydroxy-3,5,6,7,3′,4′-hexamethoxyflavone	22
5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone	10
5,6,7,3′,4′,5′-hexamethoxyflavone	20
5,6,3′,4′-tetramethoxyflavone	25
hydroxyflavones and miscellaneous	
3-*O*-methylquercetin	47[c]
quercetin	120[b,c]
tamaraxetin	70[b,d]
apigenin	20[b,c]
kaempherol	20[b,c]
flavone	166[d]
limocitrin	67[b,c]
chrysoeriol	22[b,c]
rhamnetin	22[b]
acacetin	22[b,c]

[a] Effect of compounds on monocyte production of TNFα was determined as described in Experimental Section. [b] Inhibition was also associated with >20% cytotoxicity as measured by depletion of cellular LDH activity. [c] Occurs in citrus as glycosides. [d] Does not occur in citrus, but was used to confirm the generality of the results.

donors. These studies showed that HMF was a potent inhibitor of the induced expression of not only TNFα but also IL-10 and MIP-1α (Figure 1). IC_{50} values for HMF inhibition of IL-10 and MIP-1α were 12.3 ± 3.5 and 7.3 ± 0.7 μM, respectively. In contrast, there was no inhibition at 100 μM HMF of the lipopolysaccharide-induced expression of IL-1β, IL-6, or IL-8. In fact, the expression of IL-6 was increased 30% in the presence of 100 μM HMF.

To provide insight into how HMF blocks TNFα production, monocytes were activated with bacterial lipopolysaccharides in the presence or absence of HMF, and TNFα mRNA was quantified by RT-PCR. In these experiments, mRNA encoding the constitutively expressed gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was also measured to serve as an internal standard. The ability of HMF to suppress TNF protein production correlated with the ability of HMF to suppress lipopolysaccharide-induced TNFα mRNA expression (Figure 2). Significantly, the induction of IL-1β mRNA was not effected (data not shown), consistent with the lack of effect of this compound on the production of IL-1β protein.

Flavones have been previously shown to inhibit phosphodiesterase,[17−20] the enzyme that catalyzes the hydrolysis of cyclic adenosine monophosphate (cAMP). Because phosphodiesterase inhibitors are also known to suppress TNFα production,[21] we sought to determine if the ability of HMF to suppress TNFα production correlated with an ability to inhibit phosphodiesterase. HMF was compared with flavone and myricetin, which are much less active at inhibiting TNFα production. Graded concentrations of HMF, flavone, or myricetin were added to enzyme reaction mixtures containing cAMP and type-IV phosphodiesterase semipurified from a monocyte cell line. Hydrolysis of cAMP was used to measure phosphodiesterase activity (Figure 3). Phosphodiesterase activity was inhibited 50% by 2, 11, and 16 μM with HMF, flavone, and myricetin, respectively. The order of potency was consistent with the superior

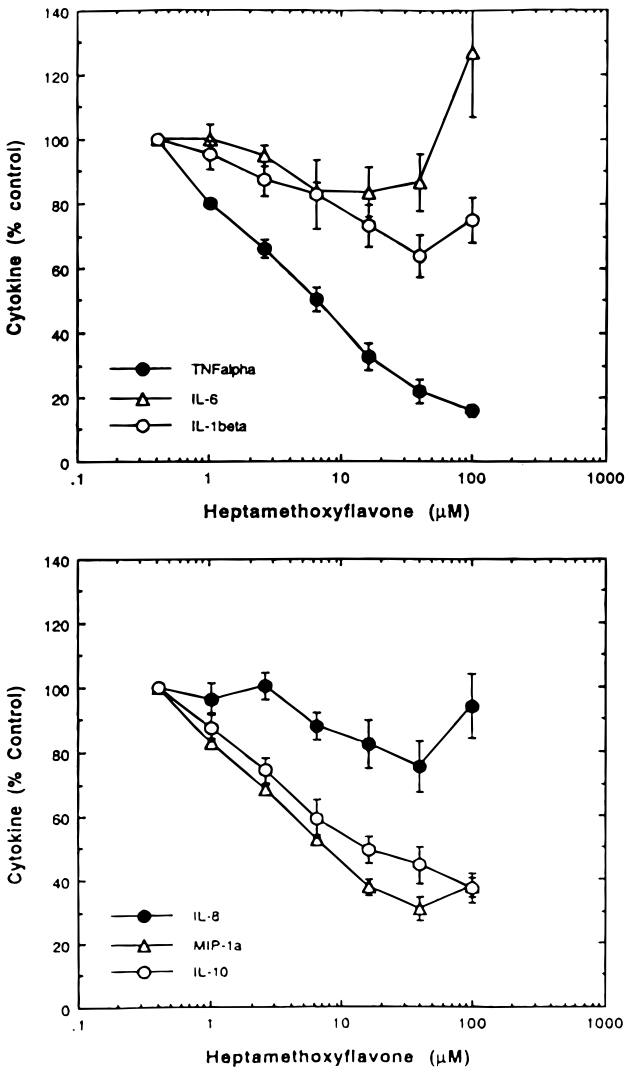

Figure 1. Effect of HMF on cytokine expression by LPS-stimulated monocytes. Adherent purified human monocytes were cultured in the presence of graded concentrations of heptamethoxyflavone for 30 min. The monocyte cultures were then adjusted to contain 20 ng/mL LPS. After 14 h of culture, TNFα, IL-6, IL-8, MIP-1α, and IL-10 were assayed in the culture supernatants and IL-1β was assayed in the cell lysates using specific ELISA assays as described in the Experimental Section. Values expressed as the percent of cytokine produced in flavone-free cultures are the means and standard errors determined using monocytes isolated from three independent donors.

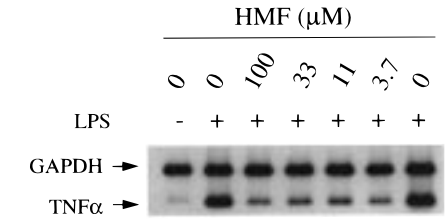

Figure 2. HMF selectively blocks expression of TNF mRNA. Primary elutriated human monocytes were cultured in the presence of graded concentrations of HMF for 30 min. The monocyte cultures were then adjusted to contain 20 ng/mL of LPS. At 90 min of culture, monocytes were harvested by centrifugation, and RNA was isolated from the cell pellet. Relative levels of TNFα and GAPDH mRNA were determined by RT-PCR as described in the Experimental Section.

ability of HMF to inhibit TNFα production. The HMF inhibition of monocyte phosphodiesterase was further demonstrated by the elevated cytosolic cAMP that occurred after treating the activated human monocytes for 30 min in the presence of 100 μM HMF, flavone, or myricetin. The

Table 2. Effect of 3,5,6,7,8,3′,4′-Heptamethoxyflavone and 3-Isobutyl-1-methylxanthine on LPS-Induced Cytokine Production in Human Monocytes[a]

cytokine	control	LPS	LPS+HMF	LPS+IBMX
TNFα	0.09 ± 0.02	59.9 ± 7.1	9.7 ± 2.2[c]	10.9 ± 2.1[c]
IL-1β	1.30 ± 0.66	87.5 ± 45.2	62.9 ± 45.2	99.1 ± 46.1
IL-6	0.18 ± 0.03	79.9 ± 29.1	90.0 ± 27.0	79.7 ± 26.5
IL-8	27.6 ± 8.37	350 ± 112	307 ± 81.2	335 ± 98
IL-10	0.007 ± 0.004	0.705 ± 0.090	0.269 ± 0.063[c]	0.651 ± 0.056
MIP-1α	1.83 ± 2.65	165 ± 39	49.2 ± 11.1[c]	33.2 ± 5.99[c]

[a] Values are ng/mL of cytokine protein and represent the mean and standard error values from analysis of PBMCs from three different human donors. [b] PBMC were cultured 14 h in the presence of media alone (control), 20 ng/mL of LPS alone (LPS), or in the presence of LPS and 100 μM HMF or IBMX. Culture supernatants were assayed by ELISA for TNFα, IL-6, IL-8, IL-10, and MIP-1α and cell lysates were assayed for IL-1β. [c] Indicates different from LPS alone ($p < 0.05$).

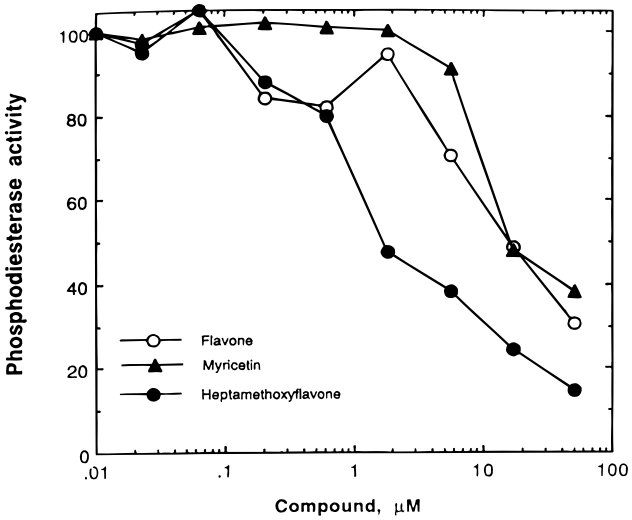

Figure 3. Inhibition of PDE activity by HMF. PDE semipurified from U937 monocytic cells was evaluated for the ability to catalyze the hydrolysis of cAMP in the presence of graded concentrations of flavone, myricetin, and HMF as described in the Experimental Section. The rates are plotted as percent of control-reactions, where no flavonoids were present.

cAMP was subsequently quantitated in cell extracts using a specific ELISA. Other cells were treated with 200 μM of 3-isobutyl-1-methylxanthine (IBMX), a known potent inhibitor of phosphodiesterase that served as a positive control. In cells cultured in media alone the levels of intracellular cAMP were 2.7 ± 0.9 pmol/10^7 cells, whereas in cells cultured in the presence of 100 μM myrecitin, flavone, HMF, or 200 μM IBMX, the levels were 3.7 ± 1.0, 3.6 ± 1.1, 5.7 ± 1.8, and 8.3 ± 1.9 pmol/10^7 cells, respectively. Hence, both HMF and IBMX caused at least a doubling of the monocyte cAMP levels, whereas the increases resulting from the presence of flavone and myricetin were much less.

To further evaluate if phosphodiesterase inhibition represents an important mechanism of action for HMF, the cytokine suppression profile of HMF was compared against the suppression profile of IBMX (Table 2). Both compounds produced a similar reduction in TNFα and MIP-1α, and neither suppressed the production of IL-1β, IL-6, and IL-8. On the basis of these similarities, we conclude that the mode of action of HMF may be similar to that of IBMX. However, distinct from HMF, IBMX did not inhibit IL-10 production. This inhibition of IL-10 production by HMF suggests that there are other yet uncharacterized aspects of the inhibitory properties of HMF toward activated human monocytes. Finally, it is of interest that polymethoxylated flavones have been shown to not inhibit protein kinase C,[22] and they exhibit minimal antioxidant activity.[23] Thus, these mechanisms, widely applicable to other flavonoids,[24] are not involved in our studies.

Experimental Section

General Experimental Procedures. Flavonoids from citrus plant material were the authentic samples reported previously.[25−28] Purity of these citrus and the noncitrus flavonoids obtained from commercial sources were confirmed >95% by HPLC analysis at 220, 285, and 330 nm. Fifty millimolar stocks of flavones were prepared in dimethyl sulfoxide and stored at −80 °C. Phenol−water-extracted *E. coli* K235 lipopolysaccharide was purchased from Sigma (St. Louis, MO).

Isolation of Human Peripheral Blood Monocytes. Peripheral blood mononuclear cells (PBMCs) were isolated from residual source leukocytes (Bonfils Blood Center, CO) by density gradient centrifugation using Ficoll-Paque Plus (Pharmacia, Sweden). Monocytes were purified further by plastic adherence (see below) or by centrifugal elutriation as described previously.[29]

Evaluation of Cytokine Suppressive Properties of Flavonoids. Fetal calf serum (FCS) (30%) in the culture media was optimal for flavonoid solubility and for cytokine expression by monocytes. To measure the impact of the flavones on cytokine production, PBMCs were resuspended to 4 × 10^6/mL in complete medium, i.e., Dulbecco's Modified Eagle Medium containing 30% FCS, 2 mM glutamine, 100 U/mL Penicillin-G, and 100 μg/mL streptomycin. A 100 μL portion of cell suspension was dispensed into each well of FALCON 96-well microtest tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) and cultured for 2 h at 37 °C and 5% CO_2. Nonadherent cells (lymphocytes) were separated from the plastic adherent cells (monocytes) by washing the wells twice with 200 μL of medium. The monocytes were cultured an additional 30 min in 100 μL of complete medium containing graded concentrations of the compound of interest, and the cultures were then adjusted to contain 10 ng/mL LPS to stimulate cytokine production. Culture supernatants were harvested 14 h later, and cells were lysed by addition of 50 μL of Dulbecco's phosphate-buffered saline containing 1% NP40. Culture supernatants were evaluated for TNFα, IL-6, IL-8, IL-10, and MIP-1α content using specific enzyme-linked immunosorbent assays (ELISA) purchased from R&D Systems. IL-1β was measured in cell lysates by ELISA. Lactate dehydrogenase content of cell lysates was measured to determine compound effects on cell viability. To 50 μL of lysate were added 200 μL of 50 mM sodium pyrophosphate buffer (pH 8.9) containing 50 mM lactic acid and 4 mg/mL β-nicotinamide adenine dinucleotide (Sigma). Rates of reduction of nicotinamide adenine dinucleotide were measured at room temperature at 340 nm.

Testing Flavones as Phosphodiesterase Inhibitors and Inducers of Cellular Cyclic Adenosine Monophosphate (cAMP). The phosphodiesterase cAMP SPA enzyme assay (Amersham, Arlington Heights, IL) was used to test the ability of flavones to inhibit cAMP hydrolysis catalyzed by phosphodiesterase. The phosphodiesterase used in this study was semipurified from U937 cells and was determined to be mostly the type-IV isoform on the basis of its sensitivity to selected isoform inhibitors.[30]

To test flavones for the ability to cause an increase in cellular cAMP, monocytes were purified from PBMC by

centrifugal elutriation. Monocytes were resuspended to 5×10^6/mL in complete medium, and 2 mL cell suspensions were dispensed into FALCON 17 × 100 mm polypropylene culture tubes (Becton Dickinson). The cells were precultured 2 h at 37 °C and 5% CO_2 and then cultured an additional 30 min following adjusting culture media to contain 100 μM of the flavone of interest or 200 μM IBMX. Cells were then collected by centrifugation and lysed in 300 μL of ice-cold aqueous solution containing 10 mM HEPES (pH 7.6) and 1% NP40. The lysates were applied to 100 mg columns of Amprep SAX resin (Amersham). The columns were then rinsed with 1 mL of methanol, and the cAMP was eluted in 600 μL of methanol containing 0.01 M HCl. The eluates were chilled on dry ice and dried under vacuum. The Amersham Biotrak cAMP EIA assay was used to measure cAMP content of residues resuspended in 400 μL of assay buffer.

Analysis of Specific mRNA Levels by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Monocytes were purified from PBMC by centrifugal elutriation. Monocytes were resuspended to 5×10^6/mL in complete medium, and 2 mL of cell suspension was dispensed into FALCON 17 × 100 mm polypropylene culture tubes (Becton Dickinson). Cultures were adjusted to contain graded concentrations of HMF and cultured 30 min at 37 °C and 5% CO_2. Cultures were then adjusted to contain 20 ng/mL LPS for an additional 90 min. Culture media was then removed, monocytes were lysed in RNAzol (Teltext Inc., Friendswood, TX), and total RNA was isolated according to the manufacturer's instructions. Relative levels of TNFα and IL-1β mRNA were then measured by RT-PCR as previously described.[29] PCR was carried out in the presence of [α-^{32}P]dCTP. Radiolabeled products were resolved by agarose gel electrophoresis and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For these reactions, the following primer sets were used: GAPDH, (5′) TGA AGG TCG GAG TCA ACG GAT TTG GT; (3′) CAT GTG GGC CAT GAG GTC CAC CAC; TNFα, (5′) ATG AGC ACT GAA AGC ATG ATC; (3′) TCA CAG GGC AAT GAT CCC AAA GTA GAC CTG CCC; IL-1β, (5′) GAC ACA TGG GAT AAC GAG GCT; (3′) ACG CAG GAC AGG TAC AGA TTC.

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NP980431J