

Luteolin Inhibits an Endotoxin-Stimulated Phosphorylation Cascade and Proinflammatory Cytokine Production in Macrophages

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ABSTRACT

Flavonoids are naturally occurring polyphenolic compounds with a wide distribution throughout the plant kingdom. In the present study, we compared the ability of several flavonoids to modulate the production of proinflammatory molecules from lipopolysaccharide (LPS)-stimulated macrophages and investigated their mechanism(s) of action. Pretreatment of RAW 264.7 with luteolin, luteolin-7-glucoside, quercetin, and the isoflavonoid genistein inhibited both the LPS-stimulated TNF- α and interleukin-6 release, whereas eriodictyol and hesperetin only inhibited TNF- α release. From the compounds tested luteolin and quercetin were the most potent in inhibiting cytokine production with an IC₅₀ of less than 1 and 5 μ M for TNF- α release, respectively. To determine the mechanisms by which flavonoids inhibit LPS signaling, we used luteolin and determined its ability to interfere with total protein tyrosine phosphorylation as well as Akt phosphorylation and nuclear factor- κ B activa-

tion. Pretreatment of the cells with luteolin attenuated LPS-induced tyrosine phosphorylation of many discrete proteins. Moreover, luteolin inhibited LPS-induced phosphorylation of Akt. Treatment of macrophages with LPS resulted in increased I κ B- α phosphorylation and reduced the levels of I κ B- α . Pretreatment of cells with luteolin abolished the effects of LPS on I κ B- α . To determine the functional relevance of the phosphorylation events observed with I κ B- α , macrophages were transfected either with a control vector or a vector coding for the luciferase reporter gene under the control of κ B *cis*-acting elements. Incubation of transfected RAW 264.7 cells with LPS increased luciferase activity in a luteolin-sensitive manner. We conclude that luteolin inhibits protein tyrosine phosphorylation, nuclear factor- κ B-mediated gene expression and proinflammatory cytokine production in murine macrophages.

Lipopolysaccharide (LPS) is an outer membrane component of Gram negative bacteria and a potent activator of monocytes and macrophages. LPS triggers the secretion of a variety of inflammatory products, such as tumor necrosis factor- α (TNF- α) (Tracey and Cerami, 1994), interleukin-6 (IL-6) (Akira et al., 1993), as well as excessive amounts of nitric oxide (NO) (Nathan and Xie, 1994), which contribute to the pathophysiology of septic shock. Increased plasma TNF- α levels during endotoxemia and Gram negative sepsis contribute to lethality as suggested by the protective effects afforded by TNF- α -neutralizing antibodies (Tracey et al., 1987). Moreover, mice with targeted disruption of either the TNF- α or the TNF- α receptor gene are resistant in models of sepsis (Pfeffer et al., 1993; Rothe et al., 1993; Pasparakis et al., 1996). In addition, there is evidence suggesting that IL-6

plays an important role in sepsis. Administration of IL-6 to rodents induces an acute phase response that consists of sepsis-like symptoms and high plasma levels of IL-6 negatively correlate with survival (Damas et al., 1992; Chai et al., 1996). More recent observations with IL-6 knockout mice suggest that targeted disruption of the IL-6 gene does not improve the survival rate of neither male nor female mice, but abolishes the fever associated with sepsis (Leon et al., 1998). LPS-treated rodents and humans with sepsis exhibit increased plasma levels of nitrite/nitrate due to the expression of the inducible isoform of NOS (Nathan and Xie, 1994). It still remains controversial whether inhibition of the production of NO has beneficial effects with regard to survival. However, studies with pharmacological inhibitors and antisense oligonucleotides suggest that inhibition of iNOS improves the responsiveness of the vasculature to vasoconstrictor agents (Szabo et al., 1994; Hoque et al., 1998).

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ABBREVIATIONS: LPS, lipopolysaccharide; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; NO, nitric oxide; iNOS, inducible nitric-oxide synthase; NF- κ B, nuclear factor- κ B; EtOH, ethanol; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; TTBS, Tween 20 in Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; Ab, antibody.

Production and release of inflammatory cytokines by LPS depends on inducible gene expression mediated by the activation of transcription factors. The transcription factor nuclear factor- κ B (NF- κ B) has been suggested to play a key role in these reactions (Baeuerle and Henkel, 1994; Baeuerle and Baltimore, 1996). Under quiescent conditions NF- κ B is sequestered in the cytosol bound to the inhibitory protein I κ B (Baeuerle and Baltimore, 1996; Israel, 2000). Exposure of cells to LPS triggers phosphorylation cascades that ultimately lead to phosphorylation and degradation of I κ B. Once I κ B dissociates from the complex, NF- κ B translocates into the nucleus where binding to specific DNA motifs in the promoter region occurs, leading to increased gene transcription.

Flavonoids are found in numerous plants and vegetables and their average daily consumption in Western diet is estimated to be 1 g (Kuhnau, 1976). This class of compounds numbers more than 4000 members and can be divided into five subcategories: flavones, flavanols, flavanones, flavonols, and anthocyanidines. Flavonoids possess antioxidant, anti-tumor, antiangiogenic, anti-inflammatory, antiallergic, and antiviral properties (Formica and Regelson, 1995; Fotsis et al., 1997; Wang et al., 1998). In addition, flavonoids inhibit tyrosine (Graziani et al., 1983; Cunningham et al., 1992) and serine kinases (Ferriola et al., 1989) by competing with ATP binding (Graziani et al., 1983). Agents with tyrosine kinase-blocking activity (such as the tyrophostins) inhibit both LPS-stimulated TNF- α production and LPS-induced lethality in mice (Novogrodsky et al., 1994). Indeed, two groups have reported on the ability of quercetin and resveratrol to inhibit LPS-induced TNF- α production (Kawada et al., 1998; Wadsworth and Koop, 1999). Based on these observations we compared the activities of a number of flavonoids on LPS-induced production of proinflammatory cytokines and investigated the mechanism of action for the most potent of these compounds.

Materials and Methods

Reagents and Cell Culture. Quercetin, genistein, myricetin, chrysin, luteolin-7-glucoside, luteolin, hesperetin, and eriodictyol were obtained from Roth Chemicalien (Karlsruhe, Germany). Flavonoids were dissolved in EtOH:DMSO (1:1, v/v) at 10 mM stock solutions. TNF- α enzyme-linked immunosorbent assay kits were from R&D Systems (Minneapolis, MN). Tissue culture plates were from Nalge Nunc International (Rochester, NY). Bradford protein dye reagent was from Bio-Rad (Muenchen, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, antibiotics, trypan blue, and LipofectAMINE were obtained from Life Technologies (Paisley, UK). The luciferase reporter gene assay kit was purchased from Boehringer-Mannheim Biochemica (Mannheim, Germany), the pNF- κ B and pTAL were obtained CLONTECH (Palo Alto, CA), nitrocellulose membrane was obtained from Bio-Rad (Hercules, CA), and enhanced chemiluminescence Western blotting analysis system from Amersham Life Science (Buckinghamshire, UK). The phosphospecific antibodies for Akt and I κ B- α , as well as the Akt and I κ B- α were from New England Biolabs (Beverly, MA). All other reagents, including LPS (*Escherichia coli* 026:B6) and the anti-phosphotyrosine antibody PT-66 were obtained from Sigma Chemical Co. (St. Louis, MO)

RAW 264.7 cells were cultured in low-glucose DMEM containing 10% fetal bovine serum supplemented with penicillin and streptomycin, at 37°C in a humidified incubator with 5% CO₂. Cells used for the nitrite assay were cultured in phenol-free DMEM.

Cytokine Measurements. RAW 264.7 cells were cultured for 2 days in 24-multiwell clusters until they reached 90 to 100% confluence and then incubated with LPS with or without pretreatment with a flavonoid. After 24 h supernatants were collected and centrifuged for 10 min in 3000 rpm in a tabletop microcentrifuge to remove nonadherent cells. After centrifugation, pellets were discarded and supernatants used for enzyme-linked immunosorbent assay in accordance to the manufacturer's instructions. RAW 264.7 cell monolayers in the multiwell plates were lysed with 1 N NaOH. Protein amounts per well were determined by the Bradford method and used to normalize the values obtained for cytokine release.

Nitrite Release. After a 24-h incubation with either LPS, or LPS in the presence of a flavonoid, supernatants were removed from the cultures. Nitrite concentration was determined by the Griess reaction. Briefly, phenol red free media were combined with an equal volume of the Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid). Optical density was measured at 550 nm using a multiwell plate reader (Lamda E; MWG Biotec, Ebersberg, Germany). A standard solution of sodium nitrite prepared in culture medium was used for this assay.

Transfections. RAW 264.7 cells were plated in six-well plates at a density of $2 \times 10^4/\text{cm}^2$ and allowed to reach 40 to 60% confluence. Cells were transfected with vector alone (pTAL) or plasmid containing the luciferase coding sequence under the control of a NF- κ B promoter (pNF- κ B). To normalize for transfection efficiency, the simian virus 40 driven lacZ gene was cotransfected with either pTAL or pNF- κ B. Transfections were performed using LipofectAMINE at a DNA/lipid of 2 μ g of plasmid DNA/3 of μ l lipid. After 24 h, cells were lysed and assayed for luciferase activity. β -Galactosidase activity was measured from different aliquots of the same lysates.

Western Blotting. RAW 264.7 cells were cultured in six-well plates, pretreated, and lysed in lysis buffer (1% Nonidet P-40, 50 mM NaCl, 0.1% SDS, 50 mM NaF, 1 mM Na₃VO₄, 50 mM Tris-HCl, 0.1 mM EGTA, 0.5% deoxycholic acid, 1 mM EDTA, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were rocked for 30 min at 4°C followed by a brief centrifugation at 14,000 rpm. Sample aliquots (35 μ g/lane) were electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane at 20 V overnight at 4°C in buffer containing 25 mM Tris and 700 mM glycine. Membranes were subsequently incubated 2 h at room temperature with 5% dry milk in buffer containing 0.1% (v/v) Tween 20 in Tris-buffered saline (TTBS) to block nonspecific binding. The following day, membranes were incubated with the primary antibody in TTBS, containing 1% milk for 2 h at room temperature, and then washed three times with TTBS for 20 min each time. Finally, membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody and washed again two times with TTBS and once with Tris-buffered saline. Immunoreactive protein bands were visualized using the enhanced chemiluminescence system.

Data Analysis and Statistics. Data are presented as means \pm S.E.M. of the indicated number of observations. Cytokine and nitrite values are expressed as nanograms per milligram of protein per 15 min or as percentage of the control value. Statistical comparisons between groups were performed using the one-way ANOVA followed by the Dunnett's or Newman-Keuls post hoc test or Student's *t* test, as appropriate. Differences among means were considered significant when $p < 0.05$.

Results

Flavonoids Inhibit TNF- α Release by Endotoxin-Activated Macrophages in Culture. RAW 264.7 cells constitutively release low levels of TNF- α (1.02 ± 0.24 ng/mg of protein/24 h). TNF- α production over a 24-h period from murine macrophages in response to increasing LPS concentrations yielded a bell-shaped curve with 10 ng/ml LPS, giv-

ing a peak of 164 ± 19 ng of TNF- α /mg of protein (data not shown). To investigate the effects of flavonoids on the LPS-induced TNF- α release, cultured mouse macrophages were pretreated with flavonoids (50 or 10 μ M) 30 min before the 24 h exposure to LPS (10 ng/ml). Myricetin and catechin showed no effect on LPS-induced TNF- α release, whereas hesperetin, luteolin-7-glucoside, and eriodictyol reduced TNF- α release approximately by 50%. Genistein, an isoflavonoid known to block LPS signaling, effectively inhibited 75% of LPS-induced TNF- α release. Quercetin and luteolin were the two most efficacious inhibitors, allowing only for minimal LPS-induced TNF- α release (Fig. 1A). Although most flavonoids were used at 50 μ M, chrysin and luteolin showed toxicity at this concentration; lower concentrations of 10 μ M were used to determine their potential to inhibit LPS-induced TNF- α release. Cell viability was greater than 90% in all treatment groups, as assessed by trypan blue exclusion (data not shown). Dose-response curves for genistein, quercetin, and luteolin showed an IC_{50} of 5, 1, and less than 1 μ M, respectively (Fig. 1B).

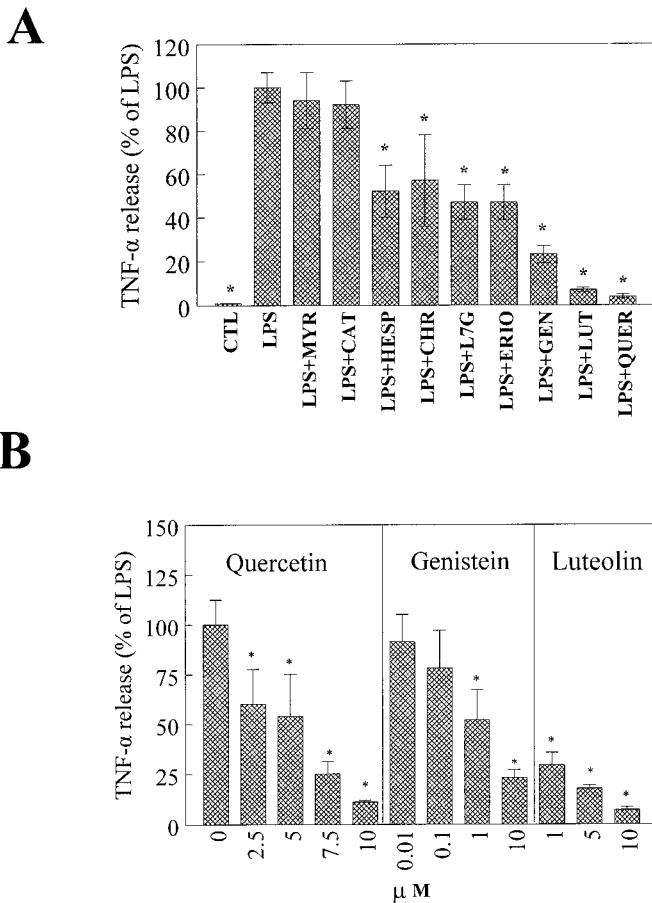


Fig. 1. A, effects of flavonoids on LPS-induced TNF- α release from mouse macrophages. Cells were pretreated for 30 min with vehicle (DMSO: EtOH; 1:1, v/v) or a flavonoid (10 or 50 μ M). At the end of pretreatment, macrophages were incubated with LPS (10 ng/ml) for 24 h and media collected and analyzed as described under *Materials and Methods*. Myricetin (myr), catechin (cat), hesperetin (hesp), luteolin-7-glucoside (L7G), eriodictyol (erio), genistein (gen), and quercetin (quer) were used at 50 μ M, whereas luteolin (lut) and chrysin (chr) were used at 10 μ M. Data are presented as means \pm S.E.M., $n = 6$ to 12; * $p < 0.05$ from LPS. B, cells were pretreated with the indicated concentration of the flavonoid for 30 min and then incubated with 10 ng/ml LPS for 24 h. Samples were analyzed as described under *Materials and Methods*. Data are presented as means \pm S.E.M., $n = 3$ to 4; * $p < 0.05$ from LPS.

To determine whether the flavonoids were able to inhibit LPS-induced TNF- α production if administered after the LPS challenge, we performed a time course experiment where quercetin or luteolin were added at different times relative to the LPS challenge (LPS addition was done at time zero). Quercetin and luteolin were both effective in blocking LPS-induced TNF- α release even if administered up to 90 or 120 min after LPS (Fig. 2).

Effects of Flavonoids on LPS-Induced IL-6 Release. To determine whether flavonoids were capable of inhibiting the release of other proinflammatory cytokines in addition to TNF- α , experiments similar to those performed for TNF- α were performed for IL-6. Quercetin, luteolin, and the isoflavonoid genistein were most effective in inhibiting IL-6 production, with luteolin-7-glucoside exhibiting a less pronounced inhibitory action and eriodictyol having no effect on IL-6 production (Fig. 3).

Effect of Luteolin and Quercetin on Nitrite Production. Nitrite released from LPS-treated cells increased in a time-dependent manner, reaching 168 ± 18.58 nmol/mg of protein at 24 h. The amount of LPS that yielded maximal nitrite release was greater (500 ng/ml) than that required for optimal TNF- α production (data not shown). To study the effect of quercetin and luteolin on nitrite production, cells were pretreated with luteolin or quercetin for 30 min and then exposed to 10 ng/ml LPS for 24 h. Under these conditions, quercetin and luteolin abolished LPS-induced nitrite release (Fig. 4). Similarly to what was observed with the TNF- α release, quercetin was able to inhibit LPS-stimulated nitrite production even when added after the addition of LPS (data not shown).

Effects of Luteolin on LPS-Induced Tyrosine and Akt Phosphorylation. To study the mechanism of action of flavonoids we tested the ability of luteolin, the most potent of the flavonoids used, to inhibit tyrosine phosphorylation. Exposure of RAW 264.7 cells to LPS led to a time-dependent increase in tyrosine phosphorylation that peaked at 20 min (Fig. 5). Pretreatment of the cells with luteolin attenuated LPS-induced tyrosine phosphorylation of many discrete proteins covering a molecular mass size from 40 to 120 kDa, as depicted in Fig. 7B. The action of luteolin on tyrosine phosphorylation was comparable to that of genistein, a known tyrosine kinase inhibitor. In addition, exposure of macro-

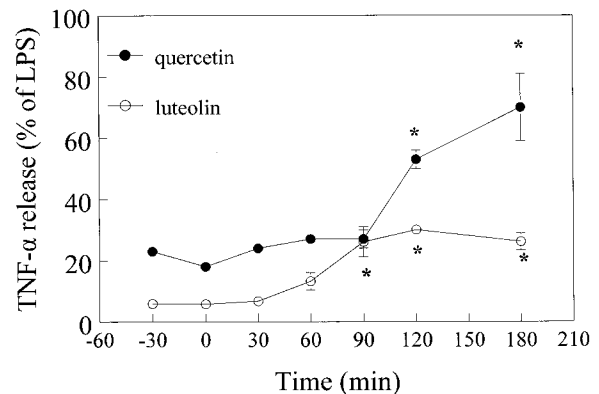


Fig. 2. Time dependence of flavonoids' action on LPS-induced TNF- α release. Time on the x-axis refers to the time of quercetin or luteolin addition relative to LPS. Cells were treated with quercetin or luteolin (10 μ M) and LPS (10 ng/ml). Data are presented as means \pm S.E.M., $n = 4$; * $p < 0.05$ from -30 min.

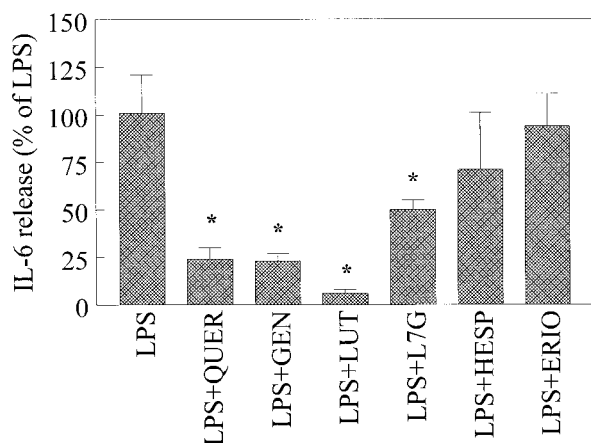


Fig. 3. Effects of flavonoids on LPS-induced IL-6 release from murine macrophages. Cells were pretreated for 30 min with vehicle (DMSO: EtOH; 1:1, v/v) or a flavonoid (10 or 50 μ M). At the end of pretreatment, macrophages were incubated with LPS (10 ng/ml) for 24 h and media collected and analyzed for IL-6 as described under *Materials and Methods*. Hesperetin (hesp), luteolin-7-glucoside (L7G), eriodictyol (erio), genistein (gen), and quercetin (quer) were used at 50 μ M, whereas luteolin (lut) was used at 10 μ M. Data are presented as means \pm S.E.M., $n = 4$ to 8; * $p < 0.05$ from LPS.

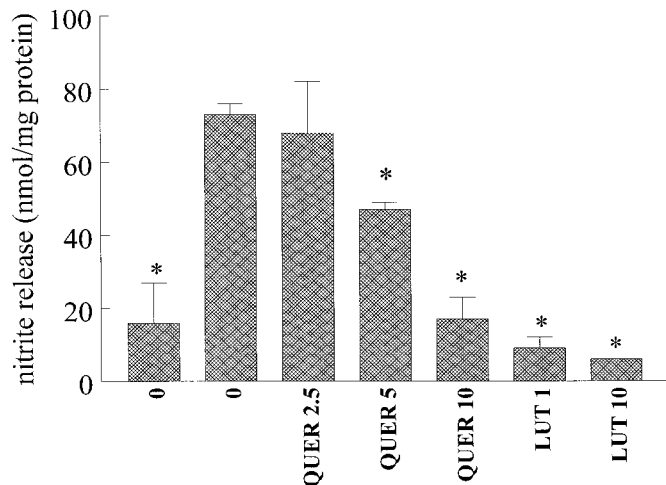


Fig. 4. Quercetin and luteolin inhibit nitrite accumulation. Cells were pretreated with the indicated amount of each compound for 30 min before being exposed to LPS (10 ng/ml). Cell culture supernatants were collected and nitrite concentration was measured using the Griess reaction. Data are presented as means \pm S.E.M., $n = 4$; * $p < 0.05$ from LPS.

phages to LPS for 20 min increased Akt phosphorylation on Ser 473, without altering total Akt levels. This effect was abolished by pretreatment with luteolin (Fig. 6).

Effects of Luteolin on NF- κ B-Mediated Promoter Activity. Activation of NF- κ B is thought to play a key role in the LPS-induced stimulated release of TNF- α , IL-6, and NO. To determine whether luteolin affects NF- κ B activation, RAW 264.7 cells were treated with LPS for 20 min and phosphorylation of the inhibitory protein I κ B- α was examined. Endotoxin increased I κ B- α phosphorylation (Fig. 7A), leading to a reduction in I κ B- α levels. Pretreatment of the cells with luteolin abolished the effects of LPS on I κ B- α . To investigate whether luteolin is able to attenuate LPS-induced NF- κ B-mediated promoter activity, we used a luciferase reporter gene expressed under the control of six κ B cis-acting elements. Incubation of transfected RAW 264.7 cells with LPS (10 ng/ml) for 24 h increased luciferase activ-

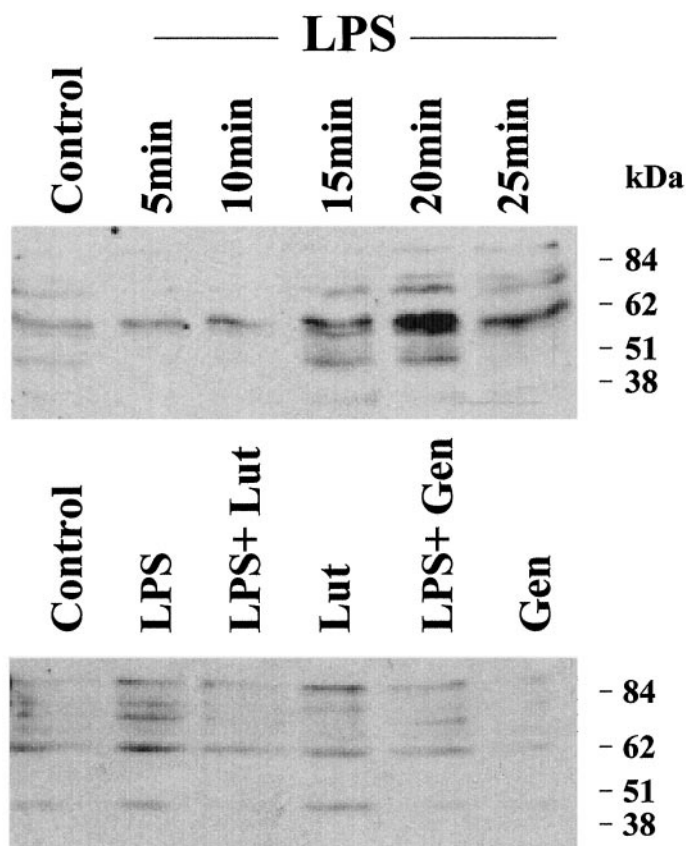


Fig. 5. Luteolin inhibits LPS-induced tyrosine phosphorylation. RAW 264.7 cells were incubated with LPS (10 ng/ml) for the indicated time. Total cell lysates were processed by SDS-PAGE and membranes blotted with an anti-phosphotyrosine Ab. Cells were pretreated with 10 μ M luteolin or 10 μ M genistein, and then exposed to LPS (10 ng/ml; 20 min) and processed as in the top panel. Equal loading (25 μ g of protein/lane) was confirmed by Poinseaux staining.

ity in a luteolin-sensitive manner (Fig. 7B), indicating that inhibition of proinflammatory cytokine expression correlates with decreased NF- κ B-stimulated promoter activity.

Discussion

Macrophages participate in host defense and are main targets for the action of LPS. To identify flavonoids that can interfere with LPS signaling and reduce the production of proinflammatory molecules, we used the macrophage cell line RAW 264.7. From the wide range of flavonoids tested myricetin and catechin showed no effect on LPS-induced TNF- α release. Similar findings for catechin have been reported as this flavan-3-ol failed to inhibit iNOS expression in LPS-treated RAW 264.7 cells and showed no effect on proliferation of human fibroblasts and keratinocytes (Fotsis et al., 1997; Kim et al., 1999). On the other hand, quercetin and luteolin were very effective in reducing the action of LPS on TNF- α release, blocking it by more than 80%. Flavonoid aglycones consist of a benzene ring (A), fused with a six-membered ring (C) that at position 2 carries a phenyl ring (B) (Table 1). Our results show that the presence of a double bond at position C2-C3 of the C ring with oxo function at position 4, along with the presence of OH groups at positions 3' and 4' of the B ring are required for optimal inhibition of LPS-stimulated TNF- α release. Chrysin, lacking OH groups

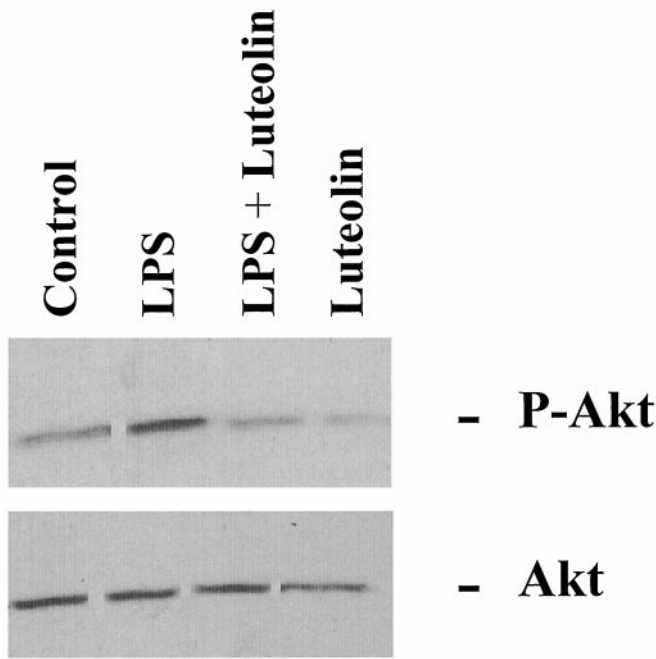


Fig. 6. Luteolin inhibits LPS-induced Akt phosphorylation. Top, cells were serum-starved overnight, pretreated with 10 μ M luteolin for 30 min, and then exposed to LPS (10 ng/ml) for 20 min before lysis. Total cell lysates were processed by SDS-PAGE and membranes blotted with an antiphosphospecific Akt Ab. Bottom, cells were treated as described in the top panel and lysed. Total cell lysates were processed by SDS-PAGE and membranes blotted with an anti-Akt Ab.

at positions 3' and 4' of the B ring, as well as eriodictyol, lacking a double bond at position C2-C3 of the C ring, were much less potent in blocking LPS-induced TNF- α production in macrophages. Addition of an OH group at position 5' of the B ring (myricetin, catechin) and elimination of the oxo group at position 4 (catechin) abolishes the biological activity. In the case of luteolin, the aglycone is more potent than the glucoside conjugate (L7G), possibly indicating that increase in water solubility attenuates the activity of the compounds.

To test whether flavonoids are able to selectively inhibit production of different proinflammatory molecules we tested the effect of some of these compounds on IL-6 and NO production. Hesperetin and eriodictyol, both lacking the double bond at position C2-C3 of the C ring, were ineffective in blocking the release of this cytokine, whereas quercetin, luteolin, and luteolin-7-glucoside inhibited IL-6 production after exposure to LPS. Our data are in line with the data of Gerritsen et al. (1995) who showed that apigenin inhibits TNF- α -stimulated IL-6 release from vascular endothelial cells. To further characterize the effects of luteolin and quercetin on proinflammatory molecule expression we tested the ability of these two flavonoids to inhibit nitrite accumulation in LPS-treated cells. Both flavonoids inhibited iNOS-mediated NO release in a concentration-dependent manner in the same concentration range observed for TNF- α release. Our results confirm previous findings showing that quercetin and luteolin are effective in blocking LPS-induced NO production (Kim et al., 1999). The difference in potency observed (higher concentrations of the flavonoids are required to inhibit NO release in the aforementioned studies) possibly reflects different culture conditions and different clonal populations of the macrophage cell line.

We chose to further investigate the mechanism of action of

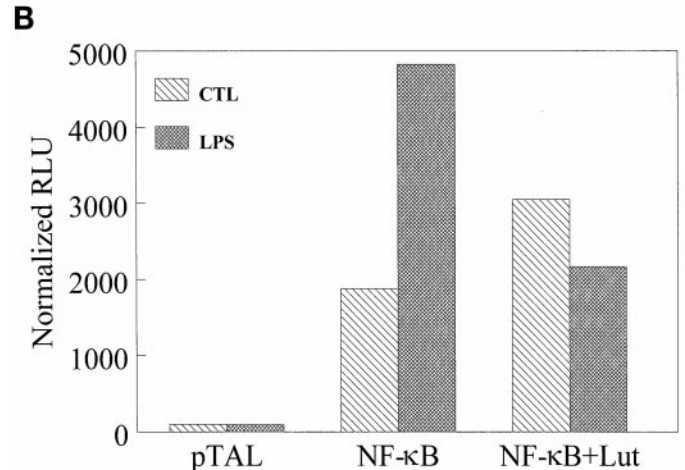
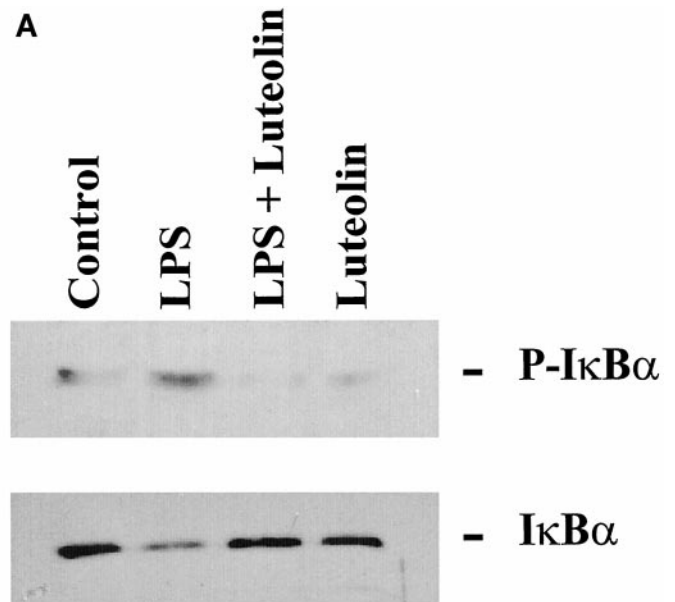
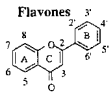





Fig. 7. Luteolin inhibits LPS-induced I κ B- α degradation and NF- κ B-mediated promoter activity. A, cells were serum-starved overnight. They were pretreated with 10 μ M luteolin for 30 min, and then exposed to LPS (10 ng/ml; 20 min) and lysed. Total cell lysates were processed by SDS-PAGE and membranes blotted with an antiphosphospecific I κ B- α Ab. Bottom, cells were treated as described in the top panel and lysed. Total cell lysates were processed by SDS-PAGE and membranes blotted with an anti-I κ B- α Ab. B, RAW 264.7 cells were cotransfected with a control plasmid (pTAL) or a plasmid containing the luciferase gene under the control of a NF- κ B promoter and a plasmid coding for the lacZ gene to normalize for transfection efficiency. Twenty-four hours after the initiation of transfection, cells were pretreated with vehicle or 10 μ M luteolin and 30 min later challenged with LPS (10 ng/ml, 24 h). Cells were then harvested and luciferase and β -galactosidase activities measured. Data are representative of observations made in one of the two independent experiments performed with similar results.

luteolin because it is the most potent inhibitor of LPS-induced TNF- α release in RAW 264.7 and very little is known about its molecular mechanism of action. Luteolin has been shown to inhibit neutral endopeptidase, xanthine/xanthine oxidase, epidermal growth factor receptor kinase activity, and autophosphorylation and to bind adenosine receptors (Huang et al., 1999; Nagao et al., 1999; Bormann and Melzig, 2000; Ingkaninan et al., 2000). LPS signaling in macrophages involves a series of phosphorylation events leading to

TABLE 1
Substances tested

Chemical Formula	Name	Substitution					
		5	7	2'	3'	4'	5'
	Crysin	OH	OH	H	H	H	H
	Luteolin	OH	OH	H	OH	OH	H
	Luteolin-7-glucoside	OH	OGLC	H	OH	OH	H
	Hesperetin	OH	OH	H	OH	OCH ₃	H
	Eriodictyol	OH	OH	H	OH	OH	H
	Quercetin	OH	OH	H	OH	OH	H
	Myricetin	OH	OH	H	OH	OH	OH
							

transcription factor activation and cytokine production. Some of the proteins involved in LPS signaling include members of the Src-family tyrosine kinases, as well as the serine/threonine kinases protein kinase A and C, mitogen-activated protein kinase, and protein kinase B/Akt (Boulet et al., 1992; Han et al., 1994; Shapira et al., 1994; Hambleton et al., 1996; Salh et al., 1998). Exposure of RAW 264.7 to LPS led to a time-dependent phosphorylation of tyrosine residues of several proteins that was inhibited by luteolin. These results are in agreement with previously published data on the inhibitory effects of quercetin and other flavonoids on both receptor and nonreceptor tyrosine kinases (Graziani et al., 1983; Cunningham et al., 1992; Huang et al., 1999). Moreover, it seems unlikely that the inhibitory action of luteolin on proinflammatory cytokine production is the result of antioxidant properties, but rather relates to its ability to restrict protein phosphorylation. This is based on the observation that myricetin and catechin, both strong protectors against oxygen-induced DNA strand breakage (Devasagayam et al., 1996), were completely ineffective in reducing LPS-stimulated TNF- α production.

In addition to their effects on protein tyrosine phosphorylation, flavonoids inhibit lipid and serine/threonine kinases, such as phosphatidylinositol 3-kinase and protein kinase C (Gamet-Payraastre et al., 1999). A pathway that links phosphatidylinositol 3-kinase with NF- κ B is mediated through activation of the serine/threonine kinase Akt (Ozes et al., 1999). Activation of Akt phosphorylates I κ B kinase- α at threonine 23, which in turn phosphorylates I κ B- α on serine 32 and 36, leading to degradation of the latter and dissociation of NF- κ B from the inhibitory complex, allowing NF- κ B to translocate into the nucleus (Israel, 2000). Exposure of the RAW 264.7 to LPS stimulated Akt phosphorylation on Ser 478. Pretreatment of macrophages with luteolin abolished the LPS-induced phosphorylation of Akt. In addition, pretreatment of cells with luteolin abolished the effects of LPS on I κ B- α phosphorylation and degradation. To test whether the inhibitory action of luteolin on I κ B- α correlates with inhibition of promoter activity we tested its ability to inhibit LPS-stimu-

lated promoter activity. In transient transfection experiments, LPS-stimulated luciferase expression through κ B response elements was abolished by pretreatment with luteolin. Wadsworth and Koop (1999) reported that quercetin inhibits LPS-induced activation of the NF- κ B complex in RAW 264.7 cells. Another flavonoid, silymarin, inhibits LPS-, but not hydrogen peroxide-induced activation of NF- κ B in U-937 cells (Manna et al., 1999). Interestingly, Gerritsen et al. (1995) demonstrated that apigenin failed to inhibit nuclear translocation of NF- κ B in endothelial cells, but was nevertheless able to inhibit TNF- α -induced β -galactosidase activity in a cell line stably transfected with a β -galactosidase reporter construct driven by κ B elements.

In summary, we have screened a number of flavonoids and have found that flavonoids such as luteolin, with a double bond at position C2-C3 of the C ring and oxo function at position 4, along with the presence of OH groups at positions 3' and 4' of the B ring, are required for optimal inhibition of LPS-stimulated TNF- α release. Such information might provide the basis for generation of more potent synthetic analogs for future use. The mechanism by which luteolin blocks the LPS-induced proinflammatory gene expression warrants further investigation. Although the inhibitory action of luteolin observed when this agent is used simultaneously with or shortly after LPS might be attributed to its effects on protein tyrosine phosphorylation and suppression of the increased transcriptional activity in response to LPS, inhibition of TNF- α release when luteolin is added much after the LPS challenge might be related to its ability to interfere with post-transcriptional and/or post-translational events. Experiments are underway to further dissect the molecular mechanism of luteolin's action.

Acknowledgment

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