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

ANGELIKI XAGORARI, ANDREAS PAPAPETROPOULOS, ANTONIS MAUROMATIS, MICHALIS ECONOMOU, THEODORE FOTSIS, and CHARIS ROUSSOS

“George P. Livanos” Laboratory, Evangelismos Hospital, Department of Critical Care and Pulmonary Services, University of Athens, Athens, Greece (A.X., A.P., A.M., M.E., C.R.); and Laboratory of Biological Chemistry, Medical School, University of Ioannina, Ioannina, Greece (T.F.)

Received May 31, 2000; accepted August 30, 2000 This paper is available online at http://jpet.aspetjournals.org

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 IC_{50} of less than 1 and 5 μM 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-κB activation. 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 IκB-α phosphorylation and reduced the levels of IκB-α. Pretreatment of cells with luteolin abolished the effects of LPS on IκB-α. To determine the functional relevance of the phosphorylation events observed with IκB-α, macrophages were transfected either with a control vector or a vector coding for the luciferase reporter gene under the control of κB 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-κB-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 (Piffner 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).

ABBREVIATIONS: LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; NO, nitric oxide; iNOS, inducible nitric-oxide synthase; NF-κB, nuclear factor-κB; 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-κB (NF-κB) has been suggested to play a key role in these reactions (Baueule and Henkel, 1994; Baueule and Baltimore, 1996). Under quiescent conditions NF-κB is sequestered in the cytosol bound to the inhibitory protein IκB (Baueule and Baltimore, 1996; Israel, 2000). Exposure of cells to LPS triggers phosphorylation cascades that ultimately lead to phosphorylation and degradation of IκB. Once IκB dissociates from the complex, NF-κB 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 tumor, antiangiogenic, anti-inflammatory, antiallergic, and antiviral properties (Formica and Regelson, 1995; Fotsis et al., 1997; Wang et al., 1998). In addition, flavonoids inhibit the production of inflammatory cytokines by LPS.

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% naphthylendiamine in 5% phosphoric acid). Optical density was measured at 550 nm using a multiwell plate reader (Lamda E; MWG Biotech, Ebersberg, Germany). A standard solution of sodium nitrite prepared in culture medium was used for this assay.

Materials and Methods
Reagents and Cell Culture. Quercetin, genistein, myricetin, chrysirin, 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 reagents, including LPS (Escherichia coli 026:B6) and the anti-phosphospecific antibodies for Akt and IκB were obtained from Amersham Life Science (Buckinghamshire, UK). The phosphospecific antibodies for Akt and IκB-α, as well as the Akt and IκB-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.
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 μM) 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. 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 IC₅₀ of 5, 1, and less than 1 μM, respectively (Fig. 1B).

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. 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 μM). 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 μM, whereas luteolin (lut) and chrysin (chr) were used at 10 μM. Data are presented as means ± 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 ± S.E.M., n = 3 to 4; *p < 0.05 from LPS.

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 μM) and LPS (10 ng/ml). Data are presented as means ± S.E.M., n = 4; *p < 0.05 from –30 min.
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-κB-Mediated Promoter Activity. Activation of NF-κB 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-κB activation, RAW 264.7 cells were treated with LPS for 20 min and phosphorylation of the inhibitory protein IκB-α was examined. Endotoxin increased IκB-α phosphorylation (Fig. 7A), leading to a reduction in IκB-α levels. Pretreatment of the cells with luteolin abolished the effects of LPS on IκB-α. To investigate whether luteolin is able to attenuate LPS-induced NF-κB-mediated promoter activity, we used a luciferase reporter gene expressed under the control of six κB cis-acting elements. Incubation of transfected RAW 264.7 cells with LPS (10 ng/ml) for 24 h increased luciferase activity in a luteolin-sensitive manner (Fig. 7B), indicating that inhibition of proinflammatory cytokine expression correlates with decreased NF-κB-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. Chrysins, lacking OH groups...
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 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 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

**Fig. 6.** Luteolin inhibits LPS-induced Akt phosphorylation. Top, cells were serum-starved overnight, pretreated with 10 μM 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.

**Fig. 7.** Luteolin inhibits LPS-induced IκB-α degradation and NF-κB-mediated promoter activity. A, cells were serum-starved overnight. They were pretreated with 10 μM 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 IκB-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-IκB-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-κB 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 μM 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.
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 NF-κB mRNA expression.

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-Payrastre et al., 1999). A pathway that links phosphatidylinositol 3-kinase with NF-κB (Gamet-Payrastre et al., 1999). Activation of Akt phosphorylates IκB kinase-α at threonine 23, which in turn phosphorylates IκB-α on serine 32 and 36, leading to degradation of the latter and dissociation of NF-κB from the inhibitory complex, allowing NF-κB 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 IκB-α phosphorylation and degradation. To test whether the inhibitory action of luteolin on IκB-α correlates with inhibition of promoter activity we tested its ability to inhibit LPS-stimulated promoter activity. In transient transfection experiments, LPS-stimulated luciferase expression through κB response elements was abolished by pretreatment with luteolin. Wadsworth and Koop (1999) reported that quercetin inhibits LPS-induced activation of the NF-κB complex in RAW 264.7 cells. Another flavonoid, silymarin, inhibits LPS-, but not hydrogen peroxide-induced activation of NF-κB in U-937 cells (Manna et al., 1999). Interestingly, Gerritsen et al. (1995) demonstrated that apigenin failed to inhibit nuclear translocation of NF-κB 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 κB 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**

We acknowledge the expert technical assistance of Athanasia Hatziastasiou.

**References**
