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Quercitrin Gallate Down-regulates Interleukin-6 Expression by Inhibiting Nuclear Factor-kB Activation in Lipopolysaccharide-stimulated Macrophages

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Abstract – Quercitrin gallate was previously isolated from *Persicaria lapathifolia* (Polygonaceae) as an inhibitor of superoxide production. In the present study, quercitrin gallate was found to inhibit interleukin (IL)-6 production in lipopolysaccharide (LPS)-stimulated macrophages RAW 264.7 with an IC_{50} value of 63 μM. Furthermore, quercitrin gallate attenuated LPS-induced synthesis of IL-6 transcript but also inhibited LPS-induced IL-6 promoter activity, indicating that the compound could down-regulate IL-6 expression at the transcription level. Since nuclear factor (NF)-kB has been shown to play a key role in LPS-inducible IL-6 expression, an effect of quercitrin gallate on LPS-induced NF-kB activation was further analyzed. Quercitrin gallate exhibited a dose-dependent inhibitory effect on LPS-induced nuclear translocation of NF-kB without affecting inhibitory kB (IkB) degradation, and subsequently inhibited LPS-induced NF-kB transcriptional activity in macrophages RAW 264.7. Taken together, quercitrin gallate down-regulated LPS-induced IL-6 expression by inhibiting NF-kB activation, which could provide a pharmacological potential of the compound in IL-6-related immune and inflammatory diseases.

Keywords – Quercitrin gallate, IL-6 expression, NF-kB activation, macrophages RAW 264.7

Introduction

Interleukin (IL)-6 was originally identified as a B-cell differentiation factor, but is now known to play an important role in the homeostasis of immune and hematopoietic systems as well as its potent ability to regulate endocrine and metabolic functions (Barton, 1997; Kamimura *et al.*, 2003). Amounts of IL-6 in the circulation are tightly regulated and maintained at a low level under normal conditions, but rapidly increased in inflammatory responses due to infection, injury, trauma or other stress (Kamimura *et al.*, 2003). A high-level production of IL-6 could generate an undesired inflammatory state, a circumstance that can cause various diseases. In fact, several reports indicate that IL-6 is implicated in the pathogenesis of a number of human disorders, including rheumatoid arthritis and inflammatory bowel disease (Wong *et al.*, 2003).

Immune cells including macrophages can recognize microbial infection through their toll-like receptors (TLRs), which plays an important role in the innate immunity (Ulevitch and Tobias, 1995). Lipopolysaccharide (LPS) is a main component of the outer membrane of Gram-negative bacteria and can be recognized by TLR4 on the immune cells (Miyake, 2004). Upon stimulation with LPS, macrophages can trigger signaling pathways to activate nuclear factor (NF)-kB. Under normal conditions, NF-kB is sequestered in the cytoplasm as an inactive complex, bound to inhibitory kB (IkB) protein (Baeuerle and Baltimore, 1988). However, signaling components downstream TLR4 stimulated with LPS can activate IkB kinase (IKK) complex, which results in the phosphorylation followed by proteolytic degradation of IkB (Magnani *et al.*, 2000; Karin and Ben-Neriah, 2000). NF-kB such as p65 or p50 subunit, freed from IkB, translocates into the nucleus, where it binds to the promoter regions of immune and inflammatory genes including IL-6 for transcriptional regulation (Tian and Brasier, 2003).

Quercitrin gallate (Fig. 1) is a naturally-occurring polyphenolic compound. Previously, we isolated the compound from *Persicaria lapathifolia* (Polygonaceae) as an inhibitor of superoxide production, and further reported its anti-complement activity (Park *et al.*, 1999; Kim *et al.*, 2000). In the present study, quercitrin gallate was found to down-regulate IL-6 expression in LPS-stimulated macrophages

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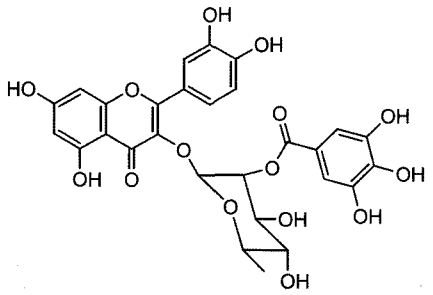

Fig. 1. Chemical structure of quercitrin gallate.

RAW 264.7, and its effect on NF-kB activation was also documented as a mechanism of the action.

Experimental

Materials – Quercitrin gallate (purity > 98%) was isolated from *Persicaria lapathifolia* as described in our previous work (Kim *et al.*, 2000). Fetal bovine serum (FBS) and Lipofectamine were purchased from Invitrogen (Carlsbad, USA). Antibodies against IkBα, NF-kB p65 or p50 subunit were obtained from Santa Cruz Biotech (Santa Cruz, USA). pNF-kB-secretory alkaline phosphatase (SEAP)-neomycin phosphotransferase (NPT) reporter construct was generously supplied by Dr. Y.S. Kim (Seoul National University, Korea), and pIL-6-luciferase (Luc) reporter construct by Dr. R.C. Schwartz (Michigan State University, USA). All other chemicals including LPS (*E. coli* 055 : B5) and pyrrolidine dithiocarbamate (PDTC) were otherwise purchased from Sigma-Aldrich (St. Louis, USA).

Cell culture – Macrophages RAW 264.7 were cultured in DMEM medium (13.4 mg/ml Dulbecco's modified Eagle's medium, 24 mM $NaHCO_3$, 10 mM Hepes, 143 U/ml benzylpenicillin potassium, 100 μg/ml streptomycin sulfate, pH 7.1) containing 10% FBS, and maintained at 37 °C in a 5% CO_2 atmosphere. The RAW 264.7 cells harboring pNF-kB-SEAP-NPT reporter construct were cultured in the same media except supplement of geneticin (500 μg/ml).

Enzyme-linked immunosorbent assay (ELISA) – Macrophages RAW 264.7 were pretreated with sample for 2 h and stimulated with LPS (1 μg/ml) for 24 h. Amounts of IL-6 in the culture media were quantified using an ELISA kit according to the supplier's protocol (Amersham-Pharmacia, San Francisco, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) – Macrophages RAW 264.7 were pretreated with sample for 2 h and stimulated with LPS (1 μg/ml) for 6 h. Total RNA of the cells was subjected to semi-quantitative RT-PCR using an RNA PCR kit (Bioneer, Taejon, Korea). Oligonucleotide sequences and RT-PCR conditions were described in our previous work (Shin *et al.*, 2004). The RT-PCR products were resolved on agarose gel by electrophoresis and stained with ethidium bromide.

Measurement of IL-6 promoter activity – Macrophages RAW 264.7 were transiently transfected with both pIL-6-Luc reporter construct and pSV-β-galactosidase control vector using Lipofectamine. The transfected cells were pretreated with sample for 2 h and stimulated with LPS (1 μg/ml) for 16 h. Lysates of the cells were subjected to luciferase assay and β-galactosidase assay using kits from Promega (Madison, USA).

Measurement of NF-kB transcriptional activity – Macrophages RAW 264.7 harboring pNF-kB-SEAP-NPT reporter construct were pretreated with sample for 2 h and stimulated with LPS (1 μg/ml) for 16 h. SEAP activity in the culture media was measured as relative fluorescence units (RFU) with emission at 449 nm and excitation at 360 nm (Moon *et al.*, 2001).

Western immunoblot analysis – Macrophages RAW 264.7 were pretreated with sample for 2 h and stimulated with LPS (1 μg/ml) for indicated times (IkBα) or 1 h (NF-kB p65 or p50). Cytoplasmic and nuclear extracts were subjected to Western blot analysis for detection of IkBα and NF-kB p65 or p50, respectively (Shin *et al.*, 2004). The blots were reacted with ECL reagent (Amersam-Pharmacia, San Francisco, USA) and then exposed to X-ray film.

Statistical analysis – Data are expressed as the mean ± standard error (SE), and analyzed using the ANOVA followed by the Dunnet test. Values of $p < 0.01$ were considered significant.

Results

Quercitrin gallate (Fig. 1) was found to inhibit IL-6 production in LPS-stimulated macrophages RAW 264.7 (Table 1). The macrophages in a resting state released 4 ± 3 ng/ml of IL-6 into the culture media during incubation for 24 h, whereas the cells markedly increased IL-6 expression, up to 83 ± 5 ng/ml, upon exposure to LPS alone (Table 1). Quercitrin gallate inhibited IL-6 production in LPS-stimulated macrophages RAW 264.7, dose-dependently, with an IC_{50} value of 63 μM (Table 1). The positive control, PDTC inhibited the LPS-induced IL-6 production with an IC_{50} value of 27 μM (Table 1). Neither quercitrin gallate nor PDTC at the effective concentrations showed significant cytotoxic effect to macrophages RAW 264.7,

Table 1. Inhibitory effect on LPS-induced IL-6 production

quercitrin gallate		PDTC	
concentration	inhibition %	concentration	inhibition %
100 μM	95.6 ± 4.4*	100 μM	80.2 ± 5.1*
75 μM	78.1 ± 3.6*	30 μM	54.5 ± 4.0*
50 μM	29.4 ± 2.9*	10 μM	37.8 ± 5.2*
25 μM	12.1 ± 4.7	3 μM	19.3 ± 6.5

Macrophages RAW 264.7 were pretreated with sample for 2 h and stimulated with LPS (1 μg/ml) for 24 h. Amounts of IL-6 in the culture media were determined. Values are mean ± SE of inhibition % obtained from three independent experiments. *$p < 0.01$ vs. LPS alone-treated group.

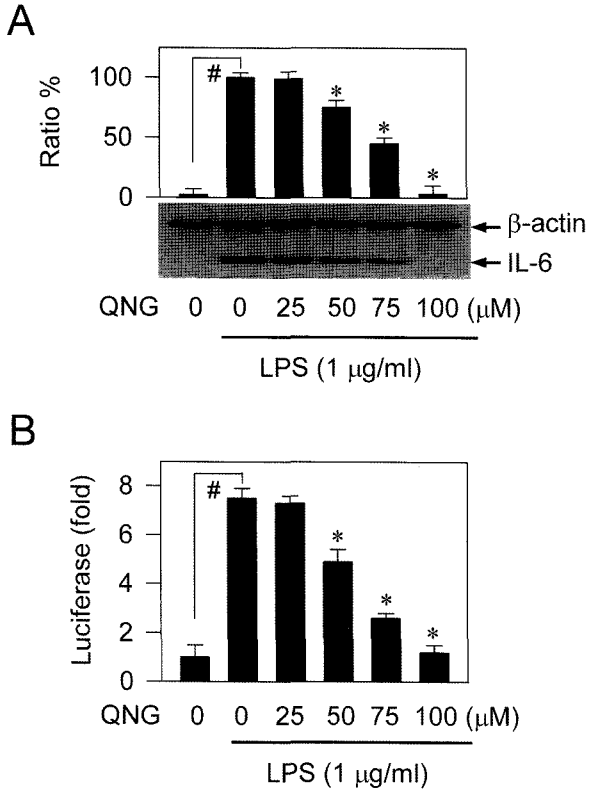

Fig. 2. Inhibitory effect on LPS-induced IL-6 expression. (A) Macrophages RAW 264.7 were pretreated with quercitrin gallate (QNG) for 2 h and stimulated with LPS for 6 h. Total RNA of the cells was subjected to semi-quantitative RT-PCR. One of similar RT-PCR results is represented and relative ratio % is also shown, where IL-6 signal was normalized to β-actin signal. (B) Macrophages RAW 264.7 were transiently transfected with pIL-6-Luc reporter construct and pSV-β-galactosidase control vector. The transfected cells were pretreated with QNG for 2 h and stimulated with LPS for 16 h. Lysates of the cells were subjected to luciferase assay and β-galactosidase assay. Luciferase expression as a reporter of IL-6 promoter activity is represented as relative fold, where luciferase activity was normalized to β-galactosidase activity. Values are mean ± SE of three independent experiments. #$p < 0.01$ vs. media alone-treated group. *$p < 0.01$ vs. LPS alone-treated group.

which was analyzed by WST-1 method (data not shown), and thus inhibitory effects of the compounds on IL-6 production were not attributable to their non-specific cytotoxicity.

To investigate whether quercitrin gallate could affect IL-6 expression, semi-quantitative RT-PCR was carried out. The IL-6 transcript was hardly detectable in resting macrophages RAW 264.7, but markedly increased upon exposure to LPS alone (Fig. 2A). Quercitrin gallate attenuated LPS-induced synthesis of IL-6 transcript with 26% inhibition at 50 μM, 54% at 75 μM, and 98% at 100 μM (Fig. 2A). However, expression of housekeeping β-actin transcript was not affected by treatment of LPS and quercitrin gallate (Fig. 2A). Transcriptional down-regulation of IL-6 synthesis by quercitrin gallate was further documented, using macrophages RAW 264.7 transiently transfected with pIL-6-Luc construct encoding IL-6 promoter (−250/+1) that was fused to luciferase gene as a reporter (Gao *et al.*, 2002). Upon exposure to LPS alone, the transfected cells increased luciferase expression to about 8-fold over the basal level (Fig. 2B). Quercitrin gallate inhibited LPS-induced luciferase expression in a dose-dependent manner, with 39% inhibition at 50 μM, 77% at 75 μM, and 96% at 100 μM (Fig. 2B).

The NF-kB activation is essential for LPS-induced expression of various pro-inflammatory mediators including IL-6 gene (Sanceau *et al.*, 1995). Since IL-6 gene contains several binding sites for NF-kB and its expression was down-regulated at the transcription level by quercitrin gallate, we decided to analyze an effect of the compound on LPS-induced NF-kB transcriptional activity, using macrophages RAW 264.7 harboring pNF-kB-SEAP-NPT construct encoding four copies of the kB motif that were fused to SEAP gene as a reporter (Moon *et al.*, 2001). Upon exposure to LPS alone, the transfected cells increased SEAP expression to 4-fold over the basal level (Table 2). Quercitrin gallate inhibited the LPS-induced SEAP expression in a dose-dependent manner with an IC_{50} value of 59 μM, and PDTC also showed an IC_{50} value of 41 μM (Table 2).

To further understand whether quercitrin gallate could affect nuclear translocation of NF-kB, Western blot analysis was carried out with nuclear extracts of LPS-stimulated macrophages RAW 264.7. The NF-kB p65 and p50 subunits in the nucleus were hardly detectable in resting macrophages RAW 264.7, but their nuclear translocations were markedly increased upon exposed to LPS alone (Fig. 3A). Quercitrin gallate inhibited LPS-induced nuclear translocations of NF-kB p65 and p50 subunits in dose-dependent manners (Fig. 3A). The

Table 2. Inhibitory effect on LPS-induced NF-kB transcriptional activity

quercitrin gallate		PDTC	
concentration	inhibition %	concentration	inhibition %
100 μM	92.5 ± 3.5*	100 μM	85.4 ± 4.1*
75 μM	71.8 ± 4.4*	50 μM	72.5 ± 3.5*
50 μM	40.1 ± 4.6*	25 μM	28.5 ± 3.5*
25 μM	14.4 ± 3.7	13 μM	13.9 ± 2.6

Macrophages RAW 264.7 harboring pNF-kB-SEAP-NPT were pretreated with sample for 2 h and stimulated with LPS (1 μg/ml) for 16 h. SEAP activity in the culture media was determined as relative fluorescence units (RFU), showing 870 ± 34 RFU in LPS alone-treated group and 219 ± 31 RFU in media alone-treated group. Values are mean ± SE of inhibition % obtained from three independent experiments. *p < 0.01 vs. LPS alone-treated group.

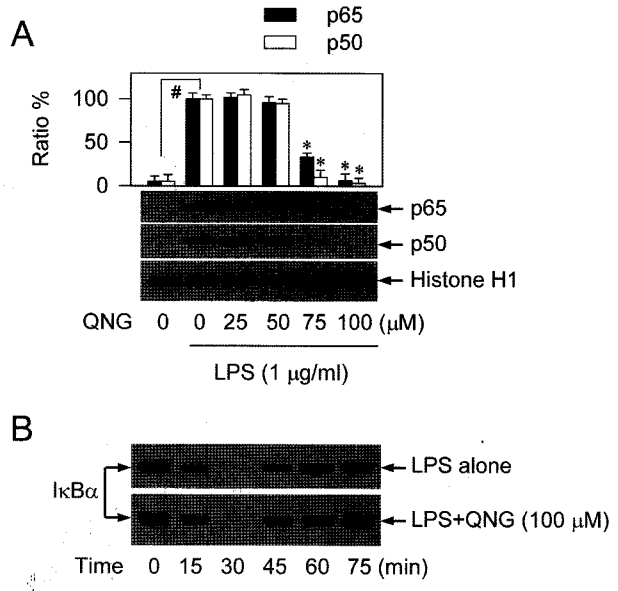

Fig. 3. Inhibitory effect on LPS-induced NF-kB activating pathway. (A) Macrophages RAW 264.7 were pretreated with quercitrin gallate (QNG) for 2 h and stimulated with LPS for 1 h. Nuclear extracts of the cells were subjected to Western blot analysis with anti-NF-kB p65 antibody or anti-NF-kB p50 antibody. One of similar Western blot results is represented and relative ratio % is also shown, where NF-kB p65 or p50 signal was normalized to histone H1 signal. Values are mean ± SE of three independent experiments. #p < 0.01 vs. media alone-treated group. *p < 0.01 vs. LPS alone-treated group. (B) Macrophages RAW 264.7 were pretreated with QNG (100 μM) for 2 h and stimulated with LPS for the indicated times. Cytoplasmic extracts of the cells were subjected to Western blot analysis with anti-IkBα antibody.

phenomenon of NF-kB translocation into the nucleus is preceded by the proteolytic degradation of IkB (Magnani *et al.*, 2000; Karin *et al.*, 2000). We next determined whether quercitrin gallate could affect LPS-induced IkBα degradation, using Western blot analysis with cytoplasmic extracts of macrophages RAW 264.7. Upon exposure to LPS alone, IkBα dramatically degraded within 30 min, and then recovered to the normal level at 75 min after LPS stimulation (Fig. 3B). Quercitrin gallate (100 μM) did not show significant inhibitory effect on LPS-induced IkBα degradation in the time-course study (Fig. 3B).

Discussion

In the present study, quercitrin gallate (Fig. 1) was found to show dose-dependent inhibitory effect on LPS-induced IL-6 production in macrophages RAW 264.7 (Table 1). Furthermore, quercitrin gallate attenuated LPS-induced synthesis of IL-6 transcript (Fig. 2A), but also inhibited LPS-induced IL-6 promoter activity (Fig. 2B), indicating that the compound could down-regulate IL-6 expression at the transcription level.

The IL-6 promoter behaves as a sophisticated biosensor for immune homeostasis, and is transcriptionally activated upon exposure to inflammatory cytokines in addition to bacterial LPS (Sanceau *et al.*, 1995; Guha and Mackman, 2001). Multiple responsive elements in the IL-6 promoter include the NF-kB-binding element between −73 and −63, the cAMP response element followed by the NF-IL6-binding site between −173 and −145, and the activator protein-1 site between −283 and −277 (Sanceau *et al.*, 1995; Hershko *et al.*, 2002). The NF-kB transcription factor has been evidenced to play a key role in the transcriptional up-regulation of LPS-inducible IL-6 gene (Sanceau *et al.*, 1995; Guha and Mackman, 2001).

Quercitrin gallate inhibited LPS-induced NF-kB transcriptional activity in a dose-dependent manner (Table 2). Further, the compound inhibited nuclear translocation of NF-kB p65 or p50 subunit in LPS-stimulated macrophages RAW 264.7 (Fig. 3A). However, quercitrin gallate did not show significant inhibitory effect on LPS-induced IkBα degradation in a time-course study (Fig. 3B). These results indicate that quercitrin gallate could affect LPS-induced NF-kB activating pathway, specifically to nuclear translocation of NF-kB, downstream IkB degradation.

Eukaryotic cells are compartmentalized by the nuclear envelope between the cytoplasm and the nucleus. The nuclear envelope contains nuclear pore complexes, which are responsible for molecular trafficking between the two compartments. NF-kB contains nuclear localizing signals that are imported into the nucleus by importin α/β heterodimers (Marelli *et al.*, 2001; Goldfarb *et al.*, 2004; Pemberton and Paschal, 2005). Importin α3 or α4 subtype binds

to nuclear localizing signals of NF-kB, and importin β is responsible for the docking followed by translocation of the importin α-cargo complex into the nucleus (Fagerlund *et al.*, 2005). Even though molecular target remains to be determined, quercitrin gallate prevented nuclear translocation of NF-kB without affecting IkBα degradation (Fig. 3). This event could result in down-regulation of IL-6 expression in LPS-stimulated macrophages RAW 264.7 (Fig. 2). Finally, this study could provide a pharmacological potential of quercitrin gallate in IL-6-related immune and inflammatory diseases.

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