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Inhibition of Interleukin-12 Production in Mouse Macrophages *via* Decreased Nuclear Factor-κB DNA Binding Activity by Myricetin, a Naturally Occurring Flavonoid

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Pharmacological inhibition of interleukin-12 (IL-12) production may be a therapeutic strategy for preventing the development and progression of disease in experimental models of autoimmunity. In this study, the effects of myricetin, a naturally occurring flavonoid present in fruits, vegetables and medicinal herbs, on the production of IL-12 were investigated in mouse macrophages stimulated with lipopolysaccharide (LPS). Myricetin significantly inhibited the LPS-induced IL-12 production from both primary macrophages and the RAW264.7 monocytic cell-line in a dose-dependent manner. The effect of myricetin on IL-12 gene promoter activation was analyzed by transfecting RAW264.7 cells with IL-12 gene promoter/luciferase constructs. The repressive effect was mapped to a region in the IL-12 gene promoter containing a binding site for NF-κB. Furthermore, activation of macrophages by LPS resulted in markedly enhanced binding activity to the NF-κB site, which significantly decreased upon addition of myricetin, indicating that myricetin inhibited IL-12 production in LPS-activated macrophages *via* the down-regulation of NF-κB binding activity.

Key words: Myricetin, Interleukin-12, Macrophage, Nuclear factor-κB

INTRODUCTION

Interleukin-12 (IL-12), a heterodimeric cytokine, is composed of two disulfide-linked subunits of 35 (p35) and 40 (p40) kDa encoded by two separate genes. Inducible expression of IL-12 has been demonstrated in phagocytic and other antigen-presenting cells in response to stimulation by a variety of microorganisms as well as their products (Ma and Trinchieri, 2001). The expression of the p40 subunit is highly inducible and regulated primarily at the transcriptional level (Kang *et al.*, 1996). Two major regulatory sites have been identified that bind inducible proteins belonging to the Ets and NF-κB families of transcription factors (Gri *et al.*, 1998).

IL-12 exerts multiple biological activities, mainly through T and natural killer cells, by inducing their production of interferon-γ, which augments their cytotoxicity, and by enhancing their proliferation potential. IL-12 production is critical for the development of T helper type 1 cells and the initiation of cell-mediated immune responses. Recent evidence has pointed to a critical role for IL-12 in the pathogenesis of rodent models of Th1-mediated autoimmune diseases, such as type-1 diabetes, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and acute graft-*versus*-host (Adorini, 1999). We recently reported that auranofin, an anti-rheumatic gold compound, inhibited IL-12 production in macrophages, leading to the deviation of $CD4^+$ T cells from the Th1 to the Th2 pathway (Kim *et al.*, 2001). Thus, pharmacological control of IL-12 production may be a key strategy in modulating specific immune-mediated diseases dominated by type-1 cytokine responses.

Myricetin, a naturally occurring flavonoid, is commonly found in tea, berries, fruits, vegetables, and medicinal herbs (Ross and Kasum, 2002). Many studies have shown that myricetin is an anti-carcinogen, although some reports indicate it can induce mutagenesis in bacteria. Myricetin also inhibits the reverse transcriptase from

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several retroviruses, and thus, may be of therapeutic use for retroviral infection (Chu *et al.*, 1992). In addition, myricetin may be of benefit as an anti-inflammatory, anti-artherosclerotic-, and anti-thrombotic agent (Landolfi *et al.*, 1984; Theoharides *et al.*, 2001), and also inhibits tumor-necrosis factor-α-induced up-regulation of intercellular adhesion molecule-1 (ICAM-1) in respiratory epithelial cells (Chen *et al.*, 2004). Some preliminary work has shown that myricetin has both hypoglycemic and hypotriglyceridimic effects in diabetic animals (Ong and Khoo, 2000).

In this study, the effect of myricetin on IL-12 production was investigated in lipopolysaccharide (LPS)-activated mouse macrophages. Herein, myricetin was demonstrated to significantly inhibit IL-12 production from LPS-activated macrophages in a concentration-dependent manner. This inhibition was, at least in part, dependent on the down-regulation of NF-κB binding activity.

MATERIALS AND METHODS

Cell culture, reagents and mice

RAW264.7 cells were cultured in RPMI 1640, supplemented with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY), at 37°C in a 5% CO_2 humidified air atmosphere. Spleen cell populations and macrophages from mice were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics. The LPS (from *E. coli* 0111:B4) and myricetin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). A 10 mg/mL stock solution of myricetin was prepared by dissolving in ethanol, and diluted into the medium, at least 400-fold, such that the final concentration of the solvent had no effect on cell growth and on IL-12 production. Female DBA/2 mice (6-8 weeks of age) were obtained from Japan SLC, Inc. (Tokyo, Japan). Pellet diets (Samyang, Daejeon, Korea) were supplied *ad libitum*. The mice were maintained in pathogen-limited conditions.

IL-12 p40 promoter constructs and transient transfection

The -689/+98 fragment of the murine IL-12 p40 promoter from pXP2 (Ma *et al.*, 1996) was subcloned into the *KpnI/XhoI* sites of the pGL3-basic luciferase vector (Promega, Madison, WI). All the deletion mutants were generated by polymerase chain reaction using an upstream primer containing the *Bam*HI site. A linker-scanning mutant was generated by a two-step polymerase chain reaction procedure with overlapping internal primers containing mutated sequences for the NF-κB site. For transfections, cells were grown for 24 h in 24-well plates, containing medium supplemented with 10% fetal bovine serum, and transfected with each of the plasmid constructs in the presence of Superfect, according to the manufacturer's protocol (Qiagen, Valencia, CA). After 12 h, the cells were washed with DMEM containing 10% fetal bovine serum, and then replenished with the same medium. The cells were harvested 24 h later and the luciferase activity assayed. The results were normalized to the *LacZ* expression.

Preparation of splenic macrophages stimulated with LPS

Spleen cells were cultured at 10^6 cells/mL for approximately 3 h at 37°C. The non-adherent cells were removed by washing with warm DMEM until visual inspection revealed a lack of lymphocytes (>98% of the cell population). The cell population consisted mostly of macrophages, as confirmed by flow cytometry (Epic V, Coulter Electronics, Hialeah, FL). The adherent cells were removed from plates by incubation for 15 min in ice-cold phosphate-buffered saline solution, with repeated rinsing. The isolated adherent cell population was stimulated with 5 μg/mL LPS, either in the absence or presence of myricetin at 1×10^5 cells per well, in 96-well culture plates for 48 h.

Cytokine assay

The quantities of IL-12 p40 in the culture supernatants were determined by a sandwich enzyme-linked immunosorbent assay (ELISA), using monoclonal antibodies specific for IL-12 p40, as previously described (Kang *et al.*, 2002). The monoclonal antibodies for coating the plates and the biotinylated second monoclonal antibodies (from PharMingen, San Diego, CA) were C17.8 and C15.6, respectively. Standard curves were generated using recombinant IL-12, with a lower limit of detection of 30 pg/mL.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from the cells, reverse-transcribed into cDNA, and PCR amplification of the cDNA then performed, as previously described (Joo *et al.*, 2002). The sequences of the PCR primers were as follows: mouse IL-12 p40 (sense, 5'CAGAAGCTAA CCATCTCCTGGTTTG3; antisense, 5'TCCGGAGTAATTTGGTG CTTCACAC3'), IL-12 p35 (sense, 5'TCAGCGTTCCAACAGCCTC3'; antisense, 5'C GCAGAGTCTC GCCATTATG3'), IL-6 (sense, 5'TGAACAACGATGATGCACTT 3'; antisense, 5'CGTAGAGAACAACATAAGTC3'), and β-actin (sense, 5'TGGAATCCT GTGGCATCCATGAAAC3'; antisense, 5'TAAAACGCAGCTCAGTAACAGTCC G3'). The PCR reactions were run for 35 cycles of 94°C (for 30 sec), 58°C (for 45 sec), and 72°C (for 30 sec), using a MJ thermal cycler (Watertown, MA). After amplification, 6 μL of the RT-PCR products were separated in 1.5% (w/v) agarose gels and stained with ethidium bromide.

Electrophoretic mobility gel shift assay

Nuclear extracts were prepared from the cells, as previously described (Dignam *et al.*, 1993). An oligonucleotide, containing a NF-κB-binding site within the immunoglobulin γ-chain (5' CCGGTTAACAGAGGGGGCTTTCCGAG 3'), was used as a probe. Specific binding was confirmed by competition experiments with 50-fold excesses of unlabeled, identical oligonucleotides or cAMP response element-containing oligonucleotides.

Statistical analysis

The Student's *t*-test and one-way analysis of variance (ANOVA) were used to determine the statistical differences between the various experimental and control groups. A difference was considered statistically significant when $P < 0.05$.

RESULTS

Myricetin inhibits IL-12 production from LPS-activated macrophages

The effect of myricetin (chemical structure, Fig. 1A) on the production of IL-12 was investigated in LPS-stimulated primary mouse macrophages. As shown in Fig. 1B, LPS readily induced the production of IL-12, as expected. However, myricetin significantly inhibited this LPS-induced IL-12 production in a concentration-dependent manner. Myricetin also inhibited the IL-12 production from RAW264.7 monocytic cell-line stimulated with LPS in a concentration-dependent manner (Fig. 2A).

Furthermore, to determine whether the inhibition of IL-12 secretion is the results of decreased mRNA production, the effects of myricetin on the expressions of IL-12 p40 and p35 mRNA were analyzed in LPS-stimulated macrophages. As shown in Fig. 2B, myricetin significantly inhibited the mRNA levels of both IL-12 p40 and p35, indicating that the inhibition of IL-12 production by myricetin occurred at the mRNA level. In contrast, treatment with myricetin did not influence the IL-6 mRNA level in LPS-stimulated macrophages, suggesting that the inhibitory effect of IL-12 production by myricetin was not the result of a general dampening of cellular activation. Furthermore, the IL-12-inhibition by myricetin did not result from a general cytotoxic effect, as the macrophages viability in all cultures remained constant throughout the incubation period in the presence of the myricetin concentrations used in this experiment, as demonstrated by the trypan blue exclusion test (data not shown).

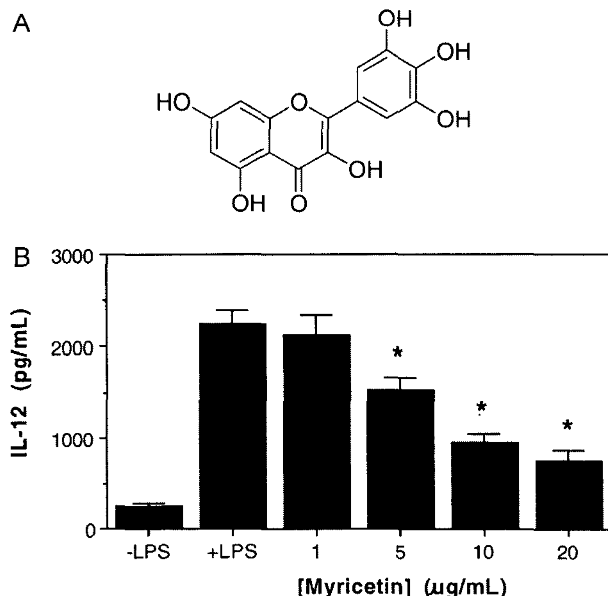

Fig. 1. Inhibition of the IL-12 production in LPS-activated primary mouse macrophages by myricetin. (A) Chemical structure of myricetin. (B) Primary macrophages were stimulated with LPS (0.5 µg/mL) for 48 h, in the absence or presence of various concentrations of myricetin, and the IL-12 levels in the culture supernatants were evaluated by an ELISA. The ELISA data represent the mean standard error (n=3). $^*P < 0.01$, relative to an untreated group.

Myricetin inhibits NFκB-mediated activation of IL-12 p40 promoter by LPS

To identify the region involved in these myricetin actions,

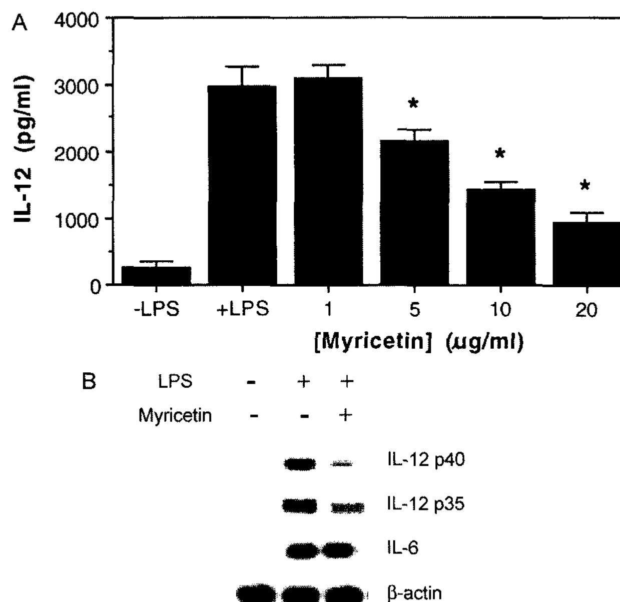

Fig. 2. Inhibition of the IL-12 production from RAW264.7 monocytic cells by myricetin. (A) RAW264.7 cells were stimulated with LPS (0.5 µg/mL) for 48 h, in the absence or presence of various concentrations of myricetin, and the IL-12 levels in the culture supernatants were determined by an IL-12 ELISA. The data represent the mean standard error (n=3). $^*P < 0.001$, relative to an untreated group. (B) RAW264.7 cells were stimulated with LPS (5 µg/mL) for 8 h, in the absence or presence of myricetin (10 µg/mL), and the RT-PCR products for IL-12 p40, IL-12 p35, IL-6 and β-actin were analyzed in 1.5 % agarose gels.

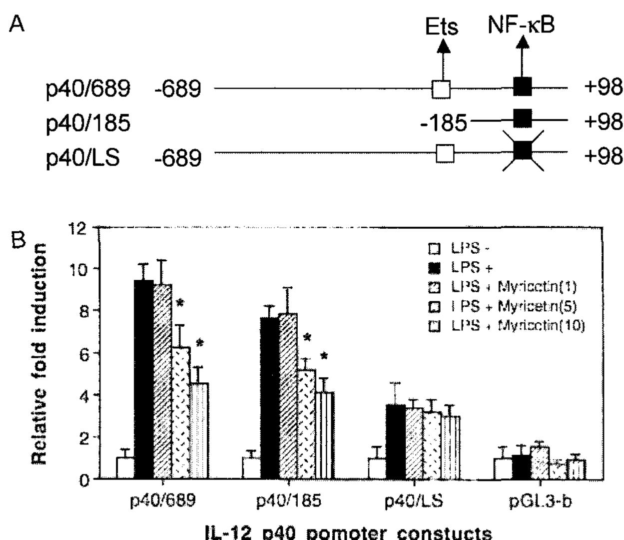

Fig. 3. Analysis of the myricetin-mediated transcriptional repression of the IL-12 p40 promoter constructs activated by lipopolysaccharide. (A) Schematic representation of the mouse p40 promoter constructs and a linker-scanning mutant for the NFκB site are as shown, along with the Ets and NFκB binding sites. The nucleotide sequence numbers for each construct are shown. (B) RAW264.7 cells were transiently transfected with mouse IL-12 p40 promoter constructs, followed by stimulation with LPS (0.5 μg/mL), either in the absence or presence of myricetin (1, 5 and 10 μg/mL). The results are expressed as the fold induction over the value obtained with the unstimulated RAW264.7 cells transfected with the -689/+98 construct, which was given an arbitray value of 1. Normalized luciferase expressions from triplicate samples are presented relative to the *LacZ* expressions. The data represent the mean standard deviations from triplicate determination. **P* < 0.05, relative to groups treated with LPS alone.

a series of luciferase reporter constructs, containing the IL-12 p40 promoter sequences from positions -689 to +98 and -185 to +98 relative to the transcription initiation site, were generated, as shown in Fig. 3A. An IL-12 p40 subunit has been shown as the highly inducible and tightly regulated component of IL-12 (Kang *et al.*, 1996). Mouse RAW264.7 monocytic cells were transfected with each of these constructs and stimulated with LPS, either in the absence or presence of myricetin, and the luciferase activity then determined. As shown in Fig. 3B, the -689/+98 full construct showed strong stimulation by LPS in the absence of myricetin, but impaired stimulation in the presence of myricetin. In particular, deleting the sequences to -185 (p40/185) did not diminish the LPS-dependent promoter activity and the inhibitory effect of myricetin was still observed, suggesting that the target site for myricetin resides within this region. To directly test the role of a NF-κB site found between -121 and -131 of the p40 promoter in the myricetin-mediated inhibitory actions, a linker scanning mutation was introduced into the NF-κB site within the context of the -689/+98 construct (p40/LS). LPS-dependent promoter activation was still observed

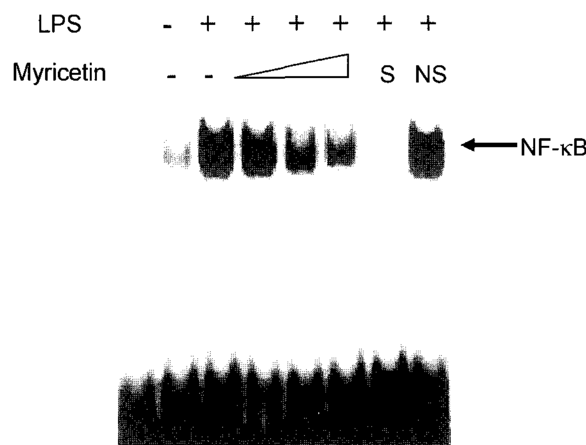

Fig. 4. Myricetin-mediated inhibition of NF-κB binding. Nuclear extracts prepared from monocytic cells stimulated with LPS, either in the absence or presence of myricetin (1, 5 and 10 μg/mL), were examined for NF-κB binding activity using the electrophoretic mobility shift assay, with a labeled oligonucleotide containing a consensus immunoglobulin-κB site. The specific binding of the NF-κB to κB sites was confirmed in nuclear extracts in the presence of an unlabeled, identical oligonucleotide (S) and nonspecific oligonucleotide (NS), respectively. The specific NF-κB complex is as indicated.

with p40/LS, although significantly reduced, consistent with the previous findings in which the NF-κB site was shown to be important for the LPS-mediated induction of the p40 promoter. However, the addition of myricetin to the LPS-stimulated cells had only a slight repressive effect with p40/LS, clearly indicating the inhibitory effect of myricetin on IL-12 production was mediated through the κB site.

NF-κB bindings to the κB site inhibited by myricetin

To gain more insight into the mechanisms of myricetin-mediated inhibition of the p40-κB function, the NF-κB binding activity present in nuclear extracts of LPS-stimulated primary macrophages, either in the absence or presence of myricetin, were analyzed. As shown in Fig. 4, the nuclear extracts from the LPS-stimulated macrophages exhibited strong NF-κB-binding activity in the electrophoretic mobility shift assay using a labeled oligonucleotide containing a consensus immunoglobulin-κB site. The binding was specific, since it was inhibited with an unlabeled, identical oligonucleotide, but not with an unrelated, nonspecific oligonucleotide, however, binding was absent with the nuclear extracts from nonstimulated cells. Nuclear extracts from macrophages stimulated by LPS in the presence of myricetin showed dose-dependent diminished NF-κB-binding activity.

DISCUSSION

Inhibiting the action of IL-12 has been shown to prevent

the development and block the progression of disease in experimental models of autoimmunity (Falcone and Sarvetnick, 1999; Prud'homme, 2000). These findings have raised great interest in identifying inhibitors of IL-12 production for the treatment of Th1-mediated diseases, such as type-1 diabetes, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease and acute graft-*versus*-host disease. In this study, myricetin was demonstrated to inhibit the IL-12 production in macrophages, in a dose-dependent manner, *via* down-regulation of the NF-κB binding activity. Recently, several compounds have been reported to inhibit IL-12 production in some antigen-presenting cells, including monocytes and macrophages. Corticosteroids have been shown to enhance the capacity of macrophages to induce IL-4 synthesis in $CD4^+$ T cells by inhibiting the IL-12 production (DeKyruff *et al.*, 1998). In addition, the angiotensin converting enzyme inhibitors, captopril and lisinopril, were also shown to suppress the IL-12 production from human peripheral blood mononuclear cells (Constantinescu *et al.*, 1998). Thalidomide, a phosphodiesterase inhibitor, inhibited the IL-12 production from human monocytes by a mechanism independent of known endogenous inhibitors of IL-12 production, such as IL-10, transforming growth factor-β, or prostaglandin E_2 (Moller *et al.*, 1997). β2-adrenergic compounds, including salbutamol, inhibited the IL-12 production from human monocytes and dendritic cells by increasing the intracellular cAMP levels, leading to inhibition of the development of Th1 cells, while promoting Th2 cell differentiation (Panina-Bordignon *et al.*, 1997). From the results presented in this report, myricetin can be added to the list of compounds that inhibit the production of IL-12 from antigen-presenting cells, such as macrophages.

The mechanism by which myricetin inhibits the IL-12 production in LPS-stimulated macrophages seems to be through the downregulation of NF-κB binding to the p40-κB site. This point was supported by several lines of evidences. First, an IL-12 p40 subunit has been shown as the highly inducible and tightly regulated component of IL-12. The inhibitory effect of myricetin was retained within a 185 bp fragment upstream of the transcription initiation site (Fig. 3B) in a series of 5 deletions of the p40 promoter, suggesting that myricetin may interfere with the inducible binding of NF-κB at position -121/-131 bp in the p40 promoter. A linker scan mutation of the p40-κB site abrogated the inhibitory effect of myricetin on the p40 promoter, indicating that this site plays a role in the transcriptional repression of the p40 gene (Fig. 3B). In addition, myricetin significantly decreased the binding activities to the κB site in LPS-activated macrophages, as demonstrated by the electrophoretic mobility assays (Fig. 4). The NF-κB-mediated inhibition of IL-12 production was in accordance with the previous observations that 1, 25-dihydroxyvitamin D_3, retinoids and chloromethyl ketones inhibited IL-12 production by downregulating the NF-κB activation and the binding into the NF-κB sequence of the IL-12 p40 gene (DAmbrosio *et al.*, 1998; Na *et al.*, 1999; Kang *et al.*, 1999). In addition, myricetin was reported to inhibit the NF-κB activity by preventing the degradations of IκBα and IκBβ through inhibition of the IKK kinase activity (Tsai *et al.*, 1999).

In conclusion, this study has shown that myricetin inhibits the IL-12 production in LPS-stimulated macrophages through the downregulation of NF-κB binding to the IL-12 p40-κB site. Myricetin-mediated inhibition of IL-12 production in macrophages may explain some of the biological effects of myricetin, including its anti-inflammatory activity.

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