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Mangiferin isolated from the rhizome of *Anemarrhena asphodeloides* inhibits the LPS-induced nitric oxide and prostagladin E_2 via the NF-κB inactivation in inflammatory macrophages

Ji-Sun Shin[1], Young-Su Noh[1], Dong-Hyun Kim[1], Young-Wuk Cho[2], and Kyung-Tae Lee[1,*]

[1]*College of Pharmacy*
[2]*College of Medical science, Kyung Hee University, 1 Hoegi-Dong, Seoul 130-701, Republic of Korea*

Abstract – This study was designed to investigate the anti-inflammatory effects of mangiferin isolated from the rhizome of *Anemarrhena asphodeloides*, a natural polyphenol, on lipopolysaccharide (LPS)-treated RAW 264.7 macrophages. Mangiferin dose-dependently inhibited LPS-induced nitric oxide (NO) and prostaglandin E_2 (PGE_2) productions in RAW 264.7 macrophages and peritoneal macrophages isolated from C57BL/6 mice. Consistent with these data, mangiferin suppressed the LPS-induced expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) at the protein and mRNA levels in a concentration-dependent manner, as determined by Western blotting and RT-PCR, respectively. In addition, the release of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), and the mRNA expression levels of these cytokines were reduced by mangiferin in a dose-dependent manner. Moreover, mangiferin effectively inhibited the transcriptional activation of nuclear factor-kappa B (NF-κB). These results suggest that the anti-inflammatory properties of mangiferin are caused by iNOS, COX-2, TNF-α, and IL-6 down-regulation due to NF-κB inhibition in RAW 264.7 macrophages.

Keywords – Mangiferin, cytokines, NO, PGE_2, NF-κB, macrophages

Introduction

Inflammation is benefic host response to a foreign challenge or tissue injury that leads ultimately to the restoration of normal tissue structure and function. A normal inflammatory response is self-limiting and involves the down-regulation of pro-inflammatory protein expression, increased expression of anti-inflammatory proteins, and a reversal in the vascular changes that facilitated the initial immune cell recruitment process (Vane *et al*., 1994; Funk *et al*., 1991; Hinz and Brune., 2002). During this response, inflammatory cells are exposed to apoptosis-inducing stimuli and subsequently undergo phagocytosis. Macrophages play significant roles in host defense mechanism.

Macrophages produce and release nitric oxide (NO), pro-inflammatory cytokines and secondary mediators such as leukotrienes (LTs) and prostaglandins (PGs); these molecules are important regulators of innate and adaptive immunity, but their overproduction contributes to the pathogenesis of several diseases such as otitis media, hearing loss, periodontitis, bacterial sepsis, rheumatoid arthritis, chronic inflammation, and autoimmune diseases (Elenkov and Chriusos., 2002; Forman and Torres., 2001, Song *et al*., 2002).

Nuclear factor-kappa B (NF-κB), the key molecule in inflammatory responses, is a generic term for a dimeric transcription factor that is formed by the dimerization of proteins in the Rel family (Jun *et al*., 1995). NF-κB activity is exerted through its ability to regulate the expression of genes that encode inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Mannel and Echtenacher., 2000). It would be valuable to develop potent inhibitors of pro-inflammatory mediators such as NF-κB inactivator for potential therapeutic use in inflammatory disease. Some natural products are candidates of potential source NF-κB inactivator.

The polyphenol mangiferin, a C-glucosylxanthone, specifically 1,3,6,7-tetrahydroxyxanthone-C2-β-D-glucoside (Aritomi and Kawasaki., 1969), has attracted considerable interest in view of its numerous pharmacological activities, including anti-tumor and anti-viral, anti-diabetic, anti-

*Author for correspondence
Fax: +82-2-9663885; E-mail: ktlee@khu.ac.kr

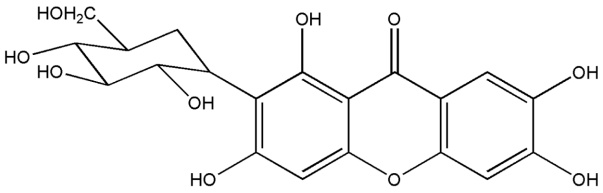

Fig. 1. Chemical structure of mangiferin isolated from the rhizome of *Anemarrhena asphodeloides*.

bone resorption and anti-oxidant activities (Guha and Mackman., 1996; Ichiki *et al*., 1998; Sanchez *et al*., 2000; Moreira *et al*., 2001). Taking into account the previous findings, it was decided to test the anti-inflammatory activities of mangiferin on the response of RAW 264.7 macrophages and peritoneal macrophages isolated from C57BL/6 mice to LPS. In this study, we reported that mangiferin inhibited LPS-induced pro-inflammatory mediators, and these effects involved in NF-κB inactivation.

Experimental

Chemicals and antibodies – Mangiferin (Fig. 1) used for this study was isolated from the rhizome of *Anemarrhena asphodeloides* as previously described (Aritomi and Kawasaki, 1969). The mangiferin isolated was checked by HPLC and was > 95% pure. Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, U.S.A.). iNOS and COX-2, β-actin monoclonal antibodies and the peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Enzyme immunoassay (EIA) kits for tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) was obtained from R&D Systems (Minneapolis, MN, U.S.A.), and luciferase assay kits was purchased from Promega (Madison, CA. U.S.A.). pNF-κB-Luc reporter plasmid was purchased from BD Biosciences (San Jose, CA, U.S.A.). FuGENE 6 transfection reagent from Roche Applied Science (Mannhei, Germany) and RNA extraction kits from Intron Biotechnology. iNOS, COX-2, TNF-α, IL-6 and β-actin oligonucleotide primers were from Bioneer (Seoul, Korea). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tertazolium bromide (MTT), L-N^6-(1-iminoethyl)lysine (L-NIL), NS-398, phenylmethylsulfonyl-l-fluoride (PMSF), *Escherichia coli* lipopolysaccharide (LPS) and all other chemicals were from Sigma (St. Louis, MO, U.S.A.).

Cell culture and sample treatment – The RAW 264.7 macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were grown at 37 °C in DMEM medium supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin sulfate (100 μg/mL) in a humidified atmosphere of 5% CO_2. Murine peritoneal macrophages were elicited by intraperitoneal injection of 2 mL of 5% thioglychollate into the peritoneal cavity of 6-10 weeks old C57BL/6 mice. After 4 days, peritoneal exudates cells were obtained by lavage with ice-cold DMEM. The cells were washed twice, resuspended in HEPES-buffered DMEM (supplemented with $NaHCO_3$, 10% FBS, and antibiotics, namely 100 units/mL penicillin, 100 μg/mL streptomycin sulfate), and were seeded in sterile disposable culture plates. Cells were incubated with the tested samples at increasing concentrations (50, 100, 200 μM) or positive chemical and then stimulated with LPS 1 μg/mL for the indicated time.

Cell viability assay – Cell respiration as an indicator of cell viability was determined on the basis of mitochondrial-dependent reduction of MTT to formazan. Cells were treated with various concentrations (from 12.5 μM to 200 μM) in presence or absence LPS (1 μg/mL). After 24 h incubation, cell viability was assessed by MTT. After 4 h of incubation at 37 °C, the medium was discarded and the formazan blue that formed in the cells was dissolved in DMSO. The optical density was measured at 540 nm.

Nitrite, PGE_2, TNF-α and IL-6 assays – Nitrite accumulation, an indicator of NO synthesis, was measured in the culture medium using the Griess reaction (Shin *et al*., 2004). PGE_2, TNF-α and IL-6 levels in macrophage culture media were quantified using EIA kits, according to the manufacture's instructions (R&D Systems, MN, USA).

Western blot analysis – Cellular proteins were extracted from control and mangiferin treated RAW 264.7 macrophages. Washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES (pH 7.0), 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM Na fluoride (NaF), 0.5 mM Na orthovanadate) containing 5 μg/mL of each of leupeptin and aprotinin, and then incubated for 20 min at 4 °C. Cell debris was removed by microcentrifugation, and supernatants were quick frozen. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacture's instructions. Total cellular proteins (40 μg) from treated and untreated cell extracts were electroblotted onto a nitrocellulose membrane following separation on a 10% SDS-polyacrylamide gel electro-

phoresis. The immunoblot was incubated 1 h with blocking solution (5% skim milk) at room temperature, and then incubated for overnight with a 1 : 1000 dilution of monoclonal anti-iNOS, a 1:1000 dilution of anti-COX-2 antibody, and a β-actin antibody (Santa Cruz Biotechnology Inc.) at 4 °C. Blots were washed twice with Tween 20/Tris-buffered saline (TTBS) and then incubated with a 1 : 1000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Blots were again washed three times with TTBS and then developed by enhanced **chemilluminescence** (Amersham Life Science, Arlington Heights, IL, U.S.A.).

RNA preparation and RT-PCR – Total cellular RNA was isolated using Easy Blue® kits (Intron Biotechnology, Seoul, Korea), according to the manufacturer's instructions. RNA (1 μg) was reverse-transcribed (RT) from each sample using MuLV reverse transcriptase, 1 mM dNTP, and oligo (dT_{12-18}) 0.5 μg/μL. PCR analyses were performed on aliquots of the cDNA preparations to detect iNOS, COX-2, TNF-α and IL-6 (using β-actin as an internal standard) gene expression using a thermal cycler (Perkin Elmer Cetus, Foster City, CA, U.S.A.). Reactions were carried out in a volume of 25 μL containing (final concentrations) 1 unit of Taq DNA polymerase, 0.2 mM dNTP, × 10 reaction buffer, and 100 pmol of 5' and 3' primers. After an initial denaturation for 2 min at 95 °C, 30 amplification cycles were performed for iNOS (1 min of 94 °C denaturation, 1 min of 58 °C annealing, and 2 min 72 °C extension), COX-2 (0.5 min of 94 °C denaturation, 0.5 min of 60 °C annealing, and 1.5 min 72 °C extension), TNF-α (1 min of 94 °C denaturation, 1 min of 58 °C annealing, and 2 min 72 °C extension) and IL-6 (0.5 min of 94 °C denaturation, 0.5 min of 50 °C annealing, and 1.5 min 72 °C extension). PCR primers used in this study are listed below and were purchased from Bioneer (Seoul, Korea): sense strand iNOS, 5'-AATGGCAACATCAG GTCGGCCATCACT-3', anti-sense strand iNOS, 5'-GCTGTGTGTCACAGAAGTCTC GAA CTC-3'; sense strand COX-2, 5'-GGAGAGACTATCAA GATAGT-3', anti-sense strand COX-2, 5'-ATGGTC AGT AGACTTTTACA-3'; sense strand TNF-α, 5'-ATGAG CACAGAAAGCATGATC-3', anti-sense strand TNF-α, 5'-TACAGGCTTGTCACTCG AATT-3'; sense strand IL-6, 5'-GAGGATACCACTCCCAACAGACC-3'; anti-sense strand IL-6, 5'-AAGTGCATC ATCGTTGTTCATACA-3'; sense strand β-actin, 5'-TCA TGAAGTGTGACGTTGAC ATCCGT-3', anti-sense strand β-actin, 5'-CCTAGAAGCA TTTGCGGTGCACGATG-3'. After amplification, portions of the PCR reaction products were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation (Shin *et al*., 2004).

Transient transfection and luciferase assay (Reporter gene assay) – RAW 264.7 macrophages were transfected using FuGENE 6 transfection reagent (Roche Applied Science, Mannhei, Germany) and pNF-κB-Luc reporter plasmid (BD Biosciences, San Jose, CA, U.S.A.), as instructed by the manufacturer's instructions. At 48 h after the start of transfection after the start of transfection, cells were pretreated with mangiferin for 1 h and stimulated with LPS (1 μg/mL). Following 24 h of stimulation, cells were lysed and the luciferase activity was determined using the Promega luciferase assay system (Promega, Madison, CA. U.S.A.) and luminometer (Perkin Elmer Cetus, Foster City, CA, U.S.A.). Luciferase activity was normalized versus sample protein concentrations.

Statistical analysis – The results were expressed as the mean ± S.D. of triplicate experiments. Statistically significant values were compared using ANOVA and Dunnett post-hoc test and *p*-values less than 0.05 were considered statistically significant.

Results

Effects of mangiferin on LPS-induced NO and PGE_2 productions in RAW 264.7 macrophages – To assess the effects of mangiferin on the LPS-induced NO and PGE_2 productions in RAW 264.7 macrophages, the culture media were harvested and NO and PGE_2 levels were measured. Mangiferin (50, 100, 200 μM) inhibited the LPS-induced NO and PGE_2 productions, and to do so in a dose-dependent manner (Fig. 2A). In addition, treatment with mangiferin at 200 μM potently reduced the LPS-induced NO and PGE_2 productions about 32% and 77%. L-NIL (10 μM) and NS398 (5 μM) were used as positive NO and PGE_2 production controls, respectively. To investigate the effect of mangiferin on cell viability, the cells were exposed to various mangiferin concentrations (from 12.5 μM to 200 μM) for 24 h in the presence or absence of LPS. Treatment of mangiferin in the presence of LPS (1 μg/mL) did not affect the viability even high concentration (200 μM) (Fig 2B). Therefore, inhibition of LPS-stimulated NO and PGE_2 productions by mangiferin was not the consequence of a cytotoxic effect on these cells.

Effects of mangiferin on LPS-induced NO and PGE_2 productions in mouse peritoneal macrophages – To confirm the effects of mangiferin on the LPS-induced NO and PGE_2 productions in peritoneal macrophages

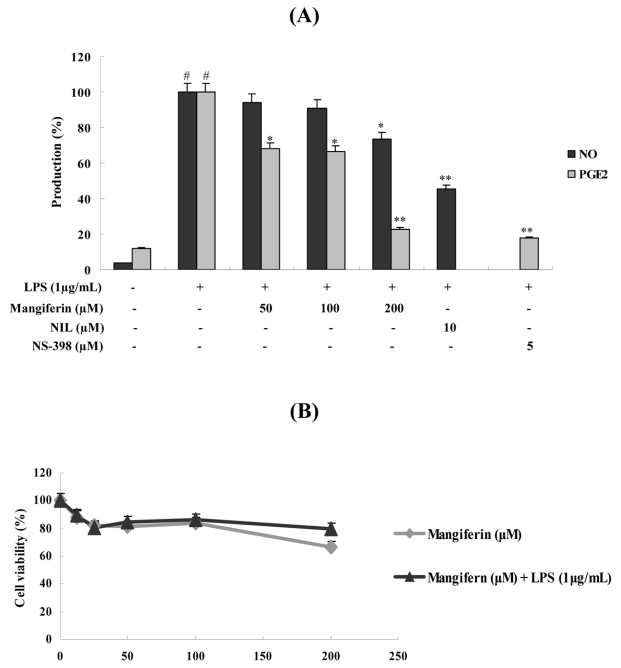

Fig. 2. Effects of mangiferin on LPS-induced NO and PGE_2 productions (A) and on cell viabilities (B) in RAW 264.7 macrophages.

(A) Cells were treated with different concentrations (50, 100, 200 μM) of mangiferin for 1 h, then with LPS (1 μg/mL), and incubated for 24 h. Control (Con) values were obtained in the absence of both LPS and tested sample. L-N^6-(1-iminoethyl) lysine (L-NIL, 10 μM) and of NS-398 (5 μM) were used as a positive controls. The values are means ± S.D. of three independent experiments. $^{\#}p < 0.05$ vs. the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the LPS-treated group; significances between treated groups were determined using ANOVA and Dunnett's post-hoc test. (B) Cells were exposed to mangiferin (from 12.5 to 200 μM), and cell viability was assessed by MTT assay after 24h incubation.

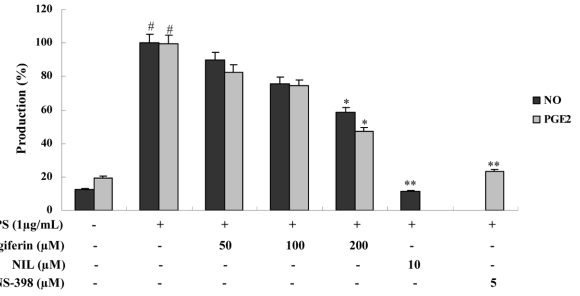

Fig. 3. Effects of mangiferin on LPS-induced NO and PGE_2 productions in peritoneal macrophages isolated from C57BL/6 mice.

Cells were treated with different concentrations (50, 100, 200 μM) of mangiferin for 1 h, then with LPS (1 μg/mL), and incubated for 24 h. Control (Con) values were obtained in the absence of both LPS and tested sample. L-N^6-(1-iminoethyl) lysine (L-NIL, 10 μM) and of NS-398 (5 μM) were used as a positive controls. The values are means ± S.D. of three independent experiments. $^{\#}p < 0.05$ vs. the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the LPS-treated group; significances between treated groups were determined using ANOVA and Dunnett's post-hoc test.

isolated from C57BL/6 mice, the culture media were harvested and NO and PGE_2 levels were measured. Mangiferin (50, 100, 200 μM) significantly inhibited the LPS-induced NO and PGE_2 productions about 42% and 47%, respectively at 200 μM mangiferin (Fig. 3). L-NIL (10 μM) and NS398 (5 μM) were used as positive NO and PGE_2 production controls, respectively. Furthermore, the cytotoxic effect of mangiferin was evaluated in peritoneal macrophages isolated from C57BL/6 mice using MTT assays, but neither affected cell viability at the concentrations used (50, 100, 200 μM) to inhibit NO and PGE_2 productions (IC_{50} value of 415.8 ± 11.0 μM).

Effects of mangiferin on LPS-induced iNOS and COX-2 protein and mRNA expressions – Several studies have demonstrated that induction of iNOS and COX-2 produces a large amount of NO and PGE_2 during endotoxemia and under inflammatory conditions (Simon., 1999). So, we investigated whether the inhibitory effects of mangiferin (50, 100, 200 μM) on NO and PGE_2 are related to iNOS and COX-2 modulation using Western blot and RT-PCR. In unstimulated RAW 264.7 macrophages, iNOS and COX-2 protein and mRNA levels were barely detectable. However, iNOS and COX-2 protein and mRNA levels were markedly expressed by LPS stimulation. Mangiferin significantly inhibited iNOS and COX-2 protein expressions in a concentration-dependent manner (Fig. 4A). Moreover, RT-PCR analysis showed that level of iNOS mRNA expression was correlated with their protein level. However, COX-2 mRNA expression was barely reduced by mangiferin (Fig. 4B). Mangiferin also had no affect on the expression of the housekeeping gene, β-actin.

Effects of mangiferin on LPS-induced TNF-α and IL-6 release and their mRNA expressions – To examine the effects of mangiferin on pro-inflammatory cytokine productions, RAW 264.7 macrophages were incubated with mangiferin in the presence of LPS. LPS-induced TNF-α and IL-6 levels were evaluated using enzyme immunoassays (EIAs) and RT-PCR. Mangiferin reduced TNF-α and IL-6 productions (Fig. 5A) and their mRNA expressions (Fig. 5B) in a concentration-dependent manner. Treatment with mangiferin at 200 μM potently reduced the LPS-induced TNF-α and IL-6 productions by 48% and 73%, respectively. These findings suggest that mangiferin inhibits various inflammatory mediators not only NO and PGE_2, but also pro-inflammatory cytokines.

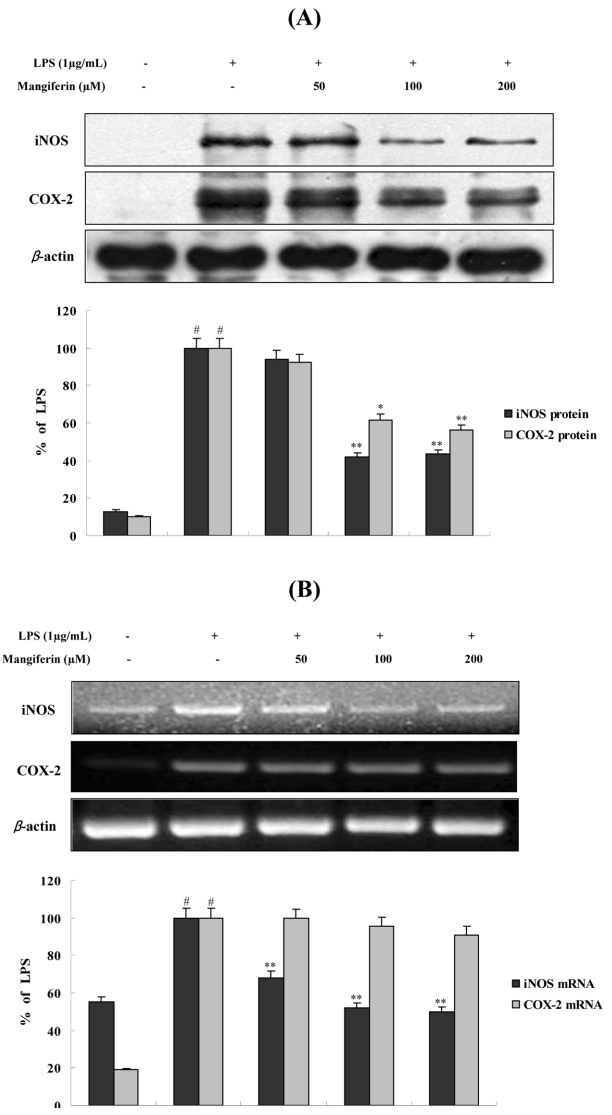

Fig. 4. Effects of mangiferin on LPS-induced iNOS, COX-2 protein (A) and their mRNA expressions (B) in RAW 264.7 macrophages.

(A) Cells were treated with different concentrations (50, 100, 200 μM) of mangiferin for 1 h, then LPS (1 μg/mL), and incubated for 24 h. Total cellular proteins (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected with specific antibodies, as described in methods. A representative immunoblot of three separate experiments is shown. (B) Total RNA was prepared for the RT-PCR analyses of iNOS, COX-2 gene expressions in RAW 264.7 macrophages pretreated with different concentrations (50, 100, 200 μM) of mangiferin for 1 h followed by LPS (1 μg/mL) treatment for 4 h. iNOS-specific sequences (807 bp) and COX-2-specific sequences (721 bp) were detected by agarose gel electrophoresis, as described in methods. PCR of β-actin was performed to verify that the initial cDNA contents of the samples were similar. The experiments were repeated three times and similar results were obtained. Density ratio of mangiferin-treated group over LPS only treated group or control group were measured by densitometer. The values are the mean ± S.D. of three independent experiments. $^{\#}p < 0.05$ vs. the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the LPS-treated group; significances between treated groups were determined using the ANOVA and Dunnett post-hoc test.

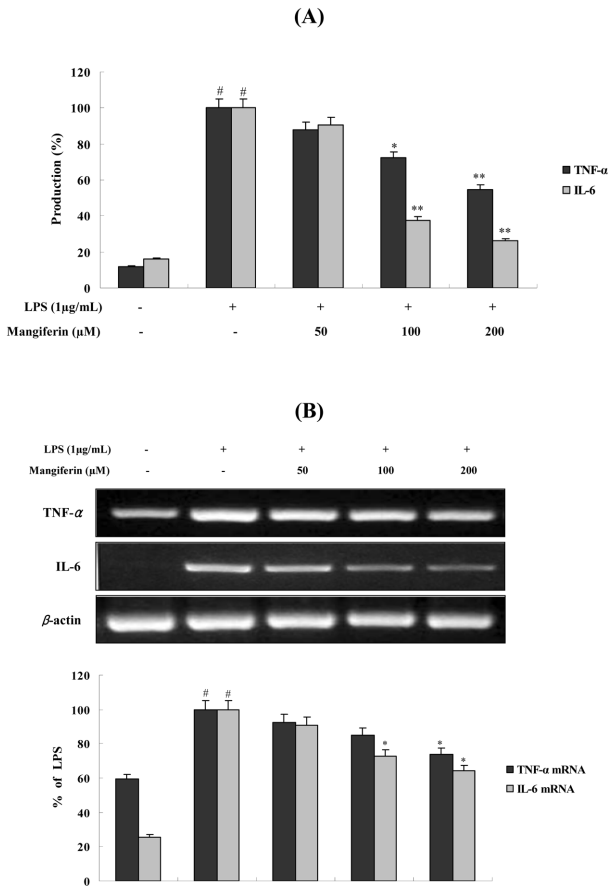

Fig. 5. Effects of mangiferin on LPS-induced TNF-α and IL-6 release (A) and their mRNA expressions (B) in RAW 264.7 macrophages.

(A) Cells were pretreated with different concentrations (50, 100, 200 μM) of mangiferin for 1 h and then LPS (1 μg/mL) was added and the cells were incubated for 24 h. Control (Con) values were obtained in the absence of both LPS and tested sample. (B) Total RNA was prepared for the RT-PCR analyses of TNF-α and IL-6 gene expressions in RAW 264.7 macrophages pretreated with different concentrations (50, 100, 200 μM) of mangiferin for 1 h followed by LPS (1 μg/mL) treatment for 4 h. TNF-α-specific sequences (351 bp) and IL-6-specific sequences (142 bp) were detected by agarose gel electrophoresis. PCR of β-actin was performed to verify that the initial cDNA contents of the samples were similar. The experiments were repeated three times and similar results were obtained. Density ratio of mangiferin-treated group over LPS only treated group or control group were measured by densitometer. The values are the mean ± S.D. of three independent experiments. $^{\#}p < 0.05$ vs. the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the LPS-treated group; significances between treated groups were determined using the ANOVA and Dunnett post-hoc test.

Effects of mangiferin on LPS-induced NF-κB activation – Since the activation of NF-κB is critically required for the activations of iNOS, COX-2, TNF-α and IL-6 by LPS (Surh *et al*., 2001; Lappas *et al*., 2002), we examined the effects of mangiferin on LPS-stimulated NF-κB-dependent reporter gene expression. We used a

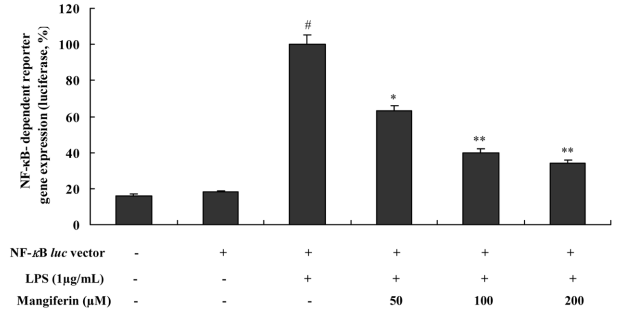

Fig. 6. Effects of mangiferin on the LPS-induced NF-κB activation RAW 264.7 macrophages.

Cells were transiently co-transfected with pNF-κB-Luc reporter and then left untreated (Con) or were pretreated with different concentrations (50, 100, 200 μM) of mangiferin. LPS (1 μg/mL) was then added and the cells were further incubated for 24 h. Luciferase activities were determined using a Promega luciferase assay system and a luminometer. Values represent means ± S.D. of three independent experiments. $^{\#}p < 0.05$ vs. the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the LPS-treated group; significances between treated groups were determined using the ANOVA and Dunnett post –hoc test.

pNF-κB-luc plasmid, which was generated by inserting four spaced NF-κB binding sites into pLuc-promoter vector. RAW 264.7 macrophages were transiently transfected with pNF-κB-luc plasmid and then stimulated with 1 μg/mL LPS either in the presence or absence of mangiferin (Fig. 6). Mangiferin treatment significantly reduced the LPS-induced increase in NF-κB-dependent luciferase enzyme expression (about 66% inhibition at 200 μM mangiferin). Taken together, the above findings show that mangiferin suppresses iNOS, COX-2, TNF-α and IL-6 expressions at least in part via an NF-κB-dependent mechanism.

Discussion

Polyphenol mangiferin is one of xanthone derivatives and consists of glucose as C-glucosylxanthone. This chemical is widely distributed in higher plants such as *Anacardiaceae* and *Gentianaceae* families, especially in the leaves and the bark (Yoshimi *et al*., 2001). Since some of natural and synthetic xanthones have shown anti-inflammatory activities (Lin *et al*., 1996; Madan *et al*., 2004; Librowski *et al*., 2005; Yamakuni *et al*., 2006), we examined the anti-inflammatory effects of mangiferin in RAW 264.7 macrophages and peritoneal macrophages isolated from C57BL/6 mice activated with LPS.

NO is short-lived free radical gas that has a variety of functions including vasodilation, neurotransmission, and tumouricidal and microbicidal activities (Nathan and Xie., 1994). A role for NO as a major mediator of text inflammation has been clearly demonstrated, and there is extensive evidence implicating NO in the pathophysiology of inflammatory processes. A number of polyphenolic phytochemicals that also shown potent antioxidant activity can inhibit iNOS gene expression and NO generation in several type of cells, including macrophages (Lin and Lin., 1997; Virgil *et al*., 1998; Xiong *et al*., 2000; Chen *et al*., 2000; Luceri *et al*., 2002; Tsai *et al*., 1999). Our results are also in line with previous results indicating that mangiferin inhibits NO production (Fig. 2A and Fig. 3) and markedly reduces both iNOS protein and iNOS mRNA level in RAW 264.7 macrophages and mouse peritoneal macrophages stimulated with LPS (Fig. 4A and B).

Moreover, a large body of evidence suggests that PGs are involved in various pathophysiological processes, including inflammation and carcinogenesis, and COX-2 is mainly responsible for the productions of large amounts of these mediators (Simon., 1999). Previous studies have shown that NO is necessary for maintaining prolonged COX-2 gene expression, and both iNOS and COX-2 expressions by macrophages generate massive productions of NO and PGE_2, both of which have cytotoxicity and pro-inflammatory activities (Perkins and Kniss., 1999). Based on this information, efforts have been made to reveal the anti-inflammatory activities of mangiferin on LPS-induced PGE_2 production in macrophages (Fig. 2A and Fig. 3). With our results that mangiferin barely reduced the expression of COX-2 mRNA level measured by RT-PCR but strongly inhibited LPS-induced COX-2 protein level (Fig. 4A and B), it can infer the influences of mangiferin on the translation stage or decrease of COX-2 protein stability. The molecular mechanism responsible for reducing the PGE_2 production and COX-2 protein level by mangiferin awaits further investigation. COX-2 expression is regulated at transcription, post-transcription and translation. COX-2 is an *N*-glycoprotein with four glycosylation sites (Nemeth *et al*., 2001; Hla and Neilson., 1992). Protein glycosylation of protein affects the stability, activity, and/or cellular localization of protein. Previous reports demonstrated that glucosamine hydrichloride inhibits COX-2 expression at protein level via a mechanism associated with the proteosome-dependent down-regulation *N*-glycosylation and turnover (Jang *et al*., 2007).

Cytokines are local protein mediators, now known to be involved in almost all important biological process, including cell growth and activation, inflammation, immunity and differentiation (Feldmann *et al*., 1996). Macrophage activation by LPS induces the secretion of

pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, TGF-β and GM-CSF (Molloy *et al*., 1993). The productions of TNF-α are crucial for the synergistic induction of NO synthesis in IFN-γ and/or LPS-stimulated macrophages (Jun *et al*., 1995). TNF-α elicits a number of physiological effects including septic shock, inflammation, cachexia and cytotoxicity (Mannel and Echtenacher., 2000). The cumulative evidence suggests that TNF-α is not only an inflammatory mediator but is also the key regulator of productions of other cytokines involved in inflammation, such as IL-1, GM-CSF, IL-6 and IL-8 (Haworth *et al*., 1991; Butler *et al*., 1995). IL-6 is also pivotal pro-inflammatory cytokine, regarded as an endogenous mediator of LPS-induced fever. In the present study, we found that mangiferin also significantly inhibits TNF-α, IL-6 release and its mRNA expressions (Fig. 5A and B). The inhibition of TNF-α and IL-6 expressions at the gene levels evidenced reductions in their protein levels in a parallel concentration-dependent manner. Thus, the inhibition of TNF-α and IL-6 release may be attributed to the suppression of their mRNA transcription. The inhibition of the LPS-stimulated expressions of these molecules in RAW 264.7 macrophages by mangiferin was not due to mangiferin cytotoxicity, as assessed by MTT assay (Fig. 2B) and the expression of the housekeeping gene β-actin.

NF-κB is known to play a critical role in the regulation of cell survival genes and to coordinate the expressions of pro-inflammatory enzymes and cytokines, such as iNOS, COX-2, TNF-α and IL-1β, IL-6 (Surh *et al*., 2001). In previous study, it revealed that mangiferin modulates the expressions of diverse genes related to the NF-κB signaling pathway, using a DNA hybridization array containing 96 NF-κB related genes (Leiro, 2004). Taking into account the previous finding, we examined influence of mangiferin in NF-κB activation to confirm that the inhibition effects of mangiferin on iNOS, TNF-α and IL-6 expressions were regulated by the NF-κB signaling pathway. Our results indicated that NF-κB activation is inhibited in a concentration-dependent manner by mangiferin (Fig. 6). From these, it is suggested that mangiferin exerts its anti-inflammatory effects in part thought transcriptional down-regulation of NF-κB.

In conclusion, we found that mangiferin is a potent inhibitor of LPS-induced NO, PGE_2, TNF-α and IL-6 productions and it acts at the transcription levels (excluding PGE_2). These effects were partially caused by the prevention of NF-κB activation in inflammatory macrophages. Therefore, we concluded that mangiferin might be value in the treatment for immunophathological disorders characterized by overproduction of pro-inflammatory mediators, such as inflammatory diseases, pain disease, athero- sclerosis or septic shock.

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