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Effects of a Tetramethoxyhydroxyflavone on the Expression of Inflammatory Mediators in LPS-Treated Human Synovial Fibroblast and Macrophage Cells

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The inhibitory effects of 5,6,3',5'-tetramethoxy 7,4'-hydroxyflavone (labeled as p7F) were elucidated on the productions of proinflammatory cytokines as well as inflammatory mediators in human synovial fibroblasts and macrophage cells. p7F inhibited IL-1β or TNF-α induced expressions of inflammatory mediators (ICAM-1, COX-2, and iNOS). p7F also inhibited LPS-induced productions of nitric oxide and prostaglandin E_2 in RAW 264.7 cells. In order to investigate whether p7F would inhibit IL-1 signaling, p7F was added to the D10S Th2 cell line (which is responsive to only IL-1β and thus proliferates), revealing that p7F inhibited IL-1β-induced proliferation of D10S Th2 cells in a dose-response manner. A flow cytometric analysis revealed that p7F reduced the intracellular level of free radical oxygen species in RAW 264.7 cells treated with hydrogen peroxide. p7F inhibited IκB degradation and NF-κB activation in macrophage cells treated with LPS, supporting that p7F could inhibit signaling mediated *via* toll-like receptor. Taken together, p7F has inhibitory effects on LPS-induced productions of inflammatory mediators on human synovial fibroblasts and macrophage cells and thus has the potential to be an anti-inflammatory agent for inhibiting inflammatory responses.

Keywords: Anti-inflammatory agent, flavone, synovial fibroblasts, inflammatory mediators, proinflammatory cytokines

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The inflammatory response, which is a complex series of vascular, leukocyte, and plasma-interactive events that occur in response to an injury, is critical in containing and ultimately eliminating infectious agents as well as promoting wound healing following tissue injury. These appropriate inflammatory responses contribute to the pathogenesis of many diseases including the response to systemic infection and systemic lupus erythematosus [3]. Chronic inflammatory diseases, such as rheumatoid arthritis (RA) or inflammatory bowel diseases, are characterized by an accumulation of immune cells such as phagocytic lymphocytes and macrophages at the site of injury [11]. Proliferation, differentiation, and activation of these cells of the hematopoietic lineage are controlled by cytokines, among which the proinflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF-α) play a key role [12]. These proinflammatory cytokines not only activate cells of the immune system but also stimulate cells of affected organs to enhance their own synthesis as well as those of other inflammatory mediators such as prostaglandins, reactive oxygen species (ROS), and nitric oxide (NO) [14]. IL-1 stimulates the proliferation of fibroblasts and production of prostaglandin (PG) E_2 and connective tissue degrading metalloproteinases by fibroblasts and chondrocyte [7, 13]. TNF-α is synthesized by monocytes, macrophages, lymphocytes, neutrophil granulocytes, mast cells, fibroblasts, and endothelial cells, usually together with IL-1 [13]. Expression of cytokines is regulated by transcription factors such as nuclear factor-*k*B (NF-κB), activating protein-1 (AP-1), and nuclear factor of activated

T cells (NF-AT). NF-κB is a transcription factor that acts as a central mediator of the immune response and regulates the transcription of various inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α as well as genes encoding adhesion molecules, immunoreceptors, hematopoietic growth factors, and growth factor receptors [10, 18, 27]. NF-κB response elements have been demonstrated to be on the promoter regions of inducible nitrogen oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which are involved in inflammatory processes by producing NO and PGs, respectively. Compounds that inhibit the activation of transcription factors and especially that of NF-κB may suppress the production of these inflammatory mediators and are regarded as promising drug candidates for the treatment of inflammatory diseases [10]. Flavonoids are substances of plant origin containing flavone (2-phenyl-4H-1-benzopyran-4-one) and are naturally occurring plant polyphenols found in abundance in diets rich in fruits, vegetables, and plant-derived beverages such as tea. Epidemiological studies have shown that the daily intake of mixed flavonoids is associated with a decreased risk of cancer and is believed to play an important role in delayed or prevented chronic inflammatory diseases [29–31, 37–41]. Plant-derived medicines have been found to be rich in polyphenolic compounds [23]. These include flavonoids, tannins, and anthraquinones and have been shown to exhibit anti-inflammatory activities. The exact mechanism of anti-inflammatory action of these compounds is not well established. Based on the above considerations, we screened anti-inflammatory materials from many herbal medicines in a previous study [23]. *Artemisia* has been traditionally used in Korea for clearing damp heat and jaundices and for treating uterine metrorrhagia and metritis. The antibacterial, antimalarial, antihelmintic, acaricidal, and hepatoprotective effects of *Artemisia absinthium* have been investigated [2, 16]. This study was focused on the anti-inflammatory effect of an isolated tetramethoxyhydroxyflavone from *Artemisia absinthium* and we elucidated that this p7F would inhibit the activation of transcription factor, NF-κB, and thus suppress the production of these inflammatory mediators and hence can be regarded as a promising drug candidate for the treatment of inflammatory diseases.

MATERIALS AND METHODS

Reagents

Lipopolysaccharide (LPS) (*Escherichia coli* 0127: E8) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and p7F (5,6,3',5'-tetramethoxy7,4'-hydroxy flavone) was isolated from the dried Korean herbal plant *Artemisia absinthium* as in our previous report [25].

Cell Culture

Macrophage cell lines, RAW 264.7 and THP1, were obtained from the American Type Culture Collection. Cells were maintained at 37.5°C and 5% CO_2 in DMEM or RPMI-1640 (for THP-1 cells) containing 10% heat-inactivated fetal bovine serum (Gibco BRL, Gaithersburg, MD, U.S.A.), 2 mM glutamate, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were plated at a density of 1.0×10^6 cells/ml and allowed to attach for 2 h. For stimulation, the medium was replaced with fresh DMEM without phenol red, and the cells were then stimulated with 0.1 μg/ml of LPS in the presence or absence of p7F for the indicated periods.

D10S Th2 Cell Culture

D10S, a subclone of the D10N Th2 cell line, was maintained in RPMI-1640 medium containing 10% FBS and 5% T cells conditioned medium as described previously [42]. For stimulation, D10S cells were washed twice with RPMI, plated at a density of 1.0×10^5 cells/ml, and then stimulated with 2 ng/ml of IL-1β in the presence or absence of p7F. After incubation for 40 h, 10 μl of cell proliferation reagent WST-1 was added to the plates. After more incubation for 0.5 to 4 h, the plates were shaken thoroughly for 1 min with a shaker. The absorbance was measured with an ELISA reader (Molecular Devices) at 490 nm. [^{3}H] Thymidine was incorporated into D10S cells at the time of stimulation with 2 ng/ml of IL-1beta in the presence or absence of p7F and the proliferation was assessed by beta counter after harvesting cells.

Synovial Cell Culture

Human rheumatoid arthritis (RA) synovial tissue samples were obtained from patients as previously described [35] with some modifications. Tissues were minced and digested with 0.2% collagenase Worthington type 1 (Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.) for 30–40 min at 37°C with agitation. Large debris was allowed to settle, and the supernatant containing single cells and small clusters was removed and centrifuged to pellet the liberated cells. The resulting cell pellets were washed twice with DMEM containing 10% FBS, supplemented with antibiotics. The synovial cells were seeded into 100-mm plastic tissue culture dishes and grown to confluence in the same medium. Fibroblast-like synovial fibroblast cells were used between passages 2 and 9.

RT-PCR

After incubation of 5×10^6 cells with p7F in the presence or absence of LPS for 12 h, total cellular RNA was extracted with TRIzol reagent (Gibco BRL). Reverse transcription was performed following the BRL protocol for Superscript II reverse transcriptase (Gibco BRL). Briefly, 5 μg of total RNA was incubated with 0.1 mM oligo-dT primers for 10 min at 65°C. This was followed by incubation with 5 mM DTT, 0.5 mM deoxynucleotide triphosphate mixture (dNTPs, Stratagene Cloning Systems Inc., La Jolla, CA, U.S.A.), and 200 U of M-MLV reverse transcriptase (RT) for 2 h at 37°C. The reverse transcription was terminated by heating for 5 min at 95°C. To remove RNA complementary to the complementary DNA (cDNA), RNase H (Gibco BRL) was added, and the sample was incubated for 20 min at 37°C. The cDNA obtained was used for the PCR. The oligonucleotide primers used were as follows: (a) hIL-1β (PCR product 802 bp), 5'-CAG-CAG-TTG-GTC-ATC-TCT-TG-3' (5' primer) and 5'-CCA-GCA-GGT-GAA-ACG-TCC-A-3' (3' primer); (b) hTNF-α (PCR product 727 bp), 5'-GGA-AAG-GAC-ACC-ATG-AGC-AC (5' primer) and 3'-ATG-TTC-GTC-CTC-CTC-ACA-GG-3' (3' primer); (c) hIL-6 (PCR product 462 bp), 5'-ATC-CTC-GAC-GGC-ATC-TCA-GCC-3' (5' primer) and 5'-CTA-CAT-TTG-CCG-AAG-AGC-CCT-3' (3' primer); (d) hIL-4 (PCR product

463 bp), 5'-ATG-GGT-CTC-ACC-TCC-CAA-CT-3' (5' primer) and 5'-TTC-AGC-TCG-AAC-ACT-TTG-AA-3' (3' primer); (e) hIL-10 (PCR product 322 bp), 5'-CTT-TCA-AAT-GAA-GGA-TCA-GC-3' (5' primer) and 5'-AAA-CTC-ACT-CAT-GGC-TTT-GT-3' (3' primer); (f) human intercellular adhesion molecule (ICAM-1; PCR product 350 bp), 5'-GGC-CTC-AGC-ACG-TAC-CTC-TA-3' (5' primer) and 5'-TGC-TCC-TTC-CTC-TTG-GCT-TA-3' (3' primer); (g) COX-2 (PCR product 334 bp), 5'-AAA-AGA-ACG-TTC-GAC-TGA-ACT-G-3' (5' primer) and 5'-AGT-ATT-CAA-ACA-TCT-TTA-CTT-TCG-TTC-3' (3' primer); (h) iNOS (PCR product 372 bp), 5'-TTT-CCT-TAC-GAG-GCG-AAG-AA-3' (5' primer) and 5'-TGA-AGT-GGT-GCA-CTC-AGC-A-3' (3' primer); and (i) GAPDH (PCR product 500 bp), 5'-ACC-ACA-GTC-CAT-GCC-ATC-AC-3' (5' primer) and 5'-TCC-ACC-ACC-CTG-TTG-CTG-TA-3' (3' primer). PCR was performed using the AccuPower PCR PreMix (Bioneer, Daejeon, Korea): initial denaturation at 94°C with an amplification profile of each cycle consisting of denaturation for 30 sec at 94°C, annealing for 30 sec at 60°C, and elongation for 1 min at 72°C, followed by final extension for 7 min at 72°C. Twenty to forty cycles of PCR were performed to determine the linearity of the PCR amplification. PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. The reproductivity of our PCR was confirmed by repeated PCR analysis of the same samples. Various RNA levels were quantitated by PhosorImager analysis (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Nitrite Assay Using Griess Reagent and Western Blot Analyses of iNOS

The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction [20]. One hundred μl of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% w/v of *N*-1-naphthyl ethylenediamine solution). Absorbance of the mixture at 550 nm was measured with an ELISA reader (Bio-Tek ELx 800) (Bio-Tek Instruments Inc., Winooski, Vermont, U.S.A.). RAW 264.7 cells (2×10^6 cells) were individually stimulated with LPS or p7F for 24 h at 37°C. The cells were washed three times with PBS. The cell pellets were suspended in a buffer consisting of 20 mM Tris (pH 7.6), 0.4 M NaCl, 0.2 mM EDTA, 20% glycerol, 1.5 mM $MgCl_2$, 2 mM DTT, 0.4 mM PMSF, and 2 μg/ml each of leupeptin, pepstatin, and aprotinin. The suspended cells were then subjected to three freeze and thaw cycles. The soluble protein was obtained by centrifugation (12,000 rpm for 30 min at 4°C). Each 50 μg of protein was electrophoresed on 8% SDS-PAGE, and transferred to polyvinylidene difluoride membrane. After blocking with PBS containing 3% skimmed milk, the membranes were incubated with a murine anti-mouse iNOS monoclonal antibody (R&D Systems, Minneapolis, MN, U.S.A.) for 2 h at room temperature. The membranes were then incubated for 1 h with a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G secondary antibody, and the blots were finally developed with the enhanced chemiluminescence (ECL) method by using the Western blot detection system ECL Plus (Amersham Pharmacia Biotech, Uppsala, Sweden) as suggested.

Measurement of PGE_2 Production and Western Blot of COX-2 Protein

RAW 264.7 cells were subcultured in 6-well plates and incubated with p7F for 24 h. One hundred μl aliquots of culture media of control and treated cells were collected, centrifuged, and stored at −70°C until tested. The level of PGE_2 released in the culture medium was quantitated using a specific enzyme immunoassay (EIA) kit (Amersham Pharmacia Biotech.). COX-2 protein was detected by Western blot as described above. In order to detect COX-2 protein, goat anti-COX-2 polyclonal antibody (BD Transduction Laboratories, Lexington, KY, U.S.A.) was used as a primary antibody and followed by the horseradish peroxidase-conjugated sheep anti-goat IgG (Amersham Pharmacia Biotech.). The immunoblot was visualized using an Amersham ECL-film (Amersham Pharmacia Biotech.).

Western Blot of IκB

RAW 264.7 cells were subcultured in 6-well plates and incubated with p7F (25 μg/ml) for 30−120 min in the presence or absence of LPS (1 μg/ml). IκB degradation was detected with Western blot using rabbit anti-IκB antibody (Cell Signaling Technology, Inc.).

Transient Transfection and Luciferase Assay

RAW 264.7 cells were seeded in a 60-mm dish. When the cells were confluent, the medium was replaced with serum-free Opti-MEM (Gibco BRL). The cells were then transfected with the pNF-κB-Luc plasmid reporter gene (Hyclone, Palo Alto, CA, U.S.A.) using Lipofectamine (Gibco BRL). After 24 h incubation, the medium was replaced with complete medium. After 24 h, the cells were trypsinized and equal numbers of cells were plated in 12-well tissue culture plates for 12 h. Cells were then treated with LPS (100 ng/ml) and/or p7F for 24 h. Each well was washed twice with cold PBS and harvested in 150 μl of lysis buffer. Aliquots of 100 μl of cell lysate were used to assay luciferase activity with the LucLite luciferase reporter gene assay kit (Packard Instrument Co., Inc., Meriden, CT, U.S.A.). Luminescence was measured in a Top Count Microplate Scintillation and Luminescence Counter in single-photon counting mode for 0.05 min/well, following adaptation for 5 min in the dark. Luciferase activities were normalized to protein concentrations.

DCFH-DA Assay

The ROS scavenging activity of p7F was measured using the oxidant-sensitive fluorescent probe DCFH-DA (2',7'-dichlorofluorescein diacetate) as described previously [9]. DCFH (2',7'-dichlorofluorescein) converted from DCFH-DA by deacetylase within the cells is oxidized by a variety of intracellular ROS to 2',7'-dichlorofluorescein (DCF), a highly fluorescent compound. RAW 264.7 cells (1×10^6 cells/ml) were pre-incubated with PBS in the presence of p7F (25 μg/ml) for 15 min at 37°C in a water bath. Twenty μl DCFH-DA was then added and an additional 15 min incubation was performed. After stimulation with 250 μM H_2O_2 for 10 min, the relative green DCF fluorescence within living cells was measured using a FACS calibur flow cytometer (Becton Dickinson, Rutherford, NJ, U.S.A.). There was no interference between p7F and DCFH-DA.

Statistical Analysis

Data are expressed as the mean+SEM. Group means were compared by analysis of variance (ANOVA) using Fisher's least significant difference. ANOVA and correlation analyses were performed with the statistical packages Statview 512+ (BrainPower, Inc., Calabasas, CA, U.S.A.).

RESULTS

Flavonoid p7F Inhibits IL-1-induced Proliferation of D10S Th2 Cells

The flavonoids used in the present study are the major components of natural herbs harboring several biological functions. In order to elucidate whether 5,6,3',5'-tetramethoxy 7,4'-hydroxy flavone p7F would inhibit IL-1-induced proliferation of D10S Th2 cells, D10S cells were cultured with IL-1β in the presence or absence of p7F and the proliferation was assessed by MTS assay and [^{3}H] thymidine incorporation, which revealed that p7F inhibited IL-1-induced proliferation of D10S cell in a dose-response manner and showed a little cytotoxicity at the high concentration of 40 μg/ml (Fig. 1).

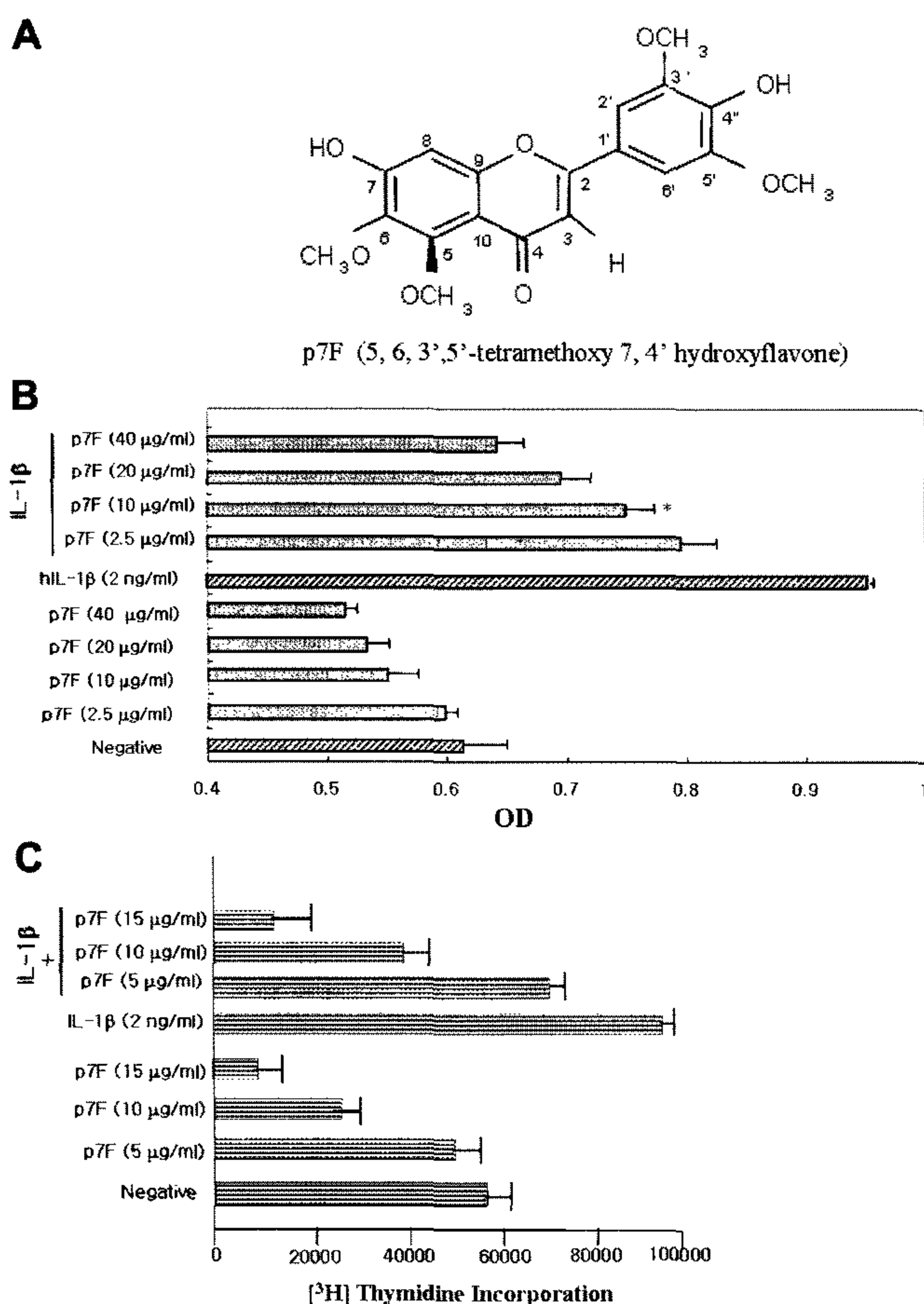

Fig. 1. Chemical structure of flavonoid p7F, 5,6,3',5'-tetramethoxy 7,4'-hydroxyflavone (**A**), and its effect on IL-1-induced proliferation of the D10S cell line, a subclone of the D10N T helper cell type 2 cell line (**B**, **C**).

On the basis of ESI-MS, UV, and NMR data, the active compound isolated from *Artemisia absinthium* was identified as 5,6,3',5'-tetramethoxy 7,4'-hydroxy flavone and labeled as p7F as previously described [25] (**A**). Cells were seeded in a 96-well plate at concentration of 5×10^5 cells/ml. After incubation for 40 h in the presence or absence of p7F, 10 μl of the tetrazolium salt WST-1 (Boerhinger Mannheim, Germany) was dispensed into these plates, and the absorbance was measured at 490 nm by an ELISA reader (**B**). The proliferation of D10S cells was evaluated by [^{3}H] thymidine incorporation as described in Materials and Methods (**C**). *Significant compared with IL-1-induced proliferation of D10S cells ($p < 0.05$). **Significant compared with non-treated D10S cells ($p < 0.01$).

Tetramethoxyhydroxyflavone p7F Inhibited TNF-α-Induced Expressions of the Proinflammatory Cytokines as Well as Inflammatory Mediators in RA Synovial Fibroblast Cells

In order to assess the effect of p7F on the production of cytokines and mediators related to the inflammatory reactions, the levels of various cytokines and mediators were measured by RT-PCR from the synovial cells. p7F downregulated the TNF-α-induced expressions of proinflammatory cytokines (IL-1β, TNF-α, and IL-6), whereas it enhanced the level of anti-inflammatory cytokines (IL-4 and IL-10) (Figs. 2B and 2C). In addition, p7F inhibited both TNF-α-induced expressions of inflammatory mediators (ICAM-1, COX-2, and iNOS) and LPS-induced TNF-α expression (Fig 2C). However, p7F showed more inhibitory effects on the TNF-α induced expression of proinflammatory cytokines and mediators than on IL-1β-induced expression of those factors (Fig. 2A). p7F increased the expression of IL-4, which was inhibited by IL-1 (Fig. 2C).

p7F Inhibited NO Production/iNOS Expression as Well as PGE_2 Production/COX-2 Gene Expression in RAW 264.7 Macrophages Stimulated by LPS

To examine whether p7F inhibited LPS-induced productions of NO and PGE_2 in RAW 264.7 cells, nitrite in the culture medium was estimated by the Griess reaction as an index for NO synthesis from the cells. p7F, even at the high concentration of 200 μg/ml, did not interfere with the reaction between nitrite and Griess reagents (data not shown). After 24 h incubation, unstimulated macrophages produced a background level of nitrite in the culture medium. After treatment with LPS (100 ng/ml) for 24 h, the concentrations of nitrite and PGE_2 in the medium increased remarkably (Figs. 3A and 3B). When RAW 264.7 macrophages were treated with p7F together with LPS for 24 h, the productions of inflammatory mediators, nitrite and PGE_2, were significantly decreased in the presence of p7F (Fig. 3). The inhibition of LPS-induced nitrite and PGE_2 by p7F was not due to their cytotoxicity on cells, because p7F at the concentration of 10 μg/ml did not decrease the cell viability (Fig. 1B). RAW 264.7 cells did not express detectable iNOS and COX-2 proteins when incubated in the medium without LPS for 24 h (Fig. 3), and the basal levels of iNOS and COX-2 proteins were not affected when incubated with p7F alone (data not shown). Upon LPS (100 ng/ml) treatment for 24 h, iNOS and PGE_2 proteins dramatically increased, and co-treatment of cells with LPS and p7F for 24 h significantly inhibited the inductions of iNOS and PGE_2 in RAW 264.7 macrophages (Fig. 3). The amount of α-tubulin protein as an internal control remained unchanged.

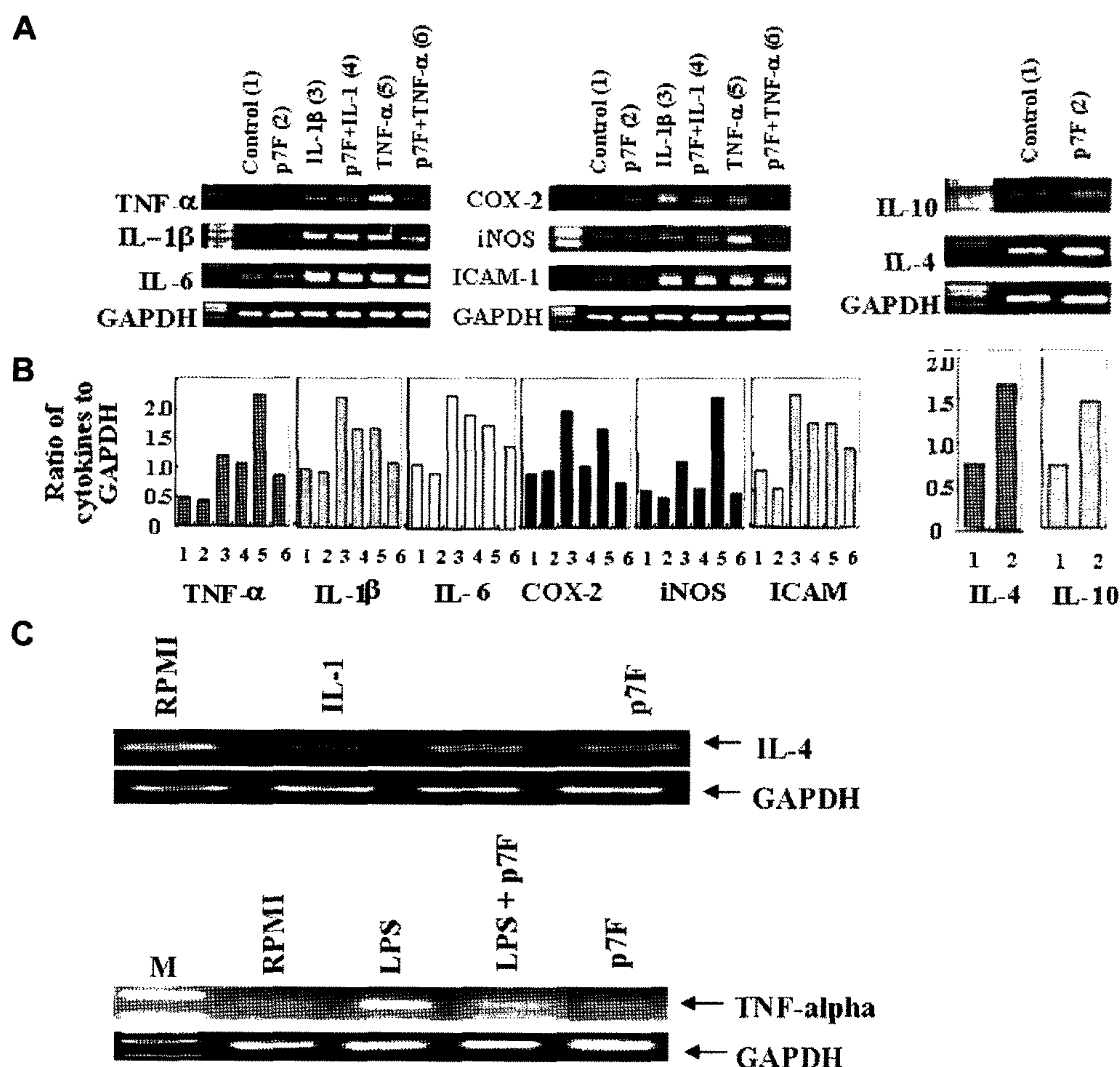

Fig. 2. The effect of tetramethoxyhydroxyflavone p7F on the productions of the proinflammatory cytokines and inflammatory mediators in RA synovial fibroblast cells.

Synovial fibroblast cells were treated with p7F and IL-1β or TNF-α for 24 h. **A**. Detection of mRNAs for the proinflammatory cytokines and inflammatory mediators were performed by RT-PCR as described in Methods. GAPDH mRNA was used as an internal control. **B**. Band intensities were quantitated by densitometry and the ratios of cytokines and GAPDH were determined. **C**. The effect of p7F on the expressions of IL-4 and TNF-α in RA synovial fibroblast cells stimulated by IL-1 or LPS.

p7F Suppressed IκB Degradation and the Activation of NF-κB in LPS-induced Macrophages

NF-κB is a transcription factor that is activated in response to stimulation by LPS, and the activation of NF-κB is an essential step in inducing iNOS and CPX-2 gene expressions in macrophages. In response to stimulation induced by LPS or other stimuli, IκB is degraded and NF-κB is then translocated into the nucleus where it is activated. Therefore, we examined whether p7F might suppress NF-κB activation in LPS-induced macrophages. To demonstrate the inhibition of NF-κB activation by p7F, a Luc-reporter plasmid containing 5 NF-κB binding sites in the enhancer element was transfected transiently and the levels of NF-κB activation were assessed. LPS induced IκB degradation after 60 min, whereas p7F inhibited LPS-induced IκB degradation (Fig. 4A) and p7F efficiently decreased the LPS-induced NF-κB luciferase activity (Fig. 4B) as expected.

p7F Reduced the Level of Intracellular ROS in RAW 264.7 Macrophages

Flavonoids are well known to have antioxidant effects. To investigate the intracelluar radical scavenging activity of p7F, the ROS level was evaluated using a DCFH-DA assay. RAW 264.7 macrophages showed relatively low levels of basal fluorescence in the absence of H_2O_2. When cells were treated with H_2O_2 for 10 min, a marked increase in the fluorescence was evident. However, the DCF fluorescence of H_2O_2-challenged macrophage significantly decreased because of the p7F pretreatment (Fig. 5).

Discussion

Our experiments suggested that p7F, an extracted flavonoid from *Artemisia absinthium*, might be effective in rheumatoid arthritis. Up to now, several flavonoids have been known

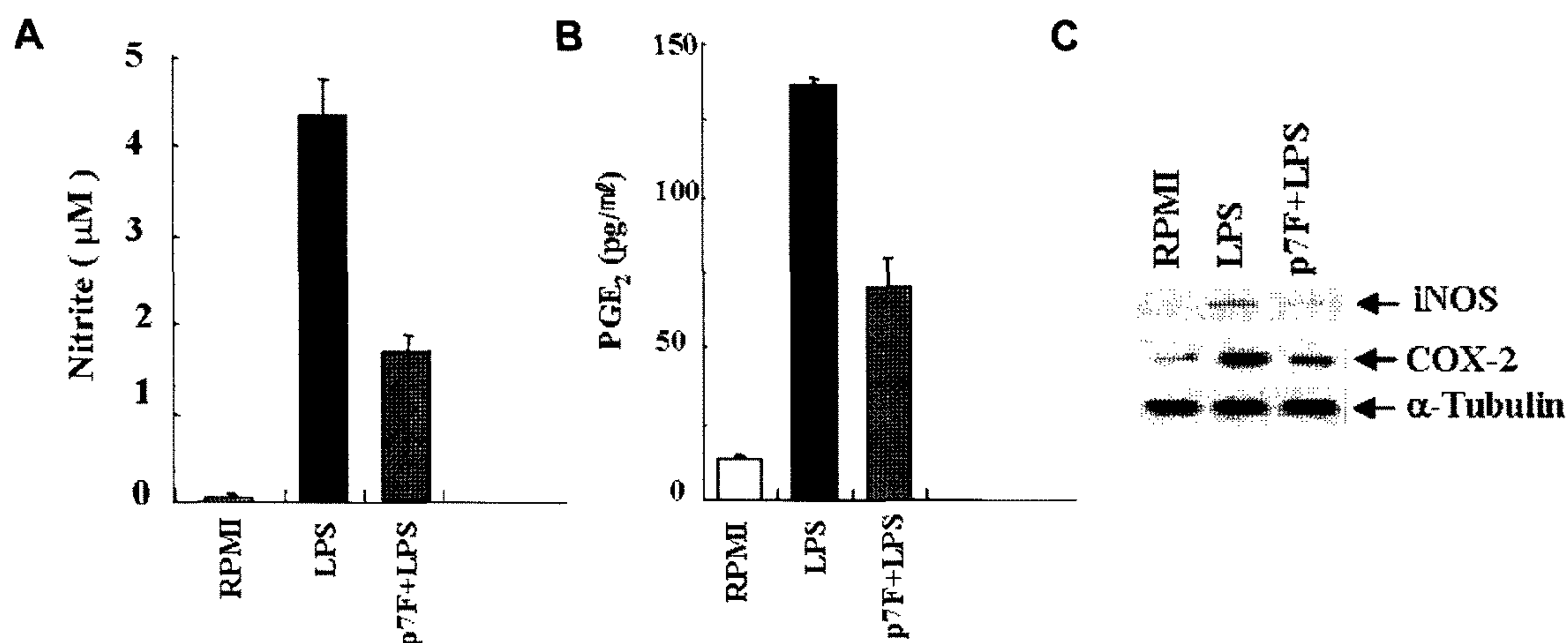

Fig. 3. Effects of p7F on LPS-induced NO production/iNOS expression as well as PGE_2 production/COX-2 gene expression in RAW 264.7 cells.

RAW 264.7 cells were treated with p7F (10 μg/ml) and LPS (100 ng/ml) for 24 h. Detection of LPS-induced nitrite synthesis was measured as an indicator of NO production according to the Griess reaction. PGE_2 synthesis was measured by an ELISA kit. iNOS and COX-2 proteins were identified by Western blotting. α-Tubulin protein was used as an internal control. *Significant compared with LPS-treated cells ($p<0.05$).

to have some beneficial effects in human. Certain flavonoids have been known to have antiarthritic effects [28]. Those are a citrus polymethoxyflavonoid, quercetin extracted from tea, onions, and apples [1, 28]. It is unclear at present whether flavonoids extracted from *Artemisia absinthium* are effective in the synovial tissue of rheumatoid arthritis.

A good therapy in chronic inflammatory diseases like RA must achieve several objectives, including the amelioration of signs and symptoms of disease and the abrogation of joint destruction, and must also be safe [8]. Acute cytotoxicity of p7F could not be found find in our experiment. Although some flavonoids have been known to be mutagenic, there are also reports that it rises as a rsult of error of the experimental system itself [26].

TNF-α, IL-1β, and IL-6 play central roles in the communication between innate and acquired immunities [24, 34]. These cytokines are produced by a variety of cells, of lymphoid and myeloid lineage, as well as by somatic tissues. These cytokines play pivotal roles in the induction of the innate immune response as well as in determining the magnitude and nature (Thl *vs* Th2) of the acquired immune response [13]. These proinflammatory cytokines also have the same actions of mechanism as described above in RA. Many studies have revealed that flavonoids suppress these proinflammatory cytokines and its mediators [9, 37, 38]. The p7F extracted in the present study also had similar actions in the above studies. The mechanism of flavonoids on the production of proinflammatory cytokines in human synovial fibroblasts and mouse macrophage cells was elucidated by the present study. We examined how p7F affects proinflammatory cytokines, inflammatory mediators, and anti-inflammatory cytokines in human synovial fibroblasts. Like other flavonoid studies, p7F downregulated the

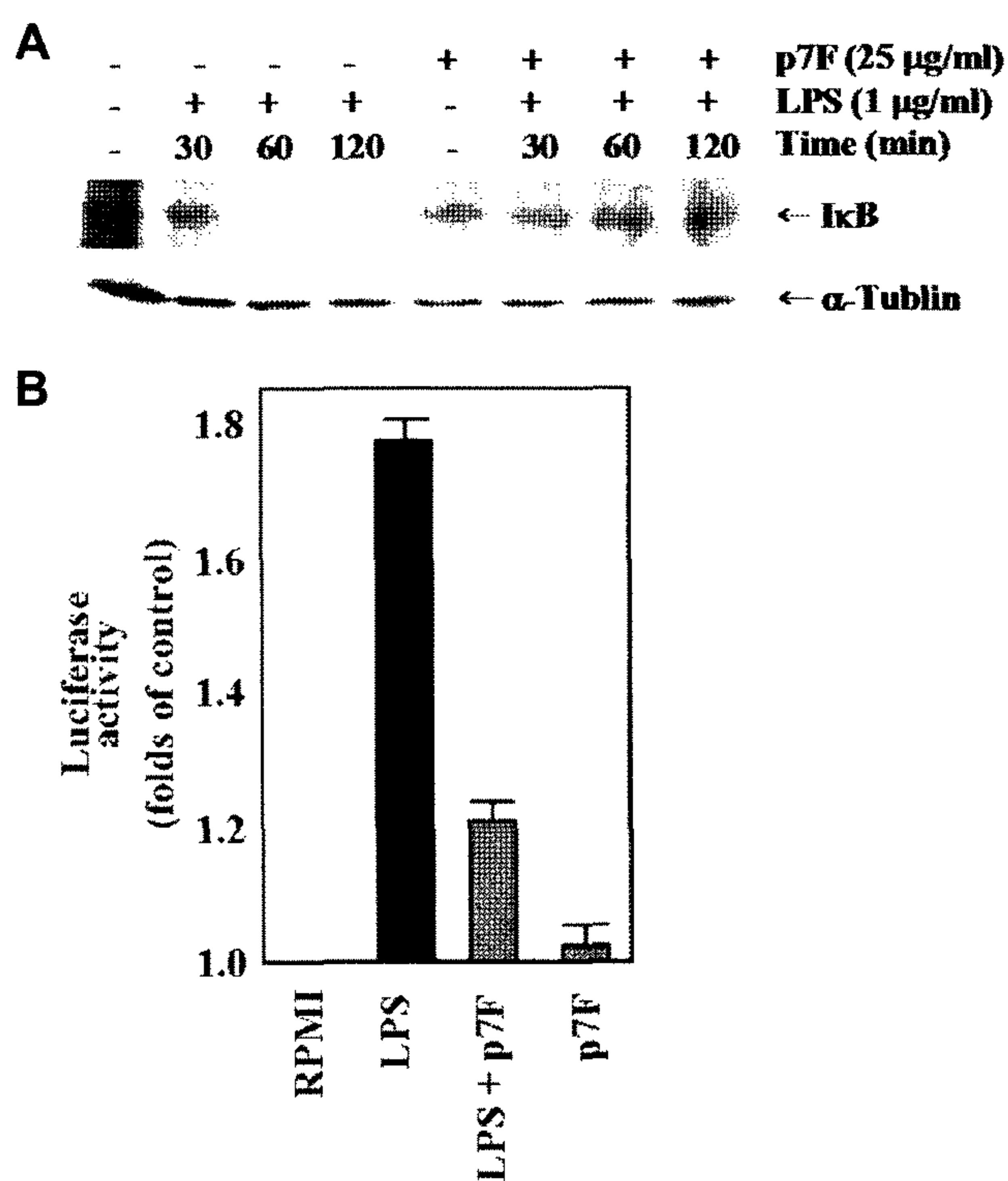

Fig. 4. Effects of p7F on LPS-induced IκB degradation and NF-κB promoter activities in machrophage cells.

A. THP-1 cells were subcultured in 6-well plates and incubated with p7F (25 μg/ml) for 30–120 min in the presence or absence of LPS (1 μg/ml). IκB degradation was detected with Western blot using rabbit anti-IκB antibody (Cell Signaling Technology, Inc.). α-Tubulin protein was used as an internal control. **B.** A reporter plasmid containing 5 NF-κB sites in its enhancer element was transiently transfected into RAW 264.7 cells for 24 h and further treated with LPS only, or LPS plus p7F for 24 h. The luciferase activity was measured by LucLite, and the control group value was defined as 1. *Significant compared with LPS-treated cells ($p<0.05$).

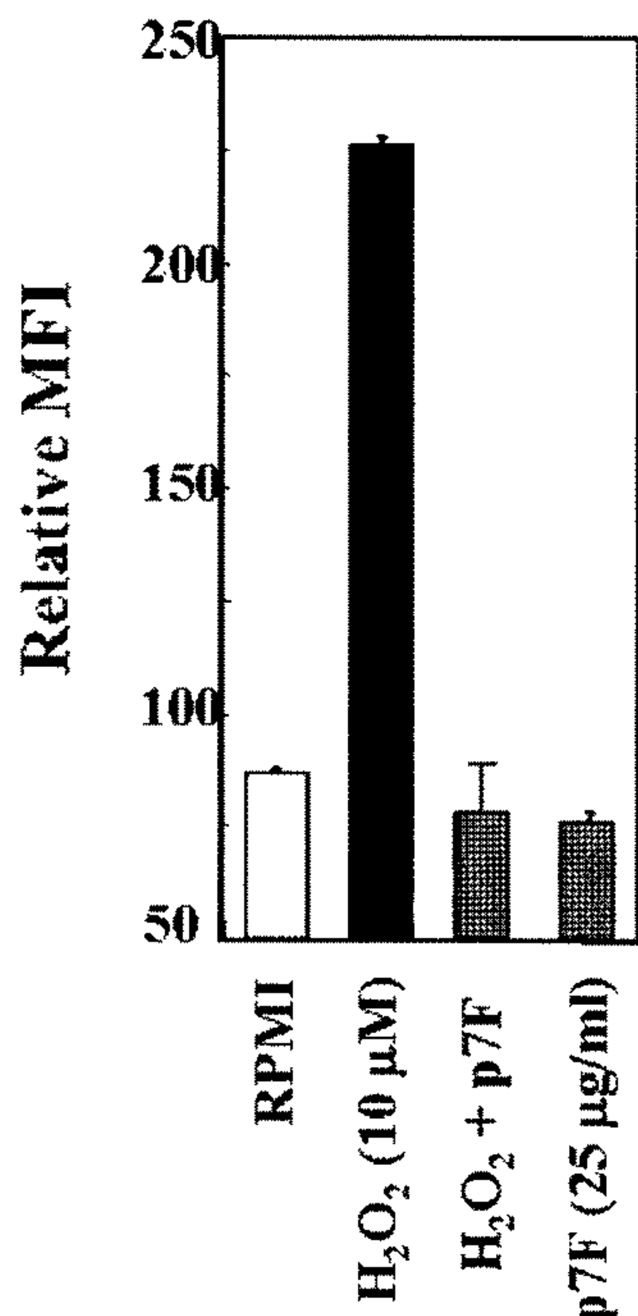

Fig. 5. The intracellular radical scavenging effect of p7F in RAW 264.7 cells.

RAW 264.7 cells were pretreated with p7F for 15 min and then 20 mM DCFH-DA was added. After 15 min incubation, the relative mean fluorescence intensity (MFI) in H_2O_2-treated cells in the presence or absence of p7F was measured by using a flow cytometer as described in Methods. *Significant compared with LPS-treated cells ($p<0.05$).

TNF-α-induced proinflammtory cytokines (IL-1β, TNF-α, IL-6) and inflammatory mediators (ICAM-1, COX-2, iNOS), whereas it enhanced the level of anti-inflammatory cytokines (IL-4, IL-10). p7F inhibited LPS-induced NO/ PGE_2 productions, iNOS/COX-2 gene expressions, and IκB degradation/NF-κB activation [21]. In addition, p7F scavenged intracellular ROS in RAW 264.7 cells. All of these results demonstrated that p7F can be used in the control of inflammatory diseases such as rheumatoid arthritis, because p7F definitely acts as anti-inflammatory agent influencing these cytokines.

COX-2, an inducible COX enzyme, is known to be a key enzyme for the production of PGE_2, which causes inflammation in rheumatoid arthritis [15, 28]. COX-2 is, therefore, an ideal target of rheumatoid diseases and osteoarthritis. In the present study, it was revealed that p7F downregulated COX-2 in human synovial fibroblasts and decreased the production of PGE_2 in RAW 264.7 macrophages. A similar observation using other flavonoids from a citrus has been reported [15, 28].

The NF-κB pathway is also a therapeutic target in inflammatory diseases because NF-κB plays an important role in the transcriptional activation of IL-1β, TNF-α, and promatrix metalloproteinases [17, 18]. NF-κB, AP-1, and NF-AT are transcription factors that regulate expression of cytokines [6, 22]. NF-κB, a pivotal mediator of the human immune system, regulates the transcription for IL-1, IL-2, IL-6, IL-8, TNF-α, cell adhesion molecules, hematopoietic growth factors, acute phase proteins, COX-2, and iNOS [5, 19, 32, 33]. Therefore, agents that inhibit activation of these transcription factors including NF-κB would have anti-inflammatory effects [4, 6]. p7F inhibited IκB degradation and is also likely to modulate NF-κB binding to the promotor gene of these cytokines. Our study revealed that TNF-independent IL-1 production and IL-1-induced D10S proliferation were also prevented with use of p7F, unlikely in some direct anti-TNF and anti-IL-1 agents. Our study also revealed that p7F inhibited not only IκB degradation but also activation of NF-κB in LPS-induced macrophages. Thus, p7F also likely targets an NF-κB pathway. However, p7F showed more inhibitory effect on the TNF-α-induced expression of proinflammatory cytokines and mediators than on the IL-1β-induced expression of those factors (Fig. 2). Anti-cytokine treatment of various animal models with inflammatory arthritis suggests that TNF-α may be more involved in inflammation, and IL-1 in the destruction of cartilage and bone [36]. It has been suggested in several studies that both TNF-α and IL-1 must be blocked for treatment of RA [8]. Thus, inhibition of the activation of NF-κB might also have better effects for control of rheumatoid arthritis [10].

In conclusion, both proinflammatory cytokines (IL-1β, TNF-α, IL-6) and inflammatory mediators (COX-2, iNOS, ICAM-1) induced by TNF-α were suppressed, whereas the expressions of anti-inflammatory cytokines such as IL-4 and IL-10 were enhanced in human RA synovial fibroblasts, demonstrating that p7F can be used as an anti-inflammatory agent in RA patients. In addition, p7F inhibited LPS-induced expressions of COX-2 and iNOS as well as efficiently decreased the LPS-induced NF-κB activation in RAW 264.7 macrophages. This mechanism appears to be eventually due to the inhibition of NF-κB activation. Thus, p7F, a new flavonoid from *Artemisia absinthium*, has the potential to be an anti-inflammatory agent for inflammatory diseases.

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