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Flavonoids Inhibit Tumor Necrosis Factor-α-Induced Up-Regulation of Intercellular Adhesion Molecule-1 (ICAM-1) in Respiratory Epithelial Cells through Activator Protein-1 and Nuclear Factor-κB: Structure-Activity Relationships

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ABSTRACT

Intercellular adhesion molecule-1 (ICAM-1) has been implicated in the processes of inflammation and carcinogenesis. Flavonoids, which are polyphenolic compounds with a wide distribution throughout the plant kingdom, have potent anti-inflammatory properties. We investigated the effects of flavonols (kaempferol, quercetin, and myricetin) and flavones (flavone, chrysin, apigenin, luteolin, baicalein, and baicalin) on the tumor necrosis factor-α (TNF-α)-stimulated ICAM-1 expression. Among those flavonoids tested, kaempferol, chrysin, apigenin, and luteolin are active inhibitors of ICAM-1 expression. Additional experiments suggested that apigenin and luteolin were actively inhibiting the IκB kinase (IKK) activity, the IκBα degradation, the nuclear factor-κB (NF-κB) DNA-protein binding, and the NF-κB luciferase activity. TNF-α-induced ICAM-1 promoter activity was attenuated using an activator protein-1 (AP-1) site deletion mutant, indicating the involvement of AP-1 in ICAM-1 expression. AP-1-specific DNA-protein binding activity was increased by TNF-α, and the supershift assay identified the components of c-fos and c-jun. Extracellular signal-regulated kinase (ERK) and p38 were involved in the c-fos mRNA expression, and c-Jun NH_2-terminal kinase (JNK) was involved in the c-jun mRNA expression. All three mitogen-activated protein kinase (MAPK) activities were inhibited by apigenin and luteolin. In comparison, kaempferol and chrysin only inhibited the JNK activity. The inhibitory effects of apigenin and luteolin on ICAM-1 expression are mediated by the sequential attenuation of the three MAPKs activities, the c-fos and c-jun mRNA expressions, and the AP-1 transcriptional activity. IKK/NF-κB pathway is also involved; however, kaempferol- and chrysin-mediated inhibitions are primarily executed through the attenuation of JNK activity, c-jun mRNA expression, and AP-1 activity. The structure-activity relationships are also explored, and the important role of –OH group at positions 5 and 7 of A ring and at position 4 of B ring is noted. Finally, our results suggested that AP-1 seems to play a more significant role than NF-κB in the flavonoid-induced ICAM-1 inhibition.

Intercellular adhesion molecule-1 (ICAM-1), a transmembrane glycoprotein of 505 amino acids, is a member of the immunoglobulin supergene family and contains five extracellular immunoglobulin-like domains that function in cell-cell and cell-matrix adhesive interactions (van de Stolpe and van der Saag, 1996). In contrast to other cell adhesion molecules, ICAM-1 is expressed in both hematopoietic and nonhematopoietic cells and mediates adhesive interactions by binding to two integrins belonging to the β2 subfamily, i.e., CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1). ICAM-1 adhesive interactions are critical for the transendothelial migration of leukocytes and the activation of T cells where ICAM-1 binding functions as a coactivation signal (Zuckerman et al., 1998). ICAM-1 is also associated with a variety of inflammatory diseases and conditions, including asthma, atherosclerosis, inflammatory bowl disease, acute respiratory distress syndrome, ischemia reperfusion injury, and autoimmune disease

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ABBREVIATIONS: ICAM-1, intercellular adhesion molecule-1; AP-1, activator protein-1; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; IκB, inhibitory protein of nuclear factor-κB; NIK, NF-κB-inducing kinase; IKK, IκB kinase; IL, interleukin; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; BCECF, 2′,7′-bis-(carboxyethyl)-5,6-carboxyfluorescein; RT-PCR, reverse transcription-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; MBP, myelin basic protein; GST, glutathione *S*-transferase; EMSA, electrophoretic mobility shift assay; Luc, luciferase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; LPS, lipopolysaccharide; PD98059, 2′-amino-3′-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; SP600125, anthra[19-*cd*]pyrazol-6(2*H*)-one.

(Sampath et al., 1979; Welty et al., 1993; Colletti et al., 1998; Kacimi et al., 1998; Stanciu and Djukanovic, 1998). ICAM-1 is present low on the cell surface of a variety of cell types, and it is up-regulated in response to a number of inflammatory mediators, including retinoic acid, virus infection, oxidant stresses, and the proinflammatory cytokines IL-1β, TNF-α, and interferon-γ (Bassi et al., 1995; van de Stolpe and van der Saag, 1996; Chen et al., 2000, 2001; Chang et al., 2002). The level of ICAM-1 expression on the surface of any given cell type depends on the concentrations of pro- and anti-inflammatory mediators, on the availability of specific receptor-mediated signal transduction pathways, and their nuclear transcription factor targeting on the ICAM-1 promoter (Cornelius et al., 1993). Nuclear transcriptional factors important to the activation of ICAM-1 expression include AP-1, NF-κB, CCAAT/enhancer-binding protein, Ets, signal transducer and activator of transcription (STAT), or SP1 (van de Stolpe and van der Saag, 1996). The major intracellular signal transduction pathways involved in the regulation of ICAM-1 expression include PKC-dependent c-Src activation of the NF-κB or the STAT1 signaling pathways (Chen et al., 2000, 2001; Chang et al., 2002, 2004; Huang et al., 2003). The abundance of signaling pathways and transcription factors involved in ICAM-1 transcription reflects the complex cell-type- and stimulus-specific regulation of the ICAM-1 gene. The modulation of ICAM-1 expression is an important therapeutic target, as shown by the beneficial effect of anti-ICAM-1 antibodies and other pharmacological agent on the progression of inflammatory responses in several in vivo studies (Barton et al., 1989; Albelda et al., 1994).

Flavonoids are polyphenolic compounds that occur ubiquitously in food plants and vegetables. The polyphenolic flavonoids, which have the diphenylpropane (C6C3C6) skeleton, include monomeric flavanols, flavanones, flavones, and flavonols. Individual differences in flavonoids result from the variation in number and arrangement of the hydroxyl groups as well as from the nature and the extent of alkylation and/or glycosylation of these groups. Some flavonoids have been found to possess various clinically relevant properties such as anti-tumor, anti-platelet, anti-ischemic, and anti-inflammatory activities (Avila et al., 1994; Gerritsen et al., 1995), and these effects are believed to come from the antioxidant properties of the related flavonoids (Rice-Evans et al., 1996). Despite numerous interest in their pharmacological activities as anti-inflammatory agents, only a few studies have investigated the role of flavonoids in the regulation of cell adhesion processes. In this study, we chose three flavonols (kaempferol, quercetin, and myricetin) and six flavones (flavone, chrysin, apigenin, luteolin, baicalein, and baicalin) (Fig. 1) to examine the inhibitory effect on the TNF-α-induced ICAM-1 expression in A549 epithelial cells, in which the molecular mechanism of this event has been explored to involve the PKC-dependent c-Src pathway in inducing IKK and NF-κB activation (Chen et al., 2001; Huang et al., 2003). The structure-activity relationship of these naturally occurring flavonoids was studied by comparing their potency in inhibiting ICAM-1 expression and inhibiting the signaling molecules involved. Our results revealed the contributions of 4′-hydroxy structure in the B ring and 5,7-metadihydroxy arrangement in the A ring, which might help the design of analogs displaying anti-inflammatory effect, and provided evidence for the correlation between the anti-inflammatory properties of flavonoids and their efficiency in inhibiting signaling molecules.

Materials and Methods

Materials. Mouse monoclonal anti-human ICAM-1 antibody and recombinant human TNF-α were purchased from R&D Systems (Minneapolis, MN). The rabbit polyclonal antibodies specific for IκBα, IKKβ, ERK2, p38, and JNK1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). T4 polynucleotide kinase and rabbit polyclonal antibodies specific for the phosphorylated forms of ERK1/2, p38, or JNK were obtained from New England Biolabs (Beverly, MA). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). 2′,7′-Bis-(carboxyethyl)-5,6-carboxyfluorescein (BCECF) was obtained from Molecular Probes (Eugene, OR). Poly(dI/dC) was obtained from Amersham Biosciences Inc. (Piscataway, NJ). Reagents for SDS-PAGE were purchased from Bio-Rad (Hercules, CA). [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Horseradish peroxidase-labeled donkey anti-rabbit second antibody and the ECL-detecting reagent were purchased from Amersham Biosciences Inc. The ICAM-1 promoter constructs, pIC1352, pIC339, and pIC1352 (ΔAP-1) (pIC1352 with the $-284/-279$ region deleted) were gifts from Dr. P. T. van der Saag (Hubrecht Laboratory, Utrecht, The Netherlands). The NF-κB-Luc and AP-Luc expression plasmids were purchased from Stratagene (La Jolla, CA).

Cell Cultures. A549 cells, an alveolar epithelial cell carcinoma, were obtained from American Type Culture Collection (Manassas, VA), and cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin in 96-well plates (ICAM-1 expression), in 6-well plates (transfection), in 6-cm dishes (IKK activity assay and RT-PCR), or in 10-cm dishes (NF-κB gel shift assay and MAPKs activation).

U937 cells, a human monocytic leukemia cell, were obtained from the Department of Microbiology (College of Medicine, National Taiwan University) and cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were split and fed every 3 to 4 days.

Quantification of ICAM-1 Expression. The level of cell surface ICAM-1 expression was determined by ELISA as previously described (Chen et al., 2000). Cells were treated with TNF-α for 4.5 h at 37°C following pretreatment with various flavonoids for 30 min. Each assay was performed in triplicate, and the basal absorbance (about 0.1 unit) was subtracted. None of the tested flavonoids affected the basal ICAM-1 expression.

Cell Adhesion Assay. A549 cells, grown in 96-well plates, were treated at 37°C with TNF-α for 4.5 h after pretreatment with flavonoids for 30 min, then washed twice with PBS. U937 cells were labeled for 30 min at 37°C with 10 ng/ml BCECF and washed twice with growth medium, then 2.5×10^5 of the labeled cell were added to the A549 monolayer in a final volume of 100 μl and incubated in a CO_2 incubator for 1 h. Nonadherent cells were removed from the plate by gentle washing with PBS, and the number of adherent cells were determined by measuring the fluorescent intensity using a CytoFluor 2300 (Millipore Corporation, Bedford, MA).

RT-PCR. Total RNA was isolated from A549 cell using Trizol reagent (Invitrogen). Reverse transcription reaction was performed using 2 μg of total RNA and reverse-transcribed into cDNA using oligo(dT) primer, then amplified 30 cycles using two oligonucleotide primers derived from published ICAM-1, c-*fos,* c-*jun*, or β-actin sequence, including 5′-TGCGGCTGCTACCACAGTGATGAT-3′ and 5′-CCATCTACAGCTTTCGGCGCCCA-3′ (ICAM-1), 5′-CAGTCAGATCAAGGGAAGCCACAGACATCT-3′ and 5′-GAATAAGATGGCTGCAGCCAAATGCCGCA3′ (c-*fos*), 5′-GAACCCCTCCTGCTCATCT-

GTCACGTTCTT-3′ and 5′-GGAAACGACCTTCTATGACGATGCCC-TCAA-3′ (c-*jun*), 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ and 5′-CTAGAAGCATTTGCGGGGACGATGGAGGG-3′ (β-actin). PCR is carried out at 94°C for 30 s, at 65°C for 30 s, and 1 min at 70°C for 34 cycles. The PCR products were subjected to 1% agarose gel electrophoresis. Quantitative data normalized to β-actin were obtained using a computing densitometer and ImageQuant software (Amersham Biosciences Inc.).

Immunoprecipitation and in Vitro Kinase Activity Assay. Following a 10-min treatment with TNF-α or 30-min pretreatment with various flavonoids before the addition of TNF-α, cells were rapidly washed with PBS, then lysed with ice-cold lysis buffer, and the IKK proteins were immunoprecipitated. Total cell extract (50 μg) was incubated for 1 h at 4°C with 0.5 μg of anti-IKKβ, anti-ERK2, anti-p38, or anti-JNK1 antibody and collected using protein A-Sepharose Cl-4B beads (Sigma-Aldrich, St. Louis, MO). The beads were then washed three times with lysis buffer without Triton X-100 and incubated for 30 min at 30°C in 20 μl of kinase reaction mixture containing 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, 1 μg of bacterially expressed GST-IκBα (1–100), 2 μg of MBP or GST-c-jun and 10 μM [γ-^{32}P]ATP. The reaction was stopped by the addition of Laemmli buffer and subjected to 10% SDS-PAGE, phosphorylated GST-IκBα (1–100), MBP or GST-c-jun being visualized by autoradiography.

Preparation of Nuclear Extracts and the Electrophoretic Mobility Shift Assay (EMSA). Control cells or cells pretreated with various flavonoids were treated with TNF-α for 1 h, then nuclear extracts were prepared as described previously (Chen et al., 2000). Oligonucleotides corresponding to the downstream NF-κB binding sequences (5′-AGC**TTGGAAATTCC**GGA-3′) or AP-1 se-

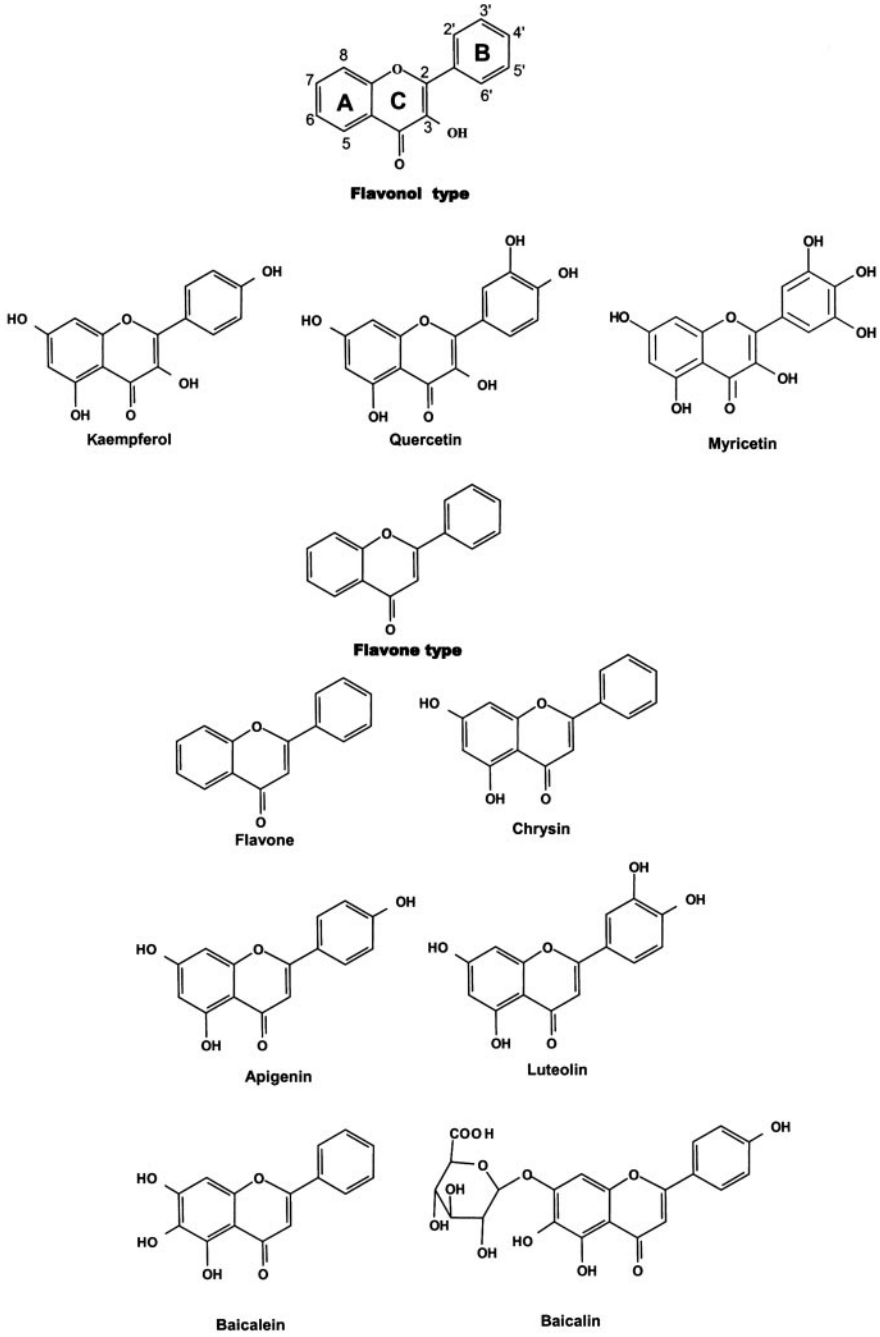

Fig. 1. Structure of the flavonoids.

quences (5′-GACCG**TGA**TTCAAGC-3′) in human ICAM-1 promoter were synthesized, annealed, and end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, and EMSA was performed as described previously (Chen et al., 2000). When supershift assays were performed, polyclonal antibodies specific for c-fos or c-jun were added to the nucleus extracts 30 min before the binding reaction.

Transient Transfection and Luciferase Assay. The human ICAM-1 firely luciferase (LUC) plasmids (pIC1352 or pIC1352ΔAP-1), or NF-κB or AP-1 luciferase reporter were transfected using Tfx-50 (Promega, Madison, WI) according to the manufacturer's recommendations. Briefly, reporter DNA (0.4 μg) and β-galactosidase DNA (0.2 μg) were mixed with 0.6 μl of Tfx-50 in 1 ml of serum-free DMEM. We used the plasmid PRK containing β-galactosidase gene driven by the constitutively active SV30 promoter to normalize the transfection efficiency. After a 10- to 15-min incubation at room temperature, the mixture was applied onto the cells. DMEM (1 ml) containing 10% FCS was added 1 h later, and the cells were grown in medium containing 5% FCS. The following day, cells were exposed to 10 ng/ml TNF-α or treated with various flavonoids for 30 min before challenge with TNF-α for 4.5 h, then cell extract was prepared, and luciferase (Promega) and β-galactosidase activity were measured. The luciferase activity of each well was normalized to β-galactosidase activity.

Preparation of Cell Extracts and Western Blot Analysis of Phosphorylated ERK1/2, Phosphorylated p38, Phosphorylated JNK, ERK2, p38, and JNK1. Following treatment with TNF-α or with various MAPK inhibitors before challenge with TNF-α for 10 min, the cells were rapidly washed with PBS, then lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM sodium fluoride, and 1 mM Na_3VO_4) as described previously (Chen et al., 2001). The lysates were subjected to SDS-PAGE using a 10% running gel. The proteins were transferred to nitrocellulose paper, and immunoblot analysis was performed as described previously (Chen et al., 2001).

Statistical Analyses. All data are expressed as mean ± S.E.M. Statistical analyses were done with Student's t test.

Results

Inhibitory Effects of Flavonoids on TNF-α-Induced ICAM-1 Expression on A549 Cells and U937 Adhesion to A549 cells. Three flavonols (kaempferol, quercetin, and myricetin) and six flavones (flavone, chrysin, apigenin, luteolin, baicalein, and baicalin) (Fig. 1) were chosen to test their inhibitory effects on TNF-α-induced ICAM-1 expression on A549 cells. Since 30 μM was the highest concentration that had no toxicity to the cells, this concentration was examined first. As shown in Fig. 2A, five showed inhibitory effects at 30 μM. The extent of inhibition for kaempferol, quercetin, chrysin, apigenin, and luteolin was 74, 61, 72, 97, and 98%, respectively. Their IC_{50} was 12.1, 14.4, 10.9, 0.82, and 0.80 μM, respectively (Fig. 2B). The action mechanism of kaempferol, chrysin, apigenin, and luteolin in inhibiting ICAM-1 expression was studied further. These four flavonoids also inhibited TNF-α-induced U937 adhesion to A549 cells in a dose-dependent manner (Fig. 2C).

Kaempferol, Chrysin, Apigenin, and Luteolin Inhibit TNF-α-Induced ICAM-1 mRNA Expression and ICAM-1 Promoter Activity. TNF-α has been shown to induce a dose- and time-dependent increase in ICAM-1 protein expression and U937 adhesion to A549 cells (Chen et al., 2001). It also induces the expression of ICAM-1 mRNA in a time-dependent manner; this effect is significant and maximal at 1 h, sustained at 3 h, and declined after 6 h (unpublished data). To determine the effects of these four flavonoids on TNF-α-induced ICAM-1 gene expression, cells were pretreated for 30 min with each of these compounds at a concentration of either 1 or 3 μM before the stimulation with TNF-α for 3 h. When ICAM-1 mRNA expression was analyzed by RT-PCR, kaempferol, cyrysin, apigenin, and luteolin

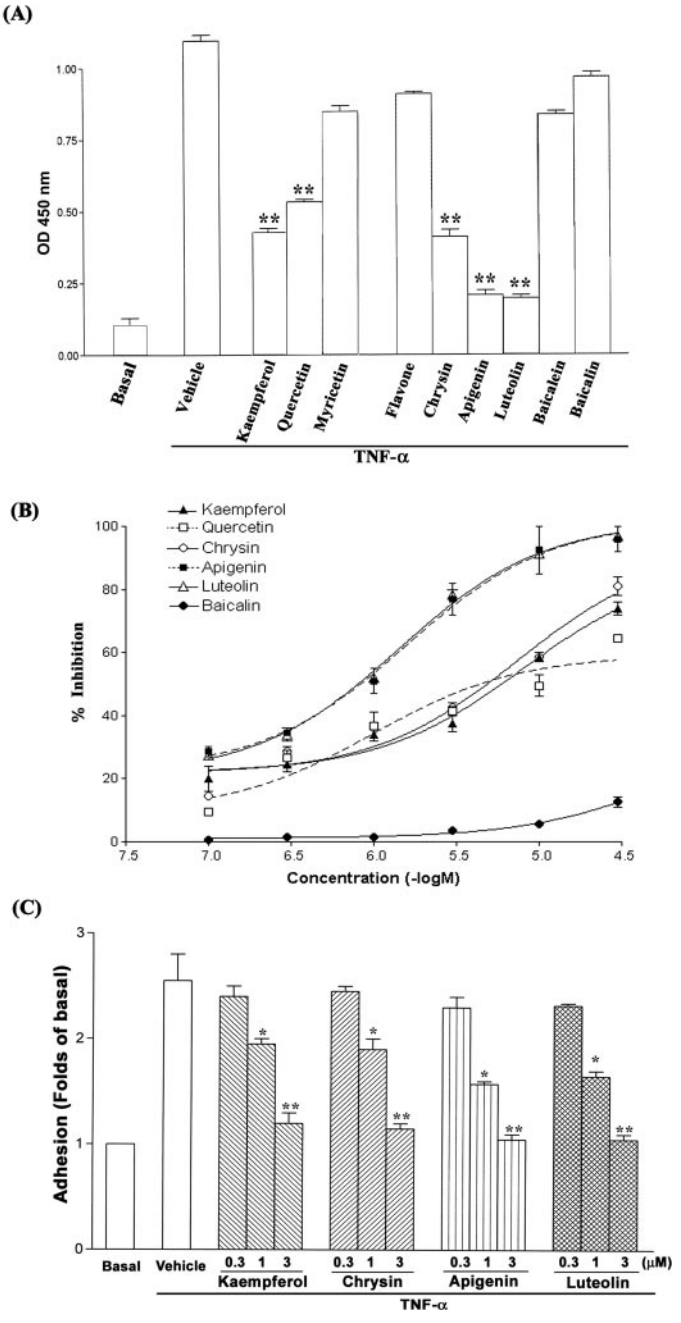

Fig. 2. Effects of various flavonoids on TNF-α-induced ICAM-1 protein expression and U937 cell adhesion to A549 epithelial cells. Cells were pretreated with vehicle or 30 μM of various flavonoids for 30 min (A) or various concentrations of flavonoids for 30 min (B) before incubation with 10 ng/ml TNF-α for 4.5 h. Surface expression of ICAM-1 was measured by ELISA using anti-ICAM-1 antibody as described under *Materials and Methods*. In C, U937 cells, labeled with BCECF, were added to A549 cells pretreated with different flavonoids for 30 min before incubation with TNF-α (10 ng/ml) for 4.5 h, and culture was continued at 37°C for 1 h, then adhesion was measured as described under *Materials and Methods*. Results are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$ compared with vehicle.

inhibited TNF-α-induced ICAM-1 mRNA expression in a dose-dependent manner (Fig. 3A).

To elucidate the mechanism involved in flavonoid-mediated inhibition of ICAM-1 expression, transient transfections were performed using a human ICAM-1 promoter-luciferase construct, pIC339. As reported previously (Chen et al., 2001), treatment with TNF-α led to an increase in ICAM-1 promoter activity, and this effect was inhibited by kaempferol, chrysin, apigenin, and luteolin in a dose-dependent manner, whereas apigenin and luteolin were more potent than kaempferol and chrysin (Fig. 3B).

Kaempferol, Chrysin, Apigenin, and Luteolin Inhibit TNF-α-induced IKK Activation, IκBα Degradation and NF-κB-Specific DNA-Protein Complex Formation and NF-κB-Luc Activity. Because TNF-α-induced ICAM-1 expression was found to involve the IKKβ/NF-κB pathway (Chen et al., 2001; Huang et al., 2003), the effects of these compounds on the TNF-α-induced IKK activity were examined. TNF-α induced IKK activation at 10-min treatments (Fig. 4A, lane 2). Among the four flavonoids tested, both apigenin and luteolin at 1 μM and 3 μM inhibited the TNF-α-induced IKK activity in a dose-dependent manner (50% and 63% inhibitions for apigenin; 54% and 67% inhibitions for luteolin) (Fig. 4A, lanes 7–10). Kaempferol and chrysin

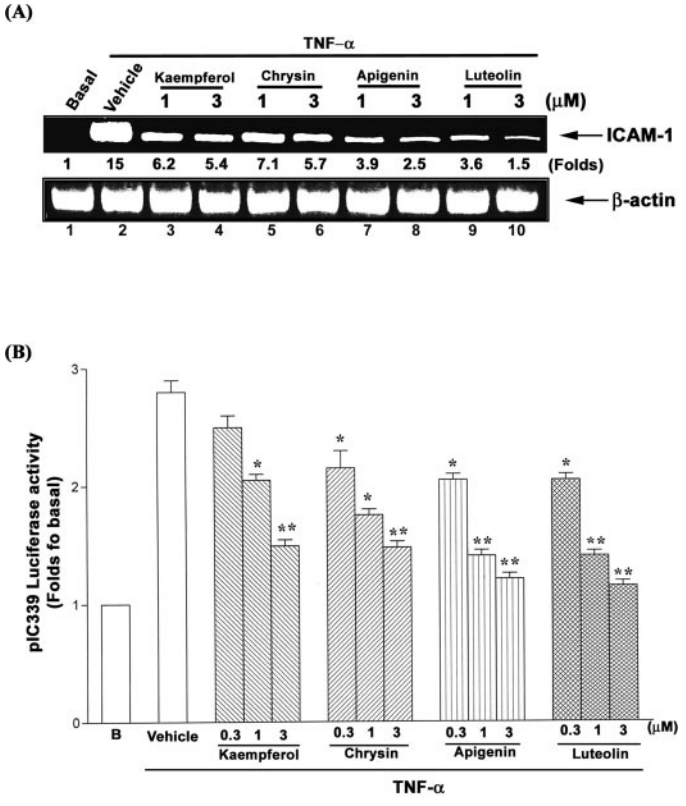

Fig. 3. Inhibitory effects of flavonoids on TNF-α-induced ICAM-1 mRNA expression and ICAM-1 promoter activity in A549 epithelial cells. In A, cells were pretreated with 1 or 3 μM flavonoids for 30 min before incubation with 10 ng/ml TNF-α for 3 h. Total RNA was analyzed by RT-PCR as described under *Materials and Methods*. In B, cells were transfected with the pIC339 luciferase expression vector, then pretreated with 0.3, 1, or 3 μM flavonoids for 30 min before incubation with 10 ng/ml TNF-α for 5 h. Luciferase activity was assayed as described under *Materials and Methods*. The results were normalized with β-galactosidase activity and expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$ compared with vehicle.

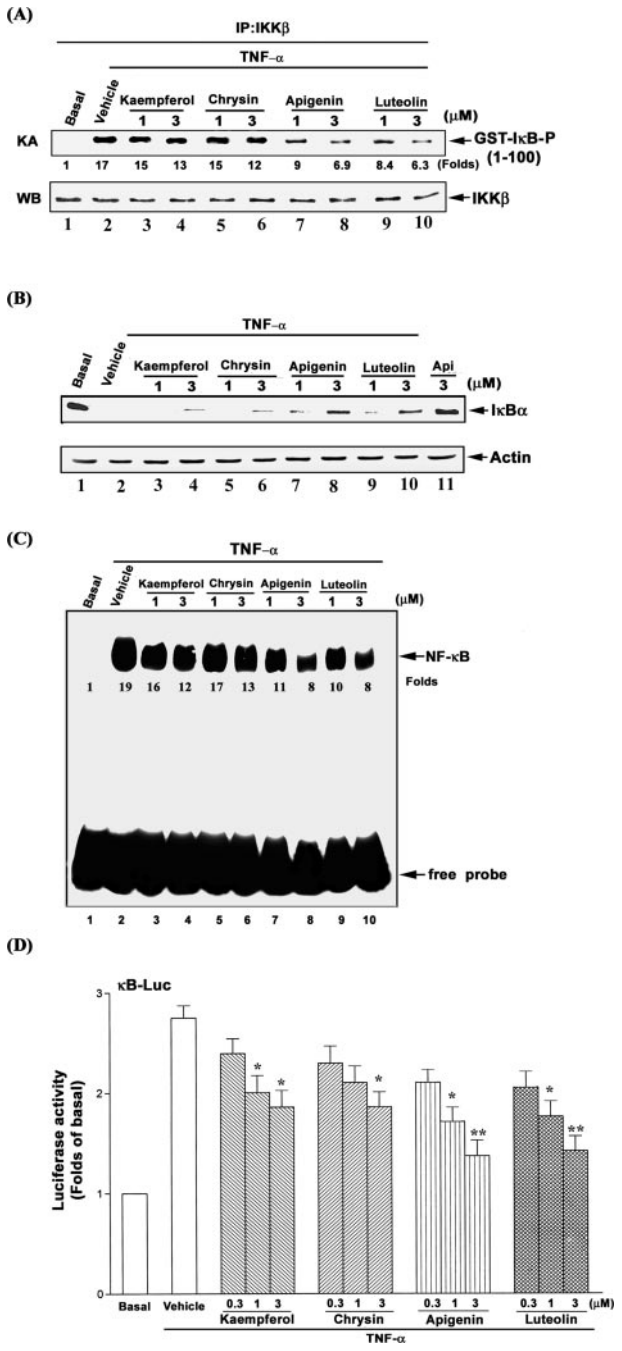

Fig. 4. Inhibitory effects of flavonoids on TNF-α-induced IKK activity, IκBα degradation, NF-κB-specific DNA-protein complex formation and NF-κB-luc activity in A549 epithelial cells. In A, cells were pretreated with 1 or 3 μM flavonoids for 30 min before incubation with 10 ng/ml TNF-α for 10 min. Whole cell lysates were immunoprecipitated with anti-IKKβ antibody. Kinase assay (KA) was performed and autoradiography of phosphorylated GST-IκBβα (1–100) was detected as described under *Materials and Methods*. The amount of IKKβ in immunoprecipitates was determined by Western blot (WB) using anti-IKKβ antibody. In B, the cytosolic levels of IκBα or actin were immunodetected using anti-IκBα or anti-actin-specific antibody. In C, cells were pretreated with 1 or 3 μM flavonoids for 1 h before incubation with 10 ng/ml TNF-α for 30 min, then nuclear extracts were prepared and NF-κB DNA-protein binding activity was determined by EMSA. In D, cells were transfected with the NF-κB-Luc expression vector, then pretreated with 0.3, 1, or 3 μM flavonoids for 30 min before incubation with 10 ng/ml TNF-α for 5 h. Luciferase activity was assayed as described under *Materials and Methods*. The results were normalized with β-galactosidase activity and expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$ compared with vehicle.

only showed minor inhibitory effects at 3 μM (25% and 31% inhibitions for kaempferol and chrysin, respectively) (Fig. 4A, lanes 4 and 6).

Phosphorylation of IκBα is necessary for its degradation and the subsequent NF-κB activation. We next examined the effects of these four compounds on TNF-α-induced IκBα degradation. The degradation of IκBα was seen after a 10-min treatment with TNF-α (Fig. 4B, lane 2). Apigenin and luteolin at 3 μM partially reversed the TNF-α-induced IκBα degradation (Fig. 4B, lanes 8 and 10), whereas kaempferol at 1 μM and chrysin at 3 μM only showed minor reversal (Fig. 4B, lanes 7 and 9 and 4 and 6). Kampferol and chrysin at 1 μM had no effect on TNF-α-induced IκBα degradation (Fig. 4B, lanes 3 and 5).

To test whether the inhibitory effect of flavonoids on IKK activity led to NF-κB inhibition, the effects of these compounds on TNF-α-stimulated NF-κB-specific DNA-protein binding activity were examined. Apigenin and luteolin at 1 and 3 μM inhibited TNF-α-induced NF-κB-specific DNA-protein binding in a dose-dependent manner (Fig. 4C, lanes 7–10). Kaempferol and chrysin at 3 μM showed minor inhibitory effects (Fig. 4C, lanes 4 and 6).

The inhibitory effects of the four flavonoids on NF-κB activation were further examined using a NF-κB-Luc construct. The results showed that apigenin and luteolin at 1 and 3 μM inhibited TNF-α-induced NF-κB luciferase activity in a dose-dependent manner (57 and 77% inhibitions for apigenin; 54 and 74% inhibitions for luteolin) (Fig. 4D). Kaempferol and chrysin displayed significant inhibition at 3 μM (49% for both) (Fig. 4D).

Role of AP-1 in TNF-α-Induced ICAM-1 Expression and the Inhibitory Effects of Kaempferol, Chrysin, Apigenin, and Luteolin. The above results showed that the extent of inhibition on TNF-α-induced NF-κB activation by the four flavonoids was not paralleled to their inhibitory effects on TNF-α-induced ICAM-1 expression, indicating that in addition to NF-κB, other transcriptional factors might be involved. The ICAM-1 promoter contains several AP-1 binding sites that may be important for ICAM-1 expression (Voraberger et al., 1991). To clarify whether AP-1 plays a role in the TNF-α-induced ICAM-1 expression in A549 cells, the human ICAM-1 promoter-luciferase constructs pIC 1352 ($-1352/+1$), which contains full-length human ICAM-1 promoter, pIC 1352 (ΔAP1), which does not contain the AP-1 site ($-279/-284$), and pIC339 ($-339/+1$), which contains the downstream NF-κB and AP-1 sites were transfected into cells to examine the TNF-α-induced promoter activity. As shown in Fig. 5, TNF-α led to a 2.5-fold increase with pIC1352 and a 2.7-fold increase with pIC339; however, TNF-α only induced a 1.6-fold increase in ICAM-1 promoter activity with pIC1352 (ΔAP1). These results indicated the requirement of AP-1 *cis*-acting element for mediating TNF-α-induced ICAM-1 expression on A549 cells.

Since AP-1 was found to involve the TNF-α-induced ICAM-1 promoter activity, AP-1 complex formation was examined by EMSA to determine whether AP-1 activity was stimulated by TNF-α. TNF-α stimulated AP-1 DNA-protein binding after 30 min treatment. The activation declined after 60 or 120 min (Fig. 6A). In the subsequent EMSA experiments, cells were treated with TNF-α for 30 min. To identify the specific subunits involved in the formation of AP-1 complex after TNF-α stimulation, supershift assays were performed in the presence of antibodies specific to either c-fos, c-jun, or p50 (NF-κB). Incubation of nuclear extracts with antibody specific for either c-fos or c-jun induced attenuation of AP-1 DNA-protein binding (Fig. 6B, lanes 3 and 4); however, no shift or attenuation occurred in the presence of antibody specific for p50, which is a component of another transcription factor NF-κB (Fig. 6B, lane 6). These results indicated the formation of c-fos and c-jun heterodimer in AP-1 complex. Excess cold AP-1 probe blocked the AP-1 DNA-protein binding (Fig. 6B, lane 5).

Since AP-1 was demonstrated to be responsible for mediating the TNF-α-induced ICAM-1 expression, and TNF-α indeed induced the AP-1 DNA-protein binding, effects of kaempferol, chrysin, apigenin, and luteolin on TNF-α-induced AP-1 DNA-protein binding and AP-1-luc activity were examined. As shown in Fig. 7A, apigenin and luteolin almost blocked TNF-α-induced AP-1 DNA-protein binding. The inhibitions at 1 μM were 84 and 88%, and those at 3 μM were both 100% for apigenin and luteolin (Fig. 7A, lanes 7–10). As for kaempferol and chrysin, the inhibitions at 1 μM were 44 and 42%, and those at 3 μM were 68 and 72%, respectively (Fig. 7A, lanes 3–6). These four flavonoids also showed sig-

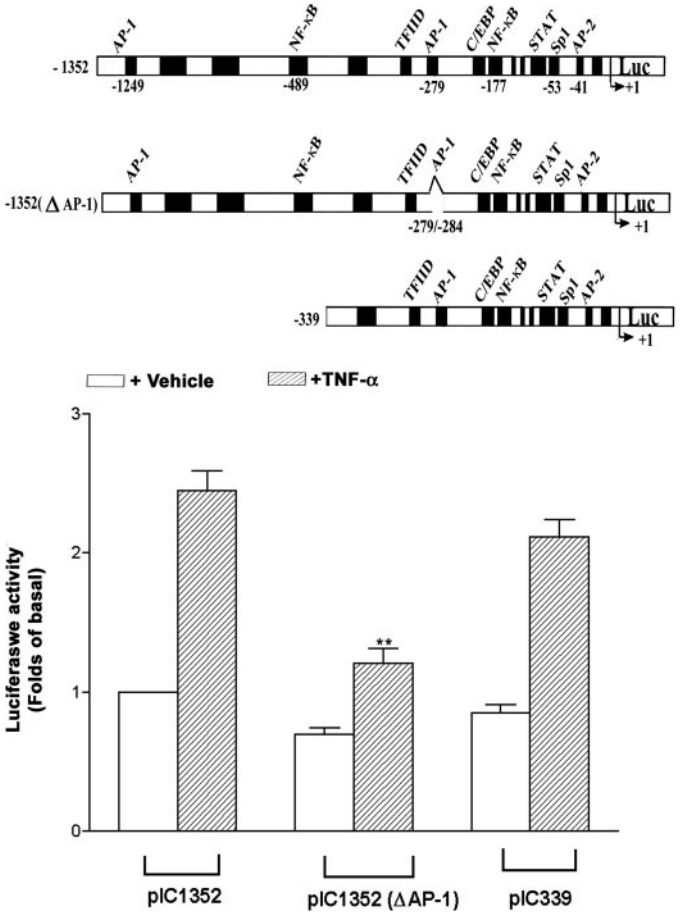

Fig. 5. AP-1-dependent activation of the ICAM-1 promoter by TNF-α. Cells were transfected with the pIC1352, pIC1352 (ΔAP-1) or pIC339 luciferase expression vector as described under *Materials and Methods*, then incubated with 10 ng/ml TNF-α for 5 h. Cell extracts were prepared and assayed for luciferase and β-galactosidase activity, then the luciferase activity was normalized to β-galactosidase activity and expressed as the mean $\pm$ S.E.M. of at least three independent experiments performed in triplicate. **, $P < 0.01$, compared TNF-α reponse with pIC1352(ΔAP-1) with pIC1352.

nificant inhibitions on TNF-α-induced AP-1 luciferase activity (Fig. 7B).

Kaempferol, Chrysin, Apigenin, and Luteolin Inhibit TNF-α-Induced c-fos and c-jun mRNA Expression and Mitogen-Activated Protein Kinases Activities. Since AP-1 complex was demonstrated to be a c-fos and c-jun heterodimer, the effect of the four flavonoids on c-fos and c-jun mRNA expression was examined by RT-PCR. As shown in Fig. 8A, apigenin and luteolin showed significant inhibition on TNF-α-induced c-fos mRNA expression (Fig. 8A, lanes 7–10), whereas kaempferol and chrysin had not much effect (Fig. 8A, lanes 3–6). As for c-jun mRNA, all the four flavonoids showed significant inhibition on the TNF-α-induced c-jun mRNA expression. However, apigenin and luteolin were more potent compared with kaempferol and chrysin (Fig. 8B, lanes 7–10 and 3–6, respectively).

Since the transcriptional activity of AP-1 has been reported to be regulated by MAPKs (Karin, 1995), the roles of ERK1/2, p38, and JNK in TNF-α-induced c-fos and c-jun mRNA expressions were examined. Similar to our previous findings (Chen et al., 2001), PD98059 (50 μM) completely blocked TNF-α-induced ERK1/2 activation without having any effect on the p38 and JNK activations, and SB203580 (30 μM) caused marked inhibition of p38 activation without affecting ERK1/2 and JNK activations (Fig. 9A, lanes 3 and 4). SP600125 inhibited JNK1/2 activation in a dose-dependent manner without any effect on ERK1/2 and p38 activations (Fig. 9A, lanes 5–7). After cells were pretreated with either 50 μM PD98059, 30 μM SB203080, 10 μM SP600125, or 25 μM SP600125, the TNF-α-induced c-fos mRNA expression was almost completely inhibited by PD98059 and SB203580 (Fig. 9B, lanes 3 and 4), whereas SP600125 had no effect (Fig. 9B, lanes 5 and 6). As for the c-jun mRNA expression, only SP600125 showed inhibitory effect (Fig. 9B, lanes 5 and 6).

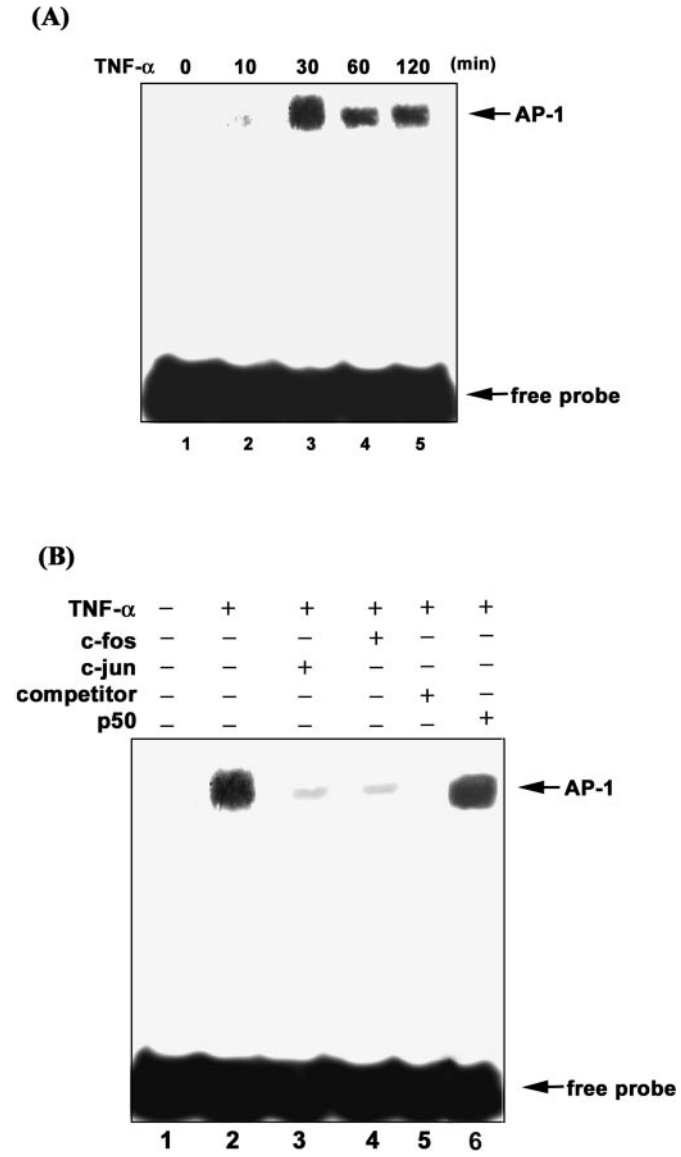

Fig. 6. Kinetics of TNF-α-induced AP-1 DNA-protein binding in A549 epithelial cells. In A, cells were treated with 10 ng/ml TNF-α for 10, 60, or 120 min, then nuclear extracts were prepared and AP-1 DNA-protein binding activity was measured by EMSA as described under *Materials and Methods*. In B, supershift assays were performed using 2 μg of the indicated antibodies, or excess of cold AP-1 probe (100 ng) was used as competitor as described under *Materials and Methods*.

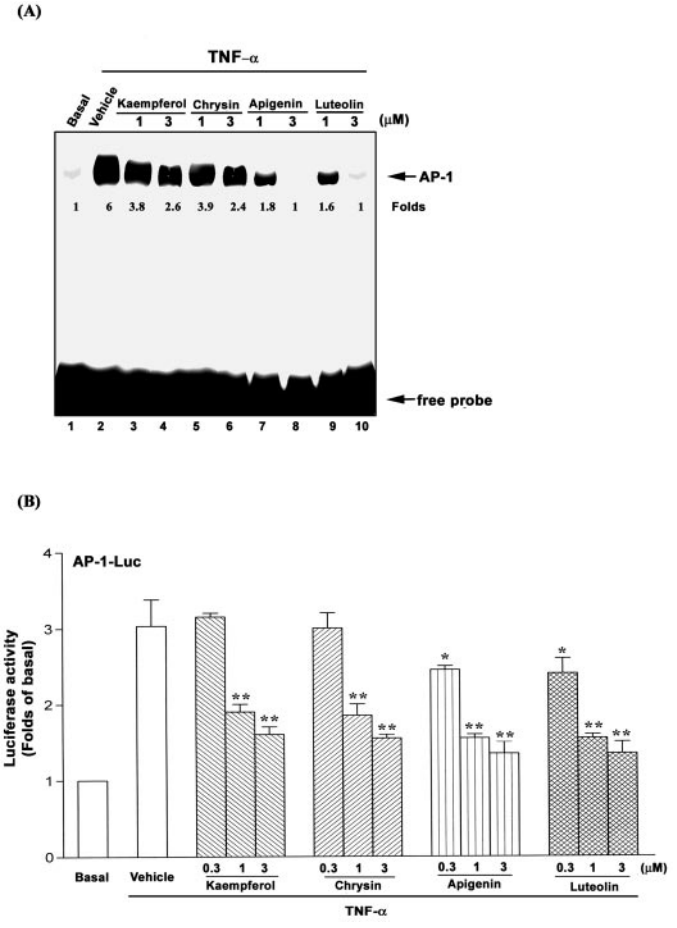

Fig. 7. Inhibitory effects of flavonoids on TNF-α-induced AP-1-specific DNA-protein complex formation and AP-1-luc activity in A549 epithelial cells. In A, cells were pretreated with 1 or 3 μM flavonoids for 1 h before incubation with 10 ng/ml TNF-α for 30 min, then nuclear extracts were prepared and AP-1 DNA-protein binding activity was determined by EMSA as described under *Materials and Methods*. In B, cells were transfected with the AP-1-Luc expression vector, then pretreated with 0.3, 1, or 3 μM flavonoids for 30 min before incubation with 10 ng/ml TNF-α for 5 h. Luciferase activity was assayed as described under *Materials and Methods*. The results were normalized with β-galactosidase activity and expressed as the mean ± S.E.M. of at least three independent experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$ compared with vehicle.

The three MAPKs were demonstrated to be involved in regulating c-fos and c-jun mRNA expressions; therefore, effects of kaempferol, chrysin, apigenin, and luteolin on their activities were examined. As shown in Fig. 10, luteolin showed the most potent inhibition on TNF-α-induced ERK, p38, and JNK activations (Fig. 10, A–C, lanes 9 and 10), and apigenin was the second (Fig. 10, A–C, lanes 7 and 8). Kaempferol and chrysin inhibited the JNK activity (Fig. 10C, lanes 3–6) while having no effect on ERK and p38 activities (Fig. 10, A and B, lanes 3–6).

Discussion

Naturally occurring flavonoids have been proposed to exert biological effects on cells through the inhibitions of different enzymes. They are considered as potential compounds for selectively blocking signal transduction pathways. Adhesion molecule ICAM-1 is known to play a central role in the regulation of cellular inflammatory responses (Albelda et al., 1994). In this study, we analyzed the effects of three flavonols and six flavones on TNF-α-induced ICAM-1 expression and found the inhibitory effects of five of them. We then studied the action mechanisms of kaempferol, chrysin, apigenin, and luteolin by examining their effects on the IKK/NF-κB pathway. We report here that in addition to the NF-κB pathway, the AP-1 seems to play a more significant role in these four flavonoid-induced down-regulation of ICAM-1 gene expression.

It is rational to study the ICAM-1 gene expression and the accompanying signaling pathways in the alveolar epithelial cells. Epithelial cells play an active role in inflammation by producing various cytokines that are involved in the late asthmatic response (Barnes, 1994). Of the potent alveolar macrophage-derived cytokines studied, TNF-α in particular has been implicated in the pathophysiology of neutrophilic infiltrating disorders, including acute lung injury from sepsis, silica-induced pulmonary fibrosis, allograft rejection, and acute respiratory tract infection (Beutler, 1995). Upon binding to its cell surface receptors, TNF-α activates the cytoplasmic form of NF-κB (Baldwin, 1996). In A549 alveolar epithelial cells, IKKβ/NF-κB pathway was required for TNF-α-induced ICAM-1 ex-

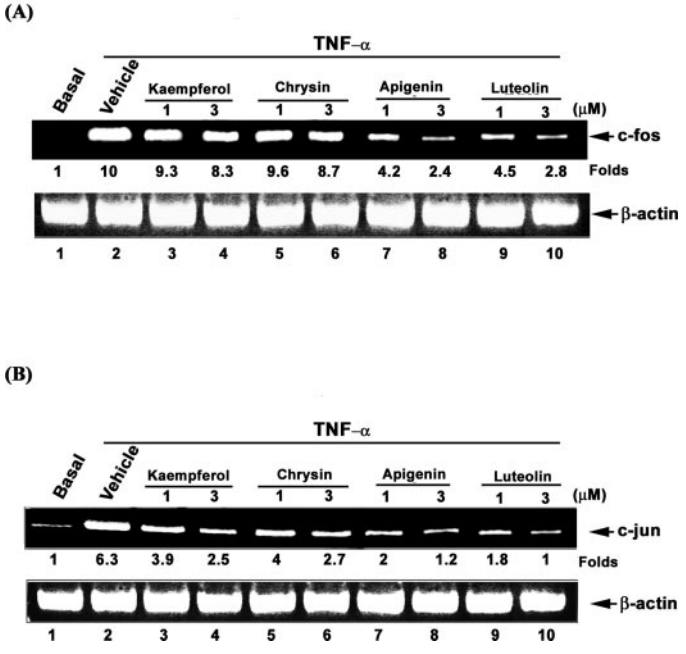

Fig. 8. Inhibitory effects of flavonoids on TNF-α-induced c-fos and c-jun mRNA expressions in A549 epithelial cells. Cells were pretreated with 1 or 3 μM flavonoids for 30 min before incubation with 10 ng/ml TNF-α for 30 min. Total RNA was analyzed by RT-PCR as described under *Materials and Methods*.

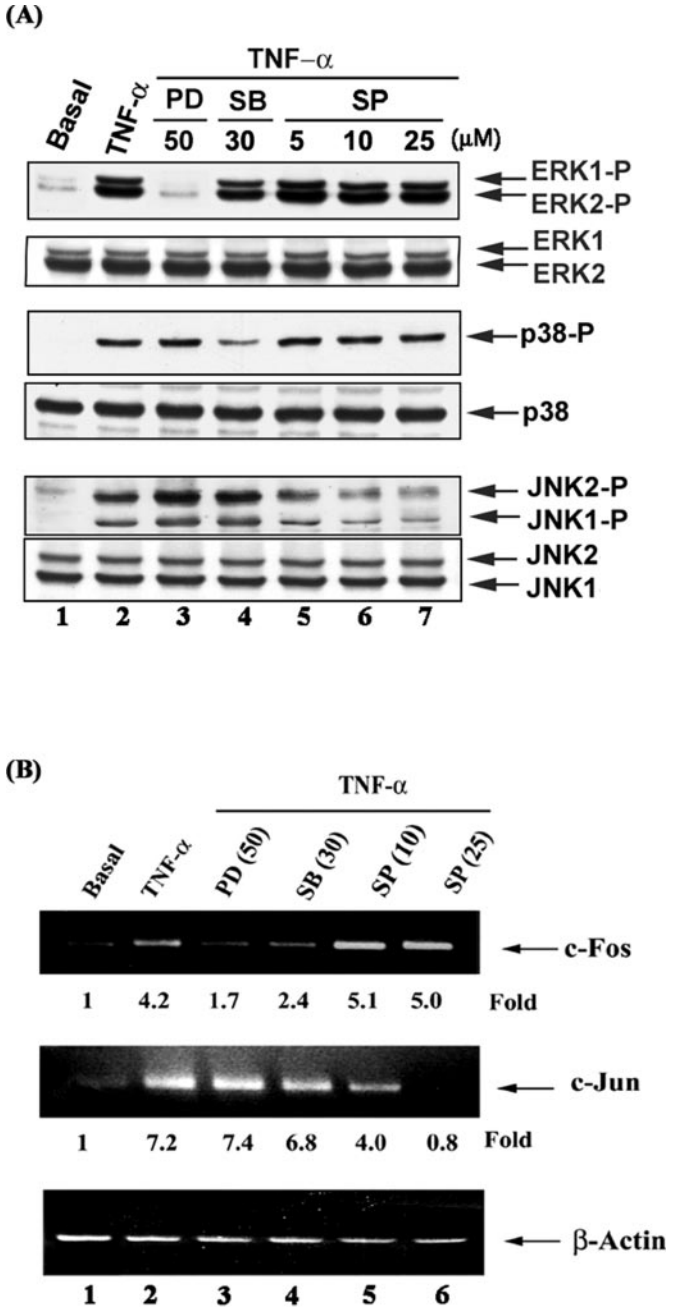

Fig. 9. Effect of PD98059, SB203580, or SP600125 on TNF-α-induced ERK1/2, p38, or JNK activation and on TNF-α-induced c-fos or c-jun mRNA expression in A549 epithelial cells. In A, cells were pretreated with 50 μM PD98059 (PD), 30 μM SB203580 (SB), or 5, 10, or 25 μM SP600125 (SP) for 30 min before incubation with 10 ng/ml TNF-α for 10 min, then whole cell lysates were prepared and subjected to Western blotting using antibodies specific for the phosphorylated form of ERK1/2, p38, JNK, or for ERK2, p38, or JNK as described under *Materials and Methods*. In B, cells were pretreated with 50 μM PD98059, 30 μM SB203580, or 10 or 25 μM SP600125 for 30 min before incubation with 10 ng/ml TNF-α for 30 min. Total RNA was analyzed by RT-PCR as described under *Materials and Methods*.

pression (Chen et al., 2001). Therefore, the inhibitory mechanisms of kaempferol, chrysin, apigenin, and luteolin were first examined for their effects on IKKβ/NF-κB pathway. The phosphorylation of IκB is a key regulatory step that dictates NF-κB activation. In nearly all instances, IKK controls IκB phosphorylation (Karin, 1999). IKK directly phosphorylates two serine residues at the N terminus of IκBα (Ser-32, Ser-36) and IκBβ (Ser-19, Ser-23). This phosphorylation event triggers the polyubiquitination of IκB and the subsequent degradation by the 26S proteasome. Among the parameters involved in IKKβ/NF-κB activation, the IKK activity, IκBα degradation, NF-κB-specific DNA-protein complex formation, and NF-κB-luc activity were examined. The results showed that apigenin and luteolin had more significant inhibitions than kaempferol and chrysin on these parameters; however, their extent of inhibitions was not paralleled with that on TNF-α-induced ICAM-1 expression. Therefore, other factors, in addition to IKKβ/NF-κB, might play a role in the flavonoid-induced inhibitions on ICAM-1 expression.

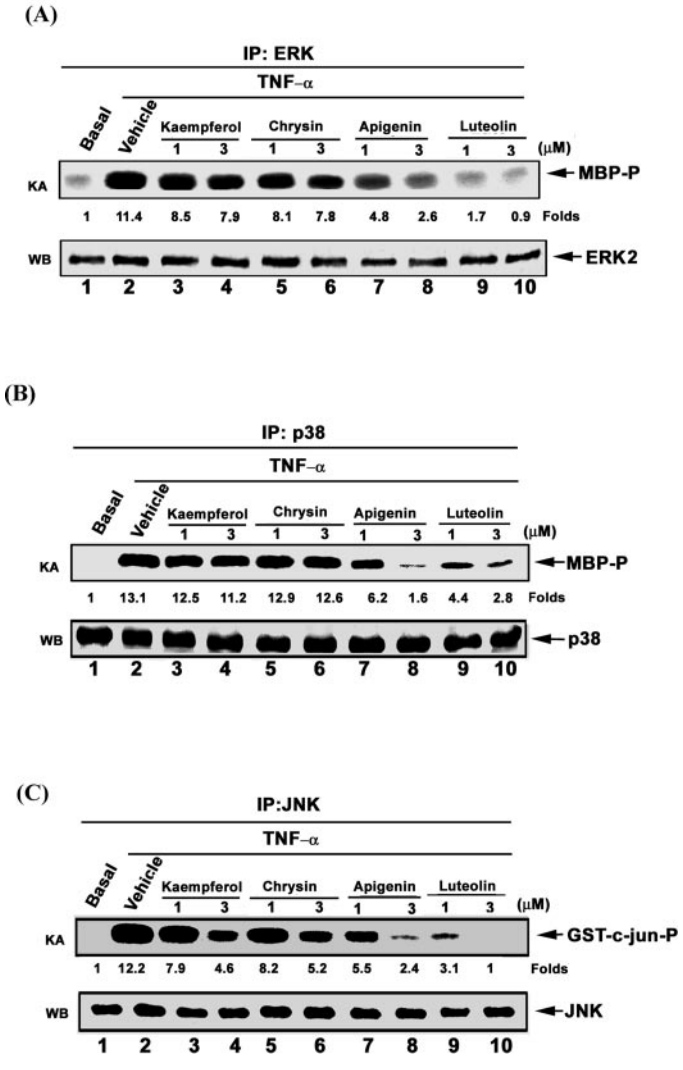

Fig. 10. Inhibitory effects of flavonoids on TNF-α-induced ERK2, p38, and JNK activities in A549 epithelial cells. Cells were pretreated with 1 or 3 μM flavonoids for 30 min before incubation with 10 ng/ml TNF-α for 10 min. Whole cell lysates were immunoprecipitated with anti-ERK2 antibody (A), anti-p38 antibody (B), or anti-JNK antibody (C), followed by kinase assay (KA). Autoradiography of phosphorylated MBP (A,B) or GST-c-jun (C) was detected as described under *Materials and Methods*. The amount of ERK2 (A), p38 (B), or JNK (C) in immunoprecipitates was determined by Western blot (WB) using anti-ERK2, anti-p38, or anti-JNK antibody, respectively.

AP-1 is another transcription factor that plays an important role in the induction of the ICAM-1 gene (Voraberger et al., 1991; Roebuck et al., 1995). TNF-α indeed elicited increases in AP-1-specific DNA-protein complex formation and AP-1-luc activity. Its role in TNF-α-induced ICAM-1 expression on A549 cells was further demonstrated by the finding that ICAM-1 promoter activity was attenuated using pIC1352 (ΔAP1) with AP-1 site (−279/−284) deletion. Kaempferol, chrysin, apigenin, and luteolin inhibited TNF-α-mediated AP-1 activation. Fos and Jun family proteins function as dimeric transcription factors that bind to AP-1 regulatory elements in the promoter and enhanced regions of numerous mammalian genes (Chinenov and Kerppola, 2001). Supershift assays identified the heterodimer of c-fos and c-jun in the TNF-α-activated AP-1 complex in A549 cells. Our data demonstrated the inhibition of the induced c-jun mRNA expression by kaempferol and chrysin, whereas both c-fos and c-jun mRNA expressions were inhibited by apigenin and luteolin.

The activity of AP-1 is regulated by those that increase the abundance of AP-1 components c-jun and c-fos and by those that stimulate their activity (Karin, 1995; Gomez del Arco et al., 1997). There are two major MAPKs signaling pathways reported to regulate AP-1 activity, one through ERK1/2 and the other through JNK. Activation of ERK results in an increase in AP-1 activity via c-fos induction, whereas JNK activation leads to the c-jun phosphorylation (Karin, 1995; Whitmarsh and Davis, 1996). TNF-α has been found to activate ERK, p38, and JNK in A549 cells (Chen et al., 2001; Fig. 9A). In addition to the specific MEK inhibitor, PD98059, and the p38 inhibitor, SB203580 used previously (Chen et al., 2001), a specific JNK inhibitor SP600125 was also used to examine their individual role in regulating the TNF-α-induced c-fos and c-jun mRNA expressions. The TNF-α-induced c-fos mRNA expression was inhibited by PD98059 and SB203580, but not by SP600125. However, c-Jun mRNA expression was inhibited by SP600125, but not by PD 98059 and SB203580. These results indicated the involvement of ERK and p38 in c-fos mRNA expression and JNK in c-jun mRNA expression. By examining the inhibitory effects of flavonoids on MAPKs activity further, it was found that kaempferol and chrysin only inhibited JNK activity, whereas luteolin and apigenin were potent in inhibiting the activities of ERK, p38, and JNK. These results explained why kaempferol and chrysin only inhibited the TNF-α-induced c-jun mRNA expression but failed to inhibit the c-fos mRNA expression, whereas luteolin and apigenin were potent in inhibiting both c-fos and c-jun mRNA expressions. Therefore, the extent of luteolin- and apigenin-induced inhibition on AP-1 activity was greater than that elicited by kaempferol and chrysin. Down-regulation of ICAM-1 expression by these four flavonoids was shown to be more consistent with their inhibitions on AP-1 DNA-protein binding, AP-1-luc activity, c-fos and c-jun mRNA expressions, and MAPK activities. The inhibitory effects of kaempferol and chrysin on ICAM-1 expression act via inhibition of the JNK activity and the c-jun mRNA expression, and those of apigenin and luteolin act via inhibition of the ERK, p38, and JNK activities and the c-fos and c-jun mRNA expressions. All lead to the inhibition of AP-1 activity; however, inhibitory effects on the NF-κB acti-

vation pathway also contribute to the ICAM-1 inhibition induced by apigenin and luteolin. Our previous finding that MAPK pathways were not relevant for TNF-α-induced ICAM-1 expression on A549 cells seemed not correct (Chen et al., 2001). The major evidences supporting this conclusion was from the inability of PD98059 and SB203580 to inhibit TNF-α-induced ICAM-1 expression, and the inability of TNF-α to induce AP-1-specific DNA-protein binding in A549 cells using a commercially available probe. In the present study, we use oligonucleotides corresponding to AP-1 sequences in the human ICAM-1 promoter and find an increase in AP-1-specific DNA-protein binding upon TNF-α stimulation. Furthermore, ICAM-1 promoter activity is attenuated using AP-1 site (−279/−284) deletion mutant. Our unpublished data also show the inhibition of AP-1 but not NF-κB luciferase activity by PD and SB compounds. Therefore, MAPK pathways do not involve the TNF-α-induced NF-κB activation that is regulated by PKC/c-Src/IKK and NF-κB-inducing kinase/IKK (Huang et al., 2003), although they involve the AP-1 activation as demonstrated in this study.

Quercetin, a flavonol with a structure similar to kaempferol, was also reported to act by attenuating the JNK pathway and the AP-1 activation to inhibit the TNF-α- or the phorbol 12-myristate 13-acetate-induced ICAM-1 expression (Kobuchi et al., 1999). However, apigenin and luteolin were reported to act via down-regulation of NF-κB pathway in macrophages to inhibit the LPS-induced inducible nitric-oxide synthase and COX-2 expressions, and the releases of TNF-α and IL-6 (Liang et al., 1999; Xagorari et al., 2001). Quercetin was mediated through the down-regulation of NF-κB to inhibit LPS-induced TNF-α production (Wadsworth and Koop, 1999). (−)Epigallocatechin-3-gallate, a flavonol, was reported to act by blocking the NF-κB activation through IKK to inhibit the endotoxin-induced TNF-α production (Yang et al., 2001). In contrast to these reports, we investigated the role of AP-1 and NF-κB concomitantly and found the greater inhibitions of kaempferol, chrysin, apigenin, and luteolin on AP-1 activity, resulting in the attenuation of ICAM-1 expression. Two synthetic α-methylene-γ-butyrolactone derivatives, CYL-19s and CYL-26z have been found to down-regulate ICAM-1 expression and tumor cell invasion through the specific suppression of IKK activity and NF-κB activation (Huang et al., 2004).

We have screened a number of flavonoids and found that apigenin and luteolin are very effective in reducing the action of TNF-α on ICAM-1 expression. Similar results were also seen in TNF-α- or LPS-induced ICAM-1 expression and TNF-α-induced E-selectin expression on human umbilical vein endothelial cells (data not shown). Flavonoid aglycones consist of a benzene ring (A), fused with a six-membered ring (C) that carries a phenyl ring (B) at position 2 (Fig. 1). Our results showed that the presence of a double bond at position C_2-C_3 of the C ring with OXO function at position 4, along with the presence of OH groups at positions 3′ and 4′ of the B ring, are required for the optimal inhibition of TNF-α-induced ICAM-1 expression by luteolin. Apigenin, having similar structure to luteolin except only one OH group at position 4′ of the B ring, showed little difference in inhibiting ICAM-1 expression, indicating that one –OH group at position 4′ of the B ring is sufficient for their actions. OH group at position 4′ of the B ring is critical for the flavone types to exert their inhibitory actions, since the potency of apigenin and luteolin is much greater than that of chrysin, which lacks OH group at position 4′ of the B ring. Flavone, having a structure similar to chrysin except lacking –OH groups at positions 5 and 7 of the A ring, was ineffective. Comparing kaempferol and quercetin, with OH group at position 3 of the C ring, to apigenin and luteolin, they were a little bit less potent in inhibiting ICAM-1 expression, indicating that the –OH group at position 3 of the C ring slightly hindered the function of flavonoids. Addition of an OH group at position 5′ of the B ring (myricetin) abolished the biological activity. Our data are in line with the findings of Xagorari et al. (2001) who showed the inhibition of LPS-stimulated TNF-α and IL-6 release by luteolin, and those of Gerritsen et al. (1995) who showed the inhibition of TNF-α-induced IL-6 release in vascular endothelial cells by apigenin.

In summary, our results demonstrated that apigenin, luteolin, kaempferol, and chrysin inhibit ICAM-1 induction and monocyte adhesion on epithelial cells. Although apigenin and luteolin were more potent than kaempferol and chrysin in inhibiting ICAM-1 expression, they did not show much difference in inhibiting monocyte adhesion to epithelial cells. It seemed that to a certain extent, ICAM-1 inhibition was enough to induce reduction of monocyte adhesion. The potency variance among these four flavonoids in inhibiting ICAM-1, NF-κB, and AP-1 promoter activities was less than that in inhibiting ICAM-1 expression, either. This implied that flavonoids might affect not only ICAM-1 gene expression but also ICAM-1 protein presentation on the cell surface. The inhibitory effects of apigenin and luteolin are mediated by the sequential attenuation of the ERK1/2, p38, and JNK activities, the c-fos and c-jun mRNA expressions, and the AP-1 transcriptional activity. IKK/NF-κB pathway is also involved in their inhibitions; however, kaempferol and chrysin primarily mediate by the attenuation of the JNK activity, the c-jun mRNA expression, and the AP-1 activity. Therefore, the transcription factor AP-1 seems to play a more significant role than NF-κB in these inhibitions. The structure-activity relationships of flavonoids are also explored and found the significance of –OH groups at positions 5 and 7 of the A ring and at position 4′ of the B ring.

References

Albelda SM, Smith CW, and Ward PA (1994) Adhesion molecules and inflammatory injury. *FASEB J* **8:**504–512.

Avila MA, Velasco JA, Cansado J, and Notario V (1994) Quercetin mediates the down-regulation of mutant p53 in the human breast cancer cell line MDA-MB468. *Cancer Res* **54:**2424–2428.

Baldwin AS (1996) The NF-κB and IκB proteins: new discoveries and insights. *Annu Rev Immunol* **14:**649–683.

Barnes PJ (1994) Cytokines as mediators of chronic asthma. *Am J Respir Crit Care Med* **150:**S42–S49.

Barton RW, Rothlein R, Ksiazek J, and Kennedy C (1989) The effect of anti-ICAM-1 on phorbol-ester-induced rabbit lung inflammation. *J Immunol* **143:**1278–1282.

Bassi V, Vitale M, Feliciello A, De Riu S, Rossi G, and Fenzi G (1995) Retinoic acid induces ICAM-1 hyperexpression in human thyroid carcinoma cell lines. *J Clin Endocrinol Metab* **80:**1129–1135.

Beutler B (1995) TNF, immunity and inflammatory disease: lessons of the past decade. *J Investig Med* **43:**227–235.

Chang YJ, Holtzman MJ, and Chen CC (2002) IFN-γ-induced epithelial ICAM-1 expression and monocyte adhesion. Involement of PKC-dependent c-Src typosine kinase activation pathway. *J Biol Chem* **277:**7118–7126.

Chang YJ, Holtzman MJ, and Chen CC (2004) Differential role of Janus family kinases (JAKs) in interferon-gamma-induced lung epithelial ICAM-1 expression: involving protein interactions between JAKs, phospholipase Cγ, c-Src, and STAT1. *Mol Pharmacol* **65:**589–598.

Chen CC, Chen JJ, and Chou CY (2000) PKCα but not p44/42 MAPK, p38, JNK is required for ICAM-1 expression mediated by IL-1β: involvement of sequential activation of tyrosine kinase, NIK, and IKK2. *Mol Pharmacol* **58:**1479–1489.

Chen C, Chou C, Sun Y, and Huang W (2001) TNF-α-induced activation of downstream NF-κB site of the promoter mediates epithelial expression and monocyte

adhesion: involvement of PKCα, tyrosine kinase and IKK2 but not MAPKs, pathway. *Cell Signal* **13:**543–553.
Chinenov Y and Kerppola TK (2001) Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. *Oncogene* **20:**2438–2452.
Colletti LM, Cortis A, Lukacs N, Kunkel SL, Green M, and Strieter RM (1998) TNF up-regulates ICAM-1 which is important in the neutrophil-dependent lung and liver injury associated with hepatic ischemia and reperfusion in the rat. *Shock* **10:**182–191.
Cornelius LA, Taylor JT, Degitz K, Li LJ, Lawley TJ, and Caughman SW (1993) A 5′-portion of the ICAM-1 gene confers tissue-specific differential expression levels and cytokine responsiveness. *J Investig Dermatol* **100:**753–758.
Gerritsen ME, Carley WW, Ranges GE, Shen CP, Phan SA, Ligon GF, and Perry CA (1995) Flavonoids inhibit cytokine-induced endothelial cell adhesion protein gene expression. *Am J Pathol* **147:**278–292.
Gomez del Arco P, Martinez-Martinez S, Calvo V, Armesilla AL, and Redondo JM (1997) Antioxidants and AP-1 activation: a brief overview. *Immunobiology* **198:**273–278.
Huang WC, Chan ST, Yang TL, Tzeng CC, and Chen CC (2004) Inhibition of ICAM-1 gene expression, monocyte adhesion, and cancer cell invasion by targeting IKK complex: molecular and functional study of novel α-methylene-γ-butyrolactone derivatives. *Carcinogenesis,* in press.
Huang WC, Chen JJ, and Chen CC (2003) c-Src-dependent tyrosine phosphorylation of IKKβ is involved in TNF-α-induced ICAM-1 expression. *J Biol Chem* **278:**9944–9952.
Kacimi R, Karliner JS, Koudssi F, and Long CS (1998) Expression and regulation of adhesion molecules in cardiac cells by cytokines: response to acute hypoxia. *Circ Res* **82:**576–586.
Karin M (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* **270:**16483–16486.
Karin M (1999) The beginning of the end: IκB kinase (IKK) and NF-κB activation. *J Biol Chem* **274:**27339–27432.
Kobuchi H, Roy S, Sen CK, Nguyen HG, and Packer L (1999) Quercetin inhibits inducible ICAM-1 expression in human endothelial cells through the JNK pathway. *Am J Physiol* **277:**C403–C411.
Liang YC, Huang YT, Tsai SH, Lin-Shiau SY, Chen CF, and Lin JK (1999) Supression of inducible COX and iNOS by apigenin and related flavonoids in mouse macrophage. *Carcinogenesis* **20:**1945–1952.
Rice-Evans C, Miller N, and Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med* **20:**933–956.
Roebuck KA, Rahman A, Lakshminarayanan V, Janakidevi K, and Malik AB (1995) H2O2 and TNF-α activate ICAM-1 gene transcription through distinct cis-regulatory elements within the ICAM-1 promoter. *J Biol Chem* **270:**18966–19874.
Sampath D, Castro M, Look DC, and Holtzman MJ (1979) Constitutive activation of an epithelial signal transducer and activator of transcription (STAT) pathway in asthma. *J Clin Investig* **103:**1353–1361.
Stanciu LA and Djukanovic R (1998) The role of ICAM-1 on T-cells in the pathogenesis of asthma. *Eur Respir J* **11:**949–957.
van de Stolpe A and van der Saag PT (1996) Intercellular adhesion molecule-1. *J Mol Med* **74:**13–33.
Voraberger G, Schafer R, and Stratowa C (1991) Cloning of the human gene for ICAM-1 and analysis of its 5′-regulatory region. Induction by cytokines and phorbol ester. *J Immunol* **147:**2770–2786.
Wadsworth TL and Koop DR (1999) Effects of the wine polyphenolic quercetin and resveratrol on pro-inflammatory cytokine expression in RAW 264.7 macrophages. *Biochem Pharmacol* **57:**941–949.
Welty SE, Rivera JL, Elliston JF, Smith CV, Zeb T, Ballantyne CM, Montgomery CA, and Hansen TN (1993) Increases in lung tissue expression of ICAM-1 are associated with hypertoxic lung injury and inflammation in mice. *Am J Respir Cell Mol Biol* **9:**393–400.
Whitmarsh AJ and Davis RJ (1996) Transcription factor AP-1 regulation by MAPK signal transduction pathways. *J Mol Med* **74:**589–607.
Xagorari A, Papapetropoulos A, Mauromatis A, Economou M, Fotsis T, and Roussos C (2001) Luteolin inhibits endotoxin-stimulated phosphorylation cascade and proinflammatory cytokine production in macrophages. *J Pharmacol Exp Ther* **296:**181–187.
Yang F, Oz HS, Barve S, de Villiers WJ, McClain CJ, and Varilek GW (2001) The green tea polyphenol (-)-Epigallocatechin-3-galate blocks NF-κB activation by inhibiting IKK activity in the intestinal epithelial cell line IEC-6. *Mol Pharmacol* **60:**528–533.
Zuckerman LA, Pullen L, and Miller J (1998) Functional consequences of costimulation by ICAM-1 on IL-2 gene expression and T cell activation. *J Immunol* **160:**3259–3268.

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