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Fisetin, an Inhibitor of Cyclin-Dependent Kinase 6, Down-Regulates Nuclear Factor-κB-Regulated Cell Proliferation, Antiapoptotic and Metastatic Gene Products through the Suppression of TAK-1 and Receptor-Interacting Protein-Regulated IκBα Kinase Activation

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ABSTRACT

Fisetin (3,7,3′,4′-tetrahydroxyflavone) exhibits anti-inflammatory and antiproliferative effects through a mechanism that is poorly understood. Although fisetin has been cocrystalized with cyclin-dependent kinase 6 and inhibits its activity, this inhibition is not sufficient to explain various activities assigned to this flavonol. Because of the critical role of the NF-κB pathway in regulation of inflammation and proliferation of tumor cells, we postulated that fisetin modulates this pathway. To test this hypothesis, we examined the effect of fisetin on NF-κB and NF-κB-regulated gene products in vitro. We found that among nine different flavones tested, fisetin was potent in suppressing tumor necrosis factor (TNF)-induced NF-κB activation. Fisetin also suppressed the NF-κB activation induced by various inflammatory agents and carcinogens, and it blocked the phosphorylation and degradation of IκBα by inhibiting IκBα (IKK) activation, which in turn led to suppression of the phosphorylation and nuclear translocation of p65. NF-κB-dependent reporter gene expression was also suppressed by fisetin, as was NF-κB reporter activity induced by TNFR1, TRADD, TRAF2, NIK, and IKK but not that induced by p65 transfection. Fisetin also inhibited TNF-induced TAK1 and receptor-interacting protein activation, events that lie upstream of IKK activation. The expression of NF-κB-regulated gene products involved in antiapoptosis (cIAP-1/2, Bcl-2, Bcl-xL, XIAP, Survivin, and TRAF1), proliferation (cyclin D1, c-Myc, COX-2), invasion (ICAM-1 and MMP-9), and angiogenesis (vascular endothelial growth factor) were also down-regulated by fisetin. This correlated with potentiation of apoptosis induced by TNF, doxorubicin, and cisplatin. Thus, overall, our results indicate that fisetin mediates antitumor and anti-inflammatory effects through modulation of NF-κB pathways.

As many as 70% of all drugs used today for the treatment of cancer were derived from or based on natural products (Newman et al., 2002). However, 80% of the world population cannot afford currently available targeted therapies. Although fruits and vegetables have been linked with reduction of risk of cancer, cardiovascular diseases, autoimmune diseases, and various other chronic illnesses (Neuhouser, 2004), neither the active components nor their mechanisms of action are well established. Identification of active ingredients in dietary plants, and the cell signaling pathways they modulate, can validate their use in various diseases (Willett, 1994).

Fisetin (3,7,3′, 4′-tetrahydroxyflavone) is a flavonoid di-

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ABBREVIATIONS: CDK, cyclin-dependent kinase; RIP, receptor-interacting protein; XIAP, X chromosome-linked inhibitor-of-apoptosis protein; TNF, tumor necrosis factor; NF-κB, nuclear factor-κB; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide; H_2O_2, hydrogen peroxide; IκBα, inhibitory subunit of NF-κB α; MMP, matrix metalloproteinase; PARP, poly-(ADP-ribose) polymerase; IAP1, inhibitor-of-apoptosis protein 1; TRAF, TNF receptor-associated factor; VEGF, vascular endothelial growth factor; ICAM-1, intercellular adhesion molecule 1; COX-2, cyclooxygenase-2; IKK, IκBα kinase; cFLIP, complementary Fas-associated death domain protein-like interleukin-1β-converting enzyme-inhibitory protein; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; SEAP, secretory alkaline phosphatase; TNFR1, TNF receptor-1; TRADD, TNFR1-associated death domain protein; TAK1, transforming growth factor-1-activated kinase; NIK, NF-κB-inducing kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

etary ingredient found in the smoke tree (*Cotinus coggygria*) and is also widely distributed in fruits and vegetables such as strawberry, apple, persimmon, grape, onion, and cucumber at concentrations of 2 to 160 μg/g (Arai et al., 2000). It exhibits a wide variety of activities, including neurotrophic (Maher, 2006), antioxidant (Hanneken et al., 2006), anti-inflammatory (Higa et al., 2003), and antiangiogenic (Fotsis et al., 1998) effects. It has been reported to suppress the proliferation of a wide variety of tumor cells, including prostate cancer (Haddad et al., 2006), liver cancer (Chen et al., 2002), colon cancer (Lu et al., 2005b), and leukemia (Lee et al., 2002) cells. How fisetin mediates these effects is not fully understood, although it has been shown to induce G_2/M arrest, decrease the activity of CDK2 and CDK4, suppress retinoblastoma protein phosphorylation, decrease the levels of cyclin D1 and cyclin E, increase the levels of p21 and p53, activate caspase-3, and inhibit DNA methyltransferases (Chen et al., 2002; Lee et al., 2002, 2005; Lu et al., 2005b; Haddad et al., 2006). Recent studies have also shown that fisetin forms a complex with human CDK6 and inhibits its activity with an IC_{50} of 0.85 μM (Lu et al., 2005a). Suppression of CDK6, however, is not sufficient to explain all activities assigned to this flavonoid, so we determined to explore other possible mechanisms. Because apoptosis, inflammation, and angiogenesis are regulated by the nuclear transcription factor nuclear factor-κB (NF-κB) (Aggarwal, 2004), we postulated that this pathway plays a major role in its activity.

NF-κB is a family of Rel domain-containing proteins present in the cytoplasm of all cells, where they are kept in an inactive state by a family of ankyrin domain-containing proteins, which includes IκBβ, IκBγ, IκBε, Bcl-3, p105, and p100. Under resting conditions, NF-κB consists of a heterotrimer of p50, p65, and IκB in the cytoplasm; only when activated and translocated to the nucleus is the sequence of events leading to activation initiated. Most carcinogens, inflammatory agents, and tumor promoters, including cigarette smoke, phorbol ester, okadaic acid, H_2O_2, and tumor necrosis factor (TNF), have been shown to activate NF-κB. The activation of NF-κB involves the phosphorylation, ubiquitination, and degradation of IκBα and phosphorylation of p65, which in turn leads to the translocation of NF-κB to the nucleus, where it binds to specific response elements in the DNA. The phosphorylation of IκBα is catalyzed by IκBα kinase (IKK), which is essential for NF-κB activation by most agents. NF-κB has been shown to regulate the expression of several genes whose products are involved in tumorigenesis (Aggarwal, 2004). These include antiapoptotic genes (e.g., cIAP1/2, survivin, TRAF1, Bcl-2, and Bcl-xL); proliferation and invasion genes (e.g., COX-2, MMP-9); genes encoding adhesion molecules, chemokines, and cell cycle regulatory genes (e.g., cyclin D1 and c-Myc).

Because of the role of the NF-κB in inflammation and cellular proliferation, we speculated that fisetin mediates its effects by modulating this pathway. Our results, derived from two cell lines, demonstrate that fisetin inhibits the NF-κB activation pathway, potentiates TNF-induced apoptosis, and down-regulates gene products that prevent apoptosis and promote inflammation and tumor metastasis.

Materials and Methods

Materials. Fisetin was purchased from Sigma-Aldrich (St. Louis, MO). A 50 mM solution of fisetin was prepared in 100% dimethyl sulfoxide, stored as small aliquots at −20°C, and then diluted as needed in cell culture medium. 3-Hydroxyflavone, quercetin, quercitrin, and kaempferol were also purchased from Sigma-Aldrich (St. Louis, MO). Taxifolin was from Calbiochem (La Jolla, CA), isorhamnetin from Fluka (Buchs, Switzerland), and myricetin from LKT Laboratories (St. Paul, MN). All these flavones were dissolved in dimethyl sulfoxide to final concentration to 100 mM. Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of 5×10^7 U/mg, was kindly provided by Genentech (South San Francisco, CA). Hoechst 33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), Tris, glycine, NaCl, SDS, and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). Penicillin, streptomycin, RPMI 1640, minimal essential medium, and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA). Phorbol 12-myristate 13-acetate (PMA), okadaic acid, epidermal growth factor (EGF), and anti-β-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies against p65, p50, COX-2, IκBα, ICAM-1, c-Myc, cyclin D1, MMP-9, PARP, cIAP-1/2, Bcl-2, receptor-interacting protein (RIP) and Bcl-xL and the annexin V staining kit were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-XIAP antibodies were obtained from BD Biosciences (San Jose, CA). Phospho-specific anti-IκBα (serine 32/36) and phospho-specific anti-p65 (serine 536) antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA). Anti-IKK-α, anti-IKK-β, and anti-FLIP antibodies were kindly provided by Imgenex (San Diego, CA).

Cell Lines. Human lung adenocarcinoma H1299 cells, human Burkitt lymphoma Daudi cells, and human embryonic kidney A293 cells were obtained from the American Type Culture Collection (Manassas, VA). H1299 cells and Daudi cells were cultured in RPMI 1640 medium, and A293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Normal human foreskin fibroblasts were cultured in minimal essential medium supplemented with 30 mM HEPES and 0.1 mM nonessential amino acids. All media were also supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

Live and Dead Assay. To measure apoptosis, we used the LIVE/DEAD cell vitality assay kit (Invitrogen), which determines intracellular esterase activity and plasma membrane integrity. We performed this assay as described previously (Takada and Aggarwal, 2004). In brief, 1×10^5 cells were incubated with 25 μM fisetin for 6 h and then treated with 1 nM TNF for 16 h at 37°C. Cells were stained with the LIVE/DEAD reagent (5 μM ethidium homodimer and 5 μM calcein-acetoxymethyl ester) and then incubated at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2; Nikon, Tokyo, Japan).

Cytotoxicity Assay. The effect of fisetin on the cytotoxic effects of TNF and chemotherapeutic reagents was determined by the modified tetrazolium salt MTT uptake method as described previously (Takada and Aggarwal, 2004). In brief, 5000 cells were incubated with fisetin for 6 h in triplicate on 96-well plates and then treated with various concentrations of reagents for 24 h at 37°C. Thereafter, an MTT solution was added to each well. After 2 h of incubation at 37°C, lysis buffer (20% SDS and 50% dimethylformamide) was added, the cells were incubated overnight at 37°C, and then the optical density was measured at 570 nm using a 96-well multiscanner (MRX Revelation; Dynex Technologies, Chantilly, VA).

Annexin V Assay. One of the early indicators of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cell's cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected using the binding properties of annexin V. To detect apoptosis, we employed annexin V antibody conjugated with the fluorescent dye fluorescein isothiocyanate. In brief, 1×10^6 cells were pretreated

with 25 μM fisetin for 6 h, treated with 1 nM TNF for 16 h, and then subjected to annexin V staining. Cells were washed, stained with fluorescein isothiocyanate-conjugated anti-annexin V antibody, and then analyzed with a flow cytometer (FACSCalibur; BD Biosciences).

Electrophoretic Mobility Shift Assay. To assess NF-κB activation, we performed EMSA as described previously (Takada and Aggarwal, 2004). In brief, nuclear extracts prepared from TNF-treated cells (1×10^6/ml) were incubated with ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (15 μg of protein with 16 fmol of DNA) from the HIV long terminal repeat, 5′-TTGTTAC-AA**GGGACTTTC**CGCTG**GGGACTTTC**CAGGGAGGCGTGG-3′ (boldface indicates NF-κB-binding sites), for 30 min at 37°C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5′-TTGTTACAA**CTCACTTTC**CGCTG **CTCACTTTC**CAGGGAGGCGTGG-3′, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with antibodies against either the p50 or the p65 subunit of NF-κB for 30 min at 37°C before the complex was analyzed by EMSA. Preimmune serum was included as a negative control. Specificity of binding was determined routinely by using an excess of unlabeled oligonucleotide for competition. The dried gels were visualized with a Storm 820 PhosphorImager, and radioactive bands were quantitated using ImageQuant software (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Western Blot Analysis. To determine the levels of protein expression in the cytoplasm or nucleus, we prepared extracts (Takada and Aggarwal, 2004) and fractionated them by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with the relevant antibody, and detected by ECL reagent (GE Healthcare). The bands obtained were quantitated using NIH Image software (http://rsb.info.nih.gov/nih-image/).

IKK Assay. To determine the effect of fisetin on TNF-induced IKK activation, IKK assay was performed by a method we described previously (Takada and Aggarwal, 2004). In brief, the IKK complex from whole-cell extracts was precipitated with antibody against IKK-α and then treated with protein A/G-agarose beads (Pierce, Rockford, IL). After 2 h, the beads were washed with lysis buffer and then resuspended in a kinase assay mixture containing 50 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 2 mM dithiothreitol, 20 μCi of [γ-^{32}P]ATP, 10 μM unlabeled ATP, and 2 μg of substrate glutathione transferase-IκBα (amino acids 1–54). After incubation at 30°C for 30 min, the reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized with a Storm820. To determine the total amounts of IKK-α and IKK-β in each sample, 30 μg of whole-cell protein was resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKK-α or anti-IKK-β antibody.

NF-κB-Dependent Reporter Gene Expression Assay. The effect of fisetin on NF-κB-dependent reporter gene transcription induced by TNF and various genes was analyzed by secretory alkaline phosphatase (SEAP) assay, with the following modification. In brief, A293 cells (5×10^5 cells/well) were plated in six-well plates and transiently transfected by the calcium phosphate method with pNF-κB-SEAP (0.5 μg). To examine TNF-induced reporter gene expression, we transfected the cells with 0.5 μg of the SEAP expression plasmid and 2 μg of the control plasmid pCMV-FLAG1 DNA for 24 h. We then treated the cells for 6 h with fisetin and then stimulated them with 1 nM TNF. The cell culture medium was harvested after 24 h of TNF treatment. To examine reporter gene expression induced by various genes, we transfected A293 cells with 0.5 μg of pNF-κB-SEAP plasmid with 1 μg of an expressing plasmid and 0.5 μg of the control plasmid pCMV-FLAG1 for 24 h, treated them with 25 μM fisetin for 6 h, and then harvested them from culture medium after an additional 24 h of incubation. Culture medium was analyzed for SEAP activity according to the protocol essentially as described by the manufacturer (Clontech, Mountain View, CA) using a Victor 3 microplate reader (PerkinElmer Life and Analytical Sciences). For the TAK1/TAB1 and RIP experiments, A293 cells (5×10^5 cells/well) were transiently transfected with the expression vectors for TAK1/TAB1, RIP, and pNF-κB-SEAP (0.5 μg) plasmids. After 24 h, cells were treated with indicated concentrations of fisetin, and conditioned medium was harvested after 24 h for SEAP activity as described above. The pSV-β-gal plasmid was used as a transfection control, and all of the transfection results were normalized against the observed β-galactosidase activity.

Immunocytochemical Analysis of NF-κB p65 Localization. The effect of fisetin on the nuclear translocation of p65 was examined by immunocytochemistry as described previously (Takada and Aggarwal, 2004). In brief, treated cells were plated on a poly(L-lysine)-coated glass slide by centrifugation (Shandon Cytospin 4; ThermoFisher Scientific, Waltham, MA), air dried, and fixed with 4% paraformaldehyde after permeabilization with 0.2% Triton X-100. After being washed in phosphate-buffered saline, the slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal anti-human p65 antibody at a 1:200 dilution. After overnight incubation at 4°C, the slides were washed, incubated with goat anti-rabbit IgG-Alexa Fluor 594 (Invitrogen) at a 1:200 dilution for 1 h, and counterstained for nuclei with Hoechst 33342 (50 ng/ml) for 5 min. Stained slides were mounted with mounting medium purchased from Sigma-Aldrich and analyzed under a fluorescence microscope (Labophot-2). Pictures were captured using a Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX) and MetaMorph version 4.6.5 software (Molecular Devices, Sunnyvale, CA).

Results

The goal of this study was to investigate the effect of fisetin on the NF-κB activation pathway induced by various carcinogens and inflammatory stimuli, on NF-κB-regulated gene expression and on apoptosis induced by cytokines and chemotherapeutic agents. The structure of fisetin is shown in Fig. 1A. The concentration of fisetin used and the duration of exposure had minimal effect on the viability of different cell lines studied as determined by trypan blue dye exclusion test (data not shown). To examine the effect of fisetin on the NF-κB activation pathway, TNF was used for most experiments, because the role of TNF in the NF-κB activation pathway has already been well characterized.

Fisetin Suppressed NF-κB Activation in a Dose- and Time-Dependent Manner. We first determined the dose and time of exposure to fisetin required to suppress NF-κB activation on human small-cell lung adenocarcinoma cells. As indicated by EMSA, fisetin suppressed TNF-induced NF-κB in a dose-dependent manner (Fig. 1B). Fisetin by itself did not activate NF-κB. Under these conditions, cells were fully viable as determined by the trypan blue dye exclusion test (data not shown). We also investigated the length of incubation required for fisetin to suppress TNF-induced NF-κB activation. Cells were incubated with 50 μM fisetin for different times and then exposed to TNF. EMSA showed that fisetin suppressed TNF-induced NF-κB activation within 6 h (Fig. 1C).

NF-κB is a complex of proteins in which various combinations of Rel/NF-κB protein constitute active NF-κB heterodimers that bind specific DNA sequences (Ghosh and Karin, 2002). Whether the band visualized by EMSA in TNF-treated cells was indeed NF-κB was determined by supershift assay. When nuclear extracts from TNF-stimulated H1299

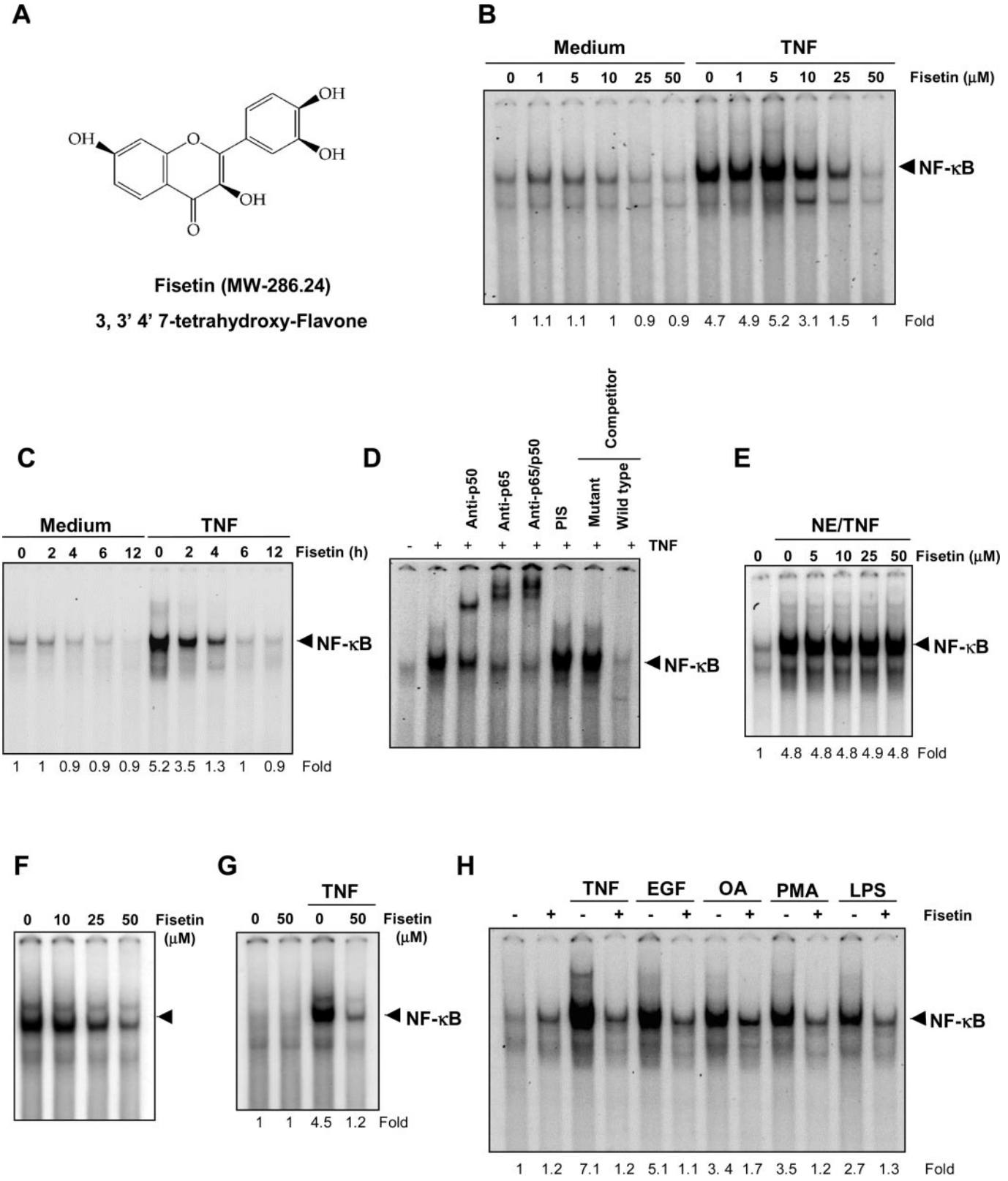

Fig. 1. A, the chemical structure of fisetin. B, effect of fisetin dose. H1299 cells were incubated with the indicated concentrations of fisetin for 6 h and treated with 0.1 nM TNF for 30 min. The nuclear extracts were assayed for NF-κB activation by EMSA. The results shown are representative of three independent experiments. C, effect of time duration. H1299 cells were preincubated with 50 μM fisetin for the indicated times and then treated with 0.1 nM TNF for 30 min. The nuclear extracts were prepared and assayed for NF-κB activation by EMSA. The results shown are representative of three independent experiments. D, NF-κB induced by TNF is composed of p65 and p50 subunits. Nuclear extracts from untreated cells or cells treated with 0.1 nM TNF were incubated with the indicated antibodies, an unlabeled NF-κB oligonucleotide probe, or a mutant oligonucleotide (*Mutant oligo*) probe. They were then assayed for NF-κB activation by EMSA. The results shown are representative of three independent experiments. E, the direct effect of fisetin on the NF-κB complex was investigated. Nuclear extracts were prepared from untreated cells or cells treated with 0.1 nM TNF and incubated

cells were treated with antibodies against the p50 (NF-κB1) or p65 (RelA) subunits of NF-κB, the major band was shifted to a higher molecular mass (Fig. 1D), thus suggesting that the TNF-activated complex consisted of p50 and p65 subunits. Preimmune serum had no effect on this band, excess (100-fold) unlabeled NF-κB caused complete disappearance of the band, and a mutant oligonucleotide of NF-κB did not affect NF-κB-binding activity.

Fisetin Did Not Directly Affect Binding of NF-κB to DNA. Some NF-κB inhibitors, such as caffeic acid phenyl ethylester (Natarajan et al., 1996) and plumbagin (Sandur et al., 2006), directly modify NF-κB to suppress its DNA binding. To determine this, nuclear extracts from TNF-treated cells were prepared and incubated with fisetin. By using EMSA, we found that fisetin did not modify the DNA-binding ability of NF-κB proteins prepared from TNF-treated cells (Fig. 1E). These results suggest that fisetin inhibits NF-κB activation by a mechanism different from that of plumbagin.

Fisetin Suppressed Constitutive NF-κB Activation. Because the signal transduction pathway mediated by NF-κB may be distinct in cell types with constitutive NF-κB activation, we also determined whether fisetin blocked constitutive NF-κB activation in Burkitt's lymphoma Daudi cells. We found that these cells displayed constitutive NF-κB activation and that fisetin suppressed it (Fig. 1F), indicating that fisetin can suppress not only inducible but also constitutive NF-κB activation.

Fisetin Suppressed NF-κB Activation in Normal Cells. Because the signal transduction pathway mediated by NF-κB may be distinct in different cell types, we also determined whether fisetin blocked TNF-induced NF-κB activation in normal human fibroblast cells. We found that TNF activated NF-κB and fisetin suppressed TNF-induced NF-κB activation in normal fibroblast (Fig. 1G), indicating that fisetin-induced suppression of NF-κB activation is not cell type-specific.

Fisetin Blocks NF-κB Activation Induced by Various Agents. Because TNF, okadaic acid, lipopolysaccharide (LPS), EGF, and PMA are known to activate NF-κB but by different mechanisms, we examined the effect of fisetin on the activation of NF-κB by these agents. All five agents activated NF-κB, and fisetin suppressed this activation (Fig. 1H). These results suggest that fisetin acts at a step in the NF-κB activation pathway that is common to all five agents.

Fisetin Inhibited TNF-Induced IκBα Degradation. Because IκBα degradation is required for activation of NF-κB by most agents, we determined whether fisetin inhibited TNF-induced NF-κB activation by inhibition of IκBα degradation. We detected TNF-induced IκBα degradation in control cells as early as 10 min but found no degradation in fisetin-pretreated cells (Fig. 2A).

Fisetin Inhibited TNF-Dependent IκBα Phosphorylation and Ubiquitination. To determine whether the inhibition of TNF-induced IκBα degradation was caused by inhibition of IκBα phosphorylation and ubiquitination, we blocked degradation of IκBα with the proteasome inhibitor *N*-acetyl-leucyl-leucyl-norleucinal. Western blot analysis using an antibody that recognizes the serine-phosphorylated form of IκBα showed that TNF-induced IκBα phosphorylation was strongly suppressed by fisetin (Fig. 2B). The results also show that TNF-induced IκBα ubiquitination was suppressed by fisetin (Fig. 2B).

Fisetin Inhibited TNF-Induced IκBα Kinase Activation. Because fisetin inhibits the phosphorylation and degradation of IκBα, we tested the effect of fisetin on TNF-induced IKK activation, which is required for TNF-induced phosphorylation of IκBα. As shown in Fig. 2C, top, TNF activated IKK and fisetin completely suppressed TNF-induced activation of IKK. Neither TNF nor fisetin had any effect on the expression of IKKα or IKKβ proteins (Fig. 2C). This finding suggests that fisetin modulates TNF-induced IKK activation.

Fisetin Inhibited TNF-Induced Phosphorylation of p65. Because phosphorylation is also required for p65 transcriptional activity, we investigated the effect of fisetin on TNF-induced phosphorylation of p65. TNF induced phosphorylation of p65 in a time-dependent manner, and fisetin suppressed it (Fig. 2D, middle).

Fisetin Inhibited TNF-Induced Nuclear Translocation of p65. Whether fisetin modulated TNF-induced nuclear translocation was examined by Western blot and by immunocytochemistry. In the nuclear fraction from the TNF-treated cells, there was a time-dependent increase in the nuclear translocation of p65, and fisetin suppressed it (Fig. 2D, top). An immunocytochemistry assay also confirmed that fisetin suppressed translocation of p65 to the nucleus (Fig. 2E).

Fisetin Repressed TNF-Induced NF-κB-Dependent Reporter Gene Expression. Because DNA binding alone does not always correlate with NF-κB-dependent gene transcription (Campbell et al., 2004), we also investigated the effect of fisetin on TNF-induced reporter gene transcription. We found that TNF produced ~6-fold increase in SEAP activity over vector control (Fig. 3A). When the cells were pretreated with fisetin, TNF-induced NF-κB-dependent SEAP expression was inhibited in a dose-dependent manner. These results indicated that fisetin inhibits the NF-κB-dependent reporter gene expression induced by TNF.

Because TNF-induced NF-κB activation is mediated through the sequential interaction of the TNF receptor with TRADD, TRAF2, NIK, and IKK, leading to the degradation of IκBα and p65 nuclear translocation, we also investigated where in the pathway fisetin suppresses gene transcription. To determine this, cells were transfected with TNFR1, TRADD, TRAF2, NIK, IKKβ, and p65 plasmids, along with the NF-κB-regulated SEAP reporter construct, incubated with fisetin, and then monitored for NF-κB dependent SEAP expression. Fisetin suppressed the NF-κB reporter activity

for 30 min with the indicated concentrations of fisetin. They were then assayed for NF-κB activation by EMSA. The results shown are representative of three independent experiments. F, fisetin inhibits constitutive NF-κB activation in Daudi cells. Cells were incubated with different concentrations of fisetin for 6 h, prepared the nuclear extracts and assayed for NF-κB activation by EMSA. G, fisetin inhibits TNF-induced NF-κB activation in normal cells. Normal human fibroblast cells were incubated with 50 μM fisetin for 6 h and then treated with 0.1 nM TNF for 30 min. The nuclear extracts were prepared and assayed for NF-κB activation by EMSA. H, fisetin blocks NF-κB activation induced by TNF, EGF, okadaic acid, PMA, and lipopolysaccharide. H1299 cells were preincubated with 50 μM fisetin for 6 h and then treated with 0.1 nM TNF for 30 min, 100 ng/ml EGF for 2 h, 500 nM okadaic acid for 4 h, 25 ng/ml PMA for 2 h, and 10 μg/ml lipopolysaccharide for 2 h. Nuclear extracts were analyzed for NF-κB activation as described under *Materials and Methods*. The results shown are representative of three independent experiments.

induced by the TNFR1, TRADD, TRAF2, NIK, and IKKβ plasmids but had no effect on the activity induced by the p65 plasmid (Fig. 3B). These results suggest that fisetin affects a step upstream of p65.

Fisetin Inhibited TAK1/TAB1-Induced NF-κB-Dependent Reporter Gene Expression. Recent studies indicate that TAK1 plays a major role in the canonical pathway through its interaction with TAB1 and TAB2 (Takaesu et al., 2003). Therefore, we investigated whether fisetin suppressed TNF-induced NF-κB activation through the inhibition of TAK1. As shown in Fig. 3C, TAK1 activated NF-κB reporter activity, and fisetin inhibited the activation.

Fisetin Inhibited RIP-Induced NF-κB-Dependent Reporter Gene Expression. It has been shown that ubiq-

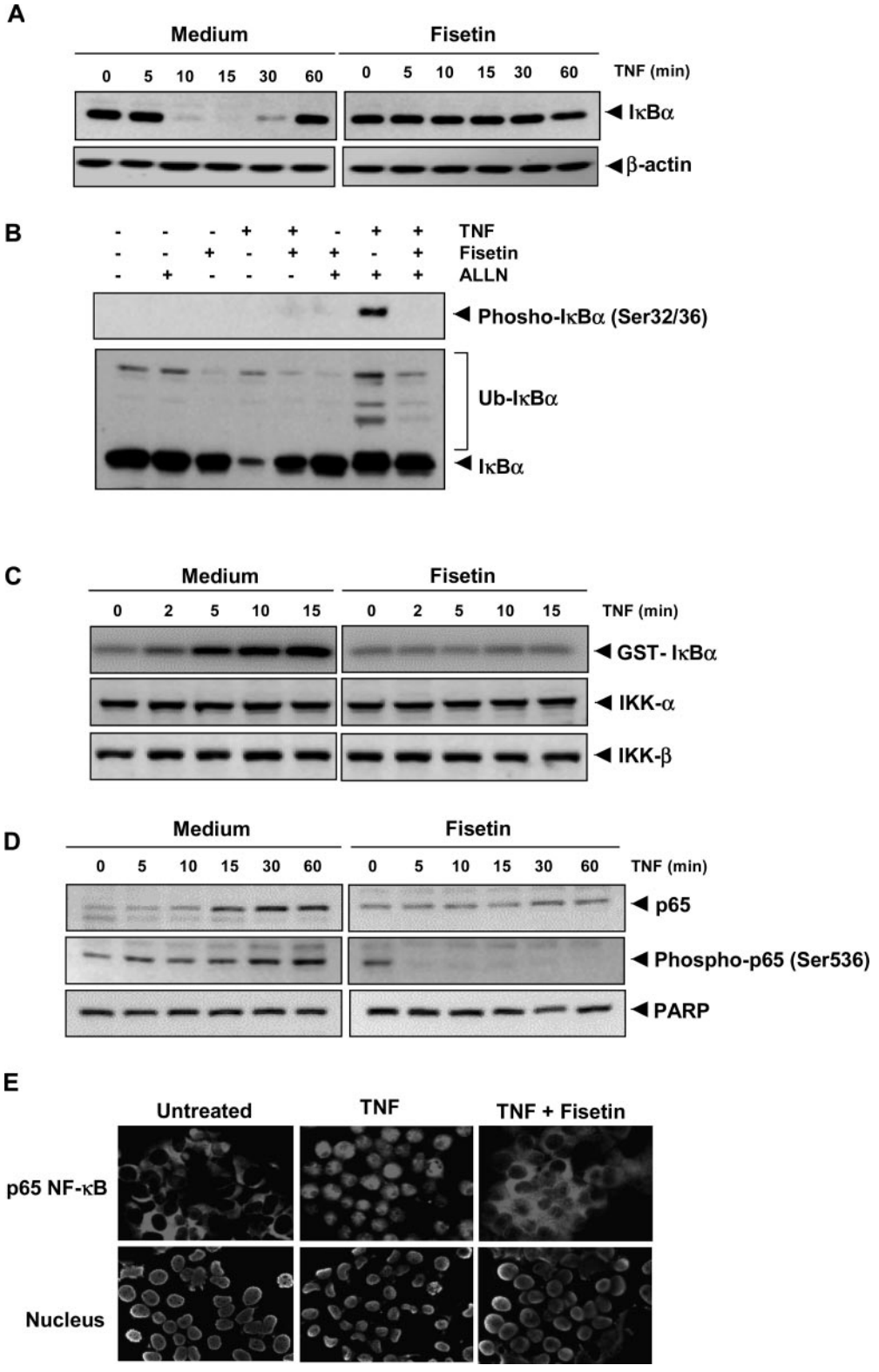

Fig. 2. A, effect of fisetin on TNF-induced degradation of IκBα. H1299 cells were incubated with 50 μM fisetin for 6 h and treated with 0.1 nM TNF for the indicated times. Cytoplasmic extracts were prepared and analyzed by Western blotting using antibodies against anti-IκBα. The results shown are representative of three independent experiments. Equal protein loading was evaluated by β-actin. B, effect of fisetin on the phosphorylation of IκBα by TNF. Cells were preincubated with 50 μM fisetin for 6 h, incubated with 50 μg/ml *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) for 30 min, and then treated with 0.1 nM TNF for 10 min. Cytoplasmic extracts were fractionated and then subjected to Western blot analysis using phosphospecific IκBα (Ser 32/36) antibody. The same membrane was reblotted with anti-IκBα antibody. C, effect of fisetin on the activation of IKK by TNF. H1299 cells were preincubated with 50 μM fisetin for 6 h, incubated with 50 μg/ml *N*-acetyl-leucyl-leucyl-norleucinal for 30 min, and then treated with 1 nM TNF for the indicated times. Whole-cell extracts were immunoprecipitated with antibody against IKK-α and analyzed by an immune complex kinase assay. To examine the effect of fisetin on the level of expression of IKK proteins, whole cell extracts were fractionated on SDS-PAGE and examined by Western blot analysis using anti-IKK-α and anti-IKK-β antibodies. The results shown are representative of three independent experiments. D, fisetin inhibits TNF-induced phosphorylation and nuclear translocation of p65. H1299 cells were either untreated or pretreated with 50 μM fisetin for 6 h at 37°C and then treated with 0.1 nM TNF for the indicated times. Nuclear extracts were prepared and analyzed by Western blotting using antibodies against phospho-specific p65 (Ser 536) and p65. The results shown are representative of three independent experiments. For loading control of nuclear protein, the membrane was blotted with anti-PARP antibody. E, immunocytochemical analysis of p65 localization. H1299 cells were first treated with 50 μM fisetin for 6 h at 37°C and then exposed to 1 nM TNF for 20 min. After cytospin, immunocytochemical analysis was done as described under *Materials and Methods*. The results shown are representative of three independent experiments.

uitination of RIP is required for TNF-induced NF-κB activation (Ea et al., 2006). Ubiquitinated RIP then interacts with TAK1/TAB1 to activate the IKK complex (Li et al., 2006). Therefore, we investigated whether fisetin suppresses TNF-induced NF-κB activation through the inhibition of RIP. As shown in Fig. 3D, RIP overexpression increased NF-κB-

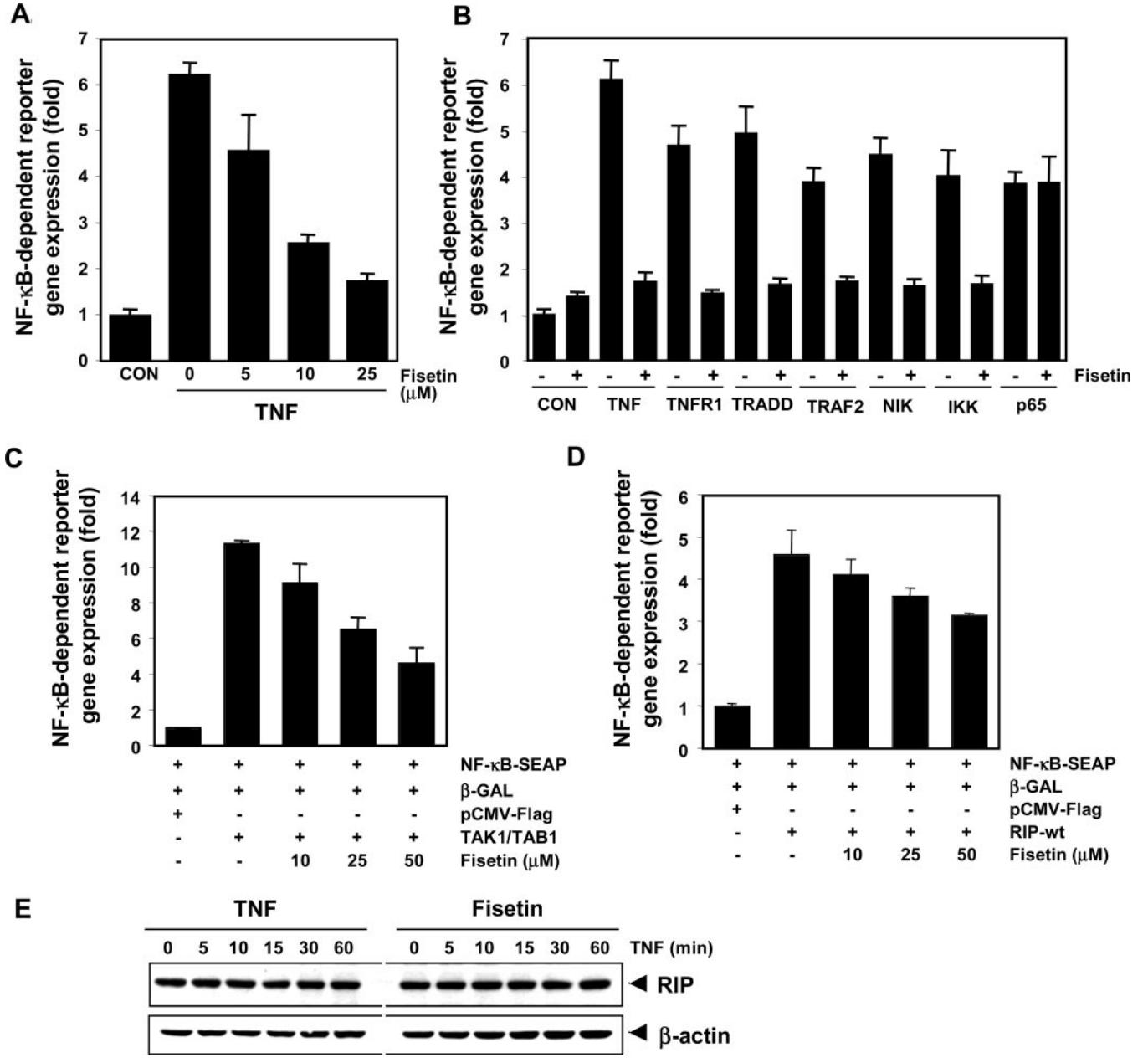

Fig. 3. A, fisetin inhibits TNF-induced NF-κB-dependent reporter gene (SEAP) expression. A293 cells were transiently transfected with a NF-κB containing plasmid linked to the SEAP gene and then treated with the indicated concentrations of fisetin. After 24 h in culture with 1 nM TNF, cell supernatants were collected and assayed for SEAP activity as described under *Materials and Methods*. Results are expressed as -fold activity over the activity of the vector control. B, fisetin inhibits NF-κB-dependent reporter gene expression induced by TNF, TNFR1, TRADD, TRAF2, NIK, and IKKβ but not by p65. Cells were transiently transfected with an NF-κB-containing plasmid alone or with the indicated plasmids. After 24 h, cells were treated with 25 μM fisetin and then incubated with the relevant plasmid for an additional 24 h. For TNF-treated cells, cells were treated with 25 μM fisetin and then incubated with 1 nM TNF for 24 h. The supernatants of the culture medium were assayed for SEAP activity as described under *Materials and Methods*. The results shown are representative of three independent experiments. C, effect of fisetin on TAK1/TAB1-induced NF-κB activation. Cells were transiently transfected with TAK1/TAB1 expression plasmid along with NF-κB-containing plasmid. After 24 h, cells were treated with the indicated concentrations of fisetin and incubated with the relevant plasmid for an additional 24 h. The supernatants of the culture medium were assayed for SEAP activity as described under *Materials and Methods*. The results shown are representative of three independent experiments. D, effect of fisetin on RIP-induced NF-κB activation. Cells were transiently transfected with RIP expression plasmid along with NF-κB-containing plasmid. After 24 h, cells were treated with the indicated concentrations of fisetin and incubated with the relevant plasmid for an additional 24 h. The supernatants of the culture medium were assayed for SEAP activity as described under *Materials and Methods*. The results shown are representative of three independent experiments. E, effect of fisetin on TNF-induced RIP expression. H1299 cells, either untreated or pretreated with 50 μM fisetin for 6 h at 37°C, were treated with 0.1 nM TNF for the indicated times, prepared the cytosolic extracts and analyzed by Western blotting using antibodies against RIP. The results shown are representative of three independent experiments. For loading control of cytosolic protein, the membrane was blotted with anti-β actin antibody.

SEAP activity by up to 5-fold, and fisetin inhibited the activation in a dose-dependent manner. The down-regulation of RIP-mediated NF-κB reporter activity was not due to down-regulation of RIP expression, because neither TNF nor TNF in combination with fisetin had any effect on RIP expression (Fig. 3E).

Fisetin Repressed the Expression of TNF-Induced NF-κB-Dependent Antiapoptotic Gene Products. Because NF-κB regulates the expression of the antiapoptotic proteins such as survivin, cIAP1/2, XIAP, Bcl-2, Bcl-xL, and TRAF-1 (Aggarwal, 2004), we investigated whether fisetin could modulate TNF-induced expression of these proteins. We found that TNF induced these gene products and that fisetin suppressed TNF-induced expression of these antiapoptotic proteins in a time-dependent manner (Fig. 4A).

Fisetin Suppressed the Expression of TNF-Induced NF-κB-Dependent Gene Products Involved in the Proliferation of Tumor Cells. Because fisetin is known to suppress the proliferation of tumor cells (Chen et al., 2002; Haddad et al., 2006), we also investigated whether fisetin could modulate NF-κB-regulated gene products involved in the proliferation of tumor cells. TNF has been shown to induce cell proliferation-regulating genes such as c-Myc, cyclin D1, and COX-2, all of which have NF-κB-binding sites in their promoters (Aggarwal, 2004). Thus, we investigated whether fisetin inhibits the TNF-induced expression of these proteins. Untreated cells and those pretreated with fisetin were examined for TNF-induced gene products by Western blot analysis using specific antibodies. Fisetin abolished TNF-induced expression of COX-2, cyclin D1, and c-Myc (Fig. 4B).

Fisetin Suppressed the Expression of TNF-Induced NF-κB-Dependent Gene Products Involved in Metastasis of Tumor Cells. We also investigated whether fisetin could modulate NF-κB-regulated gene products involved in the invasion and angiogenesis of tumor cells. TNF was found to induce the expression of gene products MMP-9, ICAM-1, and VEGF, all of which have NF-κB-binding sites in their promoters (Aggarwal, 2004), and fisetin abolished TNF-induced expression of metastatic gene products MMP-9, ICAM-1, and VEGF (Fig. 4C).

Fisetin Down-Regulated c-Myc in Burkitt's Lymphoma Cells. Burkitt's lymphoma cells, which express constitutive active NF-κB, also overexpress c-Myc protein. Whether fisetin could down-regulate the expression of c-Myc in Burkitt's lymphoma cells was examined. As shown in Fig. 4D, we found that Daudi cells overexpressed c-Myc and that treatment with fisetin down-regulated the expression (Fig. 4D).

Fisetin Potentiates Apoptosis Induced by TNF and Chemotherapeutic Agents. Because the activation of NF-κB has been shown to inhibit apoptosis induced by TNF and chemotherapeutic agents (Mayo et al., 1997; Giri and Aggarwal, 1998; Wang et al., 1998), we investigated whether fisetin affects TNF- and chemotherapeutic agent-induced apoptosis. The MTT method showed that fisetin enhanced TNF-induced cytotoxicity (Fig. 5A) and that cytotoxicity induced by doxorubicin (Fig. 5B) and cisplatin (Fig. 5C) is also potentiated by fisetin. Fisetin alone at this concentration had minimal effect. As examined by the esterase-staining method (also called LIVE/DEAD assay), fisetin up-regulated TNF-induced apoptosis from 3 to 80% (Fig. 5D). Consistent with these results, annexin V staining showed that fisetin up-regulated TNF-induced early apoptosis in a dose-dependent manner (Fig. 5E). Caspase-mediated PARP cleavage likewise showed that fisetin enhanced the apoptotic effect of TNF substantially (Fig. 5F). These results together indicate that fisetin potentiates the apoptotic effects of TNF.

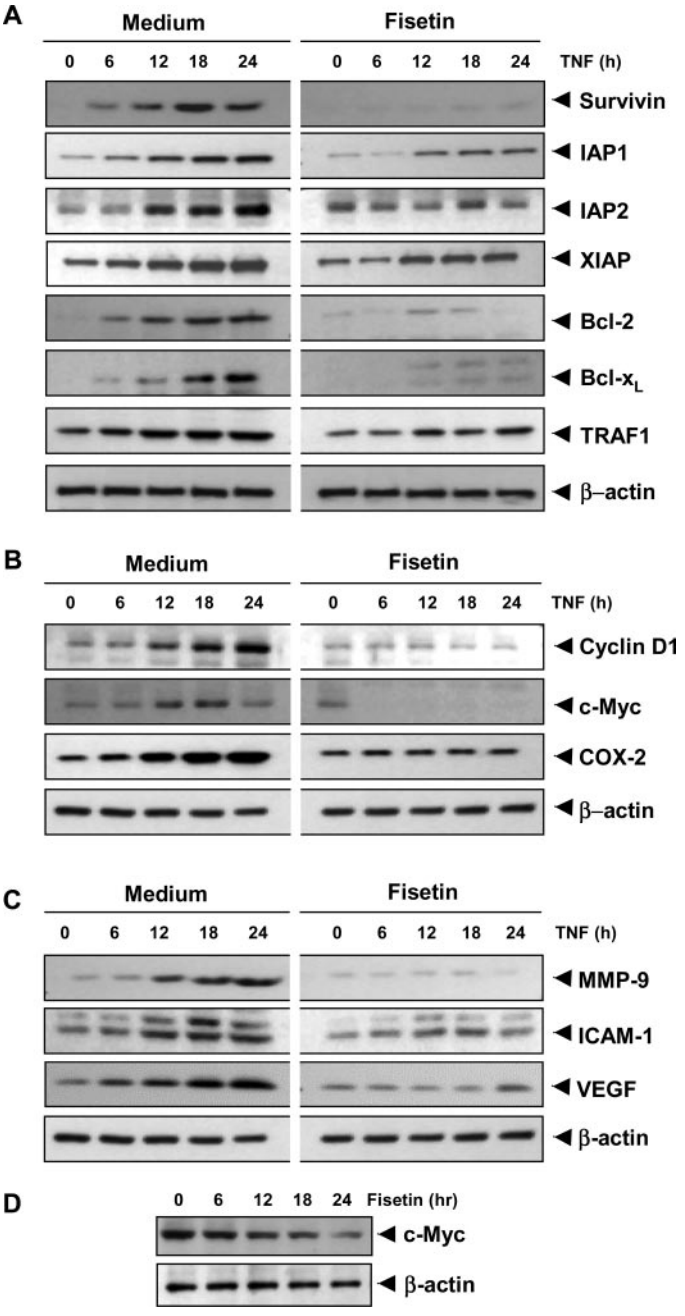

Fig. 4. Fisetin inhibits TNF-induced expression of NF-κB-dependent antiapoptotic, proliferative, and metastatic proteins. A, fisetin inhibits the expression of TNF-induced antiapoptotic proteins. B, fisetin inhibits TNF-induced cell proliferative proteins, cyclin D1, c-Myc, and COX-2. C, fisetin blocks the expression of TNF-induced metastatic proteins. H1299 cells were incubated with 25 μM fisetin for 6 h and then treated with 1 nM TNF for the indicated times. Whole-cell extracts were prepared and analyzed by Western blotting using the indicated antibodies. The results shown are representative of three independent experiments. D, fisetin inhibits c-Myc expression in Daudi cells. Cells were incubated with 25 μM fisetin for the indicated times, prepared whole-cell extracts and analyzed by Western blotting using the anti-c-Myc antibody. The results shown are representative of three independent experiments.

3,7,3′,4′-Tetrahydroxyflavone Was Most Active in Inhibiting NF-κB Activation. Fisetin is a 3,7,3′,4′-tetrahydroxyflavone. Several structural analogs of fisetin have been identified (Fig. 6A). These include 3-hydroxyflavone, myricetin, kaempferol, taxifolin, quercitrin, isorhamnetin, morin, and quercetin. Whether they could suppress TNF-induced NF-κB activation to the same extent as fisetin was investigated. None of the flavones by themselves activated NF-κB. Figure 6A shows that only fisetin suppressed TNF-induced NF-κB activation. Under these conditions, cells were fully viable as determined by the trypan blue dye exclusion test (data not shown). These results show that both the position and number of phenyl hydroxyl groups present in the flavone are critical for the suppression of NF-κB activation.

Discussion

The present study was designed to investigate the effect of fisetin on TNF-induced NF-κB activation pathway and on the NF-κB-regulated gene products that control tumor cell survival, proliferation, invasion, angiogenesis, and metastasis. We found that fisetin suppressed NF-κB activation, both

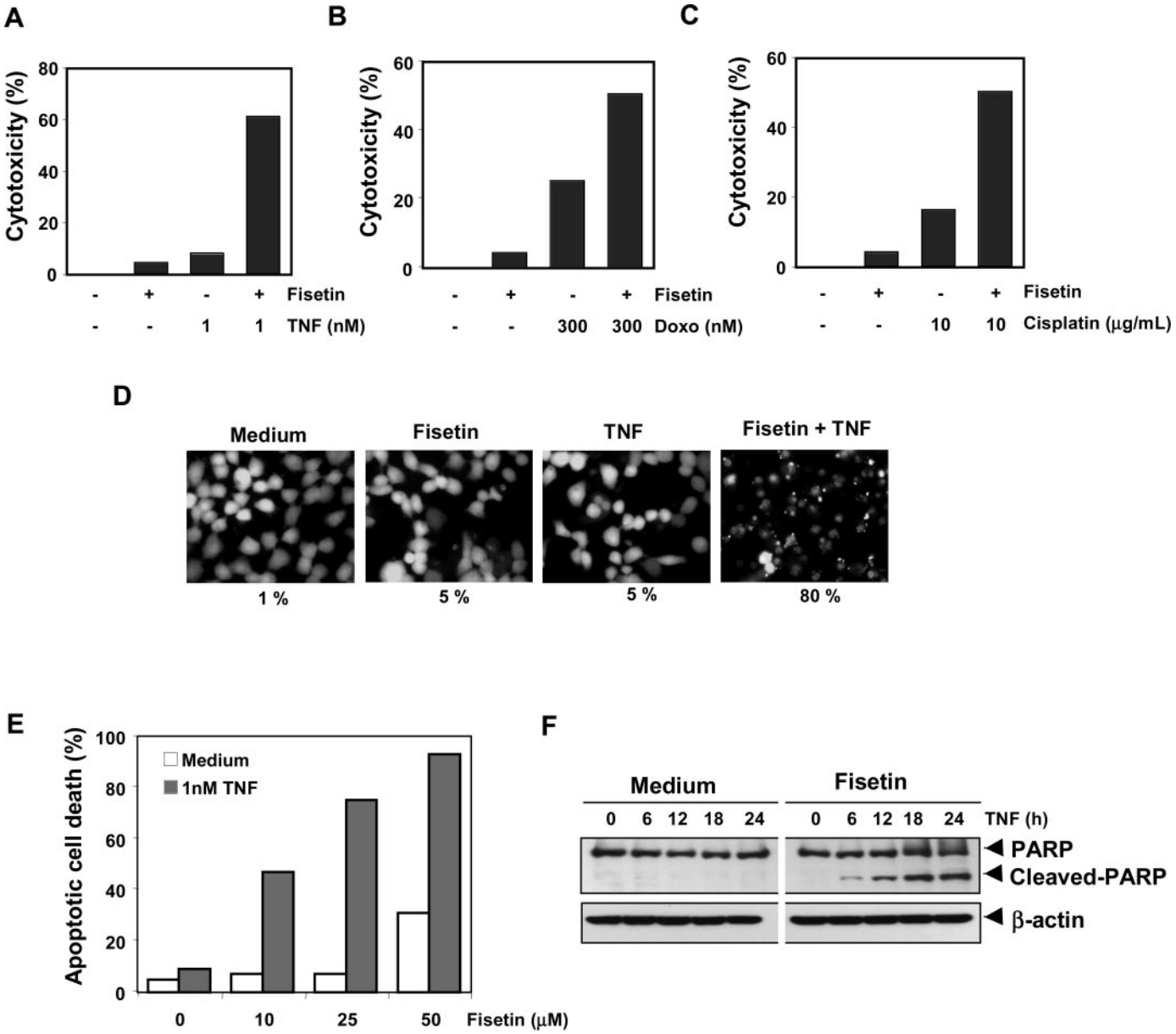

Fig. 5. Fisetin enhances TNF-induced cytotoxicity. A, B and C, fisetin enhances TNF, doxorubicin, and cisplatin-induced cytotoxicity. In total, 5000 cells were seeded in triplicate in 96-well plates. The cells were pretreated with 25 μM fisetin and then incubated with the indicated concentrations of TNF, doxorubicin, and cisplatin for 24 h. Cell viability was then analyzed by the MTT method as described under *Materials and Methods*. D, H1299 cells were pretreated with 25 μM fisetin for 6 h and then incubated with 1 nM TNF for 16 h. The cells were stained with a LIVE/DEAD assay reagent for 30 min and then analyzed under a fluorescence microscope as described under *Materials and Methods*. The results shown are representative of three independent experiments. E, cells were pretreated with indicated concentration of fisetin for 6 h and then incubated with 1 nM TNF for 16 h. The cells were incubated with a fluorescein isothiocyanate-conjugated annexin V antibody and then analyzed by flow cytometry as described under *Materials and Methods*. The results shown are representative of two independent experiments. F, cells were pretreated with 25 μM fisetin for 6 h and then incubated with 1 nM TNF for the indicated times. Whole-cell extracts were prepared and analyzed by Western blotting using an anti-PARP antibody. The results shown are representative of three independent experiments.

constitutive and that induced by various carcinogens, growth factors, and inflammatory agents. Suppression of NF-κB activation by fisetin was due to inhibition of RIP, TAK1, and IKK activation, which led to inhibition of IκBα phosphorylation and degradation, suppression of p65 phosphorylation and translocation to the nucleus, and inhibition of NF-κB-dependent reporter gene expression. Fisetin also down-regulated NF-κB -dependent gene products involved in cell proliferation, in antiapoptosis, and in invasion. This down-regulation led to the potentiation of apoptosis induced by cytokines and chemotherapeutic agents (Fig. 6B).

We found that fisetin inhibits the NF-κB activation induced by a wide variety of agents, including TNF, okadaic acid, EGF, PMA, and LPS. This suggests that fisetin must act at a step common to all of these activators. In response to most of these stimuli, NF-κB activation requires the activation of IKK. The suppression of TNF-induced IKK activation by fisetin implies that it abolishes NF-κB activation by other agents, also through a suppression of IKK activation. We also investigated how fisetin suppresses IKK activation. Several kinases, such as MEKK1 (Lee et al., 1998), MEKK3 (Yang et al., 2001), protein kinase C (Lallena et al., 1999), glycogen synthase kinase-3β (Hoeflich et al., 2000), TAK1 (Ea et al., 2006), PDK1 (Tanaka et al., 2005), and Akt (Gustin et al., 2001), have all been reported to function upstream of IKK. Recent studies, however, indicate that TAK1 plays a major role in the canonical pathway activated by cytokines through its interaction with TAB1 and TAB2. For instance, TAK1 can bind and activate IKK-β leading to NF-κB activation (Sakurai et al., 1999). RIP also is reported to play critical role in TNF-induced IKK activation and subsequent activation of NF-κB through recruiting TAK1 and MEKK3 to TNF-R1 (Blonska et al., 2005). Indeed, our study showed that both RIP-mediated and TAK1-induced NF-κB activation is inhibited by fisetin. It is unlikely, however, that RIP is a direct target of fisetin because stimuli that activate NF-κB through RIP-independent pathway (e.g., okadaic acid and PMA) are also inhibited by fisetin.

Fisetin has been shown to inhibit numerous protein kinases, including CDKs, glycogen synthase kinase-3β, tyrosine kinase activity of EGF receptor, phosphatidylinositol 3-kinase, and protein kinase C (Agullo et al., 1997; Lu et al., 2005b). This flavonoid has been shown to bind to CDK6 and inhibit its activity (Lu et al., 2005a). Although it is unlikely that inhibition of CDK6 by fisetin is involved in the inhibition of NF-κB activation pathway, other CDK inhibitors, such as flavopiridol, have also been shown to suppress NF-κB activation (Takada and Aggarwal, 2004). Our results suggest

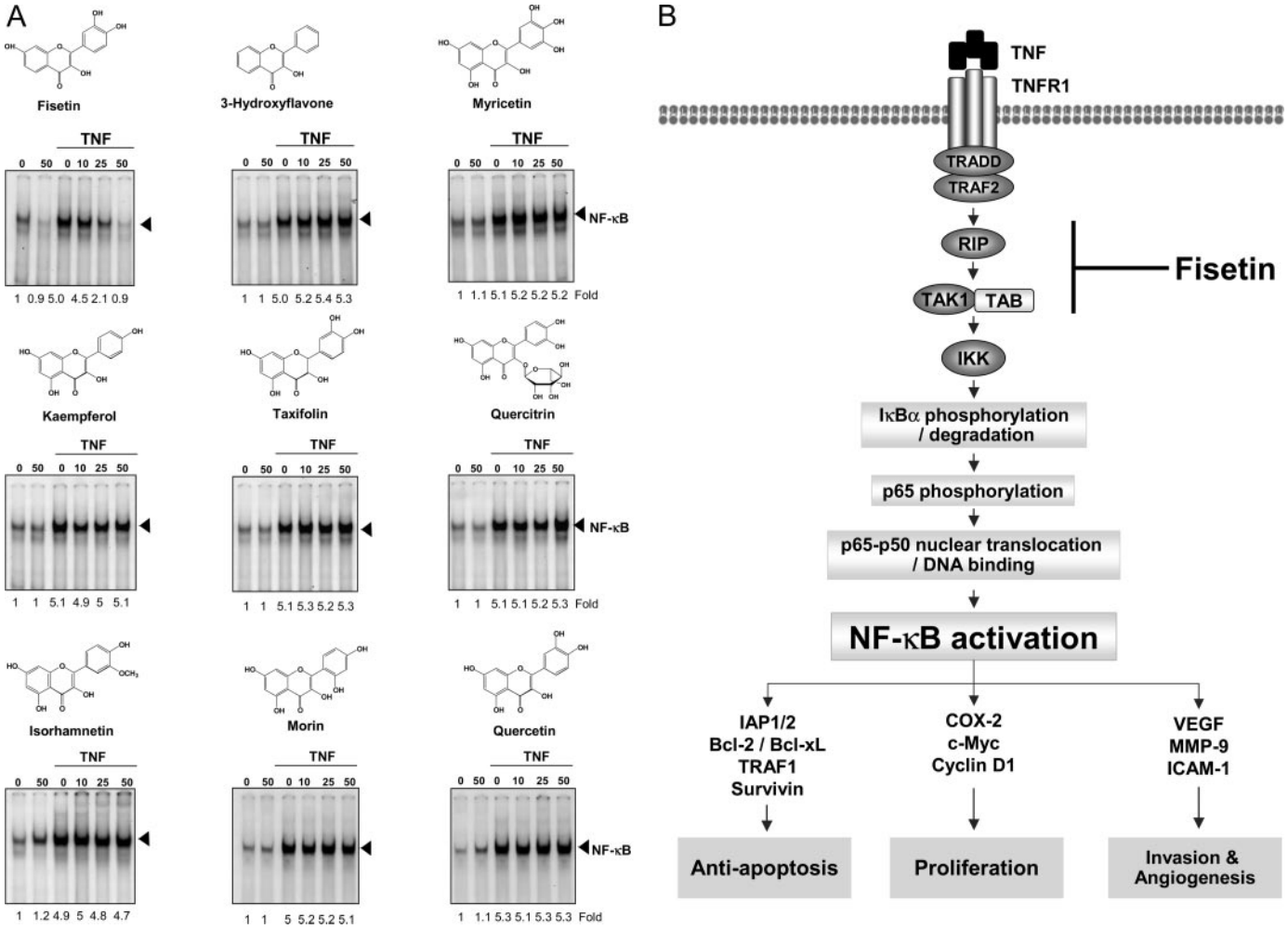

Fig. 6. *A*, effect of different concentrations of various flavones on TNF-induced NF-κB activation. H1299 cells were pretreated with different doses of fisetin, 3-hydroxyflavone, quercetin, quercitrin, kaempferol, taxifolin, isorhamnetin, morin and myricetin for 6 h before TNF treatment. Then nuclear extracts were prepared and analyzed by EMSA. B, a schematic diagram of the effect of fisetin on TNF-induced NF-κB activation and apoptosis.

that fisetin blocks IKK activation through the inhibition of TAK1. The 4-keto group and 3-hydroxy group of fisetin are hydrogen-bonded with the backbone in the hinge region between N- and C-terminal kinase domains of CDKs, producing a conformational change that leads to loss of activity of CDKs (Lu et al., 2005a). Whether suppression of all other kinases occurs through the same mechanism is not yet known. Similar to CDK6, however, among all the flavones tested, only fisetin suppressed the TNF-induced NF-κB activation. These results imply the importance of the position of phenyl hydroxyl groups and their number in the flavone for NF-κB suppressive activity.

We found that suppression of NF-κB activation correlated with the suppression of expression of the gene products survivin, IAP1, IAP2, XIAP, Bcl-2, Bcl-xL and TRAF1, all known to inhibit apoptosis. Suppression of these gene products was accompanied by enhancement of the apoptotic effects of TNF. An impressive potentiation of apoptosis by fisetin was also observed with doxorubicin and cisplatin. The increase in caspase-3-mediated PARP cleavage induced by fisetin is in agreement with previous reports (Chen et al., 2002; Lee et al., 2002). That fisetin can sensitize U-937 cells to TNF-induced apoptosis has been demonstrated before (Monasterio et al., 2004), but our results provide the mechanism of sensitization. Fisetin has been shown to suppress the proliferation of a wide variety of tumor cells, including cancers of the prostate (Haddad et al., 2006), liver (Chen et al., 2002), colon (Kuntz et al., 1999; Lu et al., 2005b), and leukemia (Lee et al., 2002). These antiproliferative effects may also be due to the down-regulation of expression of cyclin D1, c-Myc, and COX-2 as reported in our studies.

We further observed that fisetin could down-regulate the expression of MMP-9, ICAM-1, and VEGF gene products, all regulated by NF-κB. A report on the down-regulation of ELAM-1 by fisetin (Takano-Ishikawa et al., 2003) could also be due to the effect of fisetin on NF-κB activation. MMP-9 has been implicated in invasion of tumors, suggesting that fisetin may prevent tumor invasion. In addition, VEGF is a critical mediator of angiogenesis, and suppression of its expression may thus explain the previously reported antiangiogenic effect of fisetin (Fotsis et al., 1998).

Various antiinflammatory activities previously assigned to fisetin could be due to down-regulation of TNF signaling and consequent COX-2 expression as described here. Lyu and Park (2005) recently reported that fisetin can inhibit the secretion of TNFα and interleukin-2 and NO production in LPS-induced Raw 264.7 cells. This effect is also most likely mediated through suppression of NF-κB pathway as reported here. Overall, our results indicate that the antiproliferative, proapoptotic, antiangiogenic, and antimetastatic effects of fisetin may be mediated through suppression of NF-κB-regulated gene products. However, further studies are needed in animals to validate these findings for the therapeutic use of fisetin.

Acknowledgments

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MOL PHARM

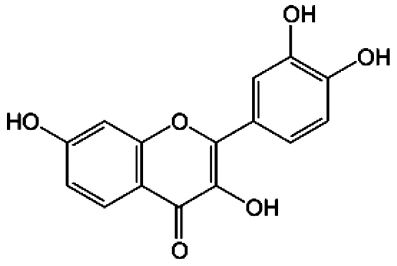
MOLECULAR PHARMACOLOGY

Correction to "Fisetin, an Inhibitor of Cyclin-Dependent Kinase 6, Down-Regulates Nuclear Factor-κB-Regulated Cell Proliferation, Antiapoptotic and Metastatic Gene Products through the Suppression of TAK-1 and Receptor-Interacting Protein-Regulated IκBα Kinase Activation"

In the above article [Sung B, Mandey MK, and Aggarwal BB (2007) *Mol Pharmacol* **71:**1703–1714], the Fisetin structure presented in Fig. 1A was incorrect. The correct structure of Fisetin is shown below.

In addition, in the second paragraph of the article, in the sentence beginning "Fisetin (3,3′,4′,7-tetrahydroxyflavone) is a flavonoid . . .," the concentration range stated is incorrect. The concentration should be "0.1 to 160 μg/g."

The authors regret these errors and apologize for any confusion or inconvenience it may have caused.



Fisetin (MW-286.24)

3,3',4',7-tetrahydroxy-flavone

Fig. 1A.