Mangiferin, a natural polyphenol is known to exhibit anti-inflammatory, antioxidant, and antiviral effects. However, the molecular mechanism underlying these effects has not been well characterized. Because NF-κB plays an important role in these processes, it is possible that mangiferin modulates NF-κB activation. Our results show that mangiferin blocks tumor necrosis factor (TNF)-induced NF-κB activation and NF-κB-dependent genes like ICAM1 and COX2. The effect was mediated through inhibition of IKK activation and subsequent blocking of phosphorylation and degradation of IκBα. In addition, mangiferin inhibits TNF-induced p65 phosphorylation as well as translocation to the nucleus and also inhibits NF-κB activation induced by other inflammatory agents like PMA, ceramide, and SA-LPS. Mangiferin, similar to the other known antioxidants, NAC and PDTC, inhibits TNF-induced reactive oxygen intermediate (ROI) generation. Since intracellular glutathione (GSH) levels are known to modulate NF-κB, we measured the levels of GSH. Mangiferin enhances glutathione level by almost 2-fold more than other antioxidants, and at the same time it decreases the levels of GSSG and increases the activity of catalase. Depletion of GSH by buthionine sulfoximine led to a significant reversal of mangiferin effect. Hence mangiferin with its ability to inhibit NF-κB and increase the intracellular GSH levels may prove to be a potent drug for anti-inflammatory and antioxidant therapy. Mangiferin-mediated down-regulation of NF-κB also potentiates chemotherapeutic agent-mediated cell death, suggesting a role in combination therapy for cancer.

Tumor necrosis factor (TNF) is one of the most multifaceted cytokines known to regulate inflammation, abnormal cell growth, immunomodulation, septic shock syndrome, etc. TNF, which is produced in the early stage of inflammation, regulates the production of other cytokines, such as IL-1β and IL-6, and thus is a major cytokine in inflammatory responses (1, 2). Several reports indicate that TNF mediates most of its effects by altering the redox state of the cell (3–7). Some reports suggest that TNF produces reactive oxygen intermediates (ROI), which in turn oxidizes intracellular glutathione, a critical change for TNF-induced apoptosis and nuclear transcription factor κB (NF-κB) activation (8–11).

Cytokines and ROI are frequent companions at sites of acute inflammation (12). Imbalance between production and elimination of ROI develops during inflammation, ischemia/reperfusion, altered metabolism, action of drugs, pollutants, etc. Such disbalance causes pathology of brain, heart, airways, parenchymatous organs (liver, kidney, and pancreas), eyes, skin, joints, etc. Exposure of the tissues to reactive oxygen species (ROS) in a variety of biological systems has documented their ability to damage lipids, proteins, and DNA. The effects of ROS could be prevented or stopped by eliminating and/or substituting antioxidant enzymes or administering non-enzyme antioxidants and scavengers.

The transcription factors thought to be involved in inflammation and allergic disorders are NF-κB, AP-1, CREB, STAT, and GATA-3. Most of the inflammatory genes overexpressed in inflammation such as those encoding proinflammatory cytokines, chemokines, adhesion molecules, and inflammatory enzymes contain κB sites for NF-κB within their promoter suggesting that these genes are controlled predominantly by NF-κB (13). NF-κB is a heterodimer of two subunits p50 (NF-κB1) and p65 (Rel A), which is normally present in the cytoplasm in an inactive state, complexed with its inhibitory subunit, IκBα. Upon phosphorylation and subsequent degradation of IκBα, the nuclear localization signal on the p50-p65 heterodimer is exposed, leading to nuclear translocation and transcription of dependent genes (14). The activation of NF-κB and its associated kinases like IκB kinase (IKK) is in most cases dependent on the production of reactive oxygen species (15–18).

GSH is important for many cellular and biochemical functions including the regulation of gene transcription, as well as the modulation of apoptosis (19, 20). The binding of many transcription factors to their cognate DNA sequences is sensitive to the redox environment. One such redox sensitive transcription factor is NF-κB (20–22). Several reports suggested that NF-κB activation is critical for the regulation of genes that are mediating cell survival and may have an anti-apoptotic role; hence, the inhibition of NF-κB sensitizes the cells to various apoptosis inducing agents, which include TNF and chemothera-

**β-δ-Glucoside Suppresses Tumor Necrosis Factor-induced Activation of Nuclear Transcription Factor κB but Potentiates Apoptosis**

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‡‡‡‡ The abbreviations used are: TNF, tumor necrosis factor; ATZ, 3-amino-1,2,4-triazole; BSO, t-buthionine sulfoximine; CE, cytoplasmic extract; COX, cyclooxygenase; γ-GCS, γ-glutamylcystein synthetase; IκBα, inhibitory subunit of κB; IκBα-CN, IκBα dominant negative; IKK, IκB kinase; mangiferin, β-δ-glucoside; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NE, nuclear extract; NIK, NF-κB-inducing kinase; PDTC, pyrillidone dithiocarbamate; PIS, pre-immune serum; PMA, phorbol myristate acetate, ROI, reactive oxygen intermediate; ROS, reactive oxygen species; SEAP, secretory alkaline phosphatase; TRADD, TNF receptor-associated death domain; TRAF, TNF receptor-associated factor; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; LPS, lipopolysaccharide; NAC, N-acetyl cysteine; IL, interleukin.
Phenolic antioxidants exhibit anti-inflammatory, anticarcinogenic, and antidiabetic activities in animals (25–27). Human beings consume antioxidants from dietary sources, either as natural components or as synthetic food additives (28, 29). The broad spectrum of the function of phenolic antioxidants suggests there are multiple targets through which they interfere with various cellular functions and protect against pathological lesions such as cancer and inflammatory diseases. Currently there is an increasing interest in therapeutic use of antioxidants to prevent tissue damage induced by overproduction of ROI, by reducing free radical formation or by scavenging or promoting the breakdown of these species (30–32). Experiments in different in vitro and in vivo systems have demonstrated the potent antioxidant action of plant polyphenols (33), and it has been suggested that they can prevent oxidative-stress-related diseases (34). Recently, the polyphenol mangiferin, a C-glucosylxanthone, specifically 1,3,6,7-hydroxyxanthone-C2-β-D-glucoside, has attracted con-
siderable interest in view of its numerous pharmacological activities, including antitumor, antiviral (35, 36), antidiabetic (37, 38), antbone resorption (39), and antioxidant activity (40). As carcinogenic, inflammatory, and growth modulatory effects of many chemicals are mediated by NF-κB, we hypothesized that the suppression of the NF-κB activation pathway accounts for mangiferin activity.

In this study, we tested the hypothesis that the anti-inflammatory effects of mangiferin are mediated through modulation of NF-κB and glutathione levels. We have shown that mangiferin inhibits NF-κB by preventing the activation of IKK followed by phosphorylation and degradation of its inhibitory subunit IκBa. Also for the first time, we have demonstrated that mangiferin increases glutathione level and inhibits TNF-activated ROI. In this report, we also prove the hypothesis that down-regulation of NF-κB by mangiferin leads to potentiation of chemotherapeutic agent-mediated apoptosis.

EXPERIMENTAL PROCEDURES

Materials

Mangiferin, glycine, pyrrolidone dithiocarbamate (PDTC), 3-amino-1,2,4-triazole (ATZ), l-buthionine sulfoximine (BSO), tocopherol, N-acetyl cysteine (NAC), 4-methyl umbelliferyl phosphate, lipopolysaccharide (LPS), bovine serum albumin, phenol myristate acetate (PMA), ceramide, Texas Red, sulfosalicylic acid, NADPH, DTNB [5,5'-dithiobis(2-nitrobenzoic acid)], glutathione reductase, 2-vinylpiridine, triethanolamine, catalase, and anticalatase antibody were obtained from Sigma Aldrich Chemicals. Penicillin, streptomycin, neomycin, RPMI 1640 medium, Dulbecco's modified Eagle's medium, fetal bovine serum, and custom synthesized NF-κB oligonucleotides (both 5'-3' and 3'-5') were obtained from Invitrogen Life Technologies and TNF from Peprotech. Antibodies against p50, p65, IκBa, IKKα, IKKβ, cyclin D1, c-Rel, ICAM1, COX2, and tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against phospho-IκBa was obtained from Cell Signaling Technologies (Beverly, MA). Mounting medium (Manassas, VA). U-937 cells were cultured in RPMI 1640 medium while others in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). RPMI 1640 medium was supplemented with L-glutamine, sodium pyruvate, nonessential amino acids, catalase, and anticatalase antibody were obtained from Sigma. The other reagents, including 3-amino-1,2,4-triazole, N-acetyl cysteine, acetyl cysteine (NAC), 4-methyl umbelliferyl phosphate, lipopolysaccharide (LPS), bovine serum albumin, phorbol myristate acetate, 1,2,4-triazole (ATZ), l-buthionine sulfoximine, tocopherol, ROI. In this report, we also prove the hypothesis that down-regulation of NF-κB by mangiferin leads to potentiation of chemotherapeutic agent-mediated apoptosis.

Cell Lines

The cell lines used in this study were as follows: U-937 (human histiocytic lymphoma), HeLa (human epithelial cells), MCF-7 (human breast cancer cell line), KB3 AN27 (murine mesencephalic cells), and Jurkat (T cell) obtained from American Type Culture Collection (Manassas, VA). U-937 cells were cultured in RPMI 1640 medium while others in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). All cells were free of mycoplasma, as detected by Gen-Probe mycoplasma rapid detection kit (Fisher Scientific).

NF-κB Activation Assay

To determine NF-κB activation, EMSA was conducted essentially as described (41). Briefly, 8 μg of nuclear extract proteins were incubated at 37 °C in a CO2 incubator. ICAM1 (A) and COX2 (B) were detected from cell extract proteins (100 μg) by Western blot analysis. As loading control, tubulin was detected by re-probing the same blot. C, mangiferin inhibits COX2-induced gene expression. U-937 cells were transfected with the COX2-Luciferase expression vector and β-galactosidase gene. After 12 h of transfection, cells were treated with different concentrations of mangiferin for 3 h and then stimulated with 0.1 nM TNF for 12 h. The luciferase and β-galactosidase enzymes activity was measured. D–G, involvement of p65 on COX2 gene expression. U-937 cells were cotransfected with the COX2-Luciferase, p65, or in combination with NF-κB-SEAP reporter construct. After 12 h of transfection, cells were treated with 10 μM mangiferin for 3 h and then stimulated with 0.1 nM TNF for 12 h. Nuclear extracts were analyzed for NF-κB by EMSA (D), culture supernatant was assayed for SEAP (E), and whole cell extracts were assayed for luciferase (F) and COX2 (G).
Effect of mangiferin on the TNF-induced activation of IkBα and nuclear factor kappa-B (NF-κB)

**Procedures.**

Cells were subjected to immunocytochemistry according to the Clontech protocol (Palo Alto, CA). In brief, U-937 cells were transiently transfected by the calcium phosphate method with 1 ml of medium containing plasmid DNA. After 12 h, cells were treated with 10 μg/ml mangiferin for 3 h and then stimulated with 0.1 nM TNF for another 12 h. Cell culture-conditioned medium was harvested, and the activity of SEAP was assayed from 96-well fluorescent plates (Fluoroscan II, Lab Systems, Needham Heights, MA) with excitation set at 360 nm and emission at 460 nm. The average number (± S.D.) of relative fluorescent light units for each transfection was then determined and reported as fold activation with respect to control vector-transfected cells. This reporter system was specific, because only TNF-induced NF-κB SEAP activity was inhibited by overexpression of IκBα mutants lacking either Ser32 or Ser36 (42). For transfection control, cells were co-transfected with β-galactosidase and the activity was assayed from each transfection.

**Western Blot for IκBα, Phospho-IκBα, p65, and Phospho-p65**

To determine the levels of IκBα and phospho-IκBα, cytoplasmic extracts were prepared from cells and resolved on SDS-polyacrylamide gels (15). To determine the levels of NF-κB p65, and its active form phospho-p65, nuclear extracts were prepared from TNF-treated cells and were resolved in 9% SDS-polyacrylamide gels. A Western blot was done using anti-IκBα, -phospho-IκBα, -p65, or -phospho-p65 and detected by chemiluminescence (Amersham Biosciences).
Immunocytochemistry

The effect of mangiferin on the nuclear translocation of p65 was examined by the immunocytochemical method as described (43) with slight modifications. Briefly, treated cells were plated on a poly-L-lysine-coated glass slide air-dried, fixed with 3% formaldehyde, and permeabilized with 0.1% of Triton X-100. After washing in phosphate-buffered saline, slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal anti-human p65 antibody at 1:100 dilutions. After overnight incubation at 4 °C, the slides were washed, incubated with goat anti-rabbit IgG-FITC (Molecular Probes) at 1:100 dilutions for 1 h, washed with PBS, and stained for cytosol with Texas Red for 10 min. Stained slides were mounted with mounting medium with DAPI (to counterstain the nucleus) purchased from Molecular Probes and analyzed under a fluorescence microscope.

IKK Assay

The IKK assay was performed by a method described previously (44). Briefly, the IKK complex from whole cell extract (300 μg) was precipitated with anti-IKKα and -IKKβ antibodies (1 μg each), followed by treatment with protein A/G-Sepharose beads (Pierce). After a 2-h incubation, the beads were washed with lysis buffer and then assayed in kinase assay mixture containing 50 mM HEPES (pH 7.4), 2 mM dithiothreitol, 20 μCi of [γ-32P]ATP, 10 mM unlabeled ATP, and 2 μg of substrate GST-IκBα (amino acids 1–54). After incubation at 30 °C for 30 min, the reaction was terminated by boiling with SDS-PAGE sample buffer for 5 min. Finally, the protein was resolved on 9% SDS-PAGE, the gel was dried, and the radioactive bands were visualized by PhosphorImager. To determine the total amounts of IKKα in each sample, 50 μg of the whole cell protein was resolved on 9% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with anti-IKKα antibody.

Measurement of GSH, GSSG, and Catalase

U-937 cells (3 × 10^6/ml) were treated with 5 mM NAC and different concentrations of mangiferin. The cells were washed with PBS and lysed with 1% Triton X-100. GSH and GSSG were measured by enzymatic recycling assay described by Anderson (45). After 50 μl of 10% sulfosalicylic acid was added to each 100-μl aliquot of cell lysate, the samples were centrifuged (10,000 × g) for 5 min at 4 °C to precipitate the protein. To assay total glutathione, a 50-μl aliquot of supernatant was added to a mixture of 0.7 ml of 0.3 mM NADPH, 100 μl of 6 mM DTNB, and 175 μl of H_2O. After adding 10 μl of 50 units/ml glutathione reductase, the rate of change in optical density at 405 nm was assessed. To assay GSSG, the GSH present in the sample was derivatized by

![Image](https://example.com/image.png)

**Fig. 5.** The effect of mangiferin on different inducers mediated NF-κB activation and TNF-induced signaling cascade. A, effect of mangiferin on PMA-, serum-activated LPS-, ceramide-, and TNF-induced NF-κB activation. U-937 cells (2 × 10^6/ml) were treated with mangiferin (10 μg/ml) for 3 h at 37 °C followed by stimulation with PMA (25 ng/ml), serum-activated LPS (100 ng/ml), ceramide (10 μM), and TNF (0.1 nM) for 30 min. NF-κB activation was assayed by EMSA from nuclear extract. B and C, mangiferin inhibits TNFR1-, TRADD-, TRAF-2, NIK-, and IKK-mediated but not p65-mediated NF-κB-dependent reporter gene expression. U-937 cells were either untreated or treated with mangiferin (10 μg/ml) for 3 h and then transiently transfected with the indicated plasmids along with an NF-κB binding site containing plasmid linked to the SEAP gene and cultured for 12 h. Cells were assayed for SEAP activity as described under “Experimental Procedures.” Results are expressed as fold activity over the vector-transfected control (B). For expression control, extracts from p65- and TRAF2 plasmid-transfected cells were used to detect p65 and TRAF2 by Western blot, respectively (C1 and C2).

Mangiferin Blocks TNF-induced Cell Signaling

U-937 cells (3 × 10^6/ml) were treated with 5 mM NAC and different concentrations of mangiferin. The cells were washed with PBS and lysed with 1% Triton X-100. GSH and GSSG were measured by enzymatic recycling assay described by Anderson (45). After 50 μl of 10% sulfosalicylic acid was added to each 100-μl aliquot of cell lysate, the samples were centrifuged (10,000 × g) for 5 min at 4 °C to precipitate the protein. To assay total glutathione, a 50-μl aliquot of supernatant was added to a mixture of 0.7 ml of 0.3 mM NADPH, 100 μl of 6 mM DTNB, and 175 μl of H_2O. After adding 10 μl of 50 units/ml glutathione reductase, the rate of change in optical density at 405 nm was assessed. To assay GSSG, the GSH present in the sample was derivatized by
adding 2 μl of 2-vinylpyridine and 6 μl of triethanolamine to a 100-μl aliquot of supernatant. After 60 min of incubation at 25 °C, GSSG was measured in the same manner as GSH. Cellular catalase values were determined using the method described in Beers and Sizer (46). Briefly, U-937 cells were treated with 10 μg/ml of mangiferin and 5 mM of NAC for 6 h. Cells were pelleted, washed with PBS, lysed in 100 μl of 1% Triton X-100, and centrifuged at 10,000 rpm for 5 min. H2O2 was used as the substrate of catalase. Catalase was assayed by the disappearance of peroxide, which was detected spectrophotometrically at 240 nm. The incubation mixture contained 50 mM potassium phosphate buffer, pH 7.4, 2 mM H2O2 and sample (enzyme source). The decrease in absorbance was recorded at 240 nm for 2 min.

Cytotoxicity Assays

MTT Assay—The cytotoxicity was assayed by the MTT dye uptake (18). Briefly, U-937 cells (10⁴ cells/well of 96-well plate) were incubated with test sample in a final volume of 0.1 ml for 72 h at 37 °C. Thereafter, 25 μl of MTT solution (5 mg/ml in PBS) was added to each well. After 2 h of incubation at 37 °C, 0.1 ml of the extraction buffer (20% SDS, 50% dimethylformamide) was added. After an overnight incubation at 37 °C, the absorbance was read at 570 nm using a 96-well multiscaner autoreader (Bio-Rad), with the extraction buffer as a blank.

Live/Dead Assay—The cytoxic effects were also determined by the Live/Dead assay (48) (Molecular Probes). Briefly, after different treatment 1 × 10⁵ cells were stained with Live/Dead reagent (5 μM ethidium homodimer, 5 μM calcine-AM) and then incubated at 37 °C for 30 min.

RESULTS

We examined the effect of mangiferin on TNF-induced signal transduction. The chemical structure of mangiferin is shown in Fig. 1. Because U-937 cells express both types of TNF receptor, and as TNF-induced responses in this cell type are well characterized in our laboratory, these cells were used for our studies. Mangiferin was dissolved in Me2SO (10 mg/ml) and further dilution was made in complete medium. At the concentration of mangiferin and duration of exposure employed in these studies, there was no effect on cell viability as detected by the trypan blue dye exclusion method (98.52 ± 2.48, 96.62 ± 4.82, and 96.24 ± 3.76 percentage of cell viability was observed at 2, 5, and 10 μg/ml mangiferin, respectively, for 3 h of incubation).

Inhibition of TNF-induced NF-κB Activation by Mangiferin—U-937 cells were pretreated for 3 h with different concentrations (0–10 μg/ml) of mangiferin and then stimulated with and without 0.1 nM TNF for 30 min. Nuclear extracts were prepared and assayed for NF-κB by EMSA. As shown in Fig. 2A, TNF induced 7-fold activation of NF-κB and mangiferin inhibited TNF-induced activation of NF-κB in a dose-dependent manner; with a complete inhibition from 10 μg/ml. Mangiferin by itself did not activate NF-κB. However, it was unable to protect the activation of NF-κB when given as post-treatment after TNF (Fig. 2C).
mangiferin on the kinetics of TNF-induced NF-κB activation. Both untreated and mangiferin-pretreated cells were incubated with TNF (0.1 nM) for different times and then assayed for NF-κB. In untreated cells, TNF activated NF-κB from 0 to 6.5-fold in a time-dependent manner with almost maximum activation at 15 min (Fig. 2D). In mangiferin-pretreated cells, activation of NF-κB was inhibited at all time points of TNF stimulation.

Activated NF-κB Inhibited by Mangiferin Consists of p50 and p65 Subunits—Various combinations of Rel/NF-κB proteins can constitute an active NF-κB heterodimer that binds to specific sequences in DNA. To show that the retarded band visualized by EMSA in TNF-treated cells was indeed NF-κB, we incubated the nuclear extracts from TNF-activated cells with antibody to either p50 (NF-κB) or p65 (Rel A) subunits and then performed EMSA. Antibodies to either subunit of NF-κB shifted the band to a higher molecular size (Fig. 2B), thus suggesting that the TNF-activated complex consisted of p50 and p65 subunits. Neither preimmune serum nor irrelevant antibodies such as anti-c-Rel or anti-cyclin D1 had any effect on the mobility of NF-κB. Excess cold NF-κB (100-fold) almost completely eliminated the band, indicating the specificity of NF-κB.

Mangiferin Represses TNF-induced NF-κB-dependent Reporter Gene Expression—Although we have shown by EMSA that mangiferin blocks the NF-κB activation, DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting the role of additional regulatory mechanisms (49). To determine the effect of mangiferin on TNF-induced NF-κB-dependent reporter gene expression, we transiently co-transfected U-937 cells with the NF-κB SEAP reporter construct and β-galactosidase then treated with mangiferin (10 μg/ml) for 3 h. Cells were then stimulated with 0.1 nM TNF for 1 h, and NF-κB was assayed from nuclear extract.
Mangiferin Inhibits TNF-induced ICAM1 and COX2 Expression and COX2-dependent Reporter Gene Expression—As mangiferin inhibited TNF-induced different biological responses, we examined the expression of NF-κB regulated genes, adhesion molecule ICAM1 and COX2. U-937 cells were treated with different concentrations of mangiferin for 3 h and then stimulated with 0.1 nM TNF for 12 h. We analyzed ICAM1 and COX2 in the cell extract proteins by Western blot. TNF induced ICAM1 (Fig. 3A) and COX2 (Fig. 3B) expression were decreased with increased concentrations of mangiferin treatment. Mangiferin alone did not show any induction of ICAM1 or COX2 expression at 10 μg/ml concentration. Upon re-probing the gels with anti-tubulin antibody, we found that the band intensities in all lanes were uniform indicating equal loading of extracted protein in the lanes.

Although we have shown that mangiferin blocks the COX2 expression, the dependent gene transcription was also assayed. To determine the effect of mangiferin on TNF-induced COX2-dependent reporter gene expression, we transiently transfected U-937 cells with COX2-Luciferase construct and then treated with different concentrations of mangiferin for 3 h. Cells were then stimulated with TNF (0.1 nM) for 12 h. An almost 7.5-fold increase in luciferase activity over the vector control was noted upon stimulation with TNF (Fig. 3C). TNF-induced luciferase activity was almost completely abolished when the cells were pretreated with 10 μg/ml mangiferin. These results demonstrate that mangiferin also represses COX2-dependent reporter gene expression induced by TNF. The β-galactosidase activity from cell extracts showed almost similar reduction of absorbance (as per Promega protocol) at 420 nm (data not shown) suggesting the transfection control for each treatment.

To further prove that COX2 is indeed NF-κB-dependent gene product, which is inhibited by mangiferin, we transiently transfected U-937 cells with COX2-Luciferase, p65, and NF-κB-SEAP constructs. As mentioned earlier, TNF induced NF-κB and SEAP activity in vector and COX2-Luciferase transfected cells about 5–6-fold, which was inhibited by mangiferin pretreatment. However, mangiferin was unable to inhibit the NF-κB and thereby the SEAP activity in p65-overexpressed cells (Fig. 3, D and E). To show the link between COX2 and NF-κB, the COX2-Luciferase was also assayed in the same set of experiments. The data prove that TNF-induced NF-κB activates COX2, which then drives the expression of its reporter luciferase activity and this was inhibited by mangiferin pretreatment. In p65-overexpressed cells, COX2 protein level increased compared with vector-transfected cells, which correlated to the expression of luciferase and its activity (Fig. 3, F and G). However mangiferin did not inhibit luciferase activity in p65 overexpressed cells as shown in SEAP expression. Since the expression of p65 has led to the expression of COX2, it is...
clear that COX2 is a NF-κB-dependent gene product.

Mangiferin Inhibits TNF-dependent Phosphorylation and Degradation of IκBα—The translocation of NF-κB to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκBα (31). To determine whether the inhibitory action of mangiferin was caused by an effect on IκBα degradation, the cytoplasmic level of IκBα proteins was examined by Western blot analysis. IκBα degradation started at 15 min of TNF (0.1 nM) treatment in U-937 cells and was completed within 30 min. The band reappeared by 120 min because of NF-κB-dependent IκBα resynthesis. The presence of mangiferin inhibited the TNF-induced IκBα degradation (Fig. 4A). To determine whether mangiferin modulates TNF-induced IκBα phosphorylation, Western blot with antibodies against the phosphorylated form of IκBα was performed. Mangiferin was found to inhibit TNF-induced phosphorylation of IκBα (Fig. 4A).

Because IκBα phosphorylation is required for IKK activation, the effect of mangiferin on IKK activation was examined. U-937 cells were pretreated with mangiferin (10 μg/ml) for 3 h and then stimulated with 0.1 nM TNF for different times. The cell extracts were prepared, and IKK activation was detected in vitro using GST-IκBα as substrate protein as described under “Experimental Procedures.” The GST-IκBα-specific signal was increased to 6-fold with time of TNF incubation, whereas mangiferin pretreatment completely suppressed the bands at all time points (Fig. 4, B1), indicating the role of mangiferin on suppression of TNF-induced IKK activation. 50 μg of extract proteins were analyzed in SDS-PAGE (9%) and probed with anti-IKKα antibody. The intensity of bands was equal in all lanes, suggesting equal expression of IKKα. To study whether mangiferin has direct interaction with IKK, cell extracts from TNF-induced cells were incubated with different concentrations of mangiferin in the cell-free system, and then IKKα was assayed using those extracts. The intensity of bands was equal in all lanes treated with different concentrations of mangiferin (Fig. 4, B2), suggesting that in a cell-free system mangiferin plays no role in inhibiting IKK activity.

Mangiferin Inhibits TNF-induced Phosphorylation and Translocation of p65 Subunit of NF-κB—To determine whether mangiferin affects the TNF-induced nuclear translocation of the p65 subunit of NF-κB, we performed Western blot analysis. Upon TNF treatment, the p65 level was increased in the nucleus with time, whereas in mangiferin-pretreated cells, TNF was unable to increase the p65 level with time (Fig. 4C). The level of phospho-p65 was increased with incubation time of TNF in the nucleus, whereas mangiferin completely inhibited TNF-induced p65 phosphorylation at all time points (Fig. 4D). Mangiferin alone had no effect in these experiments. These results indicate that mangiferin blocks the nuclear translocation of NF-κB. Inhibition of p65 translocation by mangiferin was also shown by immunofluorescence wherein mangiferin-pretreated cells did not show the p65 signal, which was otherwise shown by TNF (alone)-treated cells, in the nucleus (Fig. 4E).

Mangiferin Blocks PMA-, LPS-, Ceramide-induced Activation of NF-κB—Besides TNF, NF-κB is also activated by various other tumor promoters and inflammatory agents, including PMA, LPS, and ceramide (50) by different signal transduction pathways (51–53). We found that PMA, SA-LPS, and ceramide induced 6-, 6.5-, and 6.4-fold activation of NF-κB, respectively, and that mangiferin completely blocked the activation of NF-κB induced by those agents (Fig. 5A). These results suggest that mangiferin may act at a step in which all these agents converge in the signal transduction pathway leading to NF-κB activation.

Site of Action of Mangiferin in TNF signal Transduction Pathway—TNF-induced NF-κB activation is mediated through sequential interaction of the TNF receptor (TNFR) with associated death domain (TRADD), TRAF2, NIK followed by phosphorylation and subsequent degradation of IκBα (49, 54). To delineate the site of action of mangiferin in the TNF signaling pathway leading to NF-κB activation, cells were transfected with TNFR1, TRADD, TRAF2, NIK, IKK, and p65 plasmids, and then NF-κB-dependent SEAP expression was monitored in untreated and mangiferin-treated cells. As shown in Fig. 5B, mangiferin suppressed TNFR1-, TRADD-, TRAF2-, NIK-, IKK-induced gene expression but had little effect on p65-induced NF-κB reporter expression. The significant levels of p65 and TRAF2 in the transfected cells with p65 and TRAF2 plasmids showed the expression of the proteins by Western blot analysis (Fig. 5, C1 and C2). The specificity of the assay results is also demonstrated by the suppression of TNF-induced NF-κB reporter activity by dominant-negative IκBα plasmid. Thus, mangiferin must act at a step downstream from IKK.

Inhibition of NF-κB Activation by Mangiferin Is Not Cell Type-specific—Because NF-κB activation pathways differ in different cell types, we therefore studied whether mangiferin affects other cell types as well. It has been demonstrated that distinct signal transduction pathways could mediate NF-κB induction in epithelial and lymphoid cells (51). All the effects of mangiferin described above were conducted with U-937, a human histiocytic lymphoma. In another set of experiments, we found that mangiferin blocks TNF-induced NF-κB activation in epithelial (HeLa), MCF-7 (breast cancer cells), and IRB3 AN27 (mesencephalic cells) (Fig. 6). An almost complete inhibition in all the cell types suggests that this effect of mangiferin is not restricted to U-937 cell line.

Mangiferin Blocks TNF-induced ROI Generation—Previous reports have shown that one of the ways by which TNF activates NF-κB is through generation of ROI (15–18, 55, 56). Since mangiferin inhibits TNF mediated activation of NF-κB, we were interested to know the effect of mangiferin on the generation of ROI. This was examined by flow cytometry. U-937 cells were pretreated with different concentrations of mangiferin (1–10 μg/ml) for 3 h and then stimulated with TNF for 1 h. As indicated in Fig. 7A, TNF activated ROI, which was inhibited by mangiferin in a dose-dependent fashion. We compared our results with NAC, PDTC, and tocopherol, which are known antioxidant and modulate NF-κB activity. Our results show that mangiferin, NAC, PDTC, and tocopherol all inhibited TNF-mediated NF-κB activation (Fig. 7C). Also mangiferin like that of NAC and PDTC inhibits TNF-activated ROI generation (Fig. 7D). Thus, it is likely that mangiferin blocks TNF signaling through suppression of reactive oxygen intermediate generation.

Mangiferin Modulates Intracellular Thiol Level—Reports have shown that the replenishment of intracellular cysteine, which is required to produce reduced glutathione (GSH), the major intracellular thiol by NAC regulates NF-κB (57–59). As glutathione plays an important role in maintaining the redox status of the cell, we wanted to assay the levels of glutathione in mangiferin-pretreated cells. TNF treatment for 1 h was found to activate GSH. Mangiferin pretreatment led to increase in GSH level in a dose-dependent manner. However there was no significant change in GSH levels in TNF-treated cells (Fig. 7B). But surprisingly the level of glutathione in mangiferin-pretreated cells was higher than that of NAC- and PDTC-pretreated cells (Fig. 7E). Since the ratio of GSH/GSSH determines the redox status of a cell, we also measured the levels of GSH and GSSH in cells treated with mangiferin and NAC separately. As shown in Fig. 7, F1 and F2, cells pretreated with mangiferin show higher GSH and lower GSSH levels when...
compared with the untreated or even the NAC-pretreated cells. Thus by increasing the GSH/GSSG ratio more than the known antioxidants like NAC, Mangiferin seems to be a much more potent antioxidant.

Enhancement of Catalase Activity by Mangiferin—Catalase is one of the antioxidant enzymes whose overexpression is reported to down-regulate NF-κB levels (53). Because mangiferin inhibited NF-κB and showed antioxidant activities, we were interested to check the catalase activity also. Mangiferin was found to show a marginal increase (23%) in the catalase activity compared with untreated cells (Fig. 7, F3). Mangiferin showed a dose-dependent increase of catalase activity without changing the level of protein (Fig. 7G). Mangiferin did not alter the in vitro catalase activity (data not shown). To detect the role of GSH level and catalase activity in mangiferin-mediated response, we pretreated the cells with BSO, inhibitor of GSH biosynthesis or ATZ, irreversible inhibitor of catalase and then treated with 10 μg/ml mangiferin for 3 h. Cells were then incubated with TNF (0.1 nM) for 1 h and then GSH level or catalase activity was assayed from whole cell extract and NF-κB were assayed from nuclear extract. Mangiferin alone or in combination with TNF increased GSH level almost 4-fold, and BSO treatment inhibited this to a significant extent (Fig 7, H1). Mangiferin alone or in combination with TNF increased catalase activity by 22 and 20%, respectively. In ATZ-pretreated cells mangiferin or in combination with TNF did not activate catalase activity (Fig. 7, H2). Mangiferin inhibited TNF-induced NF-κB activation, and BSO-pretreated cells showed almost 80% protection of mangiferin-mediated down-regulation of NF-κB, whereas inhibition of catalase by ATZ did not protect mangiferin-mediated NF-κB inhibition significantly (Fig. 7I). These data suggest the potential involvement
Mangiferin Blocks TNF-induced Cell Signaling

Fig. 9. Effect of mangiferin to potentiate cell death mediated by different inducers of apoptosis. A and B, effect of mangiferin on the cell death induced by TNF. U-937 cells were treated with or without 10 μg/ml mangiferin for 3 h and then treated with different concentrations of TNF and incubated at 37 °C for 72 h. The cell viability was assayed by MTT as described under “Experimental Procedures” (A). U-937 cells were treated with or without 10 μg/ml mangiferin for 3 h and then stimulated with 1 μM TNF for 24 h. The cell viability was assayed by the Live/Dead cell assay as described under “Experimental Procedures.” C and D, effect of mangiferin on the cell death induced by various apoptotic agents. Cells were pretreated with and without mangiferin (10 μg/ml) for 3 h and then incubated with 1 μM each of cisplatin, vincristine, doxorubicin, etoposide, adriamycin, taxol, and AraC for 72 h. Cytotoxicity was assayed by the MTT assay (C). Cells were pretreated with 10 μg/ml mangiferin for 3 h and then stimulated with taxol (1 μM) and doxorubicin (1 μM) for 24 h. The cell viability was assayed by the Live/Dead cell assay (D). E–G, involvement of p65 in cell death. U-937 cells were transiently cotransfected with IκBα-DN construct, p65 construct, and NF-κB-SEAP reporter gene for 12 h. Cells were then treated with 10 μg/ml mangiferin for 3 h, followed by stimulation TNF (1 μM) for 36 h. Cell viability was assayed by MTT (E) and the Live/Dead cell (F) assay as described under “Experimental Procedures.” The IκBα-DN- and p65-transfected cells were used to detect NF-κB from nuclear extract (G1), SEAP activity from culture supernatant (G2), and IκBα and p65 were detected from whole cell extract (G3).

of GSH, but not a significant role of catalase in mangiferin-mediated down-regulation of NF-κB.

Mangiferin Induces GSH Level in γ-Glutamylcysteine Synthetase (γ-GCS)-overexpressed Cells—γ-GCS is a rate-limiting enzyme in the synthesis of GSH. Since mangiferin increases the GSH levels, we examined the effect of mangiferin in the presence of γ-GCS. For this, cells with or without overexpressed γ-GCS were treated with 10 μg/ml mangiferin and then stimulated with different concentrations of TNF, and the levels of NF-κB, ROI, and GSH were measured. Because γ-GCS is involved in synthesis of GSH, the cellular antioxidant, the cells overexpressing γ-GCS had basal levels of NF-κB and ROI but higher GSH. Mangiferin-pretreated cells as expected inhibited the TNF-induced NF-κB activation and ROI generation in vector control (neo) cells. Moreover it enhanced the ROI quenching and GSH levels in the γ-GCS-overexpressed cells. From this we suspect that mangiferin might up-regulate γ-GCS within the cells and thus enhance the GSH levels.

Mangiferin Potentiates Cisplatin-, Vincristin-, Doxorubicin-, Etoposide-, Adriamycin-, and AraC-mediated Cell Death—NF-κB is known to be involved in apoptosis, and because mangiferin was found to down-regulate NF-κB, we hypothesized that mangiferin pretreatment might potentiate the apoptotic action of other apoptotic agents though by itself at the dose of 10 μg/ml mangiferin does not cause any significant cell death. U-937 cells were preincubated with 10 μg/ml mangiferin for 3 h followed by incubation for 72 h with or without different concentrations of TNF and checked for cell viability by MTT assay and also by the fluorometric Live/Dead cell assay. Results indicate that mangiferin on its own was not toxic (cell death was only 8%) to the cells but enhances cell death mediated by TNF from 16 to 39% (Fig. 9, A and B). To extend the study to other apoptotic agents, we pretreated U-937 cells with and without 10 μg/ml mangiferin for 3 h and then treated the cells with 1 μM various apoptotic agents like cisplatin, vincristine, doxorubicin, etoposide, adriamycin, taxol, and AraC for 72 h. Cell viability was then monitored by the MTT assay and fluorometric Live/Dead cell assay. Mangiferin was found to enhance cell death mediated by these agents (Fig. 9, C and D).

To prove that the potentiation of apoptosis is caused by inhibition of NF-κB, we transiently transfected U-937 cells with IκBα-DN construct, which blocks the activation of NF-κB and the p65 construct and then looked for the cell death after...
36 h by MTT and the Live/Dead cell assay. The IκBα-DN-transfected cells, alone, showed an increase in cell death by 12% and also potentiated cell death with TNF from 42 to 50%. Mangiferin increased the cell death mediated by TNF from 42 to 53% and in IκBα-DN cells, this cell death was further increased to 57%. In p65-overexpressed cells, the cell death was not observed when cells were treated with TNF or the combination of TNF and mangiferin (Fig. 9, E and F). The IκBα-DN-transfected cells down-regulated NF-κB and its reporter gene, SEAP activity, and p65-transfected cells showed up-regulation of NF-κB by 6-fold and its reporter gene, SEAP activity (Fig. 9, G1 and G2). The higher level of expression of IκBα or p65 was observed in IκBα-DN- and p65-overexpressed cells, respectively (Fig. 9, G3). Inhibition of NF-κB (by IκBα-DN transfection) potentiated cell death mediated by various apoptotic agents, which was protected by NF-κB up-regulation (p65 overexpression) (data not shown). Overall our data clearly show the involvement of NF-κB in cell proliferation, and inhibition of this leads to cell death. Down-regulation of NF-κB sensitizes cells to chemotherapeutic agent-mediated apoptosis. Our results thus indicate that mangiferin, like other antioxidants, is not toxic to cells although through inhibition of NF-κB, it can serve as a potential drug in combination therapy with other well known chemotherapeutic agents.

**DISCUSSION**

Since mangiferin is known to elicit anti-inflammatory (61, 62), anticarcinogenic, and antidiabetic properties, and all these events occur through regulation of NF-κB, we hypothesized that these effects are mediated through suppression of NF-κB activation, an early mediator of the pleiotropic effects of the inflammatory cytokine, TNF. We chose U-937, a histiocytic lymphoma cell line for our study. Our results clearly demonstrate that mangiferin suppresses NF-κB activation induced by TNF and other inflammatory agents. The inhibition of NF-κB activation by mangiferin is correlated with the suppression of IκBα phosphorylation and degradation, p65 nuclear translocation, p65 phosphorylation, and NF-κB-dependent reporter gene transactivation. We also found that NF-κB-regulated genes involved in inflammation COX2 and ICAM1 are down-regulated by mangiferin.

There are various ways by which mangiferin might inhibit TNF-induced NF-κB activation. This involves the sequential interaction of TNF receptor with TRADD, TRAF2, and NIK, which then activates IKK (49, 54), and IKK in turn phosphorylates IκBα. NF-κB activation requires sequential phosphorylation, ubiquitination, and degradation of IκBα. Mangiferin blocks IKK activation followed by IκBα phosphorylation and degradation indicating that the mangiferin effect on NF-κB
may be due to inhibition of phosphorylation and the proteolysis of IkBα. However our findings suggest that mangiferin blocks NF-κB-dependent reporter gene expression induced by TNFα, TRAF2, NIK, and IKK, but not by p65, suggesting that mangiferin is acting by some other mechanism. Our results also indicate that mangiferin may be inhibiting these kinases through an antioxidant mechanism.

We have found that mangiferin suppresses NF-κB activation induced by a wide variety of agents, including TNF, ceramide, SA-LPS, and PMA, in U-937 cells. These results indicate that it is a broad spectrum inhibitor of NF-κB. It is also not cell type-specific because the inhibition was noticed in human cells like U-937 (human histiocytic lymphoma), HeLa (epithelial cells), MCF-7 (breast cancer cells), and also in murine IRB3 AN27 (mesenchephalic) cells.

Our results show that mangiferin inhibits ROI generation in U-937 cells. Thus, it is possible that the effects are mediated through quenching of reactive oxygen intermediates. GSH is an important cellular reductant involved in detoxifying ROI, and we observed an increase in the GSH level by mangiferin treatment. Since the GSH/GSSG ratio is fundamental to the transcriptional activation of several pro-inflammatory and antioxidant genes we also measured the GSSG levels in the same extracts. We found a subsequent decrease in the GSSG upon mangiferin pretreatment. We compared our results with NAC and PDTC that are free radical scavengers, which increase intracellular GSH levels, and are known to inhibit TNF-induced activation of NF-κB (15-18, 25). Mangiferin was found to increase the GSH/GSSG ratio more than even NAC. Thus mangiferin is also probably acting by increasing GSH and thereby down-regulating NF-κB and its downstream effects. Because one of the mechanisms of TNF-mediated NF-κB activation involves ROI generation, mangiferin could inhibit TNF-mediated action by quenching ROI generation. In this process it activates the intracellular redox-sensing GSH.

The inhibition of GSH biosynthesis using BSO mimicked the mangiferin-mediated down-regulation of NF-κB, indicating involvement of GSH in mangiferin-mediated response. Because biosynthesis of GSH involves the activation of γ-GCS, the rate-limiting enzyme, we found an increase in the level of GSH upon mangiferin treatment in γ-GCS-overexpressed cells from 3 to 6.5-fold (Fig. 8C). We therefore speculate that the mangiferin-mediated increase in GSH level is caused by activation of γ-GCS, which needs to be further investigated. Although mangiferin showed a marginal increase in the activity of catalase, an antioxidant enzyme but inhibition of catalase did not mimic the mangiferin-mediated down-regulation of NF-κB. How it induces catalase activity without changing its expression level needs to be studied further.

As chemotherapeutic agent-induced resistance is the main problem for cancer, the understanding of the basic mechanism will be helpful in addressing this problem. There was almost 20-40% survival in cisplatin-, vincristine-, doxorubicin-, etoposide-, adriamycin-, taxol-, and AraC-treated cells. These agents showed potentiation of cell killing in combination with mangiferin (Fig. 9C). Because IkBα-DN-transfected cells, which showed down-regulation of NF-κB (9G1) potentiated cell death mediated by TNFα (Fig. 9, E and F), down-regulation of NF-κB may be responsible for potentiation of apoptosis.

Our results bring into light the mechanism of action of a new plant polyphenolic compound mangiferin whose suppressive effects on NF-κB, with increase in intracellular GSH levels, may prove beneficial as an anti-inflammatory, antiangiogenic and anticarcinogenic drug. At the dose used in our study, mangiferin on its own is not toxic to the cell although at the same time suppresses the anti-apoptotic factor NF-κB and hence potentiates cell death induced by other chemotherapeutic agents. Thus mangiferin may also serve as an effective drug in combination therapy.
52. Li, N., and Karin, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13012–13017
53. Imbert, V., Rupec, R. A., Livolsi, A., Pahl, H. L., Traeneckner, E. B.-M., Mueller-Deckmann, C., Farahifar, D., Rossli, B., Aubeger, P., Basuerle, P. A., and Perren, J.-F. (1996) Cell 86, 787–798
54. Karin, M. (1998) Ann. N. Y. Acad. Sci. 851, 139–146
55. Rath, P. C., and Aggarwal, B. B. (1999) J. Clin. Immunol. 19, 350–364
56. Lo, Y. Y., Wong, J. M. S., and Cruz, T. F. (1996) J. Biol. Chem. 271, 15703–15707
57. Bowie, A. G., Moynagh, P. N., and O’Neill, L. A. J. (1997) J. Biol. Chem. 272, 25941–25950
58. Meyer, A., Buhl, R., and Magnussen, H., (1994) Eur. Respir. J. 7, 431–436
59. Rahman, I., and Mac Nee, W. (2000) Free Radi. Biol. Med. 28, 1405–1420
60. Chenguang, F., Qiang, L., Dan, R., and John, F. (2003) J. Biol. Chem. 278, 2072–2080
61. Garcia, D., Delgado, R., Ubeira, F. M., and Leiro, J. (2002) Int. Immunopharmacol. 2, 797–806
62. Sanchez, G. M., Re, L., Giuliani, A., Nunez-Selles, A. J., Davison, G. P., and Leon-Fernandez, O. S. (2000) Pharmacol. Res. 42, 565–573
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