Evodiamine Abolishes Constitutive and Inducible NF-κB Activation by Inhibiting IκBα Kinase Activation, Thereby Suppressing NF-κB-regulated Antiapoptotic and Metastatic Gene Expression, Up-regulating Apoptosis, and Inhibiting Invasion*

Yasunari Takada†§, Yoshinori Kobayashi‡, and Bharat B. Aggarwal†**

From the †Cytokine Research Laboratory, Department of Experimental Therapeutics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, the ‡Program in Immunology, The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, Texas 77030, and the §Faculty of Applied Life Sciences, Niigata University of Pharmacy and Applied Sciences, Niigata, Japan 956-8603

Evodiamine, an alkaloidal component extracted from the fruit of Evodia fructus (Evodia rutaecarpa Bentham, Rutaceae), exhibits antiproliferative, antimetastatic, and apoptotic activities through a poorly defined mechanism. Because several genes that regulate cellular proliferation, carcinogenesis, metastasis, and survival are regulated by nuclear factor-κB (NF-κB), we postulated that evodiamine mediates its activity by modulating NF-κB activation. In the present study, we investigated the effect of evodiamine on NF-κB and NF-κB-regulated gene expression activated by various carcinogens. We demonstrate that evodiamine was a highly potent inhibitor of NF-κB activation, and it abrogated both inducible and constitutive NF-κB activation. The inhibition corresponded with the sequential suppression of IκBα kinase activity, IκBα phosphorylation, IκBα degradation, p65 phosphorylation, p65 nuclear translocation, and p65 acetylation. Evodiamine also inhibited tumor necrosis factor (TNF)-induced Akt activation and its association with IKK. Suppression of Akt activation was specific, because it had no effect on JNK or p38 MAPK activation. Evodiamine also inhibited the NF-κB-dependent reporter gene expression activated by TNF, TNFR1, TRADD, TRAF2, NIK, and IKK but not that activated by the p65 subunit of NF-κB. NF-κB-regulated gene products such as Cyclin D1, c-Myc, COX-2, MMP-9, ICAM-1, MDR1, Survivin, XIAP, IAP1, IAP2, FLIP, Bel-2, Bel-α, and Bfl-1/A1 were all down-regulated by evodiamine. This down-regulation potentiated the apoptosis induced by cytokines and chemotherapeutic agents and suppressed TNF-induced invasive activity. Overall, our results indicated that evodiamine inhibits both constitutive and induced NF-κB activation and NF-κB-regulated gene expression and that this inhibition may provide a molecular basis for the ability of evodiamine to suppress proliferation, induce apoptosis, and inhibit metastasis.

Given the widespread use of diverse complementary and alternative medicine approaches by cancer patients, the research to establish the safety and efficacy is lacking. This provides many opportunities for prevention and treatment of cancer and other diseases (1). The discovery and subsequent development of novel chemical entities intoanticancer drugs are the goals of cancer researchers. Compounds from traditional Chinese medicine have in recent decades been examined for their anticancer potential, and for those compounds that do exhibit such potential, the delineation of their mechanism of action may have an enormous influence on the development of new cancer therapies.

The fruit of “Wu-Zhu-Yu” (Evodiae fructus; Evodia rutaecarpa Bentham., Rutaceae) is used in traditional Chinese medicine as a cardiotonic (2). The active constituents of this fruit are evodiamine and rutaecarpine, which are indole alkaloids found in large amounts in the Chinese medicine evodia and are the main compounds responsible for the antianoxic action (3). Evodiamine1 (see Fig. 1) has recently been shown to suppress the proliferation of a wide variety of tumor cells, including prostate cancer cells (4), leukemic T-lymphocytes (5), monocytic leukemia cells (6), melanoma cells (6, 7), cervical cancer cells (6), and mouse fibrosarcoma cells (6), but it apparently has no toxic effects against normal peripheral blood mononuclear cells (6).

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§ An Odyssey Program Special Fellow at the University of Texas M. D. Anderson Cancer Center.

** A Ransom Horne, Jr., Distinguished Professor of Cancer Research at the University of Texas M. D. Anderson Cancer Center. To whom correspondence should be addressed: Dept. of Experimental Therapeutics, Box 143, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030-4009. Tel.: 713-792-3503 (ext. 6459); Fax: 713-794-1613; E-mail: aggawra@mdanderson.org.

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* The abbreviations used and trivial name are: evodiamine, 8,13,16-tetrahydro-14-methyldino[2’3’;5,6]pyrido[2,1-b][quinazolin-5-][7H]-one; NF-κB, nuclear factor-κB; IκBα, inhibitory subunit of NF-κB; IKK, IκB kinase; TNF, tumor necrosis factor; TNFR, TNF receptor; TRADD, TNFR-associated death domain; NIK, NF-κB-inducing kinase; IL, interleukin; SEAP, secretory alkaline phosphatase; PMA, phorbol 12-myristate 13-acetate; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling; IAP, inhibitor-of-apoptosis protein; XIAP, X chromosome-linked IAP; FLIP, Fas-associated death domain protein-like IL-1β-converting enzyme-inhibitory protein; COX, cyclooxygenase; MMP, matrix metalloproteinase; TRAF, TNF receptor-associated factor, PBS, phosphate-buffered saline; ICAM, intercellular adhesion molecule; MDR, multidrug resistance protein; NO, nitric oxide; PBS, fetal bovine serum; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; PARP, poly(ADP-ribose) polymerase; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; MIT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
Evodiamine induces apoptosis in tumor cells by up-regulating Bax (5–7), down-regulating Bcl-2 (6, 7), phosphorylating Bcl-2 (5), activating caspases (5–7), and producing nitric oxide (NO) (8). Besides its antiproliferative and apoptotic effects, evodiamine suppresses the invasion and migration of human colon carcinoma cells and melanoma cells to the lung (9–12).

How evodiamine regulates cell proliferation, apoptosis, invasion, and migration is incompletely understood. We postulated that this compound mediates its effects by modulating nuclear factor κB (NF-κB), a transcription factor that plays a major role in tumorigenesis (13). NF-κB regulates several genes that mediate proliferation (e.g., Cyclin D1 and c-Myc), antiapoptosis (e.g., Survivin, tumor necrosis factor (TNF) receptor-associated factor 1 (TRAF1)), cellular Fas-associated death domain protein–like interleukin-1β (IL-1β)-converting enzyme–inhibitory protein (FLIP), inhibitor–of–apoptosis protein (IAP), X chromosome–linked IAP (XIAP), Bcl-2, and Bcl-xL, drug resistance (e.g., multidrug resistance protein 1 (MDR1)), immunomodulation (e.g., chemokines and interleukins), and metastasis (e.g., cyclooxygenase-2 (COX-2), matrix metalloproteinase-9 (MMP-9), and intracellular adhesion molecule-1 (ICAM-1)) (14–28).

NF-κB is activated by various carcinogens and inflammatory stimuli, including cigarette smoke, TNF, IL-1β, receptor activator of NF-κB ligand, phorbol 12-myristate 13-acetate (PMA), and lipopolysaccharide. NF-κB consists of a p50/p65 heterodimer, which is retained in the cytoplasm by the masking of nuclear localization sequence by IκBα, the inhibitor of NF-κB. Upon activation of NF-κB, IκBα kinase (IKK) is activated, leading to IκBα phosphorylation, ubiquitination, and degradation; therefore, release of p50/p65 the heterodimer, then translocates to the nucleus, binds to its consensus sequence, and induces gene transcription.

We postulated that evodiamine interferes with this NF-κB activation pathway and suppresses gene transcription. Because the TNF-induced NF-κB activation pathway has been well characterized, we studied the effects of evodiamine on TNF-induced NF-κB activation. In our study, evodiamine suppressed the NF-κB activation induced by a wide variety of agents irrespective of cell type, suppressed expression of various antiapoptotic and proliferative gene products, and enhanced the apoptosis and suppressed the invasive activity induced by cytokines and chemotherapeutic agents.

**MATERIALS AND METHODS**

**Reagents**—Evodiamine isolated as previously described before (29). A solution (250 μM) of evodiamine was prepared with Me2SO, stored as small aliquots at −20 °C, and diluted as needed in cell culture medium. Bacteria-derived recombinant human TNF, purified to homogeneity from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-COX-2 and anti-IKK-α antibodies were kindly supplied by Dr. C. Gary Gairola (University of Kentucky, Lexington, KY). Penicillin, streptomycin, 20 °C, and diluted as needed in cell culture medium. Anti-IKK-α, Akt, p38 MAPK, JNK1, and Cyclin D1, c-Myc, MMP-9, ICAM-1, MDR1, IAP1, IAP2, Bcl-2, Bcl-xL, BR-IA, and PARP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-COX-2 and anti-XIAP antibodies were obtained from BD Biosciences. Phospho-specific anti-IκBα (Ser-32), phospho-specific anti-p65 (Ser-536), phospho-specific anti-Akt, phospho-specific anti-p38 MAPK, and anti-activated IκBα antibodies were purchased from Cell Signaling (Beverly, MA). Anti-IKK-α, anti-IKK-β, and anti-FLIP antibodies were kindly supplied by Dr. S. M. Cookson (Chattanooga, CA).

**Cell Lines**—KB-5 (human myeloid leukemia), H1299 (human lung adenocarcinoma), Jurkat (human T-cell lymphoma), A293 (human embryonic kidney), FaDu (human squamous cell carcinoma), and U266 (human multiple myeloma) cells were obtained from American Type Culture Collection (Bethesda, MD). KB-5 cells were cultured in Iscove’s modified Dulbecco’s medium with 15% FBS, H1299, Jurkat, and U266 cells were cultured in RPMI 1640 medium, A293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, and FaDu cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS, 100 μM nonessential amino acids, 1 mM pyruvate, 6 mM l-glutamine, and l-glutathione. All culture media were also supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin.

**EMSA**—To assess NF-κB activation, we performed an electrophoretic mobility shift assay (EMSA) as described previously (31). Briefly, nuclear extracts prepared from TNF-treated cells were incubated with 32P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (15 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat 5’TTCGGCTGGGACCTGCAAGGGGCGTG3’ (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’-TTGTTTACAATCATTTTCCGGTCTGACTTTCACCAGGAGGCGTG-3’, used to examine the specificity of the experiment by 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 the p50 or p65 subunit of NF-κB for 15 min at 37 °C before the complex was analyzed by EMSA. Anti-Cyclin D1 antibody and preimmune serum were included as negative controls. The dried gels were visualized with a phosphorimager (Amersham Biosciences), and images were quantitated using ImageQuant software (Amersham Biosciences).

**Western Blot Analysis**—To determine the levels of protein expression in the cytoplasm or nucleus, we prepared extracts (32) and fractionated them by SDS-PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with each antibody, and detected with enhanced chemiluminescent reagent (Amersham Biosciences). The bands obtained were quantitated using NIH Image (National Institutes of Health, Bethesda, MD).

**Kinase Assay**—To determine the effect of evodiamine on TNF-induced IKK activation, we performed an immunocomplex kinase assay as described previously (33). Briefly, the IKK complex from whole cell extracts was precipitated with antibody against IKK-α followed by treatment with protein A/G-Sepharose beads (Pierce). After 2 h of incubation, the beads were washed with lysis buffer and quantitated using [γ-32P]ATP, 10 μM unlabeled ATP, and 2 μg of substrate glutathione S-transferase (GST)-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 phosphorimager. To determine the total amounts of IKK-α and IKK-β in each sample, 50 μg of the whole protein complex was resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and blotted with anti-IKK-α or anti-IKK-β antibodies. For JNK1, whole cell extracts were precipitated with antibody against JNK1, and performed kinase assay using GST-c-Jun (amino acids 1–79).

**NF-κB-dependent Reporter Gene Expression Assay**—The effect of evodiamine on TNF-, TNFR-, TRADD-, TRAF2-, NIK-, IKK-, and p65-induced NF-κB-dependent reporter gene transcription was analyzed by secretory alkaline phosphatase (SEAP) assay as described previously. Briefly, A293 cells were plated in 12-well plates (2 × 105 cells/well) and transiently transfected by FuGENE 6 (Roche Molecular Biochemicals, Mannheim, Germany). To examine TNF-induced reporter gene expression, we transfected the cells with 0.2 μg of the SEAP expression plasmid for 24 h. Thereafter we treated the cells for 24 h with 250 nM evodiamine and then stimulated them with 1 μM TNF for a further 24 h. To examine the expression of various gene-induced reporter genes, we transfected cells with 0.2 μg of reporter gene plasmid with each 0.5 μg of expressing plasmid for 24 h and then treated the cells with 250 nM evodiamine for 48 h. The cell culture medium was harvested and analyzed for SEAP activity according to the protocol essentially as described by the manufacturer (Clontech, Palo Alto, CA) using a Victor 3 microplate reader (PerkinElmer Life Sciences).

**Immunoprecipitation of p65 for p65 Acetylation**—To determine the effect of evodiamine on TNF-induced acetylation of p65, cells (5 × 106) were washed with LPS-free Dulbecco’s phosphate buffered saline (PBS), and lysed in a buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 2 mM NaF, 10% glycerol, 0.2% Triton X-100, 0.2 mM sodium orthovanadate, 2 μg/ml aprotinin, and 2 μg/ml leupeptin to prepare whole cell lysates. Whole cell extracts were incubated with anti-p65 antibody for 2 h and precipitated using protein A/G-Sepharose beads. After 1 h of incubation, immunocomplexes were washed with lysis buffer, boiled with SDS sample buffer for 5 min, resolved on
Evodiamine Suppresses TNF-mediated Gene Expression

**RESULTS**

The aim of the current study was to investigate the effect of evodiamine on the NF-κB activation pathway induced by various carcinogens and inflammatory stimuli and on NF-κB-regulated gene expression. The structure of evodiamine is shown in Fig. 1A.

Evodiamine Inhibits NF-κB Activation Induced by Various Carcinogens and Inflammatory Stimuli—TNF, IL-1β, PMA, okadaic acid (OA), hydrogen peroxide (H₂O₂), and cigarette smoke condensate (CSC). KBM-5 cells were incubated with 250 nM evodiamine for 24 h and then treated with 0.1 nM TNF for 30 min, 100 μg/ml IL-1β for 30 min, 15 ng/ml PMA for 1 h, 50 μM OA for 4 h, 500 μM H₂O₂ for 2 h, or 1 μg/ml CSC for 1 h. Nuclear extracts were prepared and analyzed for NF-κB activation by EMSA.

Evodiamine suppresses TNF-induced gene expression in vitro. B, evodiamine blocks NF-κB activation induced by TNF, IL-1β, PMA, okadaic acid (OA), hydrogen peroxide (H₂O₂), and cigarette smoke condensate (CSC). KBM-5 cells were incubated with 250 nM evodiamine for 24 h and then treated with 0.1 nM TNF for 30 min, 100 μg/ml IL-1β for 30 min, 15 ng/ml PMA for 1 h, 50 μM OA for 4 h, 500 μM H₂O₂ for 2 h, or 1 μg/ml CSC for 1 h. Nuclear extracts were prepared and analyzed for NF-κB activation by EMSA.

Evodiamine Inhibits NF-κB Activation Induced by Various Carcinogens and Inflammatory Stimuli—TNF, IL-1β, PMA, okadaic acid, H₂O₂, and cigarette smoke condensate are all potent activators of NF-κB, but they differ in how they activate NF-κB. We examined the effect of evodiamine on the activation of NF-κB by these agents. The results of EMSA showed that treating cells with evodiamine suppressed the activation of NF-κB induced by all six agents (Fig. 1B). The concentrations of evodiamine and NF-κB activators used and the duration of exposure had minimal effect on cell viability (data not shown). These results suggested that evodiamine acts at a step in the NF-κB activation pathway that is common to all six agents.

**Inhibition of NF-κB Activation by Evodiamine Is Not Cell Type-specific**—Because TNF is one of the most potent activator of NF-κB and how it activates NF-κB is relatively well established, we examined the effect of evodiamine on TNF-induced NF-κB
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**Inducible NF-κB activation**

![Fig. 2. Evodiamine suppression of TNF-induced NF-κB in a dose-dependent manner and in different cell lines.](Image)

Human lung carcinoma H1299 (A), human T-cell lymphoma Jurkat (B), and human embryonic kidney A293 (C) cells were treated with the indicated concentrations of evodiamine for 24 h and then with 0.1 nM TNF for 30 min. Nuclear extracts were prepared and analyzed for NF-κB activation by EMSA. Evodiamine completely abolished at 24 h (Fig. 3E).

**Constitutive NF-κB activation**

![Constitutive NF-κB activation](Image)

Evodiamine Inhibits Constitutive NF-κB Activation—As compared with normal cells, most tumor cells exhibit constitutive NF-κB activation. Whether evodiamine could inhibit constitutive NF-κB in tumor cells was also investigated. Head and neck squamous cell carcinoma FaDu and multiple myeloma U266 cells are known to express constitutive NF-κB activation. FaDu and U266 cells were treated with different concentrations of evodiamine for 24 h and then analyzed for NF-κB activation. Evodiamine inhibited the constitutive NF-κB activation in both the cell lines in a dose-dependent manner (Fig. 2, D and E).

Evodiamine Inhibits TNF-induced NF-κB Activation in a Dose- and Time-dependent Manner—To determine whether evodiamine is dose-dependent, we treated cells with up to 500 nM evodiamine and then with TNF. The EMSA results showed that evodiamine by itself did not activate NF-κB, but TNF-induced NF-κB activation was inhibited by evodiamine in a dose-dependent manner (Fig. 3A). We also investigated the length of incubation required for evodiamine to suppress TNF-induced NF-κB activation. Cells were incubated with evodiamine for up to 24 h and then exposed to TNF. EMSA results showed that TNF-induced NF-κB activation was completely abolished at 24 h (Fig. 3B). Under these conditions, cells were fully viable when treated with evodiamine (data not shown).

Results from a previous study in our laboratory showed that TNF can induce NF-κB activity more intensely at a high dose given for short period than at a low dose given for a long time (41). To determine the effect of evodiamine on NF-κB activation at high TNF concentrations, we treated cells with evodiamine and then with up to 1000 pM TNF and analyzed the resulting NF-κB activation by EMSA. In the absence of evodiamine, TNF at a concentration of 1000 pM induced NF-κB activity strongly, but in cells treated with evodiamine, TNF-induced NF-κB activation was abolished (Fig. 3C). These results showed that evodiamine is a potent inhibitor of TNF-induced NF-κB activation.

To determine whether evodiamine directly modifies the binding of the NF-κB complex to DNA, we incubated nuclear extracts from TNF-stimulated cells with evodiamine and then analyzed DNA-binding ability using EMSA. Evodiamine inhibited the DNA-binding ability of the NF-κB complex (Fig. 3D). We concluded that evodiamine inhibits NF-κB activation indirectly.

NF-κB is a complex of proteins. Various combinations of Rel/NF-κB protein constitute active NF-κB heterodimers that bind specific DNA sequences (40). To show that the band visualized by EMSA in TNF-treated cells was indeed NF-κB, we incubated nuclear extracts from TNF-stimulated cells with antibodies against the p50 (NF-κB1) or p65 (RelA) subunit of NF-κB. The antibodies shifted the band to a higher molecular mass (Fig. 3E), suggesting that the NF-κB complex consists of both p50 and p65 subunits. Neither the irrelevant anti-Cyclin D1 antibody nor preimmune serum had any effect.

Excess (100-fold) unlabeled NF-κB caused complete disappearance of the band, but a mutant oligonucleotide of NF-κB caused complete disappearance of the band, but a mutant oligonucleotide of NF-κB did not affect NF-κB binding activity.

Evodiamine Inhibits TNF-dependent IkBα Degradation—To determine whether the inhibitory activity of evodiamine was...
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Evodiamine Suppresses TNF-induced NF-κB Activation by EMSA.

Evodiamine Inhibits TNF-dependent IκBα Phosphorylation.—To determine whether inhibition of TNF-induced IκBα degradation was due to inhibition of IκBα phosphorylation, we treated cells with evodiamine and then with TNF and examined them for IκBα phosphorylation status in the cytoplasm by Western blot analysis using antibody that recognizes the serine-phosphorylated form of IκBα. TNF-induced IκBα phosphorylation was almost completely suppressed by evodiamine (Fig. 4B, lane 1). These results indicated that evodiamine inhibits both TNF-induced NF-κB activation and IκBα degradation.

Evodiamine Inhibits TNF-induced Nuclear Translocation of p65.—TNF induces the phosphorylation of p65, which is required for its transcriptional activity (42). After its phosphorylation, p65 translocates to the nucleus. TNF induced p65 phosphorylation in the cytoplasm in a time-dependent manner: p65 was phosphorylated as early as 5 min after TNF stimulation and increased up to 30 min (Fig. 4B, lane 4). In cells treated with evodiamine, TNF failed to induce p65 phosphorylation. We also determined the effect of evodiamine on TNF-induced nuclear translocation of p65. TNF induced the nuclear translocation of p65 in a time-dependent manner, and evodiamine suppressed it almost completely (Fig. 4B, lane 5). TNF also induced p65 phosphorylation in the nuclear fraction in a time-dependent manner, and evodiamine suppressed it (Fig. 4B, lane 6).

The acetylation of p65 plays a key role in IκBα-mediated activation of NF-κB transcriptional activity (43). To examine the effect of evodiamine on the acetylation of p65 by TNF, we treated cells with evodiamine and then with TNF, prepared whole cell extracts and immunoprecipitated them with anti-p65 antibody, and performed Western blot analysis using anti-acetyl-lysine antibody. TNF induced acetylation of p65 in a time-dependent manner, and evodiamine suppressed it (Fig. 4C).

Immunocytochemical analysis confirmed the suppressive effect of evodiamine on the nuclear translocation of p65. The p65 localized in the cytoplasm of untreated cells, TNF induced nuclear translocation of p65, and evodiamine suppressed the translocation (Fig. 4D).

Evodiamine Inhibits TNF-induced IKK Activation.—It has been shown that IKK is required for TNF-induced phosphorylation of IκBα (40). Because evodiamine inhibited the phosphorylation of IκBα in our study, we assessed whether it directly affects TNF-induced IKK activation. We used the proteasome inhibitor N-acetyl-Leu-Leu-norleucinal (Calbiochem) to block the degradation of IκBα (44) reading prolongation of the phosphorylated IκBα (45). Results from the immunocomplex kinase extracts were incubated with the indicated concentrations of evodiamine then analyzed NF-κB activation by EMSA. E, NF-κB induced by TNF is composed of p65 and p50 subunits. Nuclear extracts from untreated or TNF-treated KBM-5 cells were incubated with the indicated antibodies, preimmune serum (PIS), an unlabeled competitor NF-κB oligonucleotide probe, or a mutant oligonucleotide probe, and analyzed for NF-κB activation by EMSA.
assay showed that TNF activated IKK as early as 10 min after TNF treatment and that evodiamine completely suppressed this activation (Fig. 4E). Neither TNF nor evodiamine affected the expression of IKK-α or IKK-β.

To evaluate whether evodiamine suppresses IKK activity directly by binding IKK or indirectly by suppressing its activation, we incubated whole cell extracts from untreated cells and TNF-stimulated cells with anti-IKK-α antibody. After precipitation with protein A-Sepharose beads, the immunocomplexes were treated with various concentrations of evodiamine. Results from the immunocomplex kinase assay showed that evodiamine did not directly affect the activity of IKK (Fig. 4F).

This finding suggests that evodiamine modulates TNF-induced IKK activation.

Evodiamine Inhibits TNF-induced Akt Activation—It has been shown that Akt can activate IKK (48). Thus it is possible that evodiamine suppresses TNF-induced Akt activation. To examine the effect of evodiamine on the activation of Akt by TNF, we treated cells with evodiamine and then with TNF, prepared whole cell extracts, and performed Western blot analysis using anti-phospho-Akt antibody. TNF induced Akt activation in a time-dependent manner, and evodiamine suppressed it (Fig. 4G). Whether evodiamine affects the association of Akt with IKK was also examined. We prepared the
whole cell extracts from TNF-treated cells, immunoprecipitated them with anti-IKK-α antibody, and performed Western blot analysis using anti-Akt antibody. TNF induced association between IKK and Akt in a time-dependent manner, and evodiamine suppressed it (Fig. 4G).

Evodiamine Does Not Inhibit TNF-induced JNK and p38 MAPK Activation—Both JNK and p38 MAPK have been linked with NF-κB activation (47, 48). Whether these kinases activated by TNF are also affected was examined. To determine the effect of evodiamine on TNF-induced kinases, we treated cells with evodiamine and then with TNF, prepared whole cell extracts, and performed Western blot analysis using anti-phosphospecific-p38 MAPK antibody. TNF induced phosphorylation of p38 MAPK in a time-dependent manner, and evodiamine had no effect on p38 MAPK (Fig. 4H). We also prepared whole cell extracts and immunoprecipitated them with anti-JNK1 antibody and performed an immunocomplex kinase assay. TNF induced activation of JNK in a time-dependent manner, but evodiamine had no affect on the activation (Fig. 4I). These results indicate that the effects of evodiamine on Akt and IKK activation are specific.

Evodiamine Suppresses TNF-mediated Gene Expression—DNA binding does not always correspond with NF-κB-dependent gene transcription (49), and TNF-induced NF-κB activation is mediated through the sequential interaction of the TNF receptor with TRADD, TRAP2, NIK, and IKK, resulting in phosphorylation of IκBα (50, 51). To determine the effect of evodiamine on TNF-induced NF-κB-dependent reporter gene expression, we transiently transfected cells with the NF-κB-regulated SEAP reporter construct, incubated them with evodiamine, and then stimulated them with TNF. Cells were also transfected with TNFR1, TRADD, TRAP2, NIK, IKK, and p65-expressing plasmids and then monitored for NF-κB-dependent SEAP expression. We found that TNF induced NF-κB reporter activity and that this activity was inhibited by evodiamine (Fig. 5). Cells transfected with TNFR1, TRADD, TRAP2, NIK, IKK, and p65 plasmids showed NF-κB-regulated reporter gene expression; evodiamine suppressed TNFR1-, TRADD-, TRAP2-, NIK-, and IKK-induced but not p65-induced NF-κB reporter gene expression. These results suggested that the effect of evodiamine occurs at a step upstream from p65.

Evodiamine Represses TNF-induced NF-κB-dependent Receptor Gene Expression—TNF has been shown to induce the expression of the proliferative and metastatic proteins Cyclin D1, c-Myc, COX-2, MMP-9, ICAM-1, and MDR1, which have an NF-κB-binding site in their promoters (14, 15, 25–28). Whether evodiamine inhibits TNF-induced expression of these NF-κB-dependent proteins was examined. Cells were treated with evodiamine and then with TNF. We prepared whole cell extracts and analyzed them by Western blot analysis. Our results showed that TNF induced expression of all these proteins in a time-dependent manner and that evodiamine blocked it (Fig. 6A). The results provide further evidence of the role of evodiamine in blocking the expression of TNF-induced NF-κB-regulated gene products.

Evodiamine Represses TNF-induced NF-κB-dependent Antiapoptotic Gene Products—TNF has been shown to induce the expression of the antiapoptotic proteins Survivin, XIAP, IAP1/2, FLIP, Bcl-2, Bcl-xL, and Bfl-1/A1 (16, 18–24, 52). We examined whether evodiamine can modulate the TNF-induced expression of these NF-κB-dependent antiapoptotic gene products. Our results showed that TNF induced expression of all these proteins in a time-dependent manner and that evodiamine blocked it (Fig. 6B). The results provide further evidence of the role of evodiamine in blocking the expression of TNF-induced NF-κB-regulated gene products.
chemotherapeutic agent-induced apoptosis was investigated using the Live and Dead assay, the MTT assay, Western blot analysis for PARP and cleaved PARP, and flow cytometry analysis of annexin V- and TUNEL-stained cells. Results from the Live and Dead assay indicated that evodiamine up-regulated TNF-induced cytotoxicity from 4% to 32% (Fig. 7A). Evodiamine also enhanced TNF-, taxol-, and cisplatin-induced cytotoxicity as analyzed by the MTT method (Fig. 7B). Evodiamine by itself had little cytotoxic effect at this concentration (50 nM).

We investigated whether the enhanced cytotoxicity was due to increased apoptosis. TNF activated caspases, as indicated by PARP cleavage, and evodiamine potentiated the TNF-induced activity (Fig. 7C). The results of annexin V and TUNEL staining indicated that evodiamine up-regulated TNF-induced early apoptosis (Fig. 7D) and late apoptosis (Fig. 7E). The results from all assays together suggested that evodiamine enhances the apoptotic effects of TNF and chemotherapeutic agents.

**Evodiamine Suppresses TNF-induced Invasion Activity**—The mechanism of tumor metastasis has been studied, and it is known that COXs, MMPs, and adhesion molecules playing a major role in it (58). It is also known that TNF can induce the expression of tumor metastasis-related genes such as COX-2, MMP-9, and ICAM-1 (14, 25, 28). To investigate the effect of evodiamine on TNF-induced metastatic activity, which could appear as invasive activity in vitro, we subjected H1299 cells to the Matrigel invasion chamber assay. TNF induced 2.6-fold greater invasive activity, and evodiamine suppressed it (Fig. 8).

**Evodiamine Analogue Rutaecarpine Does Not Suppress TNF-induced NF-κB Activation**—Rutaecarpine lacks the methyl group of evodiamine at the N-14 position and a hydrogen at the C-13b position (Fig. 9A), has a different three-dimensional structure than evodiamine, and cannot inhibit cell proliferation or invasion (6, 9). We investigated whether rutaecarpine affects NF-κB activation. The compound was isolated as previously described (59). KBM-5 cells were treated with rutaecarpine plus evodiamine and then with TNF. Nuclear extracts were prepared and subjected to NF-κB activation by EMSA. Rutaecarpine did not suppress TNF-induced NF-κB activation (Fig. 9B).
DISCUSSION

Our goal was to determine whether the anticarcinogenic, antiproliferative, and antimetastatic effects of evodiamine were mediated by its suppression of NF-κB. We found that evodiamine suppressed NF-κB activation induced by various carcinogens, tumor promoters, and inflammatory agents irrespective of cell type. NF-κB inhibition corresponded with suppression of IKK kinase activity, and IκBα phosphorylation and degradation, and with p65 phosphorylation, nuclear translocation, and acetylation. Evodiamine also down-regulated NF-κB-regulated reporter gene transcription and the expression of gene products involved in cell proliferation, invasion, drug resistance, and antiapoptosis. This down-regulation potentiated the apoptosis induced by cytokines and chemotherapeutic agents.

Evodiamine inhibited NF-κB activation induced by TNF, IL-1β, PMA, okadaic acid, H$_2$O$_2$, and cigarette smoke condensate, suggesting that evodiamine acts at a step common to all of these activators. In response to most of these stimuli, NF-κB activation proceeds sequentially through activation of IKK, phosphorylation at serines 32 and 36 of IκBα, ubiquitination at lysines 21 and 22 of IκBα, and finally degradation of IκBα and the release of NF-κB (40). To our knowledge, our study is the first to indicate that evodiamine inhibits NF-κB activation by suppressing IKK activation. How evodiamine inhibits IKK activation was also examined. Akt has been shown to activate IKK (46). We demonstrate that evodiamine suppressed TNF-induced Akt activation as well as Akt-IKK association. These results thus indicate that evodiamine may inhibit IKK activation through suppression of Akt activation. The lack of effect of evodiamine on JNK and p38 MAPK activation indicates specificity.

Chiou et al (8), found that evodiamine suppresses NO production in cultured murine macrophages. Production of NO depends on NO synthase, whose expression is tightly regulated by NF-κB (60). The down-regulation of NO production may be mediated through the down-regulation of NF-κB, thereby leading to decreased expression of inducible NO synthase. Evodiamine has been shown to induce apoptosis through the down-regulation of Bcl-2 expression (6, 7), which is also regulated by NF-κB.

We showed that evodiamine inhibited NF-κB-regulated gene transcription and the expression of NF-κB-regulated gene products involved in cell proliferation (e.g. Cyclin D1 and c-Myc), invasion (e.g. COX-2, MMP-9, and ICAM-1), drug resistance (e.g. MDR1), and antiapoptosis (e.g. Survivin, XIAP, IAP1, IAP2, FLIP, Bcl-2, Bcl-xL, and Bfl-1/A1). We know of no other published report on the regulation of these gene products by evodiamine.

**FIG. 8.** Evodiamine suppression of TNF-induced invasion activity. H1299 cells (2.5 × 10$^4$ cells) were seeded onto the top chamber of a Matrigel invasion chamber system in the absence of serum for 12 h, and cells were then treated with 250 nM evodiamine for an additional 24 h and then exposed to 1 nM TNF for a final 24 h in the presence of 1% FBS and the evodiamine. Cells were subjected to an invasion assay. The mean value for cells treated with no evodiamine and no TNF was set to 1, and -fold differences were determined by comparing values against this set value.

**FIG. 9.** Effect of the evodiamine analogue rutaecarpine on TNF-induced NF-κB activity. A, the structure of rutaecarpine. B, KBM-5 cells were treated with the indicated concentrations of rutaecarpine along with 500 nM evodiamine for 24 h and then exposed to 0.1 nM TNF for 30 min. Nuclear extracts were prepared and analyzed for NF-κB activation by EMSA.
Cyclin D1, which is overexpressed in a variety of tumors (61), is regulated by NF-κB (15) and is required for cells to advance from the G1 phase to the S phase of the cell cycle (62). That evodiamine down-regulates Cyclin D1 expression may explain the results from earlier reports indicating that this agent induces G2/M arrest rather than G1/S arrest (4, 5). It also may explain the antiproliferative effects of evodiamine through TNF-induced NF-κB-mediated down-regulation of Cyclin D1 by evodiamine.

Our finding that evodiamine inhibited the expression of NF-κB-regulated gene products involved in cell invasion (e.g., COX-2, MMP-9, and ICAM-1) might explain the anti-invasive and antimetastatic activities of evodiamine (9–12). COX-2 has been implicated in carcinogenic processes, and its overexpression by malignant cells has been shown to enhance cellular invasion, induce angiogenesis, regulate antiapoptotic cellular defenses, and augment immunologic resistance through production of prostaglandin E2 (63). MMP-9 plays a crucial role in tumor invasion and angiogenesis by mediating degradation of the extracellular matrix, and inhibition of MMP activity has been shown to suppress lung metastasis (64). Adhesion molecules such as ICAM-1 are regulated by NF-κB activation, which may explain its antiproliferative, proapoptotic, antimetastatic, anti-inflammatory, and immunomodulatory effects.

Drug resistance is one of the major reasons for failure of cancer therapy. Neoplastic cells often develop multiple mechanisms of drug resistance during tumor progression. Bentires-Alj M et al. (65) showed that NF-κB inhibition in colon cancer cells reduced mdr1 mRNA expression and that P-glycoprotein expression led to increased apoptotic cell death in response to daunomycin treatment. Our data are consistent with this study: we found that evodiamine potentiated the apoptotic effects of cytokines and chemotherapeutic agents by down-regulating the antiapoptosis gene products survivin, XIAP, IAP1, IAP2, FLIP, Bcl-2, and Bfl-1/A1. Furthermore, the cytotoxic effects of TNF, taxol, and cisplatin were enhanced by evodiamine.

Several chemokines, interleukins, and hematopoietic growth factors are regulated by NF-κB activation (38). The immunomodulatory effects of evodiamine (8, 9, 66) may be mediated through the regulation of these cytokines. Overall, our results demonstrate that evodiamine is a potent inhibitor of NF-κB activation, which may explain its antiproliferative, proapoptotic, antimetastatic, anti-inflammatory, and immunomodulatory effects.

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