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Immunology

Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-κB

(tumor necrosis factor/okadaic acid/ceramide/phorbol ester/hydrogen peroxide)

K. NATARAJAN*, SANJAYA SINGH*, TERRENCE R. BURKE, JR.†, DEZIDER GRUNBERGER‡, AND BHARAT B. AGGARWAL*§

*Cytokine Research Section, Department of Molecular Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030; †Laboratory of Medicinal Chemistry, Division of Basic Sciences, National Cancer Institute, Bethesda, MD 20892; and ‡Institute of Cancer Research, Columbia University College of Physicians and Surgeons, New York, NY 10032-2704

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ABSTRACT **Caffeic acid phenethyl ester (CAPE), an active component of propolis from honeybee hives, is known to have antimitogenic, anticarcinogenic, antiinflammatory, and immunomodulatory properties. The molecular basis for these diverse properties is not known. Since the role of the nuclear factor NF-κB in these responses has been documented, we examined the effect of CAPE on this transcription factor. Our results show that the activation of NF-κB by tumor necrosis factor (TNF) is completely blocked by CAPE in a dose- and time-dependent manner. Besides TNF, CAPE also inhibited NF-κB activation induced by other inflammatory agents including phorbol ester, ceramide, hydrogen peroxide, and okadaic acid. Since the reducing agents reversed the inhibitory effect of CAPE, it suggests the role of critical sulfhydryl groups in NF-κB activation. CAPE prevented the translocation of the p65 subunit of NF-κB to the nucleus and had no significant effect on TNF-induced IκBα degradation, but did delay IκBα resynthesis. The effect of CAPE on inhibition of NF-κB binding to the DNA was specific, in as much as binding of other transcription factors including AP-1, Oct-1, and TFIID to their DNA were not affected. When various synthetic structural analogues of CAPE were examined, it was found that a bicyclic, rotationally constrained, 5,6-dihydroxy form was superactive, whereas 6,7-dihydroxy variant was least active. Thus, overall our results demonstrate that CAPE is a potent and a specific inhibitor of NF-κB activation and this may provide the molecular basis for its multiple immunomodulatory and antiinflammatory activities.**

Members of the transcription factor NF-κB family have been identified in various organisms, ranging from flies to mammals (for reviews, see refs. 1–3). In mammals, the most widely distributed κB-binding factor is a heterodimer consisting of p50 and p65 (Rel-A) proteins. This transcription factor plays a central role in various responses, leading to host defense through rapid induction of gene expression. In particular, it controls the expression of various inflammatory cytokines, the major histocompatibility complex genes, and adhesion molecules involved in tumor metastasis. Dysregulation of NF-κB and its dependent genes has been associated with various pathological conditions including toxic/septic shock, graft versus host reaction, acute inflammatory conditions, acute phase response, viral replication, radiation damage, atherosclerosis, and cancer (for reviews, see refs. 3 and 4).

Unlike other transcription factors, the NF-κB proteins are held in the cytoplasm in an inactive state by an inhibitory subunit called IκBα. The phosphorylation of IκB and its subsequent degradation allows translocation of NF-κB to the nucleus. This activation is induced by many agents, such as inflammatory cytokines [e.g., tumor necrosis factor (TNF), lymphotoxin, and interleukin 1], mitogens, bacterial products, protein synthesis inhibitors, oxidative stress (H_2O_2), ultraviolet light, and phorbol esters (5, 6). Agents that can downmodulate the activation of NF-κB have potential for therapeutic intervention.

Among the possibilities for such an agent is caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester (CAPE), a structural relative of flavonoids that is an active component of propolis from honeybee hives. It has antiviral, antiinflammatory, and immunomodulatory properties (7) and has been shown to inhibit the growth of different types of transformed cells (7–12). In transformed cells, CAPE alters the redox state and induces apoptosis (13). It has been reported that CAPE suppresses lipid peroxidation (14), displays antioxidant activity (15), and inhibits ornithine decarboxylase, protein tyrosine kinase (PTK), and lipoxygenase activities (16–19). CAPE can also inhibit phorbol ester-induced H_2O_2 production and tumor promotion (20, 21). Although the molecular basis for the multiple activities assigned to CAPE have not been defined, most of the activities inhibited by CAPE require the activation of NF-κB. The relationship between the activities it modulates and NF-κB prompted us to examine the effect of CAPE on the induction of this transcription factor. Our results show that CAPE is a potent and a specific inhibitor of NF-κB activation induced by different agents.

EXPERIMENTAL PROCEDURES

Materials. Penicillin, streptomycin, RPMI 1640 medium, and fetal calf serum were obtained from GIBCO. Phorbol ester and bovine serum albumin were obtained from Sigma. Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of 5×10^7 units/mg, was kindly provided by Genentech. Antibody against IκBα, cyclin D1, and the NF-κB subunits p50 and p65 and double-stranded oligonucleotides having AP-1 and Oct-1 consensus sequences were obtained from Santa Cruz Biotechnology. Ceramide (C8) was obtained from Calbiochem.

CAPE and Its Analogue. For structure-activity relationship studies, several analogues of CAPE were synthesized as described (7, 8). These analogues included ring substituents (compounds **1–3**), ester groups (compound **4**), rotationally constrained variants (compounds **5** and **6**), and saturated amide analogues (compounds **7** and **8**). Stock solutions of CAPE and its analogues were made in 50% ethanol at 1–5 mg/ml and further dilutions were made in cell culture medium.

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Abbreviations: TNF, tumor necrosis factor; CAPE, caffeic acid phenethyl ester; PTPase, protein tyrosine phosphatase; EMSA, electrophoretic mobility-shift assay; PMA, phorbol 12-myristate 13-acetate; PTK, protein tyrosine kinase; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

§To whom reprint requests should be addressed. e-mail: aggarwal@utmdacc.mda.uth.tmc.edu. FAX: 713-794-1613.

Cell Lines. The human histiocytic cell line U937 cells were routinely grown in RPMI 1640 medium supplemented with 2 mM glutamine, 50 μg/ml gentamicin, and 10% fetal bovine serum (FBS). The cells were seeded at a density of 1×10^5 cells per ml in T25 flasks (Falcon 3013, Becton Dickinson Labware) containing 10 ml of medium and grown at 37°C in an atmosphere of 95% air and 5% CO_2. Cell cultures were split every 3 or 4 days. Occasionally, cells were tested for mycoplasma contamination using the DNA-based assay kit purchased from Gen-Probe (San Diego).

Electrophoretic Mobility-Shift Assays (EMSAs). These assays were carried out as described in detail (22, 23). Briefly, 2×10^6 cells were washed with cold phosphate-buffered saline (PBS) and suspended in 0.4 ml of lysis buffer [10 mM Hepes, pH 7.9/10 mM KCl/0.1 mM EDTA/0.1 mM EGTA/1 mM DTT/0.5 mM phenylmethylsulfonyl fluoride (PMSF)/2.0 μg/ml leupeptin/2.0 μg/ml aprotinin/0.5 mg/ml benzamidine]. The cells were allowed to swell on ice for 15 min, after which 12.5 μl of 10% Nonidet P-40 was added. The tube was then vortexed vigorously for 10 s, and the homogenate was centrifuged for 30 s. The nuclear pellet was resuspended in 25 μl ice-cold nuclear extraction buffer (20 mM Hepes, pH 7.9/0.4 M NaCl/1 mM EDTA/1 mM EGTA/1 mM DTT/1 mM PMSF/2.0 μg/ml leupeptin/2.0 μg/ml aprotinin/0.5 mg/ml benzamidine), and incubated on ice for 30 min with intermittent mixing. Samples were centrifuged for 5 min at 4°C, and the supernatant (nuclear extract) was either used immediately or stored at −70°C. The protein content was measured by the method of Bradford (24).

EMSAs were performed by incubating 4 μg of nuclear extract (NE) with 16 fmol of ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotide from the HIV long terminal repeat, 5′-TTGTTACAA**GGGACTTTC**CGCTG**GGGAC-TTTC**CAGGGAGGCGTGG-3′ (25), for 15 min at 37°C. The incubation mixture included 2–3 μg of poly(dI·dC) in a binding buffer (25 mM Hepes, pH 7.9/0.5 mM EDTA/0.5 mM DTT/1% Nonidet P-40/5% glycerol/50 mM NaCl) (26, 27). The DNA–protein complex formed was separated from free oligonucleotide on 4.5% native polyacrylamide gel using buffer containing 50 mM Tris, 200 mM glycine (pH 8.5), and 1 mM EDTA (28), and the gel was then dried. A double-stranded mutated oligonucleotide, 5′-TTGTTACAA**CT-CACTTTC**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 the antibodies against either p50 or p65 subunits of NF-κB for 30 min at room temperature before the complex was analyzed by EMSA (29). Antibody against cyclin D1 was included as a negative control.

The EMSAs for AP-1, TFIID, and Oct-1 were performed as described for NF-κB using ^{32}P-end-labeled double-stranded oligonucleotides. Specificity of binding was determined routinely by using an excess of unlabeled oligonucleotide for competition as described earlier (29). Visualization and quantitation of radioactive bands was carried out by PhosphorImager (Molecular Dynamics) using IMAGEQUANT software (National Institutes of Health, Bethesda).

Western Blotting for IκBα and p65. After the NF-κB activation reaction described above, postnuclear extracts were resolved on SDS/10% polyacrylamide gels for IκBα. To determine p65 levels, nuclear and postnuclear (cytoplasmic) extracts were resolved on SDS/8% polyacrylamide gels. Proteins were then electrotransferred to Immobilon P membranes, probed with a rabbit polyclonal antibody against IκBα or against p65, and detected by Enhanced Chemiluminescence (Amersham; ref. 30).

RESULTS

In this study, we examined the effect of CAPE on the activation of the transcription factor NF-κB. We used U937 cells for these studies because their response to NF-κB activation by various stimuli has been well-characterized in our laboratory (30). The concentration of CAPE and its various analogues during the time of incubation used in our studies had cell viability greater than 98%.

CAPE Inhibits TNF-Dependent NF-κB Activation. U937 cells were preincubated for 2 h with different concentrations of CAPE and then examined for NF-κB activation by treatment of cells with TNF (0.1 nM) for 15 min at 37°C. The results in Fig. 1*A* indicate that CAPE inhibited the TNF-dependent activation of NF-κB in a dose-dependent manner, with maximum effect occurring at 25 μg/ml. No activation of NF-κB was noted in untreated cells or those treated with either the vehicle (ethanol) alone or with CAPE alone. To show that the retarded band observed by EMSA in TNF-treated cells was indeed NF-κB, nuclear extracts were incubated with antibodies either to p50 (NF-κB1) or to p65 (Rel A) subunits in separate treatments followed by EMSA. The results from this experiment (Fig. 1*B Upper*) show that antibodies to either subunit of NF-κB shifted the band to higher molecular weight, thus suggesting that the TNF-activated complex consisted of p50 and p65 subunits. Nonspecific antibody against cyclin D had no effect on the mobility of NF-κB. In addition, the retarded band observed by EMSA in TNF-treated cells disappeared when unlabeled oligonucleotide (100-fold in excess) was used but not when the mutated oligonucleotide was used (Fig. 1*B Upper*).

We also examined the kinetics of inhibition by incubating the cells with CAPE for 120, 90, 60, and 30 min before the addition of TNF, together with the addition of TNF and 5 and 10 min after the addition of TNF. The cells were treated with TNF for 15 min. TNF response was inhibited only when cells were pretreated with CAPE (Fig. 1*B Lower*). Cotreatment of cells with TNF and CAPE was not effective.

CAPE Also Blocks NF-κB Activation Induced by Phorbol Ester, Ceramide, Okadaic Acid, and Hydrogen Peroxide. NF-κB activation is also induced by the phorbol ester, phorbol 12-myristate 13-acetate (PMA), ceramide, okadaic acid, and hydrogen peroxide (31). However, the initial signal transduction pathways leading to the NF-κB activation induced by these agents differ. We therefore examined the effect of CAPE on the activation of the transcription factor by these various agents. The results shown in Fig. 2 indicate that CAPE completely blocked the activation of NF-κB induced by all four agents. These results suggest that CAPE may act at a step where all these agents converge in the signal transduction pathway leading to NF-κB activation.

CAPE Inhibits DNA Binding of NF-κB Specifically and Not Other Transcription Factors. Both L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), a serine protease inhibitor, and herbimycin A, a PTK inhibitor, have been shown to block the activation of NF-κB by their interference with the binding of NF-κB to DNA (32, 33). To determine the effect of CAPE on the binding of NF-κB to DNA, the nuclear extracts from TNF-pre-activated cells were incubated with various concentrations of CAPE. EMSA (Fig. 3*A*) showed that CAPE prevented NF-κB from binding to DNA. Since IκBα can also be dissociated from NF-κB by a mild treatment with detergent such as deoxycholate, we examined the ability of deoxycholate-treated cytoplasmic extracts to bind to the DNA with or without CAPE treatment. Here too CAPE interfered with the binding of NF-κB proteins to DNA (Fig. 3*B*).

We further tested the ability of CAPE to inhibit the binding of other transcription factors such as AP-1, TFIID, and Oct-1 to their DNA. The effect of CAPE on NF-κB binding was specific, as it did not inhibit the DNA-binding ability of the other transcription factors (Fig. 4).

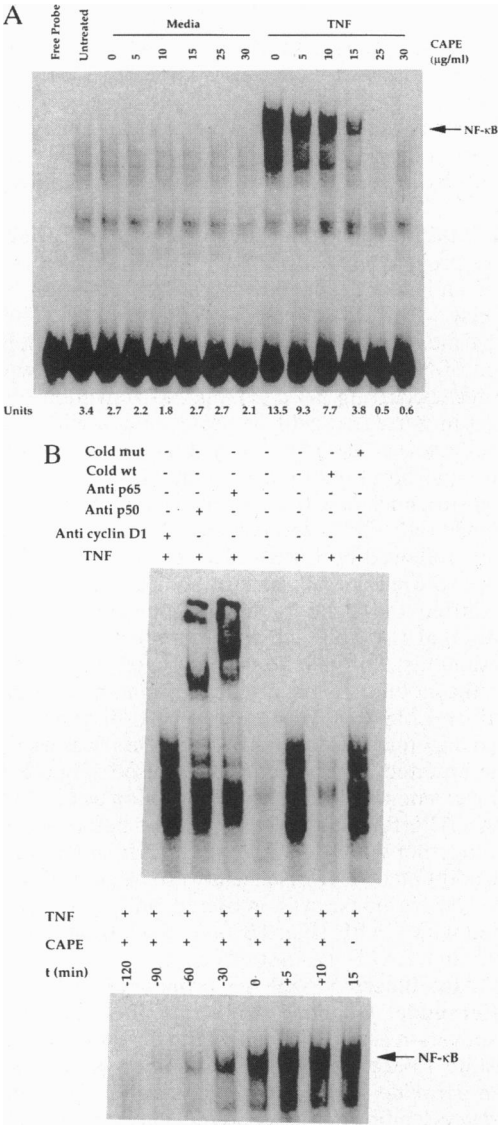

FIG. 1. Dose response and kinetics of inhibition of TNF-dependent NF-κB activation by CAPE. (*A*) U937 cells (2×10^6 per ml) were preincubated at 37°C for 2 h with indicated concentrations of CAPE followed by a 15-min incubation with 0.1 nM TNF. (*B Upper*) For supershift and specificity analysis of NF-κB activation, nuclear extracts were prepared from untreated or TNF (0.1 nM)-treated cells, incubated for 30 min with antibodies, and then assayed for NF-κB as described. (*B Lower*) Cells were preincubated at 37°C with 25 μg/ml CAPE for different times and then tested for NF-κB activation at 37°C for 15 min either with or without 0.1 nM TNF. −, CAPE was present before the addition of TNF; 0, coincubation with TNF; +, CAPE was added after TNF. After these treatments, nuclear extracts were prepared and then assayed for NF-κB as described. The arbitrary units represent the relative amounts of radioactivity present in respective bands.

CAPE does Not Inhibit TNF-Dependent Phosphorylation and Degradation of IκBα. The translocation of NF-κB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IκBα (for review, see ref. 34). To determine whether the inhibitory action of CAPE was due to an effect on IκBα degradation, the cytoplasmic levels of IκBα protein were examined by Western blot analysis. As shown in Fig. 5 *Upper*, treatment of cells with CAPE had no effect on the cytoplasmic pool of IκBα, but treatment of cells with TNF decreased the IκBα band within 5 min and completely eliminated it in 15 min; the band reappeared by 30 min. The presence of CAPE did not affect significantly the TNF-induced rate of degradation of

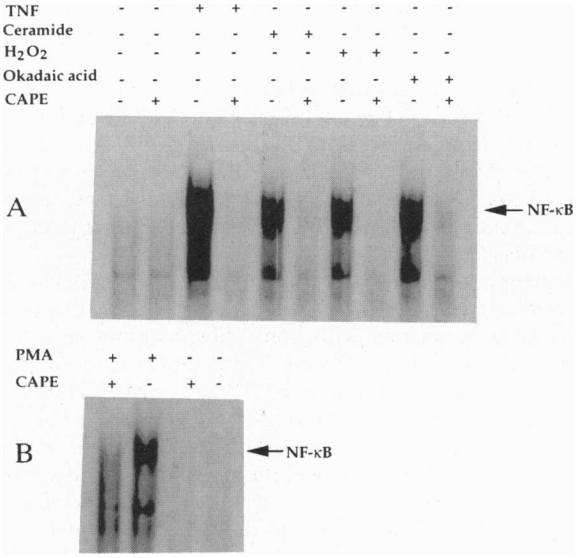

FIG. 2. Effect of CAPE on PMA-, ceramide-, okadaic acid-, and H_2O_2-mediated activation of NF-κB. (*A*) U937 cells (2×10^6 per ml) were preincubated for 120 min at 37°C with CAPE (25 μg/ml) followed by treatments at 37°C with PMA (100 ng/ml for 60 min); H_2O_2 (0.5 mM for 30 min), Ceramide-C8 (10 μM for 30 min), or okadaic acid (500 nM for 30 min) and then tested for NF-κB activation as described. (*B*) The EMSA run for PMA was separate from others.

IκBα but it did delay its resynthesis. This delay may be a feedback regulation, as the resynthesis of IκBα is dependent on NF-κB activation.

Because NF-κB activation also requires nuclear translocation of the p65 subunit of NF-κB, we examined the cytoplasmic and nuclear pool of p65 protein by Western blot analysis. As shown in Fig. 5 *Lower*, none of the treatments significantly altered the cytoplasmic pool of p65, but the TNF-induced appearance of p65 in the nucleus was blocked by CAPE. The decrease in corresponding cytoplasmic pool of p65 in TNF-treated cells was not significant, perhaps because on activation only 20% of p65 is translocated to the nucleus (35).

Reducing Agents Reverse the Effect of CAPE. It has been shown that the biological effects of pervanadate, TPCK, and

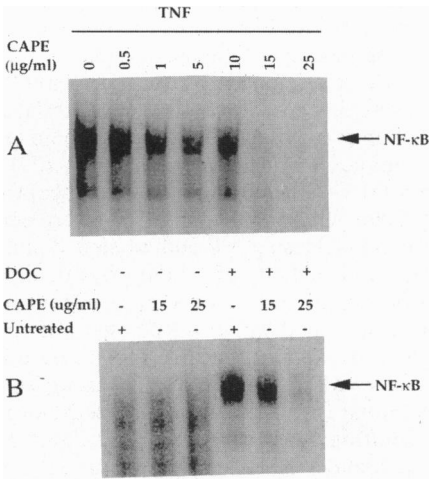

FIG. 3. Effect of CAPE on the binding of NF-κB to DNA. (*A*) Nuclear extracts prepared from TNF activated U937 cells were incubated at 37°C with indicated concentrations of CAPE for 30 min and then analyzed for NF-κB activation. (*B*) Cytoplasmic extracts from untreated cells were treated with deoxycholate (DOC) in the presence and absence of indicated concentrations of CAPE and then analyzed for NF-κB activation.

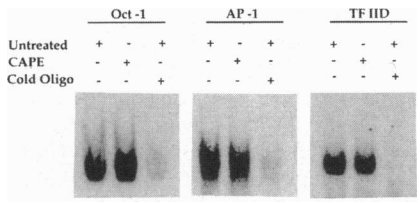

FIG. 4. Effect of CAPE on AP-1, Oct-1, and TFIID transcription factors. Cells were treated with 25 μg/ml of CAPE for 2 h at 37°C, and nuclear extracts were then prepared and used for EMSA of AP-1, Oct-1, and TFIID transcription factors as described.

herbimycin A on suppression of NF-κB activation can be reversed by reducing agents (29, 32, 33). Therefore, we examined the ability of DTT, 2,3-dimercaptopropanol (DMP), and 2-mercaptoethanol (BME) to reverse the effect of CAPE in our system. For this cells were treated with CAPE in the presence and absence of either DTT or DMP or BME and then examined for the activation of NF-κB by TNF. As shown in Fig. 6, none of the reducing agents by themselves had a significant effect on TNF-dependent activation of NF-κB, but they completely reversed the inhibition induced by CAPE. These results demonstrate the critical role of sulfhydryl groups in the TNF-dependent activation of NF-κB.

Structure-Activity Relationship Studies on CAPE. To further delineate the role of CAPE in inhibition of NF-κB activation, different analogues with four different modifications of the parent compound were used. These analogues included ring substituents (compounds **1-3**), ester groups (compound **4**), rotationally constrained variants (compounds **5** and **6**), and saturated amide analogues (compounds **7** and **8**) as shown in Fig. 7*A*. These analogues have been previously characterized for their ability to inhibit human HIV integrase and cell growth (8). Although all the compounds were active in inhibiting NF-κB activation, there were marked variations in their inhibitory ability (Fig. 7*B*). Alteration of the hydroxyl group placement from 3,4-dihydroxy pattern to 2,5-dihydroxy pattern (compound **1**) increased the potency of inhibition over

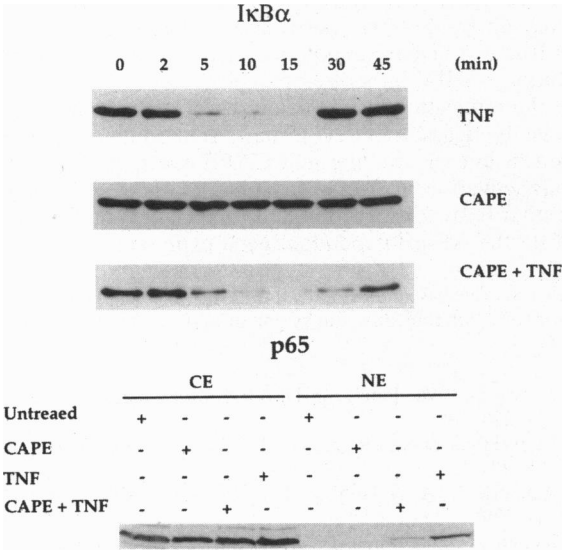

FIG. 5. Effect of CAPE on TNF-induced degradation of IκBα and on the level of p65 in cytoplasm and nucleus. (*Upper*) U937 cells (2×10^6 per ml) pretreated (for 2 h at 37°C) with or without CAPE (25 μg/ml) were incubated for different times with and without TNF (0.1 nM), and then assayed for IκBα. (*Lower*) For p65, cells pretreated (for 2 h at 37°C) with or without CAPE (25 μg/ml) were incubated for 15 min with and without TNF (0.1 nM), and then nuclear and cytoplasmic extracts prepared and assayed by Western blot analysis for p65 as described.

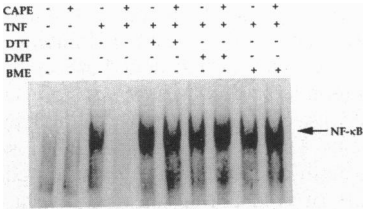

FIG. 6. Effect of DTT, BME, and DMP on the CAPE-induced inhibition of NF-κB activation. U-937 (2×10^6 per ml) were incubated for 2 h with DTT (100 μM), BME (142 μM), or DMP (100 μM) in presence and absence of CAPE (25 μg/ml), activated with TNF (0.1 nM) for 15 min, and then assayed for NF-κB activation as described.

that resulting from replacement of the hydroxyl groups of CAPE with two methyl ethers (compound **2**). However, addition of a third hydroxyl group to give 2,3,4-trihydroxy derivative (compound **3**) resulted in a loss of potency, sug-

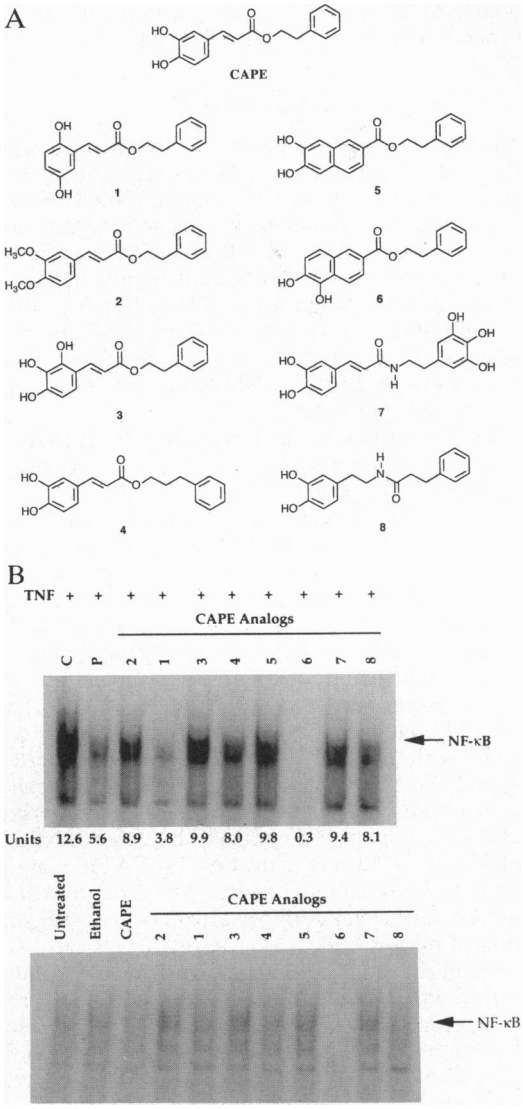

FIG. 7. Structure of different analogues of CAPE (*A*) and their effect on TNF-induced NF-κB activation (*B*). U-937 (2×10^6 per ml) were incubated for 2 h with different analogues of CAPE (25 μg/ml) at 37°C and then activated either with (*Upper*) or without (*Lower*) TNF (0.1 nM) for 15 min and assayed for NF-κB activation as described. C, TNF treatment alone; P, treatment with parent compound CAPE followed by TNF. The arbitrary units represent the relative amounts of the radioactivity present.

gesting that the number and the placement of hydroxyl groups is a critical determinant of the extent of inhibition. In the ester group of analogues, the caffeic acid portion of the molecule (3,4-dihydroxycinnmic acid) was held constant and the phenylethyl side chain was varied. An increase in the length of the alkyl spacer (compound **4**) resulted in a significant loss of inhibition.

In the rotationally constrained variants, bicyclic analogues of the two isomers of CAPE that differed in the placement of hydroxyl substituents were used. A drastic change in the inhibitory potency of the two analogues was seen. The isomer **5** was completely ineffective, whereas the isomer **6** completely abolished the binding, once again indicating that the placement of the hydroxyl groups played a critical role in inhibiting NF-κB activation. In the saturated amide analogues, the importance of the side chain bond and the ester oxygen was examined. The analogue with three additional hydroxyls in the (phenylethyl) amine ring (compound **7**) and the reverse amide analogue (compound **8**), which lacked an additional hydroxyl group, were less active than CAPE. Thus it is possible to find structural analogues of CAPE that are more active than CAPE (compound **6**), as active as CAPE (compound **1**), and less active than CAPE (compounds **2-5**, **7**, and **8**).

DISCUSSION

In an effort to find agents that downmodulate the activation of NF-κB, we investigated CAPE. The latter has been shown to be a pharmacologically safe compound with known antiinflammatory, immunomodulatory, anticarcinogenic, and antioxidant properties (7–21), and NF-κB activation is involved in most of these activities. Our results demonstrate that CAPE completely blocked the activation of NF-κB induced by a wide variety of inflammatory agents, including TNF, phorbol ester ceramide, okadaic acid, and H_2O_2. Furthermore, this effect was found to be specific for NF-κB, as other transciptional factors were not affected.

How CAPE inhibits the activation of NF-κB induced by a wide variety of agents is not clear. The roles of reactive oxygen intermediates, PTK, protein kinase C, protein tyrosine phosphatase (PTPase), proteases, and ceramide have been documented in the activation of NF-κB. As all the inducers of NF-κB used in our studies are known to produce reactive oxygen intermediates (for review, see ref. 1), and its production is shown to be critical for NF-κB activation (36, 37), it is possible that CAPE exerts its effects by inhibiting reactive oxygen intermediates production. This is consistent with the antioxidant properties assigned to CAPE (14, 20). The inhibition of PMA-induced NF-κB activation by CAPE is also in agreement with its ability to inhibit PMA-induced tumor promotion (21). Like CAPE, the inhibitors of mitochondrial electron transport that suppress reactive oxygen intermediates production also impair TNF-induced activation of NF-κB (38). The inhibition of NF-κB activation by CAPE may also be ascribed to its known ability to alter the redox state of the cell (13), as the intracellular thiols are known to regulate the activation of NF-κB (39).

The role of different TNF-activated signals including acidic and neutral sphingomyelinase-generated ceramides, proteases, serine/threonine protein kinase, PTK, PTPase, and superoxide radicals in the activation of NF-κB have been implicated (1, 29, 30, 36, 37, 40–42). Whether these signals are generated by TNF sequentially or independently of each other, however, is not understood. As CAPE can also inhibit PTK (17) and the inhibitors of PTK such as erbstatin can block TNF-dependent NF-κB activation (30), it is possible that CAPE suppresses TNF effects through PTK. Since CAPE has also been shown to inhibit protein kinase C in keratinocytes, and NF-κB activators including PMA, TNF, and H_2O_2 are known to activate protein kinase C (43), it is tempting to speculate that CAPE may blocks NF-κB induction by inhibiting protein kinase C.

Most of the above mechanisms for NF-κB activation, however, occur only in intact cells and require IκBα degradation. Our results show that CAPE inhibits NF-κB activation not by blocking the degradation of IκBα but by suppressing the interaction of NF-κB proteins with the DNA. Like CAPE, herbimycin A also inhibits NF-κB activation without interfering with the IκBα degradation (33). The inhibition of DNA binding by CAPE, however, is specific to NF-κB, as DNA binding of other transcriptional factors, including Oct-1, AP-1 and TFIID, was not affected. Similar results have been reported with TPCK (32). Like TPCK and herbimycin A, we found that CAPE-dependent inhibition of activation of NF-κB is reversed by reducing agents, suggesting that CAPE may modify a critical sulfhydryl group. It was found that herbimycin A causes a covalent modification of a key thiol at cysteine 62 present in the p50 subunit of NF-κB, resulting in inhibition of its binding to the DNA. Whether it is the same thiol on p50 that is modified by CAPE is not clear. In contrast to CAPE and herbimycin A, however, TPCK represses both the phosphorylation and degradation of IκBα (32). Previously, we have shown that, like CAPE, PTPase inhibitors also suppress TNF-induced NF-κB activation and that this inhibition can be reversed by reducing agents (29). The mechanism by which PTPase inhibitors block NF-κB activation, however, is different from CAPE and herbimycin A. Instead, PTPase inhibitors, like TPCK, inhibit IκBα degradation.

CAPE has been shown to inhibit the HIV integrase enzyme needed for integration of HIV DNA into the host genome (44). It is possible that the mechanism by which CAPE inhibits this enzyme is also the mechanism involved in inhibition of NF-κB activation. This is less likely, however, as indicated by studies with CAPE analogues. We tested several structural analogues of CAPE for inhibition of NF-κB activation. These analogues have been previously tested for their ability to inhibit HIV integrase and growth stimulation (8). The analogues that were maximally active in inhibiting NF-κB activation were different from those with maximum inhibitory activity for either HIV integrase or cell growth, suggesting a difference in the mechanism. For instance, compound **6**, one of the conformationally constrained CAPE variants (5,6 dihydroxy derivative), was more potent than native CAPE for NF-κB activation but less potent than the parent compound for inhibition of HIV integrase and cell growth.

As the replication of HIV requires NF-κB activation (1–6), our results suggest that CAPE may also modulate this activity. In conclusion, our finding that CAPE can specifically inhibit NF-κB activation by diverse inflammatory agents provides the molecular basis for its action and predicts its ability to downregulate the NF-κB-dependent gene expression.

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1. Nolan, G. P. & Baltimore, D. (1992) *Curr. Opin. Genet. Dev.* **2,** 211–220.
2. Liou, H.-C. & Baltimore, D. (1993) *Curr. Opin. Genet. Dev.* **5,** 477–487.
3. Baeuerle, P. A. & Henkel, T. (1994) *Annu. Rev. Immunol.* **12,** 141–179.
4. Siebenlist, U., Franzo, G. & Brown, K. (1994) *Annu. Rev. Cell Biol.* **10,** 405–455.
5. Baeuerle, P. A. (1991) *Biochim. Biophys. Acta* **1072,** 63–80.
6. Grilli, M., Chiu, J. J.-S. & Lenardo, M. J. (1993) *Int. Rev. Cytol.* **143,** 1–62.
7. Grunberger, D., Banerjee, R., Eisinger, K., Oltz, K., Efros, E. M., Caldwell, M., Estevez, V. & Nakanishi, K. (1988) *Experientia* **44,** 230–232.
8. Burke, T. R., Jr., Fesen, M. R., Mazumder, A., Wang, J., Carothers, A. M., Grunberger, D., Driscol, J., Kohn, K. & Pommier, Y. (1995) *J. Med. Chem.* **38,** 4171–4178.

9. Su, Z.-z., Lin, J., Grunberger, D. & Fisher, P. B. (1994) *Cancer Res.* **54,** 1865–1870.
10. Su, Z.-z., Grunberger, D. & Fisher, P. B. (1991) *Mol. Carcinog.* **4,** 231–242.
11. Hlandon, B., Bylka, W., Ellnain-Wojtaszek, M., Skrzypczak, L., Szafarek, P., Chodera, A. & Kowalevski, Z. (1980) *Arzneim. Forsch.* **30,** 1847–1848.
12. Guarini, L., Su, Z.-z., Zucker, S., Lin, J., Grunberger, D. & Fisher, P. B. (1992) *Cell. Mol. Biol.* **38,** 513–527.
13. Chiao, C., Carothers, A. M., Grunberger, D., Solomon, G., Preston, G. A. & Barrett, J. C. (1995) *Cancer Res.* **55,** 3576–3583.
14. Laranjinha, J., Vieira, O., Madeira, V. & Almeida, L. (1995) *Arch. Biochem. Biophys.* **323,** 373–381.
15. Kimura, Y., Okuda, H., Okuda, T., Hatano, T., Agata, I. & Arichi, S. (1985) *Chem. Pharm. Bull.* **33,** 2028–2034.
16. Rao, C. V., Desai, D., Kaul, B., Amin, S. & Reddy, B. S. (1992) *Chem. Biol. Interact.* **84,** 277–290.
17. Rao, C., Desai, D., Simi, B., Kulkarni, N., Amin, S. & Reddy, B. S. (1993) *Cancer Res.* **53,** 4182–4188.
18. Zheng, Z. S., Xue, G. Z., Grunberger, D. & Prystowsky, J. (1995) *Oncol. Res.* **7,** 445–452.
19. Sud'ina, G. F., Mirzoeva, O. K., Pushkareva, M. A., Korshunova, G. A., Sumbatyan, N. V. & Varfolomeev, S. D., (1993) *FEBS Lett.* **329,** 21–24.
20. Bhimani, R. S., Troll, W., Grunberger, D. & Frenkel, K. (1993) *Cancer Res.* **53,** 4528–4533.
21. Frenkel, K., Wei, H., Bhimani, R. S., Ye, J., Zadunaisky, J. A., Huang, M., Ferraro, T., Conney, A. & Grunberger, D. (1993) *Cancer Res.* **53,** 1255–1261.
22. Chaturvedi, M. M., LaPushin, R. & Aggarwal, B. B. (1994) *J. Biol. Chem.* **269,** 14575–14583.
23. Schreiber, E., Matthias, P., Muller, M. M. & Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419.
24. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
25. Nabel, G. & Baltimore, D. (1987) *Nature (London)* **326,** 711–713.
26. Collart, M. A., Baeuerle, P. & Vassalli, P. (1990) *Mol. Cell. Biol.* **10,** 1498–1506.
27. Hassanain, H. H., Dai, W. & Gupta. S. L. (1993) *Anal. Biochem.* **213,** 162–167.
28. Singh, H., LeBowitz, J. H., Baldwin, A. S., Jr., & Sharp, P. A. (1988) *Cell* **52,** 415–423.
29. Singh, S. & Aggarwal, B. B. (1995) *J. Biol. Chem.* **270,** 10631–10639.
30. Reddy, S. A. G., Chaturvedi, M. M., Darnay, B. G., Chan, H., Higuchi, M. & Aggarwal, B. B. (1994) *J. Biol. Chem.* **269,** 25369–25372.
31. Meyer, M., Schreck, R. & Baeuerle, P. A. (1993) *EMBO J.* **12,** 2005–2015.
32. Finco, T. S., Beg, A. A. & Baldwin, A. S. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11884–11888.
33. Mahon, T. M. & O'Neill, L. A. J. (1995) *J. Biol. Chem.* **270,** 28557–28564.
34. Thanos, D. & Maniatis, T. (1995) *Cell* **80,** 529–532.
35. Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D. & Miyamoto, S. (1995) *Genes Dev.* **9,** 2723–2735.
36. Eicher, D. M., Tan, T.-H., Rice, N. R., O'Shea, J. J. & Kennedy, I. C. S. (1994) *J. Immunol.* **152,** 2710–2719.
37. Schreck, R., Meier, B., Maennel, D. N., Droge, W. & Baeuerle, P. A. (1992) *J. Exp. Med.* **175,** 1181–1194.
38. Schulze-Osthoff, K., Beyaert, R., Van Dervoorde, V., Haegeman, G. & Fiers, W. (1993) *EMBO J.* **12,** 3095–3104.
39. Staal, F. J. T., Roederer, M., Herzenberg, L. A. & Herzenberg, L. A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9943–9947.
40. Schutze, S., Pothoff, K., Machleidt, T., Bercovic, D., Wiegmann, K. & Kronke, M. (1992) *Cell* **71,** 765–776.
41. Mathias, S., Dressler, K. A. & Kolesnick, R. N. (1991) *J. Biol. Chem.* **266,** 10009–10013.
42. Yang, Z., Costanzo, M., Golde, W. & Kolesnick, R. N. (1993) *J. Biol. Chem.* **268,** 20520–20523.
43. Meichle, A., Schutze, S., Hensel, G., Brunsing, D. & Kronke, M. (1990) *J. Biol. Chem.* **265,** 8339–8343.
44. Fesen, M. R., Kohn, K. W., Leteurtre, F. & Pommier, Y. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2399–2403.