Caffeic Acid Phenethyl Ester Induces Apoptosis by Inhibition of NFκB and Activation of Fas in Human Breast Cancer MCF-7 Cells*

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The transcription factor NFκB plays a role in cell survival. Apoptosis, programmed cell death, via numerous triggers including death receptor ligand binding is antagonized by NFκB activation and potentiated by its inhibition. In the present study, we found that caffeic acid phenethyl ester (CAPE), known to inhibit NFκB, induced apoptosis via Fas signal activation in human breast cancer MCF-7 cells. CAPE activated Fas by a Fas ligand (Fas-L)-independent mechanism, induced p53-regulated Bax protein, and activated caspases. CAPE also activated MAPK family proteins p38 and JNK, and suppressed CAPE-induced p53 activation, Bax expression, and apoptosis, consistent with a mechanism by which CAPE leads to Bax activation, known to be regulated by p38 and p53. The expression of dominant negative c-Jun, which inhibits the JNK signal, also suppresses CAPE-induced apoptosis, suggesting MAPKs are involved in CAPE-induced apoptosis. The expression of Fas antisense oligomers significantly suppressed the CAPE-induced activations of JNK and p38 and apoptosis as compared with Fas sense oligomers. To ascertain whether these phenomena are attributable to the inhibition of NFκB by CAPE, we examined the effect of a truncated form of IκBα (IκBΔN) lacking the phosphorylation sites essential for NFκB activation. IκBΔN expression not only inhibited NFκB activity but also induced Fas activation, Bax expression, and apoptosis. Our findings demonstrate that NFκB inhibition is sufficient to induce apoptosis and that Fas activation plays a role in NFκB inhibition-induced apoptosis in MCF-7 cells.

Programmed cell death can occur in all cells by highly efficient mechanisms, leading to the quiet disposal of millions of cells in the adult human. This efficient removal of unnecessary cells is regulated not only by cell death signals but also by those of cell survival. Any imbalance between these signals can be lethal in the development of higher organisms and likely plays a major role in pathophysiological processes as diverse as athroclerosis, heart failure, and inflammation.

Originally defined as a nuclear factor that binds to the B site of the immunoglobulin κ light chain gene enhancer in B lymphocytes (1), transcription factor NFκB is crucial for the inducible expression of many genes involved in immunity and inflammation (2). NFκB is a member of the rel multigene family and comprises five major proteins: p50, p65 (RelA), c-rel, p52, and RelB (3). The most abundant form of NFκB is the heterodimer of p50 and p65 that is retained in the cytosol by specific inhibitory proteins termed IκB (2). IκB kinases are central to NFκB activation (4–6). The IκB kinases trigger the phosphorylation of IκB on amino-terminal serine residues 32 and 36, upon which the conjugation of ubiquitin occurs, and then targets the phosphorylated IκB for degradation by proteasomes (6). The released nucleophilic heterodimer then moves to the nucleus, where both p50 and p65 contribute to NFκB DNA binding (3). The p65 subunit is responsible for transcriptional activity, resulting in the expression of NFκB-responsive target genes. Caffeic acid phenethyl ester (CAPE), a structural derivative of flavonoids, is a biologically active ingredient of honeybee propolis and a potent and specific inhibitor of NFκB activation (7).

In this study we found that CAPE, which is known to inhibit NFκB, activates the Fas death receptor in human breast cancer MCF-7 cells. We demonstrate herein that CAPE not only inhibits NFκB activity but also activates Fas in a Fas-L-independent manner. Moreover, CAPE induces Bax expression, caspase activation, DNA fragmentation, and apoptosis. We used a truncated form of IκBα (IκBΔN) lacking the phosphorylation sites essential for NFκB activation as an alternative method of inhibiting this activation. IκBΔN expression caused phenomena qualitatively identical to those observed with CAPE treatment. Taken together, these findings suggest that NFκB inhibition is sufficient to activate Fas death receptors and to induce apoptosis in human breast cancer cells.

MATERIALS AND METHODS

Materials—Anti-actin antibody, N-benzyloxy carbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (Z-VAL-FMK), Z-Leu-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone (Z-IETD-FMK), Z-Leu-Glu(OMe)-His-Asp(OMe) fluoromethyl ketone (Z-LEHD-FMK), and recombinant Fas-L (soluble) were purchased from Sigma. Anti-active JNK antibody and anti-active p38 antibody were from Promega. Polyclonal anti-Fas, Fas-L, Bax, and Fas-associated death domain (FADD) antibody were from Santa Cruz Biotechnology. 3,3'-Dithiobis(sulfosuccinimidylpropionate) was from Pierce. Fas ligand inhibitor was from Kamiya Biomedical Company.

Cell Culture—MCF-7 cells were grown in Dulbecco’s modified E-
s medium supplemented with 10% fetal calf serum at 37 °C under 5% CO2 in air.

DNA Fragmentation—DNA fragmentation was measured using a cell death detection ELISAPLUS kit (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Nuclear Morphology—Morphological study was performed as described previously (8). After CAPE treatment, the cells were collected, washed with phosphate-buffered saline, fixed with 3.7% formaldehyde for 20 min, and incubated in 2 μg/ml Hoechst33258 (Sigma) for 5 min. Nuclear morphology was examined using fluorescence microscopy.

Immunoblot Analysis—Immunoblotting was performed as described previously (9). Cells were lysed in buffer containing SDS and mercaptoethanol, and the cell lysate was then boiled. Denatured proteins were separated on a polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences). The membrane was incubated with a blocking solution (2% skim milk (Invitrogen) dissolved in phosphate-buffered saline containing 0.2% Tween 20 (TPBS) for 1 h at room temperature, washed with TPBS, and incubated for 1 h with a primary antibody dissolved in the blocking solution overnight at 4 °C. After washing, the membrane was incubated for 1 h with the respective horseradish-linked secondary antibody. Immunoreactive proteins were detected with an enhanced chemiluminescence system (Amersham Biosciences) and analyzed with image analysis software NIH-Image 1.62 for the Macintosh.

Immunofluorescence Microscopy—Immunocytochemical study was performed as described previously (10, 11). Cells were washed with phosphate-buffered saline and fixed with 3.7% formaldehyde for 20 min. Cells were permeabilized with phosphate-buffered saline containing 0.2% Triton X-100 for 5 min and then washed three times with phosphate-buffered saline. Incubation with primary antibody was carried out for 1 h at room temperature. Excess antibody was washed three times with phosphate-buffered saline. This was followed by incubation with an appropriate fluorophore-labeled secondary antibody for 1 h at room temperature in an area protected against light. After washing out the excess antibody three times with phosphate-buffered saline, we performed a mounting operation with phosphate-buffered saline containing 1 μg/ml Hoechst33258 (Sigma). Images were collected by fluorescence microscopy.

Reporter Assay—Reporter assays were performed as described previously (12). Twenty-four hours after co-transfection with p3xNFkB-Luc or p53-Luc (Stratagene) and pRL-TK (Promega) using SuperFect™ (Qiagen), the cells were treated with CAPE. Transcriptional activity in CAPE-treated cells was measured as the enzyme activity of luciferase.
which is a product of the reporter gene, using a dual-luciferase reporter assay system (Promega).

Fas Cross-linking and Immunoprecipitation—Immunoprecipitation was performed as described previously (13, 14). After treatment with CAPE, the cells were pelleted, washed twice in phosphate-buffered saline, resuspended in phosphate-buffered saline, and treated with 2 mM cross-linker 3,3′-dithiobis(sulfosuccinimidylpropionate) for 15 min on ice. The reaction was quenched with 10 mM ammonium acetate for 10 min. The cells were pelleted, washed twice in phosphate-buffered saline, and lysed in lysis buffer (20 mM Tris-Cl (pH 7.4), 140 mM NaCl, 10% glycerol, 1% Triton X-100, and 2 mM EDTA) containing a protease inhibitor mixture (Roche Molecular Biochemicals). The cell lysates were then used for immunoprecipitation in the presence of an anti-Fas antibody. Immune complexes were precipitated using protein A/G PLUS-agarose (Santa Cruz Biotechnology) and washed four times in lysis buffer. The precipitate was resuspended in buffer containing SDS and mercaptoethanol, boiled, and then immunoblot analysis was performed.

Detection of Fas-FADD Complex—Using an immunoprecipitation method, the Fas-FADD complex was detected as described previously (13, 14). After treatment with CAPE, the cells were pelleted, washed twice in phosphate-buffered saline, resuspended in phosphate-buffered saline, and treated with 2 mM cross-linker 3,3′-dithiobis(sulfosuccinimidylpropionate) for 15 min on ice. The reaction was quenched with 10 mM ammonium acetate for 10 min. The cells were pelleted, washed twice in phosphate-buffered saline, and lysed in lysis buffer (20 mM Tris-Cl (pH 7.4), 140 mM NaCl, 10% glycerol, 1% Triton X-100, and 2 mM EDTA) containing a protease inhibitor mixture. The cell lysates were then used for immunoprecipitations in the presence of an anti-Fas antibody. Immune complexes were precipitated using protein A/G PLUS-agarose and washed four times in lysis buffer. The precipitate was resuspended in buffer containing SDS and mercaptoethanol, boiled, and then immunoblotted. Half the immunoprecipitate was used for immunoblot analysis to detect the presence of FADD, and the other half was used to detect the presence of Fas.

Blockage of Fas Signaling with Fas Antisense Oligonucleotides—For antisense experiments, MCF-7 cells were transiently transfected with sense or antisense Fas oligonucleotides using SuperFect™ (Qiagen) in accordance with the manufacturer’s manual. After 24 h of transfection, the cells were treated with CAPE. Oligomer sequences for Fas sense (ATG CTG GAC TTC TGG ACC CTC) and antisense (GAG GGT CCA GTT GGC CAT GCG) oligonucleotides were designed against the ATG translation start site in Fas mRNA and were modified with phosphorothioate (Invitrogen).

Recombinant Adenovirus Vector and Transfection Efficiency—We constructed a recombinant adenovirus vector expressing the non-degraded form of the NFκB inhibitor IκBα as described previously (15). This nondenatured IκBα (IκBαN) lacks the NH2-terminal 54 amino acids of wild type human IκBα. IκBαN is reportedly not phosphorylated or proteolyzed in response to signal induction but does fully inhibit NFκB (16). To evaluate the efficiency of adenovirus-mediated gene transfer, cells were incubated with AdexlacZ at 37 °C using different multiplicities of infection (m.o.i., 1, 10, 50, and 100). β-galactosidase expression was evaluated by X-gal staining. After 48 h of incubation, AdexlacZ-infected cells were fixed with 1% glutaraldehyde in 0.1 M sodium phosphate (pH 7.0) and 1 mM MgCl2 for 15 min. Cells were washed with phosphate-buffered saline and assayed for lacZ expression by the X-gal staining method (15). Transfection efficiency was increased in an m.o.i.-dependent manner and reached ~70% at an m.o.i. of 100 (data not shown). We therefore employed an m.o.i. of 100 in all of the experiments.

RESULTS

CAPE-induced Apoptosis—We first examined the effect of CAPE on cell viability in human breast cancer MCF-7 cells. CAPE treatment for 48 h decreased cell viability in a dose-de-
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pendent manner (Fig. 1A). To investigate whether or not this decrease in cell viability was caused by apoptosis, we analyzed DNA fragmentation. An increase in histone-associated DNA fragmentation caused by 10 μM CAPE was detected at 12 h and thereafter (Fig. 1B). CAPE treatment induced nuclear fragmentation (Fig. 1C). These results show that NFκB inhibition by CAPE does induce apoptosis in MCF-7 cells. Moreover, we examined the effects of CAPE on DNA fragmentation in various cell types other than MCF-7. As shown in Fig. 1D, CAPE induced apoptosis in adherent cancer cell lines derived from different species such as human hepatocellular carcinoma HepG2 cells and rat myoblastic H9c2 cells but failed to do so in normal human fibroblast WI-38 cells.

CAPE-induced Bax Expression—To investigate the mechanisms underlying CAPE-induced apoptosis, we examined the effect of CAPE on the expressions of apoptosis-related proteins. CAPE treatment markedly decreased the expression of X-chromosome-linked inhibitor of apoptosis, one of the NFκB target genes, after 24 h (Fig. 2A), whereas Bax was induced after 12 h of CAPE treatment (Fig. 2A). Immunohistochemical analysis revealed CAPE treatment increased the amount of Bax protein in cells, the size of which was reduced by CAPE treatment (Fig. 2B, arrow).

p38-regulated Bax Induction—Bax is one of the p38 target genes. Because CAPE increased the amount of Bax, we examined the effect of CAPE on p33 transcriptional activity. As shown in Fig. 3A, CAPE activated p33 transcription 12 h after CAPE treatment. Because p33 phosphorylation is required for its activation and p33 is directly phosphorylated by certain protein kinases, including p38 which belongs to the MAP kinase superfamily (17, 18), we examined the effect of CAPE on p38 activity by detecting its phosphorylation. CAPE treatment induced p38 activation as early as 6 h; interestingly, this activation preceded the p33 activation (Fig. 3B). Moreover, SB203580, a specific p38 inhibitor, partially suppressed CAPE-induced p33 transcriptional activity in a dose-dependent manner (Fig. 3C). To investigate whether or not p38 inhibition suppressed the expression of Bax, we examined the effect of SB203580 on Bax expression. SB203580 inhibited CAPE-induced Bax expression (Fig. 3D) and CAPE-induced apoptosis (Fig. 3E) in a dose-dependent manner. Inhibition of Fas-L-induced apoptosis by SB203580 was also observed in MCF-7 cells (Fig. 3F).

JNK Activation by CAPE—JNK, a member of the MAP kinase superfamily, is involved in apoptosis. Because Bax is essential for JNK-dependent apoptosis and JNK is necessary for Bax activation (19, 20), we investigated the effect of CAPE on JNK activity. CAPE treatment induced JNK activation in a time-dependent manner (Fig. 4A). Because the mutant (Δc-Jun) lacks the transactivation domain of c-Jun, acts as a dom-

![Image](image-url)
nant negative mutant, and inhibits the JNK-mediated pathway (12), we examined the effect of CAPE-induced apoptosis. α-Jun expression partially but significantly suppressed CAPE-induced DNA fragmentation (Fig. 4B). Moreover, we also performed morphological observations. Nuclear fragmentation was detected in control vector-transfected cells (Fig. 4C, GFP-positive cells in the control group). On the other hand, nuclear fragmentation was also detected in α-Jun-nonexpressing cells (Fig. 4C, GFP-negative cells in the dominant negative group, arrow). However, no nuclear fragmentation was detected in α-Jun-expressed cells (GFP-positive cells in the dominant negative group) despite CAPE exposure (Fig. 4C). The same observation was made in MCF-7 cells undergoing recombinant Fas-L-induced apoptosis (Fig. 4D).

**Induction of Fas Aggregation by CAPE**—We examined the effects of CAPE on Fas, which is a member of the death receptor family and functions upstream from BAD, because death receptor family members cluster, thereby inducing a death signal (21, 22). Using anti-Fas antibody, CAPE caused Fas aggregation in MCF-7 cells (Fig. 5A). Because Fas aggregates, which have a high molecular mass and are SDS- and β-mercaptoethanol-resistant, are immediately formed following receptor cross-linking (23), we estimated the apparent molecular mass of Fas aggregates by immunoblotting analysis (Fig. 5B, top panel). High molecular mass aggregates formed at 24 h and thereafter when NFκB activity was almost completely inhibited. It was previously reported that maintaining cells at a low temperature blocks Fas receptor clustering by >90% (24, 25). Although the mechanism underlying the low temperature effect is not clear, changes in membrane fluidity have been suggested. It should be noted that keeping cells at a low temperature does not interfere with transcription in general (25). To clarify the role of Fas aggregation in CAPE-induced apoptosis, we preincubated cells at 4 °C and then measured DNA fragmentation. As shown in Fig. 5C, short exposure to a low temperature (4 °C) for 1 h with CAPE treatment after preincubation for 30 min at 4 °C significantly suppressed CAPE-induced DNA fragmentation (Fig. 5D). Because lowering the temperature may affect numerous other processes besides clustering, we confirmed Fas aggregation utilizing other techniques, such as an immunoprecipitation protocol. The basic principle is that a less than stoichiometric amount of antibody will be able to immunoprecipitate more Fas molecules if aggregation occurs after CAPE treatment. In contrast, a sufficient amount of antibody will immunoprecipitate the same amount of Fas molecules regardless of aggregation. At limited antibody concentrations, the amount of immunoprecipitated Fas was increased by CAPE treatment (Fig. 6A). As expected, equal amounts of Fas were immunoprecipitated under conditions of antibody excess (Fig. 6A). To determine whether this Fas aggregation functions as a Fas death system in NFκB inhibition-induced apoptosis, our attention was drawn to an adapter molecule, FADD, which
Fig. 6. Fas-L-independent activation of Fas system by CAPE. A, MCF-7 cells were treated with 10 μM CAPE for 24 h. The cells were treated with a cross-linker, lysed for immunoprecipitation using a Fas-specific antibody under antibody (Ab)-limiting conditions (top panel) or antibody excess conditions (bottom panel) as described under “Materials and Methods.” Immunoblot analyses of immunoprecipitates were performed using specific antibodies. B, MCF-7 cells were treated with 10 μM CAPE for 24 h. The cells were treated with a cross-linker and lysed, and the extract was used for immunoprecipitation (IP). Half of the immunoprecipitate was used for immunoblot analysis to detect the presence of FADD (top panel); the other half was used to detect the presence of Fas (bottom panel). C, MCF-7 cells were treated with 10 μM CAPE for the indicated times. After cell lysate preparation, immunoblot analyses were performed using specific antibodies. P denotes a positive control using a whole cell lysate of human chronic myelogenous leukemia K562 cells. D, MCF-7 cells were treated with 10 μM CAPE or 5 ng/ml recombinant Fas-L in combination with 50 or 100 μg/ml Fas-L inhibitor (FasLI) for 24 h. DNA fragmentation assay was performed as described under “Materials and Methods.” Results are presented as the means ± S.E. of three independent experiments. *, p < 0.05 compared with control. **, p < 0.05 compared with Fas-L alone.

Fig. 7. Effect of Fas antisense oligonucleotides on CAPE-induced apoptosis. MCF-7 cells were transiently transfected with sense (S) or antisense (As) phosphorothioate oligodeoxynucleotides as described under “Materials and Methods.” After cell lysate preparation, immunoblot analysis was performed using a Fas-specific antibody (A). After being transiently transfected with sense or antisense phosphorothioate oligodeoxynucleotides, MCF-7 cells were treated with CAPE for 24 h. DNA fragmentation assay was performed as described under “Materials and Methods.” Results are presented as the means ± S.E. of three independent experiments. *, p < 0.05 compared with control. **, p < 0.05 compared with CAPE of sense (B). After cell lysate preparation, immunoblot analyses were performed using specific antibodies (panels C and D).
couples the Fas death receptor to procaspase-8. As shown in Fig. 5B (bottom panel), there was no change in FADD amount with CAPE treatment. To investigate whether CAPE-mediated Fas aggregation resulted in FADD recruitment, co-immunoprecipitation experiments were performed (Fig. 6B). Immunoprecipitation of Fas followed by immunoblotting using FADD-specific antibody revealed an increase in the association of FADD with Fas. On the other hand, Fas-L expression in MCF-7 cells remained low, and no change was detectable with CAPE treatment (Fig. 6C). To investigate whether or not the multimerization of Fas and Fas-FADD complex formation required Fas-L, we examined the effect of a Fas-L inhibitor on CAPE-induced apoptosis. As shown in Fig. 6D, the Fas-L inhibitor effectively inhibited recombinant Fas-L-induced apoptosis. However, the Fas-L inhibitor did not change CAPE-induced apoptosis despite treatment with a concentration higher than that that inhibited Fas-L-induced apoptosis (Fig. 6D). We also investigated whether or not Fas really was required for CAPE-induced apoptosis. An alternate approach to knocking out Fas signaling involves transient transfections with Fas antisense oligomers. As shown in Fig. 7A, the expression of Fas antisense oligomers significantly decreased Fas compared with Fas sense oligomers. Under these conditions, we investigated the effects of expressing Fas antisense oligomers on CAPE-induced apoptosis. As a result, the expression of Fas antisense oligomers significantly suppressed CAPE-induced apoptosis compared with Fas sense oligomers (Fig. 7B). Furthermore, the activation of JNK or p38 induced by CAPE was also inhibited by the expression of Fas antisense oligomers compared with Fas sense oligomers (Fig. 7, C and D). Consequently, CAPE produces clustering of death receptors, suggesting Fas activation via a Fas-L-independent mechanism. This in turn causes apoptosis via Bax, which is regulated by MAPKs (p38/JNK) and p53.

IκBαN-induced Fas Activation and Apoptosis—To clarify whether or not the aforementioned results are attributable to the inhibition of NFκB by CAPE, we constructed a truncated form of IκBα (IκBαN) lacking phosphorylation sites essential for NFκB activation. As expected, CAPE inhibited the transcriptional activity of NFκB in a dose- and time-dependent manner in MCF-7 cells (Fig. 8, A and B). We confirmed that IκBαN expression inhibited the transcriptional activity of NFκB in a time-dependent manner, and no NFκB activity was detected at 48 h after AdexIκBαN infection (Fig. 8C). IκBαN expression in MCF-7 cells significantly increased DNA fragmentation as compared with the lacZ gene (Fig. 9A). Under these conditions, Fas aggregation was detected in cells with shrunken nuclei (Fig. 9B, arrow) and high molecular mass proteins reacted with anti-Fas antibody (Fig. 9C). These changes did not occur in LacZ-expressing cells. To determine whether IκBαN-induced apoptosis requires Fas aggregation, we preincubated cells at 4°C and then measured DNA fragmentation. Blockade of death receptor clustering at 4°C significantly inhibited IκBαN-induced DNA fragmentation (Fig. 9D). Moreover, the expression of IκBαN inhibited X-chromosome-linked inhibitor of apoptosis and induced Bax protein (Fig. 10A). Immunohistochemical analysis revealed the amount of Bax protein to be increased by IκBαN expression (Fig. 10B). Thus, IκBαN expression induced apoptosis by the same mechanism as CAPE.

Requirement of Caspase-8 and -9 for NFκB Inhibition-induced Apoptosis—To further investigate the possible requirements of both Fas aggregation and Bax action in the initiation of NFκB inhibition-induced apoptosis, we examined the effects of caspase-8 and -9 inhibitors. Z-VAD-FMK is an irreversible caspase inhibitor with a broad specificity against various caspases, Z-IETD-FMK is an irreversible specific inhibitor of caspase-8, and Z-LEHD-FMK is an irreversible specific inhibitor of caspase-9. When the cells were pretreated with each caspase inhibitor, DNA fragmentation caused by either CAPE or Z-IETD-FMK or Z-LEHD-FMK alone suppressed NFκB activity. An alternate approach to knocking out Fas, DNA fragmentation induced by CAPE was also inhibited by the expression of Fas antisense oligomers compared with Fas sense oligomers (Fig. 7, C and D). Consequently, CAPE produces clustering of death receptors, suggesting Fas activation via a Fas-L-independent mechanism. This in turn causes apoptosis via Bax, which is regulated by MAPKs (p38/JNK) and p53.

DISCUSSION

In this study, we clarified for the first time that CAPE, known to inhibit NFκB, leads to the clustering of Fas death
receptors by a Fas-L-independent mechanism and induces apo-
ptosis. UV is known to cause Fas death receptor clustering by
a Fas-L-independent mechanism (24). We have demonstrated
herein a new Fas-L-independent mechanism by which inhibi-
tion of NFκB, which controls cell survival, is sufficient to in-
duce Fas aggregation, using IκBΔN.

As a ubiquitous multifunctional signaling system, members
of the NFκB family play prominent roles in the cell death/
survival balance (26). CAPE, a flavonoid derivative, is a biolog-
ically active ingredient of honeybee propolis and strongly in-
hibits NFκB activation. We have demonstrated that apoptosis
is induced by CAPE in various cancer cell lines. However, it is
worth noting that CAPE failed to induce apoptosis in WI-38
cells, not a cancer cell line. We investigated NFκB activities in
various cell lines, including those described in this report, and
found apoptosis induction by NFκB inhibition to parallel basal
NFκB activity (data not shown). Namely, cancer cells with
high basal NFκB activity are more sensitive to NFκB inhibition
by CAPE than normal cells. This raises the possibility that
cancer cell-specific drugs could be developed if NFκB-specific
inhibitors were available for humans.

In Fas-mediated cell death, it is well known that Fas binds to
its ligand, Fas-L (28, 29). On the other hand, CAPE induced
Fas aggregation and activated Fas independently of Fas-L in
MCF-7 cells. It is also known that death receptor aggregation
results in the activation of two independent signaling path-
ways. One well characterized pathway involves the death adap-
tor molecule FADD. The other pathway is mediated through
Daxx, which enhances Fas-induced apoptosis by activating the
JNK/p38 kinase cascade via apoptosis signal-regulating kinase
1 (30–32), culminating in activation of the Bax subfamily of
Bcl2-related proteins (20) or in the phosphorylation and activa-
tion of transcription factors such as p53 (17, 18). To clarify
the role of these two pathways in CAPE-induced apoptosis, we
examined each step. First, treatment with Fas antisense oligo-
nucleotides significantly inhibited CAPE-induced cell death.
Second, immunoblotting using FADD-specific antibody clari-
fied the association of FADD with Fas, and caspase-8 inhibitor
also inhibited CAPE-induced apoptosis. These results sug-
gest that CAPE promotes Fas aggregation and the associa-
tion of Fas with FADD and subsequently activates caspase-8.
Next, we examined the role of a second pathway using Fas
antisense oligonucleotides, a p38-specific inhibitor
(SB203580), and dominant negative c-Jun. Treatment with
Fas antisense oligonucleotides suppressed CAPE-induced
cell death and JNK/p38 activation. SB203580 and dominant
negative c-Jun partially but significantly suppressed CAPE-
induced p53 activation, Bax expression, and apoptosis. Al-
though we could not exclude the involvement of some path-
way other than the Fas system, our results show both FADD/
caspase-8 and JNK/p38 play important roles in CAPE-induced cell death in MCF-7 cells.

In general, an apoptotic signal can be transmitted through either the death receptors (28, 29) or mitochondria (33, 34). Mitochondria-mediated cell death is distinguished from Fas-mediated apoptosis. Mitochondria sense the apoptotic signal and activate caspase-9 via the release of cytochrome c and Apaf-1, ultimately triggering apoptosis. In this study, CAPE induced Fas aggregation and a caspase-8 inhibitor suppressed CAPE-induced apoptosis. However, a caspase-9 inhibitor alone suppressed CAPE-induced apoptosis. These results suggest that both signals are necessary for CAPE-induced apoptosis. As for MCF-7 cells, we speculate that the death signal of caspase-8 activated by the association of Fas with FADD is transmitted to mitochondria via a second messenger such as Bid. Then, p38 activated by Fas aggregation acts on p53, followed by the expression of Bax, which is in turn activated by JNK that is activated by Fas aggregation. Subsequently, cytochrome c is released, caspase-9 is activated, and apoptosis is induced via caspases other than caspase-3.

Because a truncated form of IκBα (IκBΔN) lacking the phosphorylation sites essential for NFκB activation is a specific inhibitor of NFκB, we used this approach in addition to CAPE to obtain a line of convincing evidence that NFκB inhibition activates Fas and thereby leads to apoptosis. IκBΔN expression caused a phenomenon qualitatively identical to that observed with CAPE treatment. These results demonstrate that NFκB inhibition accounts for CAPE-induced Fas activation and apoptosis. However, it is still unclear how NFκB inhibition increases Fas aggregation/activation. As Anderson (36) as well as Rosette and Karin (37) reported, it is possible that physical perturbation of the plasma membrane or conformational

![Fig. 10. Effects of IκBΔN on expressions of apoptosis-related proteins. MCF-7 cells were infected with AdexlacZ (m.o.i. = 100) and AdexIκBΔN (m.o.i. = 100). A, three days after infection, cell lysates were prepared and immunoblotting analyses were performed using specific antibodies. B, three days after infection, intracellular localization of Bax protein was examined by immunofluorescence microscopy as described under “Materials and Methods.”](image)

![Fig. 11. Effects of caspase inhibitors on CAPE- and IκBΔN-induced apoptosis. A, after MCF-7 cells had been pretreated with various caspase inhibitors for 30 min at the indicated concentrations, cells were treated with 10 μM CAPE for 24 h. DNA fragmentation assay was performed as described under “Materials and Methods.” B, after MCF-7 cells had been pretreated with 50 μM of various caspase inhibitors for 30 min, the cells were infected with AdexlacZ (m.o.i. = 100) or AdexIκBΔN (m.o.i. = 100). Three days after infection, DNA fragmentation assay was performed as described under “Materials and Methods.” Results are presented as the means ± S.E. of three independent experiments. *, p < 0.05 compared with control. Z-VAD, IETD, and LEHD denote Z-VAD-FMK, Z- IETD-FMK, and Z-LEHD-FMK, respectively. **, p < 0.05 compared with CAPE alone or AdexIκBΔN alone.](image)
In summary, we have demonstrated that the inhibition of NFκB by CAPE is pro-apoptotic in human breast cancer MCF-7 cells and that this effect is attributable to Fas death receptor clustering.

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