Apoptosis Induction by a Novel Retinoid-Related Molecule Requires Nuclear Factor-κB Activation

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Abstract

Nuclear factor-κB (NF-κB) activation has been shown to be both antiapoptotic and proapoptotic depending on the stimulus and the specific cell type involved. NF-κB activation has also been shown to be essential for apoptosis induction by a number of agents. The novel retinoid-related molecule 4-[3-Cl-(1-adamantyl)-4-hydroxyphenyl]-3-chlorocinnamic acid (3-Cl-AHPC) activates NF-κB with subsequent apoptosis in a number of cell types. We have found that NF-κB activation is essential for 3-Cl-AHPC–mediated apoptosis. 3-Cl-AHPC activates NF-κB through IKKα kinase activation and the subsequent degradation of IκBα. IKKα kinase activation is associated with IKKα-enhanced binding to HSP90. The HSP90 inhibitor geldanamycin enhances the degradation of IKKα and blocks 3-Cl-AHPC activation of NF-κB and 3-Cl-AHPC–mediated apoptosis. In addition, inhibition of IκBα degradation using a dominant-negative IκBα inhibits 3-Cl-AHPC–mediated apoptosis. NF-κB p65 activation is essential for 3-Cl-AHPC apoptosis induction as evidenced by the fact that inhibition of p65 activation utilizing the inhibitor helenalin or loss of p65 expression block 3-Cl-AHPC–mediated apoptosis. NF-κB has been shown to be antiapoptotic through its enhanced expression of a number of antiapoptotic proteins including X-linked inhibitor of apoptosis protein (XIAP), c-IAP1, and Bcl-X_L. Whereas exposure to 3-Cl-AHPC results in NF-κB activation, it inhibits the expression of XIAP, c-IAP1, and Bcl-X_L and enhances the expression of proapoptotic molecules, including the death receptors DR4 and DR5 as well as Fas and Rip1. Thus, 3-Cl-AHPC, which is under preclinical development, has pleotrophic effects on malignant cells resulting in their apoptosis. (Cancer Res 2005; 65(11): 4909-17)

Introduction

The Rel/nuclear factor-κB (NF-κB) family of eukaryotic transcription factors is composed of a number of structurally related proteins that form homodimers and heterodimers (1). In vertebrates, the Rel/NF-κB family includes p50/p105, p52, p100, RelA (p65), c-Rel, and Rel B (2). These dimers are sequestered in the cytoplasm bound to IκBα. Upon the appropriate stimulation, IκBα is phosphorylated by the IKKα or IKKβ kinase at specific serines, which then allows IκBα to undergo proteolysis through the proteosome pathway with the subsequent NF-κB translocation to the nucleus (3). These dimers then bind to specific DNA consensus sequences in promoters and thus regulate the expression of a number of genes. Numerous stimuli have been shown to activate NF-κB. NF-κB has been found to play both an antiapoptotic role and proapoptotic role depending on the stimuli utilized and the cell type involved (4). The antiapoptotic effects of NF-κB have been shown in a number of investigations. Knockout of RelA results in an embryonic lethal phenotype in transgenic mice because of the failure of RelA to inhibit tumor necrosis factor (TNF) apoptosis of hepatocytes (5, 6). In addition, NF-κB inhibits TNF-mediated apoptosis in Jurkat T cells, primary rat and human fibroblasts, and in MCF-7 breast carcinoma cell lines (7, 8). NF-κB has also been shown to protect against chemotherapy-mediated apoptosis in a number of malignant cell lines (9).

Recent observations have implicated NF-κB activation in the induction of apoptosis (10–12). More importantly, with the development of inhibitors of NF-κB, more definite evidence has been generated documenting a role for NF-κB in the induction of apoptosis in a number of systems (13, 14). Studies have shown that etoposide-induced apoptosis in leukemic cells requires NF-κB activation and that inhibition of NF-κB activation prevents etoposide induction of apoptosis in these cells (15). Recent investigations have implicated that this paradoxical effect of NF-κB on apoptosis is dependent on the stimulus and cell type as well as which of the NF-κB family members is activated (16). It has been speculated that c-Rel and RelA function as mediators of proapoptotic and antiapoptotic signaling, respectively (ref. 16 and references within). However, other investigators have suggested that both c-Rel and RelA can function as proapoptotic agents (17–19).

We previously reported that exposure of cells to the novel compound 4-[3-Cl-(1-adamantyl)-4-hydroxyphenyl]-3-chlorocinnamic acid (3-Cl-AHPC), which binds to the retinoic acid receptor γ (RARγ) but does not activate it, results in apoptosis in cells *in vitro* as well as in an acute myelogenous leukemia mouse model with minimal toxicity (20, 21). In addition, we found that exposure to 3-Cl-AHPC activated the expression of a number of genes that have NF-κB consensus sequences in their promoters. In this report, we show that 3-Cl-AHPC activates the p65 subunit of NF-κB and that p65 activation is necessary for 3-Cl-AHPC–mediated apoptosis.

Materials and Methods

Materials/antibodies. 3-Cl-AHPC was synthesized as described previously (20, 21) and stored at −80°C in DMSO. DMEM-F12 medium, fetal bovine serum, Trizol reagent, and neomycin were purchased from Invitrogen, Inc. (Grand Island, NY). Anti-IκBα, anti–heat shock protein HSP90, anti-IKKα, anti-IKKβ, and anti–receptor interacting protein (RIP) antibodies were from Cell Signaling (Beverly, MA). Anti-Fas antibody was from BD Transduction Laboratories (San Diego, CA). Anti–X-linked inhibitor of apoptosis protein (XIAP) and anti–c-IAP1 antibodies were from R&D Systems (Minneapolis, MN); anti–Bcl-X_L, anti–c-IAP2, and HSP90β were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and anti–Trail-R1 (DR4) and anti–Trail-R2 (DR-5) antibodies were from Axxora,

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LLC (San Diego, CA). α-Tubulin antibody was from Oncogene Research Products (Boston, MA), helenalin from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA), and geldanamycin from NIH (Bethesda, MD).

Cell culture and apoptosis. The human prostate carcinoma cell lines, DU145, LNCaP, and PC-3, and the breast carcinoma cell line, MDA-MB-468, were maintained in DMEM-F12 medium as described (22). Mouse embryonic fibroblasts (MEF) derived from RelA-deficient mice fibroblast and 3T3 p65−/− and wild-type 3T3 fibroblasts were provided by Dr. Amer Beg (Department of Biological Sciences, Columbia University, New York, NY) (6). Apoptosis of cells was assessed either using acridine orange staining as previously described (22) or using Apoptosis ELISA kits obtained from Roche Diagnostic Laboratory (Indianapolis, IN). The assay was done as described in ref. (22).

Transfection and luciferase assay. For reporter gene assay, DU145 and MDA-MB-468 cells were seeded into 1×10^6 cells per Petri dish (100 × 20 mm) and transfected with 10 μg NF-κB–driven reporter (luciferase) gene construct by the calcium phosphate precipitation method (23); 36 hours following transfection, cells were treated with 1 μmol/L 3-Cl-AHPC, the proteasome inhibitor *N*-acetyl-L-leucinyl-L-leucinyl-norleucinal (LLnL; 50 μmol/L; Sigma, St. Louis, MO), and combination of both for 24 hours. Cells were harvested and reporter gene activity determined utilizing a luciferase assay. A β-galactosidase expression vector was used to normalize transfection efficiencies. IκB dominant negative–expressing cells, DU145 and MDA-MB-468 cells, were stably transfected with 8.0 μg of the vector pUSE and double mutant (S32A, S36A) IκBDN-pUSE plasmids (Upstate Biotechnology, Lake Placid, NY) per plate using LipofectAMINE Plus (Invitrogen, Carlsbad, CA) and the protocol provided by the manufacturer; 36 hours following the transfection, the cells were treated with 400 and 600 μg/mL neomycin, respectively, for selection of stable DU145 and MDA-MB-468 IκB dominant negative–expressing cell lines.

Western blots. Cells were lysed following incubation in lysis buffer [50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 0.5% Triton X-100, 2.5 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 μL/mL protease inhibitor cocktail; Sigma, St. Louis, MO] for 20 minutes at 4°C. Protein concentration was determined using the Bio-Rad assay system (Bio-Rad, Hercules, CA). Total proteins were fractionated using SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were blocked with 5% nonfat dried milk in 1× TBS buffer containing 0.1% Tween 20 and then incubated with appropriate primary antibody. Horseradish peroxidase–conjugated anti-rabbit or antimouse IgG was used as the secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) and the protein bands were developed using the enhanced chemiluminesence detection system (Amersham Pharmacia Biotech).

Immunoprecipitation studies. Total protein (1 mg) was incubated with 1 μg of appropriate antibody and 20 μL of Protein G Sepharose 4 Fast Flow bead (Amersham Pharmacia Biotech) overnight at 4°C, centrifuged, and washed thrice with TT buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 0.5% Tween 20] and twice with lysis buffer. Proteins were eluted with Laemmli sample buffer and fractionated using SDS-PAGE as described for Western blots. Coimmunoprecipitations were done as we have previously described (24).

IκB kinase assay. MDA-MB-468 and DU145 cells were treated with 1 μmol/L 3-Cl-AHPC for various times and lysed in a lysis buffer containing 50 mmol/L Tris (pH 8.0), 500 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA (pH 8.0), 10 mmol/L β-glycerophosphate, 10 mmol/L NaF, 10 mmol/L, *p*-nitro-phenyl phosphate, 300 μmol/L sodium orthovanadate, 1 mmol/L DTT, 0.1% NP40, 0.5 mmol/L PMSF, and 10 μL/mL protease inhibitor cocktail. Cell lysates were immunoprecipitated using anti-IKKα and IKKβ polyclonal antibodies and incubated with Protein G Sepharose 4 Fast Flow beads for overnight. The immunocomplexes were washed twice with lysis buffer and with kinase buffer [20 mmol/L HEPES (pH 7.5), 2 mmol/L $MgCl_2$, 10 mmol/L β-glycerophosphate, 10 mmol/L NaF, 10 mmol/L *p*-nitro-phenyl phosphate, 300 μmol/L sodium orthovanadate, 1 mmol/L DTT, 5 μL/mL protease inhibitor cocktail, and 10 μmol/L ATP]. The kinase reaction was done using an IκBα glutathione *S*-transferase fusion protein (Santa Cruz Biotechnology) as a substrate (2.5 μg) in the presence of 10 μCi per reaction [γ-^{32}P]ATP (Amersham Pharmacia Biotech). The reaction was incubated for 45 minutes at 30°C, stopped with Laemmli sample buffer, and analyzed following fractionation of the proteins by 10% SDS-PAGE by autoradiography.

Gel shift analysis. Nuclear NF-κB was assessed by the electrophoretic gel mobility shift assay using NF-κB/Rel Family Nushift kit purchased from Geneka Biotechnology, Inc. (Quebec, Canada). The assay was done according to either the manufacturer's instructions and as described previously (23). Nuclear extracts were prepared as described in ref. (23). Nuclear extracts (10 μg) were incubated with 1 μL of [γ-^{32}P]ATP-labeled NF-κB oligonucleotide (5,000 cpm) in 20 μL of binding buffer. The specificity of NF-κB DNA-binding activity was confirmed by competition with excess cold wild-type NF-κB consensus sequence or mutant NF-κB oligonucleotide (Santa Cruz Biotechnology). To identify the subunit components of activated NF-κB complexes, supershift analyses were done by additional 30-minute incubations at 4°C with polyclonal supershift antibodies against p65, p50, or c-Rel before the addition of labeled probe. DNA-protein complexes were resolved by electrophoresis in 5% nondenaturing polyacrylamide gels and analyzed by autoradiography.

RNase protection assay. MDA-MB-468 and DU145 cells were treated with 1 μmol/L 3-Cl-AHPC for 6 and 24 hours and total RNAs were extracted using Trizol reagent. Probe synthesis, RNA preparation and hybridization, RNase treatments, and gel preparation were done per the manufacturer's suggestion (PharMingen, San Diego, CA). Twenty micrograms of RNA were used for each sample and incubated with [α-^{32}P]UTP–labeled single-stranded RNA probes overnight at 56°C. The RNA-RNA complexes were resolved by electrophoresis in 5% denaturing polyacrylamide gels and autoradiography was done.

Results

4-[3-Cl-(1-adamantyl)-4-hydroxyphenyl]-3-chlorocinnamic acid exposure results in nuclear factor-κB activation. Exposure to 3-Cl-AHPC results in NF-κB activation (Fig. 1*A*). MDA-MB-468 and DU145 cells were transfected with a NF-κB reporter construct and the cells then exposed to 3-Cl-AHPC. A 2- to 3-fold increase in NF-κB activation was noted (Fig. 1*A*). NF-κB activation requires IκBα degradation, which occurs through the proteasome pathway (3, 25–28). We, therefore, determined whether exposure to 3-Cl-AHPC results in a decrease in IκBα levels and whether inhibition of the proteasome pathway inhibits 3-Cl-AHPC–mediated NF-κB activation, 3-Cl-AHPC–mediated decrease in IκBα levels, and 3-Cl-AHPC–mediated apoptosis. Decrease in IκBα levels was noted in all four cell lines within 24 hours following 3-Cl-AHPC exposure (Fig. 1*B*). We found that the proteasome inhibitor LLnL blocked not only 3-Cl-AHPC–mediated NF-κB activation and decrease in IκBα levels but 3-Cl-AHPC–mediated apoptosis as well, suggesting that NF-κB activation is required for 3-Cl-AHPC induction of apoptosis (Fig. 1*A-D*).

IκBα dominant negative inhibits 3-Cl-AHPC–mediated apoptosis. The dominant-negative IκBα reporter construct in which Ser^{32} and Ser^{36} have been mutated to alanine is resistant to phosphorylation by the IKKs; this, in turn, prevents IκBα degradation through the proteasome pathway and subsequent NF-κB activation (29–31). Utilizing this strategy, we found that expression of the IκBα dominant negative in both DU145 and MDA-MB-468 cells resulted in the inhibition of 3-Cl-AHPC–mediated degradation of IκBα (Fig. 2*A*) as well as significant inhibition of 3-Cl-AHPC induction of apoptosis but increased the basal apoptotic rate in both cell lines (Fig. 2*B*).

4-[3-Cl-(1-adamantyl)-4-hydroxyphenyl]-3-chlorocinnamic acid activates IκB kinase α. Phosphorylation of IκBα and IκBβ at specific serine residues with subsequent degradation of these molecules and NF-κB activation can be achieved through

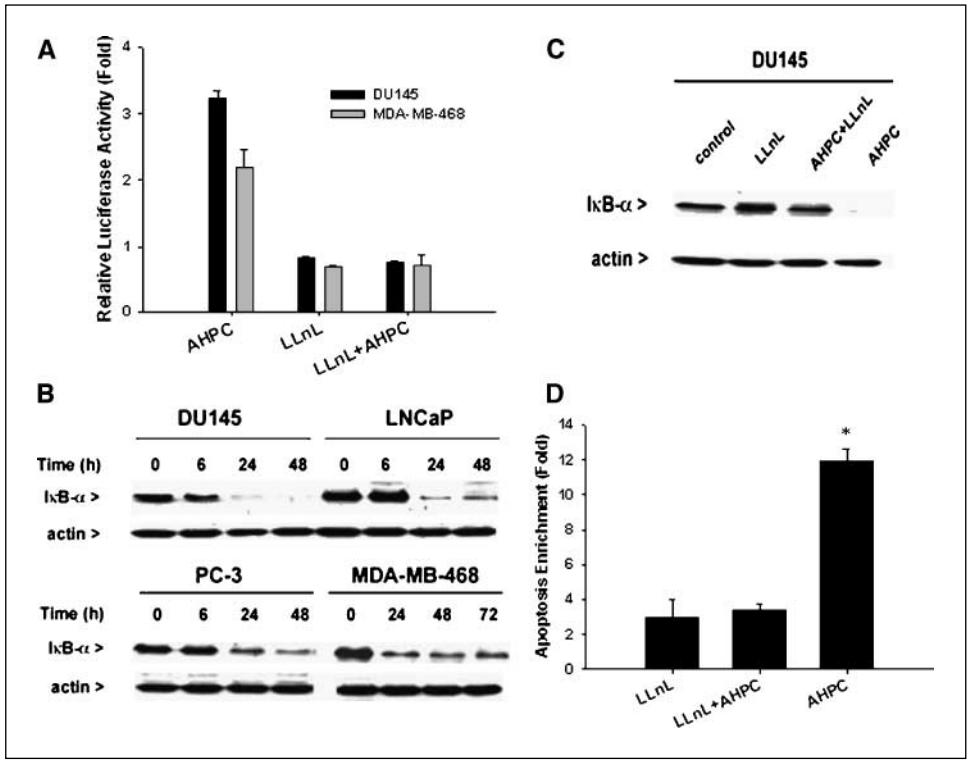

Figure 1. 3-Cl-AHPC enhances NF-κB activation, IκBα degradation, and the induction of apoptosis through the proteasome pathway. Cells were grown and exposed to 1 μmol/L 3-Cl-AHPC in the presence and absence of 50 μmol/L of the proteasome inhibitor LLnL. *A,* 3-Cl-AHPC exposure results in NF-κB activation. NF-κB activation was assessed using transient transfection with an NF-κB reporter construct. *B,* 3-Cl-AHPC enhances the degradation of IκBα. IκBα were determined using Western blots as described in Materials and Methods. *C,* exposure to LLnL inhibits 3-Cl-AHPC–mediated decrease in IκBα levels. *D,* LLnL inhibits 3-Cl-AHPC–mediated apoptosis. Apoptosis were assessed utilizing an Apoptosis ELISA kit. The results in (*C*) and (*D*) represent the mean of three independent experiments. *Bars,* SE. *Significantly greater than cells exposed to LLnL or LLnL and 3-Cl-AHPC ($P < 0.01$).

activation of the IκB kinase IKK (26–28, 32). IKK is a complex consisting of at least three different protein kinases, IKKα, IKKβ, and IKKγ (28). The ability of 3-Cl-AHPC to activate IKKα and IKKβ was examined. Cells were exposed to 3-Cl-AHPC for various times and IKK activation was assessed. Activation of IKKα but not IKKβ was observed within 24 hours of 3-Cl-AHPC exposure in both DU145 and MDA-MB-468 cells (Fig. 3*A* and *B*). A requirement for HSP90 for NF-κB activation has been suggested by several studies (33). In addition, other investigators have suggested that the interaction between HSP90 and IKKα and IKKβ enhances IKK constitutive and inducible activity resulting in NF-κB activation (34). We had previously found that 3-Cl-AHPC exposure results in increased HSP90β levels. We, therefore, examined whether exposure of DU145 and MDA-MB-468 cells to 3-Cl-AHPC results in enhanced association between IKKα and HSP90β. Incubation of DU145 and MDA-MB-468 cells with 3-Cl-AHPC for even 6 hours resulted in the enhanced association between IKKα and HSP90β (Fig. 3*C* and *D*). Incubation of cells with the specific HSP90 inhibitor geldanamycin has been shown to inhibit lipopolysaccharide and taxol-induced NF-κB activity and mitogen-induced NF-κB activity in spleen cells (35–37). Incubation of DU145 and MDA-MB-468 cells with geldanamycin resulted in the degradation IKKα and blocked constitutive NF-κB activity as well as 3-Cl-AHPC–mediated NF-κB activation and apoptosis (Fig. 3*E-G*).

Treatment with 4-[3-Cl-(1-adamantyl)-4-hydroxyphenyl]-3-chlorocinnamic acid results in activation of the p65 nuclear factor-κB subunit. We next did gel mobility shift assays to

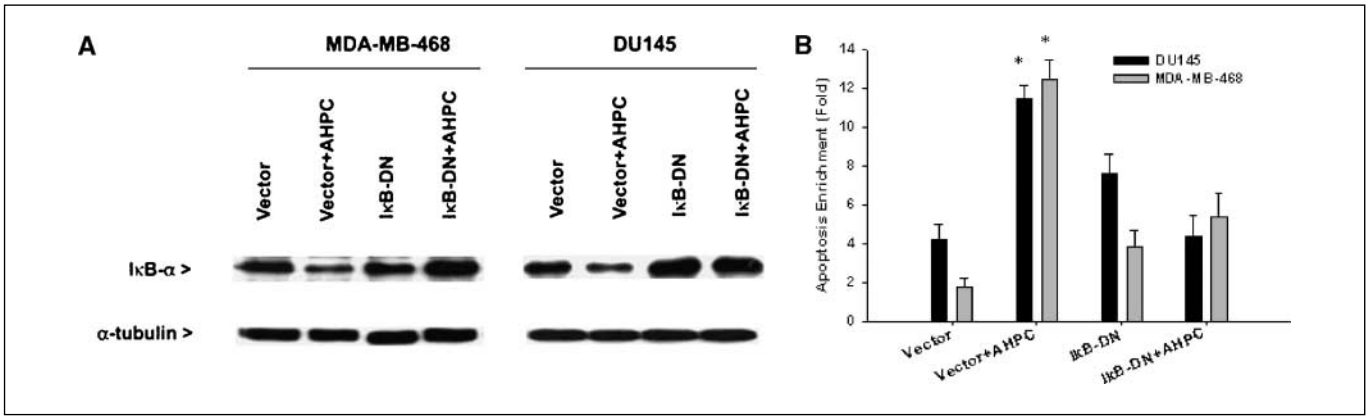

Figure 2. Dominant-negative IκBα inhibits 3-Cl-AHPC–mediated degradation of IκBα and 3-Cl-AHPC–mediated apoptosis. *A,* dominant-negative IκBα inhibits 3-Cl-AHPC degradation of IκBα. *B,* dominant-negative IκBα inhibits 3-Cl-AHPC–mediated apoptosis. Apoptosis was assessed using an Apoptosis ELISA kit. *Columns,* the mean of three independent experiments; *bars,* SE. *Significantly greater than cells transfected with vector construct alone and dominant-negative IκBα construct in the presence and absence of 3-Cl-AHPC ($P < 0.03$).

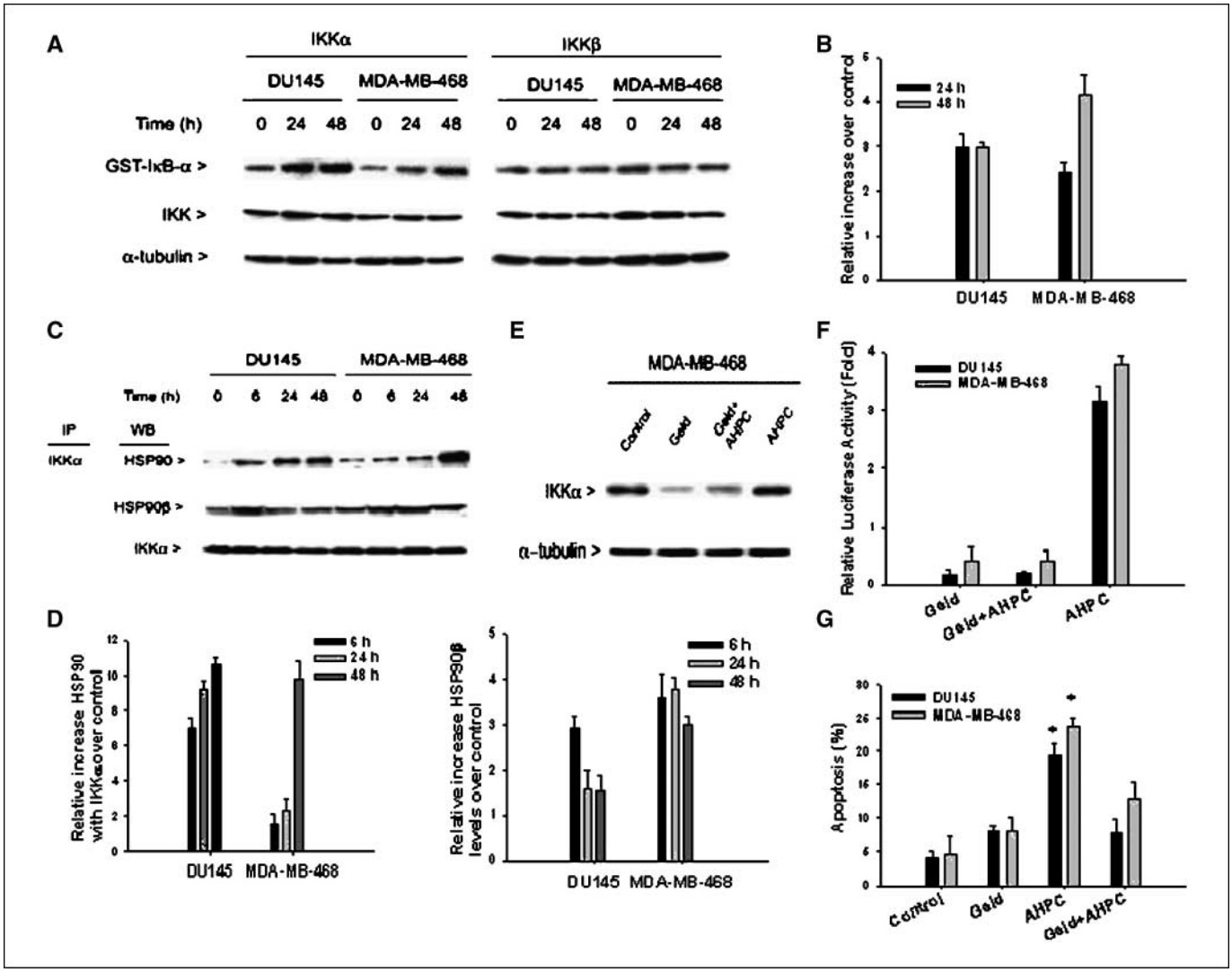

Figure 3. Exposure to 3-Cl-AHPC results in enhanced IKKα activity and the association between IKKα and HSP90β. Cells were exposed to 1 μmol/L 3-Cl-AHPC for varying times. *A,* exposure to 3-Cl-AHPC results in enhanced IKKα activity. IKKα and IKKβ activities were determined as described in Materials and Methods. *B,* quantitation of IKKα activities. *C,* association between IKKα and HSP90β was assessed using immunoprecipitation and Western blots. *D,* quantitation of IKKα associated with HSP90 and HSP90β protein levels. *E,* geldanamycin decreases IKKα levels. *F,* inhibition of 3-Cl-AHPC–mediated NF-κB activation. *G,* inhibition of AHPC-mediated apoptosis. Cells were treated with either vehicle or 1 μmol/L 3-Cl-AHPC and in the presence or absence of 1 μmol/L geldanamycin for 24 hours in cells transfected with a NF-κB reporter construct as described in the legend of Fig. 1. NF-κB activation was assessed as described in Materials and Methods. Apoptosis was assessed using acridine orange staining as described in Materials and Methods. *Significantly greater than geldanamycin and combination of geldanamycin and 3-Cl-AHPC–treated cells ($P < 0.001$).

further confirm 3-Cl-AHPC–mediated NF-κB activation and identify which of the NF-κB subunits were involved (Fig. 4*A*). Cells were incubated with 3-Cl-AHPC or vehicle for 24 hours and nuclear extracts prepared. Exposure to 3-Cl-AHPC resulted in NF-κB activation as evidenced in lane 5 by the increased binding of the extracts obtained from the 3-Cl-AHPC–exposed cells to the NF-κB consensus sequence. That this binding indeed represented NF-κB subunit binding to the labeled consensus sequence is confirmed by the fact that it was totally eliminated by the addition of excess unlabeled NF-κB consensus sequence but not by an excess of unlabeled mutant oligonucleotide (Fig. 4*A*, *lanes 3*, *4* and *6*, *7*). Supershift experiments were done to identify the activated NF-κB subunit(s). The addition of specific NF-κB p65 antibody resulted in a supershift (Fig. 4*A*; *lanes 9* and *12*) as did the addition of NF-κB p50 antibody (Fig. 4*A*; *lanes 10* and *13*). No shift in the bands were noted when c-Rel antibody was added (data not shown). These results indicate that 3-Cl-AHPC exposure resulted in the specific activation of the NF-κB p65 subunit.

p65 expression and activation is necessary for 4-[3-Cl-(1-adamantyl)-4-hydroxyphenyl]-3-chlorocinnamic acid–mediated apoptosis. To further confirm a role for the p65 subunit in 3-Cl-AHPC–mediated apoptosis, we used the sesquiterpene lactone helenalin, which covalently binds to the NF-κB p65 subunit and thus inhibits NF-κB activation (38). Incubation of cells in the presence of helenalin resulted in the inhibition of 3-Cl-AHPC–mediated apoptosis (Fig. 4*B*). This result suggested a proapoptotic role for NF-κB in 3-Cl-AHPC–mediated apoptosis and a role for the p65 subunit. To further document a role for the NF-κB p65 subunit, the ability of 3-Cl-AHPC to induce apoptosis in wild-type and p65−/− MEFs was examined (Fig. 4*C*). Loss of p65 expression completely inhibited 3-Cl-AHPC–mediated apoptosis, further

substantiating a role for p65 in 3-Cl-AHPC–mediated apoptosis (Fig. 4*C*).

4-[3-Cl-(1-Adamantyl)-4-hydroxyphenyl]-3-chlorocinnamic acid inhibits NF-κB induction of X-linked inhibitor of apoptosis protein and Bcl-X_L. Numerous investigators have shown that NF-κB–mediated antiapoptotic effects are at least partially explained by NF-κB induction of c-IAP1, c-IAP2, XIAP, and Bcl-X_L (17, 39). We have previously shown that exposure of cells to 3-Cl-AHPC results in the cleavage of Bcl-X_L and the generation of a proapoptotic molecule in leukemia cells (20, 21). We investigated the effect of 3-Cl-AHPC on c-IAP1, c-IAP2, XIAP, and Bcl-X_L in the MDA-MB-468 and DU145 cells. Incubation with 3-Cl-AHPC resulted in a marked decrease in Bcl-X_L, c-IAP1, and XIAP expression but had no effect on or c-IAP2 expression (Fig. 5*A* and *B*). Loss of expression of the NF-κB p65 subunit prevented 3-Cl-AHPC–mediated decrease in XIAP levels, suggesting that p65 expression is required (Fig. 5*C*).

4-[3-Cl-(1-adamantyl)-4-hydroxyphenyl]-3-chlorocinnamic acid induction of Fas, DR4, and DR5 expression. The ability of NF-κB to induce the expression of over 150 proteins has been documented (2). Whereas a number of these proteins seem to have antiapoptotic effects, NF-κB has also been shown to induce the expression of a number of proapoptotic proteins, including Fas, Fas ligand, and the death receptors DR4 and DR5 (4, 16, 18). Therefore, we assessed whether 3-Cl-AHPC exposure results in the induction of these proapoptotic proteins and if NF-κB activation is involved. 3-Cl-AHPC exposure indeed enhanced the expression of Fas, DR4, and DR5 mRNA levels as well as the proapoptotic Fas/TNFα–related RIP mRNA levels in the MDA-MB-468 and DU145 cells (Fig. 6*A*), but had no effect on caspase 8, TNFRp55, L32 and glyceraldehyde-3-phosphate dehydrogenase mRNA levels (Fig. 6*A*). Incubation with 3-Cl-AHPC increased the expression of Fas, DR4, and DR5 protein levels in MDA-MB-468 cells and DUI45 cells but enhanced the expression of RIP protein only in MDA-MB-468 cells (Fig. 6*B*). 3-Cl-AHPC had no effect on Fas ligand expression in either cell lines (data not shown). Expression of the IκBα dominant negative inhibited the 3-Cl-AHPC–mediated increase in the DR4 and DR5 levels, indicating that NFκ-B activation is required (Fig. 6*C*).

Discussion

The abilities of NF-κB to suppress cell death have been shown in a variety of systems (5–8, 40, 41). Activation of NF-κB by TNFα and numerous other stimuli has been shown to be necessary to protect the cells from the apoptosis cascade induced by these molecules (5, 6, 9, 42, 43). NF-κB suppression of apoptosis seems to be dependent on transcription because inhibitors of transcription as well as translation block the ability of this transcription factor to prevent cell death (6, 44). NF-κB–mediated induction of the expression of a number of proteins has been associated with apoptosis inhibition. These proteins include the cellular inhibitors of apoptosis (c-IAP), caspase 8-c-FLIP (Flice) inhibitor protein, the Bcl-2 family member BFL-1 (also known as AI), TNF receptor–associated factor-1 (TRAF1) and (TRAF2) and X1AP (45). These proteins inhibit apoptosis at multiple steps. Studies have shown that many of these factors inhibit the caspase cascade. The c-IAPs have been shown to directly bind and inhibit cellular caspases, such as caspase-3 and caspase-7, and also block the activation of procaspase-6 (46). Several investigators have suggested that c-IAP1 and c-IAP2 can also be recruited to TNFR1 signaling complex

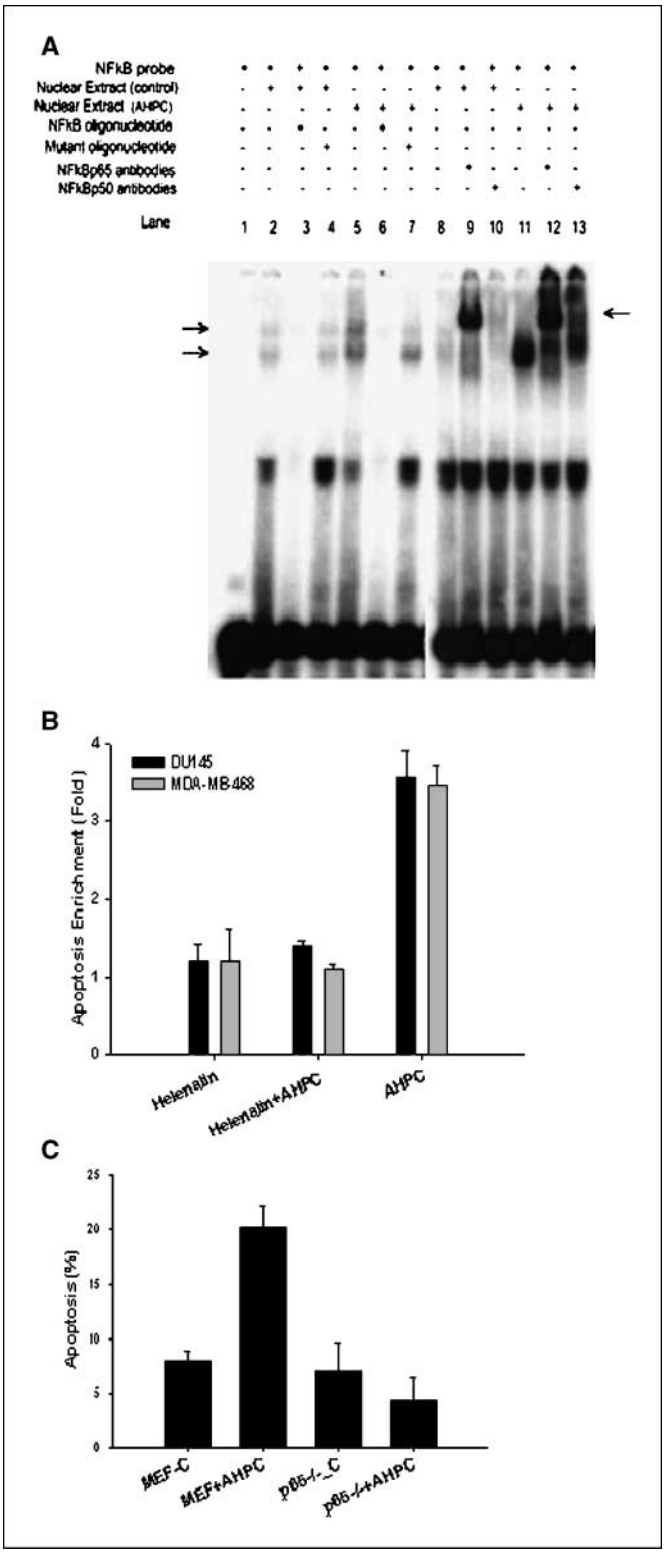

Figure 4. Exposure to 3-Cl-AHPC results in NF-κB p65 and p50 activation, which is required for 3-Cl-AHPC–mediated apoptosis. Cells were exposed to 1 μmol/L 3-Cl-AHPC for 24 hours. *A,* gel mobility shift assay demonstrating p65 and p50 activation. *Left arrows,* positions of p65 and p50 subunits. *Right arrow,* supershifted p65 band. *B,* quantification of helenalin inhibition of 3-Cl-AHPC induction of apoptosis. *C,* 3-Cl-AHPC induction of apoptosis in wild-type and p65−/− MEF cells. Apoptosis was assessed using an Apoptosis ELISA kit or acridine orange staining as described in Materials and Methods. *B* and *C, columns,* mean of three independent experiments; *bars,* SE.

through their interaction with TNFR2 and, thus, also inhibit TNF activation of caspase-8 (32, 47).

NF-κB enhanced expression of XIAP also contributes to NF-κB inhibition of caspase activity (48). XIAP has been shown to inhibit caspase-3 and caspase-7 through its IAP repeat (Bir) domain; in addition, this molecule has been shown to inhibit procaspase-9 activation through its Bir 3 domain (49, 50). We have found that exposure of cells to 3-Cl-AHPC results in activation of the p65 NF-κB subunit but decreased XIAP and c-IAP1 expression, thus inhibiting the ability of these molecules to block NF-κB activation of a proapoptotic pathway. In addition, the 3-Cl-AHPC–mediated decrease in XIAP levels seems to be dependent on NF-κB activation. NF-κB can also induce the expression of proteins that exert their antiapoptotic role via the mitochondrial-dependent pathway (45); these include the factors Bfl-1 and Bcl-X_L that inhibit apoptosis-mediated mitochondria depolarization (40). However, 3-Cl-AHPC exposure inhibits Bcl-X_L expression despite NF-κB activation.

Although the vast majority of reports have documented NF-κB as an antiapoptotic molecule, numerous studies are now emerging demonstrating the role of NF-κB in the induction of apoptosis (16–18, 51–61). A proapoptotic role for NF-κB has been shown in etoposide-induced apoptosis in human leukemia cell lines, dopamine-induced apoptosis in human breast carcinoma, and human epidermal cells as well as phorbol ester induction of apoptosis in T-cell hybridomas (4, 15, 46). NF-κB activates the transcription of vast number of genes, some of which encode for proteins that function as inducers of apoptosis (4, 15, 51). NF-κB enhances the expression of Fas, Fas ligand, and the death receptors DR4 and DR5, all of which play a role in this induction of apoptosis (4, 18). We have found that 3-Cl-AHPC enhances the expression of Fas as well as the DR4 and the DR5 death receptors and that expression of the IκBα dominant negative blocks this induction. Sun et al. (57) have previously shown that the retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid (CD437/AHPN) can enhance the expression of DR4 and DR5 in prostate carcinoma cells.

Activation of specific NF-κB subunits is necessary for the enhanced expression of these proapoptotic agents. Chen et al. (16) have found that in human breast carcinoma cells, activation of the NF-κB c-Rel subunit is essential for the enhanced expression of the DR4 and DR5 death receptors in these cells, and the induction of apoptosis and that the activation of p65 results in inhibition of DR4 and DR5 expression as well as tumor necrosis factor–related apoptosis-inducing ligand–mediated apoptosis. Ouaaz et al. (18), however, have found that p65 activation is necessary for the enhanced expression of the Fas death receptor. We have found that 3-Cl-AHPC activation of the NF-κB p65 subunit plays an essential role in 3-Cl-AHPC–mediated apoptosis. (*a*) Exposure of prostate and breast carcinoma cells to 3-Cl-AHPC results in p65 activation. (*b*) Inhibition of p65 activation utilizing the IκBα superrepressor inhibits 3-Cl-AHPC induction of apoptosis as well as 3-Cl-AHPC induction of proapoptotic molecules. (*c*) MEFs, which are p65−/− display resistance to 3-Cl-AHPC–mediated apoptosis. (*d*) The p65 activation inhibitor helenalin blocks 3-Cl-AHPC–mediated apoptosis. Interestingly, whereas expression of the IκBα dominant negative in both DU145 and MDA-MB-468 cells inhibited 3-Cl-AHPC–mediated apoptosis, it enhanced the basal apoptosis rate in the two cell lines. As suggested by numerous investigators, NF-κB can be antiapoptotic or proapoptotic depending on the cell type and the stimulus. Constitutive activation of NF-κB may very well play an antiapoptotic role in the breast and prostate carcinoma cells and, thus, the inhibition of constitutive NF-κB activation by the IκBα dominant negative may enhance the basal apoptotic rate in these cells.

3-Cl-APHC enhances the degradation of IκB through its stimulation of the IKK kinases in which IKKα and IKKβ are

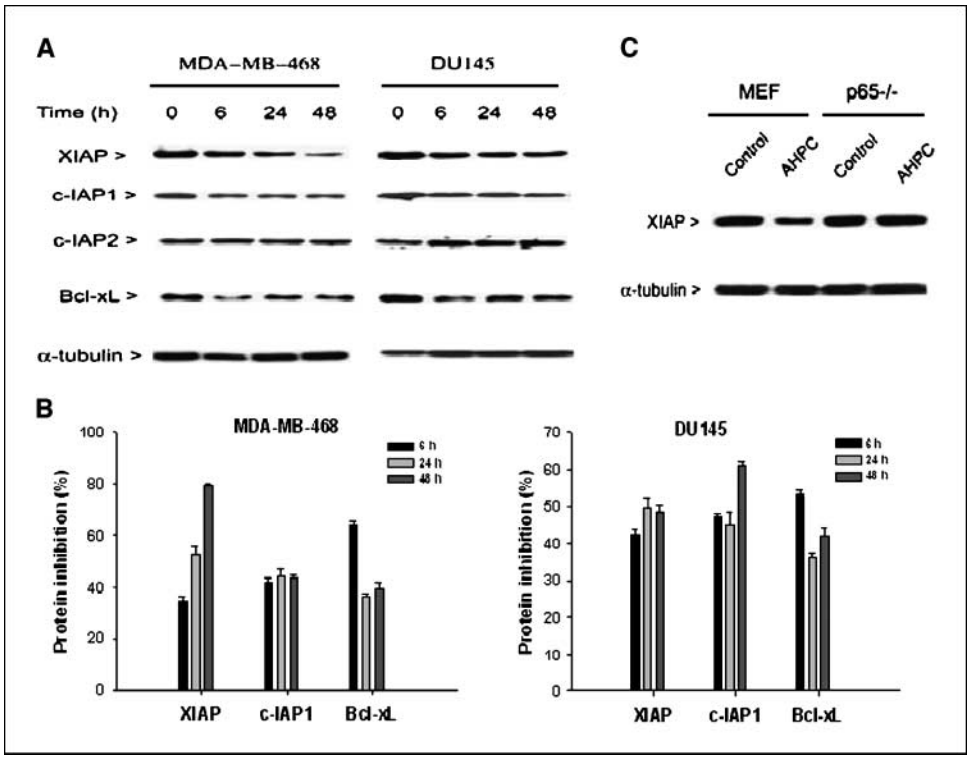

Figure 5. 3-Cl-AHPC–mediated decrease in antiapoptotic XIAP, c-IAP1, and Bcl-X_L levels. Cells were exposed to 1 μmol/L 3-Cl-AHPC for varying times. *A,* 3-Cl-AHPC–mediated decrease in XIAP, c-IAP1, and Bcl-X_L levels. *B,* quantification of XIAP, c-IAP1, and Bcl-X_L levels. Western blots were quantified by laser densitometry. *Columns,* mean of three independent experiments; *bars,* SE. *C,* 3-CL-AHPC does not decrease XIAP levels in p65−/− MEF cells.

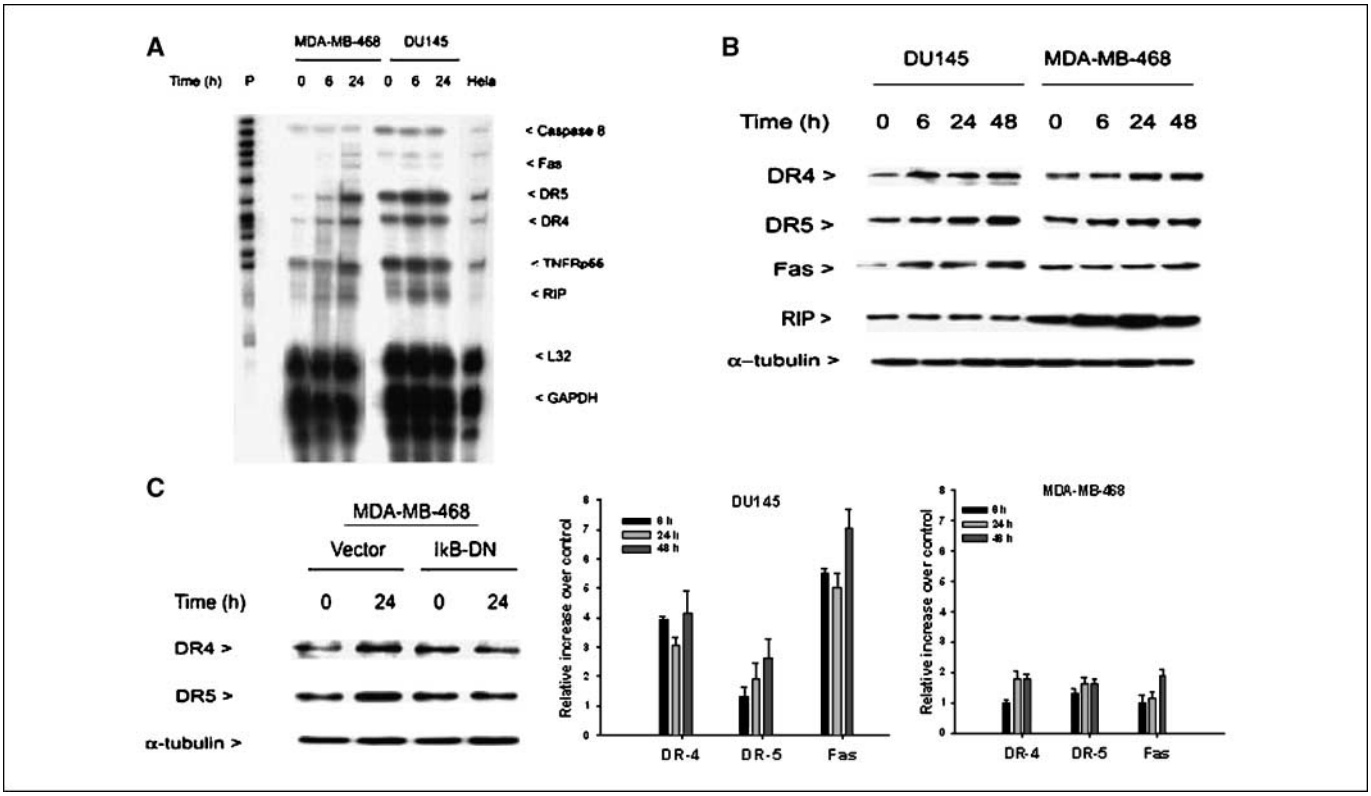

Figure 6. 3-Cl-AHPC enhances DR4, DR5, Fas, and Rip1 expression. Cells were exposed to 1 μmol/L 3-Cl-AHPC for varying times. *A,* 3-Cl-AHPC enhances Fas, DR4, DR5, and RIP mRNA levels. RNase protection assays were done as described in Materials and Methods. *B,* 3-Cl-AHPC enhances Fas, DR4, DR5, and RIP protein expression and quantification of DR4, DR5, and Fas levels. *Columns,* mean of three independent experiments; *bars,* SE. *C,* 3-Cl-AHPC does not increase DR4 and DR5 levels in MDA-MB-468 cells expressing a dominant-negative IκBα.

catalytic subunits and IKKγ serves a regulatory role. Activation of IKKα and IKKβ is a complex process requiring the dimerization of IKKα and IKKβ through their leucine zipper motifs followed by their association with IKKγ and the formation of a large complex (58–61). Stimulus-mediated activation of the IKK complex seems to require the IKKγ carboxyl terminus (59). Activation of the IKKα-IKKβ-IKKγ complex also requires the phosphorylation of IKKα or IKKβ at two conserved serines (62). Recent studies have shown that IKKα and IKKβ are not redundant (ref. 28 and references within). IKKβ activation is required for IKK activation by a number of proinflammatory stimuli, whereas IKKα is essential for IKK activation by a set of signals that do not affect the IKKβ subunit (28). In addition, not only do different stimuli activate IKKα and IKKβ, but their biological effects seem to be tissue type specific (28). We have found that exposure of breast and prostrate carcinoma cells to 3-Cl-AHPC activate IKKα with no effect on IKKβ. The exact mechanism involved is not clear. However, we found that 3-Cl-AHPC enhances the association between IKKα and HSP90β, which has been reported to enhance IKK activity (34). That this association plays a role in 3-Cl-AHPC activation of NF-κB is supported by our observation that the HSP90-specific inhibitor geldanamycin results in decreased IKKα and inhibited both 3-Cl-AHPC–mediated NF-κB activation and induction of apoptosis. Geldanamycin inhibition of IKKα and IKKβ activity has been shown to be dependent on its inhibition of HSP90 ATPase activity and not necessarily due to decreases in IKKα and IKKβ levels or interruption of the association between HSP90 and the IKKs (34).

Bayon et al. (63) have reported that the RAR antagonist 4-{3-[3-(1-adamantyl)]-4-[2-(methoxy)ethoxymethoxy]phenyl-1*E*-propen-3-oyl} benzoic acid (MX781) and the AHPN/CD437 analogue 4-{3-[3-(1-admantyl)-4-hydroxyphenyl]-1*E*-propen-1-yl}benzoic acid (CD2325) inhibited TNFα induction of both IKKα and IKKβ activities by directly interacting with these kinases. These investigators also reported that the natural RAR agonist *trans*-retinoic acid, the RAR panantagonist, and AHPN/CD437 analogue (*E*)-7-[3-(1-adamantyl)-4-methoxyphenyl]-3-methylocta-2, 4,6-trienoic acid (CD2366) and the RARβ, γ-selective antagonist and AHPN/CD437 analogue 4-[6-(1-adamantyl)-7-methoxyethoxymethoxy-2-naphthalenyl]benzoic acid (CD2665), all of which were evaluated at 6 μmol/L had no or minimal inhibitory effect on IKK activity induced by TNFα and were unable to inhibit the proliferation or induce apoptosis of prostate or lung carcinoma cells (63). In contrast to these studies, we have found that 3-Cl-AHPC stimulates IKK activity.

The introduction of a the 3-Cl ortho to the diaryl bonds resulted in the orientation of the 1-adamantyl group outside of the plane of the aromatic rings (64); this, in turn, resulted in the poor activation of RARγ by 3-Cl-AHPC and its inability to disassociate RARγ from its bound corepressors (64). The mechanism by which 3-Cl-AHPC and CD437 induces apoptosis in a number of cell types remains undefined. Numerous pathways have been implicated and gene-targeting experiments have suggested that certain pathways play specific roles in CD437-mediated apoptosis in specific cell types (57). These pathways include the mitogen-activated kinase pathway with the subsequent activation of c-Jun-NH_2-kinase (JNK) and p38 kinases, and

inhibition of the MRP-I phosphatase, which plays a role in the inactivation of JNK (65). Recently, Li et al. (66) and Lin et al. (67) have reported that exposure of cells to CD437/AHPN and 3-Cl-AHPC results in the translocation of the transcription factor TR3 from the nucleus to the mitochondria, with the binding of TR3 to Bcl-2 and the conversion of Bcl-2 to a proapoptotic molecule. There seems to be no role for the retinoid nuclear receptors in 3-Cl-AHPC–mediated apoptosis because 3-Cl-AHPC does not bind nor activate the retinoid X receptors and is an extremely poor activator of the RARs (20, 21). We have found that 3-Cl-AHPC requires NF-κB activation for maximal apoptosis induction. NF-κB activation in the presence of 3-Cl-AHPC results in the enhanced expression of a number of proapoptotic mediators and the inhibition of expression of a number of NF-κB–associated inhibitors of apoptosis. The exact mechanism by which 3-Cl-AHPC modulates the levels of these proteins remains to be discerned.

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