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Bax and Bak genes are essential for maximum apoptotic response by curcumin, a polyphenolic compound and cancer chemopreventive agent derived from turmeric, *Curcuma longa*

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Curcumin, an active ingredient of turmeric (*Curcuma longa*), inhibits proliferation and induces apoptosis in cancer cells, but the sequence of events leading to cell death is poorly defined. The objective of this study was to examine the molecular mechanisms by which multidomain pro-apoptotic Bcl-2 family members Bax and Bak regulate curcumin-induced apoptosis using mouse embryonic fibroblasts (MEFs) deficient in Bax, Bak or both genes. Curcumin treatment resulted an increase in the protein levels of both Bax and Bak, and mitochondrial translocation and activation of Bax in MEFs to trigger drop in mitochondrial membrane potential, cytosolic release of apoptogenic molecules [cytochrome c and second mitochondria-derived activator of caspases (Smac)/direct inhibitor of apoptosis protein-binding protein with low isoelectric point], activation of caspase-9 and caspase-3 and ultimately apoptosis. Furthermore, MEFs derived from Bax and Bak double-knockout (DKO) mice exhibited even greater protection against curcumin-induced release of cytochrome c and Smac, activation of caspase-3 and caspase-9 and induction of apoptosis compared with wild-type MEFs or single-knockout $Bax^{-/-}$ or $Bak^{-/-}$ MEFs. Interestingly, curcumin treatment also caused an increase in the protein level of apoptosis protease-activating factor-1 in wild-type MEFs. Smac N7 peptide enhanced curcumin-induced apoptosis, whereas Smac siRNA inhibited the effects of curcumin on apoptosis. Mature form of Smac sensitized Bax and Bak DKO MEFs to undergo apoptosis by acting downstream of mitochondria. The present study demonstrates the role of Bax and Bak as a critical regulator of curcumin-induced apoptosis and over-expression of Smac as interventional approaches to deal with Bax- and/or Bak-deficient chemoresistant cancers for curcumin-based therapy.

Introduction

Epidemiological data support the concept that naturally occurring compounds in the human diet are devoid of toxicity and have numerous long lasting beneficial effects on human health (1). Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-hepatadiene-3,5-dione; diferuloylmethane], a major constituent of turmeric derived from the rhizomes of *Curcuma spp*., has been reported to have several pharmacological effects including anti-tumor, anti-inflammatory and antioxidant properties (2–4). It increases the level of glutathione-*S*-transferase and, thus, up-regulates the synthesis of glutathione (2,5). Other beneficial effects of curcumin include wound-healing, antiviral, anti-infectious and anti-amyloidogenic properties, suggesting its use for treatment of Alzheimer's disease (6). It is used as a flavoring and coloring agent, as a food preservative and also has been used in Ayurvedic medicine for over 6000 years. Crude curcumin has a natural yellow hue and its components include curcumin, demethoxycurcumin and bisdemethoxycurcumin. Oral administration of curcumin has been shown to inhibit leukemia and solid tumors including breast, prostate, skin, colon, stomach, duodenum, head and neck and soft palate, through induction of apoptosis (7–13). However, the molecular mechanisms by which it inhibits growth and induces apoptosis in cancer cells are not well understood.

Defect in apoptosis may contribute to tumor progression and treatment resistance. Apoptosis signaling may be disrupted by deregulated expression and/or function of anti-apoptotic or pro-apoptotic molecules. Bcl-2 family members are important regulators of apoptosis that include anti-apoptotic (Bcl-2, Bcl-X_L and Mcl-1), pro-apoptotic (Bax and Bak) and the BH-3 domain-only (Bim, Bid and Bik) proteins. Bax and Bak are multidomain proteins that function as an obligate gateway for the activation of apoptosis via the mitochondrial and endoplasmic reticulum pathway (14–16). In contrast to the BH-3-only proteins, which function as transducers of the apoptotic signals upstream of mitochondria, Bax and Bak also contain BH-1 and BH-2 domains and function at the outer mitochondrial membrane (OMM) to release holocytochrome c in response to diverse stimuli (17). Consequently, we and others have shown that mouse embryonic fibroblasts (MEFs) lacking both Bax and Bak exhibited marked resistance to diverse pro-apoptotic insults, and loss of either Bax or Bak alone exerts no measurable protective effects (15,16). Furthermore, mice deleted for either Bax or Bak alone are viable, showing either defects in only a few discrete lineage (in the case of Bax) or no defects (in the case of Bak) (18,19). In contrast, mice lacking both Bax and Bak die in early embryogenesis due to failure of apoptosis in multiple developing tissues (19). Recently, it has been shown that Bax deficiency renders cancer cells resistant to several anticancer drugs acting through the mitochondria or endoplasmic reticulum stress (20,21). Overall, these findings suggest that Bax and Bak are critical for apoptosis induction.

Anticancer drugs or irradiation induce the release of mitochondrial proteins such as cytochrome c, second mitochondria-derived activator of caspases (Smac)/direct inhibitor of apoptosis protein (IAP)-binding protein with low isoelectric point (DIABLO), apoptosis-inducing factor and endonucleases G. Cytochrome c together with apoptosis protease-activating factor-1 (Apaf-1) and procaspase-9 forms the apoptosome complex (22,23). Caspase-9 subsequently activates caspase-3 that can cleave several caspase substrates leading to apoptosis (24). Smac/DIABLO contains an N-terminal 55-amino acid mitochondrial import sequence (25,26). Once released into the cytosol, Smac docks to IAPs within the baculovirus IAP repeat domains via an N-terminal motif, thereby eliminating the inhibitory effects of IAPs on caspase-3, caspase-7 and caspase-9 (27). In addition, the interaction of Smac with IAPs results in a rapid ubiquitination and subsequent degradation of released Smac, which is mediated by the ubiquitin-protein ligase (E3) function of some IAPs (25,28). Recent studies have shown that mitochondrial Smac release is suppressed by Akt, Bcl-2 and Bcl-X_L, but promoted by Bax, Bad and Bid (16,25,29). Independent of caspases, apoptosis-inducing factor and endonuclease G can induce DNA fragmentation once they are released from the mitochondria (30,31).

The objective of our study was to examine the molecular mechanisms by which Bax and Bak genes regulate apoptosis induction by curcumin in MEFs. We observed that curcumin directly altered the multidomain proteins Bax and Bak, resulting in oligomerization and activation. MEFs

Abbreviations: Apaf-1, apoptosis protease-activating factor-1; DAPI, 4′-6-Diamidino-2-phenylindole; DIABLO, direct inhibitor of apoptosis protein-binding protein with low isoelectric point; DKO, double knockout; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; IAP, inhibitor of apoptosis protein; MEF, mouse embryonic fibroblast; OMM, outer mitochondrial membrane; PARP, poly-ADP ribose polymerase; PBS, phosphate-buffered saline; Smac, second mitochondria-derived activator of caspase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

lacking Bax and Bak were resistant to curcumin-induced cytochrome c and Smac/DIOABLO release and apoptosis, suggesting that these proteins were required for curcumin-induced apoptosis.

Materials and methods

Reagents

Antibodies against Bcl-2 (SC-7382), Bax (SC-23959 for immunoprecipitation and western blotting and SC-493 for immunohistochemistry), Bak (SC-7873) and β-actin (SC-8432) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cytochrome c (Cat. no. 556433), anti-Smac/DIABLO (Cat. no. 612245), fluorescein isothiocyanate (FITC)-conjugated anti-active caspase-3 (Cat. no. 559341) and FITC-conjugated anti-poly-ADP ribose polymerase (PARP) (Cat. no. 558576) antibodies were purchased from BD Biosciences/PharMingen (San Diego, CA). JC-1 was purchased from Invitrogen/Molecular Probes (Eugene, OR). Enhanced chemiluminescence western blot detection reagents were from Amersham Life Sciences (Arlington Heights, IL). Smac siRNA and control plasmids were purchased from Imgenex (San Diego, CA). Terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling assay kit and caspase-3 activity kit were purchased from EMD Biosciences/Calbiochem (San Diego, CA). Curcumin was purchased from LKT Laboratories (St Paul, MN). Smac N7 peptide (H-AVPIAQK-P-RQI-KIWFQNRRMKWKK-OH) and control peptide (H-MKSDFYF-P-RQI-KIWFQNRRMKWKK-OH) were modified to be cell permeable by linking the lysine C-terminal to the arginine of *Antennapedia homeodomain* 16mer peptide (underlined) via a proline linker.

Cell culture

Primary MEFs derived from wild-type, Bax knockout ($Bax^{-/-}$), Bak knockout ($Bak^{-/-}$) and Bax–Bak double-knockout (DKO) mice and immortalized by transfection with a plasmid containing SV40 genomic DNA were generously provided by Dr Stanley J.Korsmeyer (Dana-Farber Cancer Institute, Boston, MA) and Dr Craig Thompson (University of Pennsylvania, Philadelphia, PA). These MEFs were previously used by us (16). MEFs were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 0.1 mM non-essential amino acids, 0.1 μM 2-mercaptoethanol and 1% antibiotic–antimycotic (Invitrogen). Normal human prostate epithelial cell line PrEC and normal human bronchial epithelial cell line Beas2B were maintained in PrGM complete medium (Cambrex, Walkersville, MD) and LHC-9 medium (Biosource, Camarillo, CA), respectively. Each cell line was maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

XTT assay

MEFs (1×10^4 in 100 μl culture medium per well) were seeded in 96-well plates (flat bottom), treated with or without curcumin and incubated for various time points at 37°C and 5% CO_2. Before the end of the experiment, 50 μl XTT (sodium 3′[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate)-labeling mixture (final concentration, 125 μM sodium XTT and 25 μM N-methyl dibenzopyrazine methyl sulfate) per well was added and plates were incubated for a further 4 h at 37°C and 5% CO_2. The spectrophotometric absorbance of the sample was measured using a microtitre plate (ELISA) reader. The wavelength to measure absorbance of the formazan product was 450 nm, and the reference wavelength was 650 nm.

Transfection

MEFs were plated in 60 mm dishes in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin–streptomycin mixture at a density of 1×10^6 cells per dish. The next day transfection mixtures were prepared. Cells were transfected with expression constructs encoding Smac/DIABLO full-length (pCDNA3-Smac-Flag), Δ55-Smac/DIABLO mature (pCDNA3-Δ55-Smac-Flag or the corresponding empty vector (pCDNA3) in the presence of an expression vector pCMV-LacZ (Invitrogen Life Technologies, Carlsbad, CA) expressing β-galactosidase. For each transfection, 2 μg of DNA was diluted into 50 μl of medium without serum. After the addition of 3 μl of LipofectAMINE (Invitrogen Life Technologies) into 50 μl Opti-MEM medium, the transfection mixture was incubated for 10 min at room temperature. Cells were washed with serum-free medium, the transfection mixture was added and cultures were incubated for 24 h in the incubator. The next day, culture medium was replaced with fresh RPMI 1640 containing 10% fetal bovine serum and 1% penicillin–streptomycin mixture and curcumin was added. At the end of incubation, cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested for analyses of apoptosis.

Measurement of apoptosis

Apoptosis was measured by the terminal deoxynucleotidyl transferase-mediated nick end-labeling method, which examines DNA strand breaks during apoptosis. Briefly, 1×10^5 cells were treated with curcumin at the indicated doses for various time points at 37°C. Thereafter, cells were washed with PBS, air-dried, fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After washing, cells were incubated with reaction mixture for 60 min at 37°C. Stained cells were mounted and analyzed under a fluorescence microscope (Olympus America, Center Valley, PA) using a SPOT camera (SPOT Diagnostic Instruments, Sterling Heights, MI). In some cases, the data were confirmed by staining cells with *4*-6-diamidino-2-phenylindol-dihydrochloride as described previously (16). Cells were counted by the counter 'blinded' to sample identity to avoid experimental bias.

Cellular fractionation

Preparation of mitochondrial and cytosolic fractions were performed by re-suspending cells in ice-cold buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 μg/ml phenylmethylsulfonyl fluoride, 8 μg/ml aprotinin and 2 μg/ml leupeptin, pH 7.4) (26). Cells were broken with 20 strokes of a Dounce homogenizer on ice, and the suspension was centrifuged at 2000 *g* for 10 min to remove nuclei. The supernatant was spun at 10 000 *g* for 25 min at 4°C, and the resulting mitochondrial pellets were layered over a 1–2 mM sucrose step gradient [10 mM Tris (pH 7.6), 5 mM EDTA, 2 mM DTT and 1× protease inhibitor cocktail) and centrifuged at 4°C for 30 min at 22 000 *g*. Mitochondria were collected at the 1–1.5 M interphase. The supernatant from the previous step was spun to obtain the cytoplasmic S100 fraction.

Western blot analysis

Cell pellets were lysed in RIPA buffer containing 1× protease inhibitor cocktail, and protein concentrations were determined using the Bradford assay (Bio-Rad, Philadelphia, PA). Cell lysates (20–50 μg) were electrophoresed in 12.5% sodium dodecyl sulfate–polyacrylamide gels and then transferred onto nitrocellulose membranes. After blotting in 5% non-fat dry milk in Tris-buffered saline, the membranes were incubated with primary antibodies at 1:1000 dilution in Tris-buffered saline–Tween 20 overnight at 4°C, and then secondary antibodies conjugated with horseradish peroxidase at 1:5000 dilution in Tris-buffered saline–Tween 20 for 1 h at room temperature. Protein bands were visualized on X-ray film using an enhanced chemiluminescence system.

Analysis of Bax conformational change

Wild-type MEFs were treated with 20 μM curcumin for 4 h and lysed using a solution containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 1% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate and protease inhibitor cocktail. Aliquots containing 1 mg lysate protein in 0.5 ml lysis buffer were incubated overnight at 4°C with 2 μg anti-Bax monoclonal antibody 6A7. Protein G-agarose beads (50 μl) were then added to each sample, and the incubation was continued for 2 h at 4°C. The immunoprecipitated complexes were washed thrice with lysis buffer and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblotting using polyclonal anti-Bax antibody.

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

Mitochondrial energization was determined by retention of JC-1 dye (Molecular Probes) as we described earlier (16,32). Briefly, drug-treated cells (5×10^5) were loaded with JC-1 dye (1 μg/ml) during the last 30 min of incubation at 37°C in a 5% CO_2 incubator. Cells were washed in PBS twice. Fluorescence was monitored in a fluorimeter using 570 nm excitation/595 nm emission for the J-aggregate of JC-1. $\Delta\Psi_m$ was calculated as a ratio of the fluorescence of J-aggregate (aqueous phase) and monomer (membrane-bound) forms of JC-1. Alternatively, mitochondrial membrane potential was also measured by fluorescence microscopy.

Immunocytochemistry

For Bax translocation, wild-type MEFs were cultured on coverslips and treated with curcumin (20 μl) or dimethyl sulfoxide (DMSO) for 12 h. After staining with MitoTracker Red, MEFs were incubated with anti-Bax antibody (1:1000 dilution) for 2 h followed by incubation with FITC-conjugated secondary antibody (1:1000 dilution) for 1 h. MEFs were washed with PBS, stained with 4′-6-Diamidino-2-phenylindole (DAPI) (1 μg/ml) for 1 h at room temperature, and examined under a fluorescence microscope.

For active caspase-3 and cleaved PARP, MEFs were grown on fibronectin-coated coverslips (Beckton Dickinson, Bedford, MA), washed in PBS and fixed for 15 min in 4% paraformaldehyde. Cells were permeabilized in 0.1% Triton X-100, washed and blocked in 10% normal goat serum. MEFs were incubated with either anti-active caspase-3–FITC antibody or anti-PARP–FITC antibody (1:200) for 4 h at 4°C. Cells were then washed and incubated with DAPI (1 μg/ml) for 1 h at room temperature. Cells were washed and coverslips were mounted using Vectashield (Vector Laboratories, Burlington, CA). Isotype-specific negative controls were included with each staining.

Statistical analysis

Analysis of variance was used to test the significance of differences in measured variables between control and treated groups followed by Bonferroni's test for multiple comparisons. Statistical significance was determined at the 0.05 level.

Results

Curcumin induced apoptosis in wild-type MEFs but not in normal human lung and prostate epithelial cells

We first sought to examine the apoptosis-inducing potential of curcumin in SV40-transformed MEFs and compare its effect with human normal lung and prostate epithelial cells. SV40-transformed MEFs become immortalized, a phenomenon commonly observed in cancer cells. Curcumin induced apoptosis in MEFs in a dose- and time-dependent manner (Figure 1A and B). By comparison, although curcumin was effective in inducing apoptosis in MEFs, it had no effect on human normal lung epithelial Beas2b and prostate epithelial PrEC cells (Figure 1C and D). These data suggest that curcumin induces apotosis in SV40-transformed MEFs, but had no effect on normal epithelial cells.

Curcumin-induced apoptosis in wild-type MEFs was associated with induction of Bax and Bak

Previous studies have shown that pro-apoptotic multidomain proteins Bax and Bak are induced in cells treated with curcumin, but the significance of induction of these proteins in curcumin-induced apoptosis has not been examined. Several studies including ours have shown that Bax and Bak are important mediators of cell death as Bax and Bak DKO cells were resistant to multiple apoptotic inducers (16,33,34). In most cells Bax is normally localized in the cytosol, whereas Bak is mostly localized in the OMM and remains inactive in healthy cells. BH-3-only Bcl-2 proteins such as tBid and Bim, upon activation or up-regulation by apoptotic stimuli, cause translocation of Bax to the OMM and subsequent confirmational changes and oligomerization of Bax and Bak (15,35). Furthermore, Apaf-1 is required for the formation of apoptosomes that activate caspase-9 leading to apoptosis (24). We therefore examined the consequences of inducing Apaf-1, Bax and Bak in SV40-transformed MEFs. Curcumin induced Apaf-1, Bax, Bak and Apaf-1 in MEFs in a dose-dependent manner (Figure 2A).

The Bax activation requires N-terminal exposure, which results in translocation and oligomerization of Bax on the OMM (36,37). We therefore examined whether curcumin-induced activation of Bax to initiate the process of apoptosis by different but complementary approaches. First, we determined whether curcumin treatment causes conformational change of Bax. The conformational change was assessed by immunoprecipitation of Bax using a monoclonal antibody (6A7). This antibody recognizes an epitope at the N-terminus of the protein, which becomes exposed only after a change in conformation of Bax. The immunoprecipated complex was then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblotting using anti-Bax polyclonal antibody. Curcumin treatment caused a change in conformation of Bax at 4 h after exposure (Figure 2B). Activation of Bax upon curcumin treatment was confirmed by mitochondrial fractionation and immunochemical studies using wild-type MEFs. Although Bax was absent in the mitochondrial fraction of untreated control cells, curcumin induced translocation of Bax into the mitochondria in a time-dependent manner (Figure 2C). Bax translocation in the mitochondria was detected as early as 4 h and increased over time with maximum translocation at 12 h. We further confirmed the activation/translocation of Bax by immunohistochemistry (Figure 2D). In DMSO-treated control MEFs, the Bax staining was restricted to the cytosol. On the other hand, the mitochondria in curcumin-treated MEFs were stained yellow-orange due to merge of green fluorescence (Bax immunostaining) and red fluorescence (mitotracker red staining), indicating translocation of Bax from cytosol to the mitochondria. To the best of our knowledge, our study is the first published report to indicate Bax activation in curcumin-treated cells.

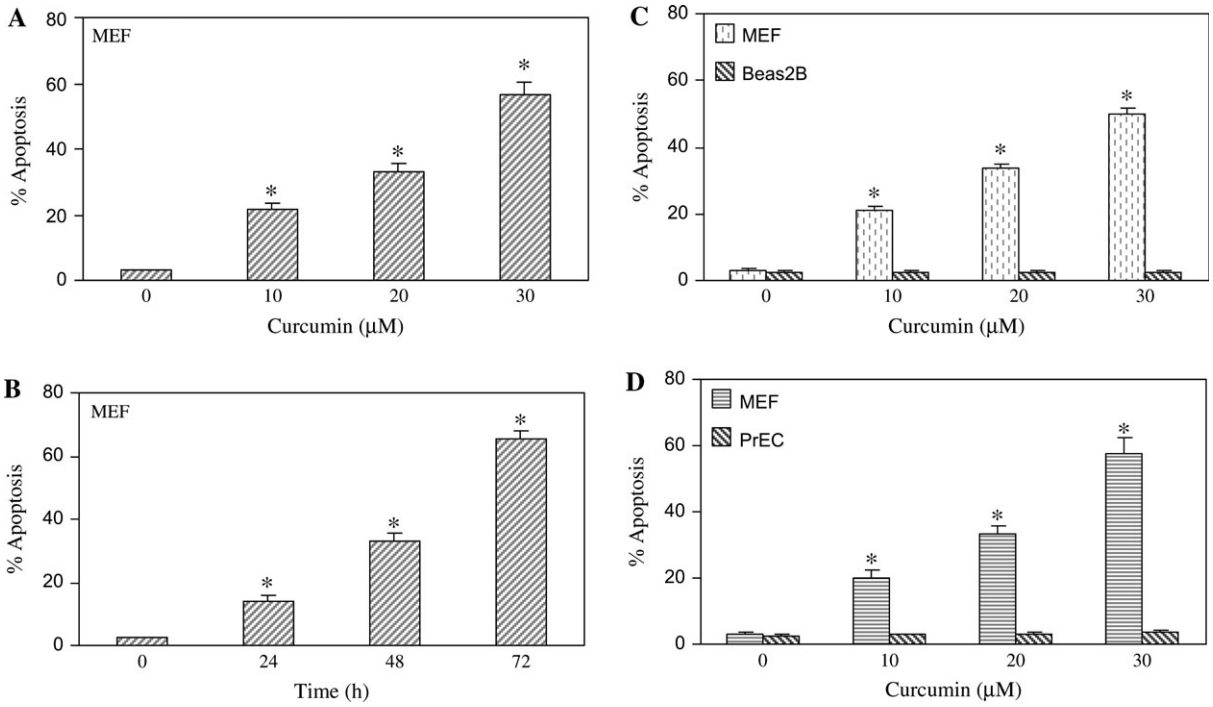

Fig. 1. Effects of curcumin on SV40-transformed MEFs, human normal lung epithelial Beas2B and normal prostate epithelial PrEC cells. (**A**) MEFs were treated with various doses of curcumin (0–30 μM) for 48 h, and apoptosis was measured by DAPI staining. Data represent mean ± SE. *Significantly different from respective control. (**B**) MEFs were treated with curcumin (20 μM) for 48 h, and apoptosis was measured by DAPI staining. (**C**) MEFs and human normal lung epithelial (Beas2B) cells were treated with various doses of curcumin (0–30 μM) for 48 h, and apoptosis was measured by DAPI staining. (**D**) MEFs and human normal prostate epithelial (PrEC) cells were treated with various doses of curcumin (0–30 μM) for 48 h, and apoptosis was measured by DAPI staining.

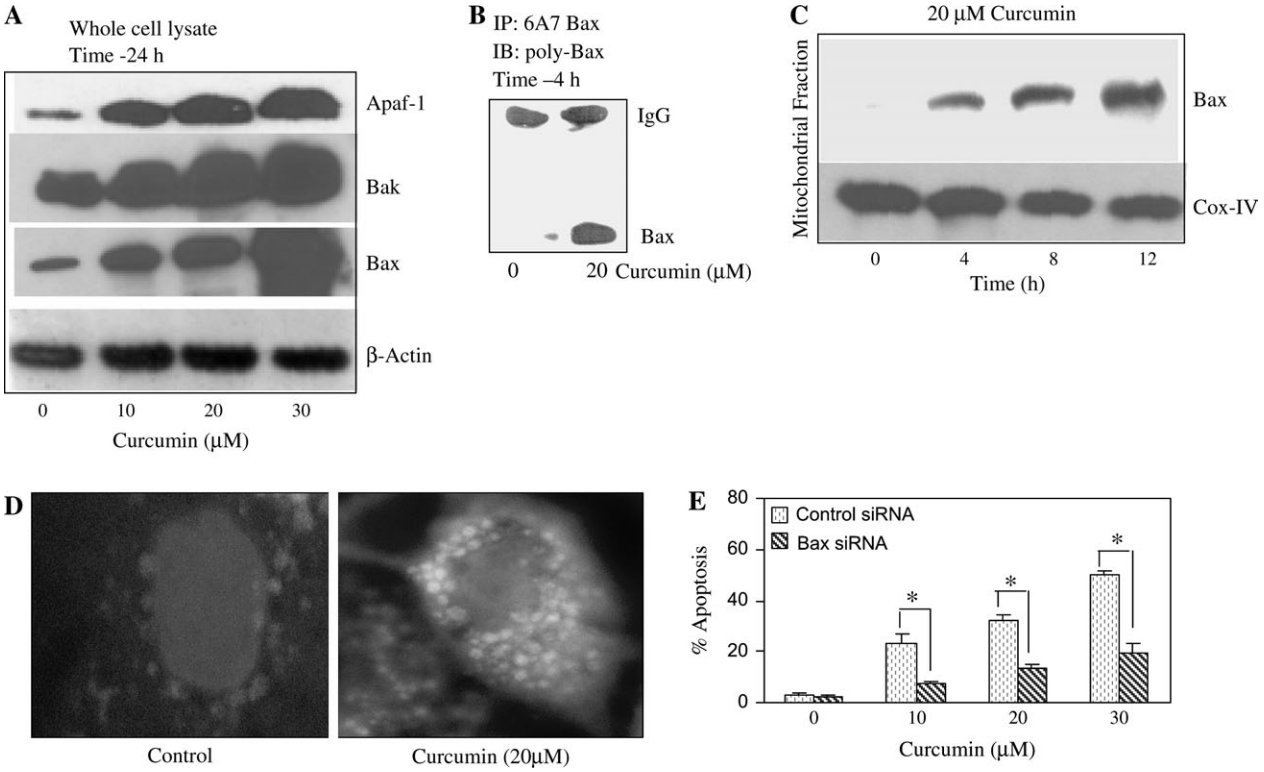

Fig. 2. Effects of curcumin on the induction of Apaf-1, Bax and Bak proteins, and on the activation of Bax in wild-type MEFs. (**A**) MEFs were treated with or without curcumin (0–30 μM) for 48 h. The expressions of Apf-1, Bak and Bax were examined by the western blotting. β-Actin was used as a loading control. (**B**) Analysis of conformational change of Bax using lysates from wild-type MEFs treated with curcumin (20 μM) for 4 h. Bax was immunoprecipitated from equal amounts of lysates proteins using anti-Bax monoclonal antibody 6A7. Immunoprecipitated complexes were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblotting using anti-Bax polyclonal antibody. (**C**) Translocation of Bax to mitochondria. Wild-type MEFs were treated with curcumin (20 μM) for 0, 4, 8 and 12 h. Mitochondrial fractions were prepared as described in Materials and Methods. Mitochondrial proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblotting using anti-Bax polyclonal antibody. Anti-Cox-IV antibody was used as loading control. (**D**) Immunohistochemistry for analysis of Bax localization. Wild-type MEFs were treated for 12 h with DMSO or 20 μl curcumin. Cells were then stained with anti-Bax antibody (green fluorescence), Mitotracker Red (red fluorescence) and DAPI (blue fluorescence). Merged images are shown, which indicate yellow-orange staining of mitochondria in curcumin-treated MEFs due to merge of green and red fluorescence. Mitochondria in DMSO-treated control were stained red. (**E**) Bax siRNA inhibits curcumin-induced apoptosis. Wild-type MEFs were transiently transfected with either control siRNA or Bax siRNA plasmids and treated with various doses of curcumin (0–30 μM). Apoptosis was measured by DAPI staining.

We next examined the contribution of Bax in curcumin-induced apoptosis by inhibiting Bax expression by RNAi technology (Figure 2E). As before, curcumin induced apoptosis in wild-type MEFs in a dose-dependent manner. Inhibition of Bax by siRNA inhibited >50% Bax-induced apoptosis. These data suggest that Bax plays an important role in cell death caused by curcumin.

Curcumin induces apoptosis in wild-type, Bax$^{-/-}$ and Bak$^{-/-}$ MEFs, but not in DKO MEFs

BAX and BAK are essential regulators of pro-apoptotic signaling, and the disruption of apoptosis is linked to the development of cancer (16,38). Bax and Bak genes play a major role in apoptosis by regulating the release of mitochondrial proteins such as cytochrome c and Smac/DIABLO (15,16). We have shown previously that Bax$^{-/-}$ and Bak$^{-/-}$ DKO MEFs were completely resistant to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis (16). To investigate the role of BAX and BAK in curcumin-induced apoptosis, MEFs from wild-type, BAX$^{-/-}$, BAK$^{-/-}$ or BAK and BAK-deficient (DKO) mice were used. As shown in Figure 3, curcumin induced apoptosis in wild-type MEFs at 24 h, and these effects of curcumin on apoptosis were further enhanced at 48 h. Curcumin-induced apoptosis was significantly inhibited in Bax$^{-/-}$ MEFs or Bak$^{-/-}$ MEFs compared with wild-type MEFs at 24 and 48 h. Interestingly, depletion of Bax and Bak genes completely inhibited curcumin-induced apoptosis in Bax$^{-/-}$ and Bak$^{-/-}$ DKO MEFs. Resistant of DKO MEFs to curcumin-induced apoptosis was confirmed by microscopic analysis of cells with condensed nuclei following staining with DAPI. Representative microscopic images for DAPI staining in wild-type and DKO MEFs following a 24 h exposure to DMSO (control) or 20 μM curcumin are shown in Figure 3B. Consistent with the results of the DNA fragmentation assay (Figure 3A), apoptotic cells with condensed nuclei were visible in wild-type MEFs cultured in the presence of curcumin. On the other hand, the Bax and Bak DKO MEFs were significantly more resistant to nuclear condensation by curcumin when compared with wild-type MEFs. These data suggest that deletion of either Bax or Bak did not completely block curcumin-induced apoptosis, and both Bax and Bak genes are required for inducing cell death by curcumin.

Curcumin causes drop in mitochondrial membrane potential, release of cytochrome c and Smac/DIABLO to cytosol in wild-type, Bax$^{-/-}$ and Bak$^{-/-}$ MEFs, but not in DKO MEFs

During apoptosis, engagement of the mitochondrial pathway involves the permeabilization of the OMM, which leads to the release of cytochrome c and other apoptogenic proteins such as Smac/DIABLO, apoptosis-inducing factor, EndoG, Omi/HtraA2 and DDP/TIMM8a (39–42). OMM permeabilization depends on activation, translocation and oligomerization of multidomain Bcl-2 family proteins such as Bax or Bak. Mitochondria fulfill a wide array of functions dedicated to the energetic metabolism as well as the control of cell death (38). These functions imply that mitochondria can be activated by a variety of signals and can integrate them to trigger a process called mitochondrial

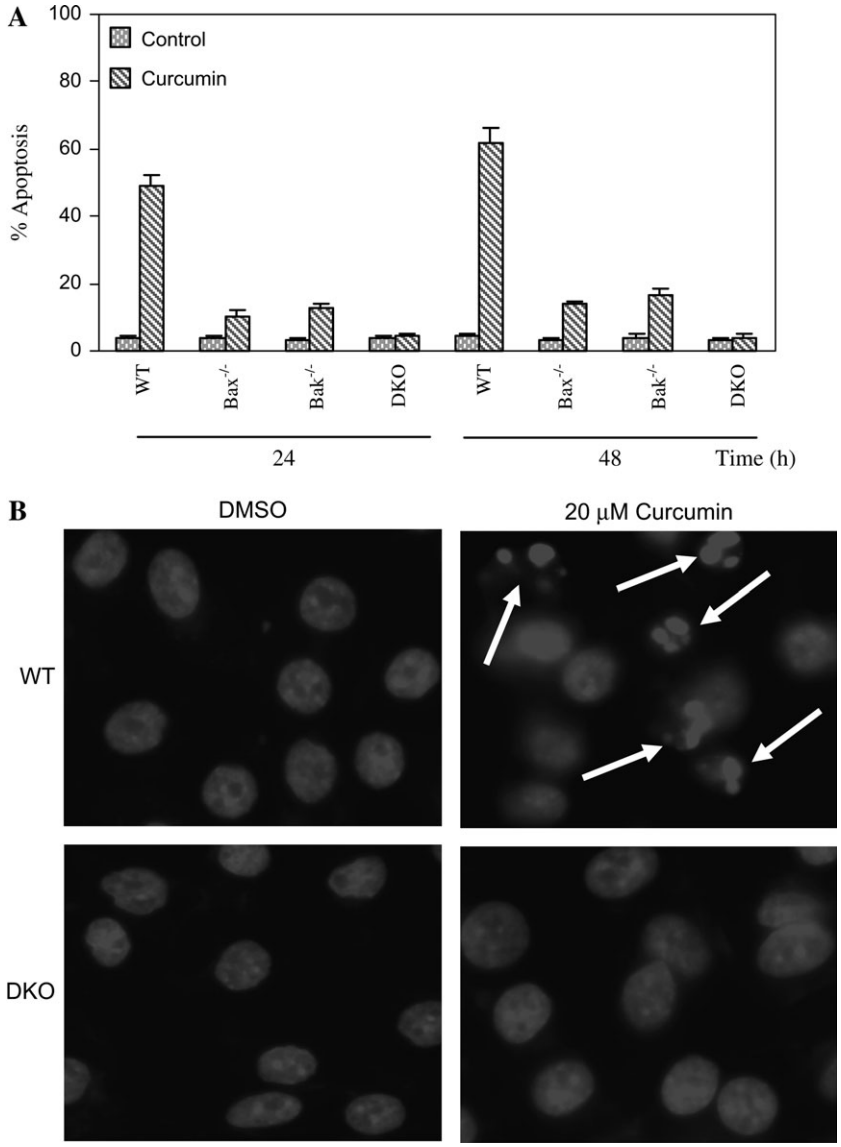

Fig. 3. MEFs derived from Bax and Bak DKO mice were significantly more resistant to curcumin-induced apoptosis. (**A**) Wild-type, $Bax^{-/-}$, $Bak^{-/-}$ and $Bax^{-/-}$ and $Bak^{-/-}$ DKO MEFs were treated with or without 20 μM curcumin for 24 and 48 h, and apoptosis was measured. (**B**) Representative DAPI staining for detection of apoptotic cells with condensed nuclei following a 24 h exposure of wild-type and DKO MEFs to DMSO (control) or 20 μM curcumin. Arrows represent apoptotic cells with condensed nuclei.

membrane permeabilization, which induces the ultimate events of apoptosis. We therefore assessed the loss of mitochondrial membrane potential in MEFs. As shown in Figure 4A, curcumin induced a massive drop in mitochondrial membrane potential in wild-type MEFs at 24 and 48 h. Curcumin had little but significant effect in dropping mitochondrial membrane potential in $Bax^{-/-}$ or $Bak^{-/-}$ MEFs. By comparison, mitochondrial membrane potential did not change in DKO MEFs. The drop in mitochondrial membrane potential in wild-type and single-knockout MEFs was slightly higher at 48 h compared with 24 h of curcumin treatment.

Loss of membrane potential leads to opening of the permeability transition pore leaking the inner components (e.g. cytochrome c and Smac/DIABLO) into cytosol, which provide the executing signals for apoptosis. We therefore measured the release of cytochrome c and Smac/DIABLO from mitochondria to cytosol in MEFs treated with curcumin. Treatment of wild-type MEFs with curcumin resulted in the release of cytochrome c and Smac/DIABLO from mitochondria to cytosol (Figure 4B). Curcumin also caused the release of cytochrome c and Smac/DIABLO in $Bax^{-/-}$ and $Bak^{-/-}$ MEFs, but significantly lower than wild-type MEFs. Interestingly, curcumin had no effect on the release of mitochondrial cytochrome c and Smac/DIABLO in DKO MEFs.

Our previous study has demonstrated that over-expression of Smac/DIABLO sensitized cells by regulating IAPs (16). Since curcumin induced Smac/DIABLO release from mitochondria to cytosol, we examined whether Smac/DIABLO regulates curcumin-induced apoptosis in MEFs. Control peptide had no effect on apoptosis (Figure 4C). Pre-treatment of MEFs with Smac N7 peptide enhanced curcumin-induced apoptosis in a dose-dependent manner. We have confirmed the role of Smac/DIABLO, in another approach, by inhibiting its expression with plasmid expressing Smac siRNA. Inhibition of Smac expression by Smac siRNA inhibited curcumin-induced apoptosis (Figure 4D). These data suggest that mitochondrial protein Smac/DIABLO plays a crucial role in curcumin-induced apoptosis in MEFs.

DKO MEFs were completely resistant to activation of caspase-3 and capase-9, and cleavage of PARP by curcumin

Apoptotic signal transmission to the mitochondria results in the efflux of a number of potential apoptotic regulators to the cytosol that trigger

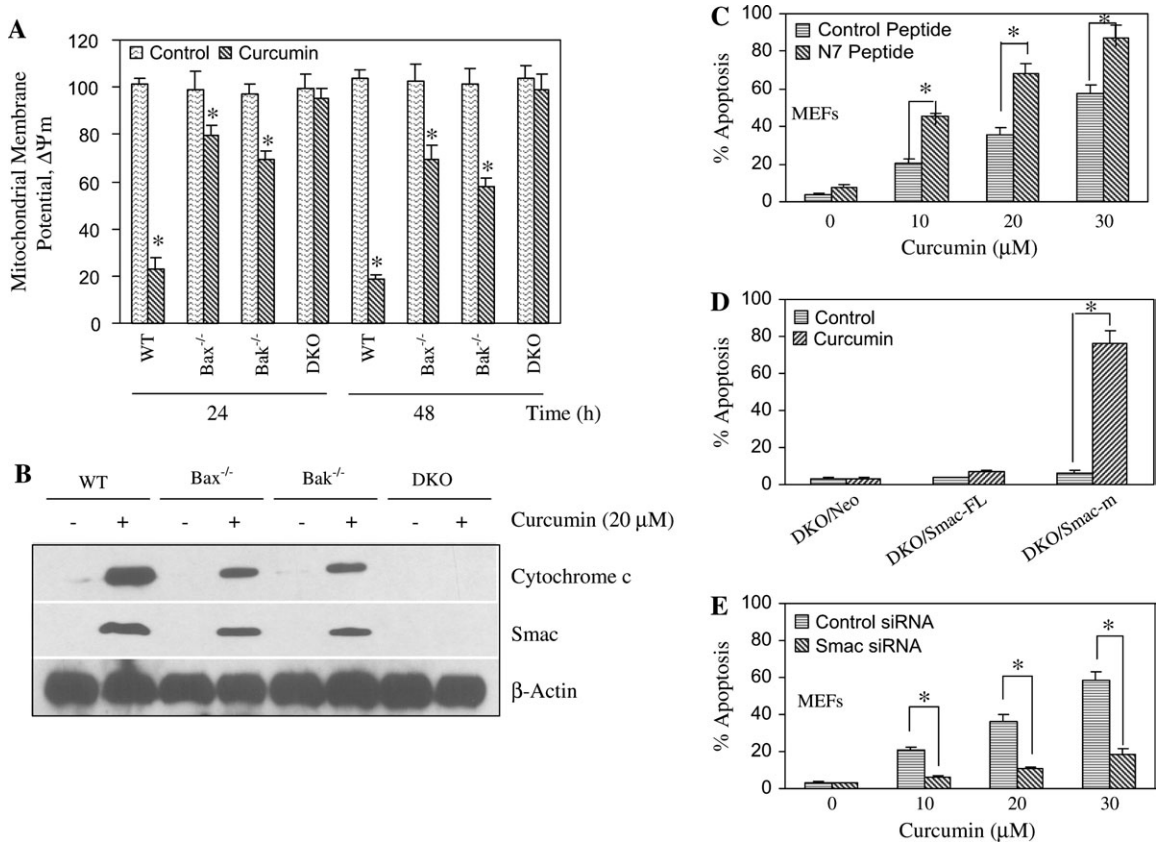

Fig. 4. Effects of curcumin on mitochondrial function and apoptosis. (**A**) Wild-type, $Bax^{-/-}$, $Bak^{-/-}$, and $Bax^{-/-}$ and $Bak^{-/-}$ DKO MEFs were treated with or without 20 μM curcumin for 24 and 48 h, and mitochondrial membrane potential was measured as described in Materials and Methods. Data represent mean ± SE. *Significantly different from respective control. (**B**) Curcumin treatment caused release of apoptogenic molecules from mitochondria to cytosol in wild-type, $Bax^{-/-}$ and $Bak^{-/-}$ MEFs, but not in $Bax^{-/-}$ and $Bak^{-/-}$ DKO MEFs. Immunoblotting of cytochrome c and Smac/DIABLO using cytosolic fractions from wild-type MEFs treated with 20 μM curcumin. (**C**) Enhancement of curcumin-induced apoptosis by Smac N7 peptide. Wild-type MEFs were pre-treated with either 25 μM control Smac peptide or Smac N7 peptide for 2 h, and treated with or without curcumin (20 μM) for 48 h. Apoptosis was measured by terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling assay as per the manufacturer's instructions. Data represent mean ± SE. *Significantly different from respective control ($P < 0.05$). (**D**) Mature Smac/DIABLO, but not full-length Smac/DIABLO, sensitizes DKO MEFs to undergo apoptosis by curcumin. DKO MEFs were transfected with plasmid containing neo, full-length Smac (Smac-FL) or mature Smac (Smac-M cDNA) in the presence of plasmid (pCMV-LacZ) encoding the β-galactosidase (β-Gal) enzyme. After transfection, cells were treated with or without curcumin (20 μM) for 24 h. There were no differences in transfection efficiency among groups. Apoptosis was measured by DAPI staining. Data represent mean ± SE. *Significantly different from respective control ($P < 0.05$). (**E**) Inhibition of curcumin-induced apoptosis by Smac siRNA. Wild-type MEFs were transiently transfected with either control plasmid or Smac siRNA plasmid in the presence of plasmid (pCMV-LacZ) encoding the β-galactosidase (β-Gal) enzyme. There were no differences in transfectiojn efficiency among groups. Cells were treated with or without curcumin (20 μM) for 48 h. Apoptosis was measured by terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling assay as per manufacturer's instructions. Data represent mean ± SE. *Significantly different from respective controls ($P < 0.05$).

caspase activation and lead to cell destruction (38,43). Since activation of caspase-9 and -3 results in PARP cleavage and apoptosis, we measured the activation (by cleavage) of caspase-9 and -3 and cleavage of PARP in MEFs treated with curcumin (Figure 5A). Curcumin induced cleavage of caspase-9, casapse-3 and PARP in wild-type MEFs. By comparison, curcumin had a slight but significant effects on cleavage of caspase-9, casapse-3 and PARP in $Bax^{-/-}$ or $Bak^{-/-}$ MEFs. Interestingly, no cleavage of caspase-9, casapse-3 and PARP was observed in DKO MEFs. These data suggest that curcumin induces apoptosis through activation of caspase-9, caspase-3 and cleavage of PARP.

Casapse-9 is activated through mitochondrial-dependent pathway, whereas the activation of caspase-3 can occur through both mitochondrial-dependent and -independent apoptotic pathways (44). We therefore examined whether inhibition of caspase activity attenuates curcumin-induced apoptosis in MEFs (Figure 5B). Interestingly, curcumin-induced apoptosis was inhibited by a pan caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk) and caspase-3 inhibitor (z-DEVD-fmk) in wild-type MEFs. These data suggest that curcumin induced apoptosis through regulation of caspase-3 and caspase-8 in MEFs.

We have shown previously that the activation of caspase-3 by stress stimuli leads to cleavage of several substrates including PARP (33,45–47). We therefore confirmed the above findings of the activation of caspase-3 and cleavage of PARP by immunofluorescence technique (Figure 5C and D). We have used antibodies that recognize active caspase-3 or cleaved PARP. Curcumin induced activation of caspase-3 (green fluorescence) and cleavage of PARP (red fluorescence). In apoptotic cells, translocation of caspase-3 to nucleus was observed. Thus, these data confirmed the above findings that curcumin induces apoptosis through activation of caspases and cleavage of PARP.

Discussion

Recent studies have shown that curcumin not only offers protection against chemically induced cancer in animal models but also suppresses proliferation of cancer cells *in vitro* and *in vivo*. Furthermore, the inhibitory effects of curcumin against proliferation of cultured cancer cells are attributed to cell cycle arrest and apoptosis. Our own study has revealed that curcumin inhibits PI3-K–Akt pathway, down-regulates Bcl-2 and Bcl-X_L and up-regulates Bax and Bak in prostate cancer LNCaP cells (48). Despite these advances, however,

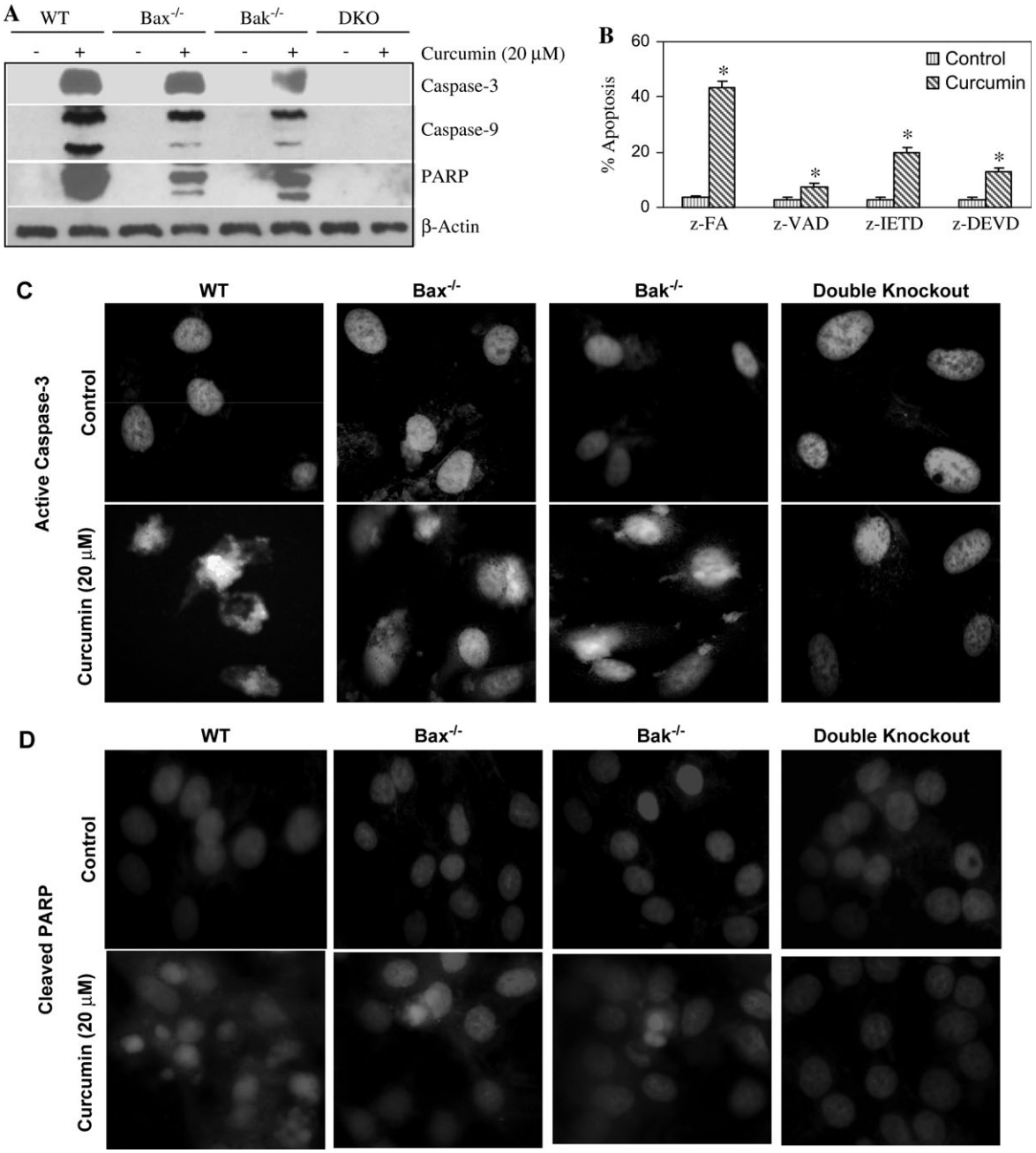

Fig. 5. Curcumin induces caspase-3, caspase-9 and PARP cleavage in wild-type, $Bax^{-/-}$, $Bak^{-/-}$, but not in DKO MEFs. (**A**) MEFs were treated with curcumin for 24 h, and cells were harvested. Cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblotting with anti-caspase-3, anti-caspase-9 and anti-PARP antibodies. Anti-β-actin antibody was used as a loading control. (**B**) MEFs were pre-treated with 25 μM z-FA, pan caspase inhibitor z-VAD-fmk (z-Val-Ala-Asp-fluoromethylketone), z-IETD-fmk or z-DEVD-fmk for 2 h, and treated with or without curcumin (20 μM) for 24 h. Apoptosis was measured by DAPI staining. *Significantly different from respective controls ($P < 0.05$). (**C**) Curcumin induces caspase-3 activity as measured by immunofluorescence microscopy. MEFs were seeded in chambered slides and treated with or without curcumin (20 μM) for 24 h. Cells were fixed, permeabilized and stained with DAPI (for nuclear staining) and FITC-conjugated anti-active caspase-3 antibody. For better visualization, the red color was assigned to nucleus. Red color = nucleus, green color = active caspase-3 and yellow color = translocation of caspase-3 to the nucleus. (**D**) Curcumin induces PARP cleavage as measured by immunofluorescence microscopy. MEF cells were seeded in chambered slides and treated with or without curcumin (20 μM) for 24 h. Cells were fixed, permeabilized and stained with DAPI (for nuclear staining) and FITC-conjugated anti-PARP antibody that recognizes cleaved PARP. Blue color = nucleus and red color = cleaved PARP. The green color of FITC was changed to red for better visualization.

the mechanisms of curcumin-induced apoptosis remains poorly defined. Our study provides first experimental evidence to indicate that pro-apoptotic members of Bcl-2 family Bax and Bak play a critical role in regulation of cell death by curcumin.

The Bcl-2 family of proteins plays a key role in regulating apoptosis at the level of mitochondrial cytochrome c release (49,50). Once released from mitochondria, cytochrome c interacts with Apaf-1, leading to caspase-9 activation and subsequent cleavage and activation of caspase-3, spurring the demise of the cell. Anti-apoptotic Bcl-2 proteins, such as Bcl-2 and Bcl-X_L, function to prevent cytochrome c release by counteracting the effects of pro-apoptotic members, which are divided into two subgroups based on the presence of Bcl-2

homology (BH) domains: the BH-3-only family (e.g. Bid and Bim) and the BH-1, -2 and -3 multidomain proteins (e.g. Bax and Bak). The BH-3-only family activates the multidomain proteins, mainly Bax and Bak, either directly or indirectly by engaging the anti-apoptotic proteins (16,51,52). The exact mechanism of direct activation remains unclear, but it appears that Bax and Bak interact with certain BH-3-only molecules, such as Bid, inducing them to undergo conformational changes and oligomerize and permeabilize membranes which causes release of apoptogenic molecules from mitochondria to cytosol (51,53,54). Previous studies, including those from our laboratories, indicated that curcumin-induced apoptosis in different cellular systems was associated with induction of Bax and Bak protein expression. Alternatively, the indirect mechanism involves BH-3-only-family members binding to and occupying the anti-apoptotic proteins, thereby derepressing Bax and Bak (51,52). Neutralization or removal of anti-apoptotic proteins may be necessary to initiate pro-apoptotic signals and, ultimately, Bax or Bak activation (55). However, release from anti-apoptotic molecules may not be sufficient to activate Bax or Bak without an additional activation step. These findings lead to an emerging model, where not only do apoptotic signals often converge on the multidomain proteins but also the activation of these proteins is regulated on multiple levels to determine precisely when to engage an apoptotic program and commit a cell to die.

Once activated, the multidomain pro-apoptotic molecules Bax and Bak permeabilize the OMM to allow release of cytochrome c and Smac/DIABLO (15,16). The present study was designed to experimentally test the role of Bax and Bak in apoptosis induction by curcumin using SV40-transformed MEFs derived from Bax and/or Bak knockout mice. SV40-transformed MEFs become immortalized, a phenomenon similar to cancer cells which have higher proliferative rate than normal cells. Our data indicate that both Bax and Bak are required for apoptosis induction by curcumin because (i) curcumin treatment causes a dose- and time-dependent increase in protein levels of both Bax and Bak in wild-type MEFs; (ii) the MEFs derived from Bax and Bak knockout mice were resistant to curcumin-induced apoptosis when compared with wild-type MEFs; (iii) the protection against curcumin-induced apoptosis in DKO is greater than in cells lacking either Bax or Bak and (iv) curcumin treatment causes release of apoptogenic molecules and caspase-3 activation in wild-type MEFs but not in DKO. Consistent with these results, Smac N7 peptides enhanced curcumin-induced apoptosis, whereas inhibition of Smac by siRNA inhibited curcumin-induced apoptosis in wild-type MEFs. We and others have shown previously that Bax and Bak are required for apoptosis through the mitochondrial pathway as $Bax^{-/-}$ $Bak^{-/-}$ DKO cells were resistant to several apoptotic stimuli (15,16). These studies suggest that Bax and Bak proteins may have overlapping functions because the single-knockout cells exhibited significant apoptosis in response to several stress stimuli including curcumin (current study). Although Bax and Bak are generally activated by BH-3-only proteins, Bax activation has been artificially achieved *in vitro* by detergent-induced conformational changes and oligomerization (56). Thus, direct activation of the multidomain proteins may be possible with a conformation-altering stimulus. Cells subjected to hyperthermic conditions face a barrage of protein damage. In addition to denaturing proteins, heat may induce conformational changes that expose previously concealed regions (57). This scenario may mirror the ability of the BH-3-only protein Bid to alter Bax conformation by exposing a normally buried hydrophobic domain, presumably resulting in oligomerization and subsequent membrane insertion of Bax (58). A recent study has shown that heat can induce conformational change of Bax and Bak, and thus may be possible to activate Bax and Bak independently of BH-3-only proteins (59). Further studies are needed to determine the mechanism by which curcumin treatment causes induction of Bax and/or Bak protein expression.

During apoptosis, Smac/DIABLO is released from mitochondria to cytosol as a mature protein lacking N-terminus 55-amino acid residues constituting mitochondrial targeting sequences (25). The mature form of cytosolic Smac/DIABLO inhibits the interaction between BIR3 (baculovirus IAP repeat) of XIAP with caspase-9 and linker-BIR2 with caspase-3 or -7, and relieves the inhibitory effects the XIAP on these caspases thereby allowing apoptosis to proceed (60–62). BIR antagonistic action of Smac/DIABLO is due to its binding to BIR3 and linker-BR2 in a mutually exclusive manner with caspase-9, and caspase-3 and -7, respectively. We have recently shown that TRAIL failed to release Smac/DIABLO from mitochondria to cytosol in MEFs lacking Bid, Bak, Bax or both Bax and Bak. Furthermore, ectopic over-expression of Smac/DIABLO or pre-treatment of cells with Smac N7 peptide sensitizes TRAIL-resistant DKO MEFs to undergo apoptosis. DKO MEFs, lacking mature Smac/DIABLO in the cytosol, are resistant to TRAIL due to strong XIAP–caspase-3 interaction, and mature Smac/DIABLO removes the inhibition of XIAP. Mature form of Smac/DIABLO will be required to remove the inhibitory effects of XIAP thereby allowing apoptosis to proceed. In the present study, although curcumin failed to cause the release of cytochrome c in DKO MEFs, these cells were sensitized by the mature form of Smac, suggesting that Smac can induce apoptosis in the absence of cytochrome c and bypass mitochondria. MEFs lacking other components of cell death pathway such as cytochrome c (63), Apaf-1 (64,65) and caspase-9 (66) were sensitive to death receptor-mediated apoptosis probably due to functional Smac/DIABLO. The Smac/DABLO knockout mice were viable, grew and matured normally without showing any histological abnormalities (67). Furthermore, $Smac/DIABLO^{-/-}$ cells were sensitive to various apoptotic stimuli *in vitro*, and hepatocytes in these knockout mice underwent apoptosis by Fas (67). These studies are in agreement with our data where over-expression of Smac enhances apoptosis and inhibition of Smac by siRNA did not completely inhibit curcumin-induced apoptosis in wild-type MEFs, suggesting the existence of a similar molecule capable of compensating for a loss of Smac/DIABLO function.

Polyphenolic phytochemicals such as curcumin is a promising candidate for cancer prevention. Like non-steroidal anti-inflammatory drugs, they suppress carcinogenesis in the $ApcMin^{+}$ mouse model. Clinical pilot studies of curcumin show that it is safe at doses of up to 3.6 g daily, and that the levels of curcumin which can be achieved in the gastrointestinal tract exert pharmacological activity. Thus, it is highly probable that the concentrations of curcumin needed to cause cell death may be achievable. More clinical evaluation will help establish whether curcumin is safe and efficacious alternatives to NSAIDs.

In conclusion, our results indicate that curcumin treatment causes induction of Bax and Bak protein expression, conformational change of Bax and mitochondrial translocation of Bax to trigger the release of apoptogenic molecules such as cytochrome c and Smac/DIABLO from mitochondria to cytosol leading to activation of caspases and apoptosis. Furthermore, curcumin-mediated caspase activation is probably amplified due to induction of Apaf-1. Finally, we provide experimental evidence to prove that both Bax and Bak are essential for curcumin-induced apoptosis, and over-expression of Smac/DIABLO as interventional approach to deal with Bax- and/or Bak-deficient chemoresistant cancers for curcumin-based therapy.

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