Bax and Bak Are Required for Apoptosis Induction by Sulforaphane, a Cruciferous Vegetable–Derived Cancer Chemopreventive Agent

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Abstract

Sulforaphane, a constituent of many edible cruciferous vegetables, including broccoli, effectively suppresses proliferation of cancer cells in culture and in vivo by causing apoptosis induction, but the sequence of events leading to cell death is poorly defined. Here, we show that multidomain proapoptotic Bcl-2 family members Bax and Bak play a critical role in apoptosis induction by sulforaphane. This conclusion is based on the following observations: (a) sulforaphane treatment caused a dose- and time-dependent increase in the protein levels of both Bax and Bak and conformational change and mitochondrial translocation of Bax in SV40-transformed mouse embryonic fibroblasts (MEF) derived from wild-type mice to trigger cytosolic release of apoptogenic molecules (cytochrome c and Smac/DIABLO), activation of caspase-9 and caspase-3, and ultimately cell death; (b) MEFs derived from Bax or Bak knockout mice resisted cell death by sulforaphane, and (c) MEFs derived from Bax and Bak double knockout mice exhibited even greater protection against sulforaphane-induced cytochrome c release, caspase activation, and apoptosis compared with wild-type or single knockout cells. Interestingly, sulforaphane treatment also caused a dose- and time-dependent increase in the protein level of Apaf-1 in wild-type, Bax+/−, and Bak+/− MEFs but not in double knockout, suggesting that Bax and Bak might regulate sulforaphane-mediated induction of Apaf-1 protein. A marked decline in the protein level of X-linked inhibitor of apoptosis on treatment with sulforaphane was also observed. Thus, it is reasonable to postulate that sulforaphane-induced apoptosis is amplified by a decrease in X-linked inhibitor of apoptosis level, which functions to block cell death by inhibiting activities of caspases. In conclusion, the results of the present study indicate that Bax and Bak proteins play a critical role in initiation of cell death by sulforaphane. (Cancer Res 2005; 65(5): 2035-43)

Introduction

Epidemiologic data continue to support the premise that dietary intake of cruciferous vegetables may be protective against the risk of various types of cancers (1–4). Laboratory studies suggest that cancer-protective effect of cruciferous vegetables may be due to isothiocyanates that occur as thioglucoside conjugate (glucosinolates) in a variety of edible plants, including broccoli, cabbage, watercress, etc. (5–8). Isothiocyanates are generated due to hydrolysis of corresponding glucosinolates through catalytic mediation of myrosinase, which is released on damage of the plant cells during processing (e.g., cutting or chewing) of cruciferous vegetables (7). Isothiocyanates, including phenethyl-isothiocyanate and benzyl-isothiocyanate, have been shown to offer significant protection against cancer in animal models induced by a variety of chemical carcinogens (5, 6, 8–13).

Sulforaphane [1-isothiocyanato-4-(methylsulfinyl)-butane; CH3-SO-(CH2)4-N=C=S] is a naturally occurring member of the isothiocyanate family of cancer chemopreventive agents that has attracted particular attention due to its potent anticancer effects (14–19). For example, sulforaphane was shown to offer statistically significant protection against 9,10-dimethyl-1,2-benzanthracene-induced mammary tumorigenesis in rats (15). Interestingly, sulforaphane exhibited bactericidal activity against clinical isolates as well as antibiotic-resistant strains of Helicobacter pylori and inhibited benzo[a]pyrene-induced forestomach cancer in mice (18). Eradication of H. pylori in human gastric xenografts implanted in nude mice on sulforaphane administration was also documented (19). Sulforaphane as well as its N-acetylcysteine conjugate given during the postinitiation period significantly inhibited azoxymethane-induced colonic aberrant crypt foci formation in rats (17). The mechanism by which sulforaphane inhibits chemically induced cancers is believed to involve impairment of carcinogen metabolism due to inhibition of cytochrome P450–dependent monooxygenases and/or induction of phase II detoxification enzymes, such as glutathione transferase (reviewed in refs. 5, 6, 8).

More recent studies, including those from our laboratory, have indicated that sulforaphane can suppress proliferation of cancer cells in culture and in vivo by inhibiting cell cycle progression and/or causing apoptosis induction (20–29). Growth inhibition, cell cycle arrest, and/or apoptosis induction by sulforaphane has been documented in human colon, leukemia, medulloblastoma, and prostate cancer cells (20–29). Gamet-Payrastre et al. (20) were the first to show that treatment of HT29 human colon cancer cells with sulforaphane resulted in G2-M-phase cell cycle arrest and apoptosis induction. Although the mechanism of cell cycle block was not thoroughly examined by these investigators, the sulforaphane-induced apoptosis in HT29 cells was associated with induction of Bax protein expression and cytosolic release of cytochrome c (20). Recent studies from our laboratory have offered novel insights into the mechanism of cell cycle block by sulforaphane (29). Using PC-3 human prostate cancer cells as a model, we showed that sulforaphane-induced G2-M-phase cell cycle arrest was associated with rapid and sustained activation of checkpoint kinase 2, which promoted Ser286 phosphorylation of cell division cycle 25C leading to its translocation from nucleus to the cytosol (29). The net result of these effects was accumulation of Tyr15-phosphorylated (inactive) cyclin-dependent kinase 1 (29), which together with the B-type cyclins plays an important role in regulation of G2-M progression (30). Although significant progress has been made toward our understanding of the signal transduction pathways.
responsible for inhibition of cell cycle progression (20, 24–27, 29), the mechanism of sulforaphane-induced apoptosis is poorly characterized. For example, apoptosis induction by sulforaphane in different cellular systems is associated with induction of Bax protein expression (20, 21, 25, 28), yet studies that could experimentally test the role of this protein in cell death are lacking.

In the present study, we used SV40-transformed mouse embryonic fibroblasts (MEFs) derived from wild-type and Bax and/or Bak knockout mice (31) to gain insights into the role of these multidomain proapoptotic Bel-2 family members in cell death caused by sulforaphane. The present study indicates that both Bax and Bak are essential for apoptosis induction by sulforaphane and that sulforaphane treatment causes activation and mitochondrial translocation of Bax to trigger cytosolic release of apoptogenic molecules.

**Materials and Methods**

### Reagents

Sulforaphane (>99% pure) was purchased from LKT Laboratories (St. Paul, MN). Tissue culture medium, penicillin-streptomycin antibiotic mixture, and fetal bovine serum were from Life Technologies (Grand Island, NY). Propidium iodide and 4′,6-diamidino-2-phenylindole (DAPI) were from Sigma (St. Louis, MO). RNase A was from Promega (Madison, WI), and the kit for quantitation of cytoplasmic histone-associated DNA fragmentation was from Roche Diagnostics (Mannheim, Germany). Antibodies against Bax (clone N-20), Bak (clone G-23), Smac/DIABLO (clone V-17), and Apaf-1 (clone H-324) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against caspase-3, caspase-9, and poly(ADP-ribose)polymerase (PARP) were from Cell Signaling Technology (Beverly, MA). Antibodies against cytochrome c and X-linked inhibitor of apoptosis (XIAP) were from BD PharMingen (San Diego, CA). Anti-actin antibody was from Oncogene Research Products (Boston, MA).

Anti-Bax monoclonal antibody 6A7 that recognizes an epitope on the NH₂-terminus (between amino acids 12 and 24) of conformationally changed (activated) Bax protein was purchased from BD PharMingen.

### Cell Culture and Cell Survival Assay

Primary MEFs derived from wild-type, Bax knockout (Bax<sup>−/−</sup>), Bak knockout (Bak<sup>−/−</sup>), 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; ref. 31). MEFs were maintained in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 0.1 mmol/L nonessential amino acids, 0.1 mmol/L 2-mercaptoethanol, and antibiotics. Normal human bronchial epithelial cell line BEAS2B and normal prostate epithelial cell line PrEC were cultured in tissue culture medium, penicillin-streptomycin, and antibiotic mixture, and fetal bovine serum were from Life Technologies (St. Paul, MN). Tissue culture medium, penicillin-streptomycin, and fetal bovine serum were from Life Technologies (St. Paul, MN). Tissue culture medium, penicillin-streptomycin, and fetal bovine serum were from Life Technologies (St. Paul, MN).

### Apoptosis Assays

**Apoptosis Detection Assays.** Apoptosis induction in sulforaphane-treated MEFs, normal epithelial cells (BEAS2B or PrEC), and cancer cells (H1299 or LNCAp) was assessed by analysis of cytoplastic histone-associated DNA fragmentation using a kit from Roche Diagnostics according to the manufacturer’s instructions or flow cytometric analysis of cells with sub-G<sub>0</sub>/G<sub>1</sub> DNA content following staining with propidium iodide as described by us previously (28, 29) or microscopic analysis of apoptotic cells with condensed nuclei following staining with DAPI as described previously (32).

**Immunoblotting.** MEFs were treated with the desired concentrations of sulforaphane or DMSO (control) as described above and lysed as reported by us previously (28). The cell lysate was cleared by centrifugation at 14,000 × g for 15 minutes. Supernatant proteins were resolved by 12.5% SDS-PAGE and transferred onto polyvinylidene difluoride membrane. After blocking with 5% nonfat dry milk in TBS containing 0.05% Tween 20, the membrane was incubated with the desired primary antibody for 1 hour at room temperature. The membrane was then treated with appropriate secondary antibody, and the immunoreactive bands were visualized using enhanced chemiluminescence method. Each membrane was stripped and reprobed with anti-actin antibody to normalize for differences in protein loading. Change in protein level was assessed by densitometric scanning of the immunoreactive bands followed by correction for actin loading control.

**Immunohistochemistry for Localization of Cytochrome c.** MEFs were cultured on coverslips and treated with 30 μmol/L sulforaphane or DMSO (control) for 8 hours. The MEFs were then washed with PBS and stained for 1 hour at 37°C with 100 μmol/L mitochondria-specific dye MitoTracker Red (Molecular Probes, Eugene, OR). After washing with PBS, MEFs were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The MEFs were incubated with normal goat serum (1:20 dilution, Sigma) in PBS for 45 minutes. Subsequently, the MEFs were treated with anti-cytochrome c antibody (1:200 dilution) for 2 hours, washed with PBS, and incubated with Alexa Fluor 488–conjugated secondary antibody (1:1,000 dilution, Molecular Probes) for 1 hour. After washing, cells were treated with DAPI (1 μg/mL) for 5 minutes to stain DNA. The cells were visualized under a Leica DC300F fluorescence microscope.

**Analysis of Bax Conformation Change.** Wild-type MEFs were treated with 40 μmol/L sulforaphane for 4, 12, or 24 hours and lysed using a solution containing 10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1% CHAPS, 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 (40 μL, Santa Cruz Biotechnology) were then added to each sample, and the incubation was continued for 2 hours at 4°C. The immunoprecipitated complexes were washed thrice with lysis buffer and subjected to SDS-PAGE followed by immunoblotting using polyclonal anti-Bax antibody.

**Immunohistochemistry for Localization of Bax.** Wild-type MEFs were cultured on coverslips and treated with sulforaphane (40 μmol/L) or DMSO for 12 hours. After staining with MitoTracker Red, MEFs were incubated with anti-Bax antibody (1:1,000 dilution) for 2 hours followed by incubation with Alexa Fluor 488–conjugated secondary antibody (1:1,000 dilution) for 1 hour. MEFs were washed with PBS, stained with DAPI, and examined under a fluorescence microscope.

**Statistical Analysis.** One-way ANOVA 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

Sulforaphane-Induced Apoptosis in Wild-type MEFs Was Associated with Induction of Bax and Bak Proteins. Previous studies have shown that apoptosis induction by sulforaphane in different cellular systems is associated with induction of Bax protein expression (20, 21, 25, 28), but the role of this protein in cell death has not been experimentally verified. In the present study, we used MEFs derived from wild-type, Bax knockout (Bax<sup>−/−</sup>), Bak Knockout (Bak<sup>−/−</sup>)...
sulforaphane treatment on viability of wild-type MEFs was assessed to investigate the role of these proteins in apoptosis induction by sulforaphane. First, we determined the sensitivity of wild-type MEFs to cell killing and apoptosis induction by sulforaphane to assess suitability of these cells for our work. The effect of sulforaphane treatment on viability of wild-type MEFs was determined by trypan blue dye exclusion assay (Fig. 1A). Similar to other cellular systems, the viability of wild-type MEFs was reduced significantly in the presence of sulforaphane in a concentration-dependent manner (Fig. 1A). Next, we addressed the question whether reduced viability of wild-type MEFs in the presence of sulforaphane was due to apoptosis induction. Apoptosis was assessed by analysis of cytoplasmic histone-associated DNA fragmentation, which has emerged as a sensitive method for detection of apoptotic cell death. As can be seen in Fig. 1B, a 24-hour treatment of wild-type MEFs with sulforaphane resulted in a concentration-dependent and statistically significant increase in cytoplasmic histone-associated DNA fragmentation. For instance, the DNA fragmentation in wild-type MEFs treated with 20 and 40 μmol/L sulforaphane was increased by ~6- and 8-fold, respectively, compared with control (Fig. 1B). These results indicated that reduced viability of wild-type MEFs in the presence of sulforaphane was indeed due to apoptosis induction.

We raised the question whether sulforaphane-induced cell death in wild-type MEFs was associated with induction of Bax and Bak protein expression. We explored this possibility by determining the effect of sulforaphane treatment on levels of Bax and Bak proteins by immunoblotting, and representative immunoblots are shown in Fig. 1C. Treatment of wild-type MEFs with sulforaphane resulted in a concentration- and time-dependent increase in the protein levels of both Bax and Bak. For example, a 24-hour treatment of wild-type MEFs with 20, 30, and 40 μmol/L sulforaphane caused an ~42%, 80%, and 155% increase in Bax protein level, respectively, when compared with control (Fig. 1C). In time course experiments using 40 μmol/L sulforaphane, the induction of Bax and Bak proteins was evident as early as 4 to 8 hours after treatment and increased gradually with increasing exposure time. Collectively, these results indicated that sulforaphane-mediated apoptosis in wild-type MEFs was associated with induction of Bax and Bak proteins.

Next, we raised the question whether sulforaphane-induced apoptosis is selective for cancer cells, which is a highly desirable feature of potential chemopreventive agents. We addressed this question by determining the effect of sulforaphane treatment on cytoplasmic histone-associated DNA fragmentation in BEAS2B normal bronchial epithelial cell line, PrEC normal prostate epithelial cell line, H1299 human lung cancer cells, and LNCaP human prostate cancer cells. As can be seen in Fig. 1D and E, both cancer cells (H1299 and LNCaP) were significantly more sensitive to sulforaphane-induced cytoplasmic histone-associated DNA fragmentation compared with BEAS2B and PrEC normal epithelial cells. These results indicated that normal epithelial cells were significantly more resistant to apoptosis induction by sulforaphane compared with cancer cells.

**Bax and Bak DKO MEFs Were Resistant to Cell Death by Sulforaphane.** We compared sensitivities of Bax and Bak knockout MEFs toward sulforaphane-induced apoptosis to determine the contribution of these proteins in cell death caused by this agent. The wild-type MEFs were included in the analysis for direct comparison. Apoptosis induction by sulforaphane was assessed by flow cytometric analysis of subdiploid cells, which is a characteristic feature of cells undergoing apoptosis. As shown in Fig. 2A, a 24-hour treatment of wild-type MEFs with 30 μmol/L sulforaphane caused a >30-fold increase in percentage of subdiploid cells compared with DMSO-treated control. The sulforaphane-mediated increase in subdiploid fraction was much less pronounced in Bax−/− and Bak−/− mice immortalized by transfection with a plasmid containing SV40 genomic DNA (31) to investigate the role of these proteins in apoptosis induction by sulforaphane. First, we determined the sensitivity of wild-type MEFs to cell killing and apoptosis induction by sulforaphane to assess suitability of these cells for our work. The effect of sulforaphane treatment on viability of wild-type MEFs was
Bak\(^{-/-}\) MEFs compared with wild-type MEFs. Combined knockdown of Bax and Bak offered even greater protection against sulforaphane-induced appearance of cells with sub-G\(_0\)/G\(_1\) DNA content (Fig. 2A).

Resistance of DKO MEFs to sulforaphane-induced cell death was confirmed by microscopic analysis of cells with condensed nuclei following staining with DAPI, which is another method of apoptosis detection. Representative microscopic images for DAPI staining in wild-type and DKO MEFs following a 24-hour exposure to DMSO (control) or 30 \(\mu\)mol/L sulforaphane are shown in Fig. 2B. Consistent with the results of DNA fragmentation assay (Fig. 1B) and flow cytometric analysis of subdiploid cells (Fig. 2A), apoptotic cells with condensed nuclei were visible in wild-type MEFs cultured in the presence of sulforaphane. On the other hand, the Bax and Bak knockout MEFs, especially the DKO, were significantly more resistant to nuclear condensation by sulforaphane when compared with wild-type MEFs (Fig. 2C).

**Sulforaphane Treatment Caused Cytosolic Release of Apoptogenic Molecules in Wild-type and Single Knockout MEFs but Not in DKO.** Release of apoptogenic molecules, including cytochrome c and Smac/DIABLO from mitochondria to the cytosol, is a critical event in apoptosis induction by a variety of stimuli (33–37). Once in the cytosol, cytochrome c binds to apoptosis protease activation factor-1 and recruits and activates procaspase-9 in the apoptosome (33, 34, 38, 39) Active caspase-9 cleaves and activates executioner caspases, including caspase-3 (38, 39). Smac/DIABLO promotes apoptosis by neutralizing caspase inhibitory effects of inhibitor of apoptosis family of proteins (38). We determined the effect of sulforaphane treatment on cytosolic release of cytochrome c and Smac/DIABLO by immunoblotting using wild-type MEFs to determine if apoptosis induction in our model was associated with cytosolic release of apoptogenic molecules. As can be seen in Fig. 3A, sulforaphane treatment resulted in release of cytochrome c from mitochondria to the cytosol, which was evident as early as 1 hour after treatment. The sulforaphane-mediated release of cytochrome c from mitochondria to the cytosol was observed at lower concentrations as well (data not shown). Sulforaphane treatment also caused cytosolic release of Smac/DIABLO, which was prominent only at 24-hour time point.

Next, we raised the question whether sulforaphane-mediated release of cytochrome c is regulated by Bax and/or Bak. We addressed this question by determining cytosolic release of cytochrome c in MEFs treated for 24 hours with DMSO (control) or 30 \(\mu\)mol/L sulforaphane. As shown in Fig. 3B, treatment of wild-type MEFs with sulforaphane resulted in cytosolic release of cytochrome c, which was not observed in corresponding DMSO-treated control. The sulforaphane-mediated release of cytochrome c was also observed in MEFs derived from Bax or Bak knockout mice. On the other hand, a similar treatment of DKO MEFs with sulforaphane failed to cause cytosolic release of cytochrome c (Fig. 3B).

The effect of sulforaphane treatment on mitochondrial/cytosolic distribution of cytochrome c was further investigated by immunohistochemistry (Fig. 4). In control (DMSO-treated) wild-type MEFs or MEFs lacking Bax or Bak, cytochrome c was primarily localized in the mitochondria as evidenced by a yellow-orange staining of the mitochondria due to merge of green fluorescence (cytochrome c staining) and red fluorescence (MitoTracker Red staining). Representative images for cytochrome c, MitoTracker Red, and DAPI staining in DMSO-treated wild-type MEF are shown in Fig. 4 (top). The mitochondria in sulforaphane-treated wild-type MEFs were stained red, and cytochrome c staining (green fluorescence) was mostly restricted to the cytosol, indicating release of cytochrome c from mitochondria to the cytosol on treatment with sulforaphane. Consistent with the results of cytochrome c immunoblotting, the mitochondria of sulforaphane-treated Bax and Bak knockout MEFs were also stained red due to cytosolic localization of cytochrome c. On the other hand, the mitochondria of sulforaphane-treated DKO MEFs exhibited yellow-orange staining resembling sulforaphane-treated wild-type MEFs. These results clearly indicated that sulforaphane-induced apoptosis in our model

![Figure 2](cancerres.aacrjournals.org)
was triggered by cytosolic release of cytochrome \(c\) and that both Bax and Bak were required for this effect.

**Sulforaphane Treatment Caused Cleavage of Caspases in Wild-type MEFs but Not in DKO MEFs.** Caspases are aspartate-specific cysteine proteases that exist as latent zymogens (38, 39). On activation by apoptotic stimuli, caspases can systematically cleave key cellular proteins, including DNA repair enzyme PARP (38, 39). The mitochondria-mediated caspase cascade (also known as intrinsic pathway) involves mitochondrial membrane permeability change that triggers release of cytochrome \(c\) and other proapoptotic molecules from mitochondria to the cytosol (38–40). Because cytochrome \(c\) release was observed in sulforaphane-treated wild-type MEFs, we determined the effect of sulforaphane treatment on proteolytic cleavage of caspase-9 and caspase-3 by immunoblotting using wild-type MEFs. As can be seen in Fig. 5A, sulforaphane treatment caused proteolytic cleavage of both caspase-9 and caspase-3 in a concentration-dependent manner. In time course experiments using 40 \(\mu\)M sulforaphane, proteolytic cleavage of caspase-9 was evident as early as 2 hours after treatment, whereas caspase-3 cleavage was not observed until 12 hours (Fig. 5B). Activation of caspase-3 leads to cleavage and inactivation of key cellular proteins, including the DNA repair enzyme PARP. We therefore determined the effect of sulforaphane treatment on cleavage of PARP to confirm caspase-3 activation. As can be seen in Fig. 5C, PARP cleavage was observed in sulforaphane-treated wild-type MEFs.

The effect of sulforaphane treatment (30 \(\mu\)M/L, 24 hours) on cleavage of caspase-3 and PARP was compared using wild-type and Bax and/or Bak knockout MEFs (Fig. 5D). Treatment of wild-type MEFs as well as the MEFs derived from Bax or Bak knockout mice with sulforaphane resulted in proteolytic cleavage of caspase-3, which was barely seen in DKO MEFs (Fig. 5D). Consistent with these results, sulforaphane-induced cleavage of PARP was much more pronounced in wild-type MEFs and MEFs derived from Bax or Bak single knockout mice when compared with DKO. Some cleavage of PARP in the DKO MEFs is expected because sulforaphane is able to induce residual cell death in these cells (Fig. 2). These results indicate involvement of a nonmitochondrial component in sulforaphane-induced apoptosis in DKO.

**Sulforaphane Treatment Caused Induction of Apaf-1 and Down-regulation of XIAP Protein Expression.** Activation of caspase-9 is regulated by Apaf-1, which facilitates recruitment of procaspase-9 to the apoptosome (37–40). The XIAP protein inhibits activity of caspases (41). To determine possible involvement of Apaf-1 and XIAP in apoptosis induction by sulforaphane, we determined its effect on levels of above proteins by immunoblotting using wild-type MEFs. As shown in Fig. 6A, a 24-hour treatment of wild-type MEFs with sulforaphane resulted in a dose-dependent induction of Apaf-1 protein expression. The sulforaphane-mediated increase in Apaf-1 protein level was evident as early as 8 hours after treatment (data not shown). Interestingly, the sulforaphane-treated wild-type MEFs also exhibited a concentration-dependent reduction in XIAP protein level, especially at higher concentrations of sulforaphane (Fig. 6A). Next, we raised the question whether sulforaphane-mediated change in Apaf-1 or XIAP protein levels was regulated by Bax or Bak. As can be seen in Fig. 6B, the sulforaphane-mediated induction of Apaf-1 protein expression was observed in wild-type and Bax and Bak single knockout MEFs but not in the MEFs derived from DKO mice. On the other hand, the sulforaphane-mediated decline in XIAP protein level was observed in wild-type as well as Bax and Bak knockout MEFs, including DKO (Fig. 6B). These results suggested that sulforaphane-mediated induction of Apaf-1 protein might be regulated by Bax and Bak proteins.

**Sulforaphane Treatment Caused Mitochondrial Translocation of Bax.** In normal cells, the Bax protein exists in an inactive form in the cytosol but can be induced to change conformation and translocate to the mitochondria in response to certain apoptotic stimuli (42, 43). The activated Bax protein oligomerizes on the outer mitochondrial membrane and induces release of apoptogenic molecules to the cytoplasm (42, 43). Because cytosolic release of cytochrome \(c\) was observed in sulforaphane-treated wild-type MEFs, we raised the question whether sulforaphane treatment caused activation of Bax to initiate the cell death process. We tested this possibility by two different but complementary approaches. First, we determined whether sulforaphane treatment causes conformational change of Bax. The conformational change was assessed by immunoprecipitation of Bax using a monoclonal antibody (6A7) that recognizes an epitope at the NH2 terminus of the protein, which becomes exposed only after a change in conformation of Bax. The immunoprecipitated complex was then subjected to SDS-PAGE followed by immunoblotting using anti-Bax polyclonal antibody. As can be seen in Fig. 6C, sulforaphane treatment caused a change in conformation of Bax that was evident as early as 4 hours after exposure and gradually increased with increasing exposure time. Activation of Bax on treatment with sulforaphane was confirmed by immunohistochemistry using wild-type MEFs (Fig. 6D). In MEFs treated for 12 hours with DMSO (control), the mitochondria were stained red and Bax immunostaining was generally restricted to the cytosol. On the other hand, the mitochondria in sulforaphane-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 sulforaphane-treated cells.

Discussion

Evidence is accumulating to indicate that sulforaphane not only offers protection against chemically induced cancer in animal models (15, 17, 18) but also suppresses proliferation of cancer cells in culture as well as in vivo (20–29). Studies have indicated that inhibitory effect of sulforaphane against proliferation of cultured cancer cells is attributable to cell cycle block as well as apoptosis induction (20–29). A novel mechanism of chemoprevention by sulforaphane involving inhibition of histone deacetylase was also suggested recently (26). Our own work has revealed that sulforaphane treatment causes apoptosis in PC-3 human prostate cancer cells in association with activation of caspases (28). Despite these advances, however, the mechanism of sulforaphane-induced apoptosis remains poorly defined. For instance, the signaling pathways upstream of caspase activation in sulforaphane-induced cell death are not characterized. The present study provides experimental evidence to indicate that Bcl-2 family proapoptotic proteins Bax and Bak play a critical role in regulation of cell death by sulforaphane.

The Bcl-2 family proteins have emerged as critical regulators of the mitochondria-mediated apoptosis by functioning as either promoters (e.g., Bax and Bak) or inhibitors (e.g., Bcl-2 and Bcl-xL) of the cell death process (44–48). Antiapoptotic Bcl-2 family members (Bcl-2 and Bcl-xL) possess four conserved BH domains (BH1-BH4) and mainly prevent the release of apoptogenic molecules (e.g., cytochrome c) from mitochondria to the cytosol by forming heterodimer with proapoptotic proteins, such as Bax (44–48). The proapoptotic Bcl-2 family proteins, which can be subdivided into the Bax subfamily of multidomain proteins (e.g., Bax and Bak) or BH3-only subfamily (e.g., Bid and Bim), induce mitochondrial membrane permeabilization and release of apoptogenic molecules from mitochondria to the cytosol (44–48). Previous studies, including those from our laboratory, indicated that sulforaphane-induced apoptosis in different cellular systems was associated with induction of Bax protein expression (20, 21, 25, 26). The present study was designed to experimentally test the role of Bax and Bak in apoptosis induction by sulforaphane using SV40-transformed MEFs derived from Bax and/or Bak knockout mice. Data presented herein indicate that both Bax and Bak are required for apoptosis induction by sulforaphane. This conclusion is based on the following observations: (a) sulforaphane treatment causes a dose- and time-dependent increase in protein levels of both Bax and Bak.

Figure 4. Sulforaphane treatment caused translocation of cytochrome c from mitochondria to the cytosol in wild-type MEFs but not in DKO. Immunohistochemistry for analysis of cytochrome c localization in wild-type MEFs and in Bax- and/or Bak-deficient MEFs following 8-hour exposure to DMSO (data for wild-type MEFs are shown) or 30 μmol/L sulforaphane. Green, red, and blue fluorescence, staining for cytochrome c, mitochondria, and nucleus, respectively. Images were merged to detect mitochondrial/cytosolic distribution of cytochrome c.
in wild-type MEFs, (b) the MEFs derived from Bax and Bak knockout mice exhibit increased resistance to apoptosis induction by sulforaphane when compared with wild-type MEFs, and (c) the protection against sulforaphane-induced apoptosis in DKO is greater than in cells lacking either Bax or Bak. Consistent with these results, sulforaphane treatment causes release of apoptogenic molecules and caspase-3 activation in wild-type MEFs but not in DKO. Our data also suggest that Bax and Bak proteins may have overlapping functions because the single knockout cells exhibited significant apoptosis in response to treatment with sulforaphane. Thus, sulforaphane resembles various other agents that require both Bax and Bak proteins to initiate the cell death, including...
tunicamycin, staurosporine, etoposide, and tumor necrosis factor-related apoptosis-inducing ligand (31, 49). The kinetics of sulforaphane-mediated induction of Bax and Bak proteins was comparable, suggesting that these proteins may have common regulatory mechanism. However, further studies are needed to determine the mechanism by which sulforaphane treatment causes induction of Bax or Bak protein expression.

In normal cells, the Bax protein exists in an inactive form mainly in the cytosol but can be induced to change conformation and translocate to the mitochondria in response to certain apoptotic stimuli (42, 43). The conformationally changed Bax protein oligomerizes on the outer mitochondrial membrane and induces release of apoptogenic molecules to the cytoplasm (42, 43). Recent studies have indicated that microtubule-damaging agents cause Bax activation to trigger the cell death (50). Because sulforaphane was shown recently to disrupt tubulin polymerization (25), we reasoned that apoptosis induction by this phytochemical may be due to Bax activation. The results of the present study indicate that sulforaphane treatment indeed causes conformational change and mitochondrial translocation of Bax. To the best of our knowledge, the present study is the first published report to show Bax activation in sulforaphane-induced cell death.

Apaf-1 is a critical regulator of mitochondria-mediated activation of caspase-9, whereas XIAP functions to inhibit caspases (38–41). The present study reveals that sulforaphane treatment causes an increase in protein level of Apaf-1 and a decrease in XIAP protein level. Thus, it is reasonable to postulate that sulforaphane-mediated activation of caspases and apoptosis in our model is probably amplified by induction of Apaf-1 and down-regulation of XIAP. It is interesting to note that sulforaphane-mediated induction of Apaf-1 was not observed in MEFs lacking both Bax and Bak. These results suggest that Bax and Bak may regulate sulforaphane-mediated induction of Apaf-1, but additional studies are needed to systematically explore this possibility. Notably, the sulforaphane-mediated decline in XIAP protein level was not influenced by the presence or absence of Bax and/or Bak.

Cell growth inhibition and apoptosis induction by sulforaphane in SV40-transformed MEFs (present study) as well as in other cells, including colon, Jurkat T leukemia, and prostate cancer cells, have been observed at 10 to 40 μmol/L concentrations (20–29). A fundamental question, which remains unanswered, is whether the micromolar concentrations of sulforaphane needed to trigger cell death are achievable in humans. Although the answer to this question awaits pharmacokinetic data in humans using pure sulforaphane, the pharmacokinetic variables for sulforaphane were determined recently in rats after oral dosing (50 μmol; ref. 51). Sulforaphane was detectable in the plasma after 1 hour, peaked around 20 μmol/L at 4 hours after dosing, and declined with a half-life of ~2.2 hours (51). The isothiocyanates, including sulforaphane, are mainly excreted as thiol conjugates in the urine (51–53). Interestingly, the thiol conjugates of sulforaphane retain chemopreventive activity (8, 12, 13). Thus, it is highly likely that the concentrations of sulforaphane needed to cause cell death may be achievable.

In conclusion, the results of the present study indicate that sulforaphane treatment causes induction of Bax and Bak protein expression and conformational change and mitochondrial translocation of Bax to trigger the release of apoptogenic molecules from mitochondria to the cytosol leading to activation of caspases and cell death. The sulforaphane-mediated caspase activation is probably amplified due to induction of Apaf-1 and down-regulation of XIAP protein. Furthermore, we provide experimental evidence to indicate that both Bax and Bak are essential for sulforaphane-induced cell death.

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