Sulforaphane-induced Cell Death in Human Prostate Cancer Cells Is Initiated by Reactive Oxygen Species*

Received for publication, November 3, 2004, and in revised form, March 3, 2005
Published, JBC Papers in Press, March 11, 2005, DOI 10.1074/jbc.M412443200

Shivendra V. Singh‡‡, Sanjay K. Srivastava‡, Sunga Choi‡, Karen L. Lew‡, Jedrzej Antosiewicz‡, Dong Xiao‡, Yan Zeng‡, Simon C. Watkins‡, Candace S. Johnson‡, Donald L. Trump‡, Yong J. Lee‡, Hui Xiao‡, and Anna Herman-Antosiewicz‡

From the ‡Department of Pharmacology and University of Pittsburgh Cancer Institute, the ¶Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213 and ¶Roswell Park Cancer Institute, Buffalo, New York 14263

We have shown previously that sulforaphane (SFN), a constituent of many edible cruciferous vegetables including broccoli, suppresses growth of prostate cancer cells in culture as well as in vivo by causing apoptosis, but the sequence of events leading to cell death is poorly defined. Using PC-3 and DU145 human prostate cancer cells as a model, we now demonstrate, for the first time, that the initial signal for SFN-induced apoptosis is derived from reactive oxygen species (ROS). Exposure of PC-3 cells to growth-suppressive concentrations of SFN resulted in ROS generation, which was accompanied by disruption of mitochondrial membrane potential, cytosolic release of cytochrome c, and apoptosis. All these effects were significantly blocked on pretreatment with N-acetylcysteine and overexpression of catalase. The SFN-induced ROS generation was significantly attenuated on pretreatment with mitochondrial respiratory chain complex I inhibitors, including diphenyleneiodonium chloride and rotenone. SFN treatment also caused a rapid and significant depletion of GSH levels. Collectively, these observations indicate that SFN-induced ROS generation is probably mediated by a nonmitochondrial mechanism involving GSH depletion as well as a mitochondrial component. Ectopic expression of Bcl-xL, but not Bcl-2, in PC-3 cells offered significant protection against the cell death caused by SFN. In addition, SFN treatment resulted in an increase in the level of Fas, activation of caspase-8, and cleavage of Bid. Furthermore, SV40-immortalized mouse embryonic fibroblasts (MEFs) derived from Bid knock-out mice displayed significant resistance toward SFN-induced apoptosis compared with wild-type MEFs. In conclusion, the results of the present study indicate that SFN-induced apoptosis in prostate cancer cells is initiated by ROS generation and that both intrinsic and extrinsic caspase cascades contribute to the cell death caused by this highly promising cancer chemopreventive agent.

Epidemiological data continue to support the premise that dietary intake of cruciferous vegetables may reduce the risk of different types of malignancies, including cancer of the prostate (1–4). The anticarcinogenic effect of cruciferous vegetables is attributed to isothiocyanates (ITCs)1 that occur naturally as thioglucoside conjugates (glucosinolates) in a variety of edible plants including watercress, broccoli, cabbage, and so forth (reviewed in Refs. 5–7). Organic ITCs are generated due to hydrolysis of corresponding glucosinolates through catalytic mediation of myrosinase (6). Naturally occurring ITCs, including phenethyl-ITC and benzyl-ITC, have been shown to offer significant protection against cancer in animal models induced by a variety of chemicals including tobacco smoke-derived carcinogens (reviewed in Refs. 5 and 7).

Sulforaphane (SFN; 1-isothiocyanato-4-(methylsulfinyl)-butane), a naturally occurring member of the ITC family of chemopreventive agents, has received particular attention because of its anticancer effects (8–12). This phytochemical is a potent inducer of the phase 2 enzymes implicated in carcinogen detoxification (13) and a competitive inhibitor of CYP2E1, which is involved in the activation of carcinogenic chemicals (14). Cancer chemoprevention by SFN or its N-acetylcycteine (NAC) conjugate has been observed against 9,10-dimethyl-1,2-benz[a]anthracene-induced mammary tumorigenesis in rats (9), azoxymethane-induced colonic aberrant crypt foci formation in rats (11), and benzo[a]pyrene-induced forestomach cancer in mice (12).

Evidence is accumulating to indicate that SFN can suppress proliferation of cancer cells in culture by causing cell cycle arrest and apoptosis induction (15–21). Growth inhibition, cell cycle arrest, and/or apoptosis induction by SFN has been observed in human colon, leukemia, and prostate cancer cells (15–21). Most interestingly, the NAC conjugate of SFN was shown recently to inhibit histone deacetylase activity (22). Our own work has revealed that orally administered SFN at a dietary achievable dose significantly retards growth of PC-3 human prostate cancer xenografts in athymic mice without causing weight loss or any other side effects (20).

Recent studies have offered novel insights into the mecha-

* This work was supported in part by United States Public Health Service Grants CA101753 and CA076348 from NCI, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Hillman Cancer Center, Research Pavilion Ste. 2.32A, University of Pittsburgh Cancer Institute, 5117 Centre Ave., Pittsburgh, PA 15213. Tel.: 412-623-3263; Fax: 412-623-7828; E-mail: singhs@msx.upmc.edu.

† This work was supported in part by United States Public Health Service Grants CA101753 and CA076348 from NCI, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: ITCs, isothiocyanates; SFN, sulforaphane; NAC, N-acetylcycteine; ROS, reactive oxygen species; DAPI, 4',6-diamidino-2-phenylindole; HE, hydroethidine; H2DCFDA, 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate; TMRM, tetramethylrhodamine methyl ester; DPI, diphenyleneiodonium chloride; SOD, superoxide dismutase; MEFs, mouse embryonic fibroblasts; PARP, poly(ADP-ribose) polymerase; DCF, 2',7'-dichlorofluorescein; MeSO2, dimethyl sulfoxide; PBS, phosphate-buffered saline; Cox IV, cytochrome c oxidase (complex IV); EGFP, enhanced green fluorescence protein; BSA, bovine serum albumin; ANOVA, analysis of variance; DMEM, Dulbecco’s modified Eagle’s medium; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; Ad, adenoviral; FADD, Fas-associated death domain; WT, wild type.
nism by which SFN inhibits cell cycle progression (15, 16, 18, 19, 21). Our own work revealed that the SFN-mediated G2-M phase cell cycle arrest in human prostate cancer cells was associated with activation of checkpoint kinase 2, which promoted Ser17 phosphorylation of Cdc25C leading to its translocation from the nucleus to the cytosol (21). The net result of these effects was accumulation of Tyr15-phosphorylated (inactive) cyclin-dependent kinase 1 (21), which together with the B-type cyclins plays an important role in the regulation of G2-M transition. Thus, significant progress has been made toward our understanding of the mechanism of cell cycle arrest by SFN (15, 16, 18, 19, 21). On the other hand, the signaling pathway by which SFN initiates the cell death process is poorly defined. An understanding of the mechanism of SFN-induced apoptosis is critical for its clinical development because this knowledge could lead to identification of mechanism-based biomarkers potentially useful in future clinical trials.

Previous studies have documented down-regulation of Bcl-2 in SFN-treated cells (16, 18–20), yet studies are lacking that could directly test the role of this anti-apoptotic protein in cell death caused by SFN. Similarly, activation of caspases on treatment with SFN has been reported (20), but the mechanism of caspase activation remains elusive. Using PC-3 and DU145 human prostate cancer cells as a model, we demonstrate the following: (a) cell death caused by SFN is initiated by reactive oxygen species (ROS); (b) the SFN-induced apoptosis is significantly attenuated by ectopic expression of Bcl-xL but not Bcl-2; and (c) both intrinsic and extrinsic caspase cascades contribute to the cell death caused by SFN.

**EXPERIMENTAL PROCEDURES**

Reagents—SFN (purity >99%) was purchased from LKT Laboratories (St. Paul, MN). Reagents for cell culture including F-12K Nutrient Mixture, DMEM, penicillin, streptomycin antibiotic mixture, and serum were purchased from Invitrogen. The kits for determination of catalase (catalog number 707002) and superoxide dismutase (catalog number 706002) activities and glutathione (GSH) levels (catalog number 703002) were purchased from Cayman Chemical (Ann Arbor, MI). Propidium iodide, NAC, RNaseA, 4,6-diamidino-2-phenylindole (DAPI), and rotenone were from Sigma; hydroethidine (HE), 6-carboxyfluorescein diacetate (H2DCFDA), and tetramethylrhodamine methyl ester (TMRM) were from Molecular Probes (Eugene, OR); and diphenyleneiodonium chloride (DPI) was from Calbiochem. The combined SOD/catalase mimetic EUK-134 was a gift from Dr. Natasha Kyprianou (University of Maryland School of Medicine, Baltimore, MD) and cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acids, 0.1 mM sodium pyruvate, and antibiotics. Primary mouse embryonic fibroblasts (MEFs) derived from wild-type and BID knock-out (Bid−/−) 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) (23). The MEFs were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, and antibiotics. Each cell line was maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO2. The effect of SFN on cell survival was determined by trypan blue dye exclusion assay as described by us previously (20, 24).

**Measurement of ROS**—Intracellular ROS generation was measured by flow cytometry following staining with HE and H$_2$DCFDA, which have been shown to be somewhat specific for detection of O$_2^\cdot$ and H$_2$O$_2$, respectively (25, 26). The HE is oxidized to ethidium bromide, whereas H$_2$DCFDA is cleaved by nonspecific cellular esterases and oxidized in the presence of H$_2$O$_2$ and peroxidases to yield fluorescent 2′,7′-dichlorofluorescein (DCF). Briefly, 5 × 10$^4$ cells were plated in 60-mm dishes, allowed to attach overnight, and exposed to different concentrations of SFN for specified time intervals. Staining solution of SFN was prepared in dimethyl sulfoxide (Me$_2$SO), and an equal volume of Me$_2$SO was added to the controls. The cells were counterstained with 2 μM HE and 5 μM H$_2$DCFDA for 30 min at 37 °C. The cells were collected, and the fluorescence was analyzed using a Coulter Epics XL Flow Cytometer. In some experiments, cells were pretreated with NAC, DPI, or rotenone prior to SFN exposure and analysis of ROS generation.

**Glutathione Assay**—The effect of SFN treatment on the intracellular level of GSH was determined using a kit from Cayman Chemical (Ann Arbor, MI). Briefly, PC-3 cells (1 × 10$^6$) were plated in 25 mm flasks, allowed to attach overnight, and exposed to Me$_2$SO (control) or SFN (20 or 40 μM) for 6 h at 37 °C. The cells were collected, washed with PBS, and pelleted by centrifugation at 500 × g for 6 min. The cell pellet was resuspended in cold 50 mM potassium phosphate (pH 7) containing 1 mM EDTA, sonicated, and centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant fraction was used for determination of catalase or SOD activity using kits from Cayman Chemical according to the manufacturer’s instructions.

**Measurement of Mitochondrial Membrane Potential**—Mitochondrial membrane potential was measured using fluorescein lipophilic cationic dye TMRM, which accumulates within mitochondria in a potential-dependent manner. Briefly, cells (5 × 10$^5$) were plated in 60-mm culture dishes, allowed to attach overnight, exposed to desired concentrations of SFN for specified time period, and collected by trypsinization. The cells were then resuspended in growth medium and stained with 0.5 μM TMRM for 15 min at 37 °C in the dark. The cells were washed twice with ice-cold PBS, and the fluorescence was measured using a Coulter Epics XL flow cytometer. Carbonyl cyanide 3-(trifluoromethoxy)phenylhydrazone (FCCP; 25 μM), an uncoupler of mitochondrial oxidative phosphorylation, was used as a positive control.

**Electron Microscopy**—Transmission electron microscopy to determine the effect of SFN on mitochondrial morphology was performed essentially as described previously (27). Briefly, PC-3 cells (2 × 10$^5$) were plated in 6-well plates and allowed to attach overnight. The cells were then treated with either Me$_2$SO (control) or 40 μM SFN for 6 h at 37 °C. The cells were fixed in 1% paraformaldehyde, 0.05% Tween 20, and 5–10% (v/v) nonfat dry milk and then exposed to the desired primary antibody for 1 h at room temperature. Following treatment with the appropriate secondary antibody, the blots were stripped and reprobed with anti-actin antibody to correct for differences in protein loading. The change in protein level was determined by densitometric scanning of the immunoreactive bands followed by correction for actin control. The immunoblotting for each protein was performed at least twice using independently prepared lysates, and the results were similar.

**SOD and Catalase Activity Determination**—PC-3 cells (5 × 10$^5$) were plated in T25 flasks, allowed to attach overnight, and exposed to Me$_2$SO (control) or SFN (20 or 40 μM) for 6 h at 37 °C. The cells were collected, washed with PBS, and pelleted by centrifugation at 500 × g for 6 min. The cell pellet was suspended in 0.1 M potassium phosphate (pH 7) containing 1 mM EDTA, sonicated, and centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant fraction was used for determination of catalase or SOD activity using kits from Cayman Chemical according to the manufacturer’s instructions.
ROS Generation in SFN-induced Apoptosis

RESULTS

SFN Treatment Caused ROS Generation in PC-3 Cells—We have shown previously that SFN exhibits significant activity against proliferation of PC-3 cells in culture as well as in vivo by causing apoptotic cell death (20). In the present study, we used the same cell line to test the hypothesis that SFN-induced apoptosis is initiated by ROS, which are implicated in apoptosis induction by different stimuli, including ionizing radiation and hyperoxia (29, 30). This hypothesis stemmed from our previous studies (21) demonstrating SFN-mediated activation of checkpoint kinase 2, which is an intermediary of DNA damage checkpoint and is activated by pro-oxidants, including ionizing radiation (reviewed in Ref. 31). Intracellular ROS generation in control (MeSO4-treated) and SFN-treated PC-3 cells was assessed by flow cytometry following staining with HE and H2DCFDA. Representative histograms for ethidium bromide and DCF fluorescence in PC-3 cells treated for 6 h with MeSO4 or 40 μM SFN are depicted in Fig. 1A. The SFN-treated PC-3 cells exhibited a dose-dependent increase in DCF fluorescence compared with control (Fig. 1B). For instance, the DCF fluorescence in PC-3 cells treated for 6 h with 10, 20, and 40 μM SFN was increased by about 2.4-, 3.1-, and 6.2-fold, respectively, compared with vehicle-treated control (Fig. 1B). In time course experiments using 40 μM SFN, a statistically significant increase in DCF fluorescence relative to MeSO4-treated control was evident as early as 1 h after treatment and increased gradually with increasing exposure time (Fig. 1C).

It is interesting to note that the ethidium bromide fluorescence was higher in vehicle-treated control than in the SFN-treated PC-3 cells (Fig. 1A). To gain insights into the mechanism of SFN-mediated suppression of ethidium bromide fluorescence, we determined the effect of SFN treatment on the protein level as well as the enzyme activity of SOD. As can be seen in Fig. 1D, the protein level of Cu,Zn-SOD and Mn-SOD was about 1.9- and 3.2-fold higher, respectively, in PC-3 cells treated for 6 h with 40 μM SFN compared with vehicle-treated control. Moreover, treatment of PC-3 cells with 40 μM SFN for 6 h resulted in a modest but statistically significant increase in SOD activity compared with control (Fig. 1E). On the other hand, SFN treatment did not affect catalase protein level or catalase activity (Fig. 1, D and F). Collectively, these results suggested that the SFN-mediated suppression of ethidium bromide fluorescence in PC-3 cells was due to induction of SOD. Mechanism of ROS Generation by SFN—Mitochondrial respiratory chain is considered a major source of ROS in cells (32). Mitochondrial ROS are generated due to incomplete reduction of oxygen primarily by redox cycling of ubiquinone from 1 to 5% electrons that escape during normal oxidative phosphorylation (32). To clarify whether ROS generation by SFN involved mitochondria, we determined the effects of DPI and rotenone on SFN-induced ROS generation. DPI is an inhibitor of NADPH oxidase, but it can also inhibit other flavoprotein oxidoreductases such as xanthine oxidase and mitochondrial respiratory chain complex I. Because DPI inhibits mitochondrial respiratory chain complex I, it increases superoxide anion formation (33). The DPI-mediated increase in HE oxidation (an indicator of superoxide anion formation) was also observed in our model (results not shown). As can be seen in Fig. 2A, although DPI treatment alone did not affect DCF fluorescence, the SFN-induced oxidation of H2DCFDA (increase in DCF fluorescence) was partially but markedly (by about 50% compared with SFN treatment alone) blocked on pretreatment with 10 μM DPI. These results suggested that the SFN-induced ROS generation in PC-3 cells might involve mitochondria. We confirmed this possibility by using rotenone, which is a specific inhibitor of the mitochondrial

90%), and embedded in Epon (dodecyl succinic anhydride, nadic methyl anhydride, scioxy 812 resin, and dimethylandimethylenomethyl; Energy Beam Sciences). Semi-thin (300 nm) sections were cut using a Reichert Ultracut, stained with 0.5% toluidine blue, and examined under a light microscope. Ultrathin sections (65 nm) were stained with 2% uranyl acetate and Reynolds’s lead citrate and examined on a JEOL 1210 transmission electron microscope at ×50,000 magnification.

Apoptosis Assays—Apoptosis induction by SFN was assessed as follows: (a) by fluorescence microscopic analysis of cells with condensed and fragmented DNA following staining with DAPI; (b) quantitation of cytoplasmic histone-associated DNA fragmentation; and (c) flow cytometric analysis of cells with sub-G1-G1 DNA content following staining with propidium iodide. For DAPI assay, cells (2 × 10⁴) were plated on coverslips, allowed to attach overnight, and exposed to MeSO4 or SFN. The cells were washed with PBS and fixed with 3% paraformaldehyde for 1 h at room temperature. The cells were washed three times with PBS, processed for immunoblotting with 1% Triton X-100 for 4 min, washed again, and stained by incubation with 1 μg/ml DAPI for 30 min. The cells with condensed and fragmented DNA (apoptotic cells) were scored using a Leica DC300F fluorescence microscope at ×40 magnification. Cytoplasmic histone-associated DNA fragmentation was determined using a kit from Roche Applied Science according to the manufacturer’s recommendations. The sub-G1-G1 fraction was quantified by flow cytometry as described previously (31).

Catalase Overexpression—Adenoviral constructs for enhanced green fluorescence protein (Ad-EGFP) or catalase (Ad-catalase) have been described previously (28). The DU145 cells (2 × 10⁴) were grown in 6-well plates, allowed to attach, infected with Ad-EGFP (5 multiplicity of infections) or Ad-catalase (20 multiplicity of infections), and treated with MeSO4 or 40 μM SFN for the specified time points. The cells were then fixed for analysis of ROS generation, immunoblotting, or apoptosis as described above.

Transfection of PC-3 Cells with Bcl-XL—PC-3 cells were transfected with pSFFV-Bcl-XL and pSFFV-neo plasmids (a generous gift from Dr. Stanley J. Korsmeyer) using Lipofectamine 2000 (Invitrogen). Transfected cells were grown in media containing 800 μg of G418/ml for 3 weeks. Several G418-resistant clones were expanded and screened for the Bcl-XL clone by immunoblotting. The clone with the highest expression (clone 18) was selected for functional studies and maintained in the presence of 500 μg of G418/ml.

Determination of Caspase Activity—Caspase-8 and caspase-9 activities were determined by using a kit from Clontech and R & D Systems (Minneapolis, MN), respectively, according to the instructions provided by the manufacturer. Briefly, 2 × 10⁴ cells were plated and allowed to attach overnight. The medium was replaced with fresh complete medium containing 40 μM SFN. After incubation for the specified time point at 37 °C, the cells were trypsinized, washed with PBS, and lysed using 50 μl of manufacturer supplied lysis buffer. The reaction mixture contained 50 μl of cell lysate, 50 μl of reaction buffer, 5 μl of caspase-8- or caspase-9-specific peptide substrate conjugated to 4-nitroanilide. The reaction mixture was incubated at 37 °C for 3 h. Absorbance at 405 nm, due to release of 3-nitroanilide, was measured using a Labsystem Multi-Scan Plus plate reader. In some experiments, the cells were pre-treated with specified concentration of NAC or EUK-134 prior to addition of SFN and determination of caspase activity.

Immunohistochemistry for Cytochrome c Localization—The MEFs derived from wild-type and Bcl-2−/− mice were cultured on coverslips and treated with 30 μM SFN or MeSO4 (control) for 8 h. The MEFs were then washed with PBS and stained for 1 h at 37 °C with 100 nM of the mitochondria-specific dye MitoTracker Red (Molecular Probes, Eugene, OR; catalog number M7513). After washing with PBS, MEFs were fixed with 1% glutaraldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 15 min. After washing with PBS and BSA buffer (PBS containing 0.5% bovine serum albumin (BSA) and 0.15% glycine), the MEFs were incubated with normal goat serum (1:20 dilution with BSA buffer, Sigma; catalog number G9023) for 45 min. Subsequently, the MEFs were washed with BSA buffer and treated with anti-cytochrome c antibody (1:400 dilution with BSA buffer) for 2 h, washed with BSA buffer, and incubated with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, catalog number A11001) for 1 h. After washing, cells were treated with DAPI, mounted, and visualized under a Leica DC300F fluorescence microscope.

Statistical Analysis—Paired t test or one-way ANOVA was used to test the significance of difference in measured variables between control and treated groups (followed by Bonferroni’s, Dunnett’s, or Tukey’s multiple comparison test). Statistical significance was determined at the 0.05 level.
respiratory chain complex I (34). Similar to DPI, rotenone treatment alone caused an increase in ethidium bromide fluorescence (results not shown) but did not affect DCF fluorescence (Fig. 2A). On the other hand, the SFN-mediated increase in DCF fluorescence was significantly inhibited on pretreatment with rotenone (Fig. 2B). Because both DPI and rotenone inhibited SFN-induced ROS production, we concluded that mitochondria are involved in this process.

Because SFN is an electrophilic molecule capable of reacting with cellular nucleophiles, including GSH (35), we reasoned that SFN treatment might cause GSH depletion to exacerbate oxidative stress. We therefore determined the effect of SFN treatment on intracellular levels of GSH by using PC-3 cells, and the results are shown in Fig. 2C. Indeed, SFN treatment caused a rapid decline in the level of GSH. For instance, the GSH levels in PC-3 cells treated for 3 and 6 h with 40 μM SFN were reduced by about 90 and 94%, respectively, compared with control (Fig. 2C). These observations led us to conclude that ROS generation on treatment with SFN probably involves both a nonmitochondrial mechanism and a mitochondria-mediated component. However, further studies are needed to determine the precise mechanism of SFN-induced ROS generation involving mitochondria.

SFN Treatment Disrupted Mitochondrial Membrane Potential in PC-3 Cells—We have shown previously (20) that SFN-induced apoptosis in PC-3 cells is associated with activation of caspase-3 and caspase-9. Activation of caspase-9 in response to different stimuli is associated with disruption of the mitochondrial membrane potential leading to release of cytochrome c and other apoptogenic molecules from mitochondria to the cytosol (36, 37). Once in the cytosol, cytochrome c binds to apoptosis protease-activating factor 1 (Apaf-1) and recruits...
and activates caspase-9 in the apoptosome (36, 37). Active caspase-9 cleaves and activates executioner caspases, including caspase-3 (37, 38). Because SFN treatment caused activation of caspase-9 in PC-3 cells (20), we sought to determine whether SFN-induced apoptosis was associated with disruption of the mitochondrial membrane potential. The effect of SFN treatment on mitochondrial membrane potential was determined by flow cytometry following staining with a cationic lipophilic dye TMRM, which accumulates within mitochondria in a potential-dependent manner. Representative histograms for TMRM fluorescence in PC-3 cells following a 3-h treatment with Me2SO (control) or 40 μM SFN are shown in Fig. 3A. As can be seen in Fig. 3A, FCCP (positive control) as well as SFN treatments caused a marked decrease in TMRM retention compared with vehicle-treated control. In time course experiments using 40 μM SFN, disruption of PC-3 mitochondrial membrane potential was evident as early as 2 h after treatment (Fig. 3B). Furthermore, SFN treatment caused release of cytochrome c from mitochondria to the cytosol in a concentration- and time-dependent manner as judged by immunoblotting using mitochondria-free cytosolic fractions (Fig. 3C).

Next, we determined the effect of SFN (40 μM, 6 h) on mitochondrial morphology by transmission electron microscopy, and representative electron micrographs from control and SFN-treated PC-3 cells are shown in Fig. 3D. The mitochondria of SFN-treated PC-3 cells exhibited more or less similar morphology as the Me2SO-treated controls. We did not observe either mitochondrial swelling or any other changes in mitochondrial morphology in SFN-treated PC-3 cells. Collectively,
these results indicated that SFN treatment disrupted mitochondrial membrane potential leading to cytosolic release of cytochrome c without causing any visible changes in mitochondrial morphology, at least after 6 h of treatment.

**NAC Pretreatment Protected against SFN-induced Cell Death**—We raised a question whether ROS production contributed to the cell death caused by SFN. We addressed this question by determining the effect of NAC, a known antioxidant, on SFN-induced ROS generation, mitochondrial membrane potential disruption, and apoptosis induction. As can be seen in Fig. 4A, PC-3 cells treated with SFN exhibited >6-fold increase in DCF fluorescence compared with MeSO-treated control, which was reduced by about 78% on pretreatment with NAC. To determine whether SFN-induced ROS generation was upstream of mitochondrial membrane potential disruption, we measured TMRM retention in PC-3 cells treated with NAC and/or SFN. The SFN-mediated disruption of mitochondrial membrane potential was abolished on pretreatment with NAC (Fig. 4B). In addition, NAC pretreatment conferred significant protection against SFN-induced release of cytochrome c to the cytosol as well as cleavage of PARP and procaspase-9 (Fig. 4C). The NAC treatment alone neither caused PARP cleavage nor reduced the level of procaspase-9 (Fig. 4D). Proteolytic cleavage of procaspase-9 is expected to yield 37–39-kDa intermediates, which we were unable to detect even though the level of procaspase-9 was consistently reduced on treatment with SFN (Fig. 4C). It is possible that the caspase-9 antibody used in the present study did not recognize cleaved intermediates. We therefore determined the activity of caspase-9 by using lysates from PC-3 cells treated with SFN and/or NAC. As can be seen in Fig. 4D, SFN treatment caused an approximate 2.6-fold increase in caspase-9 activity compared with vehicle-treated control, which was nearly fully blocked on pretreatment with NAC. To determine the contribution of ROS to cell death, we determined the effect of NAC pretreatment on SFN-induced apoptosis by measuring cytoplasmic histone-associated DNA fragmentation. As shown in Fig. 4E, the NAC pretreatment offered significant protection against SFN-induced cytoplasmic histone-associated DNA fragmentation. The protective effect of NAC against SFN-induced apoptosis was also observed in the DAPI assay (results not shown). Collectively, these results indicated that SFN-induced apoptosis in PC-3 cells was initiated by ROS, which caused disruption of the mitochondrial membrane potential leading to release of cytochrome c to the cytosol and activation of caspase-9.

**Catalase Overexpression Attenuated ROS Generation and Apoptosis Induction by SFN in DU145 Cells**—Because SFN is an electrophilic molecule capable of reacting with cellular nucleophiles including GSH (35), we raised the question whether NAC-mediated protection against cell death in PC-3 cells (Fig. 4E) was simply because of reduced intracellular accumulation of free SFN. We explored this possibility by determining the effect of adenovirus-mediated transduction of catalase, an H$_2$O$_2$ scavenger, on SFN-induced ROS generation and apoptosis using DU145 human prostate adenocarcinoma cells. We opted to use DU145 cells instead of PC-3 for these studies for two reasons. First, we reasoned that the use of DU145 cells would allow us to test whether the positive correlation between SFN-induced ROS production and apoptosis observed in PC-3 cells was restricted to this cell line. Second, we had used DU145
Data are mean adenoviral vectors containing EGFP (Ad-EGFP) or catalase (Ad-catalase) of cells with high DCF fluorescence in DU145 cells infected with generation and cytochrome c. Densitometric scanning of the infected DU145 cells (Fig. 5B). Furthermore, the SFN-mediated cleavage of PARP was more pronounced in Ad-EGFP-infected DU145 cells, as evidenced by a reduction in the level of 116-kDa full-length PARP and appearance of 89-kDa cleaved intermediate, than in cells infected with Ad-catalase (Fig. 6C). Collectively, these results indicated that the positive correlation between SFN-induced ROS generation and cell death was not restricted to the PC-3 cell line.

Role of ROS in SFN-induced Activation of Caspase-8—We have shown previously (20) that SFN treatment causes cleavage of procaspase-8 in PC-3 cells, and that SFN-induced apoptosis is significantly inhibited in the presence of the caspase-8-specific inhibitor benzoyloxycarbonyl-IETD-fluoromethyl ketone. In the present study, we sought to determine whether SFN-mediated activation of caspase-8 was dependent on ROS generation. We explored this possibility by determining the effect of SFN treatment (40 μM, 24 h) on cleavage of caspase-8 by immunoblotting using DU145 cells infected with Ad-EGFP and Ad-catalase. As can be seen in Fig. 6D, the SFN-mediated cleavage of procaspase-8 was observed in both Ad-EGFP- and Ad-catalase-infected DU145 cells, but this effect was relatively more pronounced in Ad-EGFP-infected DU145 cells than in catalase-overexpressing cells (Fig. 6D). These results suggested that the caspase-8 activation on treatment with SFN might also be dependent on ROS generation. We confirmed this possibility by determining the effect of EUK-134 (a combined SOD and catalase mimetic) on SFN-mediated activation of caspase-8. As shown in Fig. 6E, treatment of PC-3 cells with SFN (40 μM, 24 h) resulted in an approximate 52% increase in caspase-8 activity compared with Me2SO-treated control (p < 0.05). The SFN-induced activation of caspase-8 in PC-3 cells was significantly attenuated on pretreatment with EUK-134 (Fig. 6E). Caspase-8 activation was not observed in cells exposed to EUK-134 alone (Fig. 6E). We also determined the effect of EUK-134 on SFN-induced cytoplasmic histone-associated DNA fragmentation, and the results are shown in Fig. 6F. Similar to the results using NAC (Fig. 4E), the SFN-induced cytoplasmic histone-associated DNA fragmentation was nearly fully blocked on pretreatment with EUK-134 (Fig. 6F). Collectively, these results implicated ROS in SFN-induced activation of caspase-8.

Bcl-2 Overexpression Failed to Confer Significant Protection against SFN-induced Cell Death—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 (39–41). SFN-induced apoptosis in different cellular systems, including PC-3 cells, has been shown to be associated with down-regulation of Bcl-2 (16, 18–20). However, studies are lacking that could experimentally test the contribution of Bcl-2 in cell death caused by SFN. We addressed this question by comparing sensitivities of PC-3 cells stably transfected with a Bcl-2 plasmid and empty vector toward SFN-induced cell death. As shown in Fig. 7A, the level of Bcl-2 protein was about 15-fold higher in PC-3/Bcl-2 cells compared with PC-3/neo. We also performed Bcl-2 immunoblotting using cytosolic and mitochondrial fractions prepared from PC-3/neo and PC-3/Bcl-2 cells following a 24-h treatment with Me2SO (control) or 40 μM SFN. As can be seen in Fig. 7B, the Bcl-2 protein was present in the mitochondrial fraction of both PC-3/neo and PC-3/Bcl-2 cell lines. The blot was reprobed with an antibody against Cox IV to ensure equal protein loading as well as to rule out cross-contamination of the mitochondrial and cytosolic fractions.
fractions. Similar to our previous data in untransfected PC-3 cells (20), SFN treatment caused about 30–60% reduction in Bcl-2 protein level in PC-3/neo and PC-3/Bcl-2 cells (Fig. 7 B).

Sensitivities of PC-3/neo and PC-3/Bcl-2 cells toward apoptosis induction by SFN was compared by examining cytoplasmic histone-associated DNA fragmentation, sub-diploid fraction, and PARP cleavage. As can be seen in Fig. 7 C, SFN treatment caused a dose-dependent increase in cytoplasmic histone-associated DNA fragmentation in both PC-3/neo and PC-3/Bcl-2 cells. The blot was stripped and reprobed with anti-actin antibody to normalize for differences in protein loading. D, immunoblotting for caspase-8 using lysates from Ad-EGFP and Ad-catalase-infected DU145 cells following a 24-h treatment with SFN (40 μM) and without pretreatment with combined SOD/catalase mimic EUK-134. PC-3 cells were pretreated for 2 h with either Me2SO or 30 μM EUK-134. The cells were then either left untreated (Me2SO and EUK-134 alone treatment groups) or exposed to 40 μM SFN for 24 h. E, caspase-8 activity using lysates from PC-3 cells treated with SFN (40 μM) and without pretreatment with combined SOD/catalase mimic EUK-134. Data in E are mean ± S.E. (n = 3). *, significantly different between the indicated groups by one-way ANOVA followed by Bonferroni’s multiple comparison test (p < 0.05) (B, E, and F).

**Fig. 6. Catalase overexpression protected against SFN-induced apoptosis.** A, fluorescence microscopic images of DAPI-stained Ad-EGFP and Ad-catalase-infected DU145 cells following a 16-h treatment with Me2SO (control) or 40 μM SFN. B, cytoplasmic histone-associated DNA fragmentation in Ad-EGFP and Ad-catalase-infected DU145 cells following a 24-h exposure to Me2SO (control) or 40 μM SFN. C, immunoblotting for PARP using lysates from Ad-EGFP and Ad-catalase-infected DU145 cells following a 24-h treatment with Me2SO (control) or 40 μM SFN. The blot was stripped and reprobed with anti-actin antibody to normalize for differences in protein level. D, immunoblotting for caspase-8 using lysates from Ad-EGFP and Ad-catalase-infected DU145 cells following a 24-h treatment with Me2SO (control) or 40 μM SFN. The blot was stripped and reprobed with anti-actin antibody to normalize for differences in protein level. E, caspase-8 activity using lysates from PC-3 cells treated with SFN (40 μM) and without pretreatment with combined SOD/catalase mimic EUK-134. PC-3 cells were pretreated for 2 h with either Me2SO or 30 μM EUK-134. The cells were then either left untreated (Me2SO and EUK-134 alone treatment groups) or exposed to 40 μM SFN for 24 h. F, cytoplasmic histone-associated DNA fragmentation in PC-3 cells following a 24-h exposure to 40 μM SFN with or without 2 h of pretreatment with 30 μM EUK-134. Data in B, E, and F are mean ± S.E. (n = 3). *, significantly different between the indicated groups by one-way ANOVA followed by Bonferroni’s multiple comparison test (p < 0.05) (B, E, and F).
tion compared with vector-transfected control. For example, the DNA fragmentation in PC-3/neo cells following a 24-h treatment with 40 \( \mu \text{M} \) SFN was higher by about 4.3-fold in comparison with Me\(_2\text{SO}\)-treated control. A similar treatment of PC-3/Bcl-xL cells with SFN resulted in an increase of only about 1.7-fold in cytoplasmic histone-associated DNA fragmentation over vehicle-treated control (Fig. 8B). Consistent with these results, the Bcl-xL-overexpressing PC-3 cells were significantly more resistant toward cell killing by SFN compared with PC-3/neo as determined by trypan blue dye exclusion assay (Fig. 8C). For instance, the survival of PC-3/neo cells was reduced by about 97% following a 24-h treatment with 40 \( \mu \text{M} \) SFN compared with the Me\(_2\text{SO}\)-treated control, whereas about 50% of the Bcl-xL-overexpressing cells were viable following a similar treatment with SFN (Fig. 8C). Together, the present study indicates that ectopic expression of Bcl-xL, but not Bcl-2, offers statistically significant protection against cell death caused by SFN.

**SFN Treatment Caused Bid Cleavage and Increased Fas Protein Level in PC-3 Cells—**

Activation of caspase-8 is caused by the extrinsic pathway involving death receptor CD95 (also known as APO-1 or Fas)-mediated signaling (42, 43). Activation of the death receptor initiates trimerization of Fas and recruitment of the adapter molecule Fas-associated death domain (FADD) and procaspase-8 to form the death-inducing signaling complex (42). This leads to autoproteolytic cleavage of procaspase-8. Active caspase-8 can directly cleave and activate caspase-3. Alternatively, caspase-8 can cleave Bid, a pro-apoptotic Bcl-2 member, and truncated Bid translocates to the mitochondria and induces release of cytochrome c (44, 45). Depending on apoptosis signal and cell type, the death-receptor/caspase-8 pathway can either play a direct initiating role or an indirect amplifying role in execution of cell death. Because SFN treatment caused activation of caspase-8 in PC-3 cells, we determined cleavage of Bid by immunoblotting. As shown in Fig. 9A, SFN treatment indeed caused Bid cleavage in PC-3 cells that was prominent at 8–16 h post-treatment. In addition, SFN-treated PC-3 cells exhibited an increase in the protein level of Fas, which was evident as early as 4 h after treatment with 40 \( \mu \text{M} \) SFN (Fig. 9B). It is interesting to note that the protein level of FADD was reduced on treatment with SFN especially at the 16- and 24-h time points (Fig. 9B), which may explain the weak and delayed activation of caspase-8 in SFN-treated PC-3 cells (Fig. 6E). The effect of SFN treatment on protein levels of Bok and Bim EL, which are promoters of apoptosis (46, 47), was also
The level of Bim EL protein was increased by about 3.2–4.9-fold compared with PC-3/neo (lane 1) or Bcl-xL (lanes 2–5). Lanes 2–5 contained lysate proteins from different clones. The clone with the highest level of Bcl-xL protein (clone 18 in lane 3) was used for the experiments described below. B, analysis of cytoplasmic histone-associated DNA fragmentation in PC-3/neo and PC-3/Bcl-xL cells following a 24-h exposure to 20 or 40 μM SFN. Data (mean ± S.E., n = 3) relative to vehicle-treated control are shown. *, significantly different compared with PC-3/neo (p < 0.05). C, effect of SFN treatment on viability of PC-3/neo and PC-3/Bcl-xL cells following a 24-h exposure to Me2SO or indicated concentrations of SFN as determined by trypan blue dye exclusion assay. Data are mean ± S.E. (n = 3). *, significantly different compared with PC-3/neo (p < 0.05).

**Fig. 9.** SFN treatment caused Bid cleavage and increased Fas protein level in PC-3 cells. Immunoblotting for Bid and truncated Bid (tBid) (A), Fas and FADD (B), and Bok and Bim EL (C) using lysates from PC-3 cells treated with 40 μM SFN for the indicated time periods. The blots were stripped and reprobed with anti-actin antibody to ensure equal protein loading.

The relative resistance of Bid-/- MEFs toward SFN-induced cell death was confirmed by analysis of the sub-diploid fraction (Fig. 10B). Activity measurements indicated that caspase-8 acts upstream of Bid in SFN-induced apoptosis because SFN-mediated activation of caspase-8 was observed in WT as well as Bid-/- MEFs (Fig. 10C). We compared the cytosolic release of cytochrome c by immunoblotting using mitochondria-free cytosolic fractions from control and SFN-treated WT and Bid-/- MEFs, and the results are shown in Fig. 10D. The SFN-mediated cytosolic release of cytochrome c was relatively more pronounced in WT MEFs than in the MEFs derived from Bid knock-out mice (Fig. 10D). Some release of cytochrome c in SFN-treated Bid-/- MEFs is expected because apoptosis induction by SFN also involves mitochondria-mediated activation of caspase-9 (Fig. 4D).

The effect of SFN treatment on localization of cytochrome c was also determined by immunohistochemistry (Fig. 10E). In Me2SO-treated (control) WT MEFs, cytochrome c was primarily localized in the mitochondria as evidenced by a yellow-orange staining of the mitochondria due to the merging of green (cytochrome c) and red fluorescence (MitoTracker Red). On treatment with SFN, a large number of mitochondria in WT MEFs were stained red, whereas cytochrome c staining (green fluorescence) was observed in the cytosol, indicating release of cytochrome c from mitochondria to the cytosol. Consistent with the results of cytochrome c immunoblotting (Fig. 10D), some mitochondria of Bid-/- MEFs were also stained red on treatment with SFN due to cytosolic localization of cytochrome c. However, the fraction of cytochrome c-deficient (red) mitochondria on treatment with SFN was much higher in WT MEFs than in the MEFs derived from Bid knock-out mice (Fig. 10E). Based on these results, we conclude that SFN treatment causes caspase-8-mediated Bid cleavage, which triggers cytosolic release of cytochrome c. Because Bid-/- MEFs are not fully resistant to apoptosis induction by SFN, it is reasonable to conclude that SFN-induced cell death is mediated by both death-receptor and mitochondrial caspase cascades that converge on cytosolic release of cytochrome c to trigger activation of caspase-9.
Evidence is accumulating to indicate that SFN can suppress growth of cultured cancer cells as well as tumor xenografts in vivo by causing cell cycle arrest and apoptosis (15–21). Recent studies from our laboratory have offered novel insights into the mechanism by which SFN blocks cell cycle progression (21), but the signaling pathway leading to cell death was poorly defined. The data presented herein indicate that SFN-induced ROS generation is an upstream signaling event that initiates the cell death. Because ROS act as upstream signaling molecules to initiate the cell death, this hypothesis is further supported by our findings that pretreatment of PC-3 cells with NAC not only prevents ROS generation but also confers near-complete protection against SFN-induced mitochondrial membrane potential disruption, cytochrome c release, and cell death. Moreover, catalase overexpressing DU145 cells are significantly more resistant toward SFN-induced ROS generation and apoptosis when compared with vector-infected control cells. ROS generation in apoptosis induction by some agents has been shown to occur downstream of the release of cytochrome c (48, 49), which is not the case in our model because SFN-induced
release of cytochrome c is significantly inhibited by pretreatment with NAC (Fig. 4C) as well as by overexpression of catalase (Fig. 5B). Collectively, these results indicate that ROS act upstream of cytochrome c release during SFN-induced cell death in our model.

The present study suggests that SFN-mediated ROS generation is probably caused by a nonmitochondrial component and a mitochondrial mechanism because SFN-induced ROS production is markedly inhibited in the presence of mitochondrial respiratory chain complex I inhibitors DPI and rotenone (Fig. 2, A and B). We also found that SFN treatment causes a rapid and marked depletion of intracellular GSH levels. Depletion of GSH level is likely to exacerbate SFN-induced oxidative stress. However, other possibilities, which warrant further investigation, also exist to explain ROS generation in SFN-treated cells. For example, the SFN-mediated induction of cytochrome P450 protein level was shown previously to correlate positively with oxidative stress in the lungs of SFN-treated rats (50). Studies have also suggested that \(-\text{N}=\text{C}=\text{S}\) moiety of ITCs can undergo spontaneous hydrolysis leading to ROS production (51). Whether or not ROS generation in SFN-treated cells is cytochrome P450-dependent or caused by hydrolysis of the isothiocyanate moiety requires further experimentation. Because of its electrophilic nature, it is possible that SFN, similar to other alkylating electrophile agents (52), may directly and covalently modify nucleophilic sites, especially the sulfhydryl groups, in mitochondrial respiratory chain enzymes or mitochondrial membranes. Further studies are needed to explore systematically this possibility as well. Nonetheless, the present study provides experimental evidence to demonstrate that SFN-induced apoptosis is initiated by ROS generation.

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 (39–41). Differential interaction among Bcl-2 protein family members as well as their association with other cellular proteins regulates the cell death process (39–41). For example, Bcl-2 normally blocks apoptosis by forming heterodimer complex with proapoptotic proteins such as Bax. The 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 (39–41). On the other hand, 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), induce mitochondrial outer membrane permeabilization and release of apoptogenic molecules from mitochondria to the cytosol (53–55). Previous studies including those from our laboratory have indicated that SFN-induced cell death in different cellular systems is associated with induction of Bax protein and/or down-regulation of Bcl-2 (15, 16, 18–20, 56). Recently, we experimentally tested the role of the multidomain proapoptotic Bcl-2 family members Bax and Bak in SFN-induced apoptosis using SV40-transformed MEFs derived from WT and Bax and/or Bak knock-out mice (56). We found that SFN treatment causes a dose- and time-dependent increase in the protein levels of both Bax and Bak, as well as a conformational change and mitochondrial translocation of Bax in WT MEFs to trigger the release of apoptogenic molecules from mitochondria to the cytosol leading to activation of caspase-9 and -3, and ultimately cell death. The MEFs derived from Bax or Bak knock-out mice resist cell death caused by SFN, but the MEFs derived from Bax and Bak double knock-out mice exhibit even greater protection against SFN-induced cytochrome c release, caspase activation, and apoptosis compared with either WT or single knock-out fibroblasts (56). These observations provided experimental evidence to indicate that Bax and Bak proteins play an important role in SFN-induced cell death (56). In the present study, we extended these observations and investigated whether Bcl-2 offers protection against the cell death caused by SFN using PC-3 cells stably transfected with Bcl-2. To our surprise, Bcl-2 overexpression failed to confer significant protection against cell death caused by SFN as judged by different apoptosis assays including flow cytometric analysis of subdiploid cells, DNA fragmentation assay, and PARP cleavage. On the other hand, Bcl-xL-overexpressing PC-3 cells were found to be significantly more resistant to SFN-induced apoptosis compared with vector-transfected control (Fig. 8, B and C). These observations indicate that the SFN-induced apoptosis, at least in PC-3 cells, is protected by overexpression of Bcl-xL but not Bcl-2. Further studies are needed to determine whether the lack of protection by Bcl-2 overexpression observed in PC-3 cells is restricted to this cell line or applicable to other prostate cancer cells.

Bim is another BH3-only proapoptotic Bcl-2 family member that is expressed in three alternatively spliced forms designated as Bim S (~16 kDa), Bim L (~18 kDa), and Bim EL (~23 kDa) (47). All three forms are capable of inducing cell death, but Bim S is the most potent inducer of apoptosis (47). The pro-apoptotic activity of Bim isoforms is controlled by binding to the dynamin complex, which sequesters them to the cytoskeleton-associated motor complexes (57). Bok (Bcl-2-related ovarian killer), on the other hand, contains the conserved BH1–BH3 domains and is highly expressed in reproductive tissues (46). Similar to other proapoptotic multidomain Bcl-2 members, Bok promotes apoptosis by selectively heterodimerizing with some anti-apoptotic Bcl-2 members, including Mcl-1 and Bfl-1 (46). We found that SFN treatment caused an increase in the protein levels of Bim EL and Bok, which was prominent at the 16–24-h time points. We could not detect the Bim S or Bim L isoforms during immunoblotting of either control or SFN-treated PC-3 cell lysate proteins. We propose that SFN-induced apoptosis in prostate cancer cells is probably amplified due to induction of Bok and Bim EL.

We have previously shown that the SFN-induced apoptosis in PC-3 cells is significantly attenuated on pretreatment with caspase-8-specific inhibitor benzylxoycarbonyl-IETD-fluoromethyl ketone (20). The present study reveals that the activation of caspase-8 in SFN-treated cells is ROS-dependent and accompanied by induction of Fas protein level. The induction of Fas protein expression is evident as early as 4 h after treatment (Fig. 9B). Most interestingly, the protein level of FADD, which facilitates caspase-8 activation at the death-inducing signaling complex, is reduced significantly in cells exposed to SFN (Fig. 9B), which may explain the weak and delayed activation of caspase-8 on treatment with SFN. It is interesting to note that SFN-mediated activation of caspase-8 is significantly attenuated in the presence of SOD/catalase mimetic EUK-134 (Fig. 6E), which suggests that ROS may act upstream of caspase-8 activation in our model. Because ROS can induce Fas expression (58), it is reasonable to postulate that the initial signal for activation of caspase-8 on treatment with SFN also derives from ROS.

Caspase-8 activation can cause cleavage of BID, which is a BH3-only proapoptotic Bcl-2 family member that is exclusively localized in the cytoplasm. The cleaved BID, however, translocates to the mitochondria and triggers cytochrome c release and activation of caspase-9 (44). In the present study, we sought to determine whether apoptosis induction by SFN is regulated by BID. We addressed this question by examining BID cleavage as well as by comparing sensitivity of SV40-immor-
talized MEFs derived from WT and Bid knock-out mice toward SFN-induced apoptosis. We found that treatment of PC-3 cells with SFN results in Bid cleavage. Moreover, the MEFs lacking Bid protein are partially but statistically significantly more resistant toward SFN-induced apoptosis compared with WT MEFs (Fig. 10, A and B). The present study also indicates that caspase-8 acts upstream of Bid because activation of caspase-8 on treatment with SFN was observed in both WT and Bid knock-out MEFs (Fig. 10C). We also observed that the SFN-induced release of cytochrome c is significantly more pronounced in WT MEFs than in the Bid deficient fibroblasts, as judged by immunoblotting (Fig. 10D) and immunohistochemistry (Fig. 10E), suggesting that Bid regulates SFN-mediated release of cytochrome c to the cytosol. Based on these observations, we conclude that Bid protein is indeed involved in the regulation of SFN-induced cell death.

Growth inhibition and apoptosis induction by SFN have been observed at 10–40 µM concentrations (15–21). A fundamental question is whether the micromolar concentrations of SFN needed to trigger cell death are achievable in humans. Even though the answer to this question awaits pharmacokinetic studies in humans using pure SFN, the pharmacokinetic parameters for SFN were determined recently in rats orally dosed with 50 µmol of SFN (59). SFN was detectable in the plasma after 1 h, peaked around 20 µmol at 4 h after dosing, and declined with a half-life of about 2.2 h (59). Previous studies have also indicated that ITCs including SFN can accumulate in mammalian cells in up to millimolar concentrations (60). Thus, it is highly likely that the concentrations of SFN needed to cause cell death may be achievable in humans.

In conclusion, as summarized in Fig. 11, the present study demonstrates that human prostate cancer cells undergo apoptosis in response to treatment with SFN through a mitochondria-mediated pathway that requires ROS generation upstream of disruption of mitochondrial membrane potential, cytochrome c release, and activation of caspase-8/9. We also found that Bid protein plays an important role in regulation of SFN-induced cell death. In addition, we demonstrate that Bel-2, but not Bcl-2, confers significant protection against the cell death caused by SFN. To the best of our knowledge, the present study is the first published report to provide direct experimental evidence linking the ROS generation with the initiation of SFN-induced apoptosis.

Acknowledgments—We are grateful to Dr. Stanley J. Korsmeyer for the generous gift of pSFFV-Bcl-xL and pSFFV-neo plasmids and the MEFs derived from WT and Bid knock-out mice. The assistance of Kamayani Singh in the preparation of figures is also appreciated.

REFERENCES

1. Verhoeven, D. T., Goldbohm, R. A., van Poppel, G., Verhagen, H., and van den Brandt, P. A. (1996) Cancer Epidemiol. Biomarkers Prev. 5, 733–748
2. Cohen, J. H., Kristal, A. R., and Stanford, J. L. (2000) J. Natl. Cancer Inst. 92, 61–68
3. Zhang, S. M., Hunter, D. J., Rosner, B. A., Giovannucci, E. L., Colditz, G. A., Speizer, F. E., and Willett, W. C. (2000) Cancer Epidemiol. Biomarkers Prev. 9, 477–485
4. Ambrosone, C. B., Cazzaniga, A., Budak, K., Haskell, P. N., and Miller, R. A. (1996) Cancer Epidemiol. Biomarkers Prev. 5, 553–560
5. Hecht, S. S. (2002) Drug. Metab. Rev. 35, 395–431
6. Fahey, J. W., Zalewski, A. T., and Talalay, P. (2001) Phytochemistry 56, 5–51
7. Conaway, C. C., Yang, Y. M., and Chung, P. L. (2002) Curr. Drug Metab. 3, 233–235
8. Zhang, Y., Talalay, P., Cho, C. G., and Posner, G. H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2399–2403
9. Zhang, Y., Kessler, T. W., Cho, C. G., Posner, G. H., and Talalay, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3147–3150
10. Fahey, J. W., Zhang, Y., and Talalay, P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10367–10372
11. Chung, F. L., Conaway, C. C., Ras, C. V., and Reddy, B. S. (2000) Cancer Res. 51, 2287–2291
12. Fahey, J. W., Hatzios, X., Dolan, P. M., Kessler, T. W., Scholtus, I., Stephenson, K. K., Talalay, P., and Loo, A. S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7610–7615
13. Brooks, J. D., Paton, V. G., and Vidanes, G. (2001) Cancer Epidemiol. Biomarkers Prev. 10, 949–954
14. Barcelo, S., Gardiner, J. M., Gescher, A., and Chipman, J. K. (1996) Cancer Epidemiol. Biomarkers Prev. 5, 13–20
15. Misiewicz, I., Skupinska, K., and Kasprzycka-Guttman, T. (2003) Oncol. Rep. 10, 2045–2050
16. Wang, L., Liu, D., Ahmed, T., Chung, F. L., Conaway, C., and Chiao, J. W. (2004) Int. J. Oncol. 24, 187–192
17. Jackson, S. J. T., and Singletary, K. W. (2004) Cancer Res. 64, 1426–1433
18. Kim, Y., Yoon, H., and Kim, B. K. (2002) Carcinogenesis 23, 581–586
19. Misiewicz, I., Skupinska, K., and Kasprzycka-Guttman, T. (2003) Oncol. Rep. 10, 2045–2050
20. Singh, A. V., Xiao, D., Lew, K. L., Dhir, R., and Singh, S. V. (2004) Cancer Res. 64, 63–90
21. Singh, S. V., Herman-Antosiewicz, A., Singh, A. V., Lew, K. L., Srivastava, S. K., Kamath, R., Brown, K. D., Zhang, L., and Baskaran, R. (2004) J. Biol. Chem. 279, 25813–25822
22. Myzak, M. C., Karplus, P. A., Chung, F. L., and Dashwood, R. H. (2004) Cancer Res. 64, 5767–5774
23. Wei, M. C., Zong, W. X., Cheng, E. H., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., and Korsmeyer, S. J. (2001) Science 292, 727–730
24. Xiao, D., Choi, S., Johnson, D. E., Vogel, V. G., Johnson, C. S., Trump, D. L., Lee, Y. J., and Singh, S. V. (2004) Oncogene 23, 5594–5606
25. Rothe, G., and Valet, G. J. (1990) J. Leukocyte Biol. 47, 440–448
26. Narayanan, P. K., Goodwin, E. H., and Lehnert, B. E. (1997) Cancer Res. 57, 3963–3971
27. Watkins, S. C., and Cullen, M. J. (1987) J. Neurol. Sci. 82, 181–192
28. Song, J. J., Rhee, J. G., Suntharalingam, M., Walsh, S. A., Spitz, D. R., and Lee, Y. J. (2002) J. Biol. Chem. 277, 46566–46575
29. Leach, J. K., Van Tuyle, G., Lin, P. S., Schmidt-Ullrich, R., and Mikkelsen, R. B. (2001) Cancer Res. 61, 3894–3901
30. Buccellato, L. J., Tso, M., Akinci, O. I., Chandel, N. S., and Budinger, G. R. S. (2004) J. Biol. Chem. 279, 6753–6760
31. Yang, J., Yu, Y., Hamrick, H. E., and Duerksen-Hughes, P. J. (2003) Carcinogenesis 24, 1571–1580
32. Li, N., Ragheb, K., Lawler, G., Sturgis, J., Rajwa, B., Melendez, J. A., and Robinson, J. P. (2003) Free Radic. Biol. Med. 34, 465–477
33. Genova, M. L., Pich, M. M., Bernacchia, A., Bianchi, C., Biondi, A., Bovina, C., Falasca, A. I., Formiggini, G., Castelli, G. P., and Lenaz, G. (2004) Ann. N. Y. Acad. Sci. 1011, 86–100
34. Ricci, J. E., Waterhouse, N., and Green, D. R. (2003) Cell Death Differ. 10, 488–492
35. Kelm, R. H., Danielson, U. H., Zhang, Y., Talalay, P., and Mannervik, B. (1995) Biochem. J. 311, 453–459
36. Green, D. R., and Reed, J. C. (1998) Science 281, 1309–1312
37. Hengartner, M. O. (2000) Nature 407, 770–776
38. Thornberry, N., and Lazebnick, Y. (1998) Science 281, 1312–1316
39. Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D., and Korsmeyer, S. J. (1990) Nature 346, 334–336
40. Reed, J. C. (1997) Semin. Hematol. 34, 9–19
41. Chao, D. T., and Korsmeyer, S. J. (1998) Annu. Rev. Immunol. 16, 395–419
42. Ashkenazi, A., and Dicit, V. M. (1998) Science 281, 1305–1308
43. Rischel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Pawliwa, M., Krammer, P. H., and Peter, M. E. (1995) EMBO J. 14, 5579–5588
44. Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998) Cell 94, 491–501
45. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998) Cell 94, 481–490
46. Hsu, S. Y., Koppia, M. C., McGee, E., Lemoli, M., and Hseuh, A. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12401–12406
47. O’Connor, L., Strasser, A., O’Reilly, L. A., Hausmann, G., Adams, J. M., Cory, S., and Huang, D. C. (1998) EMBO J. 17, 384–395
48. Cai, J., and Jones, D. P. (1998) J. Biol. Chem. 273, 11401–11404
49. Gottlieb, E., Vander Heiden, M. G., and Thompson, C. B. (2000) Mol. Cell. Biol. 20, 5680–5689
50. Paolini, M., Poreco, P., Canistro, D., Valgimuigili, L., Pedulli, G. F., Iori, R., Croce, C. D., Cantelli-Forti, G., Legator, M. S., and Abdel-Rahman, S. Z. (2004) Carcinogenesis 25, 61–67
51. Murata, M., Yamashita, N., Inoue, S., and Kasahishki, S. (2000) Free Radic. Biol. Med. 28, 797–805
52. Kobliakov, V. A., Karamysheva, A. F., Ozrina, R. D., and Iaguzhinskii, L. S. (1976) Biokhimija 41, 1497–1503
53. Adams, J. M., and Cory, S. (1998) Science 281, 1322–1326
54. Martinou, J. C., and Green, D. R. (2001) Nat. Rev. Mol. Cell. Biol. 2, 63–67
55. Zong, W. X., Lindsten, T., Ross, A. J., MacGregor, G. R., and Thompson, C. B. (2003) Genes Dev. 15, 1481–1486
56. Choi, S. A., and Singh, S. V. (2005) Cancer Res. 65, 2035–2043
57. Puthalakath, H., Huand, D. C., O’Reilly, L. A., King, S. M., and Strasser, A. (1999) Mol. Cell 3, 287–296
58. Denning, T. L., Takaishi, H., Crowe, S. E., Boldogh, I., Jevnikar, A., and Ernst, P. B. (2002) Free Radic. Biol. Med. 33, 1841–1850
59. Hu, R., Hebb, V., Kim, B. R., Chen, C., Winnik, B., Buckley, B., Sotropoulos, P., Tolias, P., Hart, R. P., and Kong, A. N. T. (2004) J. Pharmacol. Exp. Ther. 310, 263–271
60. Ye, L., and Zhang, Y. (2001) Carcinogenesis 22, 1987–1992
Sulforaphane-induced Cell Death in Human Prostate Cancer Cells Is Initiated by Reactive Oxygen Species
Shivendra V. Singh, Sanjay K. Srivastava, Sunga Choi, Karen L. Lew, Jedrzej Antosiewicz, Dong Xiao, Yan Zeng, Simon C. Watkins, Candace S. Johnson, Donald L. Trump, Yong J. Lee, Hui Xiao and Anna Herman-Antosiewicz

J. Biol. Chem. 2005, 280:19911-19924.
doi: 10.1074/jbc.M412443200 originally published online March 11, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M412443200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 60 references, 28 of which can be accessed free at http://www.jbc.org/content/280/20/19911.full.html#ref-list-1