

Research Paper

Cellular Responses to Cancer Chemopreventive Agent D, L-Sulforaphane in Human Prostate Cancer Cells Are Initiated by Mitochondrial Reactive Oxygen Species

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Purpose. Present study was undertaken to elucidate the mechanism of cellular responses to D,L-sulforaphane (SFN), a highly promising cancer chemopreventive agent.

Methods. Mitochondrial DNA deficient Rho-0 variants of LNCaP and PC-3 cells were generated by culture in the presence of ethidium bromide. Apoptosis was assessed by analysis of cytoplasmic histone-associated DNA fragmentation and activation of caspase-3. Immunoblotting was performed to determine the expression of apoptosis- and cell cycle-regulating proteins. Generation of reactive oxygen species (ROS), mitochondrial membrane potential (MMP), and cell cycle distribution were measured by flow cytometry.

Results. The Rho-0 variants of LNCaP and PC-3 cells were significantly more resistant to SFN-induced ROS generation, apoptotic DNA fragmentation, disruption of MMP, cytosolic release of cytochrome *c*, and G2/M phase cell cycle arrest compared with corresponding wild-type cells. SFN-induced autophagy, which serves to protect against apoptotic cell death in PC-3 and LNCaP cells, was also partially but markedly suppressed in Rho-0 variants compared with wild-type cells. SFN statistically significantly inhibited activities of mitochondrial respiratory chain enzymes in LNCaP and PC-3 cells.

Conclusion. These results indicate, for the first time, that mitochondria-derived ROS serve to initiate diverse cellular responses to SFN exposure in human prostate cancer cells.

KEY WORDS: chemoprevention; prostate cancer; sulforaphane.

INTRODUCTION

Epidemiological data continues to lend support to the idea that dietary intake of cruciferous vegetables may lower

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the risk of different types of malignancies including cancer of the prostate (1–4). Anticarcinogenic effect of cruciferous vegetables is attributed to organic isothiocyanates (ITCs), which are released upon processing (cutting and chewing) of these vegetables due to myrosinase-mediated hydrolysis of corresponding glucosinolates (5,6). Broccoli is a rather rich source of the ITC compound (–)-1-isothiocyanato-(4*R*)-(methylsulfinyl)-butane (L-SFN), which together with its synthetic racemic analogue D,L-sulforaphane (SFN) has received particular attention due to remarkable anticancer properties. For example, L-SFN and SFN are equipotent inducers of phase 2 drug metabolizing enzyme quinone reductase in Hepa1c1c7 murine hepatoma cells (7). Exposure of prostate cancer cells to L-SFN resulted in transcriptional up-regulation of γ -glutamylcysteine synthetase light subunit and glutathione transferases (8). The L-SFN or synthetic SFN has been shown to afford significant protection against 9,10-dimethyl-1,2-benzanthracene-induced mammary carcinogenesis in rats, azoxymethane-induced colonic aberrant crypt foci in rats, and benzo[*a*]pyrene-induced forestomach cancer in mice (9–11). Dietary feeding of SFN inhibited malignant progression of lung adenomas induced by tobacco carcinogens in A/J mice (12).

More recent studies including those from our laboratory have documented novel cellular responses to SFN exposure

in cultured human cancer cells, including G2/M phase cell cycle arrest, induction of apoptosis and autophagy, inhibition of histone deacetylase, protein binding, and sensitization of cells to TRAIL-induced apoptosis (13–25). The SFN-mediated signal transduction pathways culminating in growth arrest and apoptosis induction have been extensively studied in human prostate cancer cells (15–17,19–21,25). For example, we have shown previously that the SFN-induced apoptosis is selective towards prostate cancer cells and correlates with generation of reactive oxygen species (ROS) (18,19). The SFN-induced apoptosis in prostate cancer cells was accompanied by depletion of intracellular glutathione levels and blunted by antioxidants (19). Pretreatment of prostate cancer cells with antioxidants including *N*-acetylcysteine and a combined mimetic of superoxide dismutase and catalase (EUK134) as well as adenovirus-mediated transduction of catalase conferred significant protection against SFN-induced ROS generation, apoptotic DNA fragmentation, cytosolic release of cytochrome *c*, collapse of mitochondrial membrane potential, and caspase activation (19). These results pointed towards critical role of ROS in signal transduction by SFN (19). We have also shown previously that oral gavage of SFN significantly retards growth of PC-3 human prostate cancer xenografts in nude mice and inhibits prostate carcinogenesis and pulmonary metastasis in a transgenic mouse model of prostate cancer (26,27).

Despite these advances, however, the mechanism by which SFN causes ROS production remains elusive. In the present study we generated mitochondrial DNA deficient Rho-0 variants of LNCaP and PC-3 cells to determine possible involvement of mitochondria in anticancer signal transduction by SFN. We now demonstrate, for the first time, that cellular responses to SFN exposure (cell cycle arrest, apoptosis induction and autophagy) in human prostate cancer cells are initiated by the mitochondria-derived ROS due to inhibition of mitochondrial respiratory chain (MRC) enzymes.

MATERIALS AND METHODS

Reagents

SFN (purity >99%) was synthesized as described by Conaway *et al.* (12). SFN was stored at -20°C and found to be stable for at least 4 months as judged by high-performance liquid chromatography (12). Reagents for cell culture including F-12K Nutrient Mixture, RPMI 1640 medium, penicillin and streptomycin antibiotic mixture, and serum were purchased from GIBCO (Grand Island, NY). Hydroethidine (HE), 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were purchased from Molecular Probes (Eugene, OR) whereas 4',6-diamidino-2-phenylindole (DAPI) was obtained from Sigma (St. Louis, MO). The ELISA kit for quantitation of cytoplasmic histone-associated DNA fragmentation was from Roche Diagnostics (Mannheim, Germany). The antibody against cytochrome *c* oxidase subunit IV (COXIV) was from Molecular Probes; the anti-cytochrome *c* antibody was from BD Pharmingen (Palo Alto, CA); antibodies against Bax, Bak, cyclinB1, Tyr15 phosphorylated cyclin-dependent kinase

1 (cdk1), and Ser10 phosphorylated histone H3 were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-catalase antibody was from Calbiochem (Gibbstown, NJ); antibody against microtubule-associated protein 1 light chain 3 (LC3) was from Cell Signaling (Danvers, MA); and anti-Bcl-2 antibody was from DAKO Cytomation (Carpinteria, CA).

Cell Lines and Generation of Rho-0 Variants

Monolayer cultures of PC-3 cells were maintained in F-12K Nutrient Mixture supplemented with 7% non-heat inactivated fetal bovine serum and antibiotics. The LNCaP cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2.4 mg/mL glucose, 1 mmol/L sodium pyruvate, and antibiotics. Both cell lines were maintained at 37°C in an atmosphere of 5% CO_2 and 95% air. The Rho-0 variants of LNCaP and PC-3 cells were generated and maintained as described previously by King and Attadi (28) with some modifications. Briefly, the cells were cultured in complete medium supplemented with 1 mmol/L sodium pyruvate, 1 mmol/L uridine and 2.5 $\mu\text{mol/L}$ ethidium bromide over a period of 7 weeks. Cells cultured in parallel in medium without ethidium bromide were used as controls (wild-type cells).

Immunocytochemical Analysis for COXIV

Wild-type and Rho-0 variants of LNCaP and PC-3 cells (1×10^5) were plated on coverslips and allowed to attach by overnight incubation. Cells were first treated with 200 nmol/L MitoTracker Red at 37°C for 30 min to stain mitochondria. After washing with PBS, the cells were fixed with 2% paraformaldehyde overnight at 4°C and permeabilized using 0.1% Triton X-100 in PBS for 10 min. The cells were washed with PBS, blocked with 0.5% bovine serum albumin (BSA) in PBS for 1 h, and incubated with anti-COXIV antibody overnight at 4°C . The cells were then washed with PBS, incubated with Alexa Fluor 488-conjugated secondary antibody (1:1,000 dilution, Molecular Probes) for 1 h at room temperature. Subsequently, the cells were washed with PBS and treated with DAPI (10 ng/mL) for 5 min at room temperature to stain nuclear DNA. The cells were washed twice with PBS and examined under a Leica fluorescence microscope at $\times 40$ objective lens magnification.

Immunoblotting

The cells were treated with 20 or 40 $\mu\text{mol/L}$ SFN and lysed as described by us previously (29). The mitochondria-free cytosolic fraction for immunoblotting of cytochrome *c* was prepared as described by us previously (25). The lysate proteins were resolved by 6–12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto membrane. Immunoblotting was performed as described by us previously (21,25,29).

Measurement of MRC Enzyme Activities

Cells were plated at a density of 1×10^6 in 100-mm culture dishes, allowed to attach by overnight incubation, and treated with DMSO (control) or different concentrations of SFN for 6 h at 37°C . Cells were then harvested by scraping, washed

with PBS, and lysed. Protein concentration was determined using the Bradford reagent. Activity of complex I-linked NADH-ubiquinone oxidoreductase, complex II-linked succinate-ubiquinone oxidoreductase, and complex III-linked ubiquinol cytochrome *c* reductase was determined as described by us previously (30).

Measurement of ROS Generation

Intracellular ROS generation in DMSO-treated control and SFN-treated cells (20 μ mol/L SFN for 4 h) was measured by flow cytometry following staining with HE and H₂DCFDA as described by us previously (19). The 2',7'-dichlorofluorescein (DCF) fluorescence was measured using a Coulter Epics XL Flow Cytometer.

Determination of Apoptotic DNA Fragmentation, Cell Viability, and Caspase-3 Activation

Apoptosis induction by SFN was assessed by analysis of cytoplasmic histone-associated DNA fragmentation using a kit from Roche Diagnostics according to the manufacturer's instructions. Activation of caspase-3 was determined by flow

cytometry using a kit from Cell Signaling. The cells were treated with DMSO (control) or SFN for specified time period, and processed for flow cytometric analysis of caspase-3 activation according to the manufacturer's instructions. The effect of SFN treatment on cell viability was determined by trypan blue dye exclusion assay as described by us previously (31).

Measurement of Mitochondrial Membrane Potential (MMP) and Cell Cycle Distribution

MMP was measured using a potential-sensitive dye JC-1 (32). Stock solution of JC-1 (1 mg/mL) was prepared in DMSO and freshly diluted with the assay buffer. Briefly, cells (2×10^5) were plated in T25 flasks, allowed to attach by overnight incubation, exposed to desired concentrations of SFN for specified time periods, and collected by trypsinization. The cells were incubated with medium containing JC-1 (10 μ g/mL) for 15 min at 37°C. The cells were washed, re-suspended in 0.5 mL assay buffer, and analyzed using a Coulter Epics XL Flow Cytometer. Mitochondrial uncoupler carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; 25 μ M) was used as a positive control. The effect of SFN treatment on cell cycle distribution was determined by flow cytometry following staining

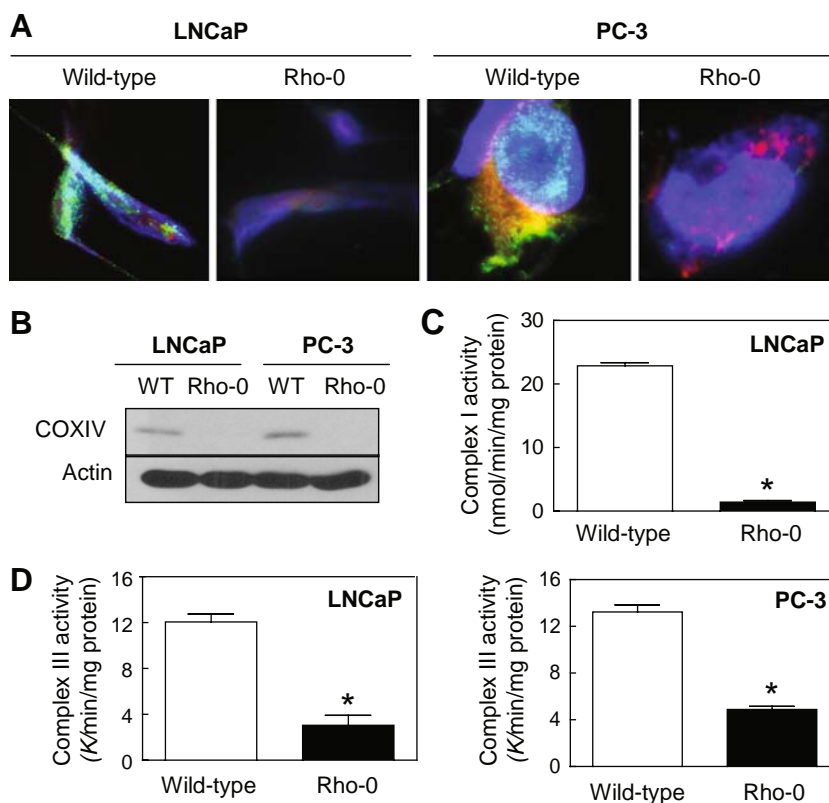


Fig. 1. Characterization of Rho-0 variants of LNCaP and PC-3 cells. **A** Fluorescence microscopic analysis of cytochrome *c* oxidase subunit IV (COXIV) expression in wild-type LNCaP and PC-3 cells and their Rho-0 variants. The staining for mitochondria (MitoTracker red), COXIV, and nuclei (DAPI) are indicated by red, green and blue fluorescence, respectively. **B** Immunoblotting for COXIV using lysates from wild-type LNCaP and PC-3 cells and their Rho-0 variants. **C** Activity of complex I using lysate proteins from wild-type and Rho-0 LNCaP cells. **D** Activity of complex III using lysate proteins from wild-type LNCaP and PC-3 cells and their Rho-0 variants. Results are mean \pm SE of three determinations. *Significantly different ($P < 0.05$) compared with wild-type cells by *t*-test.

the cells with propidium iodide essentially as described by us previously (15). Cells in different phases of the cell cycle were computed for DMSO-treated control and SFN-treated cultures.

Detection of Autophagy

Autophagy induction by SFN was assessed by (a) analysis of acidic vesicular organelles (AVOs) by fluorescence microscopy following staining with lysosomotropic agent acridine orange, (b) immunoblotting for cleavage of LC3, and (c) immunofluorescence microscopy to determine recruitment of LC3 to autophagosomes essentially as described by us previously (21).

Statistical Analysis

One-way ANOVA or *t*-test was used to determine statistical significance of difference in measured variables between control and treated groups. Difference was considered significant at $P < 0.05$.

RESULTS

Rho-0 Variants of LNCaP and PC-3 Cells were Resistant to ROS Generation and Apoptosis Induction by SFN

To determine role of mitochondria in ROS generation by SFN, we generated Rho-0 variants of LNCaP and PC-3 cells by culture in the presence of ethidium bromide. The survival of Rho-0 cells is dependent on ATP derived from anaerobic glycolysis but these cells have functional F1-ATPase (33–35). The Rho-0 cells are unable to generate ROS from MRC. Initially, we carried out experiments to confirm Rho-0 phenotype of the variant LNCaP and PC-3 cells. As can be seen in Fig. 1A, the mitochondria in wild-type cells were brightly stained with MitoTracker red and COXIV, which is encoded by the mitochondrial DNA, as revealed by fluorescence microscopy. The intensity of the MitoTracker red and COXIV staining was much weaker in Rho-0 cells than in the wild-type cells (Fig. 1A). Consistent with these results, immunoblotting revealed expression of COXIV in wild-type LNCaP and PC-3 cells but not in their Rho-0 variants (Fig. 1B). In addition, the Rho-0 variants of LNCaP and PC-3 cells exhibited significantly diminished activities of complex I (Fig. 1C) and complex III (Fig. 1D) of the MRC. These results confirmed Rho-0 phenotype of the variant LNCaP and PC-3 cells.

Next, we proceeded to determine the effect of SFN treatment on ROS generation and apoptosis induction using wild-type LNCaP and PC-3 cells and their Rho-0 variants. As expected, exposure of wild-type LNCaP and PC-3 cells to 20 $\mu\text{mol/L}$ SFN for 4 h resulted in ROS generation as evidenced by a statistically significant increase in DCF fluorescence over DMSO-treated control (Fig. 2A). The SFN-mediated increase in DCF fluorescence was not observed in the Rho-0 variants of LNCaP and PC-3 cells (Fig. 2A). The SFN treatment (20 or 40 $\mu\text{mol/L}$, 24 h) caused significant increase in cytoplasmic histone-associated DNA fragmentation in the wild-type LNCaP and PC-3 cells compared with corresponding DMSO-treated controls (Fig. 2B). The SFN-mediated cytoplasmic histone-associated DNA fragmentation was significantly lower in Rho-0 variants of LNCaP and PC-3 cells compared with corresponding

wild-type cells (Fig. 2B). The Rho-0 cells were significantly more resistant to SFN-mediated suppression of cell viability compared with the wild-type cells (Fig. 2C). These results clearly indicated that SFN-mediated apoptosis induction and growth inhibition in both LNCaP and PC-3 cells are initiated by the mitochondria-derived ROS.

ROS Acted Upstream of Caspase-3 Activation and MMP Collapse in SFN-Induced Apoptosis

Next, we determined the effect of SFN treatment on caspase-3 activation, MMP, and cytochrome *c* release using wild-type PC-3 cells and its Rho-0 variant. Twenty-four hour exposure of wild-type PC-3 cells to 20 $\mu\text{mol/L}$ SFN resulted in about ten-fold enrichment of active caspase-3 (Fig. 3A). The SFN-mediated activation of caspase-3 was only two-fold

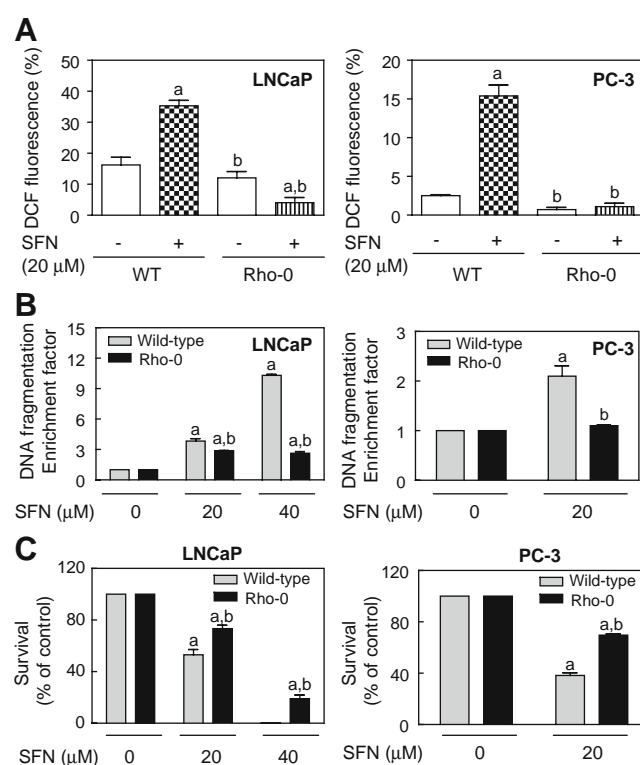


Fig. 2. Rho-0 variants of LNCaP and PC-3 cells are significantly more resistant towards ROS generation and apoptosis induction by SFN compared with wild-type cells. **A** DCF fluorescence (a measure of ROS generation) in wild-type LNCaP and PC-3 cells and their Rho-0 variants following 4 h treatment with DMSO or 20 $\mu\text{mol/L}$ SFN. Results are expressed as percentage of DCF positive cells. **B** Analysis of cytoplasmic histone-associated DNA fragmentation in wild-type LNCaP and PC-3 cells and their Rho-0 variants following 24 h treatment with DMSO or the indicated concentrations of SFN. Results are expressed as enrichment factor relative to DMSO-treated control for both wild-type and Rho-0 cells. **C** Trypan blue dye exclusion assay to assess cell viability in wild-type LNCaP and PC-3 cells and their Rho-0 variants following 24 h treatment with DMSO or SFN. Results are mean \pm SE ($n=3$). Significantly different ($P < 0.05$) compared with *a* corresponding DMSO-treated control and *b* SFN-treated wild-type cells by one-way ANOVA followed by Bonferroni's test. Each experiment was performed at least twice with triplicate measurements in each experiment. The results were consistent and representative data from a single experiment are shown.

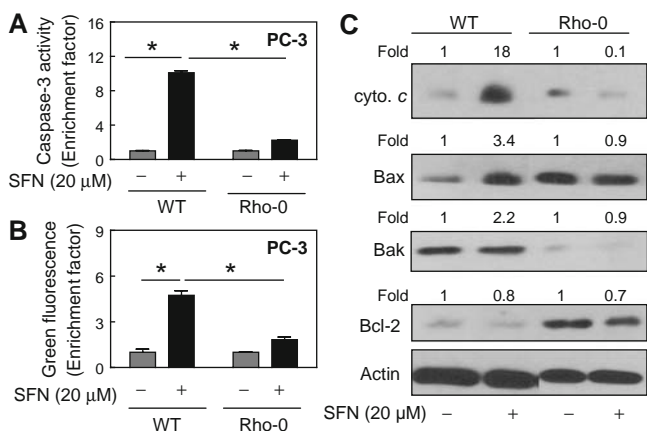


Fig. 3. ROS act upstream of mitochondrial membrane potential collapse in SFN-induced apoptosis. **A** Flow cytometric analysis of caspase-3 activation in wild-type PC-3 cells and its Rho-0 variant following 24 h treatment with DMSO or 20 μ mol/L SFN. Results are expressed as enrichment factor relative to DMSO-treated wild-type cells. **B** Analysis of mitochondrial membrane potential collapse (monomeric JC-1-associated green fluorescence) in wild-type and Rho-0 PC-3 cells following 4 h treatment with DMSO or 20 μ mol/L SFN. Results are expressed as enrichment factor relative to DMSO-treated wild-type cells. **C** Immunoblotting for cytochrome *c*, Bax, Bak, and Bcl-2 using mitochondria-free cytosolic fraction (cytochrome *c*) or whole lysates from wild-type and Rho-0 PC-3 cells treated for 24 h with DMSO or 20 μ mol/L SFN. The numbers on top of the immunoreactive bands represent change in protein levels relative to DMSO-treated control in both wild-type and Rho-0 PC-3 cells. Each experiment was performed at least twice and the results were comparable. In panels **A** and **B**, results are mean \pm SE ($n=3$). *Significantly different ($P<0.05$) between the indicated groups by one-way ANOVA followed by Bonferroni's test.

higher over DMSO-treated control in Rho-0 variant of PC-3 cells (Fig. 3A). Next, we determined effect of SFN treatment on MMP using JC-1 dye. The collapse of the MMP is characterized by green fluorescence due to accumulation of monomeric JC-1 in the cytosol (32). The SFN treatment (20 μ mol/L, 4 h) caused a marked increase in monomeric JC-1-associated green fluorescence in wild-type PC-3 cells, which was nearly completely inhibited in the Rho-0 variant (Fig. 3B). Treatment with 20 μ mol/L SFN for 24 h resulted in cytosolic release of cytochrome *c* in wild-type PC-3 cells but not in its Rho-0 variant (Fig. 3C). Collectively, these results indicated that ROS acted upstream of disruption of MMP and caspase-3 activation in SFN-induced apoptosis.

SFN-Mediated Changes in Levels of Bcl-2 Family Proteins

We addressed the question of whether resistance of Rho-0 cells to SFN-mediated apoptosis was due to lack of change in ratio of proapoptotic to anti-apoptotic Bcl-2 family protein levels. As can be seen in Fig. 3C, SFN treatment (20 μ mol/L, 24 h) caused marked induction of Bax protein level and modest decline in the levels of Bcl-2 protein in wild-type PC-3 cells. These changes were less apparent in the Rho-0 variant of PC-3 cells (Fig. 3C). Interestingly, the stress caused by depletion of mitochondrial DNA in Rho-0 PC-3 variant also resulted in up-regulation of both Bax and Bcl-2, but not Bak, in comparison with wild-type cells (Fig. 3C). These results provided further evidence that mitochon-

dria-derived ROS acted upstream of Bax induction in regulation of SFN-induced apoptotic cell death.

Critical Role of ROS in SFN-Induced G2/M Phase Cell Cycle Arrest

We designed experiments to test a hypothesis that the initial signal for SFN-mediated G2/M phase cell cycle arrest stems from ROS production. As can be seen in Fig. 4A, 24 h exposure of wild-type LNCaP cells to 20 μ mol/L SFN resulted in enrichment of G2/M fraction over DMSO-treated control. The SFN-mediated G2/M phase cell cycle arrest was not evident in the Rho-0 variant of LNCaP cells (Fig. 4A). Likewise, the Rho-0 variant of PC-3 cells was significantly more resistant to SFN-mediated G2/M phase cell cycle arrest compared with wild-type PC-3 cells (results not shown). We

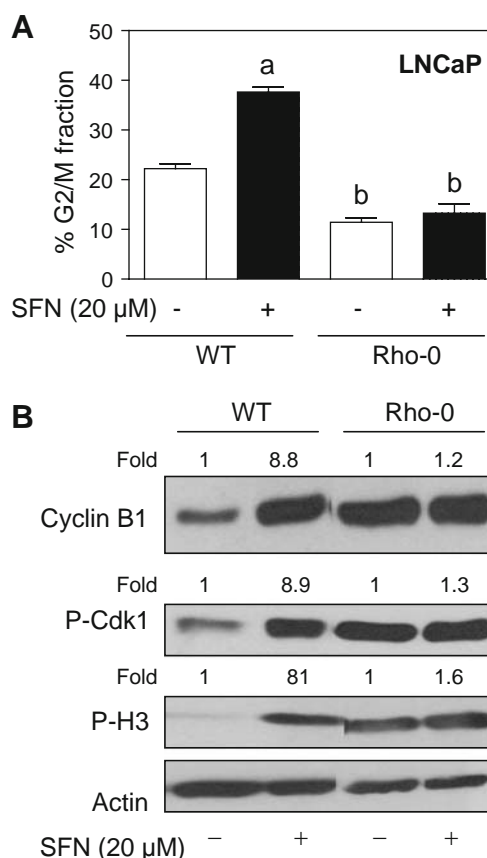


Fig. 4. SFN-induced G2/M arrest is dependent on mitochondria-derived ROS production. **A** Percentage of G2/M phase cells in wild-type LNCaP culture and its Rho-0 variant following 24 h treatment with DMSO or 20 μ mol/L SFN. Results are mean \pm SE ($n=3$). Significantly different ($P<0.05$) compared with *a* corresponding DMSO-treated control and *b* SFN-treated wild-type cells by one-way ANOVA followed by Bonferroni's test. **B** Immunoblotting for cyclin B1, Tyr15 phosphorylated (inactive) cdk1, and Ser10 phosphorylated histone H3 (a marker of mitotic cells) using lysates from wild-type and Rho-0 PC-3 cells treated for 24 h with DMSO or 20 μ mol/L SFN. The numbers on top of the immunoreactive bands represent change in protein levels relative to DMSO-treated control in both wild-type and Rho-0 PC-3 cells. Each experiment was performed at least twice and the results were comparable. Representative data from a single experiment are shown.

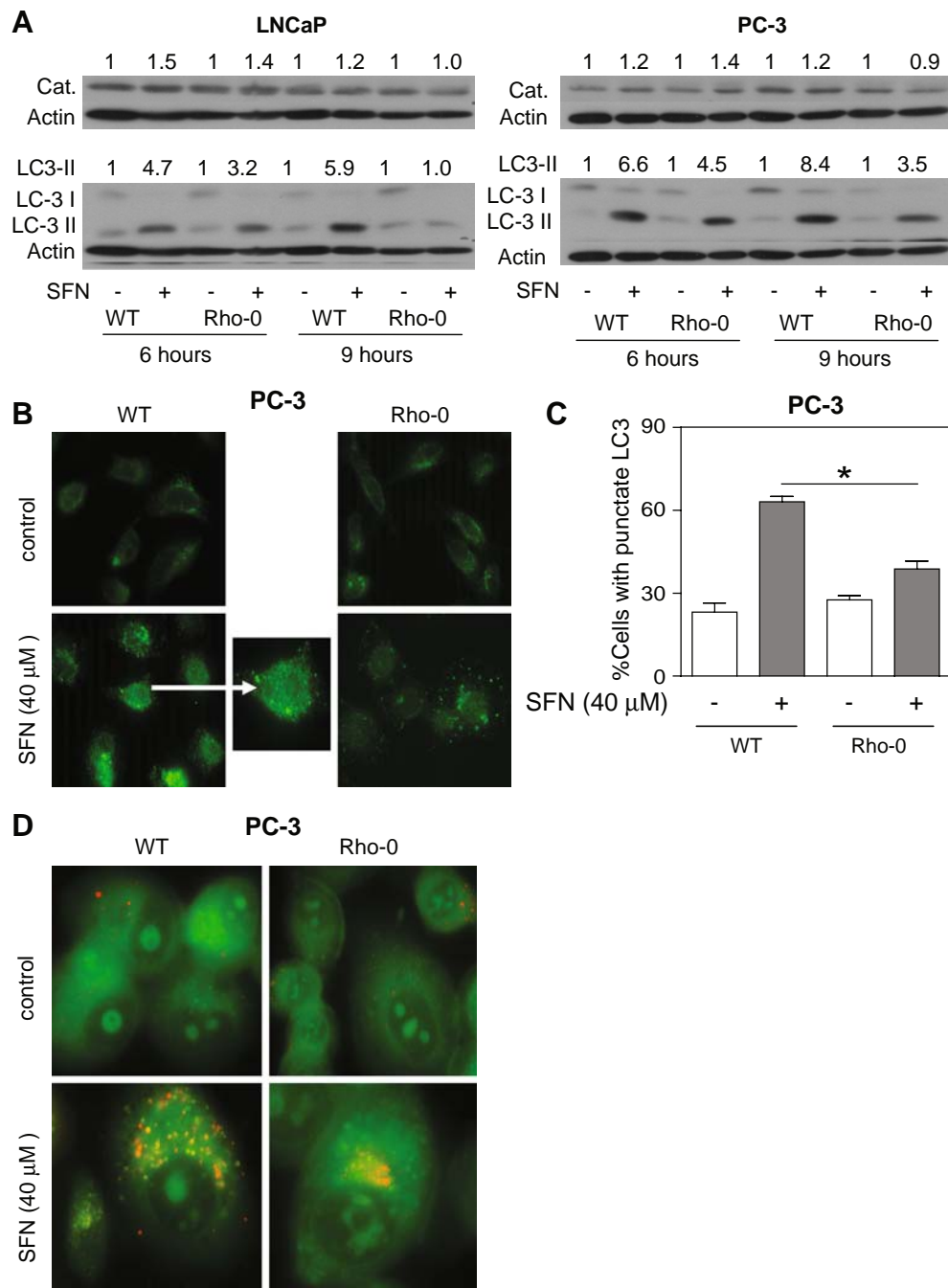


Fig. 5. SFN-induced autophagy in LNCaP and PC-3 cells is partially abrogated in Rho-0 cells. **A** Immunoblotting for catalase and cleaved LC3 (LC3-II) using lysates from wild-type LNCaP and PC-3 cells and their Rho-0 variants following 6 or 9 h treatment with DMSO or 40 μmol/L SFN. The numbers on top of the immunoreactive bands represent change in protein levels relative to DMSO-treated control in both wild-type and Rho-0 LNCaP and PC-3 cells. **B** Immunofluorescence analysis for punctate pattern of LC3 localization in wild-type PC-3 cells and its Rho-0 variant following 9 h treatment with DMSO or 40 μmol/L SFN (magnification ×100). **C** Percentage of cells with punctate pattern of LC3 localization in wild-type PC-3 culture and its Rho-0 variant following 9 h treatment with DMSO or 40 μmol/L SFN. Results are mean ± SE ($n=3$). *Significantly different ($P<0.05$) between the indicated groups by one-way ANOVA followed by Bonferroni's test. **D** Visualization of acidic vesicular organelles (yellow-orange) in wild-type PC-3 cells and its Rho-0 variant following 9 h treatment with DMSO or 40 μmol/L SFN (magnification ×100). Each experiment was performed at least twice and the results were comparable. Representative data from a single experiment are shown.

performed immunoblotting for key proteins involved in regulation of G2/M transition to confirm resistance of Rho-0 cells towards SFN-induced cell cycle arrest. As shown in Fig. 4B, the SFN treatment (20 $\mu\text{mol/L}$, 24 h) resulted in upregulation of cyclinB1 and increased Tyr15 phosphorylation (inactivation) of cdk1 in wild-type PC-3 cells, which was not observed in the Rho-0 variant (Fig. 4B). The SFN treatment also increased Ser10 phosphorylation (a marker of mitotic cells) of histone H3 in wild-type PC-3 cells but not in its Rho-0 variant (Fig. 4B). The immunoblotting for cell cycle regulatory proteins also suggested that Rho-0 cells were probably growth arrested in G2 and mitotic phases as evidenced by increased phosphorylations of cdk1 and histone H3, respectively, in DMSO-treated Rho-0 PC-3 cells (Fig. 4B). Collectively, these results indicated that (a) the mitochondrial stress resulting from depletion of mitochondrial DNA caused growth arrest even in the absence of SFN treatment, and (b) the signal transduction for SFN-induced G2/M phase cell cycle arrest was also initiated by the mitochondria-derived ROS.

SFN-Induced Autophagy Was Partially Inhibited in Rho-0 Cells

Autophagy induction is another novel cellular response to SFN exposure in cultured prostate cancer cells and serves to protect against apoptotic cell death caused by SFN treatment (21). A very recent study has implicated ROS and catalase degradation in autophagy regulation (36). We therefore raised the question of whether autophagic response to SFN in prostate cancer cells was also linked to ROS generation. As shown in Fig. 5A, SFN treatment had minimal effect on catalase protein level in wild-type LNCaP and PC-3 cells as well as in their Rho-0 variants. We determined autophagic response to SFN in wild-type LNCaP and PC-3 cells and their Rho-0 variants by analysis of processing and recruitment of LC3 and formation of AVOs, which are hallmarks of autophagy (37–41). The LC3 protein (18 kDa) is cleaved by autophagic stimuli to a 16 kDa intermediate (LC3-II) that localizes to the autophagosomes (37). As can be seen in Fig. 5A, SFN treatment resulted in cleavage of LC3 in both wild-type LNCaP and PC-3 cells. The SFN-mediated cleavage of LC3 was partially but markedly suppressed in Rho-0 variants of both cell lines (Fig. 5A). Recruitment of LC3-II to the autophagosomes is characterized by punctate pattern of its localization (21,37,38). The DMSO-treated (9 h exposure) wild-type PC-3 (Fig. 5B) and LNCaP cells (results not shown) exhibited diffuse and weak LC3-associated green fluorescence. On the other hand, the wild-type PC-3 cells (Fig. 5B) and LNCaP cells (results not shown) treated for 9 h with 40 $\mu\text{mol/L}$ SFN exhibited punctate pattern of LC3 immunostaining. The SFN-induced recruitment of LC3 to autophagosomes was much less pronounced in the Rho-0 variants of PC-3 (Fig. 5C) and LNCaP cells (results not shown). In agreement with these results, the SFN-induced (40 $\mu\text{mol/L}$, 9 h) formation of AVOs was relatively more pronounced in the wild-type PC-3 (Fig. 5D) and LNCaP cells (results not shown) than in their Rho-0 variants. The SFN-mediated recruitment of LC3 to autophagosomes in PC-3 cells stably transfected with plasmid encoding GFP-LC3 (21) was also significantly inhibited by pre-treatment with *N*-acetylcysteine (supplemental Fig. S1). Collectively these results indicated that SFN-induced autophagy was partially dependent on ROS production.

SFN Treatment Inhibited Activities of MRC

To identify the target of SFN-mediated ROS generation, we determined its effect on activities of MRC enzymes using LNCaP and PC-3 cells. SFN treatment (6 h) resulted in significant inhibition of complex-I, II, and III activities in both LNCaP (Fig. 6A) and PC-3 cell lines especially at 40–100 $\mu\text{mol/L}$ SFN (Fig. 6B). However, complex II was most sensitive to inhibition by SFN in both cell lines at 40–100 $\mu\text{mol/L}$ concentrations. Nonetheless, these results indicated that SFN treatment caused inhibition of MRC leading to ROS generation in human prostate cancer cells.

DISCUSSION

Even though possible contribution of ROS in apoptotic response to the ITC family of cancer chemopreventive agents, including phenethyl-ITC, was suggested previously (42,43), the mechanism of ROS generation by this class of chemopreventive agent was not clear. The present study indicates that ROS production in SFN-treated prostate cancer cells is mitochondria-derived. The mitochondrial DNA deficient Rho-0 variants of LNCaP and PC-3 cells are significantly more resistant to ROS

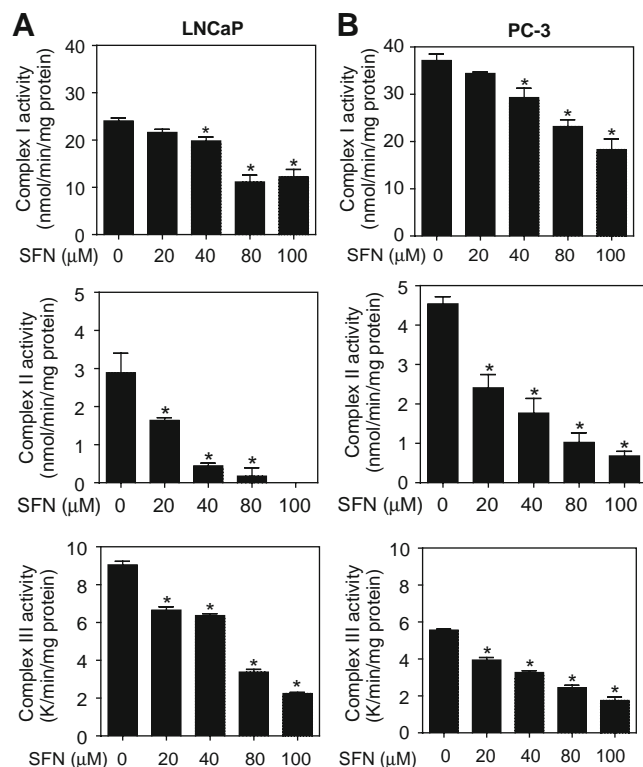


Fig. 6. SFN treatment inhibited MRC activities in LNCaP and PC-3 cells. Activities of complex I-linked NADH-ubiquinone oxidoreductase, complex II-linked succinate-ubiquinone oxidoreductase, and complex III-linked ubiquinol cytochrome *c* reductase in **A** LNCaP cells and **B** PC-3 cells treated for 6 h with DMSO (control) or the indicated concentrations of SFN. Results are mean \pm SE ($n=3$). *Significantly different ($P<0.05$) compared with control by one-way ANOVA followed by Dunnett's test. Each assay was performed at least twice using independently prepared cell extracts.

generation, growth suppression, and apoptosis induction by SFN compared with wild-type cells. Moreover, SFN treatment causes significant inhibition of MRC complex activities in both LNCaP and PC-3 cells. While further studies are needed to determine the precise mechanism of SFN-mediated inhibition of MRC enzymes, it is plausible that SFN covalently modifies critical subunit(s) of MRC complexes. This possibility is meritorious because ITC/SFN-mediated covalent modification of cellular proteins has been documented previously (24,44).

ROS function upstream of cytochrome *c* release and caspase activation by certain apoptotic stimuli such as hyperoxia (45). At the same time, generation of ROS downstream of the release of cytochrome *c* has also been described in some cellular models of mitochondria-mediated apoptosis (46,47). The present study reveals that ROS act upstream of mitochondrial changes in SFN-induced apoptosis because collapse of MMP and cytosolic release of cytochrome *c* are observed in wild-type cells but not in Rho-0 variants. Consistent with these results, the Rho-0 variant of PC-3 cell line is also resistant to SFN-mediated activation of caspase-3. Previous studies have shown that SFN-induced apoptosis correlates with induction of multidomain proapoptotic proteins Bax and Bak and down-regulation of anti-apoptotic protein Bcl-2 (18,19,25,26). We found that SFN-mediated induction of Bax, but not Bcl-2 down-regulation, is also dependent on ROS generation.

The present study shows that the G2/M phase cell cycle arrest caused by SFN exposure is initiated by mitochondria-derived ROS. The SFN-mediated G2/M phase cell cycle arrest is observed in wild-type LNCaP cells but not in its Rho-0 variant. The Rho-0 variant of PC-3 cell line is also significantly more resistant to SFN-induced G2/M phase cell cycle arrest compared with wild-type cells (results not shown). Eukaryotic cell cycle progression involves sequential activation of cdk's whose activation is dependent upon their association with regulatory cyclins (48). A complex formed by the association of cdk1 (also known as p34^{cdc2}) with cyclinB1 plays a major role in regulation of G2/M transition (48). Activity of cdk1/cyclinB1 kinase complex is negatively regulated by reversible phosphorylations at Thr14 and Tyr15 of cdk1 (48). The SFN-mediated inhibition of G2/M progression in wild-type prostate cancer cells is associated with an increase in Tyr15 phosphorylation of cdk1 suggesting inhibition of the cdk1/cyclinB1 kinase complex. Because SFN treatment causes an increase in protein level of cyclinB1 in wild-type cells, it is reasonable to conclude that the cell cycle arrest in our model is unlikely to be due to inhibition of complex formation between cdk1 and cyclinB1. Our data also suggest that the Rho-0 cells with mitochondrial stress exhibit growth arrest even in the absence of SFN treatment as evidenced by hyperphosphorylation of cdk1 at Tyr15 and accumulation of mitotic marker Ser-10 phosphorylated histone H3.

Recent studies have implicated ROS in autophagic response to various stimuli including chemical inhibitors of MRC and caspase inhibitor zVAD (36,49). For example, autophagic cell death resulting from treatment of murine L929 cell line with caspase inhibitor zVAD was shown to be associated with ROS generation and degradation of catalase (36). Interestingly, the ROS generation and catalase degradation occurred down-stream of zVAD-mediated autophagy in L929 cells (36). The results of the present study indicate that SFN-mediated autophagy, which serves to protect against

SFN-induced apoptosis at least in LNCaP and PC-3 cells (21), is partially dependent upon ROS. The SFN-mediated autophagy is relatively more pronounced in the wild-type LNCaP and PC-3 cells compared with their Rho-0 variants as judged by analysis of LC3 cleavage and recruitment and formation of AVOs. Thus, we conclude that ROS serve to partially contribute to autophagy induction by SFN in prostate cancer cells.

In conclusion the present study offers a mechanistic model for cellular response to SFN in human prostate cancer cells involving ROS, which are mitochondria-derived and serve to function upstream of apoptosis, G2/M phase cell cycle arrest, and autophagy. The translational implication of these findings is that cancer chemopreventive effect of SFN may be attenuated in the presence of anti-oxidants.

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