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Sulforaphane, a cruciferous vegetable-derived isothiocyanate, inhibits protein synthesis in human prostate cancer cells

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ABSTRACT

Sulforaphane (SFN) is a compound derived from cruciferous plants. Its anticancer properties have been demonstrated both, in cancer cell lines as well as tumors in animal models. It has been shown that SFN inhibits cell proliferation, induces apoptosis, autophagy, and sensitizes cancer cells to therapies. As induction of catabolic processes is often related to perturbation in protein synthesis we aimed to investigate the impact of SFN on this process in PC-3 human prostate cancer cells. In the present study we show that SFN inhibits protein synthesis in PC-3 cells in a dose- and time-dependent manner which is accompanied by a decreased phosphorylation of mTOR substrates. Translation inhibition is independent of mitochondria-derived ROS as it is observed in PC-3 derivatives devoid of functional mitochondrial respiratory chain (Rho0 cells). Although SFN affects mitochondria and slightly decreases glycolysis, the ATP level is maintained on the level characteristic for control cells. Inhibition of protein synthesis might be a protective response of prostate cancer cells to save energy. However, translation inhibition contributes to the death of PC-3 cells due to decreased level of a short-lived protein, survivin. Overexpression of this anti-apoptotic factor protects PC-3 cells against SFN cytotoxicity. Protein synthesis inhibition by SFN is not restricted to prostate cancer cells as we observed similar effect in SKBR-3 breast cancer cell line.

1. Introduction

Epidemiologic studies showed that high level cruciferous vegetable intake may lower the risk of cancer, including prostate cancer [1–5]. Isothiocyanates are the main chemopreventive constituents of these plants. They are generated by hydrolysis of their precursors, glucosinolates. One of the best studied isothiocyanate, abundant in broccoli and broccoli sprouts, is sulforaphane [1-isothiocyanato-4-(methyl-sulfinyl)butane], SFN. It was identified in 1992 as a potent chemopreventive agent able to induce Phase 2 detoxification enzymes [6]. Later, it was shown that SFN also acted as an inhibitor of enzymes involved in carcinogen activation [7]. Subsequent numerous studies demonstrated that SFN blocked cancer development at post-initiation phases as well, inhibiting proliferation and inducing cancer cell apoptosis [8,9]. Among reported mechanisms underlying anticancer activity of SFN there are: oxidative stress induction [10–12], DNA damage checkpoint activation [13], inhibition of histone deacetylases [14,15] or direct binding to cellular proteins, such as tubulins [16]. Importantly, signaling pathways induced in cancer cells by SFN might depend on its dose. For instance, Yeh and Yen observed that low SFN concentrations (up to 20 μM) induced MAPK-mediated activation of Nrf-2 transcription factor, thus a protective response. On the other hand, higher SFN concentrations (30–100 μM) induced apoptosis of HepG2 cells [17].

Prostate cancer is one of the most extensively studied models of SFN anticancer activity. It was demonstrated that oral administration of SFN retarded the growth of PC-3 human prostate xenografts in athymic mice and inhibited prostate carcinogenesis and pulmonary metastasis in transgenic mouse model of prostate cancer [18,19]. Based on in vitro studies using different human prostate cancer cell lines, it was shown that SFN-induced oxidative stress is caused by depletion of glutathione and elevation of reactive oxygen species (ROS) production by mitochondria which led to a drop in mitochondrial membrane potential, release of cytochrome c and caspase-dependent cell death. Antioxidants (N-acetylcysteine or mimetic of superoxide dismutase and catalase, EUK134) or overexpression of catalase significantly protected the cells against SFN-induced ROS generation and apoptosis [12]. It was later shown that mitochondria-derived ROS are generated due to inhibition of respiratory chain complexes by SFN in prostate cancer cells. Their derivatives devoid of mitochondrial DNA (Rho0 cells) were significantly more resistant to SFN-induced ROS generation, G2/M cell cycle arrest and apoptosis than wild type cells [10]. Interestingly, pro-survival autophagy observed in PC-3 and LNCaP cells treated with SFN was partially but not fully suppressed in Rho0 cells [10].

Protein synthesis is essential for cell viability and proliferation. Translation initiation is a common downstream target of signal transduction pathways deregulated in human cancers due to activation of

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oncogenes or loss of tumor suppressors which play an important role in these pathways [20]. For example, positive regulator of translation, the Akt kinase, is overactive in many human cancers, including carcinomas, glioblastoma multiforme and various hematological malignancies (reviewed in [21]). mTOR (the mammalian target of rapamycin) is a crucial regulator of translation, which together with associated proteins, raptor and mLST8 (GβL), phosphorylates the eukaryotic initiation factor 4E-binding protein (4E-BP), an inhibitor of cap-dependent translation, and the p70S6 kinase1 (S6K1) [22]. S6K1 plays a pleiotropic role in translation. For a long time it was believed that S6K1 participates in ribosomal biogenesis through activation of ribosomal protein S6 (RPS6). Other known S6K1 substrates include eukaryotic elongation factor 2 kinase (eEF2K) and eukaryotic translation initiation factor 4B (eIF4B) [23–25]. Thus, S6 kinase1 controls important stages of translation and its targeting should have detrimental effect on cancers that are dependent on the elevated rate of protein synthesis.

In the present study we demonstrate that protein synthesis inhibition is another mechanism of SFN activity. It is independent of mitochondria-derived ROS, occurs very early after SFN administration and is accompanied by a decreased mTOR-S6K1-S6 signaling. Although translation inhibition may contribute to ATP level maintenance, and thus become a protective response of cancer cells to SFN, it finally leads to depletion of short-lived survivin, the protein crucial for survival of prostate cancer cells.

2. Materials and methods

2.1. Reagents

D,L-SFN (purity 99%) was purchased from LKT Laboratories (St. Paul, MN). Tissue culture media, penicillin/streptomycin antibiotic mixture and fetal bovine serum were from GIBCO (Grand Island, NY). [^{3}H]-leucine was from Perkin Elmer (Waltham, MA). The antibodies against p-AMPK (Thr-172), p-Akt (Ser-473) and Akt were from Santa Cruz Biotechnology (Santa Cruz, CA), p-S6K1 (Thr-389), S6K1, p-S6 (Ser-235/236), ribosomal S6, survivin, PARP, 4E-BP, AMPK, calyculin A and rapamycin were from Cell Signaling Technology (Danvers, MA), the anti-β-actin, anti-GAPDH, anti-mouse and anti-rabbit antibodies conjugated with HRP, as well as DMSO, oligomycin, MG132 and PF-4708671 were from Sigma-Aldrich (St. Louis, MO). The plasmid encoding survivin was from Dr. Ritu Aneja, Department of Biology, Georgia State University, Atlanta.

2.2. Cell lines and Rho0 cell generation

Monolayer cultures of PC-3 or SKBR-3 cells were maintained in F12-K nutrient mixture medium or RPMI1640 medium, respectively, supplemented with 10% (v/v) fetal bovine serum and antibiotics. Rho0 variant of PC-3 cells (depleted of mitochondrial DNA) was generated by culturing cells for 8 weeks with 100 ng/ml ethidium bromide in complete medium supplemented with 4.5 mg/ml glucose, 100 μg/ml sodium pyruvate and 50 μg/ml uridine to compensate for the respiratory metabolism deficit as previously described [26]. To verify the mtDNA depletion, total cellular DNA was isolated and subjected to PCR using primers for mitochondrial *ND5* gene coding for NADH dehydrogenase subunit 5 (forward: TTCATCCCT GTAGCATTGTTCG and reverse: AGCGGATGAGTAAGAAGATTCC) which gives a 522 bp product. For control, fragment of the chromosomal GAPDH gene was amplified. Each cell line was maintained at 37°C in a humidified atmosphere with 5% CO_2. D, L-sulforaphane was prepared in DMSO and stored at a stock concentration of 10 mM at −20°C. Doses applied in this work (10, 20 and 40 μM) have been previously tested on PC-3 cell line as the most effective [10,12,13,27]. To inhibit PP1 and PP2A phosphatases or mTOR kinase calyculin A (final concentration 1 nM or 100 nM) or rapamycin (final concentration 100 nM) were added 30 min or 1 h before SFN treatment, respectively. To inhibit proteasome, MG132 (final concentration 1 μM) was added 2 h before SFN treatment.

2.3. Protein synthesis assay

Cells (1.5×10^5/well) were cultured in 12-well plates and treated with SFN (40 μM for indicated times or 10, 20, 40 μM SFN for 3 h) and 2 μCi/well L-[3, 4, 5-^{3}H]-leucine. Both, floating and attached cells were collected, fixed in 5% TCA at room temperature for 30 min and washed with 5% TCA for 15 min. The acid-insoluble material was dissolved in 0.1 mol/L KOH overnight at 4°C and aliquots were used to determine the radioactivity using liquid scintillation counter (Beckman LS3133P). Radioactivity of samples was normalized to cell number.

2.4. Transmission electron microscopy

Transmission electron microscopy to compare ultrastucture of parental PC-3 cells and their Rho0 derivatives was performed essentially as described previously [12,27]. Briefly, cells (1.5×10^5) were plated in 12-well plates, and allowed to attach overnight. The cells were fixed in ice-cold 2.5% electron microscopy grade glutaraldehyde in 0.1 M PBS (pH 7.3). The specimens were rinsed with PBS, post-fixed in 1% osmium tetroxide with 0.1% potassium ferricyanide, dehydrated through a graded series of ethanol (30–100%), and embedded in Epon, Fluka (USA). Semi-thin (300 nm) sections were cut using an RMC Power Tome XL Reichart Ultracut, stained with 0.5% toluidine blue and examined under a light microscope. Ultrathin sections (65 nm) were stained with 2% uranyl acetate and Reynold's lead citrate, and examined on a Philips CM100 transmission electron microscope.

2.5. Immunoblotting

Cells were treated with SFN and lysed using a solution containing 50 mM Tris, 1% Triton X-100, 150 mM NaCl, 0.5 mM EDTA, protease and phosphatase inhibitor cocktails (Roche Diagnostics, Germany). Lysates were cleared by centrifugation at 13,000 rpm for 20 min. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membrane. The membrane was blocked with 5% non-fat dry milk in phosphate buffered saline, and incubated with the desired primary antibody overnight at 4 °C. The membrane was treated with appropriate secondary antibody for 1 h at room temperature. The immunoreactive bands were detected with an enhanced chemiluminescence reagent (Thermo Scientific Pierce, Rockford, IL). The blots were stripped and reprobed with anti-β-actin or anti-GAPDH antibodies. The change in protein level was determined by densitometric analysis of the immunoreactive bands by Quantity One software (BioRad) followed by correction for the respective loading control. The immunoblotting for each protein was performed at least twice using independently prepared lysates.

2.6. Measurement of ROS generation

Intracellular ROS generation in DMSO-treated control and SFN-treated cells (40 μM SFN for 4 h) was measured by flow cytometry following staining with H_2DCFDA, which is cleaved by nonspecific cellular esterases and oxidized in the presence of H_2O_2 and peroxidases to fluorescent 2′,7′-dichlorofluorescein (DCF). Briefly, cells (5×10^5) were plated in 60 mm dishes, allowed to attach overnight, and treated with DMSO or SFN for 4 h. Cells were exposed to 5 mM H_2DCFDA for the last 30 min of incubation, collected and fluorescence was measured using a FACScan cytometer (Becton Dickinson, USA).

2.7. ATP level assay

ATP level was determined using the ATP Bioluminescence Assay Kit HS II (Roche Diagnostics) according to manufacturer recommendations. Briefly, cells were cultured in 12-well plates and treated with 40 μM SFN for indicated times. Both floating and attached cells were collected, resuspended and lysed. Aliquots were transferred to black 96-well plates and luciferase was added. The luminescence was measured in Victor^3 microplate reader.

2.8. Lactate determination

L(+)-lactate level in PC-3 cells was determined using the Lactate Assay Kit (MBL International) according to the manufacturer instruction. Briefly, 1×10^6 cells were plated and allowed to attach overnight. The following day, cells were treated with 40 μM SFN for indicated time or treated with vehicle (control). Both, floating and attached cells were collected and washed with ice-cold PBS. Cell pellet was suspended in 250 μl of ice-cold Lactate Assay Buffer and homogenized by sonication. Samples were centrifuged (13,000 rpm, 20 min) and obtained supernatant was used for subsequent assays.

2.9. Reverse transcription quantitative real-time PCR

Cells (5×10^5) were plated in 6-well plates, allowed to attach overnight and next day treated with 40 μM SFN or vehicle (DMSO) for 3 or 24 h. Both floating and attached cells were collected and washed with ice-cold PBS. Total RNA was isolated using Total RNA Mini kit (A&A Biotechnology) according to the manufacturer's instructions. A 0.5 μg of total RNA was used for first-strand cDNA synthesis using random hexamer primers and RevertAid™ H Minus Reverse Transcriptase (Fermentas). Real-time PCR was performed on LightCycler® 2.0 (Roche Applied Science) using Real-Time 2× HS-PCR Master Mix Probe (A&A Biotechnology) and probes (Roche Applied Science) according to manufacturers' instructions. Specific primers and a probe for *survivin* gene were designed using the ProbeFinder software (Roche Applied Science), and were as follows: sense 5′-CACCGCATCTCTACATTCAAGA-3′; antisense 5′-CAAGTCTGGCTCGTTCTCAGT-3′; probe #86. Primers and probe for the reference gene coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Roche Applied Science. The $2^{-\Delta\Delta ct}$ method was used to determine the relative *survivin* transcript levels after normalization to the reference gene (GAPDH).

2.10. Transient transfection with survivin-encoding plasmid

Cells were transfected with empty vector pcDNA3 or vector encoding survivin using TurboFect (Fermentas) according to the manufacturer's recommendations. After 24 h cells were treated with DMSO (vehicle) or 40 μM SFN for 24 h and cell viability, morphology and apoptosis induction (PARP cleavage) was determined. Cell viability was measured using WST1 assay for PC-3 cells growing in a 96-well plate (starting density 8×10^3/well). WST1 solution was added for the last 1 h of incubation. The absorbance was measured at 490 nm in Victor^3 microplate reader. Morphology of cells transfected with empty vector or plasmid encoding survivin, treated with DMSO or 40 μM SFN was examined under a light microscope.

2.11. Statistical analysis

Data were analyzed using GraphPad Prism software. *T*-test or one-way ANOVA followed by Bonferroni's or Dunnett's multiple comparison test was used to determine statistical significance of difference in measured variables between control and treated group or between groups, respectively. Difference was considered significant at $P<0.05$.

3. Results

3.1. Sulforaphane decreases protein synthesis in PC-3 cells

It has been previously shown that SFN inhibits proliferation of PC-3 cells in a time- and dose-dependent manner [18] and induces protective response, autophagy [27]. As cell growth inhibition and especially induction of catabolic processes, such as autophagy, are often related to perturbations in protein synthesis, we tested if SFN affects translation in prostate cancer cells. We measured [^{3}H]-leucine incorporation in control, DMSO-treated cells, and cells exposed to 40 μM SFN for different times (5, 15, 30 min or 1, 2 and 3 h). As demonstrated in Fig. 1A, SFN inhibited [^{3}H]-leucine incorporation which was evident already after 1-hour treatment. Protein synthesis measured after 3-hour exposure to SFN was inhibited in a dose-dependent manner and in samples treated with 10, 20 or 40 μM SFN it dropped to about 80, 60 and 30% of the level seen in control cells, respectively (Fig. 1B). We also determined the effect of longer time treatment with SFN on protein synthesis. As shown in Fig. 1C, [^{3}H]-leucine incorporation in cells treated with 40 μM SFN for 6, 16 or 24 h is statistically significantly lower than in respective control cells which indicates persistent translation block.

3.2. Protein synthesis block correlates with the inhibition of mTOR signaling by SFN

As translation is primarily controlled by mTOR kinase we determined the effect of SFN on phosphorylation of mTOR substrates, S6 kinase1 and 4E-BP. As shown in Fig. 2A, SFN (40 μM) treatment caused a time-dependent decrease in phosphorylation of S6K1 at Thr-389, position phosphorylated by mTOR [28]. This effect was seen very early: the amount of p-S6K1 dropped by about 20% or 50% of the level in control cells after 15 and 30 min of exposure to SFN, respectively. In cells treated for longer times (up to 3 h), phosphorylation of S6K1 at Thr-389 was kept at low level, corresponding to 10–20% of that in DMSO-treated samples. At the same time, changes in total level of S6K1 were not significant (Fig. 2A). In our model dephosphorylation of S6K1 correlated with a drop in Ser-235/236 phosphorylation of S6, the substrate of S6K1. It was evident after 1-hour treatment while changes in the total S6 level over time were not significant (Fig. 2A). Changes in S6K1 and S6 phosphorylation status were dependent on the SFN concentration: 3 h of treatment with 10, 20 or 40 μM SNF reduced threonine-389 phosphorylation of S6K1 to about 40, 30 and 10% of the control level, respectively, and serines — 235/236 phosphorylation of S6 to 70, 50 or 30%, respectively (Fig. 2B). Dephosphorylation of 4E-BP, another mTOR substrate, was observed as a drop in intensity of bands corresponding to phosphorylated (γ, upper band) and an increase in intensity of unphosphorylated (α, lower band) forms of the protein which was dependent on treatment time and dose of SFN (Fig. 2A and B).

3.3. SFN-induced dephosphorylation of S6K1 is mediated by mTOR signaling but not by calyculin A-sensitive protein phosphatases

Activation of S6K1 requires phosphorylations at multiple sites, such as series of serines and threonines in the C-terminal regulatory domain by ERK1/2, JNK1/2 or CDK1, Thr-229 in catalytic domain by PDK1, and Thr-389 in the kinase extension domain by mTOR [28,29]. Thus, status of Thr-389 of S6K1 is often regarded as a marker of mTOR activity. On the other hand, this position is dephosphorylated by class PP1 or PP2A phosphatases [30]. To elucidate which signaling pathway is affected by SFN, first we pretreated PC-3 cells with 1 nM calyculin A which inhibits PP1 and PP2A serine/threonine phosphatases. As demonstrated in Fig. 3A, 1 nM calyculin A had no effect on SFN induced dephosphorylation of S6K1, which might indicate that the observed drop in S6K1 phosphorylation is not due to enhanced phosphatases activity or that 1 nM inhibitor concentration is too low, thus not effective. On the other hand 1 nM calyculin A partially protected against SFN-induced

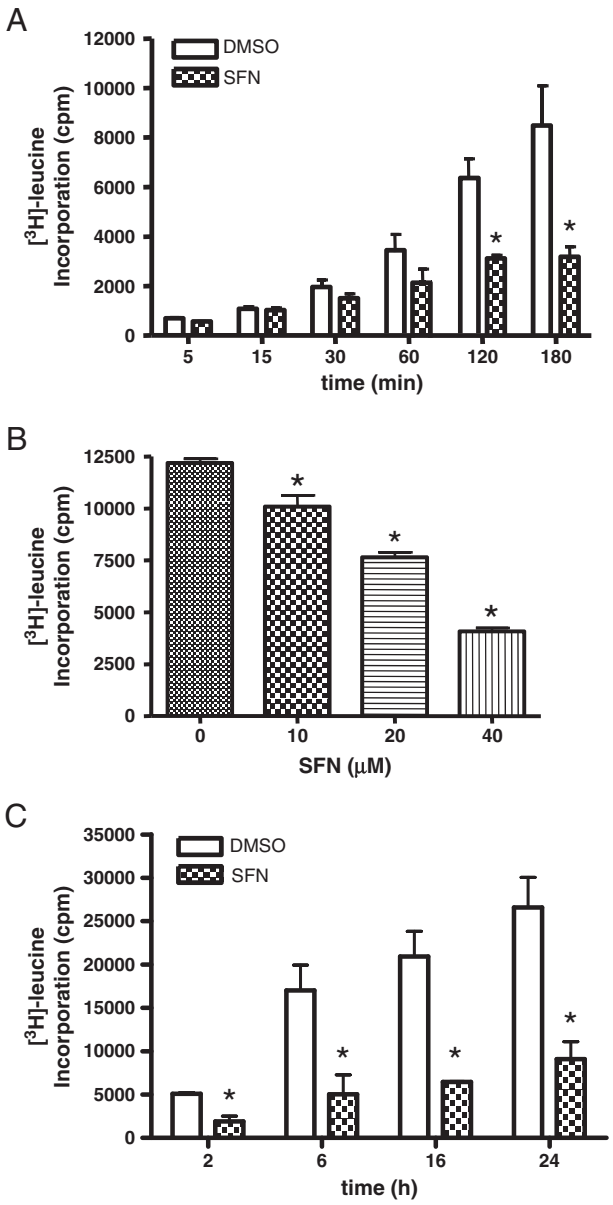

Fig. 1. SFN inhibits protein synthesis in human prostate cancer cells. PC-3 cells were treated with 40 μM SFN for indicated times (A and C) or with various concentrations of SFN for 3 h (B) in the presence of a protein precursor, [^{3}H]-leucine. Cells were harvested and radioactivity of TCA-precipitable material was estimated as described in Materials and methods. Results shown are mean ± SE of two (A and C) or three (B) independent experiments. In A and C *significantly different ($P<0.05$) compared with respective DMSO-treated control by Student's *t* test. In B *significantly different ($P<0.01$) compared with DMSO-treated control by one-way ANOVA followed by Dunnett's multiple comparison test.

dephosporylation of ribosomal S6 protein (Fig. 3A) which is also a substrate of calyculin A sensitive phosphatases [31,32]. Different sensitivity of p-S6K1, p-S6 and p-Akt to calyculin A may be due to a different affinity of calyculin A-sensitive phosphatases to the respective substrates. Moreover, compensating phosphorylation of S6 by RSK has been reported in cells devoid of S6 kinases expression [33]. However, partial S6 phosphorylation in PC-3 cells treated with 1 nM calyculin A did not suppress SFN-induced block in protein synthesis (Fig. 3B). Higher calyculin A concentration (100 nM) protected against SFN-induced S6K1 and S6 dephosphorylation, as well as caused accumulation of another PP2A substrate, Akt kinase phosphorylated at Ser-473 (Fig. 3A). However, it induced massive cell detachment and proliferation block, as incorporation of [^{3}H]-leucine was completely inhibited (Fig. 3B).

Next, we tested a hypothesis that SFN-induced drop in p-S6K1 is due to the mTOR-S6K1 signaling perturbation. PC-3 cells were treated with 40 μM SFN for 1.5 h and released to fresh medium with either DMSO or rapamycin (100 nM) for 1, 3 or 6 h. Rapamycin is a specific inhibitor of mTOR complex 1 which targets, among others, the S6 kinase1 at Thr-389 position [28,29]. SFN treatment caused dephosphorylation of S6K1 and its target, S6 (Fig. 3C). After release to control medium, we observed a gradual increase in phospho-forms of investigated proteins. However, SFN-induced hypophosphorylation of S6K1 and S6 was maintained in cells released to medium containing rapamycin. It indicates that in this model S6K1 phosphorylation at analyzed position is not mediated by any other kinase except from mTOR. Thus, SFN inhibits mTOR-mediated S6K1 phosphorylation (Fig. 3C).

3.4. Effect of SFN on protein synthesis is independent of mitochondria-derived reactive oxygen species

It has been previously demonstrated that SFN inhibits mitochondrial respiratory chain complexes in prostate cancer cells. It leads to generation of ROS by mitochondria and they play a crucial role in inducing cell cycle arrest and apoptosis [10,12]. To determine if mitochondria-derived ROS are necessary for translation inhibition we used Rho0 variant of PC-3 cells. Cells were obtained by culturing in the presence of ethidium bromide and supplements which allow cells to produce ATP at proper level through glycolysis. Lack of mitochondrial DNA was verified by amplification of mitochondrial *ND5* gene coding for NADH dehydrogenase subunit 5 (Fig. 4A) and morphology of Rho0 and parental cells by transmission electron microscopy (Fig. 4B). Presence of lipid droplets is characteristic of Rho0 cells due to impaired β-oxidation of fatty acids. Mitochondria of Rho0 cells have altered morphology and reduced cristae content compared to wild type organelle, which is consistent with the earlier reports [34]. We compared ROS level in PC-3 and Rho0 cells upon DMSO or SFN (40 μM) treatment. As expected, Rho0 cells were resistant to SFN-induced ROS formation evaluated as a mean fluorescence of DCF, which is in agreement with previously reported data for 20 μM SFN [10] (Fig. 4C). Interestingly, lack of mitochondria-derived ROS did not protect Rho0 cells from protein synthesis inhibition by SFN (Fig. 4D) and dephosphorylation of S6K1 and S6 proteins (Fig. 4E) indicating that effect of SFN on translation is independent of mitochondria-derived ROS.

3.5. Effect of SFN on energy status of PC-3 cells

As SFN inhibits enzymes of mitochondrial respiratory chain complexes I, II and III in prostate cancer cells [10], we hypothesized that it might lead to a drop in ATP level which impacts mRNA translation through AMPK-mTOR signaling. We looked at AMPK catalytic subunit which is phosphorylated at Thr-172 when the AMP:ATP ratio increases in the cells. Interestingly, SFN-induced changes in phospho-AMPK within the first minutes of the treatment were not significant. Longer treatment time caused a decrease in AMPK phosphorylated at Thr-172 which was statistically significant after 1-hour exposure to SFN (Fig. 5A). We were not able to detect any changes in ATP level measured in lysates of cells treated for various times with SFN, although inhibitor of mitochondrial respiration (oligomycin) modestly and inhibitor of glycolysis (deoxyglucose) statistically significantly decreased ATP level (Fig. 5B). Perturbation in mitochondrial ATP production might be compensated by elevated glycolysis. To test this possibility, we measured lactate production by control or SFN-treated cells. As demonstrated in Fig. 5C, there was no upregulation of glycolysis rate by SFN, rather a slight decrease (not statistically significant) in lactate production was observed. Altogether, these results indicate that even if SFN perturbs ATP production, the decrease in its

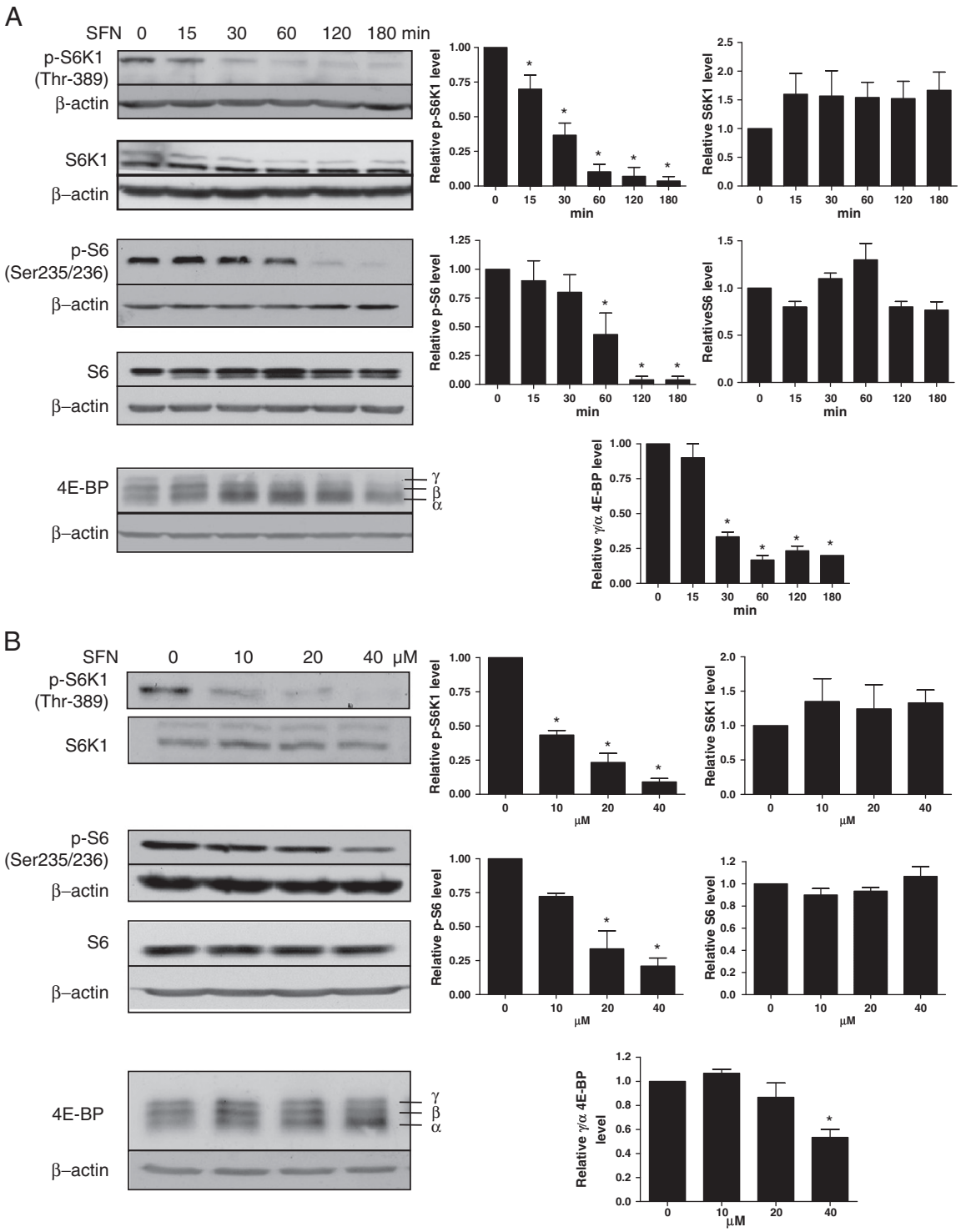

Fig. 2. SFN inhibits phosphorylation of mTOR substrates. Representative immunoblottings for p-S6K1 (Thr-389), total S6K1, p-S6 (Ser-235/236), total ribosomal S6 and total 4E-BP using lysates from PC-3 cells treated with 40 μM SFN for the indicated time periods (A) or various SFN concentrations for 3 h (B). The blots were stripped and reprobed with anti-β-actin antibody to ensure equal protein loading. Results are graphed as mean ± SE from 2 or 3 independent experiments, *significantly different ($P<0.05$) compared with DMSO-treated control by one-way ANOVA followed by Dunnett's multiple comparison test.

level is transient. We cannot exclude that inhibition of protein synthesis compensates for eventual drop in ATP.

3.6. SFN-induced survivin depletion contributes to decreased survival of PC-3 cells

Translation inhibition may cause depletion of short-lived proteins that are essential for cell survival. One of them is survivin, an inhibitor of apoptosis and mitosis regulator. It is often overexpressed in cancer cells, including prostate cancers, which is controlled by mTOR-S6K1 pathway. We determined the effect of SFN on survivin level in PC-3 cells. As shown in Fig. 6A, SFN in a dose-dependent manner decreased survivin level, and in cells treated with 40 μM SFN this drop was evident after 1 h of treatment and was maintained till 24 h (Fig. 6A). To determine if the observed changes in survivin level are due to decreased gene expression, we evaluated survivin mRNA level in control

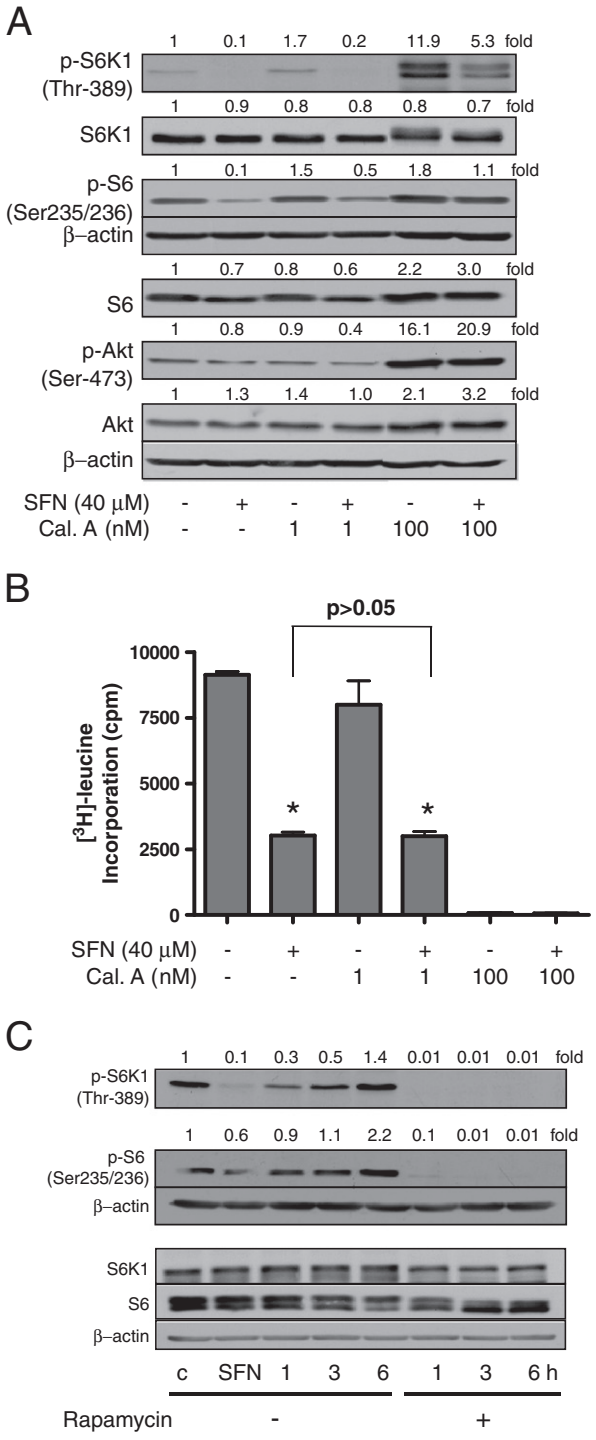

Fig. 3. SFN-induced dephosphorylation of S6K1 is mediated by mTOR signaling not calyculin A-sensitive protein phosphatases. A and B, Cells were pretreated or not with 1 or 100 nM calyculin A for 30 min. Then cells were exposed to 40 μM SFN for 2 h, harvested and immunoblotted for indicated proteins (A) or exposed to 40 μM SFN and [^{3}H]-leucine for 2 h and processed for radioactivity measurement (B). Results shown are mean ± SE (n = 3). *Significantly different (P<0.01) compared with corresponding DMSO- or DMSO and calyculin A treated controls by one-way ANOVA followed by Bonferroni's multiple comparison test. (C) Immunoblotting for p-S6K1 (Thr-389), p-S6 (Ser-235/236), total S6 and S6K1 using lysates from PC-3 cells treated with 40 μM SFN for 1.5 h and released to control medium or medium containing 100 nM rapamycin for indicated times. The blots were stripped and reprobed with anti-β-actin antibody to ensure equal protein loading. Densitometric analysis data after correction for loading control are on top of the respective immunoreactive bands. Similar results were observed in two independent experiments.

and SFN-treated cells by reverse-transcription quantitative real-time PCR. As demonstrated in Fig. 6B, 3-hour treatment with 40 μM SFN had no significant effect on survivin gene transcription as compared to control cells. To elucidate if decreased survivin level is due to enhanced proteasome activity we used a proteasome inhibitor, MG132. As shown in Fig. 6C, MG132 only partially protected against SFN-induced drop in survivin protein level. To confirm that in PC-3 cells survivin synthesis is controlled by mTOR and S6K1 we used specific inhibitors of these kinases, rapamycin and PF-470867, respectively. Treatment with 100 nM rapamycin for 3 h or 10 μM PF-470867 for 2 h caused a significant drop in survivin level (Fig. 6D). To assess a possible link between survivin downregulation and survival of cells treated with SFN, we transiently transfected PC-3 cells with a plasmid encoding survivin and cell viability was assessed by the WST1 method. Viability of cells transfected with empty vector decreased after 24-hour treatment with 40 μM SFN by 50% compared with DMSO-treated cells (Fig. 6E). Interestingly, cells expressing higher amounts of survivin showed enhanced viability compared with cells expressing only endogenous survivin, which was also observed in SFN-treated cells: their survival decreased by about 30% compared with DMSO-treated survivin overexpressing cells, and still it was 100% higher than SFN-treated cells expressing only endogenous survivin. Similar effect of survivin overexpression was evident when morphology of cells was compared in phase contrast microscopy (Fig. 6F). Protective role of survivin was due to apoptosis inhibition, as assessed by PARP cleavage, which is a marker of caspase-3 and caspase-7 activity. As shown in Fig. 6G, additional pool of survivin, even if decreased upon SFN treatment, protected against PARP cleavage by caspases.

3.7. SFN-induced protein synthesis block is not limited to PC-3 prostate cancer cells

To reconcile if translation inhibition by SFN is limited to prostate cancer cells or is a more general phenomenon we used a cell line derived from breast cancer, SKBR-3. Protein synthesis measured as [^{3}H]-leucine incorporation was inhibited by SFN in a dose dependent manner: 10, 20 or 40 μM SFN caused a drop in protein synthesis to about 80, 50 and 20% of the level seen in control cells, respectively (Fig. 7A). Similarly to prostate cancer cells, translation inhibition was accompanied by a decreased mTOR-S6K1-S6 signaling detected as a dephosporylation of S6K1 at Thr-389 and its substrate, ribosomal S6 at Ser-235/236 (Fig. 7B).

4. Discussion

Sulforaphane has been shown to inhibit proliferation of prostate cancer cells inducing G2/M cell cycle arrest and subsequent apoptosis [12,13,18]. It also activated protective autophagy [27]. In this work we show that SFN blocks protein synthesis in different cancer cells, PC-3 prostate cancer cell line and SKBR-3 breast cancer cell line. In PC-3 cells it is an early event, evident after 1-hour treatment with 40 μM drug, thus it is not a result of cell proliferation inhibition (Fig. 1A). The master regulator of translation is mTOR kinase. It is stimulated by growth factors and nutrients, and it upregulates anabolic processes, such as translation and transcription and blocks catabolic processes, like autophagy and apoptosis [22,35,36]. One of the main mTOR targets is S6 kinase1 which stimulates different stages of protein synthesis, including ribosome biogenesis, initiation and elongation of translation [23–25]. In this report we show that signal transduction between mTOR and S6K1 is inhibited by SFN. We observed a rapid decrease in phosphorylation of S6K1 at threonine 389, a residue phosphorylated by mTOR and crucial for S6K1 activation. Subsequent drop in phosphorylation of ribosomal S6 protein confirms inactivation of S6 kinase1 (Fig. 2). Lack of S6K1 might be compensated by other kinases, namely S6K2 or RSK which target serines 235/236 of ribosomal S6 upon activation by mTOR or MAPK, respectively [33]. It is

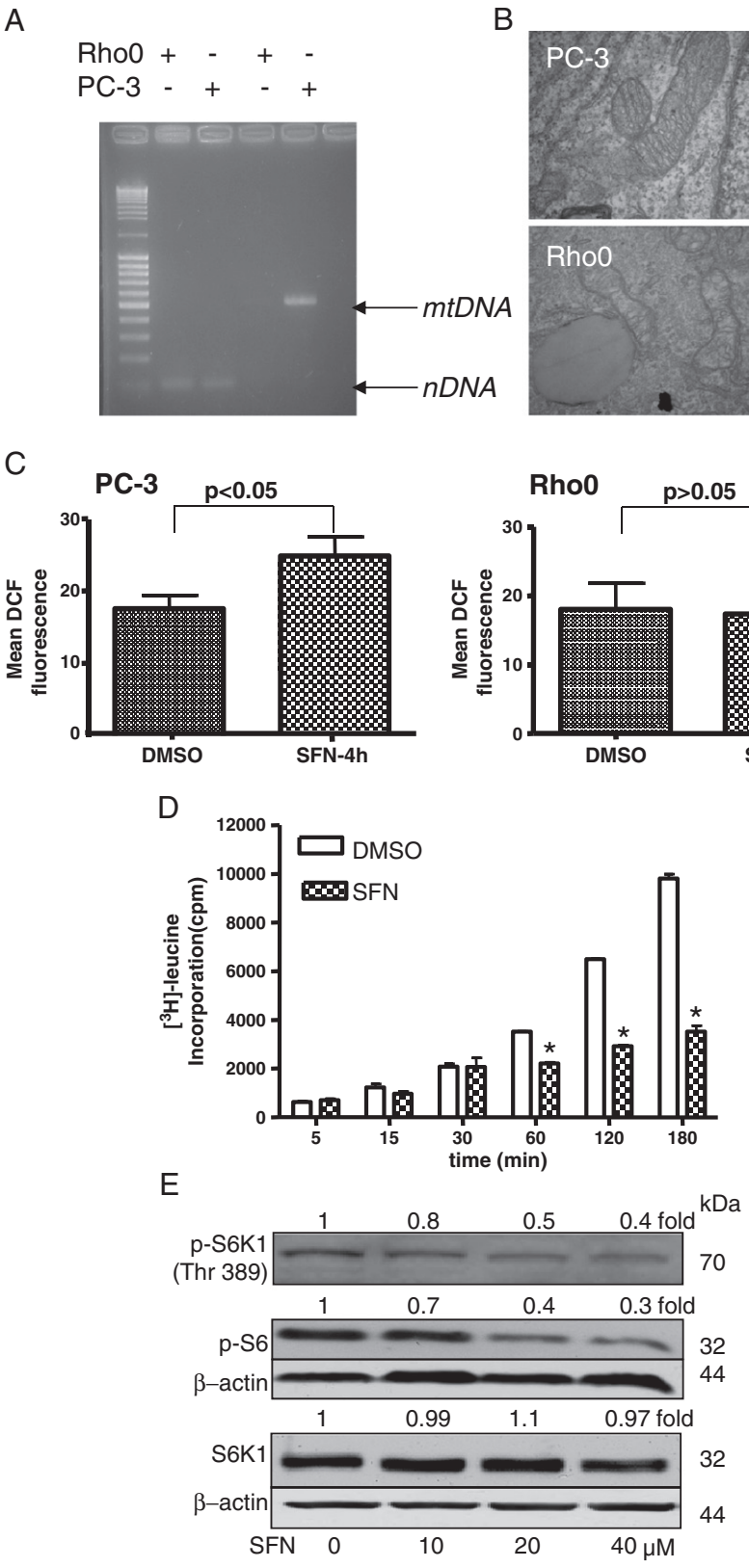

Fig. 4. SFN-induced protein synthesis block is independent of mitochondria-derived ROS. A, evaluation of Rho0 phenotype by amplification of a fragment of mitochondrially encoded NADH dehydrogenase subunit 5 gene (product length — 522 bp). As a control, fragment of GAPDH gene which is coded by nuclear DNA was amplified (product length — 90 bp). B, ultrastructure of Rho0 and parental cells in transmission electron microscopy. Note the presence of lipid droplet and mitochondria with reduced cristae content in Rho0 cells. Magnification 11,500×. C, mean DCF fluorescence (a measure of ROS generation) in wild type PC-3 cells and their Rho0 derivatives treated for 4 h with DMSO or 40 μM SFN. Results shown are mean ± SE ($n = 3$) and in the case of PC-3 cells are significantly different ($P<0.05$) by Student's *t* test. D, protein synthesis in Rho0 cells treated with DMSO or 40 μM SFN for indicated times measured by incorporation of [^{3}H]-leucine into TCA-precipitable material. Results shown are mean ± SE of two independent experiments, *significantly different ($P<0.05$) compared with respective DMSO-treated control by Student's *t* test. E, immunoblotting for p-S6K1 (Thr-389), p-S6 (Ser-235/236) and S6K1 using lysates from Rho0 cells treated with different concentrations of SFN for 3 h. The blots were stripped and reprobed with anti-β-actin antibody to ensure equal protein loading. Densitometric analysis data after correction for loading control are on top of the immunoreactive bands. Similar results were observed in at least three independent experiments.

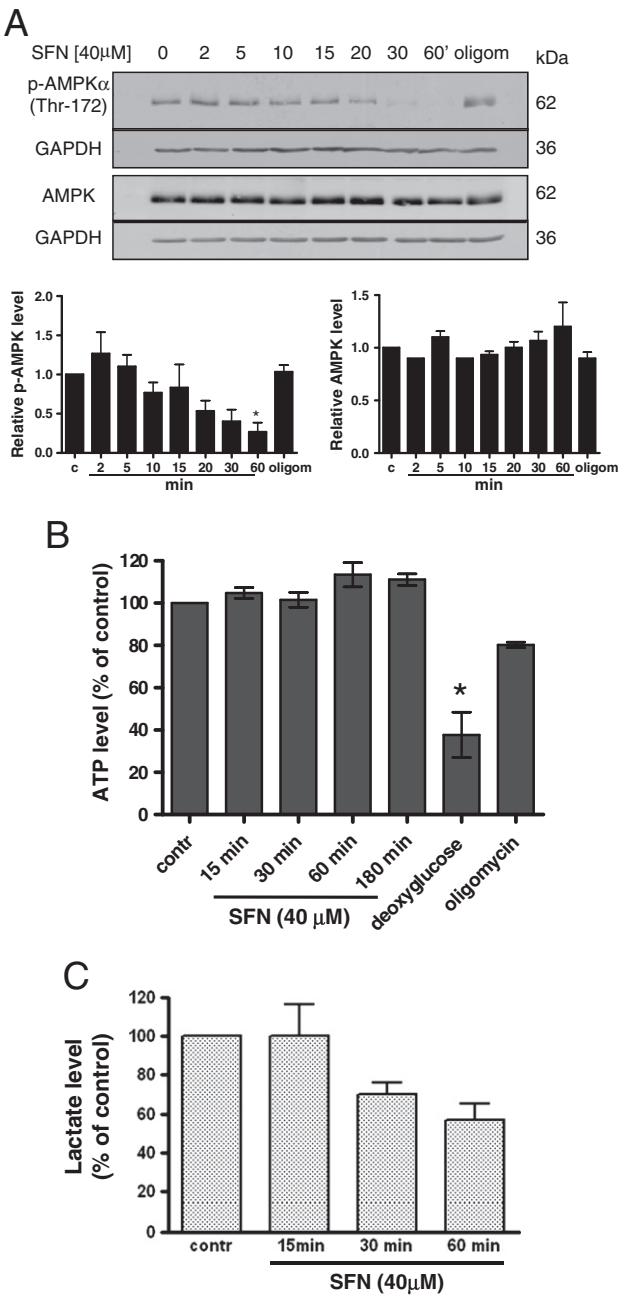

Fig. 5. Effect of SFN on energetics in PC-3 cells. A, immunoblotting for p-AMPK (Thr-172) and total AMPK using lysates from PC-3 cells treated with 40 μM SFN for indicated times or oligomycin for 30 min. The blots were stripped and reprobed with anti-GAPDH antibody to ensure equal protein loading. Results are graphed as mean ± SE from 3 independent experiments. B, ATP levels in cells treated with DMSO (control), 40 μM SFN for indicated times, 1 μM oligomycin for 30 min or 20 mM deoxyglucose for 3 h. Shown is the average (± SE) of three independent experiments, *significantly different ($P<0.01$) compared with DMSO-treated control by one-way ANOVA followed by Dunnett's multiple comparison test. C, the relative lactate level in PC-3 treated with DMSO (contr) or 40 μM SFN for indicated times. Shown is the average (± SE) of two independent experiments.

possible, that in our model such phosphorylation takes place and is revealed upon inhibition of PP1 and PP2A phosphatases (Fig. 3A). Importantly, partial protection against S6 dephosphorylation by 1 nM calyculin A did not reverse SFN-induced translation inhibition (Fig. 3B), which strongly suggests that SFN acts upstream of S6. For instance, targeting S6K1 SFN might influence different translation stages. S6K1 phosphorylates and inactivates eEF-2 kinase, which negatively regulates peptide chain elongation. Thus, inhibition of S6K1 may lead to premature translation termination [24]. Another, recently described, S6K1 substrate is a tumor suppressor, programmed cell death protein 4 (PDCD4). Phosphorylation of PCDC4 by S6K1 leads to its proteasomal degradation which correlates with cancer progression and resistance to chemotherapy [37]. PCDC4 negatively regulates protein synthesis by binding to and inhibiting the helicase activity of eIF4A, thus impairing the cap-dependent translation [38]. S6K1 also stimulates eIF4A helicase through phosphorylation and activation of eIF4B [25]. Thus, SFN-mediated inhibition of S6K1 might negatively regulate translation initiation. Besides, SFN inhibits mTOR-mediated phosphorylation of 4E-BP (Fig. 2A and B) which may lead to a block in a cap-dependent translation.

SFN-induced block in protein synthesis is reversible, at least when cells are treated for a short time: changing medium after 1.5-hour SFN treatment for drug-free medium causes gradual re-phosphorylation of S6K1 and S6. This process, however, is inhibited by rapamycin, which indicates involvement of mTOR kinase in the signaling. Early removal of SFN from the medium allows for protein synthesis continuation and recovery of cell viability to the level characteristic of control cells (data not shown).

Oxidative stress and dysfunctional mitochondria may influence mTOR-S6K1 signaling [39,40]. Earlier studies on mechanisms of SFN anticancer activity in prostate cancer cells showed that ROS play a causative role in cell death or cell cycle block, and antioxidants or overproduction of catalase significantly protected cells against these responses to SFN [12]. The main source of oxidative stress appeared to be mitochondrial respiratory chain complexes and SFN inhibiting their activities increased production of ROS [10]. We wondered if a block in protein synthesis by SFN is mediated by ROS. PC-3 cells which are devoid of mtDNA (Rho0 cells), thus they have non-functional respiratory chain, are resistant to SFN-induced oxidative stress (this work and [10]). However, we observed a protein synthesis block in Rho0 cells upon SFN treatment. It may indicate that inhibition of translation by SFN is independent of mitochondria-derived ROS. SFN-induced oxidative stress in prostate cancer cells is mediated also by a non-mitochondrial pathway, through glutathione depletion [12]. Thus, our results do not exclude involvement of mitochondria-independent ROS in the translation inhibition by SFN. The mechanism underlying the block in mTOR-S6K signal transduction is currently investigated but it is not caused by direct inhibition of mTOR catalytic activity by SFN as judged by in vitro assay (data not shown).

Recent studies demonstrated that translation inhibition is a mechanism protecting cells against energetic crisis [41,42]. SFN disturbs oxidative phosphorylation [10] which might decrease ATP level. However, we were unable to detect significant changes in ATP concentration in cells treated with SFN for different times (15 min to 3 h). It is possible that changes in ATP production are too subtle to be detected by chemiluminescence method or glycolysis is upregulated and compensates for ATP production. In fact, glycolysis rate is decreased by SFN, both in PC-3 cells (Fig. 5C) and their Rho0 derivatives (data not shown). We examined the status of AMPK, a sensitive sensor for cell energetics. It becomes activated by phosphorylation at Thr-172, when AMP:ATP ratio increases in the cells, and then it inhibits mTOR pathway. In our model AMPK phosphorylation level rather decreases – statistically significant drop is observed after 1 h of SFN treatment (Fig. 5A). Thus, it rather excludes its role in mTOR-S6K signaling inhibition, which is persistent (Fig. 2A), and indicates that there is no energetic crisis in SFN-treated cells. It is possible that inhibition of anabolic processes, such as translation (this work), and activation of catabolic processes, such as autophagy [27], by SFN allows for maintenance of proper ATP level in cells. Inhibition of mTOR pathway and protein synthesis, and thus a reduction of energy consumption, has been recently described as a mechanism preventing energetic stress in glucose deprived MEF cells [41]. It is possible that in our model, the inhibition of mitochondrial respiratory chain enzymes parallels protein synthesis block.

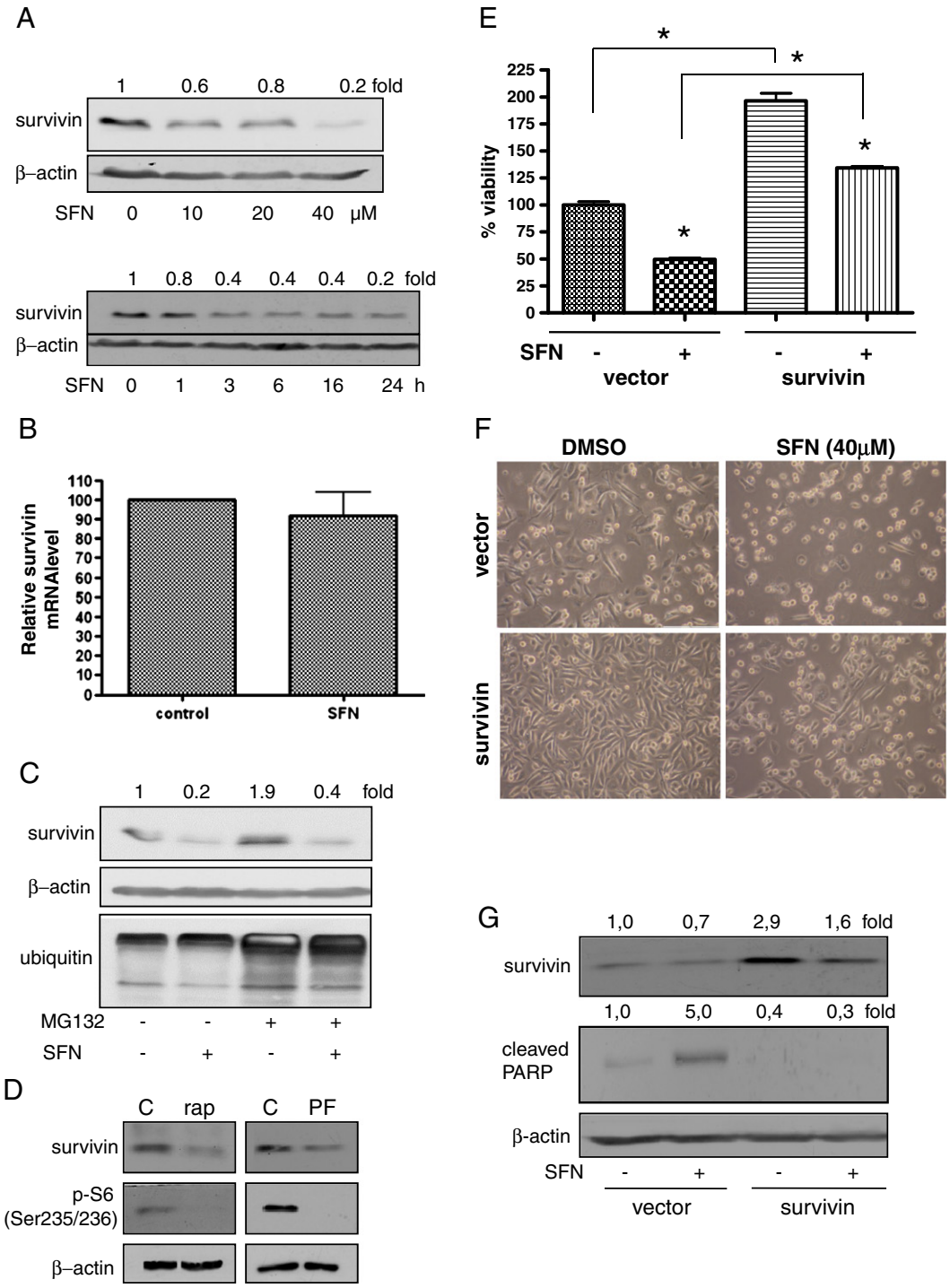

Fig. 6. Drop in survivin protein level contributes to SFN anticancer activity. A, immunoblotting for survivin using lysates from PC-3 cells treated with various SFN concentrations for 3 h or with 40 μM SFN for the indicated time periods. The blots were stripped and reprobed with anti-β-actin antibody to ensure equal protein loading. B, comparison of survivin mRNA level in PC-3 cells treated for 3 h with DMSO (control) or 40 μM SFN. Shown is the average (±SE) of two independent experiments. C, immunoblotting for survivin using lysates from PC-3 cells treated with DMSO or 40 μM SFN for 3 h pretreated or not with 1 μM MG132: D, survivin level in control cells (C) and cells treated or not with mTOR inhibitor, rapamycin (rap, 100 nM, 3 h) or S6K1 inhibitor, PF-4708671 (PF, 10 μM, 2 h). S6 dephosphorylation indicates inhibition of mTOR-S6K1 pathway. Comparison of viability (E), morphology of cells (F) or survivin level and PARP cleavage (G) in cells transfected with empty vector or vector encoding survivin and treated or not with 40 μM SFN for 24 h (E and F) or 16 h (G). The immunoblots were stripped and reprobed with anti-β-actin antibody to ensure equal protein loading. Densitometric analysis data after correction for loading control are on top of the immunoreactive bands. Similar results were observed in at least two independent experiments. Viability of PC-3 cells was measured by WST1 method, shown is the mean (±SE) of three parallel samples. *Significantly different ($P<0.001$) compared with corresponding controls or between indicated groups by one-way ANOVA followed by Bonferroni's multiple comparison test. Morphology of cells was investigated under light microscopy (400×). Similar results were observed in at least two independent experiments.

Translation inhibition by SFN may lead to depletion of short lived proteins, crucial for cell viability. One of them is an IAP family member, survivin, which regulates cell division, inhibits apoptosis and its half-life is about 30 min [43]. It is undetectable in majority of normal adult tissues, however it is often overexpressed in tumors, including prostate cancer [44]. Analysis of clinical samples has shown that expression of survivin increases from normal prostate tissue to low-grade primary carcinoma, to high-grade primary carcinoma, and is

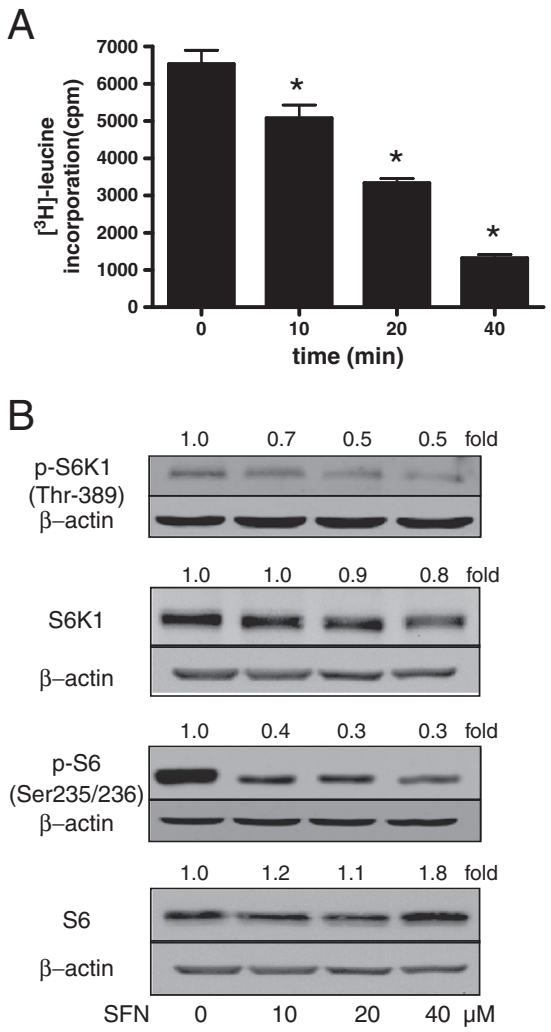

Fig. 7. SFN inhibits protein synthesis in SKBR-3 breast cancer cell line. A. SKBR-3 cells were treated with various concentrations of SFN for 3 h in the presence of a protein precursor, [^{3}H]-leucine. Cells were harvested and radioactivity of TCA-precipitable material was estimated as described in Materials and methods. Results shown are mean ±SE of three parallel samples, *significantly different ($P<0.01$) compared with DMSO-treated control by one-way ANOVA followed by Dunnett's multiple comparison test. Similar results were observed in two independent experiments. B. Immunoblotting for p-S6K1 (Thr-389), p-S6 (Ser-235/236), S6K1 and S6 using lysates from SKBR-3 cells treated with different concentrations of SFN for 3 h. The blots were stripped and reprobed with anti-β-actin antibody to ensure equal protein loading. Densitometric scanning data after correction for loading control are on top of the immunoreactive bands. Similar results were observed in at least two independent experiments.

the highest in lymph node metastases [45]. High level of survivin correlates with progression of cancer and resistance to therapies [46,47]. Its expression is regulated at multiple levels. Interestingly, the mTOR-S6K1 pathway has been shown to regulate stability and translation of survivin mRNA [48–50]. For instance, it has been shown that insulin growth factor-1 (IGF-1) induced persistence and translation of a pool of survivin mRNA in prostate cancer cells and it was mediated by mTOR-S6K1 [48]. Ablation of endogenous S6K1 by siRNA down-regulated survivin level [48]. Lowering intracellular survivin levels in cancer cells has been associated with cell cycle arrest, apoptosis and sensitization to therapies, thus it was proposed to be a novel cancer-specific treatment [51]. Here, we show that survivin level drops upon SFN treatment in PC-3 cells. Similar effect of SFN on survivin protein level has been observed in LNCaP and DU145 prostate cancer cells. Authors correlated it with suppression of STAT3 transcription factor by SFN. However direct link between STAT3 activity and survivin mRNA or protein level has not been investigated [52]. In our model a drop in survivin level is concomitant with translation inhibition and is preceded by dephosphorylation of S6K1. Moreover, it is not a result of survivin gene transcription perturbations (Fig. 6B), pointing toward post-transcriptional regulation. It has been reported that SFN stimulates ubiquitin–proteasome system activity [53,54]. However, inhibition of proteasome by MG132 only partially protected against SFN-induced drop in survivin level, while in control cells led to its accumulation (Fig. 6C). Thus, all these results indicate that SFN negatively regulates survivin protein synthesis, rather than accelerates its degradation. A decrease in survivin level strongly contributes to SFN anticancer activity as overproduction of survivin protects against SFN-induced changes in viability of cells mostly inhibiting apoptosis execution (Fig. 6E–G).

Translation is a vital process, necessary for cell growth and proliferation. Cancer cells worked out mechanisms to sustain this process at high level, ensuring sufficient levels of nutrients and energy and upregulating signaling pathways that induce translation. Thus, targeting synthesis of proteins, especially short-lived ones which are indispensable for cancer cell survival, might be an important mechanism of SFN chemopreventive and anticancer activity.

Acknowledgments

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