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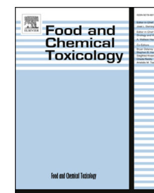

Sulforaphane induces differential modulation of mitochondrial biogenesis and dynamics in normal cells and tumor cells

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Antioxidant-based chemotherapy has been intensely debated. Herein, we show that sulforaphane (SFN) induced mitochondrial biogenesis followed by mitochondrial fusion in a kidney cell line commonly used in nephroprotective models. At the same concentration and exposure time, SFN induced cell death in prostate cancer cells accompanied by mitochondrial biogenesis and fragmentation. Stabilization of the nuclear factor E2-related factor-2 (Nrf2) could be associated with these effects in the tumor cell line. An increase in the peroxisome proliferator-activated receptor-γ co-activator-1α (PGC1α) level and a decrease in the hypoxia-inducible factor-1α (HIF1α) level would suggest a possible metabolic shift. The knockdown in the nuclear respiratory factor-1 (NRF1) attenuated the SFN-induced effect on prostate cancer cells demonstrating that mitochondrial biogenesis plays an important role in cell death for this kind of tumor cells. This evidence supports SFN as a potential antineoplastic agent that could inhibit tumor development and could protect normal tissues by modulating common processes.
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1. Introduction

In order to establish the best therapeutic strategy for each kind of tumor, a metabolic characterization should be done first in model cell lines. In some experimental cancer models it has been shown that mitochondrial-based metabolism is very important in the maintenance of cancer cell survival and malignancy (Zong et al., 2016). However, it is widely accepted that mitochondrial dysfunction contributes to cell growth and tumorigenesis. Many types of tumors rely on glycolytic metabolism to proliferate and invade (Boland et al., 2013). Like disturbing the redox status of cancer cells, shifting metabolism and promoting a mitochondria-dependent one could be an appropriate strategy for many types of cancer.

It is well known that preservation of mitochondrial function plays a major role in cytoprotection of non-cancer tissues (Guerrero-Beltrán et al., 2010; Sun et al., 2013). Recently, we found that curcumin, a bifunctional antioxidant, protected renal tubular cells against gentamicin toxicity through a mechanism that involved the nuclear factor E2-related factor-2 (Nrf2) and the regulator of mitochondrial biogenesis, the peroxisome proliferator-activated receptor-γ co-activator-1α (PGC1α) (Negrette-Guzmán et al., 2015). Likewise, it has been demonstrated that mitochondrial biogenesis is a crucial mechanism for proliferation, invasiveness, and metastasis control of several kinds of tumors (Liu et al., 2014; Onishi et al., 2014; Wang and Moraes, 2011), though others cancer types showed a noticeable adaptation when mitochondrial mass and function were increased (Alam et al., 2016; Zong et al., 2016). Mitochondrial dynamics have also been highlighted as a regulator of cell fate. In general, mitochondrial fusion has been associated with apoptosis resistance in cancer cells whereas mitochondrial fragmentation is a signal for apoptosis initiation. The GTPases mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic-nerve atrophy 1 (Opa1) are the main regulators of mitochondrial fusion

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and have been demonstrated to protect cells from mitochondrial outer membrane permeabilization and apoptosis (Jourdain and Martinou, 2009; Corrado et al., 2012; Thomas and Jacobson, 2012). The main protein in mitochondrial fission machinery, the dynamin-related protein-1 (Drp1), stimulates the oligomerization of Bcl-2-associated X protein (Bax) on the mitochondrial outer membrane, making it permeable, allowing the cytochrome *c* release and triggering intrinsic apoptosis (Montessuit et al., 2010). Mitochondrial hyperfusion has also been identified as a response to mild stress and could represent a pro-survival mechanism (Tondera et al., 2009).

Previously, mitochondrial modulation induced by the indirect antioxidant sulforaphane (SFN) on cancerous and non-cancerous cells was reviewed. SFN is an isothiocyanate derived from cruciferous vegetables and its best-known molecular target is Nrf2 (Negrette-Guzmán et al., 2013b). Nevertheless, the cytotoxicity and anti-proliferative effects of SFN on cancer cells have been linked to other mechanisms and targets, e.g., the activation of mitogen-activated protein kinases or the activation of the tumor suppressor protein p53, which can ultimately initiate an intrinsic apoptosis program (Rudolf and Cervinka, 2011; Rudolf et al., 2014). It has not been investigated if these pathways and proteins are associated to Nrf2. We proposed that both SFN-induced death in cancer cells and cytoprotection in non-cancer cells may be mediated by a differential regulation of mitochondrial biogenesis and dynamics that depends on the cell type. Mitochondrial biogenesis could be promoted by activation of Nrf2 (Negrette-Guzmán et al., 2013b). In this work, we used two characterized cell models in which SFN effects have been widely studied. It has been shown that SFN protects kidney tubular cells LLCPK1 against nephrotoxicants by means of nuclear accumulation of Nrf2, induction of an antioxidant response, preservation in mitochondrial functions, and finally, inhibition of mitochondrial apoptosis (Guerrero-Beltrán et al., 2010; Negrette-Guzmán et al., 2013a). However, SFN induces mitochondrial apoptosis in prostate cancer cells PC3 (Singh et al., 2004). Herein, we did not intend to compare redox and metabolic SFN-induced responses between tumor and non-tumor cells although this must be performed; we aimed to describe two known opposite SFN-induced effects in both (i) a non-transformed cell type which is a co-lateral target of antineoplastic agents and (ii) a tumor cell line used as a model for chemotherapy investigation. Our experiments were focused on the modulation of mitochondrial biogenesis and dynamics. Our results would boost SFN as an excellent agent in some cancer treatments. Further studies are still required.

2. Materials and methods

2.1. Reagents

SFN (S8044, a racemic mixture, R,S-sulforaphane) was purchased from LKT Laboratories (St. Paul, MN, USA); Dulbecco's Modified Eagle Medium (DMEM), Advanced Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), trypsin, and other supplements were obtained from Gibco-Thermo Fisher Scientific (Waltham, MA, USA). 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and crystal violet were purchased from Sigma-Aldrich (St. Louis, MO, USA). The probes Mito-Tracker Green FM and 5,5′,6,6’-tetrachloro- 1,1′3,3’-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), and Lipofectamine 2000 were acquired from Invitrogen-Thermo Fisher Scientific (Eugene, OR, USA). Mammalian Protein Extraction Reagent (M-PER) was purchased from Thermo Scientific-Thermo Fisher Scientific (Eugene, OR, USA). All buffers and reagents for Western blotting were obtained from Bio-Rad Laboratories (Hercules, CA, USA). The following antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA): anti-nuclear respiratory factor-1 (NRF1, sc-33771), anti-Bax (sc-493), anti-mitochondrial fission-1 (Fis1, sc-98900), anti-Drp1 (sc-32898), and anti-Nrf2 (sc-722). Anti-mitochondrial transcription factor-A (TFAM, 7495) and anti-β-actin (12262) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-mitochondrially-encoded NADH dehydrogenase-1 (MT-ND1, ab74257) and anti-PGC1α (ab54481) were provided by Abcam (Cambridge, MA, USA). Novus Biologicals (Littleton, CO, USA) provided the anti-hypoxia-inducible factor-1α (HIF1α, NB100-479) antibody. Anti-voltage-dependent anion channel (VDAC, V2139) antibody was purchased by Sigma-Aldrich (St. Louis, MO, USA). *In Situ* Cell Death Detection Kit, POD (Cat. No. 11684817 910), was acquired from Roche Applied Science (Mannheim, Germany). Dicer-substrate small interfering RNA (DsiRNA) for silencing NRF1 and control DsiRNA were obtained from IDT Technologies (Coralville, IA, USA). Universal biotinylated link, streptavidin conjugated to horseradish peroxidase (HRP), and 3,3’-diaminobenzidine (DAB) were purchased from Dako (Carpinteria, CA, USA). All other chemical and compounds used were reagent grade and commercially available.

2.2. Cell culture, treatments and cell assays

Porcine renal epithelial cells LLCPK1 (CL-101, American Type Culture Collection, Rockville, MD, USA) were grown in DMEM supplemented with 10% FBS and 1% antibiotic under permissive conditions: 37 °C and 5% CO_2 (Guerrero-Beltrán et al., 2010). Androgen-independent human prostate cancer cells PC3 (CRL-1435, American Type Culture Collection, Rockville, MD, USA) were cultured in Advanced RPMI 1640 supplemented with 3% FBS, 1% antibiotic, 1% non-essential amino acids, 1% pyruvate and 1% glutamine. Both cell lines were seeded onto 96-well plates at a density of 5×10^3 cells/well and used for experiments the next day. Cells were incubated in medium containing 1–20 μM SFN for 24–96 h in order to evaluate the effect of SFN on MTT reduction. Every 24 h, culture medium was replaced by fresh medium with SFN. MTT reduction was assessed as was done previously (Negrette-Guzmán et al., 2015). Briefly, cells were incubated in medium containing MTT (0.125 mg/mL) at 37 °C for 1 h in humidified air supplemented with 5% CO_2. Medium was then discarded and the formazan crystals deposited in each well bottom were dissolved in 100 μL of 0.1 N HCl in isopropanol. Absorbance was determined at 570 nm using a Synergy HT multimode microplate reader (Biotek Instruments Inc., Winooski, VT, USA). Based on the results from MTT assays and other previous studies (Guerrero-Beltrán et al., 2010; Negrette-Guzmán et al., 2013a), following experiments were carried out with 5 μM SFN. 5-bromo-2-deoxy-uridine (BrdU) incorporation as a measurement of cell proliferation was assessed in LLCPK1 cells using the Cell Proliferation ELISA, BrdU kit (Roche, Mannheim, Germany) and following the manufacturer instructions. For crystal violet assay, the medium was discarded at the end of treatment, cells were washed with phosphate-buffered saline (PBS) pH 7.4, then fixed with 4% paraformaldehyde pH 7.4 for 30 min and stained with 0.2% crystal violet in 6% methanol for 30 min at room temperature. Finally, each well in the plate was carefully washed with distilled water until wastewater was not colored by crystal violet. The plate was left to dry and the crystal violet staining nuclei was dissolved using 100 μL/well of 0.5% sodium dodecyl sulfate (SDS) in a solution containing 0.1 M sodium citrate in 50% ethanol. The plate was read at 570 nm in a microplate reader.

2.3. Apoptosis detection

DNA fragmentation, as a late apoptosis marker, was evaluated by terminal-deoxynucleotidyltransferase mediated dUTP-digoxigenin

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