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Research Paper

Sulforaphane is a Nrf2-independent inhibitor of mitochondrial fission

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ABSTRACT

The KEAP1-Nrf2-ARE antioxidant system is a principal means by which cells respond to oxidative and xenobiotic stresses. Sulforaphane (SFN), an electrophilic isothiocyanate derived from cruciferous vegetables, activates the KEAP1-Nrf2-ARE pathway and has become a molecule-of-interest in the treatment of diseases in which chronic oxidative stress plays a major etiological role. We demonstrate here that the mitochondria of cultured, human retinal pigment epithelial (RPE-1) cells treated with SFN undergo hyperfusion that is independent of both Nrf2 and its cytoplasmic inhibitor KEAP1. Mitochondrial fusion has been reported to be cytoprotective by inhibiting pore formation in mitochondria during apoptosis, and consistent with this, we show Nrf2-independent, cytoprotection of SFN-treated cells exposed to the apoptosis-inducer, staurosporine. Mechanistically, SFN mitigates the recruitment and/or retention of the soluble fission factor Drp1 to mitochondria and to peroxisomes but does not affect overall Drp1 abundance. These data demonstrate that the beneficial properties of SFN extend beyond activation of the KEAP1-Nrf2-ARE system and warrant further interrogation given the current use of this agent in multiple clinical trials.

1. Introduction

Sulforaphane (SFN) is an isothiocyanate compound derived in the diet most commonly from cruciferous vegetables [56]. It is generated in plants as a xenobiotic response to predation via vesicular release of the hydrolytic enzyme myrosinase from damaged cells; this enzyme converts glucosinolates to isothiocyantes [42]. Over the last two decades, SFN has been extensively characterized for its reported anticancer, antioxidant, and antimicrobial properties [57]. Much of this efficacy has been attributed to the capacity of SFN to modulate the KEAP1-Nrf2-antioxidant response element (ARE) signaling pathway, although additional activities of the compound have been identified, including the inhibition of histone deacetylase activity and cell cycle progression [29]. Nrf2 is the master antioxidant transcription factor and under conditions of homeostasis, its stability is suppressed through the action of the cytoplasmic Cullin3^{KEAP1} ubiquitin ligase complex [20]. Specifically, Nrf2 is recruited to the Cullin3^{KEAP1} ligase by binding to the dimeric substrate adaptor KEAP1 and is subsequently modified with polyUb chains that target the transcription factor for proteasome-mediated degradation. This constitutive turnover limits the half-life of Nrf2 in unstressed cells to ~15 min [30,33,46,55]. In response to numerous types of stress, most notably oxidative stress, KEAP1, a cysteine-rich protein, acts as a redox sensor, and oxidative modification of critical cysteines, particularly C151, of KEAP1 dissociates Nrf2-KEAP1 from CUL3 thereby preventing Nrf2 degradation [20,55,8]. Notably, SFN, and possibly other Nrf2 activators, mimic oxidative stress by modifying C151 of KEAP1 e.g. [21]. Stabilization of Nrf2 allows for its translocation to the nucleus where it induces the expression of a battery of Phase II antioxidant and detoxification genes. Nrf2 binds to the antioxidant response promoter elements (ARE) of its cognate target genes through heterodimerization with small Maf proteins [19]. This system presents a dynamic and sensitive response to indirect antioxidants like SFN, free radicals generated by the mitochondria [16], or other physiologic sources of oxidative stress [41].

Mitochondria are dynamic, subcellular organelles that regulate a host of cellular functions ranging from ATP production and intracellular calcium buffering to redox regulation and apoptosis [13,49]. Mitochondria also represent the principal source of reactive oxygen species (ROS) within the cell. Proper regulation of mitochondrial function is therefore necessary for optimizing ATP production to meet cellular needs while simultaneously minimizing the potentially harmful effects of excessive free radical production. A critical requirement for fine modulation of mitochondrial function is the capacity for mitochondria to function both independently as biochemical machines and as part of a vast, responsive network.

Mitochondrial network morphology and function are determined by a regulated balance between fission and fusion. Mitochondrial fission is

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required for daughter cell inheritance of mitochondria during cell division [28] as well as for the selective, autophagic degradation of depolarized or damaged mitochondria, termed mitophagy [1]. Conversely, fusion is required for complementation of mitochondrial genomes and sharing of electron transport chain components between neighboring mitochondria [54]. At the molecular level, mitochondrial fission and fusion are regulated by large, dynamin-like GTPases. Three enzymes primarily regulate fusion: Mitofusins 1 and 2 (Mfn1/2) are two-pass outer membrane proteins that mediate outer membrane fusion via heterotypic interactions between adjacent mitochondria [15,25,37], while OPA1 is an inner membrane protein that simultaneously ensures matrix connectivity by regulating the melding of inner membranes [5]. The GTPase activity of all three proteins is required for robust fusion [18,5], and OPA1 is further regulated by complex proteolysis within the mitochondrial inner membrane by the proteases OMA1 [14], PARL [6], and YME1L [45]. Importantly, intact mitochondrial membrane potential is required for efficient fusion in order to suppress integration of damaged and healthy mitochondria [26].

Mitochondrial fission is primarily catalyzed by a cytosolic protein called Dynamin-related protein 1 (Drp1/DNM1L). Drp1 is recruited from the cytosol to prospective sites of fission on the mitochondrial outer membrane [43]. The major receptors for Drp1 on the outer membrane are mitochondrial fission factor (Mff) [32] and, to a lesser extent, Fission 1 (Fis1) [51]. Additionally, a decoy receptor, MIEF1/MiD51, was discovered that acts to further limit the activity of Drp1 protein at potential fission sites [58]. Once docked at the mitochondrial outer membrane, Drp1 oligomerizes into spiral-like structures around the body of the mitochondrion and then utilizes the energy derived from GTP hydrolysis to mediate the physical scission of the mitochondrial outer and inner membranes [17]. Endoplasmic reticulum-derived tubules act as an initial constrictor of mitochondria prior to Drp1 oligomerization, underscoring the revelation that non-constricted mitochondria are wider than the permissive circumference of a completed Drp1 spiral [12]. Actin dynamics are also important for the ER-mitochondria interactions that precede mitochondrial fission [24]. In addition to its role in mitochondrial fission, Drp1 catalyzes the fission of peroxisomes [40].

Drp1 is very similar to the well-characterized dynamin protein in that both proteins contain an N-terminal GTPase domain, a Middle domain that is critical for self-oligomerization, and a C-terminal GTPase effector domain [31]. Drp1 achieves selectivity for mitochondrial membranes through a combination of interactions with its receptor proteins Mff and Fis1 and also through its affinity for the mitochondria-specific phospholipid cardiolipin via the unique B-insert domain of Drp1 [2]. Drp1 typically exists as a homotetramer in the cytoplasm, and higher order assembly at mitochondrial fission sites is mediated by the Middle domain of Drp1 [3].

Given the implicit link between mitochondrial function and the KEAP1-Nrf2-ARE pathway, we investigated the effects of Nrf2 activation on mitochondrial structure and function. We demonstrate here that SFN induces mitochondrial hyperfusion that, unexpectedly, is independent of both Nrf2 and KEAP1. This effect of SFN is through an inhibition of Drp1 function. We further demonstrate that SFN confers resistance to apoptosis that is Nrf2-independent and mimics that observed in cells depleted of Drp1. These data collectively indicate that in addition to stabilizing and activating Nrf2, SFN modulates mitochondrial dynamics and preserves cellular fitness and survival.

2. Results

2.1. Sulforaphane induces Nrf2/KEAP1-independent hyperfusion of mitochondria

In the course of studying the effects of Nrf2 activation on mitochondrial network dynamics, we discovered that treatment of immortalized, human retinal pigment epithelial (RPE-1) cells with sulforaphane (SFN), a potent activator of Nrf2 signaling, induced a robust fusion of the mitochondrial network when compared with vehicle-treated control cells (Fig. 1A and B). The morphology of the mitochondria in these cells greatly resembled that of the mitochondria in cells depleted by siRNA of endogenous Drp1, the principal mitochondrial fission factor (Fig. 1A). This result raised the intriguing idea that mitochondrial fission and fusion status responds directly to Nrf2 levels in the cell. However, stimulation of cells with other Nrf2 stabilizers and activators such as the proteasome inhibitor MG132, the pro-oxidant tBHQ, or knockdown of the Nrf2 inhibitor KEAP1 did not induce mitochondrial fusion (Fig. 1A and B). Stabilization of Nrf2 by these manipulations was confirmed by western blotting for endogenous Nrf2 (Fig. 1C). Furthermore, expression of Nrf2 was dispensable for SFN-induced mitochondrial fusion, as knockdown of endogenous Nrf2 with siRNA failed to counter this phenotype (Fig. 1D–F). Because SFN stimulates the KEAP1-Nrf2-ARE pathway by covalently modifying cysteine residues of KEAP1 [21], we knocked down KEAP1 to address whether SFN-induced mitochondrial hyperfusion is stimulated through a KEAP1-dependent, but Nrf2 independent pathway. However, depletion of KEAP1 also failed to abrogate SFN-induced mitochondrial fusion (Fig. 1G–I). In fact, SFN reversed the pro-fission morphology induced by depletion of KEAP1 (Fig. 1G, panel b versus panel d). These results indicate that SFN treatment causes mitochondrial fusion independent of the canonical KEAP1-Nrf2-ARE pathway and led us to interrogate whether SFN directly affects components of the mitochondrial fission or fusion machinery.

2.2. Sulforaphane impairs the mitochondrial association of Drp1

Based on the finding that SFN-treatment induces mitochondrial hyperfusion, we reasoned that this phenotype was either a consequence of excessive fusion activity or an inhibition of fission activity. To discriminate between these two possibilities, we compared the morphology of peroxisomes in the presence and absence of SFN. Peroxisomes are similar to mitochondria in that they are dynamic organelles the shape and length of which are constantly in flux [44]. Peroxisomes contain both Fis1 and Mff in their outer membrane and, as a consequence, are targets for Drp1-mediated fission [22,23]. However, peroxisomes do *not* utilize the fusion machinery of the mitochondrial network and consequently, do not undergo fusion [39]. Rather, peroxisomal fission is opposed by the lengthening of existing peroxisomes via de novo addition of membranes and proteins [44]. Because peroxisomes lack Mfn1/2 and OPA1, we reasoned that if SFN activates the fusion machinery rather than inhibiting the fission machinery, peroxisome length would not be affected. In vehicle-treated cells, peroxisomes are maintained as short, round, punctiform organelles (Fig. 2, panels b and d). However, SFN treatment increased peroxisome length by ~2-fold as compared to control cells (Fig. 2, panels f and h). Furthermore, many of the peroxisomes were pinched near the center, indicating a potential scission defect (Fig. 2, panel h, arrowheads). Likewise, peroxisomes in cells transfected with Drp1 siRNA were abnormally long (Fig. 2, panels j and l), confirming that Drp1 is required for peroxisomal fission and suggesting that SFN-treatment causes mitochondrial and peroxisomal phenotypes by disrupting the fission machinery.

We next determined how SFN restricts Drp1 function. Possibilities included reductions in expression levels, recruitment/retention at mitochondria, oligomerization, or enzymatic activity of the GTPase. A deficit in any one of these would result in reduced mitochondrial fission and hyperfusion. We did not detect reproducible changes in Drp1 protein levels after SFN-treatment (Figs. 1C and 3A), and therefore concluded that SFN does not alter Drp1 stability or expression, consistent with Drp1 having a half-life of >10 h [50] and our SFN treatments being of shorter duration. Next, we investigated whether SFN affected the recruitment or retention of Drp1 to mitochondria. Fractionation studies showed that SFN induced a loss of Drp1 from the