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Sulforaphane can not protect human fibroblasts from repeated, short and sublethal treatments with hydrogen peroxide

Maria Chiara Lionetti[1], Federico Mutti[1], Erica Soldati[1], Maria Rita Fumagalli[1,2], Valentina Coccé[3], Graziano Colombo[4], Emanuela Astori[4], Alessandro Miani[5,6], Aldo Milzani[4], Isabella Dalle-Donne[4], Emilio Ciusani[7], Giulio Costantini[2], Caterina A. M. La Porta[1]

[1] Center for Complexity and Biosystems, Department of Environmental Science and Policy, University of Milan, via Celoria 26, 20133 Milano, Italy
[2] Center for Complexity and Biosystems, Department of Physics, University of Milan, via Celoria 16, 20133 Milano, Italy
[3] Department of Biomedical, Surgical and Dental Sciences, University of Milan, Via Pascal 36, 20133 Milano, Italy
[4] Department of Biosciences, University of Milan, via Celoria 26, 20133 Milano, Italy
[5] Department of Environmental Science and Policy, University of Milan, via Celoria 10, 20133 Milano, Italy
[6] SIMA, Societá Italiana di Medicina Ambientale, via Monte Leone 2, 20149, Milano, Italy.
[7] Istituto Besta, Via Celoria 11, 20133 Milano, Italy
* Correspondence: caterina.laporta@unimi.it

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Abstract: A delicate balance of reactive oxygen species (ROS) exists inside the cell. When the mechanisms that control the level of ROS fail, the cell enters in an oxidative stress state, a condition that is involved in accelerating aging processes. To counteract the effects of ROS, the supplementation of antioxidants such as sulforaphane (SFN) was recently proposed. SFN is an isothiocyanate isolated from Brassica plants that modulates many critical factors of the cells and that seems to counteract aging processes. In the present work, we exposed human dermal fibroblast to short, sublethal and repeated treatments with hydrogen peroxide for eight days, without or in combination with low concentration of SFN. Hydrogen peroxide treatment does not affect the oxidative status of the cells or change the intracellular level of ROS, the number of mitochondria or thiols in total proteins. However, this treatment promotes cells from G0/G1 to S and G2-M phase, affects cell viability, increases the anti-apoptotic factor survivin and increases DNA damage, measured as number of foci positive for γ-H2AX. On the other hand, SFN alone plays a protective effect increasing the level of p53 and blocking the expansion of possible DNA damaged cells. However, SFN is not able to protect the cells from the stresses induced by hydrogen peroxide.

Keywords: oxidative stress; sulforaphane; fibrobalsts; p53

1. Introduction

Senescence is a complex process where the integrity and the structure of the nuclear scaffold changes [1]. One important factor contributing to cell senescence is oxidative stress [2].

Reactive oxigen species (ROS) are physiological by-products of mitochondria metabolism. Oxidative stress is due to an unbalanced oxidant/antioxidant status occurring in cells that could cause oxidative damage to DNA, lipids and proteins. ROS level regulates physiological functions, including signal transduction, gene expression, and proliferation, therefore they underlie physiological and pathological events [3]. For example, mitochondrial ROS may activate an adaptive response which

promotes health to extend the lifespan through diseases prevention [4]. ROS overproduction, on the other hand, hampers nuclear and mitochondrial DNA repair at multiple steps, contributing to cell genomic instability [5]. Interestingly, ROS, including hydrogen peroxide, can inhibit cell growth and induce cell death and senescence in a context-dependent manner [6]. Accordingly, a recent paper showed that low levels of ROS can improve the defense mechanisms by inducing adaptive responses, which in turn contribute to stress resistance and longevity [7]. In contrast, high levels of ROS induce ineffective adaptive responses, contributing to aging onset and progression [7]. There are many anti-ageing strategies, from the scavenger of free radicals to the enhancing of antioxidant factors, that are proposed to buffer the level of ROS.

The main goal of the present paper was to investigate the impact of short and repeated sublethal treatments with hydrogen peroxide, commonly used to mimic oxidative stress [2], alone or in combination with sulforaphane (SFN) on human primary dermal fibroblasts (hSDF) focusing on critical biological functions of the cells. Recent evidence in fibroblasts showed that concentrations between 90-360 μM of hydrogen peroxide are sufficient to induce oxidative stress and premature cellular senescence in vitro recapitulating an aging process profile [8]. Therefore, the possible protective effect of factors such as SFN on normal human cells such as human fibroblasts is not investigated in the literature yet. This kind of study appears crucial to plan possible advice in general for anti-aging purposes. We are not in fact strictly interested to skin aging but in general to develop a strategy to investigate the impact of specific factors on critical cellular functions linked to aging such as DNA damage.

SFN is a well tolerated natural compound obtained from cruciferous vegetables, which has been shown to have a cytoprotective effect through Nrf2-mediated induction of phase 2 detoxification and anti-oxidant enzymes, such as heme oxygenase-1 (HO-1), NAD[P] H:quinone oxidoreductase-1 (NQO1), superoxide dismutase (SOD), glutathione S-transferase (GST), and γ-glutamyl cysteine ligase (γ-GCL), that elevate cell defense against oxidative damage and promote the removal of potential carcinogens [9]. However, it is becoming clear that multiple mechanisms are activated in response to SFN, including the suppression of cytochrome P450 enzymes, the induction of apoptotic pathways, the suppression of cell cycle progression, the inhibition of angiogenesis and anti-inflammatory activity [9][10] [11]. Another important biological activity of SFN is the negative control of HDAC activity [9].

Altogether, our findings show that prolonged exposure to SFN alone increases p53 expression suggesting that, in the absence of exogenous oxidative stress stimuli, it plays a protective role against DNA damage. The main significance and consequence of this findings is that everyday life can lead to a short and low increase of hydrogen peroxide repeated in time and SFN is not able to counteract these effects but exerts an anti aging effect without oxidative stress changes. It is tempting to speculate that the combination of many factors rather than a single element can have a better protective effect. Further investigations will try to address these points.

2. Material and methods

2.1. Cell lines and treatments

Human primary dermal fibroblasts (hSDF) (BS PRC 41, IZSLER, Brescia, Italy) were established from a skin biopsy obtained from a healthy adult donor during a surgical procedure [12] and cultured in EMEM (Euroclone) containing 1% L-Glutamine, 1% Penicillin/Streptomycin and 10% FBS (basal medium) at 37°C in 5% CO_2 for no more than 10 passages [12]. Each experiments are carried out using cells coming form the same batch, therefore at the same passage in culture. The cells were treated with sublethal concentration of H_2O_2, for short (30min) and repeated time [2].

Briefly, subconfluent cells were plated and exposed to 15 or 25μM H_2O_2 (Fluka cod.95302) for 30 minutes at 37°C. This treatment was repeated every 48hrs for four times Fig.S1. Untreated cells were plated and grown in basal medium for all the duration of the experiment (8 days) Fig.S1. After every

treatment with H_2O_2, the cells were washed twice with sterile PBS and maintained in basal medium until the next treatment. Subconfluent cells were treated with sulforaphane (SFN, cod.S4441, Sigma) in basal medium at a final concentration of 1μM for 8 days after plating (see Fig.S1). For both conditions, medium was changed every 48h for a total of 8 days. In combined experiments with H_2O_2 and SFN, cells were maintained in basal medium containing H_2O_2 for 30min without SFN, then replaced with fresh medium containing SFN Fig.S1.

2.2. Proliferation assay

Sulforhodamine B (SRB) assay allows to quantify cellular protein content [13]. Briefly, the cells were fixed with 10% trichloroacetic acid (Sigma, cod.T6399) for 2 hours at 4°C and 0.04% (wt/vol) SRB protein-bound dye (Sulforhodamine B Sigma, cod. S1402, dissolved in 10 mMTris base solution) was added to each well and incubated at RT for 1 hour. After four washes with 1% (vol/vol) acetic acid, the samples were left to air-dry at room temperature. 100μl of 10 mM Tris base solution (pH 10.5) was added to each well and the plate was shaked on an orbital shaker for 10 min to solubilize the protein-bound dye. The absorbance at 510nm was detected using an microplate reader (BioRad).

2.3. Cell Cycle Analysis

Subconfluent cells were harvested by trypsinization, pelleted and fixed in 70% cold ethanol and subsequently stained with propidium iodide (PI, cod. P4864, Sigma) for 30 minutes at 4°C [14]. PI fluorescence was analyzed using FACS Vantage SE Becton Dickinson flow cytometry. The percentages of cells in each phase of the cell cycle were calculated using FlowJO software.

2.4. p53 level of expression by flow cytometry

Subconfluent cells were fixed 15 minutes in ice-cold methanol at -20°C, and incubated with primary antibody p53 linked to FITC at 4°C under dark condition (1:500, Abcam, ab156030) for 1h and then immediately analyzed using FACS Vantage SE Becton Dickinson flow cytometry. Analysis were conducted using FlowJo software and the expression of p53 for each sample is reported as the ratio between the intensity of fluorescence with respect to unstained cells due to autofluorescence.

2.5. Quantification of intracellular ROS by H2DCFDA

To detect ROS in cells, the cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA)(Thermo Fisher, cod.D399) has been used. The latter is converted into the highly fluorescent 2',7'-dichlorofluorescein (DCF) by the cleavage of acetate groups due to intracellular esterases and oxidation. Briefly, acetylated dye has been reconstituted in anhydrous dimethylsulfoxide (DMSO) at stock concentration of 100μM just prior to use. Cells have been incubated in 10μM dye solution in pre-warmed PBS containing calcium and magnesium for 1h at 37°C in 5% CO_2, protected from light. Following, loading buffer has been removed and cells returned to pre—warmed growth medium and incubated at the optimal temperature, for 1h at 37°C in 5% CO_2 in order to allow esterases to hydrolyze the acetate groups and render the dye responsive to oxidation. Fluorescence has been determined using Ensight microplate fluorescence reader (Perkin Elmer) using and Ex/Em: 492-495/517-527nm. Results are reported as mean fluoresce values for each sample.

2.6. Quantification of numbers of mitochondria

To quantify the numbers of mitochondria per cell, MitoTracker probe was used. The latter passively diffuses across the plasma membrane and accumulates in active mitochondria. Lyophilized MitoTracker (Thermo Fisher, cod. M7512) has been reconstituted in anhydrous dimethylsulfoxide (DMSO) to a final concentration of 1mM, then the cells have been incubated in 250 nM MitoTracher probe solution in pre-warmed growth medium for 45min at 37°C in 5% CO_2 under dark condition. Fluorescence has been detected using FACS Vantage SE Becton Dickinson flow cytometry and the

data were analysed by FlowJo software. Results are reported as the ratio between the intensity of fluorescence of each sample with respect to unstained cells due to autofluorescence.

2.7. Quantification of thiols in proteins

Total cellular proteins were obtained by cell lysis with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% TRITON X-100, 0.1% SDS, 0.5% sodium deoxycholate supplemented with protease inhibitors). The lysate was incubated on ice for 30 min and centrifuged at 10000rpm, for 10 min at 4°C to remove cell debris. The concentration of protein was assessed using BCA protein assay. To detect thiols present into proteins a biotin-maleimide assay was carried out. Briefly, 40 mM biotin-maleimide stock solution was prepared in DMSO and stored at -20°C. 1mg/mL of protein was incubated with 75μM biotin-maleimide solution for 1 hour at RT and then mixed to Laemmli sample buffer (2% SDS, 20% glycerol, and 125 mM Tris-HCl, pH6.8), boiled for 5 min at 90°C and immediately loaded on 12% SDS-PAGE gel [15]. The protein were then electroblotted onto a low-fluorescence polyvinylidene difluoride (LF-PVDF) membrane. Biotin tag was revealed using streptavidin-HRP assay as following. LF-PVDF membrane was washed with PBST (10 mM Na-phosphate, pH 7.2, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20 (Sigma Aldrich, cod. P9416) [15]and blocked for 1h in 5% (w/v) non-fat dry milk in PBST. After washing three times with PBST for 5 min, biotin tag was probed by 2-h incubation with 5% non-fat dry milk/PBST containing streptavidin-HRP (1:5,000 dilution,BioRad). Biotinylated proteins were visualized by ECL detection (cod.1705061, Biorad) using Chemidoc Touch Imaging System (Biorad). ECL signals has been normalized with respect to PVDF stain free [16].

2.8. Western Blot

Subconfluent cells were lysed in Laemmli sample buffer (2% SDS, 20% glycerol, and 125 mM Tris-HCl, pH6.8), briefly centrifuged and the protein concentration of the supernatant was measured by BCA Protein Assay Kit (cod.23225, Thermo Scientific). 15μg protein were loaded on 12% SDS-PAGE gel and transferred on PVDF using the TranBlot Turbo Transfer System Bio-Rad (cod.1704150, BioRad). After incubation of the PVDF sheet with 5% fat dry milk (cod.70166, Sigma) in PBS Tween 0.1% for 1h at room temperature, the membrane was incubated overnight at 4°C with primary antibody, anti-Nrf2 (1:2000, ADI-KAP-TF125, ENZO). The sheet has been then incubated with secondary antibodies, anti-rabbit HRP (Bio-Rad 1: 3000) or anti-Mouse HRP (Bio-Rad 1: 3000) for 1h. As housekeeping anti-βactin (1:10000, ab11003, Abcam) was used. ECL Blotting reagents (GE Healthcare, cod. RPN2109) were used at room temperature to detect chemioluminescence. The signal has been acquired using Chemidoc Touch (cod. 1708370, Bio-Rad). Densitometric analysis was carried out with ImageJ.

2.9. Immunofluorescence

Subconfluent cells plated on coverslips were fixed with 3.7% paraformaldehyde (BDH 29447) or in 100% cold methanol. For paraformaldehyde fixed cells, the cells were permeabilized with 0.1 %TritonX-100 in PBS for 15min at RT and then incubated with blocking solution (1%BSA/10% goat-serum/0.3 M glycine/0.1% Tween in PBS) for 1h at RT. The cells were incubated overnight at 4°C with the primary antibody as following: γ-H2AX (1:700, Abcam, ab2893-Phosho139, Rabbit)or anti-survivin (1:250, NB500-201, Novus Biological, Rabbit). The samples were incubated with secondary antibodies FITC anti-Rabbit (1:250, ab150077, AbCam) for 1h at RT and then mounted with Pro-long anti-fade reagent (P7481, Life Technologies) with DAPI to stain the nuclei. The images were acquired with a Leica TCS NT confocal microscope.

2.10. γ-H2AX spots counting and nuclear survivin

γ-H2AX spots inside the nuclei were counted using spot detector tool of ICY Software as described in our previous paper [17]. Briefly, we created a ROI for each nucleus and we computed the number of the marker spots inside its enabling the Scale n.3 with a sensitivity equal to 15. We also extracted the number of nuclei from the images to calculate the ratio of the number of foci per nucleus. The level

of expression of survivin fluorescence inside the nucleus was evaluated using a custom pipeline in ICY software. Briefly, we created a ROI for each nucleus and evaluated the mean intensity of Survivin signal over all the nuclear surface. DAPI channel intensity was considered in order to verify the absence possible bias due to differences between the nuclei and images.

2.11. Statistical analysis

Statistical significance analysis is performed using the Kolmogorov-Smirnov test and unpaired t-test..

3. Results

3.1. Effect of short and repeated sublethal treatment with hydrogen peroxide without or in combination with SFN on the oxidative status of hSDF

H2DCFDA is a chemically reduced form of fluorescein used as an indicator for intracellular ROS levels. The short oxidizing treatment (30min) repeated every 48hrs for 8 days with sublethal concentrations (15μM or 25μM) of hydrogen peroxide (see Fig.S1) according to [2] alone or in combination with 1μM SFN does not affect the levels of ROS measured using H2CDFDA assay or the numbers of mitochondria in the cells quantified by flow cytometry (Fig.1). These data suggest that during the 48 hours of recovering, hSDF cells implement response and adaptative mechanisms to protect against permanent injuries. Since it is known that Nfr2 is a transcription factor whose activation is induced by SNF [18] [19], we performed western blot of Nrf2 on untreated cells with respect to cells treated with SFN for 8days. As shown in Fig.S2 we found a significative increase in the level of Nrf2 in treated cells ($p<0.01$). Furthermore, we also checked the capability of these cells to be affected by high levels of ROS using H2DCFDA reduction as an indicator of the intracellular ROS level as shown in Fig.1. The treatment with 500μM hydrogen peroxide for one hour doubled the level of ROS ($p\ll0.0001$), confirming that the cells respond to hydrogen peroxide induction.

It is known that oxidative stress leads to the formation of unwanted disulphide bonds in the cytoplasm, eventually leading to impaired protein function. To face this, the cells have several mechanisms to increase the intracellular levels of thiols [20]. Notably, intracellular increased of thiol levels are strongly associated with an increased tolerance to an oxidant stress [20] since they act as extraordinarily efficient antioxidants protecting the cells against consequences of damage induced by ROS [21]. Differently, an age-dependent reduction in the amount of (free) thiols occurs in plasma proteins in healthy humans. This indicates that the efficiency of the reduced protein thiol pool as an antioxidant defense system decreases with age. The drop in the plasma level of protein thiol suggests depletion and/or impairment of the antioxidant capacity of plasma [22]. Indeed, the protein thiolation index, i.e., the molar ratio between the sum of all low molecular mass thiols bound to plasma proteins (forming, as a whole, S-thiolated proteins) and protein free thiols, is a suitable biomarker of oxidative stress [23]. Protein thiolation index shows a near linear age-dependent increase during ageing in humans and is a useful indicator of thiol-specific oxidative stress in patients with end stage renal disease on maintenance haemodialysis [24]. Under our experimental conditions, the levels of reduced thiols in total proteins measured by biotin maleimide assay do not show any significant change (Fig.1).

3.2. SFN and oxidative stress decrease cell vitality and regulate apoptosis

The short treatment (30min) with H_2O_2 repeated every 48hs for 8 days with sublethal concentrations (15μM or 25μM) (Fig.S1), impact on cell cycle profile of hSDF cells as shown in Fig.2a, leading the cells to cell cycle progression. In fact, we observed a shift of distribution towards S-phase in all the experimental conditions and a slight increase in the number of cells into G2-M phase when treated with hydrogen peroxide combined with SFN (Fig.2a).On the other hand, the treatment with alone SFN does no result in restoring the typical cell cycle pattern distribution of these cells and in combination with hydrogen peroxide does not protect from the effect due to oxidative stress (Fig.2a).

Since the cells were not synchronized, it is tempting to speculate that the number of cells that are in a certain cell cycle phase is proportional to the time that cells spend in that phase of the cell cycle. We also detect the viability of the cells with the SRB assay. As shown in Fig.2b, hSDFs viability decreases significantly with 25μM of H_2O_2 alone. The cytotoxic effect of 25μM of H_2O_2 is not prevented by the presence of SFN (Fig.2b).

To investigate whether this regime results in changes the apoptotic pathway, we analyzed the expression of a well known anti-apoptotic factor, survivin. Fig.2c and Fig.S3 show an increased level of expression of survivin in hSDF cells treated with both concentrations of hydrogen peroxide in the absence and presence of SFN. Moreover, the treatment with SFN alone does not affect survivin expression (Fig.2c and Fig.S3). We also checked every 48hs when we changed the medium of the cells with fresh one after hydrogen peroxide treatment, the presence in the medium of apoptotic cells. We found always less than 4% of apoptosis.

Finally, we checked p53 expression, a well known protein which controls the genome by orchestrating a variety of DNA-damage-response to restore genome stability and that plays a critical role in triggering apoptotic pathways in damaged cells [25]. Interestingly, the treatment with 1μM SFN alone increases significantly the level of expression of total p53 (Fig.2d). These data are in agreement with recent findings showing that p53 increases thanks to Nrf2 through NQO1 [26]. This effect disappears when the cells are exposed to both SFN and hydrogen peroxide (Fig.2d).

Effect of SFN alone or with hydrogen peroxide on DNA damage

Histone γ-H2AX is the most sensitive marker of double-stranded DNA breaks (DSB) and telomere shortening [27]. Herein we have quantified the number of γ-H2AX foci in hSDF cells after 8 days of hydrogen peroxide treatment with or without 1μM SFN. As shown in Fig.3, there is a significant increase in the number of γ-H2AX positive foci increasing the concentration of hydrogen peroxide. In SFN treated hSDF cells there is no significant change in comparison to the untreated cells (Fig.3).

Discussion

Sulforaphane (SFN) is mainly present in Cruciferae such as broccoli sprouts and cabbages. It is a very well tolerated factor, showing antioxidant properties and inhibiting histone deacetylase enzymes (HDAC) [9].

SFN seems to have a double face effect: on one side it helps the clearance of progerin in accelerating ageing [28], and on the other hand it acts as anti-tumorigenic factor targeting cancer stem cells (CSC) [10,27,29]. Furthermore, high levels of SFN (higher than 5μM) were shown to induce apoptosis in cancer cells increasing ROS [11]. However, very little is known about the effects of SFN on healthy human cells. In a recent study, it has been investigated the effect of SFN on human mesenchymal stem cells (MSCs) at different concentrations [30], resulting in contrasting effects. In fact, while low (1μM) doses of SFN for 3 days enhanced the cellular proliferation and protected the cells against apoptosis and senescence, higher (5μM) concentration had a cytotoxic effect, leading to cell cycle arrest, programmed cell death and senescence [30]. It is known that some ROS, mainly hydrogen peroxide, at sublethal concentrations act as second messenger in signaling cascades and are involved in cell proliferation and differentiation [31] [32]. It has been recently reported that moderate increases in ROS levels trigger signalling pathways involved in cell proliferation, whereas an excessive ROS increase causes oxidative stress, which in turn induces cell death and/or senescence [33].

The main goal of this paper was to investigate the combined effect of sublethal concentrations and long-term exposure to SFN and H_2O_2 on human primary normal dermal fibroblasts (hSDF) on critical cell functions and the possible protective role of SFN against negative effects of oxidative stress. Regarding to hydrogen peroxide, we have used a physiological concentration [34]. Our experimental approach leads to faithfully mimic physiologic stress conditions. In fact, in the majority of the studies present in the literature, the experimental induction of oxidative stress is achieved by short exposure of the cells to high concentration of exogenous ROS, or by long term and continuous exposure to

moderate concentration of exogenous ROS. Both of these models are unlikely to reproduce physiologic conditions, where stimuli are discontinuous and ROS exposure limited. Indeed, excluding particular pathological conditions, it is very rare to find constantly increased level of ROS in healthy people but rather occasional and short ROS levels increases, albeit for a long time [35]. The sublethal exposure to hydrogen peroxide repeated for 30min every 48h up to 8 days does not significantly change the oxidative status of the cell measured as levels of ROS, number of mitochondria and levels of thiols in total proteins. This suggests that, using our protocol, the cells are able to activate compensatory mechanisms and recover the physiological oxidative status. However, hydrogen peroxide, both alone or in combination with SFN, modifies the complex and delicate physiology of the cells since it promotes the cell to S and G2/M phases, counteracts apoptosis increasing survivin expression albeit without changing in p53 levels. Moreover, hydrogen peroxide exposure results in a higher number of γ-H2AX positive foci which quantified DNA damage.

Two interesting results are related to SFN. Firstly, SFN induces alone an increase of p53 but does not induce any DNA damages. Consistently, the presence of SFN upregulates and stabilizes p53 oscillatory physiologic behaviour probably due to its indirect effect on NRF2 and HIPK2 [36] [37] [38]. In fact, SNF decreases the ubiquitinization of Nrf2 [39], this leads to Nrf2 to translocate into the nucleus where it can accumulate and activate its target genes [38]. In particular HIPK2 is transcriptionally regulated by Nrf2 [36] and its overexpression downregulates WIP1 participating to a negative feedback loop with p53 [40] [41]. A direct consequence is an increase of p53 level and a stabilization of its oscillatory dynamics [42] [37] [41]. Moreover, in our experimental conditions, the presence of hydrogen peroxide stimulus prevent the SFN-induced increase of p53 possibly due to the activation of different response pathways p53 independent.

The second interesting result is that SFN can not counteract the effect of hydrogen peroxide in hSDFs, confirming SFN negligible scavenging capacity [43] but also suggesting the presence of a common mechanism of action that results in cell type-specific response of either cell death and survival. In non-cancer cells, which have an inherent ROS level (IRL) lower than for cancer cells, SFN exposure causes just an adaptive antioxidant response, whereas, in cancer cells, which have an IRL closer to the ROS death threshold, leads to growth inhibition and death [43]. In conclusion, our findings show that SFN is not able to protect against low concentration and repeated exposure to hydrogen peroxide in human fibroblasts cells resembling a physiological condition of everyday life. Further studies should investigate the possible effect of synergic factors to protect these kind of cellular damages. In fact, to untangle the complex network inside the cells it is necessary to investigate the co-exposure to multiple factors in a model like ours that resembles a physiological condition.

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Author Contributions: MCL, FM, ES performed the cell treatments with hydrogen peroxide and/or SFN, cell proliferation, flow cytometry, immunofluorescence, quantification of mitochondria, western blot, EC helped with flow cytometry; GC, EA, AM, IDD performed the quantification of thiols and intracellular ROS; VC obtained the cells and carried out the characterization, MRF and GC carried out the quantification of spots immunofluorescence counting; MCL, FM, AM, MRF and CAMLP designed the experiments and wrote the paper.

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Figures

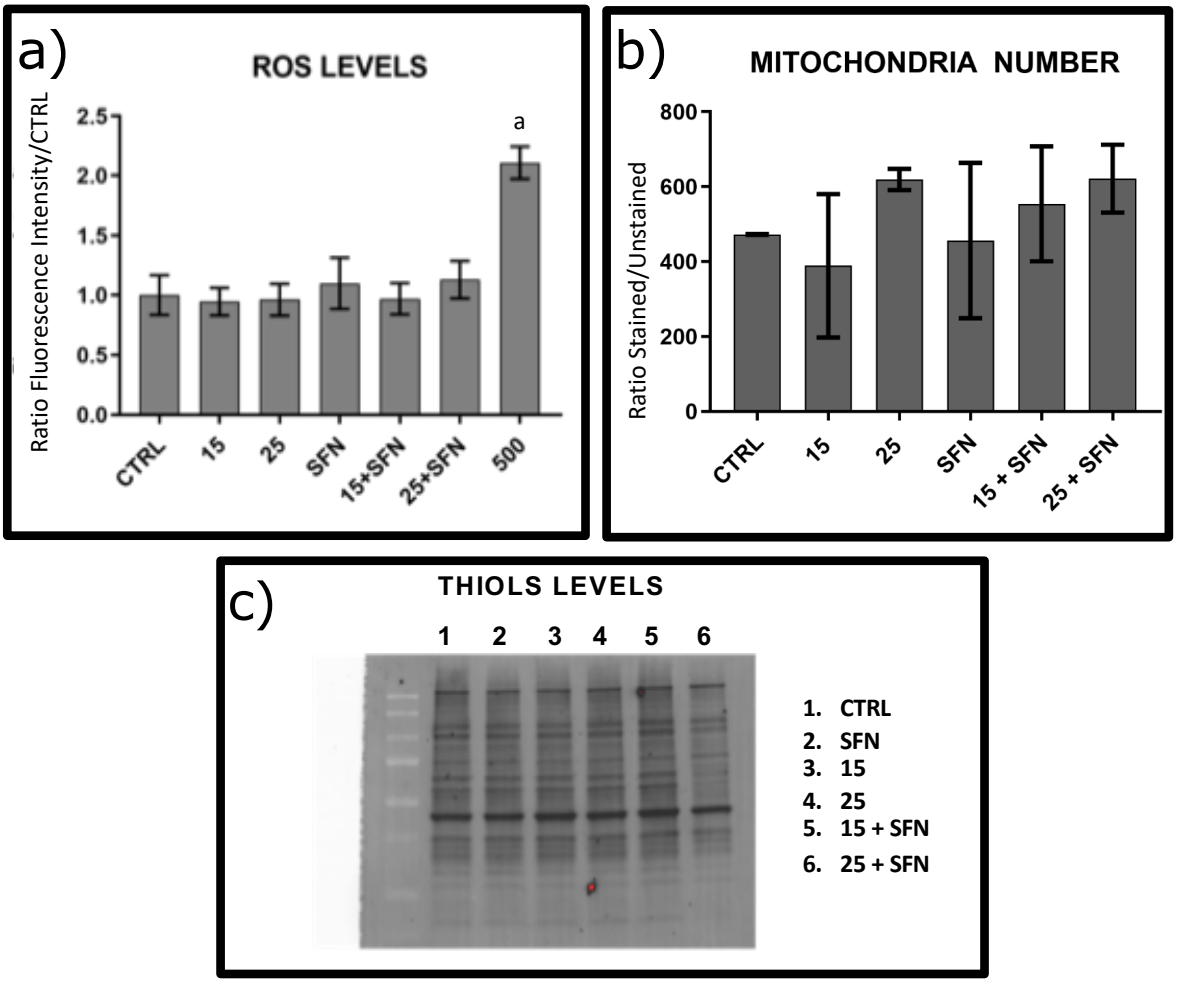

Figure 1. Evaluation of oxidative status after treatment with hydrogen peroxide at sublethal concentration repeated every 48h without or with 1μM SFN a) ROS level. Subconfluent cells treated as described in Fig.S1 and Material and Methods section with 15 or 25μM hydrogen peroxide without or with 1μM SFN (15+SFN, or 25+SFN, respectively) for 8 days were incubated in in pre-warmed PBS containing 10μM H2DCFDA (Thermo Fisher, cod.D399) for 1h at 37°C in 5% CO_2. The cells were treated with 500μM hydrogen peroxide for one hour and then the fluorescence was immediately quantified as positive control. The fluorescence was quantified by a Ensight microplate fluorescence reader (Perkin Elmer) (Ex/Em: 492-495/517-527nm). The results were reported as fluorescence intensity with respect to the fluorescence obtained by control cells (CTRL). a.u., Each bar represents the mean and the corresponding error bars of 16 independent measures for all the treatments and untreated cells with the exception of 500μM hydrogen peroxide where we carried out 4 independent measurements. a: $p<0.0001$ versus untreated cells. b) Numbers of Mitochondria. Cells treated as described in panel (a) were quantified using MitoTracker probe, which passively diffuses across the plasma membrane and accumulates in active mitochondria. Briefly, subconfluent cells were incubated with 250nM MitoTracker (Thermo Fisher, cod. M7512) for 45min at 37°C in 5% CO_2. Fluorescence has been detected using FACS Vantage SE Becton Dickinson flow cytometry and data were analyzed by FlowJo. The results were reported as the ratio between the intensity of fluorescence of each sample with respect to unstained cells due to autofluorescence. The bars are the mean with the statistic errors of three independent experiments. c) Levels of thiols into total protein. Total cellular proteins were obtained by cell homogenization with ice-cold lysis buffer. The lysate was incubated on ice for 30 min and centrifuged at 10000rpm, for 10 min at 4°C to remove cell debris. The concentration of protein was assessed using BCA protein assay. To detect thiols present into proteins a biotin-maleimide assay was carried out. 1mg/mL of protein was incubated with 75μM biotin-maleimide solution for 1 hour at RT and then mixed to Laemmli sample buffer, boiled for 5 min at 90°C and immediately loaded on 12% SDSPAGE gel. The proteins were then electroblotted onto a low-fluorescence polyvinylidene difluoride (LF-PVDF) membrane. Biotin tag was revealed using streptavidin-HRP assay. Biotinylated proteins were visualized by ECL detection (cod.1705061, Biorad) using Chemidoc Touch Imaging System (Biorad). ECL signals has been normalized with respect to PVDF stain free. This gel is representative of four independent experiments carried out.

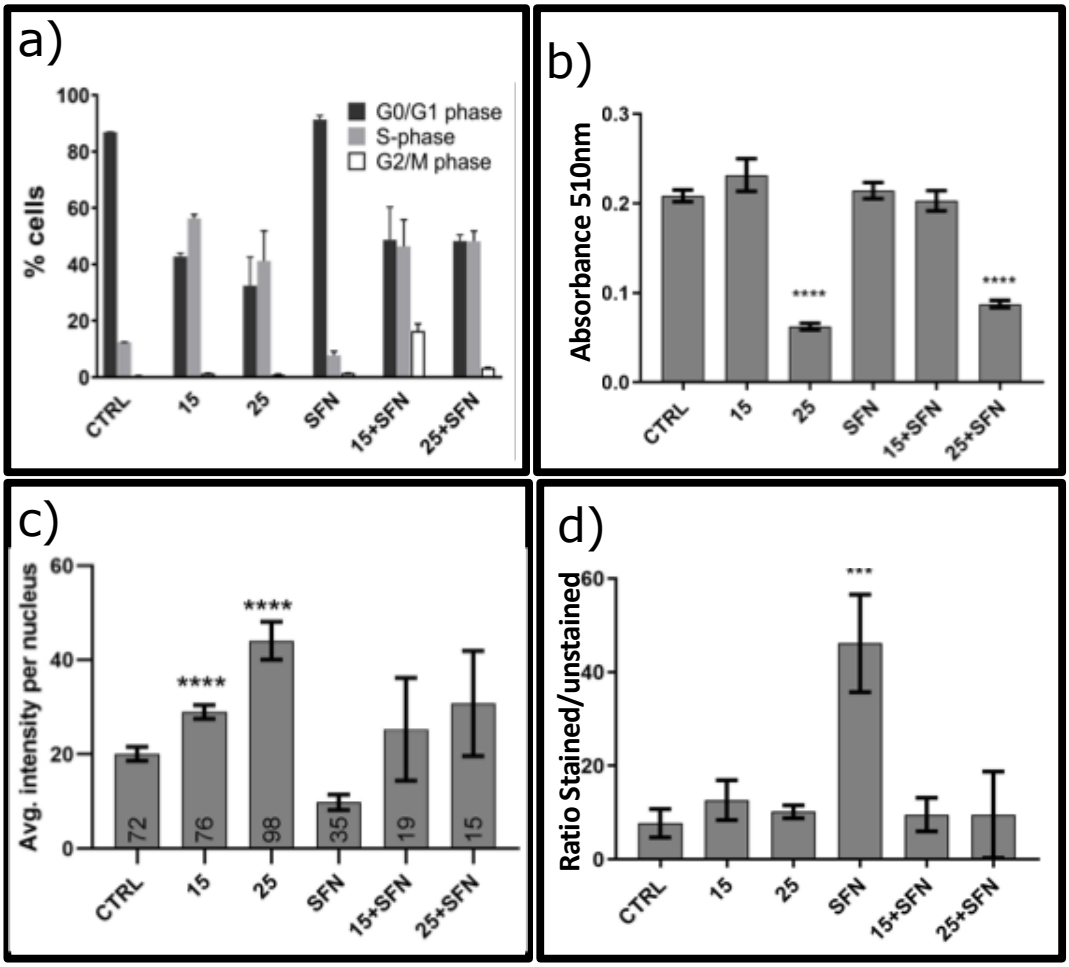

Figure 2. Effect on cell vitality, PI staining and survivin expression of sublethal concentration and repeated exposure to hydrogen peroxide without or with 1μM SFN. The subconfluent treated cells as described in Fig.S1 and Material and Methods section with 15 or 25μM hydrogen peroxide without or with 1μM SFN (15+SFN, or 25+SFN, respectively) up to 8 days. a) Propidium Iodide (PI) staining Subconfluent cells were fixed in 70% cold ethanol and stained with propidium iodide (PI, cod. P4864, Sigma) for 30 minutes at 4°C. PI fluorescence was analyzed using FACS Vantage SE Becton Dickinson flow cytometry. The percentages of cells in each phase of the cell cycle were calculated using FlowJo software.The bars show the mean and the statistic errors of two independent experiments. The results are expressed as percentage of cells into each cell cycle (G0/G1, S and G2-M phase). b) Proliferation assay. Sulforhodamine B (SRB) assay allows to quantify cellular protein content. Briefly, subconfluent cells were fixed with 10% Trichloroacetic acid (Sigma, cod.T6399) for 2 hours at 4°C. 0.04% (wt/vol). SRB protein-bound dye was added to each well and incubated at RT for 1h. 100μl of 10 mM Tris base solution (pH 10.5) was added to each well and the plate placed on an orbital shaker for 10 min to solubilize the protein-bound dye. The absorbance was detected using a Ensight microplate reader (Perkin Elmer) at 510nm. The bars are the mean and statistic errors of at least 18 independent sample. ****p<0.0001 versus untreated cells. c) Immunofluorescence of Survivin. Subconfuent cells plated on coverslips were fixed with 3.7% paraformaldehyde, permeabilized with 0.1%TRITOX-100 in PBS for 15min at RT, and incubated overnight at 4°C with anti-survivin (1:250, NB500-201, Novus Biological). The samples were incubated with the secondary antibody FITC anti-Rabbit (1:250, ab150077, AbCam) and then mounted with Pro-long anti-fade reagent (P7481, Life Technologies) with DAPI to stain the nuclei. The images were acquired with a Leica TCS NT confocal microscope. The bars show the average intensity of the nuclear fluorescence for each nucleus (see Materials and Methods). Each bar reports the average intensity and the error standard of the nuclear fluorescence of the number of nuclei reported inside the bar. d) p53 level of expression by flow cytometry. Subconfluent cells were fixed 15 minutes in ice cold methanol at -20°C, and then incubated with primary antibody anti-p53 FITC-conjugated at 4°C (1:500, Abcam, ab156030 Mouse) for 1h and then immediately analysed using FACS Vantage SE Becton Dickinson flow cytometry. Analysis were conducted using FlowJo software and the expression of p53 for each sample is reported as the ratio between the intensity of fluorescence with respect to unstained cells due to autofluorescence. Two independent experiments were carried out, each in triplicate.

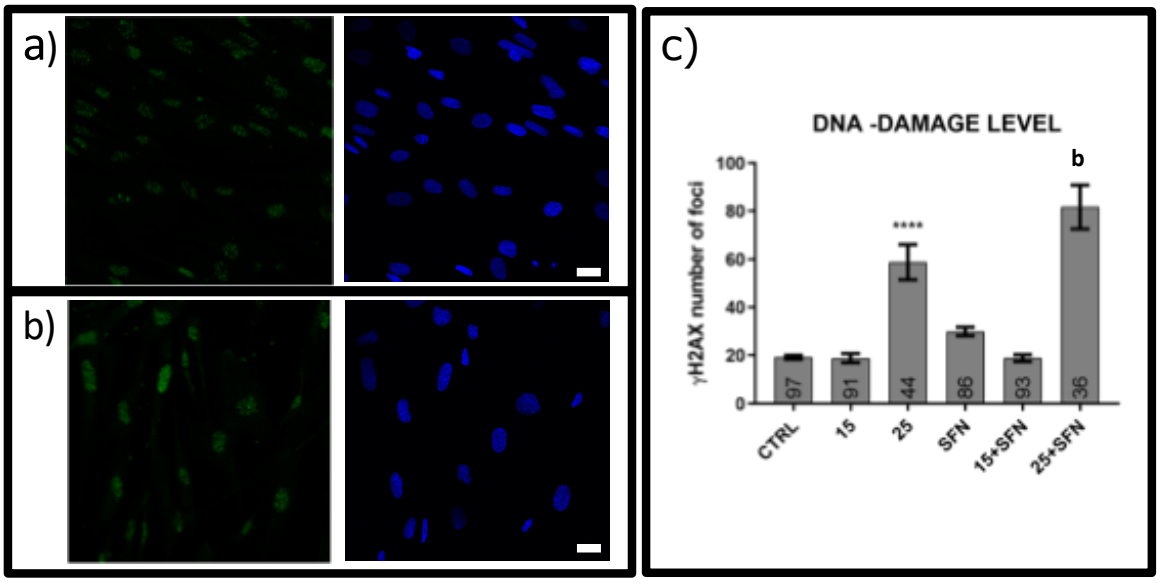

Figure 3. Effects of SFN alone or in combination with hydrogen peroxide on DNA-damage Subconfuent cells were treated as described in Fig.S1 and Material and Methods section. Panel a) shows an example of immunofluorescence for γH2AX and the correspondent nuclei stained with DAPI of untreated (CTRL, panel a) and treated cells treated with 25μM hydrogen peroxide. (panel b) Scale bar is 10μm. Briefly, the cells were fixed with 3.7% paraformaldehyd, permeabilized with 0.1%TRITOX-100 in PBS for 15min at RT and incubated overnight at 4°C with the γH2AX (1:700, Abcam, ab2893-Phosho139). The cells were then incubated with FITC anti-Rabbit (1:250, ab150077, AbCam) for 1h at RT and mounted with Pro-long anti-fade reagent (P7481, Life Technologies) with DAPI to stain the nuclei. The images were acquired with a Leica TCS NT confocal microscope. Panel c shows the quantification of γH2AX spots inside the nuclei using spot detector tool of ICY Software as described in the Materials and Method section. All the resulting values are normalized with the total number of pixels of their image, to make possible the comparison of all the nuclei, one with each other. In the bars are reported the number of cells analyzed for each conditions. ****p<0.0001 versus untreated cells; b: p<0.0001 versus SFN treated cells.

Supplementary Information

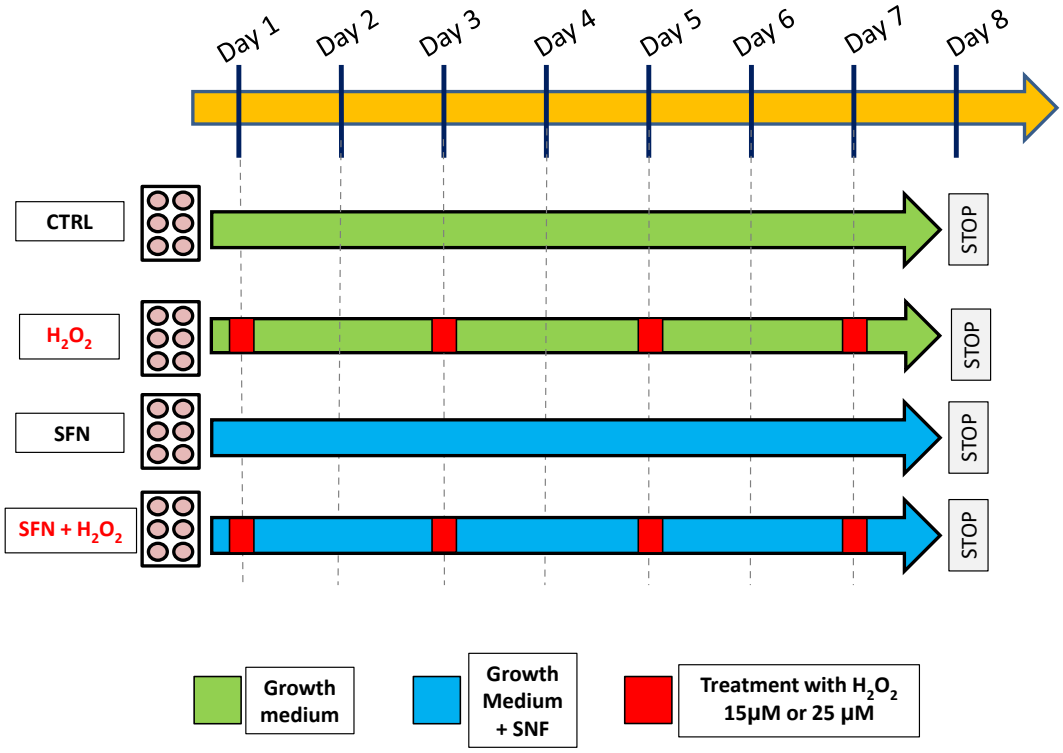

Figure S1. Schematic time line of the treatments with hydrogen peroxide alone or in combination with SFN

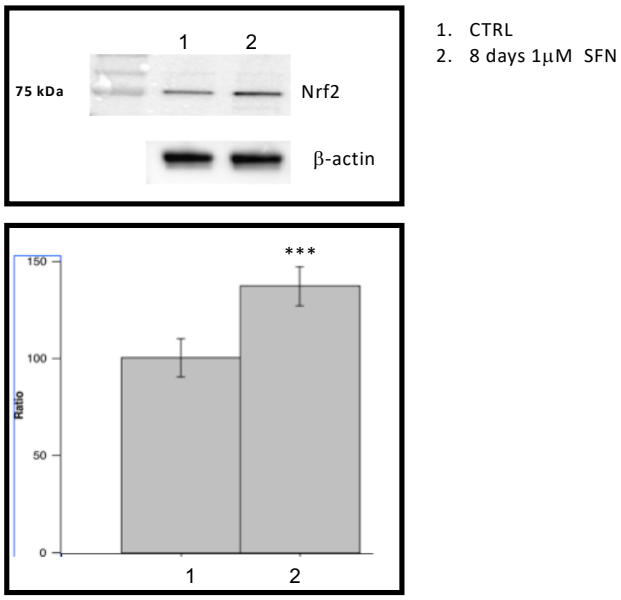

Figure S2. Level of expression of Nrf2 in SFN treated cells. 15μg protein obtained from subconfluent cells treated for 8 days with 1μM SFN accoding to Material and Method section were loaded on 12% SDS-PAGE gel and transferred on PVDF using the TranBlot Turbo Transfer System Bio-Rad. The membrane was incubated overnight at 4°C with anti-Nrf2 (1:2000, ENZO cod. ADI-KAP-TF125). Anti-*beta*actin antibody (1:10000, ab11003, Abcam) was used as housekeeping. Signals were quantified by densitometric analysis using ImageJ.***p<0.01 versus untreated cells. The gel represents a typical blot out of three blots carried out .

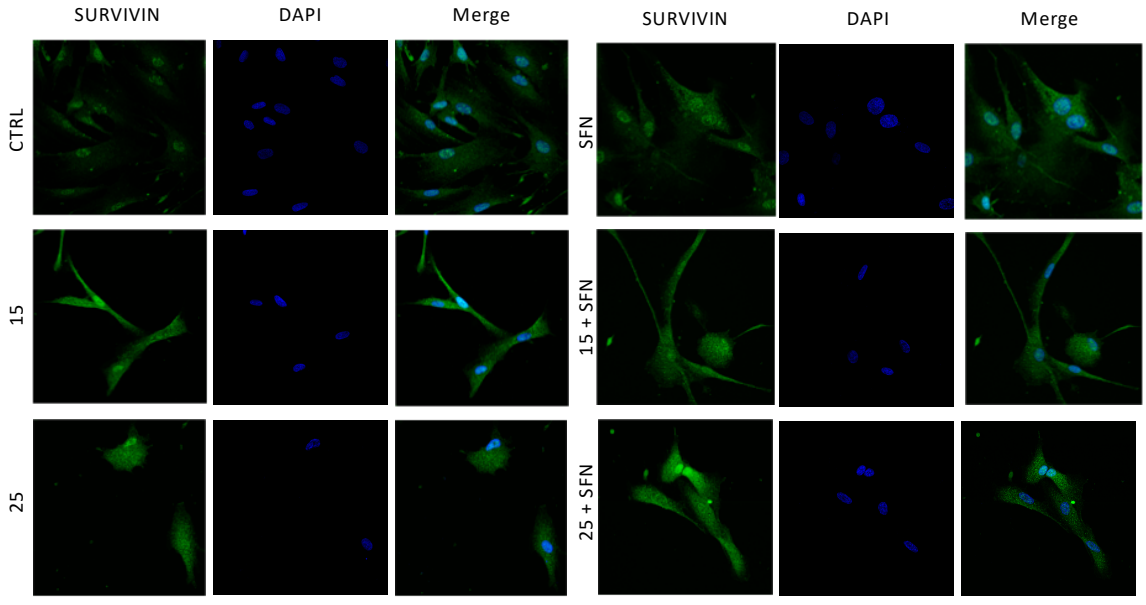

Figure S3. Immunoflorescence of survivin. Subconfuent cells (untreated or treated with hydrogen peroxide or SFN alone or in combination) were plated on coverslips, fixed with 3.7% paraformaldehyde and incubated overnight at 4°C with anti-Survivin (1:250, NB500-201, Novus Biological). The images were acquired with a Leica TCS NT confocal microscope. Here is shown the quantification of the nuclear fluorescence (see Materials and Methods). The bars show the mean and statistic errors of at least two independent experiments.