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Chapter 10

THE MULTIFACETED ROLE OF SULFORAPHANE IN PROTECTION AGAINST UV RADIATION-MEDIATED SKIN DAMAGE

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ABSTRACT

Ultraviolet radiation (UVR), the most abundant carcinogen in our environment, is the major factor in the etiology of skin damage and photocarcinogenesis. Common preventive measures, such as sunscreens and general sun avoidance, are not sufficiently effective, and skin cancer is the most common human cancer. Furthermore, cutaneous squamous cell carcinomas are among the most highly mutated human malignancies, carrying 1 mutation per ~30,000 bp of coding sequence. Such extraordinary mutation

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burden makes the possibility for success of a single-target therapy questionable and highlights the need for agents capable of affecting multiple hallmarks of cancer. UVR causes direct DNA damage, generation of reactive oxygen species (ROS), inflammation, and immunosuppression. These deleterious biological insults are counteracted by an elaborate network of cellular defense mechanisms. The isothiocyanate sulforaphane is a potent inducer of these defenses, which include cytoprotective antioxidant and anti-inflammatory enzymes and glutathione. Sulforaphane-containing broccoli sprout extracts, administered either topically or in the diet, protect SKH-1 hairless mice against UVR-mediated skin damage and tumor formation. In humans, application of these extracts to the skin of healthy subjects reduces susceptibility to erythema arising from acute exposure to UVR. Many of the protective effects of sulforaphane are due to the potent ability of the isothiocyanate to activate transcription factor Nrf2, and are lost in cells and animals that are Nrf2-deficient. In addition, sulforaphane provides Nrf2-independent protection, such as suppression of NFκB signaling and direct inhibition of the activity of macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine implicated in UVR-mediated skin damage and carcinogenesis. Thus, sulforaphane provides a paradigm for a dietary small molecule indirect antioxidant which plays a multifaceted role in protection against the damaging effects of oxidative stress and inflammation.

Keywords: chemoprotection, Keap1, MIF, Nrf2, photodamage, skin cancer

INTRODUCTION

Ultraviolet Radiation (UVR)-Induced Skin Damage and Carcinogenesis

Non-melanoma skin cancer is the most common human malignancy, with more than two million new cases diagnosed globally each year [1]. Exome sequencing of human primary cutaneous squamous cell carcinomas and matched normal tissue has revealed an extraordinary large mutation burden of about 1,300 somatic single-nucleotide variants per exome (1 per ~30,000 bp of coding sequence) [2]. Ultraviolet radiation (UVR) is the major causative factor for skin cancer. It acts as a complete carcinogen that can cause the initiation, promotion and progression of squamous cell and basal cell carcinomas. Following UVR exposure, cells display diverse damage, including direct modification of DNA bases, inflammation, morphological changes, and generation of ROS, which in turn leads to oxidative stress [3, 4]. New strategies of molecular protection are being explored as a method to protect the cells of the skin from the damaging effects of UVR, as the standard practices of sun avoidance and sunscreens have not proven adequately effective in decreasing the global incidence of skin cancer. Molecular protectors that are capable of boosting multiple intracellular defense mechanisms, thereby defending against the many types of deleterious biological insults of UVR, would be particularly advantageous [5].

The UVR-spectrum has two physiologically relevant wavelength components: UVB (280-315 nm) and UVA (315-400 nm) (Figure 1) [6]. While UVB wavelengths only represent about 5% of the solar radiation energy that reaches the surface of the Earth, they are largely responsible for causing erythema, photoaging, and cancer [1]. UVB penetrates the epidermis, which consists mainly of differentiated and proliferating keratinocytes [7]. These wavelengths damage DNA directly, promoting cross-linking between DNA bases with the formation of cyclobutane-pyrimidine dimers and pyrimidine [6-4] pyrimidone photoproducts [8, 9]. The

resulting mutations (characterized by C to T or CC to TT transitions) are known as "signatures" of sun exposure [10]. UVB wavelengths are also known to produce an acute inflammatory response culminating in edema and erythema. This response is accompanied by release of pro-inflammatory cytokines and upregulation of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), promoting prostaglandin-E2 (PG-E2) and nitric oxide (NO) synthesis [3]. This process signals the recruitment of leukocytes, leading to secondary tissue injury through the generation of reactive oxygen species (ROS) [6]. Evidence also suggests that UVB-induced DNA damage may play a role in initiating the inflammatory response and erythema by triggering IL-1 release [3].

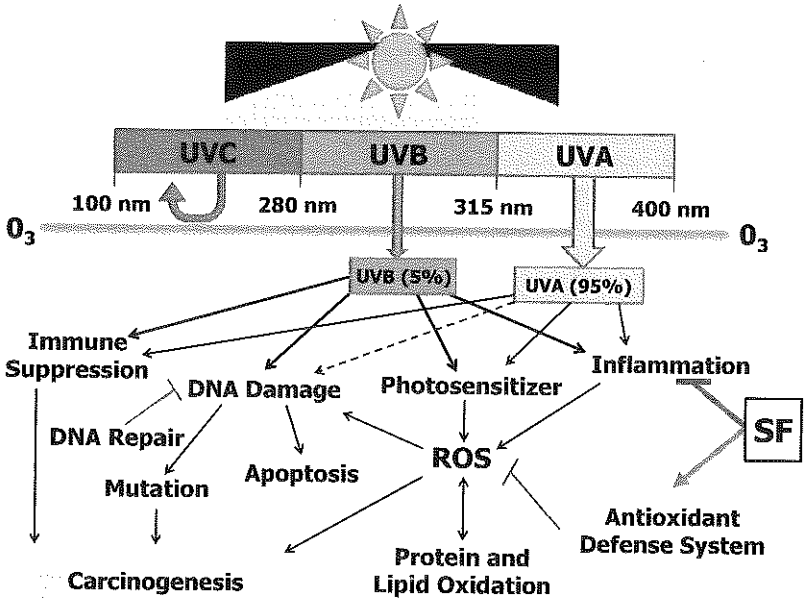

Figure 1. Sulforaphane has the potential to protect skin cells from UVR. The UVR-spectrum comprises UVC (100-280 nm), UVB (280-315 nm), and UVA (315-400 nm). UVC wavelengths are filtered by the ozone layer, thus UVB and UVA wavelengths are responsible for the physiological changes in skin and skin cells after exposure to solar radiation. These wavelengths are capable of producing direct damage to cellular macromolecules, generating ROS, increasing inflammation, as well as suppressing the immune system. These damaging effects are all important factors in the multistep carcinogenesis process. Sulforaphane (SF) has the potential to attenuate UV-induced damage by upregulating the antioxidant phase 2 defense system and suppressing pro-inflammatory processes. Modified from Svobodova et al. (2006).

UVA comprises ~95% of the terrestrial solar radiation energy, but it penetrates deeper into the dermis than does UVB, reaching the dermal fibroblast population, generating ROS and therefore indirectly oxidizing cellular proteins, lipids, polysaccharides, and DNA [4, 7]. In addition to damaging DNA indirectly resulting primarily in the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine, exposure of human skin to UVA also generates cyclobutane pyrimidine dimers, typical UVB-induced DNA lesions [11]. At high doses, UVA also initiates inflammatory responses within the skin [3]. Both UVA and UVB wavelengths thus induce inflammation, although wavelengths in the UVB region are more effective than those

in the UVA region in inducing an erythemic response [6]. This may be a result of the ability of UVB to cause direct damage to the DNA in the cells of the epidermis, subsequently triggering a release of the pro-inflammatory cytokines IL-1β and TNF-α from keratinocytes [7, 12]. In turn, these soluble factors regulate the release of IL-6 and IL-8 from dermal fibroblasts, inducing a dermal inflammatory response resulting in edema and erythema [13]. UVA and UVB wavelengths also cause DNA damage, through both direct modification and oxidative damage to DNA bases, leading to increased risk of mutations in the genome. When mutations occur in the tumor suppressor gene sequences, such as the *p53* gene, the ability of the cells to undergo apoptosis is impaired, leading to survival of cells with mutations that can potentially progress to skin cancer [4].

Exposure to UVA radiation can also sensitize the skin of those individuals who are undergoing certain therapies, notably treatments with the anti-inflammatory and immunosuppressive thiopurines. Indeed, long-term use of thiopurines (azathioprine, 6-mercaptopurine, and 6-thioguanine) is associated with increased risk (by more than 100-fold) for the development of very aggressive multiple squamous cell carcinomas of the skin [14-16]. During replication, the active metabolite of the thiopurines, 6-thioguanine nucleotide, is incorporated into DNA. Interestingly, oral treatment with azathioprine in mice leads to a much greater incorporation of 6-thioguanine in DNA of skin than liver [17]. Peter Karran and his colleagues have elegantly demonstrated that unlike guanine, 6-thioguanine absorbs in the UVA region of the solar spectrum, generating reactive oxygen species (ROS) and 6-thioguanine photo-oxidation products that damage DNA and proteins, including DNA repair proteins [18-20]. The combination of 6-thioguanine and UVA radiation is synergistically mutagenic in cells [21]. Treatment with azathioprine increases the skin photosensitivity to UVA radiation in humans [21], whereas replacement of azathioprine with mycophenolate in organ transplant recipients leads to reversal of this sensitization [22]. Therefore, oxidative stress and the inflammation produced by UVR are potential targets for intervention to alleviate the cellular damage that may lead to skin cancer.

Antioxidant Defenses

Many of the damaging processes initiated by UVR are counteracted by the intrinsic cellular antioxidant defenses. Superoxide dismutase (SOD), a widely distributed enzyme that converts superoxide to hydrogen peroxide at diffusion-controlled rates, together with catalase and peroxidases that then dispose of hydrogen peroxide, exemplify three critical endogenous enzymatic protective antioxidants. Cells are also equipped with small molecules with direct antioxidant functions, including reduced glutathione (GSH), ascorbic acid (vitamin C), tocopherols, lipoic acid, ubiquinol, and carotenes, which participate in redox reactions directly and scavenge oxidants. In addition to these housekeeping antioxidant systems, cells have evolved other elaborate and complex protective mechanisms, such as the inducible phase 2 cytoprotective enzymes and GSH, which act to detoxify harmful electrophiles and ROS [23]. In the case of excess UVR, the cells are overwhelmed by oxidative stress and the innate defenses are insufficient for protection [4]. Increasing the antioxidant capacity of the cell helps to protect it from oxidative damage. Direct antioxidants, such as (–)-epicatechin-3-gallate and carotenoids (i.e., β-carotene and lycopene), are themselves oxygen radical scavengers and protect skin cells from ROS-induced damage [24-26]. Nevertheless, their

protective effects are short-lived and they can also have pro-oxidant effects [27]. A more efficient and long-lasting protective strategy is the upregulation of the intrinsic antioxidant defense system through the Keap1/Nrf2 pathway.

The Keap1/Nrf2 Pathway

Keap1 (Kelch-like ECH-associated protein 1) mediates the ubiquitination of Nrf2 (NF-E2-related factor 2) by Cullin3 (Cul3)-based E3 ligase, and the subsequent degradation of the transcription factor by the 26S proteasome [28-32]. It does so by using a cyclic sequential attachment and regeneration mechanism whereby the Keap1-Nrf2 protein complex sequentially adopts two distinct conformations, "open," in which Nrf2 interacts with one molecule of Keap1, followed by "closed," in which Nrf2 binds to both members of the Keap1 dimer, allowing Nrf2 ubiquitination; the ubiquitinated Nrf2 is degraded by the proteasome, and Keap1 is regenerated (Figure 2) [33]. Oxidants and electrophiles modify reactive cysteine residues on Keap1 and disrupt the ability of Keap1 to target Nrf2 for degradation. Nrf2 then accumulates, translocates to the nucleus, where it dimerizes with small Maf proteins, and binds to cis-acting antioxidant response elements (AREs) in the 5'-flanking regions of Nrf2-dependent genes. These genes encode proteins and enzymes that have antioxidant and detoxification activities which promote removal of damaging ROS and reactive nitrogen species, electrophiles, toxins, and damaged proteins, lipids, and nucleic acids. Nrf2-dependent genes that provide these protective effects include: 1) conjugating enzymes, such as glutathione transferases (GSTs) and UDP-glucuronosyltransferases (UGTs); 2) enzymes involved in the GSH synthesis and utilization, including γ-glutamylcysteine synthetase (γ-GCS), glutathione peroxidase, and glutathione reductase; 3) antioxidant enzymes and proteins, such as NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HMOX1), superoxidase dismutase (SOD), catalase, thioredoxin (TRX), and thioredoxin reductase (TRXR). The coordinate upregulation of these systems, termed the phase 2 response, provides protection against a wide array of challenges and onset of disease [34].

Sulforaphane: Discovery As an Inducer of the Phase 2 Response

A natural, electrophilic compound isolated from broccoli extracts, sulforaphane (SF) [1-isothiocyanato-(4*R*)-(methylsulfinyl) butane] (Figure 3), is a potent inducer of the phase 2 cytoprotective response. The discovery and isolation of SF as an inducer was made by Yuesheng Zhang and colleagues [35] during the quest to find the factor(s) within vegetables that upregulate the phase 2 response and confer protection against carcinogenesis. The correlation between high consumption of certain vegetables and a lower risk for developing cancer had long been observed in humans and in animal studies [36-39]. These studies led to the conclusion that many green and yellow vegetables protect against colorectal cancer, lung cancer, and possibly prostate cancer. It was found that broccoli and other members of the Cruciferae family were especially rich sources of phase 2-inducer activity and that 3-day-old broccoli sprouts contain 20 to 50 times higher levels of SF than does the mature plant [40].

SF is naturally found in the form of an inert glucosinolate (β-thioglucoside *N*-hydroxysulfate) precursor called glucoraphanin. Glucosinolates, like glucoraphanin, are enzymatically converted to their biologically-active forms by thioglucoside glucohydrolases, also known as myrosinases (EC 3.2.1.147) (Figure 3) [41]. Enzyme and substrate are compartmentalized within plants and, when the plant tissue is damaged, they come in contact giving rise to a range of hydrolytic products, including SF [42-44]. Myrosinases are also present in fungi and bacteria. Of specific importance, microflora of the animal and human gastrointestinal tract can efficiently convert glucosinolates to isothiocyanates [45-48].

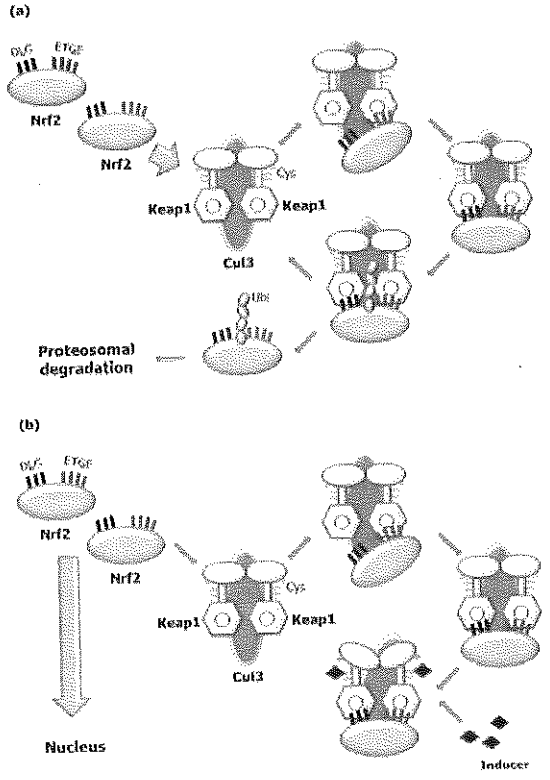

Figure 2. The cyclic sequential binding and regeneration model for Keap1-mediated degradation of Nrf2. (A) Nrf2 (purple) binds sequentially to a free Keap1 dimer (blue): first through its high-affinity ETGE motif (red) to one member of the Keap1 dimer to form the open conformation of the Keap1-Nrf2 complex, and then through its low-affinity DLG motif (black) to the second member of the Keap1 dimer to form the closed conformation of the complex. In the closed conformation, Nrf2 undergoes ubiquitination (yellow) by Cullin3 (green)/Rbx1 ubiquitin ligase. Ubiquitinated Nrf2 is released from Keap1 and degraded by the proteosome, free Keap1 is regenerated and able to bind to newly translated Nrf2, and the cycle begins again. (B) Inducers react with sensor cysteines of Keap1 (orange) leading to a conformational change resulting in inability for degradation. Consequently, the complex accumulates in the closed conformation, free Keap1 is not regenerated, and the newly-synthesized Nrf2 accumulates and translocates to the nucleus where, as a heterodimer with a small Maf transcription factor (not shown), Nrf2 binds to promoter sequences and activates expression of its target cytoprotective genes.

The central carbon of the isothiocyanate (-N=C=S) group is highly reactive with sulfhydryl groups, forming dithiocarbamate products. In animal tissues, SF is metabolized by the enzymes of the mercapturic acid pathway, ultimately forming reversible N-acetylcysteine-SF conjugates. Conjugation with glutathione, by action of cellular GSTs, sequesters SF within cells and this accumulation increases the inducer potency of SF [49-52]. Since its discovery as a phase 2 inducer, SF has been shown to be protective against many forms of cancers, traumatic injury, age-related diseases, and in many other disease and injury models. Together with the ability to initiate Nrf2-dependent phase 2 response, SF also suppresses pro-inflammatory processes and induces the heat shock response through Nrf2-dependent and -independent mechanisms [53-57]. At high concentrations, SF can also inhibit phase 1 enzymes, which activate procarcinogens to ultimate carcinogens, stimulate cell cycle arrest and apoptosis, and inhibit angiogenesis [58]. The multifunctional protective mechanisms and the natural, dietary occurrence of SF have made it a very attractive candidate as a potential therapeutic and cytoprotective agent against many forms of cellular damage, including UVR.

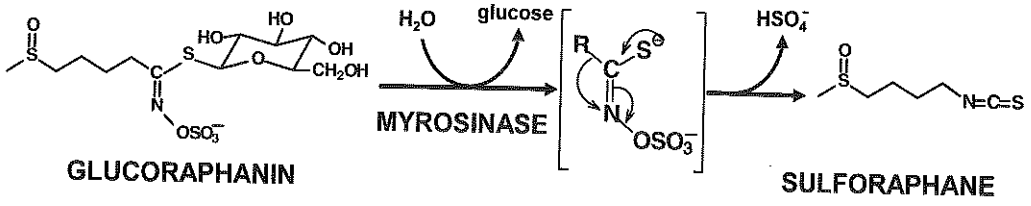

Figure 3. The general reaction in which glucoraphanin [(4-methylsulfinyl]butyl-glucosinolate; or thioglucoside *N*-hydroxysulfate, a glucosinolate], is converted to sulforaphane [1-isothiocyanato-(4*R*)-(methylsulfinyl)butane], is catalyzed by myrosinase (EC 3.2.1.147). This scheme in which a water soluble, relatively inactive, vacuole-bound precursor in a plant cell, is converted to a highly reactive, lachrymating, more lipophilic compound, is also known as the "mustard-oil bomb" - the enzyme is physically segregated from glucosinolates within the plant, but the two come into contact with each other upon tissue damage to the plant, such as by injury, cutting or chewing. The reaction involves enzymatic cleavage of the thioglucoside linkage, requires ascorbic acid as a co-factor, and is followed at neutral pH by rapid rearrangement of the unstable aglycone (a thiohydroximate-*O*-sulfonate) to form the cognate isothiocyanate. Under alternative conditions of pH, temperature, and metal cations, oxazolidine-2-thione may be formed, and with further specifier proteins a variety of other rearrangement products can also be formed (e.g., nitriles, epithionitriles, and thiocyanates).

Cell Culture and Nrf2-knockout Research

Cell culture experiments and Nrf2-knockout mice have helped to define the protective role of Nrf2 and the SF-mediated induction of the Keap1/Nrf2 pathway on the damage caused by UVR insult. SF has been shown to induce NQO1 enzyme activity, the prototypic biomarker of the Nrf2-mediated phase 2 antioxidant response, and increase GSH levels in skin cell lines and primary culture, including human HaCaT keratinocytes, murine PE keratinocytes, and primary SKH-1 mouse keratinocytes and dermal fibroblasts [59, 60]. Prior treatment of the aforementioned cells with SF for 24 h before UVA irradiation protects the cells against UVA-induced oxidative stress by reducing levels of ROS. Protection by SF is

also evident in cells that have 6-thioguanine in their genomic DNA and are hypersensitive to UVA radiation-mediated ROS formation [60]. At the same 6-thioguanine levels in their DNA, Keap1-knockout cells, in which Nrf2 is constitutively activated, are highly resistant to UVA radiation-induced oxidative stress, whereas cells that lack Nrf2 are more sensitive than their wild-type counterparts [61]. The protective effect of both SF and the Keap1-knockout genotype is completely lost in the absence of Nrf2 (i.e., in Nrf2-knockout or Keap1/Nrf2-double knockout cells), implying that protection is due to induction of Nrf2-dependent target genes [60, 61].

Curiously, in SKH-1 hairless mice the time of day of exposure to UVR is a contributing factor to its carcinogenicity, correlating with the circadian rhythmicity of the rate of excision repair in the murine skin [62]. SF does not appear to affect the extent of direct DNA damage in skin cells in culture. Primary SKH-1 keratinocytes and dermal fibroblasts treated for 24 h with 1 μM SF before broadband UVB radiation did not show reduction in the formation of cyclobutane pyrimidine dimers (CPDs) or 6,4-photoproducts (6,4-PPs) [60]. Oxidative DNA damage in skin cells caused by both UVA and UVB radiation has the potential to be affected by the SF-mediated upregulation in antioxidant capacity of the cells and has yet to be explored. Studies in Nrf2-knockout mice have shown that oxidative DNA damage and inflammation in ear tissue were increased with chronic UVB exposure as compared to wild-type mice, but this did not result in differences in tumor incidence or multiplicity between Nrf2-knockout and wild-type mice on the dorsal skin [63]. This study highlights the importance of this pathway in protection against oxidative stress and inflammation, but also indicates that there are other factors involved in addition to the Nrf2 pathway in preventing UVR-induced carcinogenesis. Another study on the impact of the Nrf2 pathway and SF treatment on UVR-induced damage also found photoprotective effects [64]. When Nrf2 wild-type and knockout mice were irradiated with 300 mJ/cm^2 UVB broad-band radiation, Nrf2-knockout animals were more susceptible than were wild-type mice to sunburn and apoptotic cell formation, increased skin thickness, and had higher levels of the pro-inflammatory cytokines, IL-1β and IL-6. When the mice were treated topically for 4 to 5 days with a low dose of SF (100 nmol), the wild-type mice showed a 43% protection against UVB-induced increase in skin thickness, whereas Nrf2-knockout mice had a variable 18% reduction in skin thickness 8 h after irradiation. These findings suggest that the Nrf2 pathway plays an important role in protection against UV-insult and that SF-mediated upregulation of this pathway could lead to reduction of oxidative DNA damage and inflammatory response induced by UVB and UVA wavelengths. Therefore, SF is a potential protective agent against UVR-induced skin damage by its ability to modulate oxidative stress, inflammation, and other skin cancer initiation and promotion effects at the molecular level.

Protection of Mitochondrial Integrity

UVR causes mitochondrial dysfunction manifesting as a decrease in mitochondrial membrane potential and an increase in superoxide production [65]. Cells isolated from Nrf2-knockout animals have higher basal levels of superoxide than do their wild-type counterparts [66]. Furthermore, under conditions of Nrf2 deficiency, mitochondria are depolarized, respiration is impaired, and cellular ATP levels are decreased. Conversely, in cells with

constitutive activation of Nrf2 (by Keap1-knockdown or knockout), the mitochondrial membrane potential, ATP levels, rate of respiration and efficiency of oxidative phosphorylation are all increased [66, 67]. Together, these findings suggest that enhanced mitochondrial activity under conditions of Nrf2 activation may be contributing to the Nrf2-mediated cytoprotection.

Mitochondria isolated from brain and liver of rats treated with a single dose of SF were resistant to opening of the mitochondrial permeability transition pore (PTP) caused by the oxidant *tert*-butyl hydroperoxide [68, 69]. Interestingly, PTP, a complex that allows the mitochondrial inner membrane to become permeable to solutes with molecular masses up to ~1500 Da, was recently found to be formed from dimers of the FoF1-ATP synthase [70]. The SF-mediated resistance to PTP opening correlated with increased antioxidant defenses, and the levels of mitochondrial GSH, glutathione peroxidase 1 (GPX1), malic enzyme 3 (ME3), and thioredoxin 2 (TRX2) were all upregulated in mitochondrial fractions isolated from the SF-treated animals [69]. Mitochondrial protein damage and impairment in respiration caused by the electrophilic lipid peroxidation product 4-hydroxy-2-nonenal were attenuated in mitochondria isolated from the cerebral cortex of SF-treated mice [71]. In rat renal epithelial cells and in kidney, SF protected against cisplatin- and gentamicin-induced toxicity and loss in mitochondrial membrane potential [72-74]. Protection against a panel of oxidants (superoxide, hydrogen peroxide, peroxynitrite) and electrophiles (4-hydroxy-2-nonenal, and acrolein), and an increase in mitochondrial antioxidant defenses were also observed when rat aortic smooth muscle cells were treated with SF [75].

In addition to protecting mitochondria by increasing the endogenous antioxidant defenses, SF also stimulates mitochondrial biogenesis, a process which is largely controlled by two classes of nuclear transcriptional regulators: transcription factors, such as nuclear respiratory factor-1 and 2, and transcriptional coactivators, such as peroxisome proliferator-activated receptor γ coactivator-1α and β (PGC1α and PGC1β) [76-80]. The Nrf2-dependent transcriptional upregulation and nuclear accumulation of nuclear respiratory factor-1 promotes mitochondrial biogenesis and protects against the cytotoxicity of the cardiotoxic anthracycline chemotherapeutic agent doxorubicin [81]. SF treatment of human fibroblasts causes an increase in mitochondrial mass and induction of PGC1α and PGC1β [82]; however, the dependence on Nrf2 has not been examined. Although further work is required to establish the precise relation between the Nrf2 status and the expression of the PGC1 coactivators, current findings suggest that preservation of mitochondrial integrity, through both increased endogenous antioxidant capacity and enhanced mitochondrial function, contributes to the cytoprotective effects of SF.

SKH-1 Mouse Studies

Several studies have shown that SF-containing broccoli sprout extracts (BSE) are capable of protecting the skin of SKH-1 hairless mice against the damaging effects of UVR and UVR-induced carcinogenesis. Among rodents, the hairless but immunologically competent SKH-1 mouse, lacking a transcriptional co-repressor essential for hair follicle regeneration, is a highly relevant model for human skin cancer [83]. After 16-20 weeks of bi-weekly exposure to relatively low-dose UVB (30 mJ/cm^2), this mouse develops multiple skin tumors during

the subsequent 12-16 weeks [84]. Topical application of a single dose of SF-containing BSE (100 nmol SF/cm^2) to the dorsal skin of these mice caused a 1.6-fold induction of NQO1 activity and increased the protein levels of NQO1, HMOX1 and GSTA1, indicating induction of the Nrf2 pathway in skin cells [85]. Treatment of SKH-1 mouse skin with the same dose of SF-containing BSE for 3 days resulted in an even greater (2.7-fold) induction of NQO1 activity [85].

To confirm that the induction of the Nrf2 pathway was solely due to the SF and not some other constituent of the BSE, as well as whether UVB wavelengths would affect induction of the Nrf2 pathway, synthetic SF and BSE containing equivalent amounts of SF were applied topically to SKH-1 hairless mouse dorsal skin at a dose of 100 nmol/cm^2, once daily for 3 days, followed by irradiation with a single dose of 700 mJ/cm^2 UVB narrow-band (311 nm) 24 h after the last topical treatment [53]. Twenty-four hours after irradiation, the NQO1 activity was found to be slightly depressed in control-treated dorsal skin compared to non-irradiated control levels, and treatment with either synthetic SF or equivalent concentrations of SF in BSE for 3 days before irradiation resulted in nearly identical ~40% induction in NQO1 activity compared to control levels. This study provides evidence that the phase 2 inducer activities of SF-containing BSE are entirely attributable to their SF content.

To determine the effect of SF on tumor formation after UV exposure, SKH-1 mice were chronically irradiated with UVB broad-band wavelengths (30 mJ/cm^2; bi-weekly), and were subsequently topically treated once daily, 5 days a week, for 11 weeks with BSE delivering 1.0 μmol SF [59]. It was found that topical treatment with SF-containing BSE resulted in a significant decrease in tumor incidence, multiplicity, and burden by ~50% when compared to control-treated animals. Another study showed that feeding daily doses of broccoli sprout powder containing 10 μmol of the SF-precursor glucoraphanin to SKH-1 mice, that had received chronic bi-weekly UVB exposure, also led to a decrease in tumor incidence, multiplicity and volume [86], indicating that dietary intake of the precursor of SF is capable of being converted to SF by the gut microflora of mice and is subsequently bioavailable to skin cells.

To address the ability of SF to provide protection against the damaging effects of UVR before UVR exposure, SKH-1 mice were pretreated with synthetic SF (1 or 2.5 μmol SF) for 1 week prior to UVB exposure and then 1 h before each irradiation, which resulted in a reduction in tumor multiplicity and burden at both doses of SF over the 25 week study [87]. In another pretreatment experiment, synthetic SF (5.6 and 14.1 μmol) was given orally once a day for 14 days and SKH-1 mice were irradiated with UVB wavelengths on days 9, 11, and 13 [88]. Oral SF pretreatment reduced gross skin thickness, number of cell layers in the epidermis and protein levels of COX-2 in the skin 24 h after the last UVB exposure. These studies in the SKH-1 hairless mouse have provided significant evidence that SF, administered both topically and orally and in the form of BSE rich in SF or pure SF, can protect the skin against the damaging effects of UVR and prevent or reduce tumor formation when treatments are given before or after UVR insult.

We have recently generated SKH-1 hairless mice in which Nrf2 is either deleted or constitutively activated by back-crossing Nrf2-knockout [89, 90] and Keap1-knockdown [91] C57BL/6 mice onto the SKH-1 hairless genetic background over six generations. Compared to wild-type, skin and liver samples isolated from Nrf2-knockout SKH-1 hairless animals have lower enzyme activity levels of the Nrf2-dependent enzyme NQO1, whereas those isolated from their Keap1-knockdown SKH-1 hairless counterparts, have higher enzyme

activity levels (Figure 4A, B). Interestingly, the contribution of Nrf2 to the basal levels of NQO1 in the skin and liver is vastly different. Thus, in wild-type animals, the NQO1 activity is 2.8-fold greater in the skin than in the liver. Furthermore, whereas the NQO1 activity in the liver of Nrf2-knockout mice is only ~20% of their wild-type counterparts (Figure 4B), the NQO1 activity in the knockout mouse skin is ~80% of that in wild-type skin (Figure 4A). In agreement with the differential effect of Nrf2 in the two organs, the levels of NQO1 in the skin and liver of Keap1-knockdown mice are ~2-fold and ~10-fold greater than that in the wild-type animals. The finding that the skin of the Nrf2-knockout mice contains higher basal levels of NQO1 than the liver could indicate the existence of compensatory mechanism(s) for the absence of Nrf2, such as those mediated by Nrf1, Nrf3, or p45NEF2 [92] in the skin that may not be present and/or activated in the liver. Indeed, the levels mRNA of Nrf3 are elevated in the skin of Nrf2-knockout mice [93, 94]. This organ-specific difference in basal levels of NQO1 suggests that skin cells may have adapted alternate antioxidant response mechanisms that help them to maintain homeostasis in the face of environmental stresses.

Primary dermal fibroblasts from SKH-1 hairless mice displayed potent and robust NQO1 induction 48 h after SF treatment (~8-fold at 5 μM SF), whereas their counterparts isolated from hairless Nrf2-knockout mice showed no inducible NQO1 activity (Figure 4C). When SKH-1 and hairless Nrf2-knockout mouse dorsal skin was treated in vivo topically with 100 nmol/cm^2 SF, once daily for 3 days, the NQO1 activity in the SKH-1 skin increased ~2-fold with the SF treatment, whereas an identical treatment of hairless Nrf2-knockout mice did not induce NQO1 (Figure 4D).

Skin isolated from Keap1-knockdown SKH-1 hairless mice has ~3-fold higher levels of Nrf2 compared to SKH-1 hairless mouse skin [94]. In addition to NQO1, the levels of GST, HMOX, and GCLC are also upregulated. Keap1-knockdown mice showed a profound reduction in induction of erythema and the inflammatory marker IL-6 expression provoked by solar-simulated UVR. Furthermore, Keap1-knockdown SKH-1 hairless mice were substantially protected against the carcinogenic effects of solar-simulated UVR. Thus, compared to SKH-1 hairless mice, the tumor incidence was reduced by 40%, and the tumor multiplicity by ~5-fold in the Keap1-knockdown SKH-1 hairless mice. The total tumor volume per mouse was also profoundly reduced (by 80%) by the genetic upregulation of Nrf2. The Nrf2-knockout and Keap1-knockdown SKH-1 hairless mice provide important tools for mechanistic studies of the role of Nrf2 in protection against photodamage and photocarcinogenesis.

HUMAN STUDIES

In healthy human subjects, topical SF treatment has been shown to upregulate the phase 2 response and guard against the erythema caused by acute exposure to UVR. When a single dose of BSE containing 170 or 340 nmol of SF was applied to small circular areas (1 cm in diameter) on the skin of three healthy human subjects, NQO1 enzyme activity increased by ~1.5-fold 24 h after treatment in a 3-mm punch biopsy taken from the center of the area, compared to control-treated skin (80% acetone, v/v) [85]. The effects of three repeated topical applications, given at 24-h intervals, was also examined with cumulative doses of 150, 300, and 450 nmol SF in BSE, resulting in an average increase of NQO1 specific activity by ~4.5-

fold for the 450 nmol SF dose over control skin 24 h after the last treatment. In an independent study, SF, and the related phenethylisothiocyanate (PEITC), were reported to upregulate the mRNA levels for Nrf2 and increase the expression of its downstream target genes NQO1, HMOX1, γGCS, and catalase, and to protect against UVR-mediated structural damage in organ cultures of explants from full-thickness human skin [95].

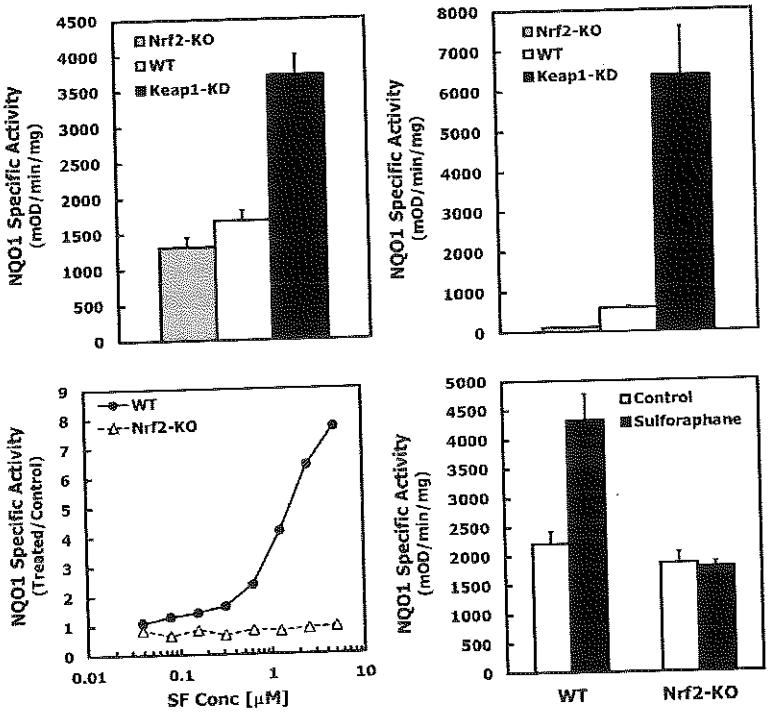

Figure 4. Compared to SKH-1 hairless mice, Nrf2-knockout SKH-1 hairless mice have lower and uninducible levels of NQO1, whereas the levels of this Nrf2-target enzyme are higher in their Keap1-knockdown counterparts. (A,B) The NQO1 enzyme activity was determined in homogenate supernatants prepared from dorsal skin (A) and liver (B) of male SKH-1 (WT), Nrf2-knockout (Nrf2-KO), and Keap1-knockdown (Keap1-KD) mice (n=3 for each genotype). Average values ± SD are shown. In the WT animals, the basal activity of NQO1 are higher in skin than in liver. The effect of the Nrf2 genotype are much more pronounced in liver than in skin. (C) Primary dermal fibroblasts were isolated from 2-day-old SKH-1 (WT) or hairless Nrf2-knockout (Nrf2-KO) mice. Cells were plated at 20,000 cells per well in 96-well plates and 24 h later were exposed to serial dilutions of SF for a further 48 h. NQO1 activity is expressed as mean ratios of treated over control specific activities using eight replicate wells for each SF concentration. The standard deviation for all points was less than 10%. SF induced dose-dependently the NQO1 enzyme activity in SKH-1 (WT) dermal fibroblasts, but not in the hairless Nrf2-knockout (Nrf2-KO) cells. (D) The dorsal skin of female SKH-1 (WT) mice or hairless Nrf2-knockout (Nrf2-KO) mice (n=3 for each genotype) was treated topically, on the right- or left-hand side of the back, with 3 daily doses of: (i) 50 μL of 80% acetone/20% water (control), and (ii) 0.5 μmol of SF (in 50 μL of 80% acetone, v/v). The animals were euthanized 24 h later. NQO1 specific activity was measured in total skin homogenate supernatants. Average values ± SD are shown. SF treatment increased NQO1 activity ~2-fold over control treatment in the SKH-1 mice, but no NQO1 induction was detected in the Nrf2-KO mice.

The potential ability of SF to protect against skin photodamage in healthy human subjects was evaluated by measuring the susceptibility to erythema arising from narrow-band (311 nm) UVB radiation [53]. Six healthy human volunteers (three males and three females) received on 3 consecutive days at 24-h intervals BSE containing 200 or 400 nmol SF or vehicle (80% acetone, v/v) applied topically to small circular areas (2 cm in diameter) on their back skin that had not been previously exposed to UVR. Twenty-four hours after the last dose, these small areas of the skin were exposed to narrow-band (311 nm) UVB radiation. Erythema development was objectively determined 24 h after irradiation by using reflectance spectroscopy and was found to be reduced by ~40% at sites that received BSE containing SF compared with vehicle-treated sites.

A randomized, double-blind, placebo-controlled study compared the response of the skin of 24 healthy human volunteers to different wavebands of monochromator UVR, solar-simulated UV waveband radiation, and visible light after topical application of extracts containing either sulforaphane (200 nmol/cm^2, active) or glucoraphanin (200 nmol/cm^2, placebo), with each subject acting as their own control [94]. The extracts were applied in a randomized fashion to the right or the left half of the mid-upper back skin of each volunteer, 3 times, 24 h apart. Small areas of the skin (approximately 1 cm^2) within the extract-treated areas were exposed to a range of doses of monochromatic UVR or visible light, or solar-simulated UVR 24 h after the last extract applications, and skin erythema responses were assessed afer a further 24 h. Erythema was determined by using both a semi-quantitative visual scale as well as erythema meter for objective quantification. Although there were no significant differences in threshold responses, i.e., minimal erythemal dose (MED), at any of the wavelengths, the mean of the sum of erythema across all doses after exposure to solar-simulated UVR for the SF-treated sites was reduced by 35% compared to the placebo-treated areas. Therefore, SF has been shown to offer a degree of photoprotection against UVB and solar-simulated UVR-induced erythema, supporting a role in the protection against the deleterious effects of solar radiation.

NRF2-INDEPENDENT MECHANISMS OF SULFORAPHANE PROTECTION

Sulforaphane also mediates Nrf2-independent events that can contribute to the protection to skin cells. SF has been shown to reduce the inflammatory response in vitro and in vivo, and this activity could play a significant role in the protective effects of SF against UV-induced inflammation. In HaCaT keratinocytes, pretreatment with SF 24 h before UVB radiation reduced the gene expression of IL-1β, IL-6, and COX-2, decreased protein expression of COX-2 and PGE2, and inhibited phosphorylation and activation of the MAPK pathway [88]. Studies have also shown that UVB radiation can induce an NF-κB-mediated inflammatory response in murine 308 keratinocytes [96]. SF treatment reduces LPS- and IFN-γ-induced NF-κB-mediated inflammation [57, 59]. Although it has not been directly demonstrated, one possibility is that SF reacts with cysteine residues in the DNA binding domain of NF-κB and/or in the IKK kinase complex (e.g., IKKβ) (Figure 5A), as has been shown for the cysteine-reactive triterpenoids [97-100]. Some aspects of the anti-inflammatory actions of SF may involve Nrf2-dependent mechanisms, as studies utilizing MEF cells and primary peritoneal macrophages isolated from Nrf2-knockout mice show SF and other Nrf2 activators

(e.g., the triterpenoid TP-225) are much less effective at inhibiting IFN-γ- and TNF-α-stimulated NO production in the Nrf2-knockout cells, compared to WT cells [55], which could indicate that this inflammatory response is through a pathway other than NF-κB (e.g., the JAK/STAT pathway).

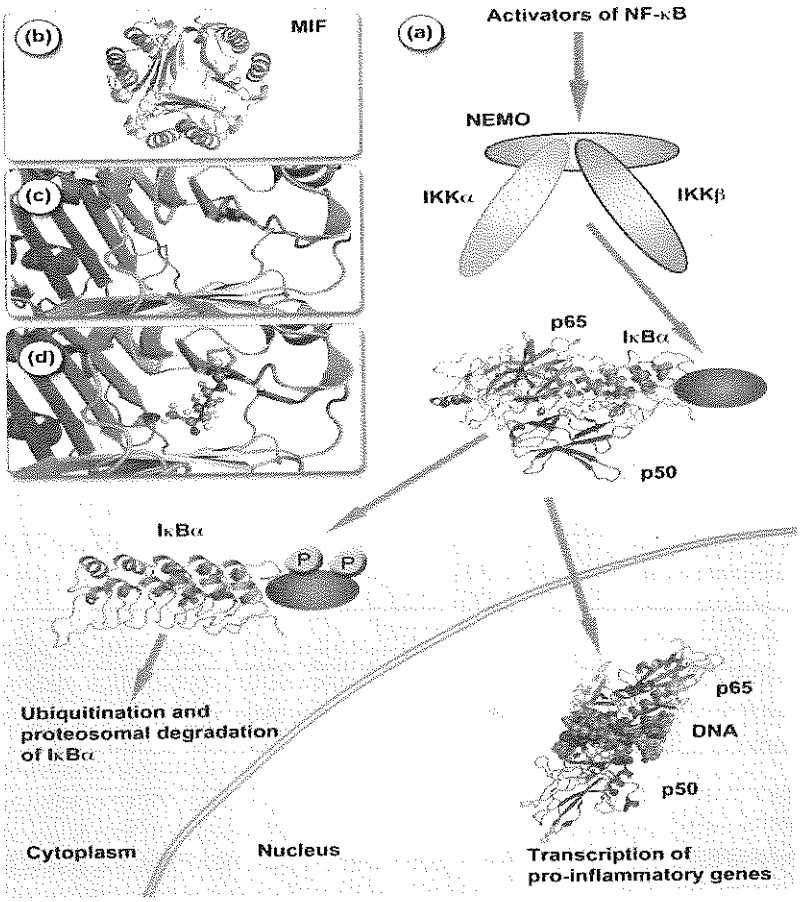

Figure 5. Examples of protein targets and processes that underlie the anti-inflammatory activities of sulforaphane. (A) The NF-κB pathway. In the absence of a stimulus, IκBα (green) negatively regulates transcription factor NF-κB by binding both subunits (p65, red, and p50, blue) of NF-κB and precludes its transfer to the nucleus (pdb data from 1IKN file). Activators of the NF-κB pathway include bacterial and viral products, inflammatory cytokines, reactive oxygen species, and ultraviolet radiation. These stimuli activate a kinase complex (IKKα, IKKβ, NEMO) resulting in IκBα phosphorylation, followed by ubiquitination and proteasomal degradation (pdb data from 1IKN file). Consequently, NF-κB translocates to the nucleus where its p50-p65 heterodimers bind specific DNA sequences of the promoter regions of its target genes (pdb data from 1LE5 file). Following transcription, NF-κB is removed from its gene promoters through association with nuclear IκBα (not shown), restoring the pre-activation state. SF may inhibit this pathway by binding to cysteine residues of the kinase complex (e.g., IKKβ) or NF-κB itself. (B) Crystal structure of the macrophage migration inhibitory factor (MIF) trimer (generated with the program Pymol based on pdb file 1MIF). (C) Closeup of the MIF protein subunit showing the N-terminal active site proline. (D) In silico model of the binding of SF to the active site N-terminal proline of MIF. To generate the model, the data from the pdb file 3CE4 and the program Pymol were used.

Another Nrf2-independent inflammatory mechanism that has been correlated with UVR-induced skin damage and carcinogenesis is the increase of the levels of macrophage migration inhibitory factor (MIF). MIF is a pleiotropic cytokine also possessing catalytic tautomerase activity, and both UVA and UVB wavelengths have been shown to increase MIF production in dermal fibroblasts and keratinocytes, respectively [101-103]. Studies in MIF-knockout and MIF-overexpressing transgenic mice have suggested that MIF plays a role in skin cancer by increasing inflammation and inhibiting the p53-dependent apoptotic process of eliminating damaged cells [104, 105]. MIF has also been shown to be increased in skin biopsies of actinic keratosis and cutaneous squamous cell carcinoma patients [103]. Inactivation of MIF catalytic activity has been implicated to have anti-inflammatory effects in vitro and in vivo [106, 107]. SF modifies MIF by binding to its N-terminal proline residue in the enzymatic active site, leading to loss of catalytic tautomerase activity and causing conformational changes in protein structure (Figure 5B-D) [108]. Studies have shown that MIF tautomerase activity was detectable in human urine, and that oral administration of broccoli sprout preparations as a source of the SF precursor, glucoraphanin, to human volunteers almost completely abolished urinary tautomerase activity [109]. This inhibitory action of SF on the catalytic activity of MIF in human subjects makes it an attractive candidate for further studies on the anti-inflammatory properties mediated by this inactivation.

Sulforaphane has also been shown to prevent UVB-induced DNA binding and activation of activator protein-1 (AP-1) both in vitro and in vivo, potentially by directly modifying cysteine residues in the DNA binding sites of cFos and cJun [87]. Activation of AP-1 by UVR leads to changes in proliferation, apoptosis and differentiation, and inhibition of AP-1 activation has been shown to reduce UVB-induced carcinogenesis [110]. Using an AP-1 reporter mouse, a topical formulation of SF in polyethylene glycol (PEG) ointment base has been recently developed and shown to be stable and effective in reducing AP-1 activation after UVR stimulation [111]. SF also modulates the heat shock response. The heat shock response is primarily regulated by the transcription factor heat shock factor-1 (HSF1), which upon stimulation dissociates from heat shock protein (Hsp) 90 complex, trimerizes, and binds to heat shock elements (HSEs) and drives expression of target genes that upregulate molecular chaperone proteins, Hsp90, Hsp72, and Hsp27, which direct refolding of denatured proteins [112]. This response could be critical in eliminating proteins that have been damaged by UVR. SF has been shown to activate HSF1, upregulating the expression of Hsp27 and increasing proteasome activity [56]. There is also evidence that SF can increase the expression of 26S proteasomal subunits through an Nrf2-dependent mechanism, which protected murine neuroblastoma cells from hydrogen peroxide-mediated toxicicity [113]. These findings indicate a possible overlap in function between the HSF1-mediated heat shock response and the Nrf2 pathway.

CONCLUSION

Sulforaphane was identified as a potent inducer of the phase 2 response, via activation of the Nrf2 pathway. SF also harnesses many other protective mechanisms, which make it an ideal candidate as a cytoprotector against the many forms of cellular damage and injury that occur in skin exposed to UVR. Many of the protective responses activated by SF are

overlapping, and crosstalk among the cellular signaling pathways is not well understood. The process of evolution has provided cells with interconnected mechanisms of protection that are wired to respond to the environment that we are now only starting to unravel. The advantages of using SF as inducer are that it is a dietary component and exerts most of its effects via the transcriptional enhancement of the synthesis of proteins, most of which are enzymes that have long-lasting, catalytic effects. There is strong indication that SF, and other inducers of the Nrf2 pathway, are protective against UVR-induced skin damage by means of reducing oxidative stress and inflammation, which ultimately can lead to carcinogenesis. The Nrf2-dependent and -independent aspects of this protection are not completely understood and the stage is set for further investigation into the mechanisms of SF-mediated protective effects against UVR and the role of the Nrf2 pathway in this protection.

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