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Sulforaphane and phenylethyl isothiocyanate protect human skin against UVR-induced oxidative stress and apoptosis: Role of Nrf2-dependent gene expression and antioxidant enzymes

Konrad Kleszczyński[a,1], Insa M.A. Ernst[b,1], Anika E. Wagner[b], Nathalie Kruse[a], Detlef Zillikens[a], Gerald Rimbach[b], Tobias W. Fischer[a,*]

[a] Department of Dermatology, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany
[b] Institute of Human Nutrition and Food Science, Christian-Albrechts-University Kiel, Hermann-Rodewald-Strasse 6, 24118 Kiel, Germany

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ABSTRACT

Chronic UVR-exposure may impair the stress response and antioxidant defense mechanisms of human skin. The transcription factor nuclear factor erythroid-2 related factor 2 (Nrf2) orchestrates the expression of genes coding for the stress response and antioxidant proteins. Here, we tested sulforaphane (SFN) and phenylethyl isothiocyanate (PEITC) for their ability to counteract UVR-induced oxidative stress and apoptosis in ex vivo human full-thickness skin combined with in vitro HaCaT keratinocytes. Investigation of Nrf2 transactivation and induction of genes coding for Nrf2-dependent phase II antioxidative enzymes (γ-glutamylcysteine-synthetase (γGCS), heme oxygenase 1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1)) was performed in HaCaT keratinocytes. Comparative investigations in human ex vivo skin were conducted for analysis of gene expression of above mentioned phase II enzymes and catalase (CAT) as well as hematoxylin/eosin (H&E) and immunofluorescence (catalase, cleaved Casp-3). UVR exposure of human skin (300 mJ/cm^2) resulted in a significant time-dependent increase of the number of sunburn cells and caspase-3 activation as biomarkers of apoptosis for up to 48 h ($p < 0.001$) and induced a significant decrease of the antioxidant enzyme catalase ($p < 0.001$). This was significantly counteracted by the pre-treatment of human skin with SFN and PEITC (5 μM and 10 μM). Mechanistic cell culture studies revealed SFN and PEITC to increase Nrf2 activity and Nrf2-dependent gene expression (γGCS, HO-1, NQO1); this was paralleled in human full skin mRNA. In conclusion, the induction of Nrf2-dependent antioxidant pathways seems to be a potential mechanism by which SFN and PEITC protect against UVR-induced oxidative stress and apoptosis in human skin.

1. Introduction

Impaired stress response and antioxidant defense negatively affect skin function and integrity. The transcription factor Nrf2 is a master switch that controls the stress response and antioxidant defense mechanisms in skin [1]. Nrf2 binds to the antioxidant response element (ARE), which is found in the promoter region of genes encoding for phase II antioxidant enzymes such as γ-glutamylcysteine-synthetase (γGCS), glutathione-S-transferase (GST), heme oxygenase 1 (HO-1) and NAD(P)H dehydrogenase, quinone 1 (NQO1) [2]. Nrf2 counteracts inflammation [3] and is also involved in the protection of skin against UVR-induced cell death [1,4]. Given the antioxidant and anti-apoptotic role of Nrf2, compounds which target the Nrf2 signal transduction pathway might be excellent candidates to maintain and/or improve skin function and integrity with regard to UV-induced oxidative stress and apoptosis. In this context, we have previously shown that the isothiocyanate sulforaphane (SFN) increases Nrf2-dependent gene expression in murine cultured fibroblasts [5]. However, little is known about the role of SFN and structurally related molecules such as phenylethyl isothiocyanate (PEITC) in stress responses and antioxidant defense in human full-thickness skin. Therefore, we evaluated whether SFN and PEITC would protect human skin against UVR-induced stress and apoptosis via induction of Nrf2-dependent phase II enzymes. We monitored, in UVR exposed intact human ex vivo skin, the number of sunburn cells and in situ protein expression of caspase-3 as biomarkers of apoptosis as well as catalase, a representative antioxidant enzyme of the oxidative stress defense of the skin.

Abbreviations: CAT, catalase; cl. casp-3, cleaved caspase-3; γGCS, γ-glutamylcysteine-synthetase; GSH, glutathione; H&E, hematoxylin and eosin; HO-1, heme oxygenase 1; NQO1, NAD(P)H quinone oxidoreductase 1; Nrf2, nuclear factor erythroid-2 related factor 2; PEITC, phenylethyl isothiocyanate; SFN, sulforaphane; UVR, ultraviolet radiation.

* Corresponding author. Tel.: +49 451 500 6743; fax: +49 451 500 2981.
E-mail address: Tobias.Fischer@uksh.de (T.W. Fischer).

[1] These authors contributed equally to this work.

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Additionally, we conducted cell culture studies in human HaCaT keratinocytes to determine the possible underlying mechanisms by which SFN and PEITC may improve stress response of human skin.

2. Materials and methods

2.1. Reagents

SFN and PEITC were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). The test compounds were dissolved in DMSO (Carl Roth GmbH, Karlsruhe, Germany) and stock solutions were stored at −80 °C until usage. L-glutamine (100×), penicillin–streptomycin solution (10,000 units of penicillin and 10 mg of streptomycin in 1 ml 0.9% NaCl) were supplied by Invitrogen (Carlsbad, CA, USA). Insulin from bovine pancreas and hydrocortisone, acetone, ethanol, Triton® X-100 and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (St. Louis, MO, USA). Normal goat serum (NGS) or normal rabbit serum (NRS) were obtained from Dako (Dako, Inc., Carpinteria, CA, USA).

2.2. Cell culture

HaCaT keratinocytes (generated by Dr. N. Fusenig, DKFZ, Heidelberg and obtained from the Institute of Applied Cell Culture, Munich, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 4 mM L-glutamine and 1 mM sodium pyruvate, 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere at 37 °C and 5% CO_2. All cell culture media and reagents were purchased from PAA Laboratories GmbH (Cölbe, Germany), plastic ware was from Sarstedt (Nümbrecht, Germany) unless specified otherwise.

2.3. Cytotoxicity measurement

Cytotoxicity in cell culture was determined via the neutral red assay [6,7]. HaCaT keratinocytes were seeded in 24-well plates (Fisher Scientific GmbH, Schwerte, Germany) at a density of 0.4×10^6 cells/well, pre-cultured for 24 h and treated with increasing concentrations of the test compounds for 24 h, respectively. Briefly, the culture medium containing the test substances was replaced with fresh serum-containing medium including 50 μg/ml neutral red (Carl Roth GmbH, Karlsruhe, Germany). After incubation for 3 h, the medium was removed and the cells were extracted using a solution comprising 50:49:1 (v/v/v) ethanol, water and glacial acetic acid. The absorbance was measured in a plate reader (Labsystems, Helsinki, Finland) at 540 nm.

2.4. Transient transfection and luciferase reporter gene assay

HaCaT keratinocytes were grown to 60–80% confluence in 24-well plates for 24 h. The keratinocytes were transiently transfected with an expression vector containing the ARE-promoter region of the gastrointestinal glutathione peroxidase 2 and the reporter gene firefly luciferase (pARE_GIGPx) kindly provided by A. Banning and R. Brigelius-Flohé (DIfE, Potsdam-Rehbruecke, Germany) [8,9] and a normalization vector phRL-TK (Promega, Mannheim, Germany) containing the renilla reniformis luciferase gene. Transfection was performed using FuGeneHD transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's instructions. Following 18 h of transfection, the keratinocytes were incubated with the test compounds for 24 h. Subsequently, cells were lysed and luciferase activity was measured using the Dual-Luciferase reporter gene assay system (Promega, Mannheim, Germany) according to the manufacturer's protocol in a Tecan Infinite 200 microplate reader (Tecan Group Ltd., Crailsheim, Germany).

2.5. RNA isolation and real-time PCR

HaCaT keratinocytes were pre-cultured in 6-well plates in normal medium for 24 h (1×10^6 cells/well). Subsequently, cells were treated with increasing, non-cytotoxic concentrations of the test compounds for 6 h. Cellular RNA was isolated with TRIsure following the manufacturer's protocol (Bioline, Luckenwalde, Germany).

For RNA extraction of human full-thickness skin, skin pieces (0.25 cm × 0.25 cm; approximately 30 mg) were homogenized using a Ultra-Turrax homogenizer (IKA GmbH, Staufen, Germany) for 3 × 30 s in 0.8 ml TriSure. Between each homogenization step, samples were chilled on ice for 1 min and perturbing connective tissue was discarded. Subsequently, RNA isolation was performed according to manufacturer's instructions via phenol-chloroform extraction. For improved PCR detection, remaining DNA was lysed using DNAse A according to manufacturer's instructions (New England Biolabs, Ipswich, USA).

Primers were designed by Primer3 software with the following sequences: CAT, F: 5′-CGTGCTGAATGAGGAACAGA, R: 5′-AGTCAGGGTGGACCTCAGTG; γGCS, F: 5′-GGCGATGAGGTGGAATACAT, R: 5′-CCTGGTGTCCCTTCAATCAT; HO-1, F: 5′-CCAGGCAGAGAATGCTGAGT, R: 5′-GTAGACAGGGGCGAAGACTG; NQO1, F: 5′-CTGATCGTACTGGCTCACTC, R: 5′-GAACAGACTCGGCAGGATAC; Nrf2, F: 5′-AAACCAGTGGATCTGCCAAC, R: 5′-GCAATGAAGACTGGGCTCTC; β-actin, F: 5′-GGATGCAGAAGGAGATCACTG, R: 5′-CGATCCACACGGAGTACTTG (MWG Biotech, Ebersberg, Germany). Real-time PCR in HaCaT samples was performed using Sensi-Mix one-step kit (Bioline GmbH, Luckenwalde, Germany). In human skin samples, two-step real-time PCR was performed according to manufacturer's instructions.

2.6. Skin organ culture

Explants were full-thickness skin samples taken from human individuals undergoing abdominoplastic surgery after informed consent. Skin samples from seven independent Caucasian individuals at the age range = 42–59 years were taken for seven independent experiments. All subjects were in good health with no evidence of acute or chronic skin or systemic disease. The tissue was delivered to laboratory within 1–1.5 h post surgery in Williams' E medium (Biochrom AG, Berlin, Germany) on ice. Upon arrival in the laboratory, the skin was defatted, cut by scalpel into sample pieces of 0.5 cm × 1.0 cm, rinsed abundantly with 1× PBS (pH 7.2) and placed epidermis up/dermis down into 6-well plates. The skin samples were placed in 2 ml Williams' E medium supplemented with 1% penicillin-streptomycin solution, 1× L-glutamine, 1% insulin from bovine pancreas and 0.1% hydrocortisone in a humidified atmosphere of 5% CO_2 at 37 °C. Samples were cultured in that manner that epidermis was kept dry above the surface of culture medium (air–liquid interface) prior to incubation with SFN or PEITC and UV irradiation. Validation of this skin model has been recently performed by our own group [10].

2.7. SFN and PEITC-incubation and UV irradiation of skin

After 24 h cultivation of skin samples in normal Williams' E medium, culture medium was replaced with fresh medium containing SFN or PEITC at final concentrations of 5, 10, 25 μM or with fresh medium without SFN/PEITC as controls. Skin pre-incubation with SFN or PEITC was performed for 24 h, and fresh solutions containing SFN/PEITC were added again 1 h before UV irradiation. After the pre-incubation phase, skin samples were washed twice with 1× PBS to remove remnants of medium and tested compounds,