Free Radical Biology and Medicine 102 (2017) 47–56

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

# SRB1 as a new redox target of cigarette smoke in human sebocytes

Ilaria Crivellari[a,b], Claudia Sticozzi[a], Giuseppe Belmonte[a], Ximena M. Muresan[a], Franco Cervellati[a], Alessandra Pecorelli[a], Carlotta Cavicchio[a], Emanuela Maioli[c], Christos C. Zouboulis[d], Mascia Benedusi[a], Carlo Cervellati[b], Giuseppe Valacchi[a,e,*]

[a] Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy
[b] Department of Biomedical and Specialist Surgical Sciences, Section of Medical Biochemistry, Molecular Biology and Genetics, University of Ferrara, Ferrara, Italy
[c] Department of Life Sciences, University of Siena, Siena, Italy
[d] Departments of Dermatology, Venereology, Allergology and Immunology, Dessau Medical Center, Dessau, Germany
[e] Department of Animal Science, Plants for Human Health Institute, North Caroline State University, NC Research Campus, Kannapolis, NC, USA

ARTICLE INFO

*Keywords:*
Hydroxynonenal
Lipids
Smoke
Cutaneous tissue

ABSTRACT

For its critical location, the skin represents the major interface between the body and the environment, therefore is one of the major biological barriers against the outdoor environmental stressors. Among the several oxidative environmental stressors, cigarette smoke (CS) has been associated with the development and worsening of many skin pathologies such as acne, dermatitis, delayed wound healing, aging and skin cancer. In our previous work we have demonstrated that CS is able to affect genes involved in skin cholesterol trafficking, among which SRB1, a receptor involved in the uptake of cholesterol from HDL, seems to be very susceptible to the oxidative stress induced by CS.

In the present work we wanted to investigate the presence of SRB1 in human sebocytes and whether CS can affect cholesterol cellular uptake via the redox modulation of SRB1.

By using a co-culture system of keratinocytes/sebocytes, we found that CS exposure induced a SRB1 protein loss without affecting sebocytes viability. The decrease of SRB1 levels was a consequence of SRB1/HNE adducts formation that leads to SRB1 ubiquitination and degradation. Moreover, the CS-induced loss of SRB1 induced an alteration of sebocytes lipid content, also demonstrated by cholesterol quantification in SRB1 siRNA experiments.

In conclusion, exposure to CS, induced SRB1 post-translational modifications in sebocytes and this might affect sebocytes/skin functionality.

## 1. Introduction

Several studies have demonstrated that more than 30% of United States population has been affected by cutaneous pathologies. Although most are not life-threatening, many skin diseases are able to influence life quality and represent a major cost for public health [1].

The tegumentary system is a complex organ that does not consists only in the cutaneous tissues, but comprises also its appendages, including glands, nails, hair. The main function of this organ system is to protect the body from the out-door insults including the environmental stressors.

Recent reports revealed that cigarette smoke (CS), ozone ($O_3$) and concentrated air particles (CAPs) can influence the skin physiology and its functions [2,3]. In the specific, CS has been shown to play a key role in diseases such as acne, dermatitis, delayed wound healing, aging and cancer [4–7].

CS is an heterogeneous mixture of gas, vapour and suspended solid particles, derived from the combustion of the cigarette itself and representing one of the most significant source of inhaled chemical pollutant. Among the several noxious effects, CS is able to induce cutaneous elastosis, upregulation of matrix metalloproteinases enzymes (MMPs 1-3) involved in the degradation of the connective tissues and to alter the transforming growth factor (TGF-β) pathway which is crucial for healthy wound closure [8,9].

CS noxious effects are mainly related to the reactive oxygen species (ROS) which are both present in the smoke and also induced by the interaction of CS components with biological systems. It has been demonstrated that CS leads to an oxidative stress condition in oral keratinocytes as shown by the increase of intracellular oxidants and by a significant decrease of GSH intracellular level [10]. An interesting

* Corresponding author at: Department of Animal Science, Plants for Human Health Institute, North Caroline State University, NC Research Campus, Kannapolis, NC, USA
*E-mail addresses:* giuseppe.valacchi@unife.it, gvalacc@ncsu.edu (G. Valacchi).

http://dx.doi.org/10.1016/j.freeradbiomed.2016.11.021
Received 30 May 2016; Received in revised form 9 November 2016; Accepted 12 November 2016
Available online 16 November 2016
0891-5849/ 

study revealed that smoke is able to induce specific facial aging in identical twins with different smoking history [11].

As mentioned above, not only the skin participates to protect our body from the out-door stressors but also its appendages have an important defensive role, as it is for the sebaceous glands that have the ability to maintain the composition of the hydrolipidic film, a mixture of cholesterol, triglycerides, wax esters and squalene [14]. In addition, sebaceous glands represent the major deliver system of vitamin E to the cutaneous tissues, protecting skin from oxidative damage [12].

Indeed, sebocytes functions are more than the production of sebum and the passive formation of the cutaneous barrier; through many paracrine, endocrine and immunological mechanisms, sebaceous glands take part in many of the homeostatic physiological functions of the skin [11,14].

Sebocytes participate in the regulation of immunological functions and inflammatory processes for their ability to produce cytokines (IL-1β, IL-6, IL-8/CXCL-8, TNFα) and lipid inflammation mediators (5-LOX, LTA4 hydrolase, LTB4, PGE2), which have a key role in the pathogenesis of several inflammatory skin diseases (i.e. acne vulgaris) [14].

New etiologic models reveal that acne can develop without the colonization of pathogen microorganisms, rather can be due to other factors (androgen hormones, PPAR activation, SP-mediated stress response) that increase the production of inflammation mediators and induce hyperseborrhea [15–18]. Moreover, many subunits of cholinergic muscarinic and nicotinic receptors have been found in sebocytes at various grade of differentiation [19]. Thus it is fascinating to hypothesize that the activation of these receptors from neural or paracrine acetylcholine or from CS nicotine, can have a role in the pathogenesis of acne or other cutaneous diseases by affecting sebocytes functionality [19].

For several years our group has been studying the effects of CS on cutaneous tissue focusing the attention on Scavenger Receptor B1 (SRB1), a transmembrane receptor well known for the cholesterol uptake from high density lipoprotein (HDL) [20] and on its modulation by CS. We have demonstrated that in human keratinocytes the modification of proteins involved in cholesterol trafficking such as SRB1 and ABCA1 can lead to skin alteration [21,22]. Whereas sebaceous glands are dragged in physiologic homeostatic functions of the skin, we believe that SRB1 could have an important role also in sebocytes function although no data have yet demonstrated its presence in this particular secretory organ.

The aim of our study was to evaluate the presence of SRB1 in sebaceous glands and to investigate its susceptibility to CS-induced oxidative damage as well as its influence on cellular lipid uptake.

## 2. Methods

### 2.1. Cell culture

HaCaT cells (gift from Dr. F. Virgili) were grown in Dulbecco's modified Eagle's medium High Glucose (Lonza, Milan, Italy), supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine, as previously described [24].

SZ95 sebocytes were grown in Sebomed® (Biochrom, Berlin, Germany), supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/L EGF (Biochrom). Cells were incubated at 37 °C for 24 hrs in 95% air/5% $CO_2$ until 80% confluency.

In order to reproduce cutaneous tissue closely to the real system in vitro, SZ95 sebocytes ($1\times10^6$ viable cells/mL) and HaCaT cells ($1\times10^5$ viable cells/mL) were co-cultured in 6 well plates (Falcon®), where HaCaT were seeded on the top of the Transwell containing a PET membrane 0,4 μm pores (BD Falcon TM). The cells were observed under an inverted microscope until co-culture cells reached a 80% density.

### 2.2. CS exposure and treatments

Prior to CS exposure of the co-culture cells, the culture medium was aspirated and fresh serum-free medium was added. In order to only expose HaCaT cells to CS, home made Teflon lid was put over the cell culture plates in the way that CS was only able to interact with keratinocytes and not directly with sebocytes. This allowed us to expose the cells for 50 min to CS. Control cells were exposed to filtered air for the same duration (50 min) after changing media.

The time and the way of exposure were chosen based on our previously published results [20,23,24] and no significant difference in the cell viability, as measured by Trypan blue exclusion, was detected between control (air) and CS treatment (data not shown). HaCaT and SZ95 cells were exposed to fresh CS in an exposure system that generated CS by burning two 3R4F Kentucky research cigarette (University of Kentucky, Kentucky, USA) (12 mg tar, 1.1 mg nicotine) using a vacuum pump to draw air through the burning cigarette and leading the smoke stream over the cell cultures as described previously by our group [24]. After the exposure (air or CS), fresh media supplemented with 10% FBS was added to the cells.

For proteasome inhibition experiment, SZ95 sebocytes were pre-treated (2 h) with MG-132 (Calbiochem, La Jolla, CA) before CS exposure with or without HDL treatment (Sigma - Aldrich®).

After treatments, cells were collected by centrifugation for the assays described below.

### 2.3. Cellular viability

Cell viability studies were performed by cytofluorimetric and LDH assays. The cytofluorimetric assay was performed by using the Muse Count & Viability Kit (Millipore, Corporation, Billerica, MA, USA) [25]. The amounts of LDH in the supernatant were determined and calculated according to the manufacture's instructions (EuroClone Milan, Italy), as previously described [26].

### 2.4. Western blot analysis

Total cell lysates were extracted in RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma–Aldrich Corp.), as described before [20]. Cells were harvested by centrifugation and protein concentration was determined by the method of Bradford (Biorad Protein assay, Milan, Italy). Samples, 30 μg of proteins, were loaded into 10% sodium dodecyl sulphate– polyacrylamide electrophoresis gels and separated by molecular size. The gels were then electro-blotted onto nitrocellulose, as previously described [27]. Membranes were incubated overnight at 4 °C with rabbit polyclonal SRB1 antibody (1:1000) (Novus Biologicals, Inc.; Littleton, CO) or rabbit polyclonal β-actin (1:1000) (Cell Signaling; Celbio, Milan, Italy). After incubation with secondary goat anti-rabbit IgG (H+L)-HRP conjugate antibody (1:10000) (BioRad, Milan, Italy), the bound antibodies were detected using chemiluminescence (BioRad, Milan, Italy). Images of the bands were digitized and the densitometry of the bands was performed using the Image-J software.

### 2.5. Quantitative real-time PCR

Quantitative real-time PCR was carried out as described in detail previously [26]. Briefly, total RNA was extracted, using an AURUM total RNA Mini Kit with DNase digestion (Bio-Rad), from $1\times10^6$ sebocytes for each experimental condition, according to the manufacturer's instructions. First-strand cDNA was generated from 1 μg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). The primer pairs (Table 1) capable of hybridization with unique regions of the appropriate gene sequence were obtained from the Real-Time PCR GenBank Primer and Probe Database Primer Bank, RTPrimerDB.