SRB1 AS A NEW REDOX TARGET OF CIGARETTE SMOKE IN HUMAN SEBOCYTES

Ilaria Crivellari[1-2], Claudia Sticozzi[1], Giuseppe Belmonte[1], Ximena M Muresan[1], Franco Cervellati[1], Alessandra Pecorelli[1], Christos C Zouboulis[3], Vinno Savelli[4], Mascia Benedusi[1], Carlo Cervellati[2], Giuseppe Valacchi[1*]

[1]Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy.

[2]Department of Biomedical and Specialist Surgical Sciences, Section of Medical Biochemistry, Molecular Biology and Genetics, University of Ferrara, Ferrara, Italy

[3]Departments of Dermatology, Venereology, Allergology and Immunology, Dessau Medical Center, Dessau, 14195, Germany.

[4] Department of Medical, Surgical and Neurosciences, University of Siena, Siena, Italy.

*corresponding author.

Abstract

For its critical location, the skin represents the major interface between the body and the environment being therefore one of the main biological barrier against all the environmental stressors. Among the several oxidative environmental stressors, cigarette smoke (CS) has been shown to be associated with the development of many skin pathologies such as acne, dermatitis, wound healing, aging, skin cancer. In our previous work we have demonstrated that CS is able to affect skin cholesterol traffic involved genes, among which SRB1, 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 cells and whether CS can affect cholesterol cellular uptake via the redox modulation of SRB1.

By using a co-colture system of kereatinocytes/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 loss of SRB1 induced an alteration of sebocytes lipids content as demonstrated by Oil-Red and SRB1 siRNA experiments.

In conclusion, exposure to CS, induced post-translational modifications of SRB1 with the consequence decrease of SRB1 in sebocytes and this may contribute to the altered sebocytes functionality as aftereffect of the of cholesterol uptake alteration, compromising therefore the skin physiology and functionality.

Introduction

Multiple studies have demonstrated that more than 30% of people in the United States are affected by cutaneous pathologies. Although some of skin diseases are not lethal or with a strong impact on health, they can influence life quality and represent a large cost for public health [1].

The tegumentary system comprises the skin and its appendages, including glands, nails, hair. This organ system main function is to protect the body from the out-door insults including among them also the environmental stressors.

Several 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]. Among these various environmental stressors, it has been demonstrated that CS can affect skin homeostasis playing a key role in diseases such as acne, dermatitis, wound healing, aging, tumors [4–6]. 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 chemical inhaled air pollution. It is able to induce cutaneous elastosis, upregulation of matrix metalloproteinases enzymes (MMPs 1-3) involved in the degradation of the connective tissue modifying the transforming growth factor (TGF-β) pathway [7]. All these damaging effects seems to be related to the release of reactive oxygen species (ROS) present or induced by CS exposure. It has been demonstrated that CS leaded to an oxidative stress condition in oral keratinocytes as it is shown by the increase of intracellular oxidants and a significant decrease of GSH intracellular level [8]. An interesting study revealed that the smoke is able to induce specific facial aging in identical twins with different smoking history [9]. Among the cutaneous defence function, the sebaceous glands have the ability to keep constant the composition of the hydrolipidic film, a mixture of cholesterol, triglycerides, wax esters and squalene, and further they represent the major deliver system of the well-known antioxidant vitamin E from dermis to the surface of the skin protecting cutaneous tissue from oxidant damaging insults [10].

Indeed sebocyte's 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 homeostatic physiological function of the skin [11].

Sebocytes participate in the regulation of immunological functions and inflammatory processes for their ability to produce several cytokines (IL-1β, IL-6, IL-8/CXCL-8, TNFα) and lipid inflammation mediators (5-LOX, LTA4-idrolasi, LTB4, PGE2), which have a key role in the pathogenesis of several inflammatory skin diseases (i.e. acne vulgaris). New etiologic models reveals that acne can develop without the colonization of pathogen microorganisms, rather can be due to other factors (androgen hormons, PPAR activation, SP-mediate stress response) that increase

the production of inflammation mediators and induce hyperseborrhea [12–15]. Moreover, many subunits of cholinergic muscarinic and nicotinic receptor have been found in sebocytes at various grade of differentiation [16]; thus it is fascinating to hypothesize that the activation of these receptor from neural or paracrine acetylcholine or from CS nicotine, can have a role in the pathogenesis of acne or other cutaneous diseases affecting sebocyte's functionality [16].

Since years our group is 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) [17] and its modulation by CS. We have demonstrated that in human keratinocytes the modification of cholesterol traffic-involved proteins such as SRB1 and ABCA1 lead to the skin physiology alteration [18,19]. Whereas sebaceous glands are dragged in physiologic homeostatic function of the skin, we believe that SR-B1 could have an important role also in sebocyte's function but at the moment, no data have demonstrated its presence in this particular secretory organ.

Therefore 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.

Methods

Cell culture

HaCaT cells (a cell line 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 (Valacchi et al., 2001).

SZ95 cells (a cell line gift from Prof. Zouboulis) 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 24hrs in 95% air/5% CO_2 until 80% confluency.

In order to reproduce cutaneous tissue closely the real system in vitro, SZ95 (1 x 10^6 viable cells/mL) and HaCaT (1 x 10^5 viable cells/mL) were co-cultured in 6 well plate (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 sebocytes/keratinocytes co-culture cells reached a 80% density.

CS exposure and treatments

Prior to CS exposure of the co-colture cells, media was aspirated and fresh serum-free medium was added. In order to expose only HaCaT cells to CS, home made Teflon lid was put over the cell culture plates in the way that CS was able only to interact with keratinocytes and not directly with the sebocytes. This allow 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 method of exposure were chosen based on our previous work [17,20,21]. HaCaT and SZ95 cells were exposed to fresh CS in an exposure system that generated CS by burning two research cigarettes (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 [21]. After the exposure (air or CS), fresh media supplemented with 10% FBS was added to the cells.

For proteasome inhibition experiment, SZ95 cells were pre-treated (2hrs) with MG-132 (Calbiochem, La Jolla, CA) before CS exposure with or without HDL treatment (28 mg/mL Sigma - Aldrich®).

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

Cellular Viability

Cell viability studies were performed by cytofluorimetric and LDH assay. The cytofluorimetric assay was performed by using Muse Count & Viability Kit (Millipore, Corporation, Billerica, MA,

USA) [22]. The amounts of LDH in the supernatant were determined and calculated according to the kit instructions (EuroClone Milan, Italy) as previously described [23].

Western blot Analysis

Total cell lysates were extracted in RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma–Aldrich Corp.) as described before [17]. Cells were harvested by centrifugation and proteins concentration was determined by the method of Bradford (Biorad Protein assay, Milan, Italy). Samples, 30µg 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 by Fortino et al. [24]. Membranes were incubated overnight at 4°C with anti- SRB1 (Novus Biologicals, Inc.; Littleton, CO) or β-actin (Cell Signalling; Celbio, Milan, Italy). After incubation with secondary antibody, the bound antibodies were detected using chemiluminescence (BioRad, Milan, Italy). Images of the bands were digitized and the densitometry of the bands were performed using Image-J software.

Quantitative real-time PCR

Quantitative real-time PCR was carried out as described in detail previously [23]. Briefly, total RNA was extracted, using an AURUM total RNA Mini Kit with DNase digestion (Bio-Rad), from 1×10^6 sebocytes for each experimental condition, according to the manufacturer's istructions. 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.

RPL13A	F : 5'-cctaagatgagcgcaagttgaa- 3' R : 5'-ccacaggactagaacacctgctaa-3'	60.2	203	97.3	39	Pattyn *et al*. 2006
RPL11A	F : 5'- tgcgggaacttcgcatccgc-3' R : 5'- gggtctgccctgtgagctgc-3'	60.1	108	96.5	39	GenBank Accession NM 000975.2
GAPDH	F : 5'- tgacgctggggctggcattg -3 R : 5'- ggctggtggtccaggggtct -3'	60	134	94.6	39	GenBank Accession NM 002046.3

SRB1	F : 5'- aatctgtcgcaggcattgga -3' R : 5'- caggaccttggctccggatt -3'	60.2	225	97.5	39	GenBank Accession NM 01082959.1

Table 1 Primers sequencing for housekeeping and SRB1 gene, with amplified size.

Immunocytochemistry

SZ95 cells were grown on coverslips at a density of 1,6 x 10^5 cell/mL, and after CS exposure fixed in 4% paraformaldehyde for 30 min at 4°C as previously described [18]. After permeation and block in BSA 5%, cells were incubated for 1 hr with primary antibody, followed by 1 hr with secondary antibodies. Nuclei were stained with 1 mg/mL DAPI (Molecular Probes) for 1 min after removal of secondary antibodies. Coverslips were mounted onto glass slides using anti-fade mounting medium 1,4 diazabicyclooctane in glycerine (DABCO) and examined by the Leica light microscope equipped with epifluorescence at 40x magnification. Negative controls for immunostaining experiments were performed by omitting primary antibodies. Images were acquired and analyzed with Leica AF CTR6500HS (Microsystems).

Oil Red O Staining

SZ95 cells were seeded on coverslips, exposed to CS and after 6 hrs incubated with 50 and 100 µg/mL HDL for 12-24hrs. After changing media and washing with PBS, SZ95 cells were fixed with 4% paraformaldehyde for 30 min at room temperature, washed with H_2O and 60% isopropanol. Fixed cells were stained with freshly prepared Oil Red O solution for 10 min. Extra stain was remove, samples were washed with distilled water and 60% isopropanol and incubated with Hematoxylin. Coverslips were mounted onto glass slides using glycerol-water 9:1 and than images were acquired with a Leica AF CTR6500HS microscope (Microsystems). The quantification was performed using Image-J software.

SRB1 siRNA Transfection

For siRNA studies, SZ95 cells were transfected with SRB1 siRNA according to the manufactured protocol (Ambion®, USA). Briefly, 1,6 x 10^5 cell/mL were transfected with solution containing 15pmol SRB1 siRNA and 2 µl Lipofectamine® transfection agent. After 24 hrs cells were treated with HDL and than fixed for Oil Red O staining at the time points previously described.

Immunohistochemistry

Human skin tissues were fixed in 10% NBF (neutral-buffered formalin) for 24 hrs at room temperature. Sections (4 mm) were deparaffinized in xylene and rehydrated in alcohol gradients. After dewaxing, sections were incubated overnight at 4°C with anti-SRB1 (Novus Biologicals, Inc.; Littleton, CO). Then slides were washed three times with PBS and endogenous peroxidase was blocked with 3% hydrogen peroxide in absolute methyl alcohol for 30 min at room temperature. Finally, the slides were incubated with EnVision+System-HRP (DAKO, Glostrup, Denmark) for 45 min at room temperature. The reaction products were stained with diaminobenzidine (DAB), counterstained with Mayer's Hematoxylin and after drying were mounted with Eukitt mounting medium.

Statistical Analysis

For each of the variables tested, two-way analysis of variance (ANOVA) was used. A significant effect was indicated by a P-value<0.05. Data are expressed as mean ± S.D. of triplicate determinations obtained in 5 independent experiments.

Results

SRB1 expression in human skin sebaceous glands

To evaluate the presence of SR-B1 in sebaceous glands, immunohistochemistry analysis was performed in samples from human skin biopsies. As shown in Fig. 1 a strong SRB1 cytoplasm positivity was demonstrated in sebaceous glands. Moreover SRB1 presence was further confirmed also in basal keratinocytes, as previously reported by our group [19].

CS exposure did not affect sebocytes viability

As shown in figure 2A, CS was able to decrease keratinocytes viability after 6 hrs CS exposure in the co-culture system where only HaCaT where directly exposed to CS. The viability was reduced by 40% in keratinocytes after 24 hrs CS exposure. On the other hand, CS did not affect sebocytes viability. These data were also confirmed by LDH assay as well (figure 2B).

CS exposure decreased SRB1 protein levels in sebocytes

Next we have evaluated whether SRB1 protein expression was modulated by CS exposure in SZ95 cells. As it is shown in Figure 3 SRB1 protein levels decreased 12 hrs after CS exposure, reaching the maximum decrease at 24 hrs time point (loss of 75% respect to control). Of note that SRB1 protein decrease was not a consequence of sebocytes cell death since their viability was not affected by CS (see Fig. 2).

CS exposure increased SRB1 gene expression

To investigate whether the decrease of SRB1 protein was at the transcriptional level, SRB1 gene expression experiments were performed. Figure 4 showed an increase of SRB1 mRNA level already after 6 hrs CS exposure (20%). This effect was then even more enhance at the later time points (12 and 24 hrs) with circa 2 fold increase.

CS exposure induced HNE/SRB1 adducts

It has been demonstrated that many toxic effect of CS can be linked to the generation of aldehydes, such as 4-hydroxinonenal (HNE). Therefore we have evaluated HNE adducts levels in sebocytes exposed to CS by using immunocytochemistry analysis. As shown in Fig. 5 after CS exposure there was an increase of HNE protein adducts levels (red colour). This increase was already evident 6 hrs after the exposure to CS. Then, we evaluated whether the formation of HNE protein adducts induced by CS could affect SRB1. As shown in Figure 5, after CS exposure, the levels of HNE

increased dramatically (red colour, left column) with a concomitant decrease of SRB1 (green colour, central column). The co-localization (yellow) appreciable in the right column showed the presence of HNE adducts on SRB1. These data suggested that CS induced a covalent modification of SRB1 with HNE.

CS exposure induced Ubiquitin/SRB1adducts and SRB1 degradation via proteasome

A consequence of a cellular oxidative damage is the activation of the proteasome machinery to remove oxidized damaged proteins. Therefore we evaluated whether CS exposure induced ubiquitination in SZ95 cells. Immunocytochemistry (Figure 6A) showed an increase of Ubiquitin (Ub) expression (red colour left column) combined with a decrease of SRB1 (green colour, central column) after CS exposure. The co-localization (yellow) appreciable in the right column showed the presence of Ubiquitin and SRB1 (right column yellow), suggesting that CS is able to induce the formation of Ub/SRB1 adducts. To confirmed the proteasome involvement in SRB1 degradation after CS exposure, we next treated sebocytes with MG-132, a well known proteasome inhibitor. CS exposed cells in the presence of MG132 did not show changes in SRB1 levels (Figure 6B).

CS exposure decreased lipidic content

Since one of the most important role of SRB1 is cholesterol uptake by HDL, we investigated whether the decrease in SR-B1 protein level by CS exposure could have any effect on lipid content in sebocytes. Figure 7 shown that, when cells were treated with different concentrations of HDL (50 and 100 μg/mL), lipid cell content decreased by 20% after 12 and 24 hrs from CS exposure.

As a proof of concepts we performed a SRB1 silencing experiment in sebocytes. As it is shown in Figure 8, cells (SZ95) knockout for SRB1 and treated with HDL showed a clear decrease of lipid content, indicating a role of this receptor on cellular lipid content uptake.

Discussion

The present study is the continuous of our previous work [19] in which we have analyzed the mechanisms involved in SRB1 modulation in human keratinocytes by CS. We want to extend this study also to sebocytes as these cells play a key role in protecting the skin from the outdoor stressors by their ability to produce sebum. In addition, being SRB1 involved in cholesterol cellular trafficking its exogenous and endogenous modulation in sebocytes is of undoubtedly importance.

At today the presence of SRB1 in sebaceous glands has not yet been determined. Therefore the first step of our study has been to localize this receptor in human skin with focus on sebocytes. As it was hypothesized, SRB1 was present in sebocytes and this in is line with what has been suggested by Thiele et al.[10] that sebaceous glands are the main responsible for vitamin E secretion in skin. Indeed, among the several functions related to SRB1 there is also its ability to be involved in lipophilic antioxidant trafficking such as Vitamin E and A [25].

Cigarette contains circa 5000 toxic and carcinogenic compounds (carbon monoxide, benzene, acetaldehyde, methanol, hydrocyanic acid, ammonium, acrolein, formaldehyde, tar, vinyl chloride, 2-naphthylamine, nicotine) [26]; its damaging effects on respiratory and cardiovascular systems have been well demonstrated and now also its noxious effect on cutaneous tissues have been studied [27,28]. Since its location, cutaneous tissue is exposed to CS directly during expiration, and indirectly through the harmful substances itself included, which after the absorption from respiratory apparatus can reach the skin by blood flow. CS exposure has a fundamental impact on cutaneous aging, leading to the so called “smokers face” which underline the particular features that are induced by CS exposure in skin [4].

CS exposure leads to the production of free radicals with the consequent oxidative biological tissue damage [2,29,30], the decrease of cutaneous antioxidant concentration as α-tocopherol [31], the alteration of lipidic film composition in which are immersed human keratinocytes [18,19,31]. Among several lipid components of the skin, cholesterol is essential for cell membranes and the skin physiological functions [19,32]. Scavenger Receptor B1 (SRB1) is one of the main players that are involved in the selective uptake of tissues cholesteryl ester (CE) [33] and its presence in both keratinocytes and sebocytes makes this receptor as one of the main skin cholesterol player.

Sebocytes are localized between dermis and epidermis forming one of the ancillary organ of the skin, the sebaceous gland. Recent study revealed that sebaceous gland is not only a passive “memento” with the unique function to produce sebum, but it is also responsible of the physical-chemical barrier function of the skin against environmental insults and acts as a cutaneous “organ” at neuronal-immune-endocrine level for the presence of several types of receptors [11]. To mimic the cutaneous morphology characterized by the epidermal keratinocytes in contact with the

underlying dermis where sebaceous glands are localized, our studies were performed in keratinocytes/sebocytes co-culture. SRB1 is highly expressed in human epidermis, as previously demonstrated [19]; and for the first time the presence of this receptor in sebaceous glands was also demonstrated in the present work. On these bases, the cell culture model was exposed on CS and then evaluated the cellular viability. CS exposure induced a decrease of keratinocytes viability in a time dependent manner, on the contrary, the sebocytes viability was not affected by CS. This was not surprising because, as the experimental model showed, only keratinocytes were directly exposed to CS, whereas sebocytes were covered and isolated by home made Teflon lid.

The decreased SRB1 levels induced by CS could be a consequence of both transcriptional and translational effects. As it concerns transcriptional modifications, there was a significant increase of the SRB1 mRNA which can be a consequence of the protein loss activating a positive feedback on gene transcription to replenish the decrease in the protein level.

As mentioned before, CS is one of the most toxic insult to which skin can be exposed and it has been shown that second hand smoke (passive smoke) can be even more toxic than the mainstream smoke, as a consequence of its chemical composition [34]. CS can affect tissues mainly by the large amount of reactive oxygen species and reactive nitrogen species present in its phases that leads to membrane peroxidation and formation of toxic molecules such as high reactive α,β-unsaturated aldehydes of which HNE is one of the most reactive. This aldehyde is able to form covalent protein binding with amino acids residues such as lysine, histidine and cysteine presents in the proteins, leading to the alteration of protein function [35]. In our experimental procedure, CS was able to induce not only an increase of HNE but also a co-localization between HNE and SRB1 showing that SRB1 can be one of the protein target of α,β -unsaturated aldehydes. In addition, the increased levels of HNE protein adducts has been connected with both skin aging and inflammation [36].

It is well known that during an oxidative event cells can eliminate damaging or oxidized protein by proteasome machinery [37] that starts the protein ubiquitination, which is another protein post-transcriptional modification due to the covalent bond of one or more Ubiquitin monomers on the target protein. We found an increased ubiquitin expression after CS, and its expression co-localized with SR-B1, indicating that CS was able to induce SRB1/Ub. The involvement of the proteasome machinery in the decreased levels of SRB1 protein was confirmed by the use of the proteasome inhibitor MG132, which was able to prevent SRB1 loss. This effect is in line with the one observed in human keratinocytes after CS exposure, suggesting that both cutaneous cells, sebocytes and keratinocytes, respond similar to CS insults as it concern SRB1 levels. Therefore it is possible that CS exposure can decrease SRB1 levels in different cutaneous cells promoting a more intensive and disseminate effect in the skin.

The loss of SRB1 can affect the ability of cells to uptake lipids and vitamin E [25]. Indeed SRB1 KO animals have shown to have very large HDL particles, increased plasma levels of cholesterol and very low levels of tissue tocopherol [38].

Our results confirm the role that SRB1 has on cellular lipid uptake, in fact sebocytes exposed to CS and then treated with several doses of HDL showed impairment in lipid cellular uptake that can consequently affect the skin functionality. By the use of SRB1 knockout experiments we were abel to confirm its role in lipid uptake also in sebocytes.

Indeed, as hypothesized, when SRB1 was silenced, the cellular lipid content was almost abolished respect to the wild type cells treated with HDL. These last sets of data confirm that SRB1 play an important role in the transport of cholesterol also in sebocytes.

This study can also suggest a possible role of SRB1 in acne pathogenesis induced by CS. The literature has suggested a role of CS in acne development. For instance, an interesting study highlighted the connection between smoking and post-pubertal non inflammatory acne (APAA) and the increase in sebum peroxidation as well as the reduction of the antioxidant vitamin E [39]. Among the thousands compounds present in CS there are also the polycyclic aromatic hydrocarbons (PAHs), several of which have been identified to have chloracnegenic potential [40,41]. Shelley and Kligman were the first able to experimentally induce localized acne in healthy subjects with daily topical application of penta- and hexachloronaphthalene, further demonstrating the inherently acnegenic potential of these organic compounds [42]. Among the several functions that have been attributed to SRB1 there is also its role in bacteria recognition [23,43] therefore it is possible that the loss of SRB1 in sebocytes will lead to alteration of the sebaceous gland composition, impariment in skin bacteria recognition and decreased antioxidants levels (vitamin E) and together with the increased oxidative stress induced by CS could make skin tissues more susceptible to infections.

In conclusion, we evidenced a new target of CS in human sebocytes suggesting that the loss of SRB1 protein induced from CS, via post-translational modifications, reduced the uptake of cholesterol from HDL. That could lead to the alteration of sebum lipid composition and to the impairment of protective sebum function against oxidative environmental insult such as CS. Although SRB1 is not the unique protein involved in cholesterol pathway, these results represent a new direction to follow to better understand the molecular modifications affecting sebocytes that occur in pathologies linked with CS exposure and lipidic profile alteration.

Aknowledgments

The authors would like to thank Dr. Angela Pignatelli and Mr. Andrea Margutti for technical support.

Legend

Figure 1. Immunohistochemistry of human skin biopsies. The anti SRB1 antibody stains the cytoplasm of the cells of sebaceous glands in normal human skin biopsies. DAB, Original Magnification x400 (A,B) and x200 (C,D).

Figure 2. Keratinocytes/sebocytes viability after CS exposure. The citotoxicity of the two different cellular lines was measured by cytofluorimetric assay (A) and by LDH assay (B). TritonX represent 100% of cell death (100% of LDH release from the cells). Data are the average of five different experiments *p<0.05).

Figure 3. SRB1 protein expression after CS exposure in human sebocytes. Cells were exposed to CS for 50 min, than harvested at different time points (0-24 hrs) and the protein expression was measured by Western blot. Representative Western blot of five different experiments is depicted in the top panel. Quantification of the SRB1 bands is shown in the bottom panel. Data are expressed in arbitrary units (averages of five different experiments, *p<0.05). β-actin was used as a loading control.

Figure 4. SRB1 gene expression after CS exposure in human sebocytes. mRNA was extracted from the sebocytes after CS exposure and the gene expression was measured by Real Time PCR. Data are expressed in % $2^{-\Delta\Delta CT}$, in arbitrary units and are the average of five different experiments (*p<0,05).

Figure 5. HNE/SRB1 protein adducts after CS exposure in human sebocytes. Cells were exposed to CS and fixed at different time points (0-24hrs). Immunocytochemistry of SZ95 cells shown localization of HNE –adducts (left column, red color), SRB1 (central column, green color) and HNE/SRB1 adducts (right column, yellow color). Images are merged in the right panel and the yellow color indicates overlap of the staining. Quantification is shown in the panel B. Data are the average of five different experiments (*p<0,05).

Figure 6A. Ubiquitin/SRB1 adducts after CS exposure in human sebocytes. Cells were exposed to CS and fixed at different time points (0-24 hrs). Immunocytochemistry of SZ95 cells showing localization of UB–adducts (left column, red color), SRB1 (central column, green color) and UB/SRB1 adducts (right column, yellow color). Images are merged in the right panel and the yellow color indicates overlap of the staining. Quantification is shown in the panel B. Data are the average of five different experiments (*p<0,05).

Figure 6B. SRB1 protein expression after proteasome inhibition and CS exposure in human sebocytes. Cells were pretreated with MG-132 (proteasome inhibitor), exposed to CS for 50 min, harvested at different time points (0-24 hrs) and protein expression was measured by Western blot. The Western blot shown in the top is representative of five different experiments. Quantification of the SRB1 bands is shown in the bottom panel. Data are expressed as arbitrary units (averages of five different experiments, *$p<0.05$). β-actin was used as loading control.

Figure 7. Sebocytes lipid content after CS exposure. Imagines on the left represented the Oil Red staining which shown the lipid drops into human sebocytes after the treatment with HDL and CS exposure. Scale Bar, 50 μm. Quantification of the cholesterol uptake HDL-mediated is shown in the right panel. Values are the area of the lipid drops (%). Data are the average of five different experiments (*$p<0,05$).

Figure 8. Lipid content after SRB1 silencing in human sebocytes. Imagines on the left represented the Oil Red staining which shown the lipid drops in human sebocytes. Cells were silenced for SRB1 and than treated with HDL. Scale Bar, 50 μm. Quantification of the cholesterol uptake HDL-mediated is shown in the right panel. Values are the area of the lipid drops (%). Data are the average of five different experiments (*$p<0,05$).

References

[1] D.R. Bickers, H.W. Lim, D. Margolis, M.A. Weinstock, C. Goodman, E. Faulkner, C. Gould, E. Gemmen, T. Dall, American Academy of Dermatology Association, Society for Investigative Dermatology, The burden of skin diseases: 2004 a joint project of the American Academy of Dermatology Association and the Society for Investigative Dermatology., J. Am. Acad. Dermatol. 55 (2006) 490–500. doi:10.1016/j.jaad.2006.05.048.

[2] G. Valacchi, C. Sticozzi, A. Pecorelli, F. Cervellati, C. Cervellati, E. Maioli, Cutaneous responses to environmental stressors., Ann. N. Y. Acad. Sci. 1271 (2012) 75–81. doi:10.1111/j.1749-6632.2012.06724.x.

[3] N.D. Magnani, X.M. Muresan, G. Belmonte, F. Cervellati, C. Sticozzi, A. Pecorelli, C. Miracco, T. Marchini, P. Evelson, G. Valacchi, Skin Damage Mechanisms Related to Airborne Particulate Matter Exposure., Toxicol. Sci. 149 (2016) 227–36. doi:10.1093/toxsci/kfv230.

[4] D. Model, Smoker's face: an underrated clinical sign?, Br. Med. J. (Clin. Res. Ed). 291 (n.d.) 1760–2.

[5] I.E. El-Hakim, M.A. Uthman, Squamous cell carcinoma and keratoacanthoma of the lower lip associated with "Goza" and "Shisha" smoking., Int. J. Dermatol. 38 (1999) 108–10.

[6] G. Valacchi, S.U. Weber, C. Luu, C.E. Cross, L. Packer, Ozone potentiates vitamin E depletion by ultraviolet radiation in the murine stratum corneum., FEBS Lett. 466 (2000) 165–8.

[7] L. Yin, A. Morita, T. Tsuji, Alterations of extracellular matrix induced by tobacco smoke extract., Arch. Dermatol. Res. 292 (2000) 188–94.

[8] K. Avezov, A.Z. Reznick, D. Aizenbud, Oxidative damage in keratinocytes exposed to cigarette smoke and aldehydes., Toxicol. In Vitro. 28 (2014) 485–91. doi:10.1016/j.tiv.2014.01.004.

[9] H.C. Okada, B. Alleyne, K. Varghai, K. Kinder, B. Guyuron, Facial changes caused by smoking: a comparison between smoking and nonsmoking identical twins., Plast. Reconstr. Surg. 132 (2013) 1085–92. doi:10.1097/PRS.0b013e3182a4c20a.

[10] J.J. Thiele, S.U. Weber, L. Packer, Sebaceous gland secretion is a major physiologic route of vitamin E delivery to skin., J. Invest. Dermatol. 113 (1999) 1006–10. doi:10.1046/j.1523-1747.1999.00794.x.

[11] B.I. Tóth, A. Oláh, A.G. Szöllosi, G. Czifra, T. Bíró, "Sebocytes' makeup": novel mechanisms and concepts in the physiology of the human sebaceous glands., Pflügers Arch. Eur. J. Physiol. 461 (2011) 593–606. doi:10.1007/s00424-011-0941-6.

[12] M.D. Farrar, E. Ingham, Acne: inflammation., Clin. Dermatol. 22 (n.d.) 380–4. doi:10.1016/j.clindermatol.2004.03.006.

[13] I. Kurokawa, F.W. Danby, Q. Ju, X. Wang, L.F. Xiang, L. Xia, W. Chen, I. Nagy, M. Picardo, D.H. Suh, R. Ganceviciene, S. Schagen, F. Tsatsou, C.C. Zouboulis, New developments in our understanding of acne pathogenesis and treatment., Exp. Dermatol. 18 (2009) 821–32. doi:10.1111/j.1600-0625.2009.00890.x.

[14] C.C. Zouboulis, Acne and sebaceous gland function., Clin. Dermatol. 22 (n.d.) 360–6. doi:10.1016/j.clindermatol.2004.03.004.

[15] C.C. Zouboulis, A. Eady, M. Philpott, L.A. Goldsmith, C. Orfanos, W.C. Cunliffe, R. Rosenfield, What is the pathogenesis of acne?, Exp. Dermatol. 14 (2005) 143–52. doi:10.1111/j.0906-6705.2005.0285a.x.

[16] C.C. Zouboulis, J.M. Baron, M. Böhm, S. Kippenberger, H. Kurzen, J. Reichrath, A. Thielitz, Frontiers in sebaceous gland biology and pathology., Exp. Dermatol. 17 (2008) 542–51. doi:10.1111/j.1600-0625.2008.00725.x.

[17] G. Valacchi, P.A. Davis, E.M. Khan, R. Lanir, E. Maioli, A. Pecorelli, C.E. Cross, T.

Goldkorn, Cigarette smoke exposure causes changes in Scavenger Receptor B1 level and distribution in lung cells., Int. J. Biochem. Cell Biol. 43 (2011) 1065–70. doi:10.1016/j.biocel.2009.05.014.
[18] C. Sticozzi, A. Pecorelli, G. Belmonte, G. Valacchi, Cigarette smoke affects ABCAl expression via liver X receptor nuclear translocation in human keratinocytes., Int. J. Mol. Sci. 11 (2010) 3375–86. doi:10.3390/ijms11093375.
[19] C. Sticozzi, G. Belmonte, A. Pecorelli, B. Arezzini, C. Gardi, E. Maioli, C. Miracco, M. Toscano, H.J. Forman, G. Valacchi, Cigarette smoke affects keratinocytes SRB1 expression and localization via H2O2 production and HNE protein adducts formation., PLoS One. 7 (2012) e33592. doi:10.1371/journal.pone.0033592.
[20] E.M. Khan, R. Lanir, A.R. Danielson, T. Goldkorn, Epidermal growth factor receptor exposed to cigarette smoke is aberrantly activated and undergoes perinuclear trafficking., FASEB J. 22 (2008) 910–7. doi:10.1096/fj.06-7729com.
[21] G. Valacchi, G. Rimbach, C. Saliou, S.U. Weber, L. Packer, Effect of benzoyl peroxide on antioxidant status, NF-kappaB activity and interleukin-1alpha gene expression in human keratinocytes., Toxicology. 165 (2001) 225–34.
[22] A. Cortelazzo, R.L. Lampariello, C. Sticozzi, R. Guerranti, C. Mirasole, L. Zolla, G. Sacchetti, J. Hajek, G. Valacchi, Proteomic profiling and post-translational modifications in human keratinocytes treated with Mucuna pruriens leaf extract., J. Ethnopharmacol. 151 (2014) 873–81. doi:10.1016/j.jep.2013.11.053.
[23] G. Valacchi, C. Sticozzi, G. Belmonte, F. Cervellati, J. Demaude, N. Chen, Y. Krol, C. Oresajo, Vitamin C Compound Mixtures Prevent Ozone-Induced Oxidative Damage in Human Keratinocytes as Initial Assessment of Pollution Protection., PLoS One. 10 (2015) e0131097. doi:10.1371/journal.pone.0131097.
[24] V. Fortino, E. Maioli, C. Torricelli, P. Davis, G. Valacchi, Cutaneous MMPs are differently modulated by environmental stressors in old and young mice., Toxicol. Lett. 173 (2007) 73–9. doi:10.1016/j.toxlet.2007.06.004.
[25] G. Valacchi, E. Maioli, C. Sticozzi, F. Cervellati, A. Pecorelli, C. Cervellati, J. Hayek, Exploring the link between scavenger receptor B1 expression and chronic obstructive pulmonary disease pathogenesis., Ann. N. Y. Acad. Sci. 1340 (2015) 47–54. doi:10.1111/nyas.12714.
[26] R. Talhout, T. Schulz, E. Florek, J. van Benthem, P. Wester, A. Opperhuizen, Hazardous compounds in tobacco smoke., Int. J. Environ. Res. Public Health. 8 (2011) 613–28. doi:10.3390/ijerph8020613.
[27] A. Morita, Tobacco smoke causes premature skin aging., J. Dermatol. Sci. 48 (2007) 169–75. doi:10.1016/j.jdermsci.2007.06.015.
[28] A. Morita, K. Torii, A. Maeda, Y. Yamaguchi, Molecular basis of tobacco smoke-induced premature skin aging., J. Investig. Dermatol. Symp. Proc. 14 (2009) 53–5. doi:10.1038/jidsymp.2009.13.
[29] C. Sticozzi, F. Cervellati, X.M. Muresan, C. Cervellati, G. Valacchi, Resveratrol prevents cigarette smoke-induced keratinocytes damage., Food Funct. 5 (2014) 2348–56. doi:10.1039/c4fo00407h.
[30] A. Valavanidis, T. Vlachogianni, K. Fiotakis, Tobacco smoke: involvement of reactive oxygen species and stable free radicals in mechanisms of oxidative damage, carcinogenesis and synergistic effects with other respirable particles., Int. J. Environ. Res. Public Health. 6 (2009) 445–62. doi:10.3390/ijerph6020445.
[31] G. Valacchi, V.T. Vasu, W. Yokohama, A.M. Corbacho, A. Phung, Y. Lim, H.H. Aung, C.E. Cross, P.A. Davis, Lung vitamin E transport processes are affected by both age and environmental oxidants in mice., Toxicol. Appl. Pharmacol. 222 (2007) 227–34. doi:10.1016/j.taap.2007.04.010.
[32] J. van Smeden, J.A. Bouwstra, Stratum Corneum Lipids: Their Role for the Skin Barrier

Function in Healthy Subjects and Atopic Dermatitis Patients., Curr. Probl. Dermatol. 49 (2016) 8–26. doi:10.1159/000441540.
[33] W.-J. Shen, J. Hu, Z. Hu, F.B. Kraemer, S. Azhar, Scavenger receptor class B type I (SR-BI): a versatile receptor with multiple functions and actions., Metabolism. 63 (2014) 875–86. doi:10.1016/j.metabol.2014.03.011.
[34] S. Schick, S. Glantz, Philip Morris toxicological experiments with fresh sidestream smoke: more toxic than mainstream smoke., Tob. Control. 14 (2005) 396–404. doi:10.1136/tc.2005.011288.
[35] G. Poli, R.J. Schaur, W.G. Siems, G. Leonarduzzi, 4-hydroxynonenal: a membrane lipid oxidation product of medicinal interest., Med. Res. Rev. 28 (2008) 569–631. doi:10.1002/med.20117.
[36] N. Tanaka, S. Tajima, A. Ishibashi, K. Uchida, T. Shigematsu, Immunohistochemical detection of lipid peroxidation products, protein-bound acrolein and 4-hydroxynonenal protein adducts, in actinic elastosis of photodamaged skin., Arch. Dermatol. Res. 293 (2001) 363–7.
[37] A.M. Pickering, K.J.A. Davies, Degradation of damaged proteins: the main function of the 20S proteasome., Prog. Mol. Biol. Transl. Sci. 109 (2012) 227–48. doi:10.1016/B978-0-12-397863-9.00006-7.
[38] A. Rigotti, B.L. Trigatti, M. Penman, H. Rayburn, J. Herz, M. Krieger, A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism., Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 12610–5.
[39] B. Capitanio, J.L. Sinagra, M. Ottaviani, V. Bordignon, A. Amantea, M. Picardo, Acne and smoking., Dermatoendocrinol. 1 (2009) 129–35. doi:10.4161/derm.1.3.9638.
[40] J.P. Tindall, Chloracne and chloracnegens., J. Am. Acad. Dermatol. 13 (1985) 539–58.
[41] Q. Ju, C.C. Zouboulis, L. Xia, Environmental pollution and acne: Chloracne., Dermatoendocrinol. 1 (2009) 125–8.
[42] W.B. SHELLEY, A.M. KLIGMAN, The experimental production of acne by pentaand hexachloronaphthalenes., AMA. Arch. Derm. 75 (1957) 689–95.
[43] G. Schäfer, R. Guler, G. Murray, F. Brombacher, G.D. Brown, The role of scavenger receptor B1 in infection with Mycobacterium tuberculosis in a murine model., PLoS One. 4 (2009) e8448. doi:10.1371/journal.pone.0008448.

Fig. 1

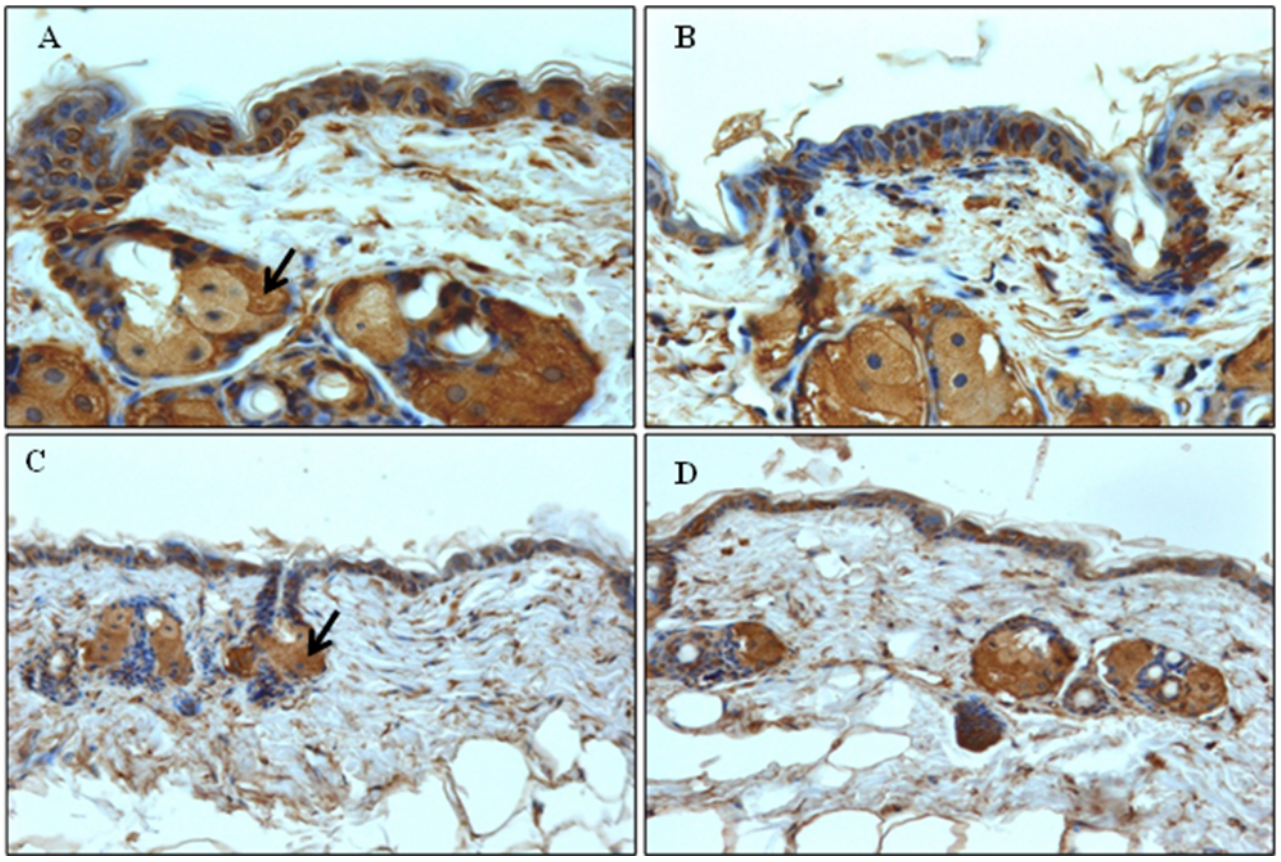


Fig. 2

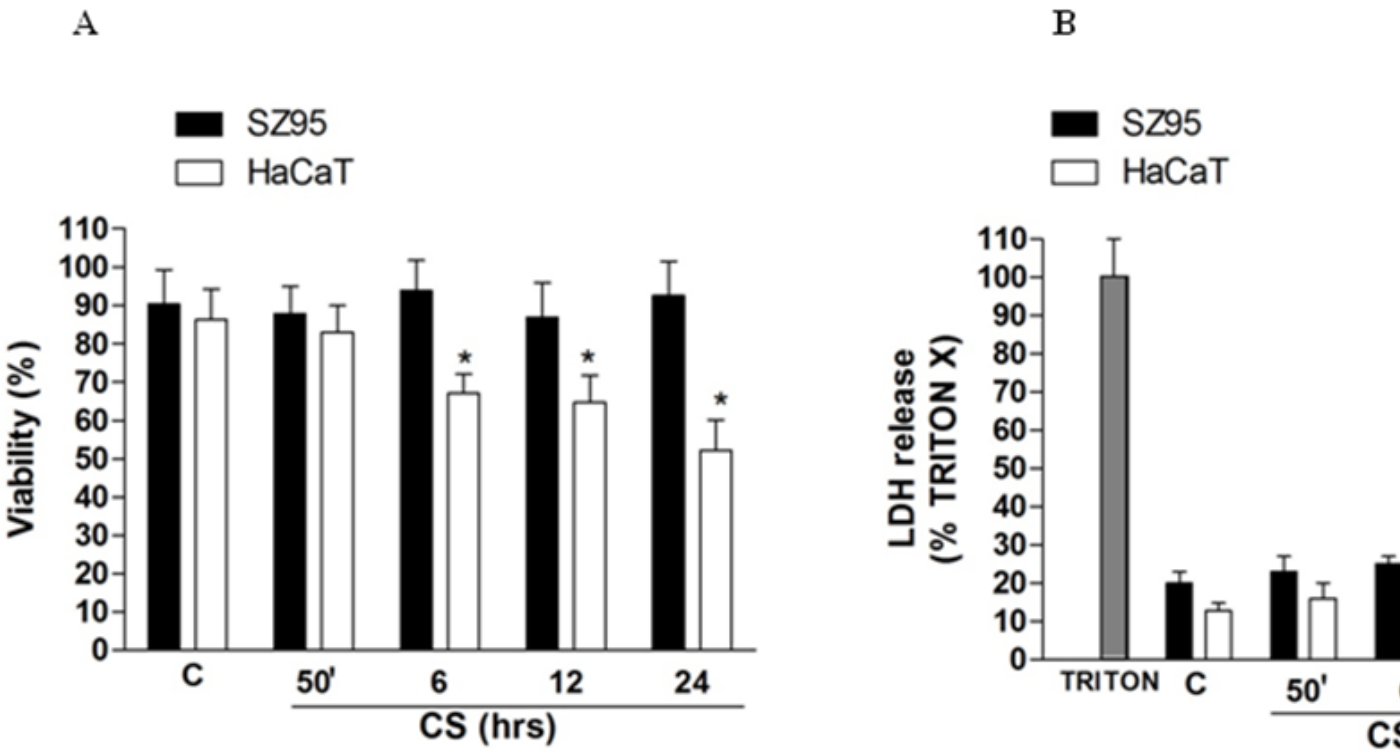

A
SZ95
HaCaT
Viability (%)
110
100
90
80
70
60
50
40
30
20
10
0
C
50'
6
12
24
CS (hrs)
*
*
*
B
SZ95
HaCaT
LDH release
(% TRITON X)
110
100
90
80
70
60
50
40
30
20
10
0
TRITON
C
50'

Fig. 3

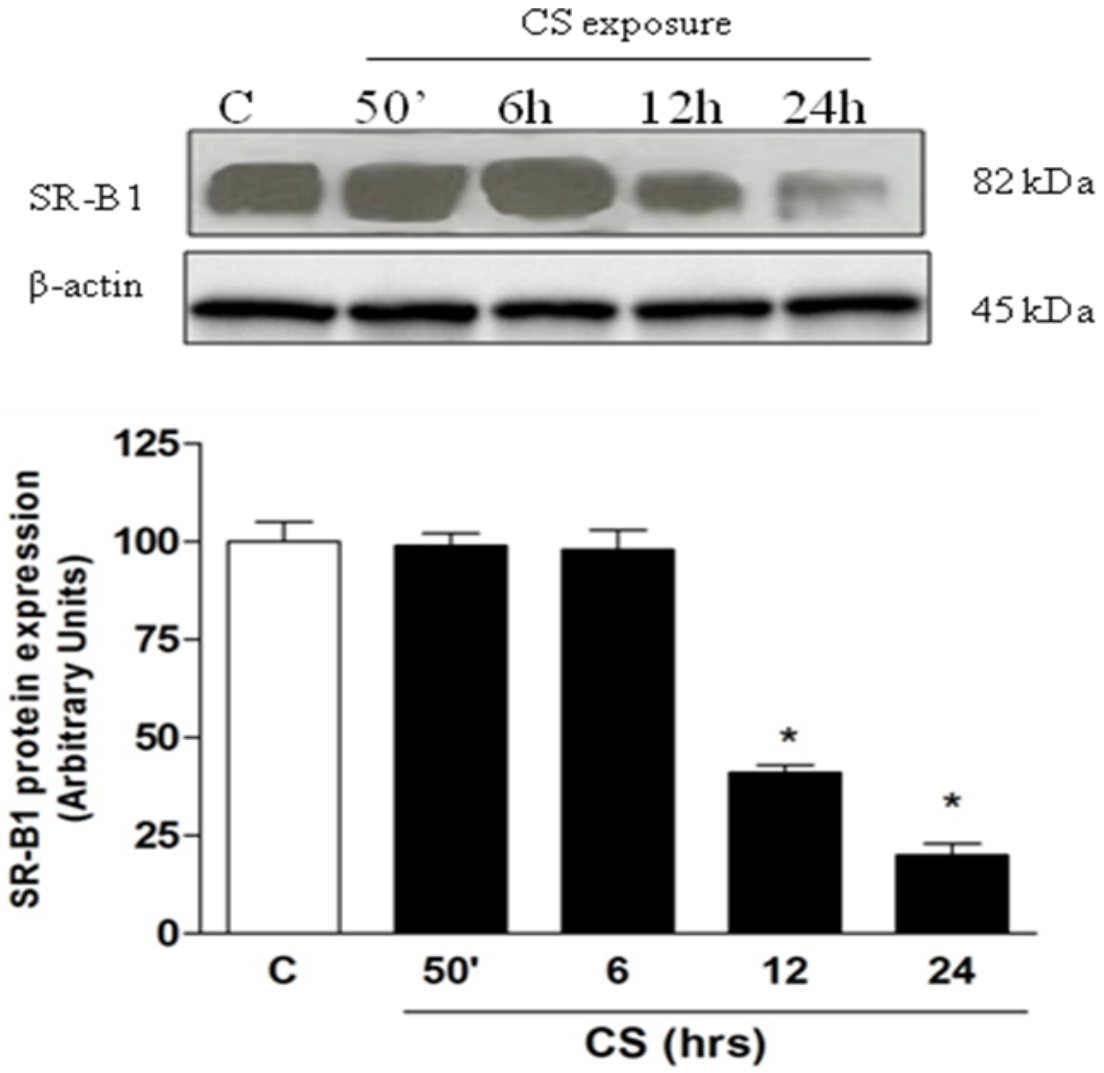

CS exposure
C
50'
6h
12h
24h
SR-B1
82 kDa
β-actin
45 kDa
SR-B1 protein expression (Arbitrary Units)
125
100
75
50
25
0
*
*
C
50'
6
12
24
CS (hrs)

Fig. 4

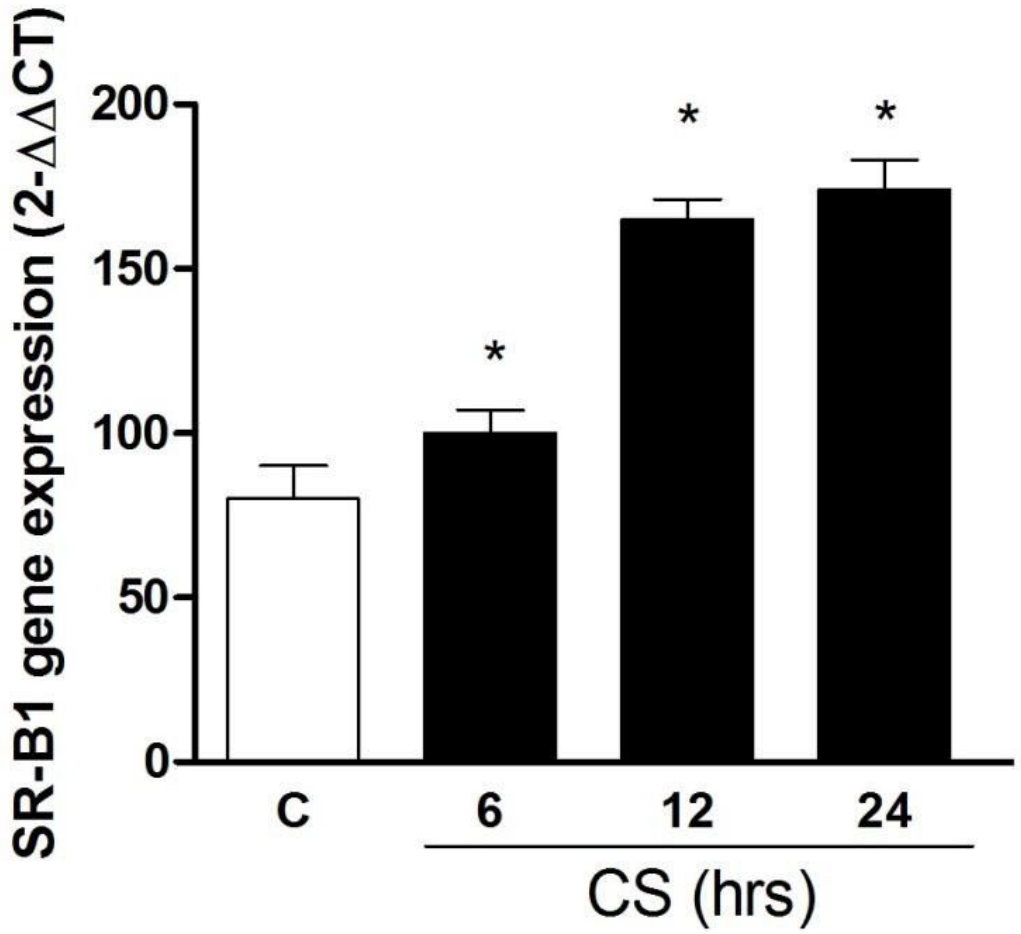
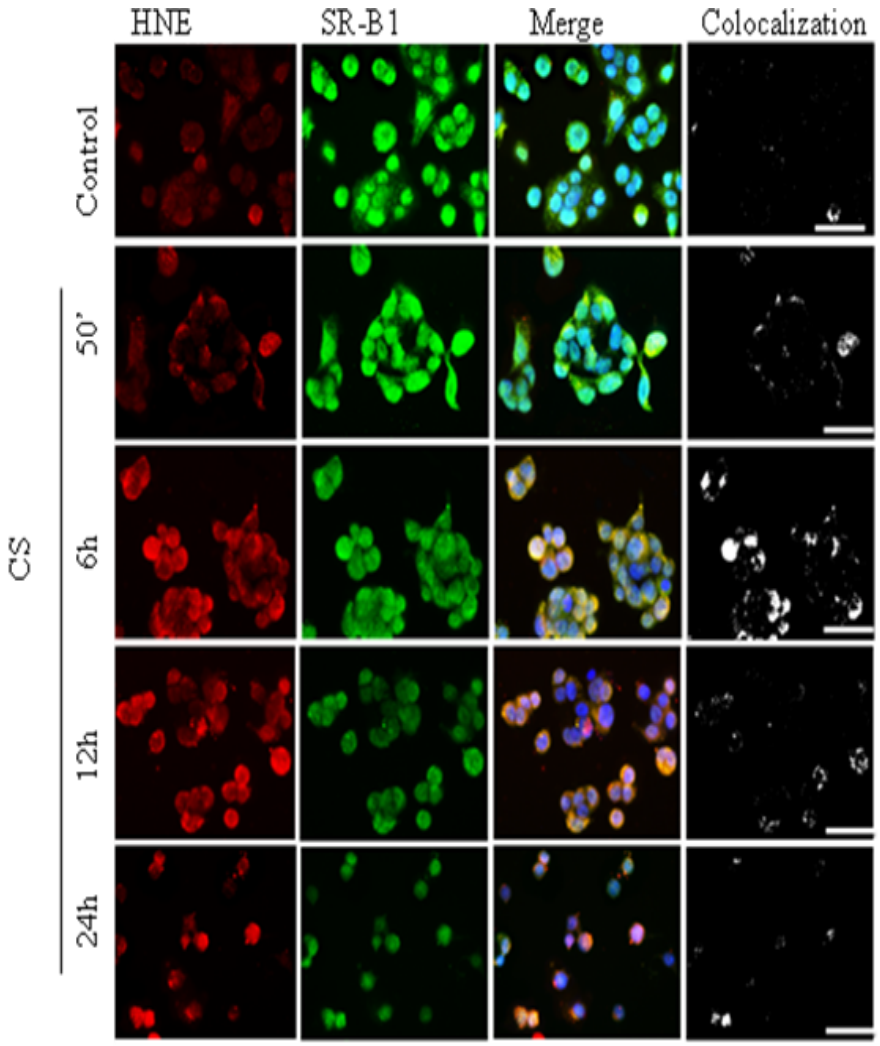


Fig. 5


HNE
SR-B1
Merge
Colocalization
Control
CS
50'
6h
12h
24h

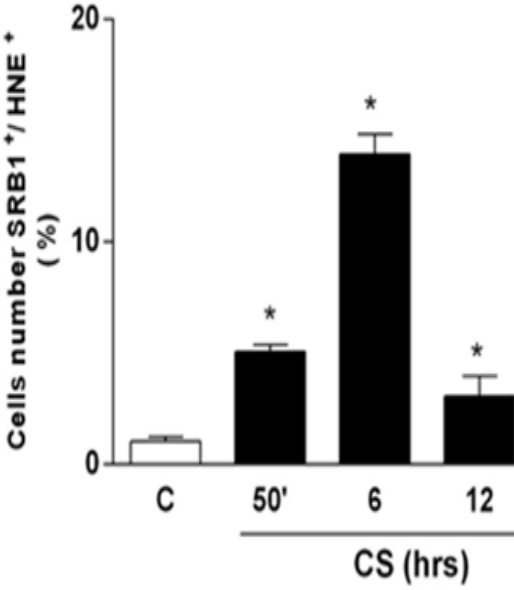
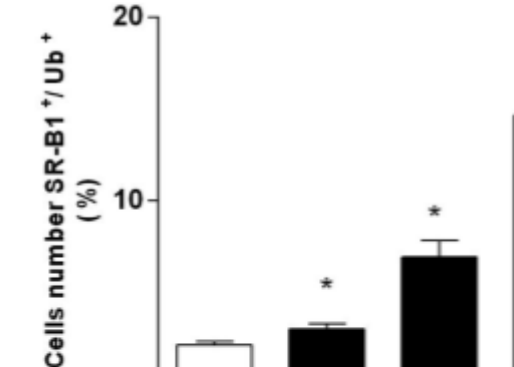

Cells number SRB1 +/ HNE +
(%)
20
10
0
C
50'
6
12
CS (hrs)

Fig. 6A

Fig. 6B


Cells number SR-B1 +/ Ub +
(%)
20
10

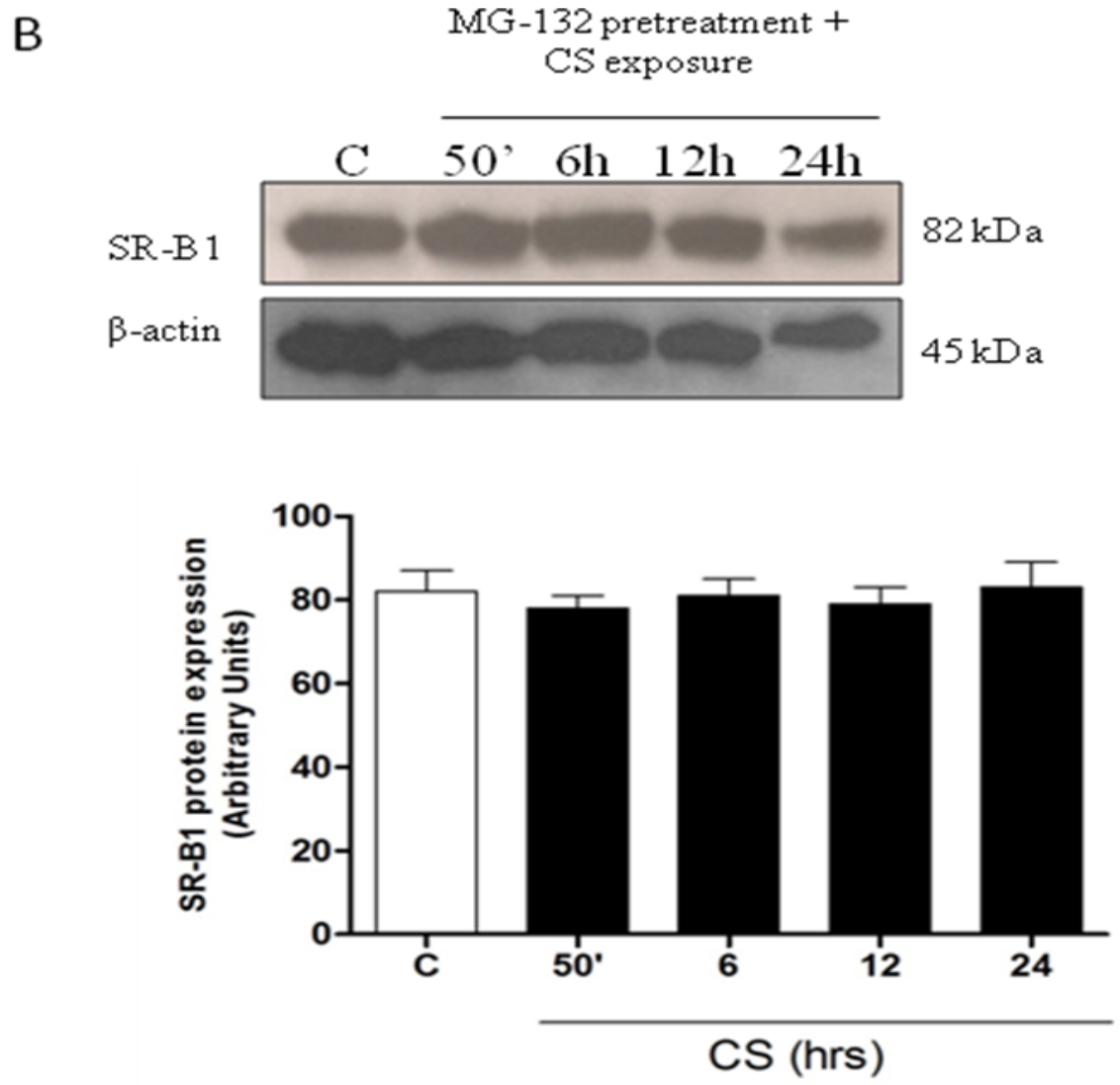
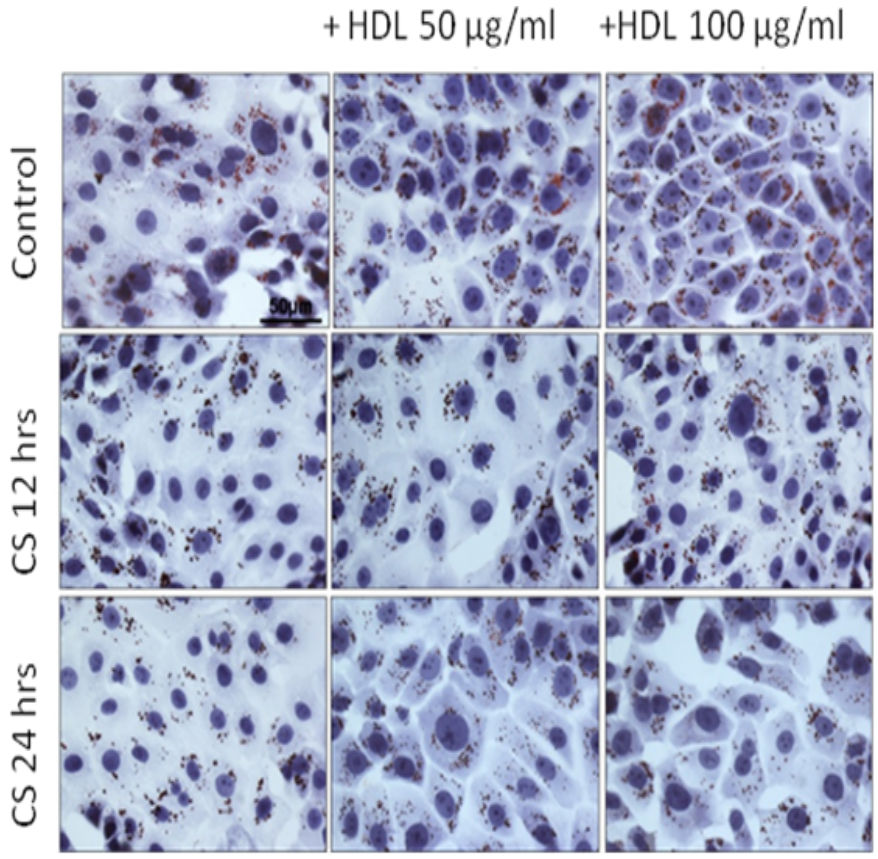

B
MG-132 pretreatment +
CS exposure
C
50'
6h
12h
24h
SR-B1
β-actin
82 kDa
45 kDa
SR-B1 protein expression
(Arbitrary Units)
100
80
60
40
20
0
C
50'
6
12
24
CS (hrs)

Fig. 7


+ HDL 50 µg/ml
+HDL 100 µg/ml
Control
CS 12 hrs
CS 24 hrs
50µm

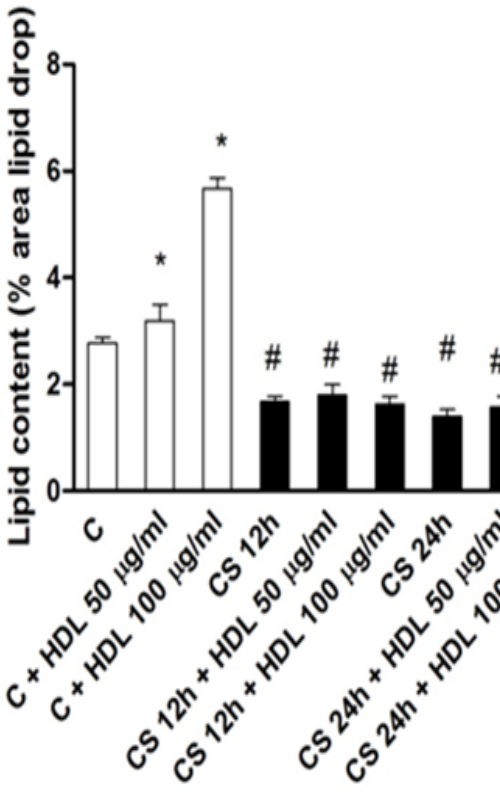
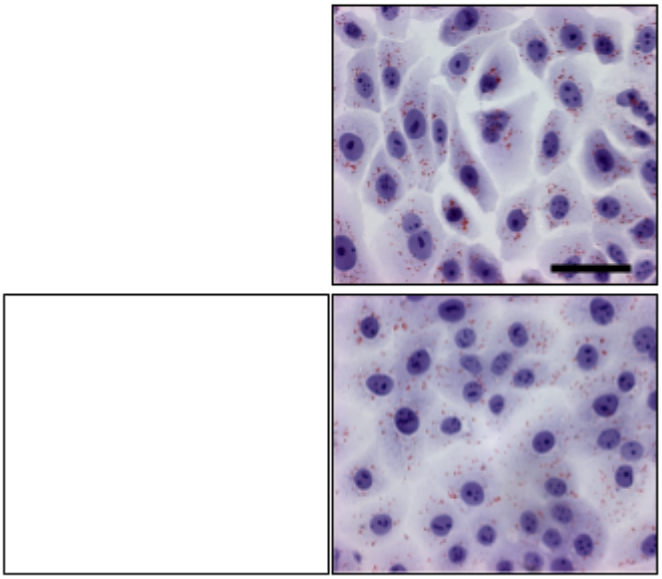
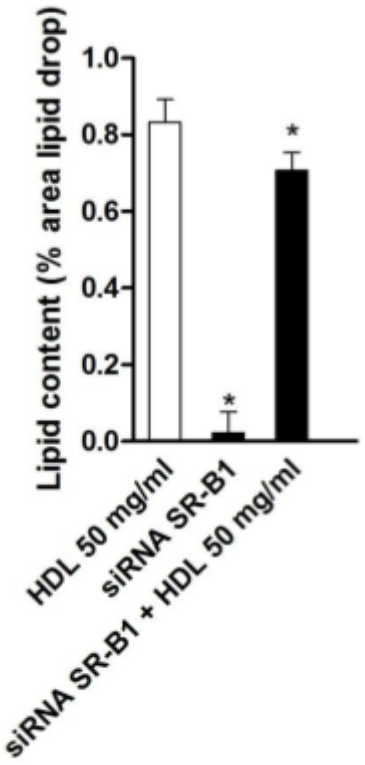

Lipid content (% area lipid drop)
0
2
4
6
8
*
*
#
#
#
#
C
C + HDL 50 µg/ml
C + HDL 100 µg/ml
CS 12h
CS 12h + HDL 50 µg/ml
CS 12h + HDL 100 µg/ml
CS 24h
CS 24h + HDL 50 µg/ml
CS 24h + HDL 100

Fig. 8




Lipid content (% area lipid drop)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
HDL 50 mg/ml
siRNA SR-B1
siRNA SR-B1 + HDL 50 mg/ml