Scavenger Receptor B1 involvement in COPD pathogenesis

Carlo Cervellati¹, Paolo Casolari², Alessandra Pecorelli³, Claudia Sticozzi⁴, Francesco Nucera⁵, Alberto Papi², Gaetano Caramori⁵*, Giuseppe Valacchi³,⁶,⁷*

¹Department of Translational Medicine and for Romagna, University of Ferrara, Via Luigi Borsari 46, Ferrara, 44121, Italy;
²Interdepartmental Study Center for Inflammatory and Smoke-Related Airway Diseases, Cardiorespiratory and Internal Medicine Section, University of Ferrara, Ferrara, Italy;
³Department of Environmental Sciences and Prevention, University of Ferrara, Via Luigi Borsari 46, Ferrara, 44121, Italy;
⁴Department of Life Sciences, University of Siena, Via Aldo Moro 2, 53100 Siena, Italy;
⁵Pneumologia, Dipartimento di Scienze Biomediche, Odontoiatriche e delle Immagini Morfologiche e Funzionali (BIOMORF), Università di Messina, Messina, Italy;
⁶Department of Animal Science, Plants for Human Health Institute, North Carolina State University, Kannapolis, NC 28081, USA;
⁷Department of Food and Nutrition, Kyung Hee University, Seoul 02447, Korea.

* Correspondence:
Giuseppe Valacchi: gvalacc@ncsu.edu
Gaetano Caramori: gaetano.caramori@unime.it

Running title: SRB1 and COPD
Abstract

Objective: Chronic obstructive pulmonary disease (COPD) is one of the main causes of morbidity and mortality in the United States. Oxidative stress due to cigarette smoking seems to be one of the major driving mechanisms in COPD pathogenesis. Since the scavenger receptor BI (SRB1) appears to play a key role in mediating the uptake for α-tocopherol and other antioxidants in lung tissue, we aimed to investigate its role in COPD pathogenesis.

Methods: Lung tissue from 12 subjects: 6 of these had a diagnosis of COPD in a stable clinical state, the others 6 were current (n=1) or ex-smokers (n=5) with normal lung function (controls). 4-hydroxynonenal (4HNE)-SRB1 Adducts were detected by immunoprecipitation. The concentration of α-tocopherol concentrations were determined by HLPC.

Results: SRB1 levels were lower in COPD patients and these results parallel with lower levels of vitamin E in lung tissue found in COPD patients. This effect can be the consequence of oxidative post-translational modifications, confirmed by the binding of the peroxidation product 4-hydroxynonenal (4HNE) to SRB1 possibly leading to its degradation.

Conclusions and significance: The loss of SRB1 may be involved in lung α-tocopherol content decrease with the consequence of making lung tissue more susceptible to oxidative damage as suggested by the SRB1-4HNE adducts formation, and more prone to COPD development. Thus, our findings suggest a novel role of SRB1 in pathomechanisms underlying COPD.
Keywords: Oxidative stress, cigarette smoke, 4-hydroxy-2-nonenal

Introduction

It is well established that cigarette smoking (CS) is a risk factor for many chronic diseases mainly affecting cardiovascular and respiratory systems (PDQ Screening and Prevention Editorial Board, 2002). At today it has been well demonstrated that CS is the main cause for developing COPD, although not all the smokers are affected by this pathology evidencing a possible genetic predisposition (Cho et al., 2022).

COPD is a major health issue and one of the three most common causes of death worldwide (Adeloye et al., 2022). The abnormal inflammatory response to chronic exposure to CS components is believed to be the key component of COPD pathogenesis (Caramori et al., 2016). However, inflammation is not the only pathogenic mechanism underlying COPD. Oxidative stress, have been also shown to play a primary role in COPD onset and clinical progression (NUCERA et al., 2022). This chronic inflammation of the lower airways is characterized by a progressive loss of lung parenchyma and an accelerated decline of organ function.

Cigarettes smoke contains over 4,700 compounds in gaseous and particulate states that are able to induce oxidative stress to cells and its toxic effect is mainly due to the presence of oxidants, including volatile electrophilic compounds such as α,β-unsaturated aldehydes (Szparaga et al., 2021). Among these, 4-hydroxy-2-nonenal (HNE), a lipid peroxidation product, is highly reactive and potentially toxic. These aldehydes, form covalent adducts with various proteins, thus affecting a variety of biochemical processes, including transcription factor activation, gene and protein expression, production of inflammatory cytokines and cell death (Sharma et al., 2022).

Clinical trials with antioxidants have not been able to definitely prove the ability of micronutrients to prevent tobacco related diseases such as COPD (Barnes, 2020). The reason behind these controversial results could depend from both, genetic background and from the personal ability to uptake micronutrients in the cells of the lung.

Two decades ago, Actonet al. (Acton et al., 1996) identified the Scavenger Receptor class B1 (SRB1) as a HDL receptor. This transmembrane protein mediates the selective uptake of HDL cholesteryl esters and facilitates the trafficking of these lipids in the tissues (Gillard et al., 2018). Additional functions of this receptor have been shown, such as its ability to uptake the lipophilic antioxidant α-tocopherol, which suggests an indirect role of the receptor in cell defensive mechanisms against oxidative stress challenges. More specifically, it has been observed...
that SRB1 KO mice had 64% less vitamin E levels in the lung (Mardones et al., 2002) and therefore being more susceptible to oxidative damage. In addition, SRB1 is very susceptible to oxidative damage, that induces its degradation via proteasome (Crivellari et al., 2017; Sticozzi et al., 2013).

Therefore, we have hypothesized that upon lung exposure to SHS and its attendant OS, a positive feedback loop is induced where the lipid soluble antioxidants consumption increases (to fight against oxidative stress) and SRB1 expression diminish as a consequence of oxidative damage. This combination results in reduced lung vitamin E contents, making the target tissue even more vulnerable to further insults.

These premises provide the rationale of the current study which addresses the hypothesis that the expression of SRB1 is down-regulated in COPD lung as effect of oxidative burden potentially caused by CS. We found that the level of SRB1 is significantly lower in COPD patients compared to control smokers with normal lung function, and this alteration maybe occur via oxidative post-translational modification.
Material and methods

2.1. Study approval

All patients were recruited from the Respiratory Diseases Clinic of the University Hospital of Ferrara (www.ospfe.it). The diagnosis of COPD was based on the GOLD (www.goldcopd.org) criteria [a compatible history and spirometry, a post bronchodilator forced expiratory volume in 1 second (FEV$_1$/forced vital capacity (FVC) ratio < 70%)].

Patients were included whether: 1) had pulmonary function test report within 3 days 2) with diagnosis stable COPD (for more than 2 weeks) 3) did not report any treatment with oxygen, antibiotics, glucocorticoids, and theophylline within the last 1 month. The exclusion criteria were: 1) treated with immunosuppressive drugs in the past month; 2) other airflow-limited diseases (3) with severe diseases; (4) with infectious diseases other than the respiratory system.

We obtained and studied peripheral lung tissue from 12 subjects: 6 of these had a diagnosis of COPD in a stable clinical state, the others 6 were current (n=1) or ex-smokers (n=5) with normal lung function (controls).

The study conformed to the Declaration of Helsinki and was approved by the ethics committees of the University Hospital of Ferrara, Italy; written informed consent was obtained from each participant, and nonneoplastic peripheral lung tissue sampling was performed during lung resection surgery for a suspected malignancy according to the guidelines of the local ethics committee.

2.2 Harvesting and preparation of lung samples.
Lung tissue preparation was performed as previously detailed (Valacchi et al., 2007). Briefly, lung tissue biopsies were homogenized at 4 °C in RIPA buffer (150 mM NaCl2, 50 mM Tris–HCl, pH 7.4, 1% NP-40, 1 mM EDTA, 1 mM EGTA, 0.1% sodium dodecyl sulfate, 5 mM dithiothreitol, 5 mM NaF, 1 mM phenylmethyl sulfonyl fluoride, 10 mg/ml leupeptin, 10 μg/ml aprotinin, 10 mg/ml iodoacetamide) (Merck KGaA, Darmstadt, Germany), incubated on ice for 1 h and separated by centrifugation at 15,000 rpm for 10 min. Supernatants were stored at −80 °C until further processing.

2.3 Western blot analysis

Total cell lysates were extracted in RIPA buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM EGTA, 0.1% SDS, 5 mM N-ethylmaleamide (Merck KGaA, Darmstadt, Germany), protease and phosphatase inhibitor cocktails (Merck KGaA, Darmstadt, Germany) as described before (Valacchi et al., 2007). Briefly, 60 μg of boiled proteins were loaded onto 10% sodium dodecyl sulphate–polyacrylamide electrophoresis gels. The gels were electro-blotted onto nitrocellulose membranes and then blocked for 1 h in 3% milk. Membranes were incubated overnight at 4 °C with the primary antibody SRB1 (Novus Biologicals, Inc., Littleton, CO, USA) and with horseradish peroxidase-conjugated secondary antibody (BioRad, Milan, Italy). The blots were stripped and re-probed with β-actin (Cell Signalling; Celbio, Milan, Italy) as the loading control. Images of the bands were digitized and the densitometry of the bands were performed using Image-J software.

2.4 Immunoprecipitation of SRB1 and detection of HNE adducts

The antibody for SRB1 (5 μg) (Thermo Fisher Scientific Inc., Waltham, MA USA) was pre-coupled to 50 μL of magnetic Dynabeads Protein G (Novex, Life Technologies). Excess antibody was washed by placing the tube on a DynaMag™ magnet and removing the supernatant. Then, cell protein extracts (500 μg) were incubated with the antibody-coated beads for 10 min at RT. After washing, the immunocomplexes were mixed with reducing sample buffer, boiled and analyzed by SDS/PAGE and immunoblotting with 4HNE antibody (Millipore Corporation, Billerica, MA, USA).
2.5 Quantification of α-tocopherol

α-tocopherol concentrations were determined by HLPC using a Waters Spherisorb ODS2 C-18 (4.6 × 100 mm, 3-Am particle size) column with electrochemical detection as described by Valacchi et al (Valacchi et al., 2000). Tissue AT was extracted following saponification with alcoholic potassium hydroxide in the presence of 1% ascorbic acid. AT was detected electrochemically using an oxidizing potential of 500 mV and quantitated by calculation from a standard curve of authentic AT standards.
Results

The main characteristics of the study subjects are shown in Table 1.

SRB1 has been shown to be involved in many regulatory functions, including the ability to indirectly protect from the oxidative stress-related damage caused by CS. Owing to this, we assessed the SRB1 protein levels in the peripheral lung tissue of both COPD patients and control smokers with normal lung function, using a specific SRB1 antibody able to recognize both, the mature (82 KDa) and the immature form (not glycosylated form, 66 KDa) of SRB1. As shown in Fig. 1, COPD patients have high levels of SRB1 immature form and very low level of the functional form (82 KDa).

Increased levels of peroxidation (Sticozzi et al., 2014) and oxidative stress (OS) (Prieux et al., 2020; Solak et al., 2005) have been widely related to the high levels of pro-oxidants contained in CS. In particular, we have previously shown that post-translational modification via formation to 4HNE protein adducts deeply affect SRB1, by increasing the rate subsequent ubiquitination and proteasome degradation, as we showed in previous studies (Ferrara et al., 2022; Sticozzi et al., 2013). For this reason, we evaluated the presence of this highly reactive aldehyde and the eventual formation of adducts with SRB1 in lung tissue from COPD patients and control subjects. As shown in Fig. 2 A, in COPD peripheral lung tissues there was a significant increase in 4HNE protein adducts (top panel) resulting in an increment of almost 50% (bottom panel). Immunoblotting assay (Fig. 2B upper panel) evidenced that the interaction between SRB1 and 4HNE was clearly stronger in COPD patients compared to the control smokers with normal lung function resulting in almost 2-fold increase (Fig.2B bottom panel)


Loss of SRB1 due to oxidative modification could affect vitamin E uptake in lung cells, being this receptor a key-player in this process (Valacchi et al., 2011). To address this hypothesis, we measured the levels of α-tocopherol in peripheral lung tissue of COPD and control smokers with normal lung function. As showed in Table 2, the levels of this lipophilic vitamin were significantly lower in COPD peripheral lung tissue respect to control smokers with normal lung function.

[insert Table 2]
Discussion

In the present study, we found for the first time that COPD patients have lower levels of mature (and biologically functional) SRB1 in the peripheral lung parenchyma compared to control smokers with normal lung function. The collected data suggest that this alteration may be the result of oxidative stress-mediated post-translational modification which, as previously shown in other experimental settings, may increase the rate of proteasomal degradation of this receptor.

We have decided to focus our attention to SRB1 in COPD for two may reasons: 1) evidence suggests that SRB1 may be involved in many physiological processes in lung, including some that are altered in COPD  2) SRB1 is highly vulnerable to oxidative challenges caused to CS, the main risk factor and pathogenic player of COPD (Valacchi et al., 2015).

SRB1 is mostly known and studied for its critical role in reverse cholesterol transport (RCT) and HDL homeostasis (Shen et al., 2018). The possible role of HDL in the development of COPD has been proposed, on the basis on the observed epidemiological association between the clinical severity of the disease and low levels of these lipoproteins (Valacchi et al., 2015; Vicol et al., 2022; Zafirova-Ivanovska et al., 2016). However, these data are mixed and, in any case, did not support a role of SRB1 in the etiology and pathogenesis of COPD, but rather on the frequent cardiovascular complications of the lung disease.

There are several mechanisms, besides the well-characterized chronic inflammation of the airways, that are involved in COPD onset and progression, and SRB1 may play a role in some of them. This multifunctional receptor is able to recognize a vast variety of ligands, including apoptotic cells, seemingly facilitating their disposal, and pathogens (Gillard et al., 2018) (10.1016/j.jacl.2018.04.001). Notably, the increase in apoptotic alveolar epithelial and endothelial cells in the lung tissue of COPD patients has been referred as a potential upstream event in COPD pathogenesis (Demedts et al., 2006). Moreover, lungs of patients affected by this disease are abnormally sensitive to respiratory virus and bacteria infections, which can greatly worsen the prognosis (D’Anna et al., 2021). In particular, preclinical evidence suggests that downregulation of SRB1 may facilitate the entry and the replication of virus. SRB1 may also be able to recognize bacteria and also to orchestrate neutrophilic
host defense response to inhaled noxious compounds, included those present in COPD patients (Gowdy et al., 2015).

This receptor may also be indirectly involved in the regulation of redox homeostasis in lung cells. Abnormal elevation in reactive oxygen species (ROS) has been well-documented in COPD and may occur as direct consequence of inhaled toxicants and/or as result of activation of leukocytes and epithelial cells (Barnes, 2022).

A functional SR-B1 seems to contribute to preserve the oxidative balance by, among others, mediating the uptake of α-tocopherol and carotenoids, from HDL and other lipoproteins. This vitamin seems to play an important protective role in human lung. Accordingly, large trials have shown that α-tocopherol supplements significantly decrease the risk of developing COPD and other chronic pulmonary diseases (Agler et al., 2011) and higher vitamin E intake prevents COPD development (Liu et al., 2023). Preclinical evidence points to direct link between SRB1 and intracellular levels of the potent lipophilic antioxidant. Indeed, it has been shown that in SR-B1-null mice a significant increase in levels of circulating α-tocopherol is accompanied by a concomitant reduction in several organs, including lung (Mardones et al., 2002). Owing the important contribution in the antioxidant defensive mechanism, the reduction in the levels of α-tocopherol observed in stable COPD lungs could result in their major vulnerability to oxidative challenge.

Decline in α-tocopherol could be a cause of the detected decrease of functional SRB1 in diseased lungs. Our hypothesis is that, the increase in oxidative stress (witnessed by the observed increase in 4-HNE protein adducts levels) may lead to the observed post-translational change related to the covalent binding with 4HNE. This highly reactive aldehyde tends to form covalent bounds with amino acids residues such as lysine, histidine and cysteine presents in the proteins (Pecorelli et al., 2016). The loss of SRB1 may be ascribed to this modification. Indeed, we have previously shown that this significantly accelerates proteosome-mediated degradation of the receptor in cultured cells following cigarette-smoking or ozone exposure (Sticozzi et al., 2012, 2018, 2020).
Declaration of interest
The authors declare no conflicts of interest

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Author contribution statement
Please include a statement concisely specifying the contribution of each co-author. Use author initials to indicate contributions, for example:
CC conceived the study and wrote the paper; GV conceived the study and reviewed the manuscript; AP and CS performed experiments and analyzed data; GC, conceived the original idea and reviewed the final draft; AP, supervised the project and reviewed the final draft; PC and FN, supervised the project, analyzed the data and reviewed the final draft
**Figure legends**

**Fig. 1** Peripheral lung parenchyma tissue samples of COPD patients showed low levels of mature SR-B1 compared to those of control smokers with normal lung function. Data are expressed as mean ± S.E.M. (\* P < 0.05). (A) Western blot (top panel) is a representative of 5 different patients B) SR-B1 bands quantification is shown in the right panel. Data are expressed in arbitrary units (averages of five different experiments, \* P < 0.05). β-Actin was used as loading control.

**Fig. 2:** COPD peripheral lung tissue showed high levels of HNE protein adducts (A) and the presence of HNE adducts on SR-B1 (B) respect to controls (A) Lung lysates were immunoblotted for HNE adducts. Showed is a representative Western blot of five experiments from five different patients. (B) Samples were immunoprecipitated with SRB1 Ab, and immunoblotted with anti-HNE. Western blot is representative of five independent experiments.
References


Figure 1. Protein levels of mature and immature forms of SRB1 in lungs from COPD and healthy controls.
Table 1. Characteristics of subjects for the study on peripheral lung parenchyma. Data expressed as mean ± SEM.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>Age</th>
<th>Sex</th>
<th>Smoking history</th>
<th>Pack-years</th>
<th>Chronic bronchitis</th>
<th>FEV₁</th>
<th>FEV₁/FVC %</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>F</td>
<td>Ex-smokers</td>
<td>Current smokers</td>
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<td></td>
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<tr>
<td>Control smokers</td>
<td>6</td>
<td>71.2±3.9</td>
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<td>2</td>
<td>5</td>
<td>1</td>
<td>35.8±5.4</td>
<td>0</td>
</tr>
<tr>
<td>COPD</td>
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<td>5</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>45.5±3.2</td>
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**Table 2:** COPD lungs showed lower levels of α-tocopherol respect to control smokers with normal lung function

<table>
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<tr>
<th></th>
<th>Control smokers with normal lung function</th>
<th>COPD</th>
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<tr>
<td>α-tocopherol (nmol/mg tissue)</td>
<td>70 ± 2.34</td>
<td>46 ± 4.56 (p&lt; 0.01)</td>
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Data expressed as mean ± SEM.