Carbocysteine regulates innate immune responses and senescence processes in cigarette smoke stimulated bronchial epithelial cells

Elisabetta Pace\textsuperscript{a,∗}, Maria Ferraro\textsuperscript{a}, Liboria Siena\textsuperscript{a}, Valeria Scafidi\textsuperscript{a}, Stefania Gerbino\textsuperscript{a}, Serena Di Vincenzo\textsuperscript{a,b}, Salvatore Gallina\textsuperscript{c}, Luigi Lanata\textsuperscript{d}, Mark Gjomarkaj\textsuperscript{a}

\textsuperscript{a} Institute of Biomedicine and Molecular Immunology, National Research Council, Via Ugo La Malfa, 153, 90146 Palermo, Italy
\textsuperscript{b} Scienze e Biotecnologie Mediche e Sperimentali-Pneumologia Sperimentale e Clinica-Università degli Studi, Palermo, Italy
\textsuperscript{c} Dipartimento di Biomedicina Sperimentale e Neuroscienze Cliniche- Sezione di Otorinolaringoiatria, Università degli Studi di Palermo, Palermo, Italy
\textsuperscript{d} Medical Affair, Domè SPA Milan, Milan, Italy

HIGHLIGHTS

- In bronchial epithelial cells smoke alters innate immune and senescence processes.
- Carbocysteine is an anti-oxidant drug.
- Carbocysteine may revert the inflammatory and senescence processes present in smokers.

ABSTRACT

Cigarette smoke represents the major risk factor for chronic obstructive pulmonary disease (COPD). Cigarette smoke extracts (CSE) alter TLR4 expression and activation in bronchial epithelial cells. Carbocysteine, an anti-oxidant and mucolytic agent, is effective in reducing the severity and the rate of exacerbations in COPD patients. The effects of carbocysteine on TLR4 expression and on the TLR4 activation downstream events are largely unknown. This study was aimed to explore whether carbocysteine, in a human bronchial epithelial cell line (16-HBE), counteracted some pro-inflammatory CSE-mediated effects. In particular, TLR4 expression, LPS binding, p21 (a senescence marker), IL-8 mRNA and release in CSE-stimulated 16-HBE as well as actin reorganization in neutrophils cultured with supernatants from bronchial epithelial cells which were stimulated with CSE and/or carbocysteine were assessed. TLR4 expression, LPS binding, and p21 expression were assessed by flow cytometry, IL-8 mRNA by Real Time PCR and IL-8 release by ELISA. Actin reorganization, a prerequisite for cell migration, was determined using Atto 488 phallloidin in neutrophils by flow cytometry and fluorescence microscopy.

CSE increased: (1) TLR4, LPS binding and p21 expression; (2) IL-8 mRNA and IL-8 release due to IL-1 stimulation; (3) neutrophil migration. Carbocysteine in CSE stimulated bronchial epithelial cells, reduced: (1) TLR4, LPS binding and p21; (2) IL-8 mRNA and IL-8 release due to IL-1 stimulation; (3) neutrophil chemotactic migration. In conclusion, the present study provides compelling evidences that carbocysteine may contribute to control the inflammatory and senescence processes present in smokers.

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Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by chronic inflammation and premature lung aging and is mainly caused by cigarette smoke exposure. Airway epithelium is emerging as a regulator of innate immune responses to a variety of insults including cigarette smoke (MacNee, 2011).

A key component of the innate immunity and of the innate defence mechanisms is represented by the Toll like receptor (TLR) family (Adem and Ulevitch, 2000). TLRs, predominantly expressed by monocytes/macrophages and neutrophils (Adem and Ulevitch, 2000), are also expressed by lung and bronchial epithelial cells (Sha et al., 2004). A recent hypothesis regarding COPD pathogenesis suggests as “step 1 of the disease” the activation of innate responses by injured tissue components (Cosio et al., 2009). Products derived from epithelial cell injury can act as ligands for TLR4 and TLR2, thus amplifying inflammatory responses

Abbreviations: CSE, cigarette smoke extracts; CARB, carbocysteine; TLR4, toll like receptor 4; IL-1, interleukin-1.

* Corresponding author. Tel.: +39 91 680 9148; fax: +39 91 680 9122.
E-mail address: pace@ibim.cnr.it (E. Pace).

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within the airways. In this regard, it has been demonstrated that upon cigarette smoke extracts (CSE) stimulation, airway epithelium is able to release increased concentrations of IL-8 and the prevalence of IL-8 may in turn sustain the influx of neutrophils into the airways thus triggering innate immune responses (Pace et al., 2008). Neutrophils represent the predominant cell type in COPD patients since neutrophil elastase (NE) promotes mucous cell metaplasia in chronic bronchitis and is actively involved in the protease/anti-protease imbalance, a phenomenon which leads to lung tissue destruction and emphysema (O’Donnell et al., 2006).

Oxidative stress induced by chronic smoke exposure is considered to be a crucial event in the COPD pathogenesis (Brusselle et al., 2011) also inducing cell senescence. Premature senescence of airway epithelial cells is characterized by p21 over-expression, by increased IL-8 expression (Amsellem et al., 2011), by impaired repair processes and by exacerbated inflammation after airway injury (Zhou et al., 2011).

Carbocysteine (CARB), an anti-oxidant and mucolytic agent, is effective in reducing the severity and the rate of exacerbations in COPD patients (Zheng et al., 2008). The clinical efficacy of carbocysteine seems to be more related to its anti-oxidant and anti-inflammatory effects than to its mucolytic activity (Rahman and MacNee, 2012).

Limited information is available on the effects of CARB on the innate responses as well as on the inflammatory and senescence processes in cigarette smoke stimulated bronchial epithelial cells. The present work was aimed to evaluate the effects of CARB on TLR4 expression/activation, IL-8 production/release, neutrophil chemo-attraction, p21 expression in bronchial epithelial cells stimulated with CSE.

**Materials and methods**

**Preparation of cigarette smoke extracts (CSE)**

Commercial cigarettes (Marlboro) were used in this study. Cigarette smoke solution was prepared as described previously (Su et al., 1998). Each cigarette was smoked for 5 min and two cigarettes were used per 50 ml of PBS to generate a CSE–PBS solution. The CSE solution was filtered through a 0.22 µm-pore filter to remove bacteria and large particles. The smoke solution was then adjusted to pH 7.4 and used within 30 min of preparation. This solution was considered to be 100% CSE and diluted to obtain the desired concentration in each experiment. The concentration of CSE was calculated spectrophotometrically measuring the OD at 254 nm and the concentration of CSE was 100 µg/ml.

**Stimulation of epithelial cell lines**

16-HBE, an immortalized normal bronchial epithelial cell line (Cozenz et al., 1992) was used in this study. 16HBE was cultured at density of 100,000 cells/cm² in 6 well polystyrene plates (BD Falcon, Franklin Lakes, New Jersey). 16-HBE was maintained in MEM (Gibco, BRL, Germany), supplemented with 10% fetal calf serum (Gibco) and 0.5% gentamicin (Gibco). Cells cultures were maintained in a humidified atmosphere of 5% CO₂ in air at 37 °C. Cell lines were cultured in the presence of CSE (Pace et al., 2008; Chiappara et al., 2011) and in the presence and in the absence of CARB (Dompè, Italy) for 18 h. CSE concentration and time of incubation were selected on the basis of previous findings (Pace et al., 2008). In some experiments cells were stimulated also with IL-1 beta (30 ng/ml).

**Carbocysteine (CARB) treatment**

CARB (10⁻⁶M) was added 1 h before CSE or CSE + IL-1 beta cell stimulation. The concentration and time of pre-incubation of CARB were selected on the basis of previous works (Caravaglia et al., 2008; Pace et al., 2013). At the end of stimulation, cells were collected for further evaluations. Three replicates were performed for each experiment.

**Cell necrosis and cell apoptosis of 16-HBE**

Cell apoptosis in the presence of the used CSE concentration (20%) was evaluated by staining with annexin V-fluorescein isothiocyanate and propidium iodide (PI) using a commercial kit (Bender MedSystem, Vienna, Austria) following the manufacturer’s directions. Cells were analysed using a FACScan Plus (Becton Dickinson, Mountain View, CA) analyzer equipped with an Argon ion laser (Innova 70 Coherent) and Consort 32 computer support. The PI and annexin V negative cells (viable cells) were present in the lower left quadrant; the PI positive cells (necrotic cells) were present in the upper left quadrant; the PI and annexin V double positive cells (late apoptotic cells) were present in the upper right quadrant and the single annexin V positive cells (early apoptotic cells) were present in the lower right quadrant.

**Expression of TLR4 or p21 in 16-HBE**

To evaluate the expression of TLR4 or p21, cells were fixed with PBS containing 4% paraformaldehyde for 20 min at room temperature. Fixed cells were washed twice in permeabilization buffer (PBS containing 1% PBS, 0.3% saponin, and 0.1% Na azide) for 5 min at 4 °C and incubated in the dark (30 min, 4 °C) with specific rabbit anti-human TLR4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The samples were then incubated with a Fluorescein Isothiocyanate (FITC) conjugated anti-rabbit IgG (Dako) or with mouse anti-human p21 antibody (sc-817; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by FITC-conjugated goat anti-mouse (DAKO) and then evaluated by flow-cytometry. Negative controls were performed using rabbit or mouse immunoglobulins negative control (Dako). Data are expressed as percentage of positive cells.

**Binding of LPS**

The binding of LPS was assessed using ALEXA fluor LPS. In particular, 16-HBE stimulated as described above, were incubated with ALEXA fluor LPS for 30’ and the binding of LPS was evaluated by flow-cytometry. Data are expressed as percentage of positive cells.

**cDNA expression array**

Total RNA was isolated from the cells using TRIzol reagent (Invitrogen, California, USA) and RNA purification was performed with RNaseasy Mini kit (Qiagen INC, CA, USA). Human Cytokine Gene Array, to analyze simultaneously 114 genes (SuperArray Inc.,Bethesda, MD, USA) was used to compare the gene expression profiles of the collected samples as previously described (Pace et al., 2012).

**Real time PCR analysis of IL-8 mRNA by 16-HBE**

Real time PCR was performed as previously described (Pace et al., 2006). The 16-HBE cells were stimulated with CSE and IL-1 (30 ng/ml) for 18 h and total cellular RNA extracted using TriZol reagent (Invitrogen), was reverse-transcribed to cDNA, using M-MLV-RT and oligo(dT)₁₂–₁₈ primer (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR of human IL-8 gene was carried out on ABI PRISM 7900 HT Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA) using specific FAM-labeled probe and primers (Applied Biosystems, TaqMan Assays on Demand). GAPDH gene expression was used as endogenous control for normalization. Relative quantitation of mRNA was carried out with comparative CT method.

**Measurement of IL-8**

The concentrations of IL-8 were determined with an enzyme-linked immunosorbsent assays (ELISA) (Quantikine; R&D Systems, Minneapolis, MN).

**Isolation of neutrophils**

Neutrophils were purified from peripheral blood of normal donors using dextran sedimentation as previously described (Pace et al., 2004). In particular, peripheral blood was centrifuged at 1200 rpm for 15 min, plasma was removed and a same volume of PBSX was added to samples. The diluted blood was mixed with dextran and left for 1 h at room temperature. The upper phase was collected and centrifuged at 1200 rpm for 15 min. After the lysis of red blood cells and after Ficoll–Hypaque (Phar-macia) gradient centrifugation the pellet containing the neutrophils was recovered.

**Determination of actin reorganization and immunofluorescence**

Actin reorganization was analysed by flow cytometry and by fluorescence microscopy. Neutrophils (5 x 10⁴/300 µl) were stimulated with supernatants from unstimulated and CSE stimulated bronchial epithelial cells for 10 min. Neutrophils were then washed with PBS 1X, fixed with PBS containing 4% paraformaldehyde and permeabilised with 0.1% Triton X-100 in PBS and thereafter incubated with FITC-Phalloidin (200 ng/ml) in the dark for 45 min at room temperature. The cells were washed in PBS and resuspended in PBS and analysed by flow cytometry with FACS Calibur (Becton Dickinson, Mountain View, CA) or by fluorescence microscope Axioskop 2 Zeiss microscope (Heidelberg, Germany).
Data are expressed as mean counts ± standard deviation. Kolmogorov–Smirnov Normality test was initially performed to assess whether parametric analyses of data could be performed. Comparison between different experimental conditions was evaluated by paired t test. \( p < 0.05 \) was accepted as statistically significant.

**Results**

Effects of CARB on TLR4 expression by bronchial epithelial cells

CSE 20% did not induce necrosis or apoptosis in bronchial epithelial cells [Fig. 1]. CSE 20% increased the expression of TLR4 in CSE stimulated bronchial epithelial cells and CARB was able to reduce this effect [Fig. 2].

Effects of CARB on LPS binding expression by bronchial epithelial cells

CSE increased the binding of LPS in CSE stimulated bronchial epithelial cells and CARB was able to reduce this effect [Fig. 3].

**Effects of CARB on pro-inflammatory cytokine profile by bronchial epithelial cells**

As showed in Fig. 4 a different expression profile of inflammatory cytokines in CSE and IL-1 beta + CSE stimulated cells was observed. In IL-1 beta and CSE stimulated cells, among the checked genes, in the two experiments performed, C3, CCL2 and IL-8 were the only cytokines whose expression was increased. CARB was able to reduce the observed increase. We decided to further study the effect on IL-8 since this cytokines plays an important role in neutrophil chemotaxis and since it is increased in smokers (Llinàs et al., 2011).

**Effects of CARB on IL-8 mRNA expression by bronchial epithelial cells**

Real time PCR experiments confirmed the effects of CARB on IL-8 mRNA expression in bronchial epithelial cells and the ability of CARB to reduce the increased expression of IL-8 mRNA upon
CSE and IL-1 beta stimulation (Fig. 5). CSE alone did not induce a relevant increase in IL-8 mRNA.

**Effects of CARB on IL-8 release by bronchial epithelial cells**

Bronchial epithelial cells treated with CSE or with IL-1 beta alone showed significantly increased IL-8 release as compared to controls. Exposure of the cells to CSE together with IL-1 beta resulted in an additive effect on enhancing the IL-8 release, which could be reduced by CARB (Fig. 6).

**Effects of CARB on actin reorganization in neutrophils**

Since CARB affected IL-8 expression and release and since IL-8 acts as chemoattractant for neutrophils, the effect on actin reorganization in neutrophils was assessed. CSE + IL-1 beta increased and CARB was able to reduce the actin reorganization in neutrophils (Fig. 7A–C).

**Effects of CARB on p21 expression by bronchial epithelial cells**

The effect of CARB on p21 was assessed. CSE increased the expression of p21 in CSE stimulated bronchial epithelial cells and CARB was able to reduce this effect (Fig. 8).

**Discussion**

Cigarette smoking is a major risk factor in COPD with chronic airway inflammation as a key feature. COPD is a glucocorticoid resistant condition characterized by altered innate immune responses, increased oxidative stress and airway neutrophilia (Brusselle et al., 2011; Jog et al., 2007).

Antioxidant agents, such as carbocysteine, scavenge free radicals and oxidants and reduce numbers of exacerbations per year in COPD patients (Zhou et al., 2011). In bronchial epithelial cells exposed to cigarette smoke, carbocysteine protects these cells reducing ROS production and increasing GSH and HO-1 (Pace et al., 2013). The present study extends the results on the antioxidant and on the cytoprotective role of carbocysteine from an our
previous study demonstrating for the first time that this drug exerts anti-inflammatory activities reducing TLR4 expression/activation, IL-8 expression/release, neutrophil chemotaxis and p21 expression in bronchial epithelial cells stimulated with CSE.

TLRs establish the inflammatory setting in response to infections or tissue damage and provides a low-grade activation of the innate immune system for day-to-day lung structure stability (Zhang et al., 2006). High grade activation of TLR signaling leading to increased production of cytokines and reactive oxidant contributes to experimental emphysema (Zhang et al., 2006). Data herein reported, confirm that CSE exposure increases TLR4 expression and the LPS binding by bronchial epithelial cells (Pace et al., 2008) leading to increased pro-inflammatory responses. Carbocysteine counteracts these CSE mediated effects reducing both TLR4 expression

Fig. 7. Effects of carbocysteine on actin reorganization in neutrophils. 16-HBE (n = 3) cells were cultured in the presence and in the absence of CSE (20%) and IL-1 beta (30 ng/ml) and of CARB (10^{-4} M) for 18 h. The supernatants were collected and used for stimulating neutrophils from normal donors. For assessing actin reorganization in unstimulated and activated neutrophils phalloidin expression was evaluated by flow cytometry (A)–(B) and by fluorescence microscopy (C). (A) Data are expressed as percentage of phalloidin positive cells ± SD. *p < 0.05. (B) Representative histogram plots are also shown.

Fig. 8. Effects of CARB on p21 expression by bronchial epithelial cells. 16-HBE (n = 3) cells were cultured in the presence and in the absence of CSE (20%) and of CARB (10^{-4} M) for 18 h and then were used for assessing p21 expression by flow cytometry (see Materials and methods for details). (A) Data are expressed as percentage of p21 positive cells ± SD. *p < 0.05. (B) Representative histogram plots are also shown.
and LPS binding. TLR4 stimulation is an event critically involved in IL-8 production. CSE pre-treatment followed by TLR4 agonist stimulation increased IL-8 release from primary epithelial cells of smokers (Comer et al., 2013) and CSE-induced IL-8 production was inhibited by an antibody against TLR4 (Mortaz et al., 2011). IL-8 mRNA levels increased upon CSE exposure in a concentration- and time-dependent manner, and such an effect was accompanied by IL-8 secretion (Moretto et al., 2012). In a previous study from Wyatt et al. (1999) it was demonstrated that CSE 5% after 2 h of incubation may induce IL-8 release (Wyatt et al., 1999). In the present study CSE after 18 h of incubation did not induce IL-8 mRNA but induced IL-8 release. It is conceivable that after 18 h of incubation while the increase in protein persists the effect on the m-RNA is not visible any more.

Furthermore, inflammasome activation due to TLR4 induces the release of IL-8 and IL-1 beta (Mortaz et al., 2011). Since CSE modifies both TLR4 expression and activation it is conceivable that these events, inducing inflammasome activation, further increase the IL-1 beta release and in turn IL-8 release. In the present study, IL-1 beta dramatically increased the expression of IL-8 mRNA in CSE-stimulated bronchial epithelial cells and carbocysteine is able to reduce this increase. These data confirm and extend previous data on another anti-oxidant, n-acetylcysteine that inhibits the TNF-alpha/IL-1beta-stimulated IL-8 release from a different airway cell line (Radomska-Leśniewska et al., 2006).

Carbocysteine could reduce IL-8 mRNA expression and release increasing Nrf2 activity. In this regards it has been demonstrated that: (1) the regulatory region of IL-8 gene contains binding sites for Nrf2 (Gruber et al., 2010); (2) carbocysteine increases Nrf2 nuclear expression in CSE-stimulated bronchial epithelial cells (Pace et al., 2013); (3) sulforaphane activating Nrf2 pathways inhibits CSE-induced IL-8 release (Starrett and Blake, 2011).

IL-8 is an important neutrophil chemoattractant known to be elevated in the airways of cigarette smokers and in patients with COPD (Linnäs et al., 2011). The increased neutrophil chemotactic activity upon CSE stimulation may be also due to other molecules. In this regards in the present study we observed an increased expression of C3 and CCL2, molecules contributing to neutrophil accumulation.

Neutrophils are cells crucially involved in COPD pathogenesis and symptoms. In this regard, a recent study has been demonstrated that cough frequency was related to current cigarette consumption and percentage of sputum neutrophils (Sumner et al., 2013).

Cell migration and phagocytosis ensue from extracellular-initiated signaling cascades that orchestrate dynamic reorganization of the actin cytoskeleton. Filamentous actin (F-actin) provides tracks on which intracellular organelles move using molecular motors. Polymerization of actin cytoskeleton is an essential element for neutrophil motility, with F-actin formation at the leading edge guiding cell locomotion (Karlsson et al., 2012). The formation of F-actin in the whole cytoplasm could be identified using phalloidin. Neutrophils are largely resistant to glucocorticoid activity. In this regard it has been demonstrated that array neutrophils have a low expression of glucocorticoid receptor (Plumb et al., 2012). Pre-treatment with the antioxidant n-acetylcysteine effectively attenuated neutrophil chemotaxis and elastase release (Milara et al., 2012).

Herein, CSE increase phalloidin expression while carbocysteine counteracts this effect, suggesting the utility of this drug also in limiting the accumulation of neutrophils within the airways.

The accumulation of neutrophils within the airways of smokers with or without COPD can be due to increased cell recruitment (Bildberg et al., 2012) in addition to reduced cell apoptosis (Pace et al., 2011; Zhang et al., 2012). This reduced neutrophil apoptosis may be due, as recently demonstrated, to the direct effect of cigarette smoke in blocking Akt deactivation (Xu et al., 2013) or in increasing the expression of TLR4 (Pace et al., 2011) in neutrophils. Future experiments could clarify the effects of carbocysteine in neutrophil apoptosis.

Furthermore, cigarette smoke, by inducing oxidative stress, alters the activity of proteins associated with cell proliferation and cell apoptosis, resulting in reduced reparative mechanisms and accelerated epithelial cell senescence in the lung. Phenomena implicated in COPD pathogenesis. Accordingly, the incidence of COPD increases with age thus further supporting the concept that chronic inflammation and cellular senescence are intertwined in the pathogenesis of premature aging (MacNee, 2011). When epithelial cells are chronically exposed to cigarette smoke they differentiate in a functional senescent phenotype with a reduced reparative potential and with higher pro-inflammatory properties. Secretion of IL-8 and p21 over-expression are both characteristics of a senescence-associated phenotype (Fujii et al., 2012). p21 (WAF1/CIP1) is highly responsive to oxidative stress and may play a role in the cellular processes involving the imbalance of cell proliferation/apoptosis (Tomita et al., 2002). It has been previously demonstrated that alveolar macrophages and bronchial epithelial cells from smokers have reduced cell death as well as increased cytoplasmic p21 expression (Steinman et al., 1994). In bronchial airways of current smokers with and without COPD, cigarette smoke alters the expression and sub-cellular distribution of p21 (Chiappara et al., 2013). This event, in turn, may play an important role in the imbalance between apoptosis, proliferation, and differentiation of bronchial epithelial cells leading to squamous metaplasia, an alteration associated with increased airway obstruction of COPD and with increased risk of lung cancer in smokers (Chiappara et al., 2013). CSE increase and carbocysteine is able to reduce the expression of p21 in CSE stimulated bronchial epithelial cells supporting the concept that this drug may also be useful to limit the senescence induced by cigarette smoke exposure. It is conceivable that the increased expression of p21 due to cigarette smoke exposure is due to TLR4 activation. In this regard it has been demonstrated that gial cells upon stimulation with LPS, the agonist of TLR4, upregulate p21 expression (Tusell et al., 2009).

In conclusions, taken together the data herein presented support a new role of carbocysteine as anti-inflammatory and anti-senescence drug in smokers. In vivo studies further exploring the molecular mechanisms involved in anti-inflammatory anti-senescence effects of carbocysteine in smokers are needed.

Conflict of interest statement

Although E. Pace received research funds from Dompè and Luigi Lanata is employed by Dompè, these relationships did not influence authors’ objectivity. For the other authors no conflict of interest exits.

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