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Oxidative stress and innate immunity responses in cigarette smoke stimulated nasal epithelial cells

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ABSTRACT

Cigarette smoke extracts (CSE) may play a significant role in diseases of the upper airway including chronic rhinosinusitis. Even short term exposure of cigarette smoke has adverse effects on mitochondrial functions and redox homeostasis in tissues which may progress to further complications associated with chronic smoking. Cigarette smoke alters toll-like receptor 4 (TLR4) expression and activation in bronchial epithelial cells. Carbocysteine is an anti-oxidant and mucolytic agent. The effects of carbocysteine on CSE induced oxidative stress and on associated innate immune and inflammatory responses in nasal epithelial cells are largely unknown. The present study was aimed to assess in CSE stimulated nasal epithelial cells (RPMI 2650) the effects of carbocysteine (10^{-4} M) on: cell survival, intracellular reactive oxygen species (ROS) production, TLR4 expression, LPS binding and neutrophil chemotaxis (actin reorganization). We found that CSE increased ROS production, TLR4 expression, LPS binding and neutrophil chemotaxis and all these events were counteracted by pre-incubating CSE stimulated RPMI 2650 cells with carbocysteine. In conclusion, the present study provides compelling evidence that carbocysteine may be considered a promising therapeutic strategy in chronic inflammatory nasal diseases.

1. Introduction

The "field of injury" hypothesis proposes that the entire respiratory system of an individual reacts to environmental insults such as tobacco smoke in a similar manner (Gower et al., 2011).

The expression of several detoxification genes was commonly altered by smoking in nasal, oral and bronchial compartment supporting the presence of a relationship between gene expression in extra- and intrathoracic airway epithelial cells (Sridhar et al., 2008). This relationship may be useful to set up a non-invasive biomarker for tobacco exposure in nasal compartment to be used as a non-invasive screening or as a diagnostic tool for those subjects with high susceptibility to smoking-induced lung diseases (Sridhar et al., 2008). Airway epithelium including nasal epithelium is emerging as a regulator of innate immune responses to a variety of insults including cigarette smoke (Rackley and Stripp, 2012).

A key component of the innate immunity and of the innate defence mechanisms is represented by the toll like receptor (TLR) family (Pace et al., 2008). In this regard, it has been demonstrated that cigarette smoke is able to increase TLR4 expression and the release of IL-8 and neutrophil chemotaxic activity in bronchial epithelial cells (Pace et al., 2008) thus amplifying the inflammatory and innate immune responses.

Carbocysteine (CARB), an anti-oxidant and mucolytic agent, is effective in reducing the severity of symptoms in COPD patients (Zheng et al., 2008). The clinical efficacy of carbocysteine seems to be more linked to its anti-oxidant and anti-inflammatory effects than to its mucolytic activity (Rahman and MacNee, 2012). In bronchial epithelial cells stimulated with CSE, carbocysteine reduces ROS production, increases cytoprotective events including GSH and HO-1 expression and counteracts the effects of CSE in reducing HDAC2 activity thus providing new mechanisms that are not shared with steroids, whose activity is impaired in models with high oxidative stress (Pace et al., 2013). Limited information is available on the effects of carbocysteine in innate immunity response of nasal cellular models with elevated oxidative stress due to cigarette smoke exposure.

Therefore, we sought to understand whether, in nasal epithelial cells stimulated with cigarette smoke extracts (CSE), CARB was

Abbreviations: CSE, cigarette smoke extracts; CARB, carbocysteine; ROS, reactive oxygen species; TLR4, toll like receptor 4; LPS, lipolysaccharide.

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effective in controlling oxidative stress and in modulating innate immunity responses.

2. Materials and methods

2.1. Reagents and antibodies

Carbocysteine was provided by Dompè (Milan, Italy). Annexin V binging test was purchased from Bender MedSystem (Vienna, Austria). Non-fluorescent dichlorofluorescein diacetate (DCFH-DA) and FITC- Phalloidin were purchased from Sigma Aldrich (St Louis, MO). PE anti-human TLR4 antibody was purchased from eBioscience (Santa Cruz, CA). Negative controls were from DAKO (Glostrup, Denmark). ALEXA fluor 488-labeled LPS from Molecular Probes (Eugene, Oreg).

2.2. Preparation of cigarette smoke extracts (CSE)

Commercial cigarettes (Marlboro Red label) were used in this study. Cigarette smoke solution was prepared as described previously (Su et al., 1998). Each cigarette was smoked through a vacuum flask for 5 min and one cigarette was used per 25 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 experiments. The concentration of CSE was calculated spectrophotometrically measuring, as previously described (Luppi et al., 2005), the OD of the 100-fold diluted solution at a wavelength (320 nm) at which the maximal absorbance was detected. The pattern of absorbance, among different batches, showed very little differences and the mean OD of the different batches was 1.37 ± 0.16. The presence of contaminating LPS on undiluted CSE was assessed by a commercially available kit (Cambrex Corporation, East Rutherfort, New Jersey, USA) and was below the detection limit of 0.1 EU/ml.

2.3. Culture of nasal epithelial cell lines

RPMI 2650 cell line (ATCC-CCL-30), purchased from American Type Culture Collection (ATCC; Rockville, Md) was used in this study as a model of nasal epithelial cells as previously described (Pace et al., 2012a; Salib et al., 2005).

Cells were cultured in complete culture medium (MEM minimum essential media containing 10% FCS , L-glutamin 2 mM, gentamicin 50 mg/ml, MEM NEAA 0.5%, sodium pyruvate 1nM, HEPES 1%) (Gibco, BRL, Germany). Cell cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C.

Cell lines were cultured in the presence of CSE (5%, 10%) and in the presence and in the absence of CARB (10^{-4} M, 10^{-3} M) Garavaglia et al., 2008; Pace et al., 2013. Two different time points (4 and 18 h) were tested in preliminary ROS expression experiments. At the end of stimulation, cells were collected for further evaluations. Two replicates of each independent experiment were performed and analyzed.

2.4. Cell necrosis and cell apoptosis

Necrosis and apoptosis of cells exposed to CSE (5%, 10%) and to CARB were 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 analyzed using a FACStar Plus (Becton Dickinson, Mountain View, CA) analyzer equipped with an Argon ion Laser (Innova 70 Coherent) and Consort 32 computer support. The PI negative and annexin V negative cells (i.e. viable cells) were present in the lower left quadrant; the PI cells (i.e. necrotic cells) were present in the upper left quadrant; the PI and annexin V double positive cells (i.e. late apoptotic cells) were present in the upper right quadrant and the single annexin V positive cells (i.e. early apoptotic cells) were present in the lower right quadrant.

2.5. Analysis of intracellular reactive oxygen species (ROS)

Intracellular ROS were measured by the conversion of the DCFH-DA in a highly fluorescent compound, DCF, by monitoring the cellular esterase activity in the presence of peroxides as previously described (Bruno et al., 2011). The ROS generation was assessed by uptake of 1 μM DCFH-DA, incubation for 10 min at room temperature in the dark, followed by flow cytometric analysis.

2.6. Expression of TLR4 in RPMI 2650

To evaluate the expression of TLR4, 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% FBS, 0.3% saponin, and 0.1% Na azide) for 5 min at 4 °C, incubated in the dark (30 min, 4 °C) with specific PE anti-human TLR4 antibody and then evaluated by flow-cytometry. Negative controls were performed using mouse PE immunoglobulins negative control (Dako). Percentages of positive cells were determined from forward (FS) and sideways (SS) scatter patterns, after gating on the cells, excluding debris. Non specific binding and background fluorescence were quantified by analyzing negative control.

2.7. Binding of LPS

The binding of LPS was assessed using ALEXA fluor LPS as previously described (Pace et al., 2008). In particular, RPMI 2650, 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.

2.8. Isolation of neutrophils

Neutrophils were purified from peripheral blood of normal donors using dextran sedimentation as previously described (Pace et al., 2004).

2.9. Determination of actin reorganization and immunofluorescence

Actin reorganization was analyzed by flow cytometry and by fluorescence microscopy. Neutrophils (5×10^5/300 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 permeabilized 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, resuspended in PBS and analyzed by flow cytometry with FACS Calibur (Becton Dickinson, Mountain View, CA) or by fluorescence microscope Axioskop 2 Zeiss microscope (Heidelberg, Germany).

2.10. Statistics

Data are expressed as mean counts ± standard deviation. Kolmorogov–Smirnov Normality test was initially performed to assess whether parametric analyses of data could have been performed. Comparison between different experimental conditions