

# Comparative cytoprotective effects of carbocysteine and fluticasone propionate in cigarette smoke extract-stimulated bronchial epithelial cells

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Received: 20 January 2013 / Revised: 25 March 2013 / Accepted: 27 March 2013 / Published online: 12 April 2013  
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**Abstract** Cigarette smoke extracts (CSE) induce oxidative stress, an important feature in chronic obstructive pulmonary disease (COPD), and oxidative stress contributes to the poor clinical efficacy of corticosteroids in COPD patients. Carbocysteine, an antioxidant and mucolytic agent, is effective in reducing the severity and the rate of exacerbations in COPD patients. The effects of carbocysteine on CSE-induced oxidative stress in bronchial epithelial cells as well as the comparison of these antioxidant effects of carbocysteine with those of fluticasone propionate are unknown. The present study was aimed to assess the effects of carbocysteine ( $10^{-4}$  M) in cell survival and intracellular reactive oxygen

species (ROS) production (by flow cytometry) as well as total glutathione (GSH), heme oxygenase-1 (HO-1), nuclear-related factor 2 (Nrf2) expression and histone deacetylase 2 (HDAC-2) expression/activation in CSE-stimulated bronchial epithelial cells (16-HBE) and to compare these effects with those of fluticasone propionate ( $10^{-8}$  M). CSE, carbocysteine or fluticasone propionate did not induce cell necrosis (propidium positive cells) or cell apoptosis (annexin V-positive/propidium-negative cells) in 16-HBE. CSE increased ROS production, nuclear Nrf2 and HO-1 in 16-HBE. Fluticasone propionate did not modify intracellular ROS production, GSH and HDCA-2 but reduced Nrf2 and HO-1 in CSE-stimulated 16-HBE. Carbocysteine reduced ROS production and increased GSH, HO-1, Nrf2 and HDAC-2 nuclear expression/activity in CSE-stimulated cells and was more effective than fluticasone propionate in modulating the CSE-mediated effects. In conclusion, the present study provides compelling evidences that the use of carbocysteine may be considered a promising strategy in diseases associated with corticosteroid resistance.

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**Keywords** Cigarette smoke · Airway epithelial cells · Reactive oxygen species

## Abbreviations

CSE	Cigarette smoke extracts
CARB	Carbocysteine
FP	Fluticasone propionate
ROS	Reactive oxygen species
GSH	Glutathione
HO-1	Heme oxygenase-1
Nrf2	Nuclear-related factor 2
HDAC-2	Histone deacetylase 2

## Introduction

The airway epithelium is emerging as a regulator of innate immune responses to a variety of insults including cigarette smoke. In this regard, it has been demonstrated that upon cigarette smoke extracts (CSE) stimulation, the airway epithelium is able to release increased concentrations of IL-8, but reduced IFN-gamma-inducible protein 10 (Pace et al. 2008a), thus amplifying the inflammatory and immune responses.

Chronic obstructive pulmonary disease (COPD) is mainly caused by cigarette smoke exposure, and oxidative stress induced by chronic smoke exposure is considered to be a crucial event in the COPD pathogenesis (Brusselle et al. 2011). The inflammatory events present in COPD are largely resistant to corticosteroids (Adcock et al. 2010). Oxidative stress contributes to the low response to corticosteroids through the downregulation of histone deacetylase (HDAC) activity, a crucial event in the trans-repression activities of corticosteroids (Barnes and Adcock 2009; Malhotra et al. 2011; Barnes 2011). HDACs, deacetylating histones which bind to inflammatory gene promoters, limit the access of the transcriptional machinery to these genes, thus repressing their transcription (Barnes 2011).

Carbocysteine (CARB), an antioxidant 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 antioxidant and anti-inflammatory effects than to its mucolytic activity (Rahman and Macnee 2012). Limited information is available on the comparative effects between carbocysteine and a potent corticosteroid, fluticasone, in cellular models with elevated oxidative stress due to cigarette smoke exposure.

The present work was aimed to understand whether, in bronchial epithelial cells stimulated with CSE, CARB was similar or superior to fluticasone propionate (FP) in controlling oxidative stress and antioxidant responses and whether it was able to modulate histone deacetylase 2 (HDAC-2) expression and activity.

## Materials and methods

### Preparation of cigarette smoke extracts

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 20 ml of PBS to generate a CSE-PBS solution. The CSE solution was filtered through a 0.22- $\mu$ m 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 as previously described (Luppi et al. 2005) at the wavelength of 320 nm. The pattern of absorbance, among different batches, showed very little differences, and the mean OD of the different batches was  $1.37 \pm 0.16$ . The presence of contaminating LPS on undiluted CSE was assessed by a commercially available kit (Cambrex Corporation, East Rutherford, NJ) and was below the detection limit of 0.1 EU/ml.

### Stimulation of epithelial cell lines

16-HBE, an immortalised normal bronchial epithelial cell line (Cozens et al. 1992), RPMI 2650a, a nasal epithelial cell line (Pace et al. 2012a), and a mucin-producing human NCI-H292 airway epithelial cell line (Nogawa et al. 2009) were used in this study.

16-HBE was maintained in MEM (Gibco, BRL, Germany) supplemented with 10 % foetal calf serum (Gibco) and 0.5 % gentamicin (Gibco). Cell cultures were maintained in a humidified atmosphere of 5 % CO<sub>2</sub> in air at 37 °C. Cell lines were cultured as previously reported in the presence of CSE (5 and 10 %; Pace et al. 2008a) and in the presence and absence of FP ( $10^{-4}$ ,  $10^{-7}$ ,  $10^{-8}$  and  $10^{-9}$  M; Sigma-Aldrich, St. Louis, MO) and CARB ( $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  and  $10^{-8}$  M; Dompè, Italy; Garavaglia et al. 2008). Three time points were initially tested: 2, 4 and 18 h. CARB was added 1 h before CSE cell stimulation. RPMI 2650 and H292 were cultured for 18 h in the presence of CSE (10 %) and in the presence and absence of FP ( $10^{-8}$  M; Sigma-Aldrich) and of CARB ( $10^{-4}$  M), respectively. At the end of stimulation, cells were collected for further evaluations. Three replicates were performed for each experiment.

### Cell necrosis and cell apoptosis

Cell necrosis and cell apoptosis were evaluated, as previously described (Pace et al. 2007), 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 FACS Calibur (Becton Dickinson, Mountain View, CA) analyser 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.

### Analysis of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) were measured by the conversion of the non-fluorescent dichlorofluorescein diacetate (DCFH-DA; Sigma) in a highly fluorescent compound, DCF, by monitoring the cellular esterase activity in the presence of peroxides, as previously described (Bruno et al. 2011). ROS generation was assessed by the uptake of 1  $\mu$ M DCFH-DA, incubation for 10 min at room temperature in the dark, followed by flow cytometric analysis.

### Measurement of cellular glutathione content

Intracellular total glutathione (GSH) content was assessed in cell extracts as previously reported (Rahman et al. 2006). Briefly, cell extracts were prepared in 0.1 M potassium phosphate extraction buffer containing 0.6 % (w/v) sulfosalicylic acid, 0.1 % (v/v) Triton X-100 and 5 mM EDTA. After harvesting and resuspension in the extraction buffer, cells were sonicated in ice-cold water and underwent two cycles of freezing and thawing. Supernatants/extracts were collected by centrifugation and used for the following colorimetric assay: 10  $\mu$ l of the extracts was incubated in the presence of 60  $\mu$ l 0.6 mg/ml 5,5'-dithiobis(2-nitrobenzoic acid) and of 60  $\mu$ l of 250 U/ml glutathione reductase for 30 s at RT; 50  $\mu$ l of 0.6 mg/ml  $\beta$ -NADPH was added and 2-nitro-5-thiobenzoic acid formation was immediately evaluated by measuring the absorbance at 412 nm in a microplate reader. The concentration of GSH in the cell extracts was calculated using a standard curve, normalised for the total protein content and expressed as nanomoles per milligram protein.

### Western blot

The expression of heme oxygenase-1 (HO-1), nuclear-related factor 2 (Nrf2) and HDAC-2 was evaluated by western blot analysis as previously described (Pace et al. 2012b). The concentration of proteins in cell lysates was measured using the Bradford assay. The following primary antibodies were used: rabbit polyclonal anti-human HO-1 (Assay Design, SPA-896, Anna Arbor, MI), rabbit polyclonal anti-human Nrf2 and mouse anti-human HDAC-2 (sc-81599, Santa Cruz) and rabbit anti- $\beta$ -actin (Sigma). To study Nrf2 and HDAC-2 nuclear translocation, the protein extracts were treated to separate the cytoplasmic and nuclear protein fractions as previously described (Pace et al. 2008b) by using a commercial kit following the manufacturer's directions (Pierce, Rockford, IL). Secondary anti-rabbit and anti-mouse antibodies were purchased from Sigma. Revelation was performed with an enhanced chemiluminescence system (GE Healthcare, Chalfont St. Giles, UK) followed by autoradiography. Beta-actin (Sigma) was used

as the housekeeping protein to normalise differences in protein loading.

### Immunoprecipitation and HDAC-2 activity

HDAC-2 activity was assessed as previously described (Malhotra et al. 2011). HDAC2 was immunoprecipitated from cell lysates (100  $\mu$ g) by overnight incubation with anti-HDAC-2 at 4 °C and then incubated with protein G-agarose beads.

Beads were extensively washed with lysis buffer and resuspended in reducing sample buffer for western blot analysis. HDAC-2 activity was measured using a commercial kit (Enzo Lifesciences, Farmingdale, NY, USA). An acetylated lysine substrate is incubated with samples with HDAC activity. Deacetylation sensitises the substrate such that treatment with the HDAC developer in the second step releases a fluorescent product. The assay was performed exactly as recommended by the manufacturer. Fluorescence was measured in a microplate reader using Ex 355 and Em 460.

### Statistics

Data are expressed as the mean counts  $\pm$  standard deviation. Comparison between different experimental conditions was evaluated using paired *t* test. A value of  $P < 0.05$  was accepted as statistically significant.

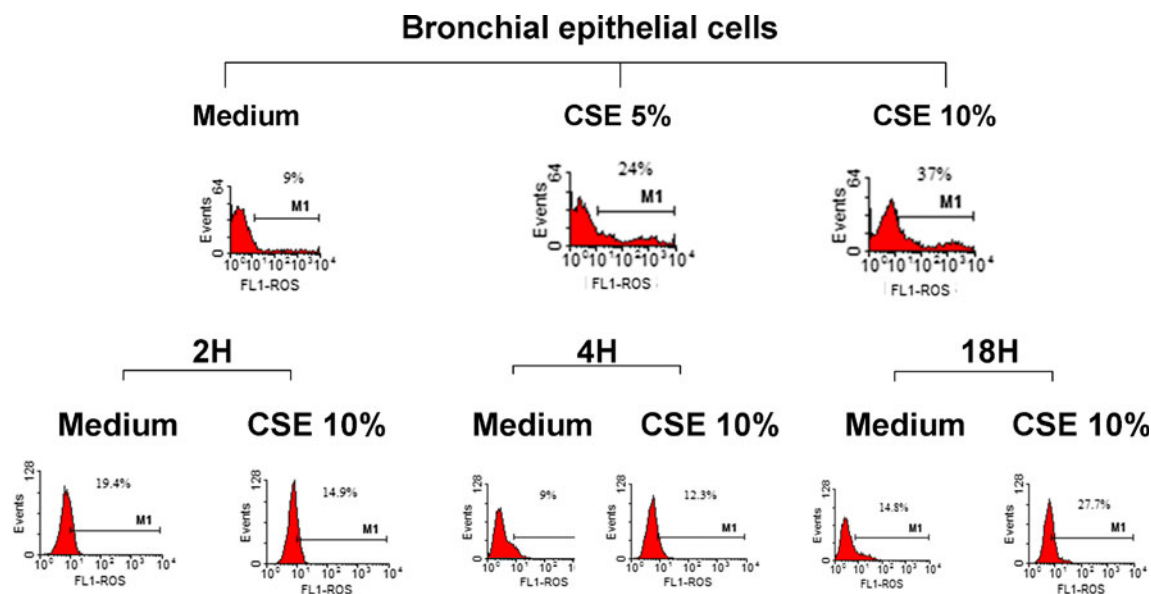
## Results

### Effects of CSE on ROS production in bronchial epithelial cells

The sources of the increased oxidative stress in COPD patients derive from the increased burden of inhaled oxidants such as cigarette smoke and from the increase in ROS generated by several inflammatory, immune and structural airways cells (Faux et al. 2009). We tested the effect of CSE on ROS production in bronchial epithelial cells at three different time points (2, 4 and 18 h) to test whether a short- or a long-term incubation was suitable for evaluating ROS formation. The highest ROS production was observed with CSE 10 % and at 18 h (Fig. 1). We then selected CSE 10 % and 18 h of incubation for assessing ROS in the presence of CARB and FP.

### Effects of FP and CARB on ROS formation in bronchial epithelial cells

We next tested the effects of FP and carbocysteine on ROS formation in bronchial epithelial cells. In a preliminary dose-response experiment, three different concentrations of CARB ( $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  and  $10^{-8}$  M) and of FP ( $10^{-4}$ ,

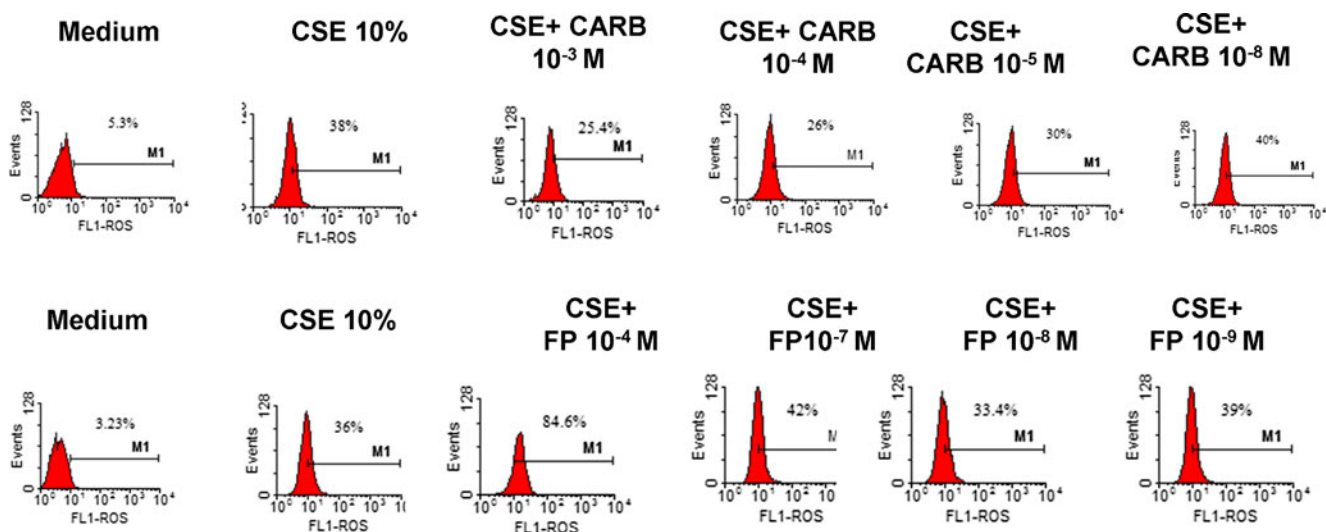


**Fig. 1** Effects of CSE in ROS production in bronchial epithelial cells. 16-HBE cells were cultured in the presence and absence of CSE (5 and 10 %) for 2, 4 and 18 h and then were used for assessing ROS

production using flow cytometry (see “Materials and methods” for details). Representative histogram plots from two experiments are shown

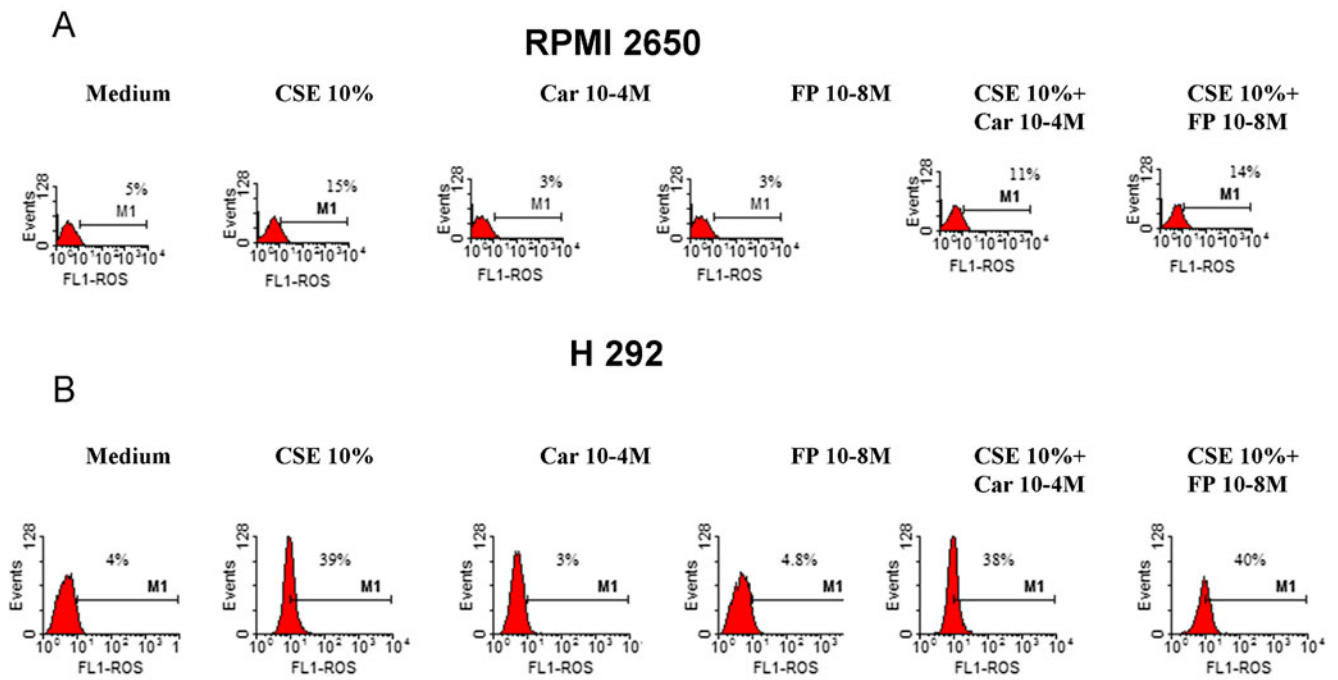
$10^{-7}$ ,  $10^{-8}$  and  $10^{-9}$  M) were tested. Since the obtained results did not show any relevant difference between CARB concentrations of  $10^{-3}$  and  $10^{-4}$  M (Fig. 2), the lower concentration of CARB of  $10^{-4}$  M was selected. At all the tested concentrations, FP was unable to limit ROS production. On the contrary, the concentration of FP of  $10^{-4}$  M induced relevant ROS production. The concentration of FP of  $10^{-8}$  M was selected because it was associated with the lowest ROS production. The effect of CARB and FP on ROS

formation in a nasal epithelial cell line (RPMI 2650) and in another airway epithelial cell line (H292) was also tested. The effect of CARB ( $10^{-4}$  M, for 18 h) on ROS formation in RPMI 2650 and in H292 was modest (Fig. 3a, b), and we decided to continue the study on the bronchial epithelial cell line because it was more sensitive to CARB effects. In bronchial epithelial cells, CARB significantly reduced CSE-induced ROS production, and this effect was significantly higher than the effect exerted by FP (Fig. 4).



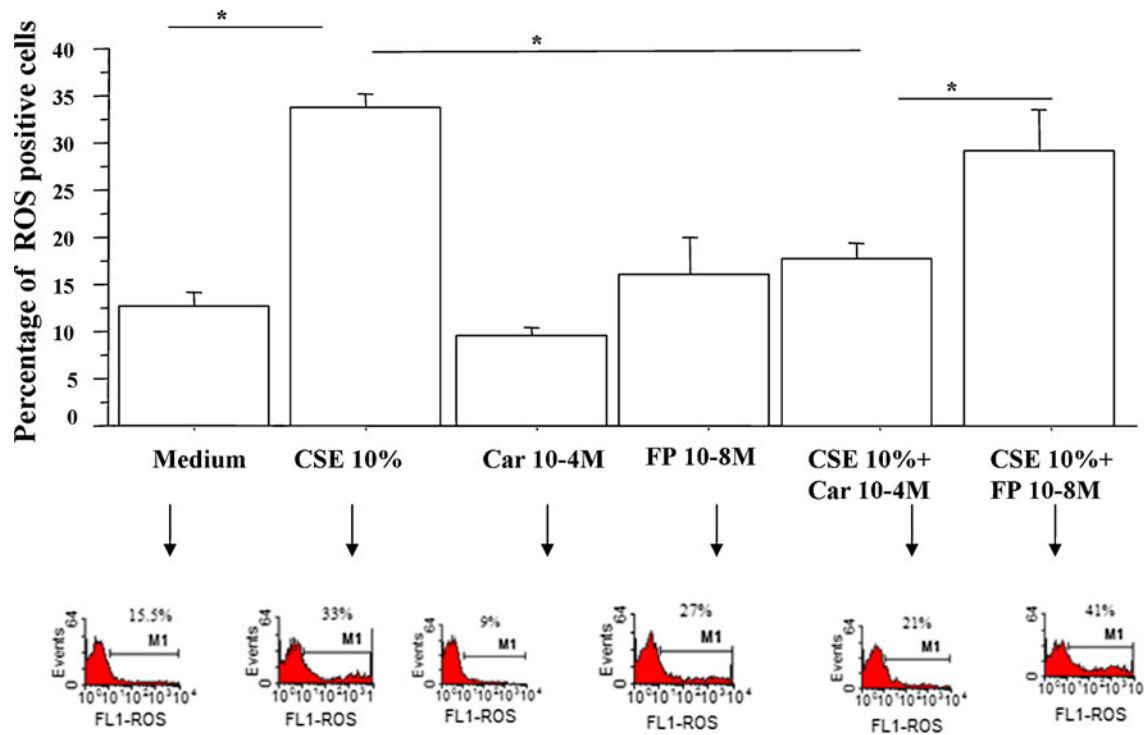
**Fig. 2** Dose–response experiments for CARB and FP. 16-HBE cells were cultured in the presence and absence of CSE (10 %), CARB ( $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  and  $10^{-8}$  M) and FP ( $10^{-4}$ ,  $10^{-7}$ ,  $10^{-8}$  and  $10^{-9}$  M) for

18 h and then were used for assessing ROS production using flow cytometry (see “Materials and methods” for details). Representative histogram plots from two experiments are shown



**Fig. 3** Effects of CARB and FP on ROS production in nasal and airway epithelial cells. Nasal epithelial cells (*RPMI 2650*) (a) and airway epithelial cells (*H292*,  $n=2$ ) (b) cells were cultured in the presence and absence of CSE (10 %), CARB ( $10^{-4}$  M) and FP

( $10^{-8}$  M) for 18 h and then were used for assessing ROS production using flow cytometry (see “Materials and methods” for details). Representative histogram plots are shown



**Fig. 4** Effects of CARB and FP on ROS production in bronchial epithelial cells. 16-HBE ( $n=6$ ) cells were cultured in the presence and absence of CSE (10 %), CARB ( $10^{-4}$  M) and FP ( $10^{-8}$  M) for 18 h and then were used for assessing ROS production using flow

cytometry (see “Materials and methods” for details). Data are expressed as the percentage of ROS-positive cells  $\pm$  SD. Representative histogram plots are also shown

### Effects of CSE, FP and CARB on necrosis and apoptosis of bronchial epithelial cells

We tested whether, at the used concentrations, CSE (5 and 10 %), FP ( $10^{-8}$  M) and CARB ( $10^{-4}$  M) induced cell apoptosis or necrosis in bronchial epithelial cells using the PI/annexin V binding method. Neither CSE (5 or 10 %) nor FP ( $10^{-8}$  M) or CARB ( $10^{-4}$  M) induced relevant numbers of necrotic (PI-positive) or apoptotic (annexin V-positive) cells (Fig. 5).

### Effects of FP and CARB on GSH expression in bronchial epithelial cells

GSH is one of the most important defensive mechanisms against oxidative stress (Biswas and Rahman 2009; Ghezzi 2011). The effect of CARB and FP on GSH expression in CSE-stimulated bronchial epithelial cells was explored. CSE, CARB and FP alone did not significantly increase GSH expression in bronchial epithelial cells. CARB, but not FP, significantly increased GSH expression in CSE-stimulated bronchial epithelial cells (Fig. 6).

### Effects of FP and CARB on HO-1 expression in bronchial epithelial cells

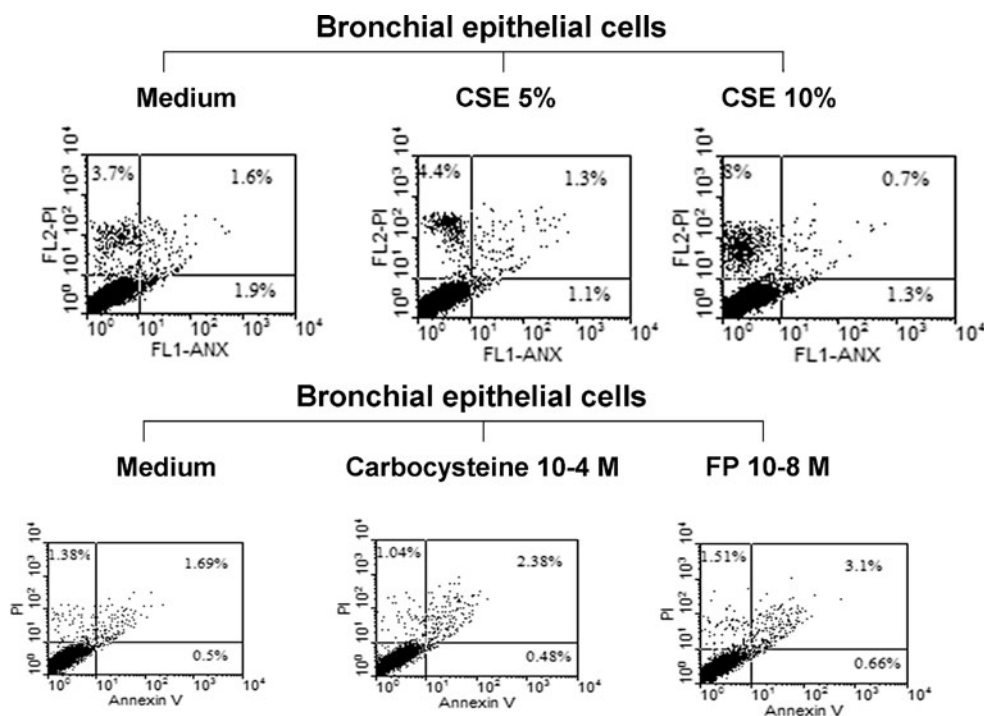
The inducible stress protein HO-1 has been implicated in cytoprotection against the toxic action of CSE (Dolinay et al. 2012; Raval and Lee 2010). The effect of CARB and FP

on HO-1 expression in CSE-stimulated bronchial epithelial cells was explored. CSE increased HO-1 expression in bronchial epithelial cells. CARB and FP alone did not significantly induce HO-1 expression. FP significantly reduced HO-1 expression in CSE-stimulated bronchial epithelial cells. CARB significantly increased HO-1 expression in CSE-stimulated bronchial epithelial cells, and its effect was opposite to the effect exerted by FP (Fig. 7a, b).

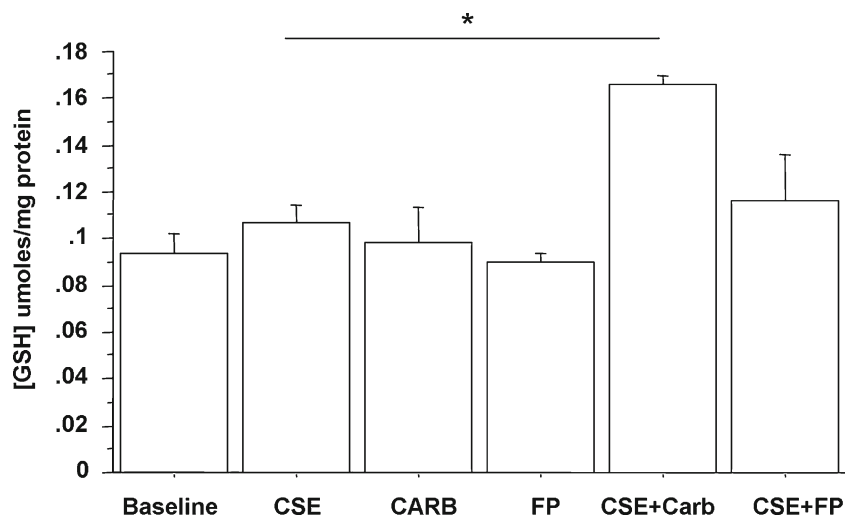
### Effects of FP and CARB on Nrf2 and HDAC-2 nuclear expression in bronchial epithelial cells

Since CSE affects Nrf2 nuclear translocation and reduces the expression of HDAC-2 (Malhotra et al. 2011), the effects of carbocysteine and FP on nuclear Nrf2 and HDAC-2 expression in CSE-stimulated bronchial epithelial cells were explored. CSE increased nuclear Nrf2 expression in bronchial epithelial cells. CARB significantly increased Nrf2 nuclear expression in CSE-stimulated bronchial epithelial cells. Nuclear Nrf2 was significantly higher in cells treated with both CARB and CSE than in cells treated with FP and CSE (Fig. 8a, b). With regard to HDAC-2 nuclear expression, CSE reduced the nuclear expression of HDAC-2. CARB and FP alone did not affect nuclear HDAC-2 expression. CARB, but not FP, slightly, but significantly, increased HDAC-2 nuclear expression in CSE-stimulated bronchial epithelial cells (Fig. 9a, b).

**Fig. 5** Effects of CSE, CARB and FP on the necrosis and apoptosis of bronchial epithelial cells (16-HBE). 16-HBE cells were cultured in the presence and absence of CSE (5 and 10 %), CARB ( $10^{-4}$  M) and FP ( $10^{-8}$  M) for 18 h and then were used for assessing cell necrosis and cell apoptosis by the annexin V/PI method using flow cytometry (see “Materials and methods” for details). Representative dot plots showing the percentage of annexin V, PI and annexin V/PI-positive 16-HBE at baseline and following the exposure of CSE (5 and 10 %), CARB ( $10^{-4}$  M) and FP ( $10^{-8}$  M) are shown



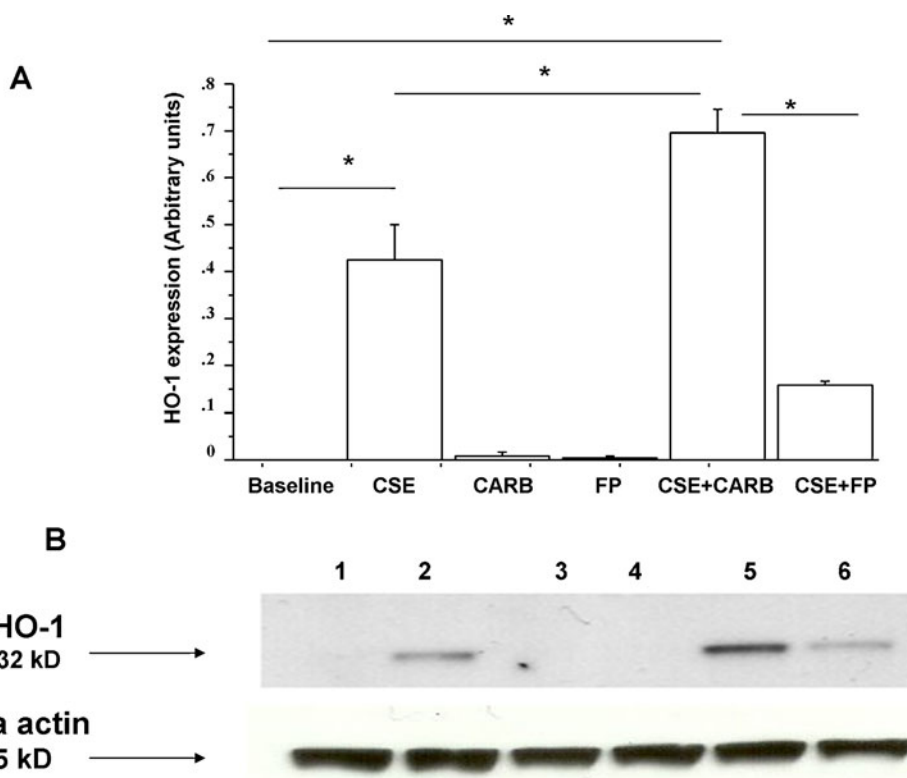
**Fig. 6** Effects of CARB and FP on GSH expression in bronchial epithelial cells. 16-HBE ( $n=6$ ) cells were cultured in the presence and absence of CSE (10 %), CARB ( $10^{-4}$  M) and FP ( $10^{-8}$  M) for 18 h and then were used for assessing GSH content (see “Materials and methods” for details). Data are expressed as micromoles GSH per milligram proteins  $\pm$  SD



Effects of FP and carbocysteine on HDAC-2 activity in bronchial epithelial cells

Cigarette smoke extracts induce posttranslational modifications (nitrosilation) of HDAC-2, leading to inactivity (Yang

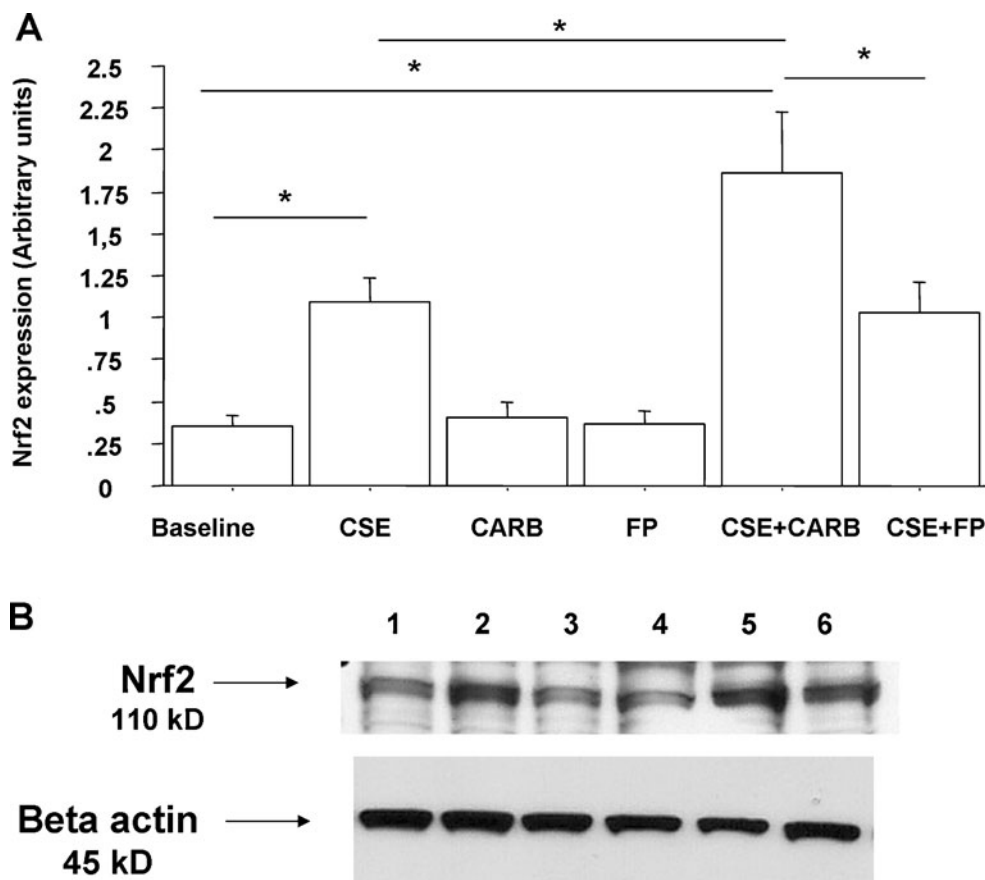
et al. 2006). The effects of carbocysteine and FP on HDAC-2 activity in CSE-stimulated bronchial epithelial cells were evaluated. CSE reduced HDAC-2 activity. Carbocysteine, but not FP, was able to partially counteract the effects of CSE on HDAC-2 activity in bronchial epithelial cells (Fig. 9c).



**Fig. 7** Effects of CARB and FP on HO-1 expression in bronchial epithelial cells. 16-HBE ( $n=5$ ) cells were cultured in the presence and absence of CSE (10 %), CARB ( $10^{-4}$  M) and FP ( $10^{-8}$  M) for 18 h. Total proteins were extracted and analysed for HO-1 expression using western blot analysis. Membranes were then stripped and incubated with rabbit polyclonal anti- $\beta$ -actin. **a** Densitometric analysis of HO-1 expression. Signals corresponding to HO-1 on the various western

blots were semi-quantified by densitometric scanning, normalised and expressed after correction with the density of the band obtained for beta-actin. Data are expressed as arbitrary units  $\pm$  SD. **b** Representative western blot performed on nuclear extracts. Lane 1, baseline; lane 2, CSE 10 %; lane 3, CARB ( $10^{-4}$  M); lane 4, FP ( $10^{-8}$  M); lane 5, CSE 10 % + CARB ( $10^{-4}$  M); lane 6, CSE 10 % + FP ( $10^{-8}$  M)

**Fig. 8** Effects of CARB and FP on Nrf2 nuclear expression in bronchial epithelial cells. 16-HBE ( $n=5$ ) cells were cultured in the presence and absence of CSE (10 %), CARB ( $10^{-4}$  M) and FP ( $10^{-8}$  M) for 18 h. Total proteins were extracted and nuclear proteins were collected and analysed for Nrf2 expression by western blot analysis. Membranes were then stripped and incubated with rabbit polyclonal anti- $\beta$ -actin. **a** Densitometric analysis of Nrf2 expression. Signals corresponding to Nrf2 on the various western blots were semi-quantified by densitometric scanning, normalised and expressed after correction with the density of the band obtained for  $\beta$ -actin. Data are expressed as arbitrary units  $\pm$  SD. **b** Representative western blot performed on nuclear extracts. Lane 1, baseline; lane 2, CSE 10 %; lane 3, CARB ( $10^{-4}$  M); lane 4, FP ( $10^{-8}$  M); lane 5, CSE 10 % + CARB ( $10^{-4}$  M); lane 6, CSE 10 % + FP ( $10^{-8}$  M)



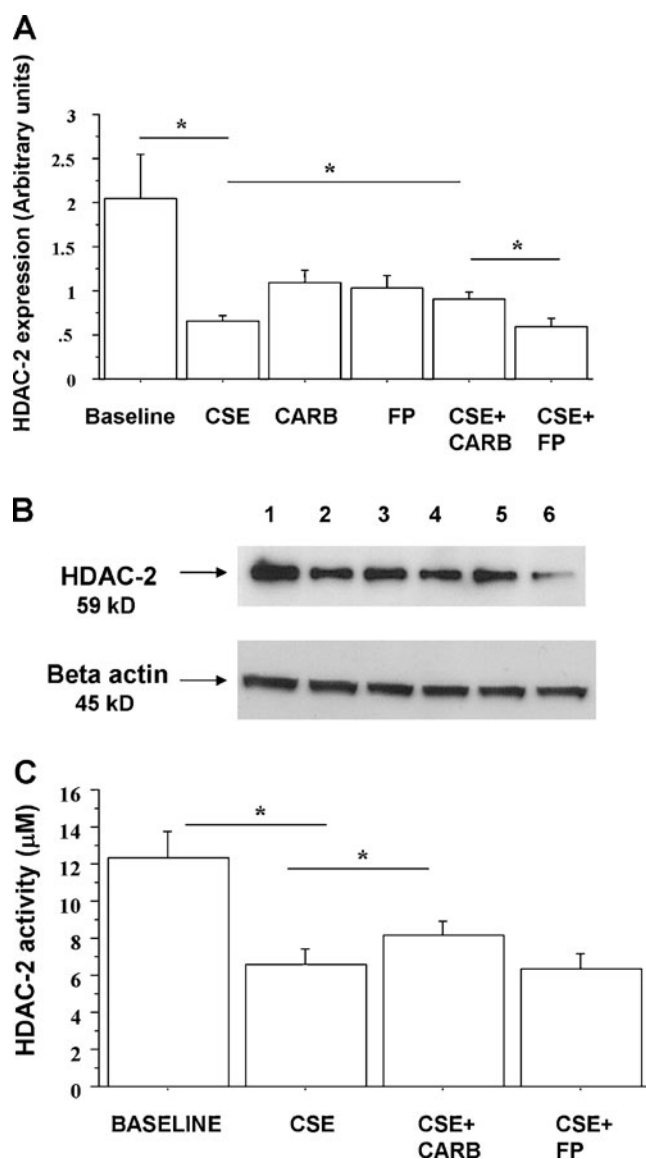
## Discussion

The bronchial epithelium has a central role in coordinating many of the responses that are observed in COPD (Puchelle et al. 2006). Cigarette smoke is the major risk factor for airflow limitation in COPD. Cigarette smoke, promoting oxidative stress- and nitrosative stress-induced posttranslational modifications, leads to HDAC2 inactivity and to corticosteroid resistance, phenomena both frequently observed in COPD patients. The main aims and the novelty of this work were to provide data to support the use of CARB to control oxidative stress and to improve the antioxidant (GSH) as well as the protective (Nrf2 and HO-1) mechanisms in CSE-exposed airway epithelial cells. The present study demonstrates that, in bronchial epithelial cells, CARB counteracts the oxidative stress induced by CSE exposure, by increasing GSH, HO-1, Nrf2 and HDAC-2 nuclear expressions and activity, and is more potent in these effects than fluticasone propionate. Furthermore, the finding that CARB increases nuclear translocation and activity of HDAC-2 in cigarette smoke-stimulated bronchial epithelial cells supports a new potential role in improving corticosteroid activity, suggesting the combined use of FP and carbocysteine to better control inflammation and oxidative stress, both important features of COPD pathogenesis.

Increased oxidative burden and antioxidant imbalance generated by inhaled oxidants or by endogenous sources are involved in cellular and tissue damage related to the pathogenesis of many acute and chronic respiratory diseases, including COPD. For this purpose, strategies aimed at reducing oxidative burden or increasing antioxidants are central in the therapy of COPD.

Antioxidant agents, such as carbocysteine, scavenge free radicals and oxidants (Zheng et al. 2008). COPD patients treated with carbocysteine experienced fewer numbers of exacerbations per year (Zheng et al. 2008). This effect may be linked to the reduced adherence of bacterial pathogens to airway epithelial cells (Rahman and Macnee 2012) and to the downregulation of ICAM-1, a receptor for the major group of rhinovirus (Yasuda et al. 2006). Moreover, carbocysteine inhibiting cell apoptosis and promoting the reversal of the imbalance of proteolytic and anti-proteolytic enzyme activities protects against the development of CSE-induced emphysema in rats (Hanaoka et al. 2011). To counteract oxidative stress, cells enlist a battery of antioxidant systems, and carbocysteine in a lung “epithelial-like” cell line (WI-26VA4) (Garavaglia et al. 2008) induces an increase in the chloride and GSH anionic fluxes, along with an increase in GSH permeability. Here, it has been demonstrated that, in bronchial epithelial cells stimulated with CSE





**Fig. 9** Effects of carbocysteine and FP on HDAC-2 nuclear expression in bronchial epithelial cells. 16-HBE ( $n=6$ ) cells were cultured in the presence and absence of CSE (10 %), CARB ( $10^{-4}$  M) and FP ( $10^{-8}$  M) for 18 h. Total proteins were extracted and nuclear proteins were collected and analysed for HDAC-2 expression by western blot analysis. Membranes were then stripped and incubated with rabbit polyclonal anti- $\beta$ -actin. **a** Densitometric analysis of HDAC-2 expression. Signals corresponding to HDAC-2 on the various western blots were semi-quantified by densitometric scanning, normalised and expressed after correction with the density of the band obtained for beta-actin. Data are expressed as arbitrary units $\pm$ SD. **b** Representative western blot performed on nuclear extracts. Lane 1, baseline; lane 2, CSE 10%; lane 3, CARB ( $10^{-4}$  M); lane 4, FP ( $10^{-8}$  M); lane 5, CSE 10% + CARB ( $10^{-4}$  M); lane 6, CSE 10% + FP ( $10^{-8}$  M). **c** In some experiments ( $n=3$ ), nuclear proteins were immunoprecipitated and then assessed for HDAC activity. Data are expressed as micromolar $\pm$ SD using as reference a curve of the Fluor-de-Lys deacetylated standard (provided with the kit)

(10 %), ROS production started to increase after 4 h and reached higher levels after 18 h of incubation, and for this

reason, this last time point was selected for all subsequent experiments.

Carbocysteine, but not FP, was able to counteract the oxidant activities of CSE, reducing ROS production. However, CARB, at the selected concentration ( $10^{-4}$  M), resulted less potent in controlling ROS formation in a nasal epithelial cell line and in another airway epithelial cell line (Nogawa et al. 2009). Furthermore, in bronchial epithelial cells, carbocysteine, but not FP, was able to counteract the oxidant activities of CSE, also increasing GSH, HO-1 and Nrf2. HO-1 is an enzyme that catalyses the degradation of the heme group into iron, biliverdin, and carbon monoxide and acts as a stress response protein (Morse and Choi 2002). This enzyme is highly inducible in response to oxidants and inflammatory molecules and might serve as a protective mechanism against oxidative stress and inflammation-induced lung injury. In this regard, it has been demonstrated that acrolein, an aldehyde present also in cigarette smoke, induces HO-1 in bronchial epithelial cells (Zhang and Forman 2008). An imbalance between HO-1 and inducible nitric oxide synthase may be associated with the development of severe impairment in chronic obstructive pulmonary disease patients (Maestrelli et al. 2003). In addition, it is noteworthy that *n*-acetylcysteine is able to induce HO-1 also in the presence of CSE in alveolar “epithelial-like” cells (Kosmider et al. 2011). Herein, we show that carbocysteine is able to further increase the expression of HO-1 in CSE-stimulated bronchial epithelial cells while FP has an opposite effect. The increased expression of HO-1 is normally linked to the inactivation of the Kelch-like ECH-associated protein 1 (Keap1), resulting in nuclear accumulation of the transcriptional regulator Nrf2. CSE oxidises Keap1 thiol groups, resulting in the dissociation of Nrf2 from Keap1 and the translocation of Nrf2 into the nucleus leading to increased HO-1 mRNA and protein in human macrophages (Goven et al. 2009). It has also been previously demonstrated that during keratinocyte differentiation, HO-1 may be modulated via a Nrf2-independent pathway (Ballatori et al. 2009).

The data herein reported confirm the effect of CSE in increasing the nuclear translocation of Nrf2 and show a significant increase of Nrf2 nuclear expression in CSE-stimulated cells treated with CARB compared to FP. The observed reduced nuclear expression of Nrf2 in CSE-stimulated cells treated with FP confirms previous observations concerning a suppressive role of glucocorticoids in Nrf2-dependent antioxidant response (Kratschmar et al. 2012). Furthermore, in bronchial epithelial cells stimulated with CSE, CARB was able to further increase Nrf2 nuclear expression, suggesting that the further increased expression of HO-1 in this condition was dependent on the modulation of the Nrf2 pathway. In the present study, carbocysteine improves the cytoprotective events against oxidative stress,

increasing Nrf2 and HO-1 expression and GSH content. Interestingly, the increased GSH content of cells treated with both CSE and carbocysteine further supports the cytoprotective role of carbocysteine. GSH is a major low molecular weight antioxidant thiol and its levels decline in both ageing and COPD (Drost et al. 2005). GSH levels can control both inflammation and oxidative stress, two major factors contributing to COPD.

The increase of GSH induced by carbocysteine in bronchial epithelial cells stimulated with CSE may also promote, as previously reported, the denitrosylation of proteins by means of a trans-nitrosylation reaction (Benhar et al. 2009). Exogenous GSH treatment of macrophages from individuals with COPD restores HDAC-2 activity and induces a concomitant decrease in *S*-nitrosylation of HDAC2 (Malhotra et al. 2011).

HDAC plays a critical role in the regulation of inflammatory responses (Shakespeare et al. 2011). Class I HDACs (HDAC-1, HDAC-2, HDAC-3 and HDAC-8) mediate the deacetylation of histones proteins. Upon the deacetylation of histones, chromatin condensation and transcriptional repression occur, while the deacetylation of transcription factors alters protein–protein interactions and DNA binding, thus regulating the transcriptional programme (Shakespeare et al. 2011). The decrease in HDAC-2 activity in alveolar macrophages and in lung epithelial cells, which occurs concomitantly with COPD progression, is responsible for the amplified inflammation and glucocorticoid resistance observed in these patients (Cosio et al. 2004). The anti-inflammatory response induced by glucocorticoids is linked to the trans-repression of NF- $\kappa$ B-dependent cytokine gene expression (Ito et al. 2006). HDAC-2 is a critical component for the trans-repression of NF- $\kappa$ B transcriptional activity by deacetylating histones in the pro-inflammatory gene promoters and deacetylating the glucocorticoid receptor (Ito et al. 2006). Moreover, HDAC-dependent mechanisms contribute to the maintenance of the adult lung structure since in animal models HDAC inhibition leads to emphysema (Mizuno et al. 2011). Pretreatment of A549, an adenocarcinomic alveolar basal epithelial cell line, with *N*-acetyl-L-cysteine attenuated the oxidant-mediated reduction in HDAC activity (Moodie et al. 2004). According to these data, we now demonstrate that carbocysteine is able, differently from FP, to increase HDAC-2 nuclear translocation and to improve HDAC-2 activity in CSE-stimulated bronchial epithelial cells. Additional experiments will be required in the future to better clarify whether the increased HDAC-2 activity caused by carbocysteine could improve the response to corticosteroids in models with elevated oxidative stress.

In conclusion, the present study demonstrates that, 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 HDAC-2 activity, thus providing new mechanisms that are not shared with steroids, whose activity is impaired in models with high oxidative stress.

The main aims and the novelty of this work were to provide data to support the use of CARB to control, as well known, the oxidative stress and to improve the antioxidant (GSH) and the protective (Nrf2 and HO-1) events in CSE-exposed airway epithelial cells. Furthermore, the finding that CARB increases, in cigarette smoke-stimulated bronchial epithelial cells, the nuclear translocation and activity of HDAC-2 supports a new potential role in improving corticosteroid activity. Taken together, these data support a combined use of FP and carbocysteine to better control inflammation and oxidative stress, both important features of COPD pathogenesis.

**Acknowledgments** This work was mainly supported by the Italian National Research Council and by Dompè, Italy. Elisabetta Pace declares that she has had access to and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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