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N-ACETYLICYSTEINE RESTORES ENDOGENOUS ANTIOXIDANT SYSTEM IN HUMAN BRONCHIAL EPITHELIAL CELLS EXPOSED TO CIGARETTE SMOKE EXTRACT.

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ABSTRACT

Recent studies have revealed that treatment with N-acetyl-l-cysteine (NAC) improved airway function in Chronic Obstructive Pulmonary Disease (COPD) patients and lowered the frequency of acute exacerbations. In this study, we investigated the biochemical mechanisms through which NAC exerts its protective effects against bronchial epithelium cells exposed to cigarette smoke. To evaluate the efficacy of NAC to counteract the deleterious effects induced by cigarette smoke, we treated human bronchial epithelial cells (16 HBE) with NAC alone and in combination with cigarette smoke extract (CSE). After treatment, we measured reactive oxygen species (ROS) formation, effect on cell viabilty, glutathione resources (contents and genes), inflammatory cytokines expression (IL-1 β, IL-6 and TNF-α) and antioxidant genes expression SOD1, SOD2, iNOS, HO-1, NRF2, IRE-1α and PGC-1α**.** NAC treatment was able to counteract free radicals (ROS) formed and reduce viability after CSE induced oxidative damage. Moreover, NAC increased the GSH's resources and gluathione metabolism gene such as GPX, GCLC and GT, restored the gene expression of SOD1, SOD2, reduced expression of pro-inflammatory cytokines, and was able to positively regulate expression of iNOS HO-1, NRF2, IRE-1α and PGC-1α**.** Our results demonstrated that NAC treatment restores endogenous antioxidant defenses in human bronchial epithelial cells exposed to cigarette smoke extract suggesting its use in the therapeutic strategy of patients with COPD.

1. Introduction

The airway epithelium is crucial for maintaining pulmonary homeostasis since it acts not only as a physical barrier but also driving a significant response to inhaled pathogens and chemicals triggering complex molecular events in immune and stromal cells (1-4). Epithelial integrity and function is impaired following chronic exposure to various inhaled toxic agents, including cigarette smoke, leading to the formation of oxidative stress mediators such as reactive oxygen species (ROS) and hydrogen peroxide (5).

Several studies have demonstrated that cigarette smoke upregulates ROS levels in airway epithelial cells and dramatically increases systemic levels of oxidative stress in smokers and COPD patients (6, 7). Therefore, cigarette smoke-induced endogenous ROS in airway epithelial cells may play an important role in the pathogenesis of COPD and may represent a possible pharmacological target for its treatment(8). Other detrimental effects associated to chronic exposure to cigarette smoke include modification of the metabolism of several drugs, including those anesthetics used as muscle relaxants, and may also affect the sensitivity of the central nervous system to psychoactive drugs, e.g. benzodiazepines and anesthetics (9, 10).

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Therefore, antioxidant pharmacological strategies have a strong rationale to mitigate various effects of oxidative stress leading to chronic degenerative diseases and other adverse effects. With regard to this, previous studies have revealed that treatment with a high-dose of the thiol-group bearing mucolytic and antioxidant N-acetyl-l-cysteine (NAC) improved small airway function in COPD patients and lowered the frequency of acute exacerbations(11, 12). N-Acetylcysteine (NAC) is a well-known thiol compound that possesses a free sulfhydryl group through which it reduces disulfide bonds conferring antioxidant effects. NAC is the N-acetyl derivative of the amino acid L-cysteine, and is a precursor of glutathione (13). In addition, N-acetylcysteine reduces neutrophil chemotaxis reducing the number and activity of bronchoalveolar neutrophils and alveolar macrophages in smokers(14).
In the present study, we explored the impact of cigarette smoke extract (CSE) on the main antioxidant systems in epithelial cells and the pharmacological effects of NAC in such a pathophysiological cascade.

2. Material and Methods

Cell cultures

Human bronchial epithelial cells (16HBE) were purchased from ATCC. The cells were maintained in culture medium D-MEM (Gibco, BRL, Germany), supplemented with 10% of fetal bovine serum (FBS) (Gibco), 0.5% gentamycin (Gibco), 1% glutamine (Gibco) and incubated in a humidified atmosphere of 5% CO_2 at 37 ° C. 16HBE cells were treated for 24 hours in the presence or absence of N-Acetyl Cysteine (NAC 1 mM) (Sigma-Aldrich, Milan, Italy).

Preparation of cigarette smoke extract (CSE)

For the *in vitro* studies, we used cigarettes containing 10 mg of tar, 0.7 mg nicotine and 10 mg of carbon monoxide per cigarette. The derived-cigarette smoke was bubbled through a vacuum pump in 10 ml of phosphate buffered saline (PBS). The obtained solution was filtered through a 0.22 μm filter and then diluted to be used in each experiment. The concentration of CSE used in all experimental procedures was 10% (v/v) as previously described.

Measurement of cell viability

Cells were cultured in 96-well plates and treated for 24 hours with CSE in the presence or absence of NAC. Successively, the medium was replaced by a solution containing bromide 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium (MTT) and incubated for 3 hours at 37 °C. Finally, dimethylsulfoxide (DMSO) was added to all wells and the plate was read in a multiplate reader (Bio-Tek, Milan, Italy) at λ=570 nm.

Measurement of reactive oxygen species (ROS)

Reactive oxygen species formation (ROS) was determined cytofluorimetrically using the MUSE Oxidative Stress kit (Millipore, Milan, Italy). Briefly, 10 microliters of cell suspension in 1X Muse assay buffer was added to 190 μL of working solution Reagent MUSE® Oxidative Stress. The samples were vortexed for 3-5 seconds and then incubated for 30 minutes at 37°C, subsequently read by Muse ™ Cell Analyzer (Millipore, Milan, Italy).

RNA extraction and real-time PCR

Trizol reagent (Life technology, Milan, Italy) was used to extract total RNA. The extracted mRNA was subsequently converted into cDNA through a kit containing the reverse transcription (Life technology). The quantitative analysis was performed using the One-Step Real-Time PCR instrument using the SYBR Green PCR master mix (Life Technologies). The primer sequences are shown in Table I. The level of expression of its mRNA was calculated using the comparative method ΔΔCt-2 as previously described (15-17).

Gene	Primer Forward	Primer Reverse
GAPDH	AGACACCATGGGGAAGGTGA	TGGAATTTGCCATGGGTGGA
GCLC	ACTTCATTTCCCAGTACCTTAACA	CCGGCTTAGAAGCCCTTGAA
GPX	CAGTCGGTGTATGCCTTCTCG	GAGGGACGCCACATTCTCG
GST	CTGGGCTTCGAGATCCTGTG	GGCAGACAAACTTCCACTGTC
HO-1	GTGCCACCAAGTTCAAGCAG	CACGCATGGCTCAAAAACCA
IL-1β	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
IL-6	GAAAGCAGCAAAGAGGCACT	TTTCACCAGGCAAGTCTCCT
NOS2	TTCAGTATCACAACCTCAGCAAG	TGGACCTGCAAGTTAAAATCCC
NRF2	TCAGCGACGGAAAGAGTATGA	CCACTGGTTTCTGACTGGATGT
PGC-1⟨	GGTGCAGTTTTGCCAAGGAG	TTCCTTGGGGTCCAGACAGA
SOD1	TGGTTTGCGTCGTAGTCTC	CCAAGTCTCCAACATGCCTCT
SOD2	GCATCAGCGGTAGCACCA	GCAACTCCCCTTTGGGTTCT
TNFα	AAGCACACTGGTTTCCACACT	TGGGTCCCTGCATATCCGTT
IRE-1α	ACCAGCGTGGTGATAGTTGG	CGTGCAATGACGTCTTCGTG

Table 1. PCR primers used

Statistical analysis

Data were presented as mean ± standard deviation (SD) of n=4 experiments performed in triplicate. The statistical significance (p <0.05) of differences between experimental groups was determined by analysis of *t*-test and ANOVA (Post hoc test: Newman-Keuls).

3. Results

Effect of CSE on cell viability and ROS formation

In the first set of experiments, we showed that CSE significantly reduced cell viability when compared to control (Figure 1A) (p<0.05). Interestingly, NAC abolished the toxic effects of CSE restoring cell viability to control levels (Figure 1 A). With these results, we consistently showed that CSE treatment resulted in a significant increase in ROS formation (Figure 1B). Furthermore, NAC treatment abolished ROS formation when compared to CSE treated cells (Figure 1B). Interestingly, NAC treatment also reduced ROS formation when compared to untreated cells.

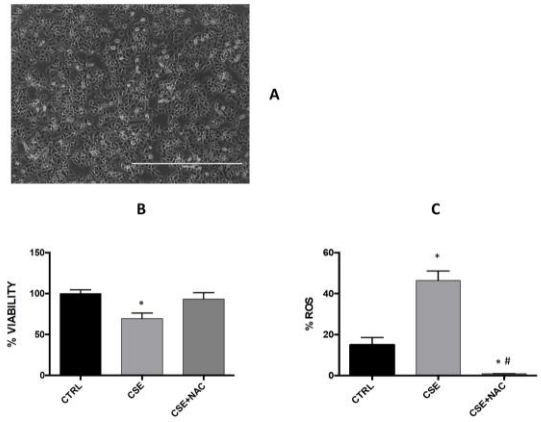

Figure 1. A) Microphotography of 16HBE cells in confluence B) Viability assay measured by MTT assay after 24 hours of treatment. C) Effect of CSE and NAC on the ROS production analyzed by flow cytometric assay. (*p<0.05 to CTRL; #p<0.05 to CSE).

Effect of CSE on glutathione oxidation and metabolism

Consistently, with above presented results, CSE treatment resulted in a significant decrease of intracellular reduced glutathione content when compared to untreated cells (Figure 2A). Furthermore, NAC treatment significantly increased GSH content when compared to CSE treated cells even though it was not restored to control levels (Figure 2A). Interestingly, CSE significantly reduced CGLC gene expression whereas NAC treatment increased its expression when compared to both CSE and untreated cultures (Figure 2B). Our results also showed that CSE treatment resulted in a notable increase of GPX gene expression which was restored to control levels following NAC treatment (Figure 2C). Finally, CSE treatment resulted in a significant increase of GST gene expression and such increase was prevented by NAC treatment (Figure 2D).

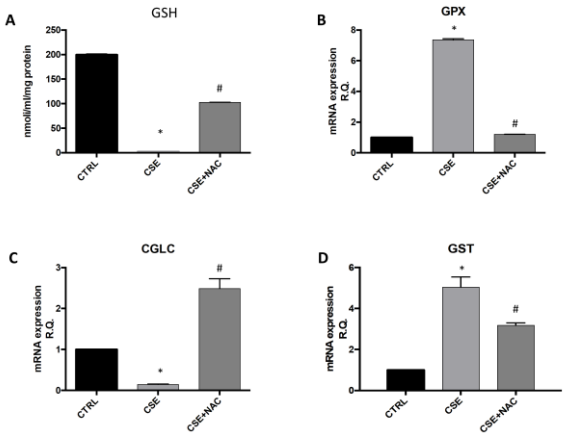

Figure 2. Effect of CSE and NAC on Glutathione content and gene levels of GPX, GCLC and GT. (*p<0.05 to CTRL; #p<0.05 to CSE)

NAC induces SOD1, SOD2 and iNOS gene expression

Figure 3 shows that CSE treatment was able to increase SOD1 (Figure 3A) when compared to untreated cells, while it caused a remarkable decrease of SOD2 (Figure 3B) and iNOS (Figure 3C) gene expression. Furthermore, NAC treatment resulted in a significant increase of SOD1 gene expression when compared to both CSE or untreated cells. Similar effects were also observed for SOD2 and NOS2 gene expression following NAC treatment (Figure 3B and 3C).

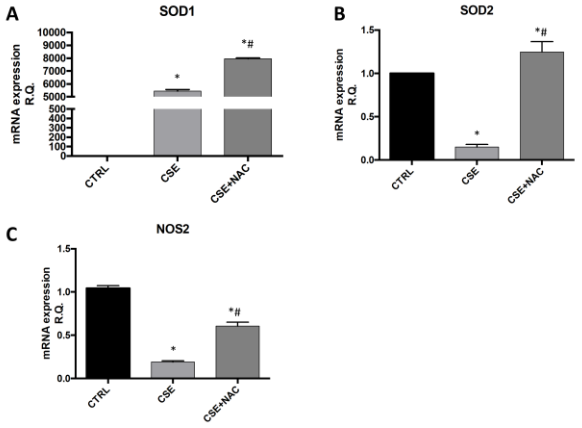

Figure 3. Effect of CSE and NAC on SOD1, SOD2 and iNOS gene expression. (*p<0.05 to CTRL; #p<0.05 to CSE)

CSE induces NRF2, HO-1, IRE-1α and PGC-1α genes expression

Figure 4 shows that CSE significantly increased nuclear transcription factor erythroid-2 (NRF2) gene levels and such increase was more evident following NAC cotreatment (Figure 4A). Consistently with NRF2 increase, we observed a remarkable increase of HO-1 gene expression. In particular, HO-1 was upregulated following CSE treatment when compared to untreated cells (Figure 4B) but no significant change was observed following NAC cotreatment when compared to CSE alone (Figure 4B). Similarly, CSE treatment also resulted in a notable increase in endoplasmic reticulum stress as measured by a significant increase in inositol-requiring enzyme-1α (IRE-1α) gene expression (Figure 4C). Treatment with NAC significantly reduced ER stress when compared to CSE treated cells even though it was unable to be restored to control levels (Figure 4C). Finally, CSE resulted in a significant increase in peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) gene expression whereas NAC treatment restored it to control levels (Figure 4D).

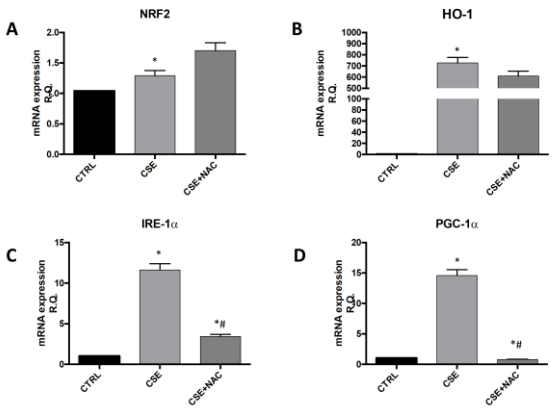

Figure 4. Effect of CSE and NAC on HO-1, NRF2, PGC-1α and iNOS gene expression analyzed by Real Time PCR. (*p<0.05 to CTRL; #p<0.05 to CSE)

Expression of cytokines genes IL-1β, IL-6 *and* ***TNFα***

Consistently, with increased oxidative and ER stress, CSE resulted in a significant increase of inflammation as measured by IL-1β, IL-6 and TNFα expression. In particular, CSE treatment resulted in a notable increase of IL-1β, IL-6 and TNFα (Figure 5A, 5C) and such increase was abolished by NAC treatment. Interestingly, no significant effect was observed for IL-6 expression following NAC treatment (Figure 5B).

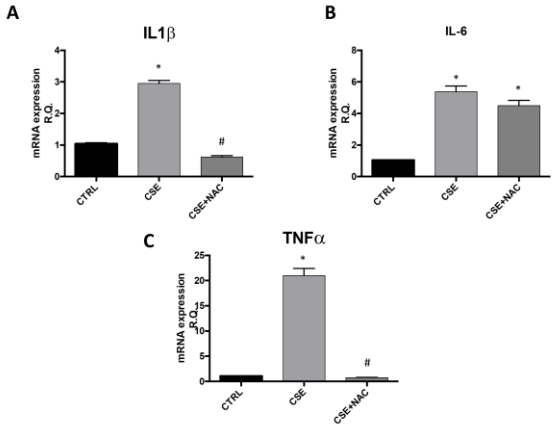

Figure 5. Effect of CSE and NAC on cytokines genes IL-1β, IL-6 and TNFα analyzed by Real Time PCR. (*p<0.05 to CTRL; #p<0.05 to CSE)

4. Discussion

In this study, we investigated the biochemical mechanisms through which N-acetylcysteine exerts its protective effects against bronchial epithelium cells, formerly used in analogous experimental models(18-21), exposed to cigarette smoke with particular regards to oxidative and ER stress and inflammation.

Our results showed that NAC have a direct scavenging effect on ROS by increasing GSH levels but also exhibited indirect effects by upregulating key elements orchestrating the cellular redox balance (i.e. HO-1, SOD, IRE-1α). Several mechanisms underlie CSE mediated toxicity, among which oxidative stress seems to play a major role. Protein folding in ER and formation of ROS are closely related, and it has become clear that the activation of UPR (unfolded protein response) that occurs in response to exposure to oxidative stress is an adaptive mechanism that preserves cell function and survival. Cigarette smoke also causes oxidative stress, which has been reported to induce apoptosis, necrosis, and other types of damage in cells (22, 23). Previous studies showed that cigarette smoking induced endoplasmic reticulum (ER) stress through oxidative stress and complement activation in the retinal pigment epithelium of mice (24) through an alternative pathway, resulting in ER-stress-mediated lipid accumulation. Our results are consistent with these observations, showing that CSE results in significant reduction of cell viability and increased oxidative stress and inflammation as measured by ROS production and inflammatory cytokines IL-1β, IL-6 and TNF-α. Furthermore, NAC treatment restored ROS and cytokines to control levels, thus resulting in a significant reduction of CSE toxicity. We next correlated the induction of oxidative and ER stress with the CSE-induced depletion of GSH. We showed that treatment with CSE resulted in a strong increase in HO-1 and IRE-1-α and such effect was abolished by NAC treatment for IRE-1-α but not for HO-1. These results are in agreement with a recent report by Rahman and colleagues (25), where in primary small airway epithelial cells cigarette smoke induced an initial decrease, followed by an increase, in GSH levels. Furthermore, a previous study found that mRNA encoding the catalytic subunit of γ-glutamylcysteine ligase (GCL), the rate-limiting enzyme of GSH biosynthesis, was also upregulated after exposure to CSE(26). However, with higher percentages of CSE, GSH levels were unable to recover possibly because of the overwhelming oxidant burden to the cells exerted by the CSE. With regard to this, our results showed that NAC treatment reduced IRE-1α but surprisingly showed no effect on HO-1 expression. In particular, our previous studies showed that under certain experimental conditions these two events may be related since thapsigargin, a well known chemical chaperone and inhibitor of ER stress, prevents HO-1 upregulation (27, 28). Consistent with this hypothesis is the fact that CSE and NAC cotreatment does not restore NrF2 activation, a well known HO-1 transcription factor. Similarly, CSE significantly induced SOD1 expression whereas it decreased SOD2. Superoxide dismutases (SODs), which catalyze the dismutation of superoxide into oxygen and hydrogen peroxide, provide the first line of defense against free radicals (29). CSE significantly reduced SOD2 expression and thus may account for impaired mithocondrial function leading to increased ROS formation. On the other hand, cytoplasmic SOD1 is upregulated in response to increased oxidative stress. Consistently with these observations, our results showed that peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) is activated in response to oxidative stress induced by CSE. PGC-1α is a transcriptional coactivator, whose role is to regulate cellular energy metabolism (30). PGC-1α expression is influenced by several factors including reactive oxygen species and reactive nitrogen species; its function is necessary for the activation of many detoxifying enzymes, including GPX1 and SOD2 (31). While previous studies show the regulation of PGC-1α expression by antioxidant species(32, 33), we proved in our experiment the efficacy of NAC on decreasing the expression of this gene.

In conclusion, our results have shown that NAC treatment restores endogenous antioxidant defenses in human bronchial epithelial cells exposed to cigarette smoke extract suggesting its use in the therapeutic strategy of patients with COPD.

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