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INFLUENCE OF *N*-ACETYLCYSTEINE ON ICAM-1 EXPRESSION AND IL-8 RELEASE FROM ENDOTHELIAL AND EPITHELIAL CELLS

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Chronic obstructive pulmonary disease (COPD) is characterized by chronic airway inflammation. The initial step in the inflammatory process is overexpression of adhesion molecules, which leads to excessive transmigration of neutrophils. One of these adhesion molecules is ICAM-1 which is elevated in COPD patients. In this study we evaluated the influence of *N*-acetylcysteine (NAC) (0.01 mM-30 mM) on the cytokine-induced (TNF-α/IL-1β) expression of the ICAM-1 adhesion molecule and on IL-8 release in endothelial (ECV-304) and bronchial epithelial (H292) cell lines. The methodology used consisted of immunochemistry for the assessment of surface ICAM-1 and ELISA method for that of soluble ICAM-1 and IL-8. NAC inhibited the TNF-α/IL-1β-stimulated ICAM-1 expression and IL-8 release from both cell lines in a concentration dependent manner. The most effective concentrations were 30 mM and 20 mM (99 and 90% inhibition respectively, $P<0.01$). We conclude that NAC is an effective inhibitor of TNF-α/IL-1β- stimulated ICAM-1 and IL-8 release in endothelial and epithelial cells. This fact highlights the anti-inflammatory potential of NAC in COPD.

Key words: *COPD, inflammation, ICAM-1, interleukin-8, N-acetylcysteine*

INTRODUCTION

Intercellular adhesion molecule-1 (ICAM-1) is an inducible cell adhesion glycoprotein of the immunoglobin supergene family expressed on the surface of

a variety of cell types. By interacting with β2-integrins on the surface of leukocytes, it plays a crucial role in the leukocyte transmigration process. It is constitutively present on the surface of a wide variety of cell types including fibroblasts, leukocytes, epithelial and endothelial cells, and is up-regulated in response to a number of inflammatory mediators such as TNF-α, interleukin-1β, and other stimuli e.g. H_2O_2, retinoic acid, and viral infection (1, 2). Leukocyte recruitment to the sites of inflammation depends on the expression and function of cell adhesion molecules (3). There are two forms of ICAM-1; the membrane-associated ICAM-1 (mICAM-1) that is fundamental for the adhesion of leukocytes to endothelial cells, and a soluble form of ICAM-1 (sICAM 1) that exists in the human serum (4) as a result of a proteolytic cleavage releasing the soluble ectodomain from the cell surface (5).

One of the factors that may influence ICAM-1 expression is IL-8 (6, 7), which also is influenced by ICAM-1 (8). IL-8 is a crucial cytokine in the pathogenesis of COPD. It is released by human bronchial epithelial (9) and endothelial cells (10) and it is involved in the activation of inflammatory cells in the lungs. IL-8 release is controlled by various mechanisms (11) that overlap with the ICAM-1 controlling pathways, involving TNF-α and IL-1β activation with subsequent activation of p38, JNK, and NF-κB (2, 11). Increased levels of IL-8 were found in the sputum supernatants of stable and exacerbated COPD patients (12, 13). This increase is associated with an elevation in the adhesion molecule expression in smokers (12, 14) and patients with COPD (12). Therefore, both ICAM-1 and IL-8 could be considered as therapeutic targets that, when blocked, might inhibit the excessive transmigration of neutrophils and hence limit the inflammatory process in COPD.

N-acetylcysteine (NAC) is an antioxidant that has also been observed to exert some anti-inflammatory effects (15, 16). It is widely used in COPD as a mucolytic agent and an antioxidant. However, its efficacy in this disorder is debatable (17-19). As far as the cell adhesion is concerned, Riise et al (20) showed that NAC decreased the adhesion of bacteria to the epithelial cells. On the other hand, some studies show enhanced cell adhesion properties after treatment with NAC because of its possible influence on the cell cytoskeleton (21). Moreover, the actions of NAC on ICAM-1 and IL-8 have been reported to coincide, which supports the existence of common regulating pathways (22).

The influence of NAC on the ICAM-1 expression on endothelial and epithelial cells has not yet been fully studied. In this study we evaluated the influence of NAC on the cytokine-induced expression of the ICAM-1 adhesion molecule and on IL-8 release in endothelial (ECV-304) and bronchial epithelial (H292) cell lines.

MATERIAL AND METHODS

The study was approved by an institutional Ethics Committee.

Reagents

Tissue culture media, glutamine, fetal calf serum, trypsin/EDTA solution, Dulbecco's Phosphate Buffered Saline (PBS) were purchased from Invitrogen (Paisley, UK); tumour necrosis factor-α (TNF-α) and interleukin-1 β (IL-1 β) from Peprotech (Rocky Hill, NJ, USA); ECV-304, human endothelial cell line and H292, human bronchial epithelial cell line, were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). All other chemicals used were reagent grade and obtained from Merck (Darmstadt, Germany).

Cell culture

Endothelial cells (ECV-304) and epithelial cells (H292) were cultured in M199 and RPMI media, respectively, supplemented with 10% heat-inactivated human serum, glutamine (2mM ECV, 0.2 mM H292), mixture of penicillin and streptomycin (final concentration 100 U/ml and 10 mg/ml, respectively) in culture flasks. The doubling time was approximately 36 h for ECV and 48 h for H292. At confluence, the cells were detached using trypsin/EDTA solution for 5 min, washed, and neutralized with cell culture medium. They were preincubated with concentrations of NAC ranging from 0.01 mM to 30 mM. The time of preincubation for ECV 304 and H292 cells was 0.5 h, 2 h, or 6h. After preincubation with NAC, cells were washed twice in cell culture medium and then stimulated with TNFα and IL-1β (both at 10 ng/ml) for 4 h (23-25). Cytospins were made and air-dried before further analysis.

For ICAM-1 immunostaining, sections were incubated with peroxidase blocker for 5 min and washed with PBS + 0.1% albumin. Subsequently, cells were incubated with anti-ICAM-1 monoclonal antibody (ICAM-1 CD54 clone 6.5B5) (1:50) for 30 min at 25°C, washed, and incubated with labeled polymer for 30 min, followed by 10 min chromogen incubation, according to the manufacturer's instructions (EnVision System; DAKO, Denmark). ICAM-1-positive cells were identified using light microscopy (magnification - 100x). The results are expressed as the percentage of ICAM-1 positive cells. Soluble ICAM-1 and IL-8 in the supernatants were measured using ELISA (Biosource International, Camarillo, CA, USA).

Statistical analysis

Results are expressed as means ±SE. Statistical differences between the evaluated parameters were determined by non-parametric one-way analysis of variance (Kruskal-Wallis test) followed by Dunn's test. Correlations between parameters were estimated with Spearman R coefficient value. $P<0.05$ was considered significant for all statistical comparisons. Statistical calculations were performed using GraphPad Prism version 4.00 for Windows, GraphPad Software (San Diego, CA, USA).

RESULTS

The viability of the epithelial and endothelial cells, estimated by trypan blue exclusion, always exceeded 85% after a 6-hour incubation with NAC and was always >90 % after 0.5 and 2- hour incubation.

NAC inhibits ICAM-1 expression in endothelial and epithelial cells

The basal (no cytokine induction) percentage of the ICAM-1 positive endothelial cells was 35.2 ±0.7%, while after cytokine induction, the expression rose to 74.5

±3.2%. NAC inhibited the number of ICAM-1 positive cells in a concentration-dependent manner. The strongest inhibition was seen at a concentration of 30 mM of NAC for ECV (0.6 ±0.2% positive cells) (*Fig. 1*) and for H292 (0.5 ±0.3% positive cells) (*Fig. 2*). No influence of NAC on ECV cells was found at concentrations =10 mM. In the case of H292 cells, even lower concentrations (3 mM and 10 mM) still exerted inhibitory effects. There was no significant difference between 0.5, 2 and 6-hour incubation times for both cell lines (*Fig. 1* and *Fig. 2*).

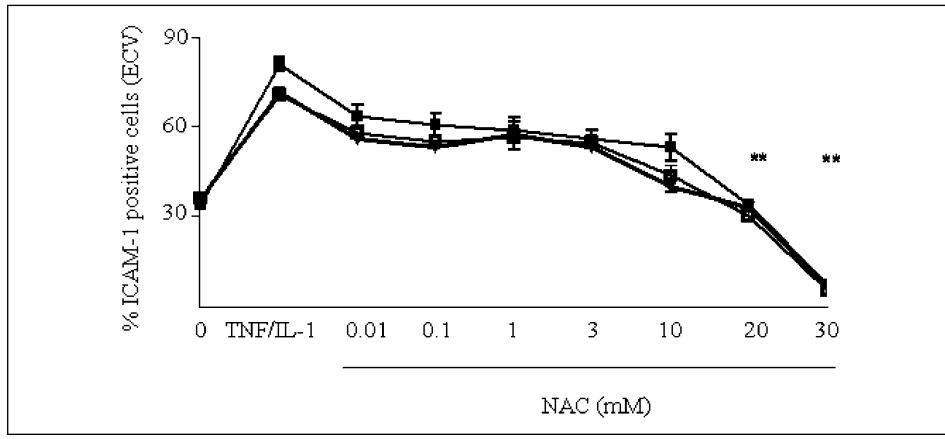

Fig. 1. Inhibition of TNF-α / IL-1β- induced ICAM-1 expression in endothelial cells (ECV-304) by increasing concentrations of *N*-acetylcysteine. Data are % of positive cells; n=3 in duplicate. Solid squares - 0.5 h, open squares – 2 h, and solid triangles - 6 h of NAC incubation. *P<0.05 compared with positive control.

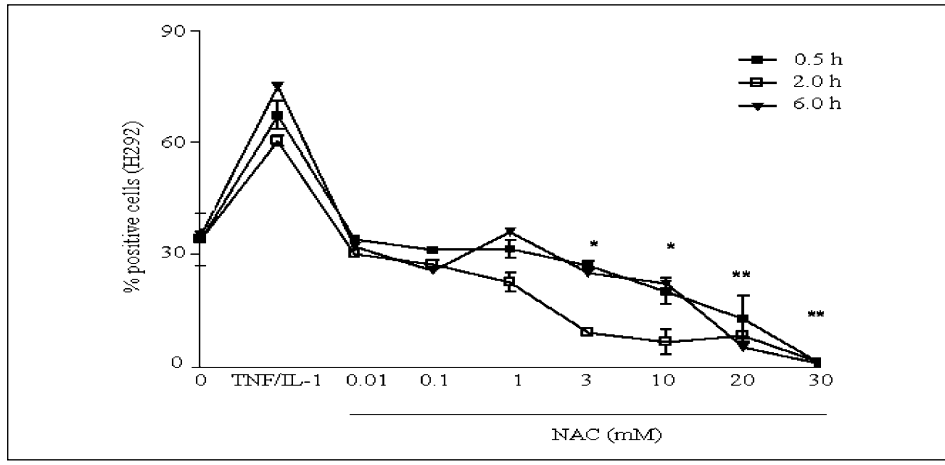

Fig. 2. Effects of incubation of epithelial cells (H292) with increasing NAC concentrations on TNF-α/IL-1β- induced ICAM-1 expression. Data are % of positive cells; n =3 in duplicate. Solid squares - 0.5 h, open squares - 2 h, and solid triangles - 6 h of NAC incubation. *P< 0.05 compared with positive control.

NAC inhibits soluble ICAM-1 in endothelial cell supernatants

The soluble ICAM-1 (sICAM-1) measured in supernatants of the endothelial cells also was decreased by >90% after incubation with NAC at a concentration of 30 mM. At 10 mM, the inhibition was 77%. Concentrations =1 mM did not have a significant effect on sICAM-1 (inhibition <20%) (*Fig. 3*). Moreover, there was a positive correlation between mICAM-1 and sICAM-1 measured in supernatants in the ECV cells (r^2=0.9, P<0.01) (*Fig. 4*).

NAC inhibits IL-8 release from endothelial and epithelial cells.

There was a decrease in IL-8 release from both cell lines after NAC incubation (30 mM) (*Fig. 5*) correlating with the decrease in the percentage of ICAM-1 positive cells (for ECV cells: r^2=0.8, P<0.05).

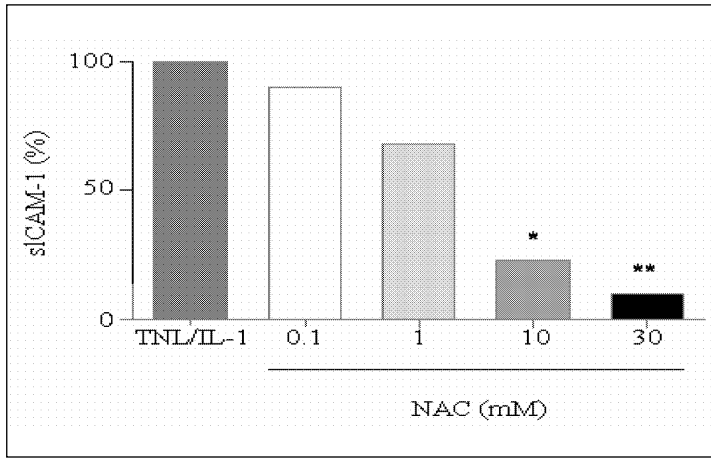

Fig. 3. Decreases in soluble ICAM-1 in ECV supernatants after preincubation with increasing concentrations of NAC compared with the positive control (TNF-α/IL-1β) taken as 100%. *P<0.05, **P<0.01 compared with the positive control (TNF-α/IL-1β).

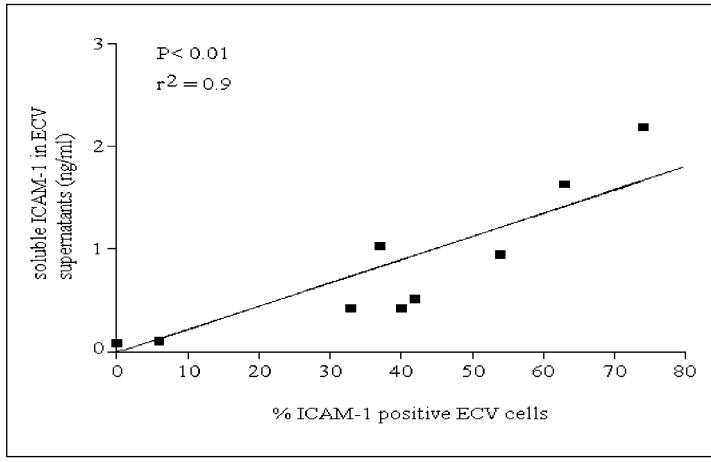

Fig. 4. Correlation between soluble ICAM-1 (sICAM-1) in supernatants from ECV culture and membrane-associated mICAM-1 (% of positive cells).

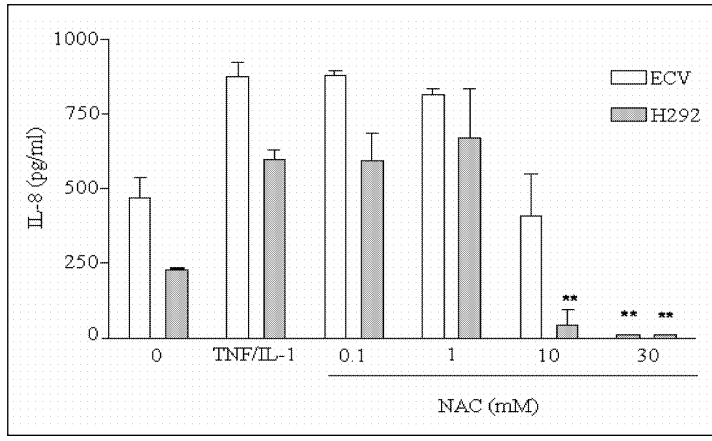

Fig. 5. Decreases in IL-8 release from ECV and H292 cells after incubation with increasing concentration of NAC. *P<0.05, **P<0.01 compared with the positive control (TNF-α/IL-1β).

DISCUSSION

The present study demonstrates that the pre-treatment of endothelial and epithelial cells with NAC efficiently inhibits the IL-1β and TNF-α- induced ICAM-1 expression as well as IL-8 release. This inhibition is more evident at high concentrations (>10 mM) for both cell lines, but there is also a slight decrease in ICAM-1 expression at concentrations lower than 10 mM in the epithelial cell line.

Other *in vitro* studies report the inhibitory effect of NAC on a battery of inflammatory cytokines and inflammatory cell accumulation and migration. These effects are observed only at high concentrations of NAC and these observations are in agreement with the present results. Namely, NAC has been observed to inhibit matrix metalloproteinase -9 (MMP-9), IL-8, IL-6, TNF-α, and NF-κB activation in human fetal membranes at concentrations =5mM (26). Moreover, it was also reported to inhibit IL-8-induced upregulation of CD11b at concentrations >10 mM in a dose-dependent manner (27). The factors that play an important role in the regulation of IL-8 and ICAM-1, namely, p38 MAPK (28) and NF-κB (26), were also shown to be attenuated by NAC and this fact would explain the observed parallel inhibitory effect directed toward IL-8 and ICAM-1.

Since high concentrations of NAC may be achieved after its intravenous/intraperitoneal administration *in vivo* (the infusion of 150mg/kg within 15 min results in a peak of 554mg/L (3.4 mM) (29)), some anti-inflammatory effects of NAC could be expected in such studies. Nevertheless, these reports are equivocal. NAC did not affect ICAM-1 increase during ischemia/reperfusion study in rats (30), but it attenuated the increase in circulating ICAM-1 after reperfusion in humans undergoing liver transplantation (31). Furthermore, it decreased the IL-8 in sepsis patients with no effect on ICAM-1 (32).

On the other hand, when administered orally at 600 mg/day NAC fails to reach the concentration that proved to be necessary for its anti-inflammatory effects *in vitro*. Peak plasma concentrations of NAC following oral administration of 600 mg reached 4.6 μM after 60 min and 2.5 μM after 90 min (33). Moreover, no NAC was detectable 10-12 hours after oral administration (34). This fact would exclude any direct anti-inflammatory action of NAC after oral administration. Nevertheless, there are reports suggesting some anti-inflammatory actions in COPD following an oral dose of 600 mg/day. Van Overveld et al (35) reported a decrease in neutrophil chemoattractant properties of the COPD patients' sputum after 8 weeks of NAC treatment at 600 mg/day. Since the main chemoattractants for neutrophils in sputum are IL-8 and LTB4, it could be suspected that NAC would decrease the concentration of those chemoattractants in the sputum. In a previous study, conducted in stable COPD patients, we observed modulation of the interrelationship between inflammatory and oxidative stress markers by NAC, implying that some inflammation regulating pathways are influenced by it (36). It would be plausible that NAC affects the inflammatory response in two ways. Firstly, through direct effects on the signaling pathways, the effect observed at high concentrations, and secondly, through a sustained effect on the cellular concentration of thiols. The latter effect would be observed after long-term NAC administration (37) and might lead to changes in the redox status of cells (38) and thus to modulation of the redox-sensitive transcription factor activation (39). This may result in the attenuation of inflammatory responses (40, 41).

In summary, we investigated the inhibition of ICAM-1 expression and IL-8 release by NAC. Only concentrations >1 mM of NAC successfully decreased the number of the ICAM-1 positive cells and IL-8 release *in vitro*. The reports suggesting some anti-inflammatory action at lower concentrations of NAC, which are reached following an oral dose of 600 mg/day, require further elucidation. Therefore, identification of the pathways and factors that are influenced by NAC would contribute to the development of better preventive and therapeutic strategies that may help slow down the process of the lung destruction caused by excessive inflammation and oxidative stress.

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