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# *N*-acetylcysteine reduces chemokine release *via* inhibition of p38 MAPK in human airway smooth muscle cells

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N-*acetylcysteine reduces chemokine release* via *inhibition of p38 MAPK in human airway smooth muscle cells. W.A. Wuyts, B.M. Vanaudenaerde, L.J. Dupont, M.G. Demedts, G.M. Verleden. ©ERS Journals Ltd 2003.*

**ABSTRACT: Reactive oxygen species are involved in the activation of several mitogen-activated protein kinases (MAPKs), key-players in the production of several cytokines. Therefore the current study investigated whether *N*-acetylcysteine (NAC), an antioxidative agent, inhibits the interleukin (IL)-1β-induced expression and production of eotaxin and monocyte chemotactic protein (MCP)-1 in human airway smooth muscle cells (HASMC).**

**NAC (10 mM) decreased the expression of eotaxin and MCP-1, by 46±11% (n=7) and 87±4% (n=6), respectively; the eotaxin release was inhibited by 75±5% (n=7), whereas the MCP-1 release was decreased by 69±4% (n=10). NAC (1 mM) also decreased the IL-1β-induced activation of p38 MAPK.**

**Compared with unstimulated cells, a four-fold increase in 8-isoprostane production in IL-1β-stimulated HASMC was observed, which could be inhibited by NAC in a concentration-dependent way, with a maximum inhibition of 39±12% with 1 mM NAC.**

**The present study demonstrated that *N*-acetylcysteine inhibits the interleukin-1β-induced eotaxin and monocyte chemotactic protein 1 expression and production due to a decreased activation of p38 mitogen-activated protein kinase. This study has also shown that *N*-acetylcysteine decreases the interleukin-1β-induced production of reactive oxygen species, as suggested by a reduction in the 8-isoprostane production.**
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Asthma is a complex disease associated with airflow obstruction and bronchial hyperresponsiveness. Recent studies have shown substantial inflammation in bronchial biopsy specimens from patients with asthma, even those with mild disease. These inflammatory changes can occur throughout the central and peripheral airways and often vary with the severity of the disease [1]. Several cells have been shown to play a crucial role in the airway inflammation in asthma, such as mast cells, eosinophils and lymphocytes [2]. These inflammatory cells are attracted to the locus of inflammation by cytokines with a chemotactic function, the so-called chemokines, such as eotaxin, which specifically attracts eosinophils, whereas monocyte chemotactic protein-1 (MCP-1) attracts several types of inflammatory cells, such as monocytes and lymphocytes. The present authors have already demonstrated that interleukin (IL)-1β-stimulated human airway smooth muscle cells (HASMC) are able to express and produce these chemokines *in vitro* [3]. They have also previously identified several elements, such as p38 mitogen-activated protein kinase (MAPK), cJun N-terminal kinase (JNK) and p42/p44 extracellular signal-regulated kinase (ERK) that are involved in IL-1β-induced eotaxin, MCP-1 and MCP-3 expression and release in HASMC *in vitro* [4].

It has become clear that oxidative stress plays an important role in the onset and progression of inflammation. Recently, more evidence regarding the involvement of oxidative stress in the pathophysiology of asthma has emerged. Indeed, F2-isoprostanes are shown to be increased 2 h after allergen challenge in urine of patients with atopic asthma [5]. 8-Isoprostane is a prostaglandin (PG)$F_2$-like compound and is produced *in vivo* by the free radical-catalysed peroxidation of arachidonic acid. It has been used as a biomarker of oxidative stress [6]. An increase in 8-isoprostane has been found in breath condensate of mild, moderate and severe asthma patients in comparison with normal subjects [6]. The thiol reducing agent *N*-acetylcysteine (NAC) is known as a direct scavenger of reactive oxygen species (ROS), but also as an indirect antioxidative agent, acting by increasing intracellular glutathione [7]. NAC has been commonly used in studies to establish the role of ROS in gene expression and signalling pathways of MAPK activation by cytokines [8].

It has been observed previously that ROS are involved in the activation of p38 MAPK and other key elements of the signal transduction in several cell types [9–11].

The current study investigated whether NAC could attenuate the IL-1β-induced chemokine expression/production in HASMC and, moreover, which signalling mechanisms may be involved. Therefore, the study also investigated whether there is an effect of NAC on the activation of p38 MAPK, JNK, or ERK.

## Materials and methods

### *Culture of human airway smooth muscle cells*

HASMC were isolated and cultured from explants of human bronchial smooth muscle, as described previously [3]. Airway tissue was obtained from patients undergoing surgery for lung carcinoma in accordance with procedures approved by the local ethical committee. These patients never received any chemotherapeutics before the operation. None of the patients had clinical characteristics of asthma. Primary cell cultures used for the experiments showed >95% of cells staining for smooth muscle actin. After reaching confluence the cells were washed with phosphate-buffered saline and incubated with serum-free Dulbecco's Modified Eagle Medium, L-glutamine (2 mM), penicillin (100 $U \cdot mL^{-1}$), streptomycin (100 $\mu g \cdot mL^{-1}$) and amphotericin B (1.25 $\mu g \cdot mL^{-1}$). All experiments were carried out between passage three and six. The HASMC were obtained from four different patients.

### *Experimental protocol*

HASMC were stimulated with IL-1β (10 $ng \cdot mL^{-1}$) alone or in combination with different concentrations of NAC (0.01–10 mM), added 30 min before IL-1β. Messenger ribonucleic acid (mRNA) was measured after 4 h and proteins were measured after 24 h of stimulation with IL-1β, the previously published optimal time intervals for these measurements [3]. Samples used for 8-isoprostane measurement were stimulated for 24 h.

### *Measurement of eotaxin protein by use of enzyme-linked immunosorbent assay*

MCP-1 and eotaxin protein were measured in supernatant of cultured HASMC. This was done with a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Europe Ltd, Abingdon, UK), as specified by the manufacturer. These ELISA kits are highly specific; there is no significant interference between any of the cytokines being investigated. The sensitivity of these assays is high, with a lower detection limit of 5.0 $pg \cdot mL^{-1}$ for both MCP-1 and eotaxin.

### *Northern blot analysis*

Total ribonucleic acid (RNA) was isolated by phenol/chloroform extraction and isopropanol precipitation as described previously by CHOMCZYNSKI *et al.* [12]. Hybridisation was performed as described by PYPE *et al.* [3] with a 227 base pair (bp) fragment specific to the human eotaxin complementary deoxyribonucleic acid (cDNA; A. Yokoyama, Ehime, Japan), a 170 bp fragment specific to the human MCP-1 cDNA (G. Opdenakker, Rega Institute, Leuven, Belgium), and a 1,200 bp cDNA fragment specific to rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA purchased from Clontech (Heidelberg, Germany). These experiments were performed in duplicate. The duplicate values were averaged.

The RNA levels were expressed as the ratio of chemokine mRNA to GAPDH mRNA.

### *Immunoblot analysis of p38 mitogen-activated protein kinase, cJun N-terminal kinase and extracellular signal-regulated kinase*

After extraction of cytosolic proteins, the threonine and tyrosine phoshorylation of p38 MAPK, JNK and ERK were analysed by Western blot analysis, using phospho-p38 MAPK, JNK and ERK polyclonal antibodies that only react with the phosphorylated form of the MAPK studied. The assay was performed as described previously [13]. Phospho-p38 MAPK, phospho-JNK, phospho-ERK, p38 MAPK, JNK and ERK polyclonal antibodies were diluted as recommended by the manufacturer (New England Biolabs, Beverly, MA, USA).

After the adequate exposure time, the autoradiographs were developed and analysed by a laser densitometer. The levels were expressed as the ratio of phosphorylated to unphosphorylated MAPK.

### *Measurement of 8-isoprostane by use of an enzyme immunoassay kit*

Samples used for 8-isoprostane were immediately frozen and stored at -80°C and measured within 2 months. The concentrations of 8-isoprostane were measured with a specific enzyme immunoassay (EIA) kit. The antiserum used in the EIA has 100% cross-reactivity with 8-isoprostane and 0.2% cross-reactivity each with $PGF_{2\alpha}$, $PGF_{3\alpha}$, $PGE_1$ and $PGE_2$, and 0.1% cross-reactivity with 6-keto-$PGF_{1\alpha}$. The detection limit of the assay is 4 $pg \cdot mL^{-1}$.

### *Statistics*

All data are presented as mean±SEM. Statistical analysis was performed using the Mann-Whitney U-test.

## Results

### *The effect of* N*-acetylcysteine on interleukin-1β-induced eotaxin expression and production in human airway smooth muscle cells*

IL-1β produced an increase in eotaxin expression that peaked after 4 h with a maximum effect of 10 $ng \cdot mL^{-1}$ [3]. Pre-incubation for 30 min with NAC (0.01–10 mM) inhibited the IL-1β-induced expression of eotaxin mRNA in a concentration-dependent manner. NAC (10 mM) induced a maximal reduction of 46±11% (n=7, $p<0.05$), when compared with IL-1β alone (fig. 1a and b).

The eotaxin protein release reached a peak (18±2 $ng \cdot mL^{-1}$) after 24 h of stimulation. NAC led to a concentration-dependent decrease in the IL-1β-induced eotaxin protein production, resulting in a decrease of 75±5% with 10 mM NAC (n=7, $p<0.05$; fig. 1c).

### *The effect of* N*-acetylcysteine on interleukin-1β-induced monocyte chemotactic protein-1 expression and production in human airway smooth muscle cells*

MCP-1 mRNA expression was decreased by NAC in a concentration-dependent manner, with a maximal reduction of 87±4% (n=6, $p<0.01$) at a concentration of 10 mM (fig. 2a and b).

The MCP-1 protein release reached a peak (96±11 $ng \cdot mL^{-1}$) after 24 h of stimulation. Incubation with NAC results in

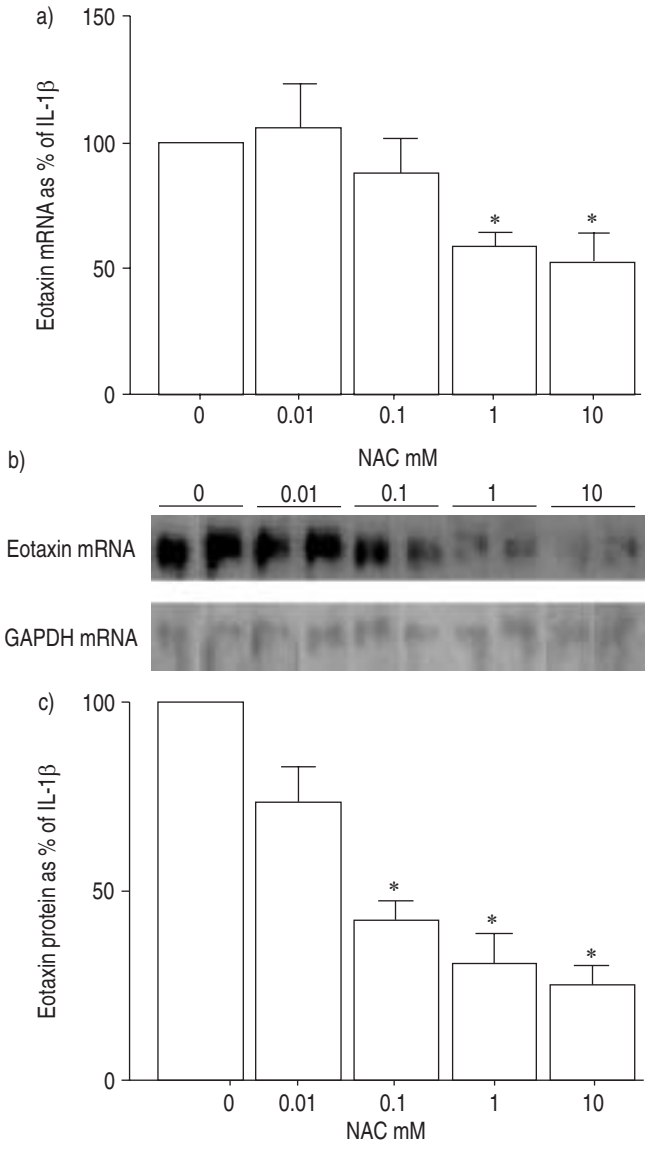


Fig. 1. – Human airways smooth muscle cells (HASMC) stimulated with interleukin (IL)-1β (10 ng·mL$^{-1}$) and coincubated with different concentrations of *N*-acetylcysteine (NAC) (0.01–10 mM), messenger ribonucleic acid (mRNA) and protein were measured after 4 and 24 h, respectively. a) Shows the effects of different concentrations of NAC (0.01–10 mM) on eotaxin mRNA expression. b) One representative blot is shown (from n=7) for eotaxin mRNA, presenting the results of one experiment in duplicate. The mRNA levels were expressed as the ratio of chemokine mRNA to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. c) Shows the same data for eotaxin release (n=7). For b) and c), data are expressed as a percentage of the control response to IL-1β alone and are represented as mean±SEM of duplicate values from independent experiments using cells cultured from four different donors, cell passages three to six. *: $p<0.05$.

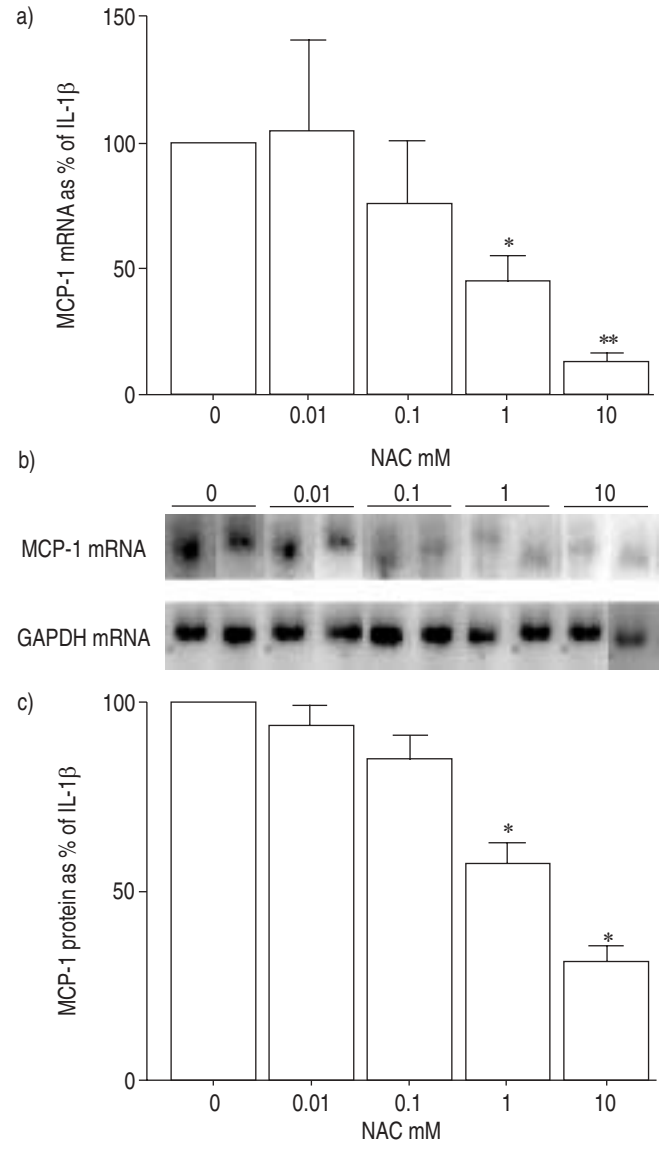


Fig. 2. – a) This figure shows the effects of different concentrations of *N*-acetylcysteine (NAC) (0.01–10 mM) on monocyte chemotactic protein (MCP)-1 messenger ribonucleic acid (mRNA) expression (all reactions stimulated with interleukin (IL)-1β (10 ng·mL$^{-1}$)). b) One representative blot is shown (from n=6), presenting the results of one experiment in duplicate. The RNA levels were expressed as the ratio of chemokine mRNA to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. c) Shows the same data for MCP-1 release (n=10). For b) and c), data are expressed as a percentage of the control response to IL-1β alone and are represented as mean±SEM of duplicate values from independent experiments using cells cultured from four different donors, cell passages three to six. *: $p<0.05$; **: $p<0.01$.

a concentration-dependent decrease in the IL-1β-induced MCP-1 protein production, resulting in a decrease of 69±4%, with 10 mM NAC (n=10, $p<0.05$; fig. 2c).

### *Interleukin-1β-induced activation of p38 mitogen-activated protein kinase in human airway smooth muscle cells is decreased by* N-*acetylcysteine*

Immunoblot analysis showed no phosphorylated p38 MAPK in unstimulated HASMC. The level of phosphorylated tyrosine of p38 MAPK in cells stimulated with 10 ng·mL$^{-1}$ IL-1β peaked between 5–15 min and was comparable with baseline after 60 min.

In order to examine the effect of NAC on IL-1β-induced p38 MAPK activation, cells were pre-incubated with 1 mM NAC for 30 min and then stimulated for 5, 15, 30, 60 or 120 min with IL-1β. Phosphorylated p38 MAPK was measured at these time points. Pre-incubation with NAC significantly inhibited the phosphorylated p38 MAPK level when compared with cells treated with IL-1β alone. There was no difference in the nonphoshorylated p38 MAPK at the

different time points measured (fig. 3a and b), suggesting that the amount of protein blotted was equal for the different samples.

### N-*acetylcysteine did not affect the interleukin-1β-induced cJun N-terminal kinase and extracellular signal-regulated kinase activation*

IL-1β (10 ng·mL$^{-1}$) also induced phosphorylation of threonine and tyrosine of JNK and ERK, with a peak activation after 15–30 min. Thereafter, the activation decreased and disappeared after 60 min. There was no difference either in JNK nor in ERK activation, compared with control levels after adding 1 mM NAC. There was a slight increase in the amount of unphosphorylated JNK after 15 min, but this was of no importance due to the fact that the ratio of phosphorylated and unphosphorylated protein was used (fig. 3c–f).

### *The effect of interleukin-1β and* N-*acetylcysteine on the production of 8-isoprostane in human airway smooth muscle cells*

The addition of IL-1β (10 ng·mL$^{-1}$) induced a four-fold increase ($p<0.0001$, n=10) in the production of 8-isoprostane when compared with unstimulated HASMC (fig. 4).

The release of 8-isoprostane from IL-1β-stimulated HASMC was inhibited by NAC in a concentration-dependent manner with a maximum decrease of 39±12% ($p<0.05$, n=5) with 1 mM NAC (fig. 4).

## Discussion

In this study an inhibitory effect of NAC on the IL-1β-induced eotaxin and MCP-1 expression and release in HASMC *in vitro* was observed. In addition, there was an inhibitory effect of NAC on the IL-1β-induced activation of p38 MAPK, although there was no effect of NAC on the

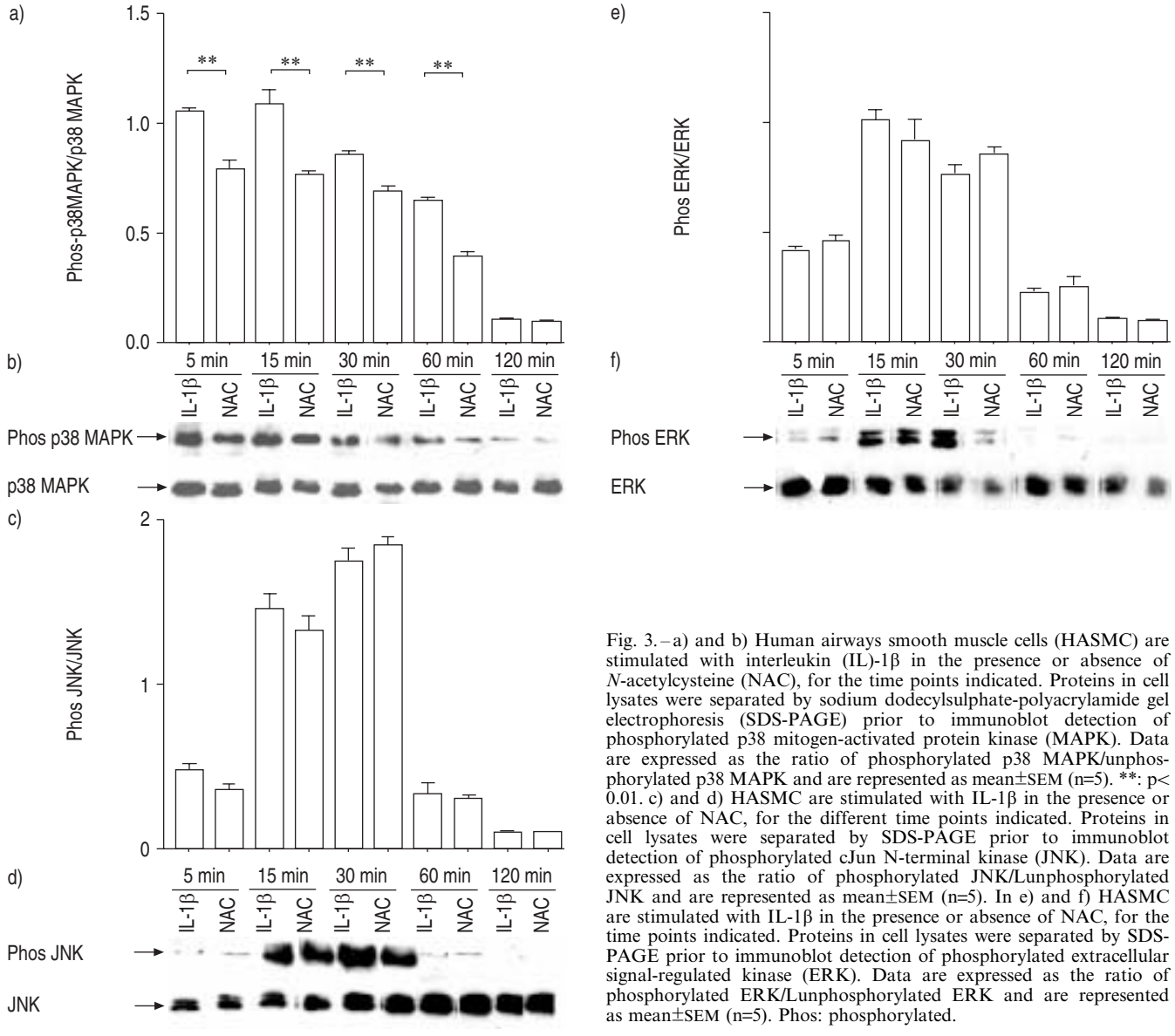


Fig. 3.– a) and b) Human airways smooth muscle cells (HASMC) are stimulated with interleukin (IL)-1β in the presence or absence of *N*-acetylcysteine (NAC), for the time points indicated. Proteins in cell lysates were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) prior to immunoblot detection of phosphorylated p38 mitogen-activated protein kinase (MAPK). Data are expressed as the ratio of phosphorylated p38 MAPK/unphosphorylated p38 MAPK and are represented as mean±SEM (n=5). **: $p<0.01$. c) and d) HASMC are stimulated with IL-1β in the presence or absence of NAC, for the different time points indicated. Proteins in cell lysates were separated by SDS-PAGE prior to immunoblot detection of phosphorylated cJun N-terminal kinase (JNK). Data are expressed as the ratio of phosphorylated JNK/Lunphosphorylated JNK and are represented as mean±SEM (n=5). In e) and f) HASMC are stimulated with IL-1β in the presence or absence of NAC, for the time points indicated. Proteins in cell lysates were separated by SDS-PAGE prior to immunoblot detection of phosphorylated extracellular signal-regulated kinase (ERK). Data are expressed as the ratio of phosphorylated ERK/Lunphosphorylated ERK and are represented as mean±SEM (n=5). Phos: phosphorylated.

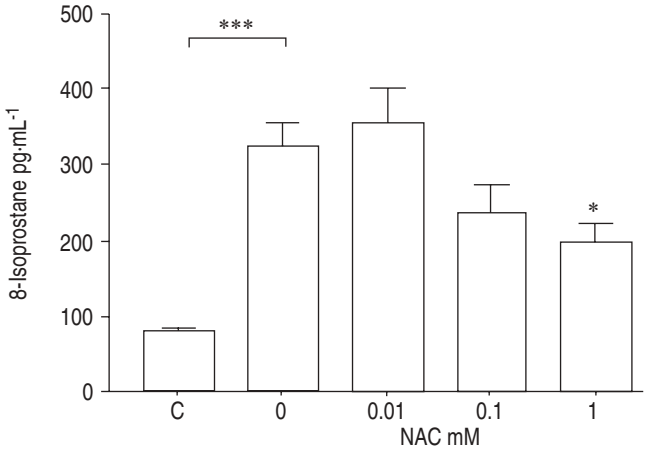


Fig. 4. – The effect of different concentrations of *N*-acetylcysteine (NAC) (0.01 mM, 0.1 mM and 1 mM) on 8-isoprostane release from cells stimulated with interleukin (IL)-1β (10 ng·mL$^{-1}$) is shown. In the negative control (C), no NAC or IL-1β were used. Data are represented as mean±SEM of duplicate values from independent experiments using cells cultured from four different donors, cell passages three to six. *: $p<0.05$; ***: $p<0.001$.

IL-1β-induced ERK, nor on the JNK activation. Furthermore, the present study has shown that IL-1β is able to increase the formation of 8-isoprostane, which was significantly decreased after addition of NAC. This is, to the best of the authors' knowledge, the first time that NAC has been shown to inhibit the IL-1β-induced eotaxin and MCP-1 expression and production in HASMC. It was described previously that NAC inhibits tumour necrosis factor (TNF)-α-induced IL-8 production in human pulmonary vascular endothelial cells [14]. Also in cultured human synovial cells, it was observed that NAC inhibits the TNF-α-mediated expression of IL-8 and MCP-1 [15]. The mechanisms of this inhibition, however, remain unclear.

From previous studies it is known that p38 MAPK, JNK and ERK are key-players in the IL-1β-induced expression and production of chemokines. In general, the MAPK pathways have crucial roles in mediating signals triggered by cytokines, growth factors and environmental stress, and are involved in cell proliferation, cell differentiation and cell death. MAPKs are activated by the dual phosphorylation of threonine and tyrosine residues by upstream kinases, collectively termed MAPK kinases (MKKs). MKK3, MKK4 and MKK6 phosphorylate p38 MAPK on the Thr-Gly-Tyr motif [16]. As a consequence of this, the current authors investigated whether NAC exerts any effect on these elements of the MAPK family. The current study demonstrated an inhibition of p38 MAPK activation in HASMC, whereas there was no effect of NAC on the activation of JNK or ERK.

In the literature, the effect of NAC on p38 MAPK is cell type-dependent; in human bronchial epithelial cells, NAC did not affect influenza virus infection-induced increases in p38 MAPK phosphorylation [17], but in other cell types such as in human pulmonary vascular endothelial cells, there is some evidence that NAC affects the activation of p38 MAPK [14, 18].

A possible role in this inhibition can be attributed to ROS. An increase in the production of ROS in bronchoalveolar lavage fluid (BALF) has been shown in adults and children with distinct asthma severity and acute exacerbations of asthma [19, 20].

Different sources of ROS are possible in asthma. Environmental factors such as diesel exhaust particles, cigarette smoke and ozone are able to increase the generation of ROS in the airways [21]. It has also been shown that inflammatory cells have the capability of producing ROS [22]. Constitutive airway cells such as epithelial cells are a third source of ROS [23]. In contrast to the conventional idea that ROS are mostly triggers for oxidative damage of biological structures, it is clear that a low physiological concentration of ROS can regulate cell signalling and gene expression, with important effects on inflammation, proliferation, and apoptosis [24]. There is a lot of evidence in the literature that ROS are also involved in the activation of MAPKs [25].

Indeed, ROS seem to be involved in the activation of p38 MAPKs in different cell types, such as vascular smooth muscle cells [11, 26, 27] and bronchial epithelial cells, stimulated with diesel exhaust particles [18].

There is ample evidence that oxidative injury occurs in allergic inflammation. In patients with atopic asthma, $F_2$-isoprostanes, free radical-catalysed products of arachidonic acid and urinary excretion of 15-$F_2$t-isoprostane-M increased at 2 h after allergen challenge [5]. $F_2$-isoprostanes were found to be increased in BALF 24 h after segmental instillation of the allergen [28]. The current authors have now shown for the first time that the production of 8-isoprostane is increased in IL-1β-stimulated HASMC. This indicates that IL-1β is able to increase oxidative stress and, hence, ROS in HASMC *in vitro*.

The present study has also demonstrated that NAC is able to reduce the IL-1β-induced production of 8-isoprostane in HASMC.

Thus, it is clear that ROS in physiological concentrations are important cofactors in different physiological and pathophysiological processes, and possibly also in the IL-1β-induced activation of p38 MAPK. A possible explanation for the observations of the current study could be that IL-1β induces an increase in ROS, which in turn activate p38 MAPK. NAC may then reduce the production of ROS and, as a consequence, the production of 8-isoprostane. This may then explain the reduced activation of p38 MAPK and the inhibition of the expression and release of eotaxin and MCP-1 by NAC in IL-1β-stimulated HASMC.

NAC is known as a drug that easily enters the cell and because of its -SH group, it is able not only to scavenge ROS, such as hydrogen peroxide and the hydroxyl radical, but it can also easily be deacetylated to cysteine, an important precursor of cellular glutathione synthesis. Therefore NAC scavenges oxidants both directly and indirectly [7, 29–31].

Although the pathogenesis of allergic inflammation is complex, in the last few years it has become clear that decreasing the attraction of inflammatory cells could be an important element in treatment of the allergic inflammation process. The present study showed that NAC decreases IL-1β-induced eotaxin and MCP-1 expression and production.

Enhancement of antioxidant defence mechanisms, therefore, seems a rational therapeutic option. Antioxidant therapy, including NAC, has been reported to be useful in the treatment of acute lung injury [32, 33]. Understanding the key elements of the redox control mechanism of IL-1β-induced eotaxin and MCP-1 expression and production by HASMC, may indicate a new strategy in controlling airway inflammation. The current study provides some *in vitro* evidence that NAC, an antioxidative agent that has been used for many years in Europe as a mucolytic drug, could also be useful in the treatment of more chronic inflammatory diseases such as asthma, however, it appears debatable whether or not orally administered NAC can produce a sustained increase in glutathione levels sufficient to increase the antioxidant capacity of the lungs. Although major side effects of NAC are rather infrequent [34], caution is needed, since intravenous administration of NAC has been reported to cause adverse reactions, such as bronchoconstriction, which happens more frequently in asthmatics [35]. It is not

known, at the present time, whether NAC is capable of producing a beneficial effect in controlling the airways inflammation *in vivo*. However, if NAC, a relative harmless molecule, is able to exert any anti-inflammatory effect, this can be used in combination with existing, potent, but potentially more harmful, drugs. This hypothesis, however, needs further investigation.

To conclude, the current study showed that *N*-acetylcysteine decreased the interleukin-1β-induced eotaxin and monocyte chemotactic protein-1 expression and release in human airway smooth muscle cells in a concentration-dependent manner. In this study, *N*-acetylcysteine was able to reduce the activation of p38 mitogen-activated protein kinase, induced by interleukin-1β. The current study also demonstrated that interleukin-1β was able to increase the 8-isoprostane production in human airway smooth muscle cells, which could be decreased by *N*-acetylcysteine in a concentration-dependent way.

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