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Inhibitory effects of *N*-acetylcysteine on the functional responses of human eosinophils *in vitro*

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Clinical and Experimental Allergy

Summary

Background Oxidative stress appears to be relevant in the pathogenesis of inflammation in allergic diseases like bronchial asthma. Eosinophils are oxidant-sensitive cells considered as key effectors in allergic inflammation.

Objective The aim of this work was to study the effects of the clinically used antioxidant *N*-acetyl-L-cysteine (NAC) on the functional responses of human-isolated eosinophils.

Methods Human eosinophils were purified from the blood of healthy donors by a magnetic bead separation system. The effects of NAC were investigated on the generation of reactive oxygen species (chemiluminescence and flow cytometry), Ca^{2+} signal (fluorimetry), intracellular glutathione (GSH; flow cytometry), p47^{phox}–p67^{phox} translocation (Western blot) and eosinophil cationic protein (ECP) release (radioimmunoassay).

Results NAC (0.1–1 mM) inhibited the extracellular generation of oxygen species induced by *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) and eotaxin (in the presence of IL-5) with $-\log\text{IC}_{50}$ values of 3.61 ± 0.03 and 3.36 ± 0.09 , respectively. Also, the intracellular generation of hydrogen peroxide was virtually abolished by NAC (0.5–1 mM). NAC (1 mM) did not alter the fMLP-induced Ca^{2+} signal but augmented the eosinophil content of reduced GSH and inhibited p47^{phox}–p67^{phox} translocation. NAC inhibited the release of ECP (~90% inhibition at 1 mM) from fMLP-activated eosinophils.

Conclusion Inhibition by NAC of human eosinophil functions *in vitro* is potentially useful in the treatment of allergic inflammation.

Keywords eosinophil cationic protein, human eosinophils, *N*-acetylcysteine, p47^{phox}–p67^{phox}, reactive oxygen species

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Introduction

There is increasing evidence that enhanced levels of reactive oxygen species (ROS) and/or defective endogenous antioxidant defences are involved in allergic diseases like bronchial asthma [1–3]. Eosinophils are effector cells in allergic inflammation and asthma [4]. They generate a variety of proinflammatory mediators including oxidant and non-oxidant [e.g. eosinophil cationic protein (ECP)] cytotoxic materials [5, 6]. Thiol-sensitive redox regulation is important in eosinophil function [7]. Indeed, the activity of NADPH oxidase is higher in eosinophils compared with other phagocytes whereas their intracellular glutathione (GSH)

concentration is half that of neutrophils [8]. Hence, eosinophils are sensitive to oxidant stress, and antioxidant drugs may modulate eosinophil function and have a potential role in the pharmacologic treatment of allergic inflammation.

Although initially used as a mucolytic, *N*-acetyl-L-cysteine (NAC) is a thiol compound that acts directly as a free radical scavenger and precursor in reduced GSH synthesis, thus protecting cells from oxidant damage [9, 10]. Experimental studies indicate that oral administration of NAC is beneficial in animal models of allergic asthma [11] and NAC has proved to be clinically useful in pulmonary diseases linked to oxidative stress [12]. However, bronchoconstriction has been occasionally reported

after inhalation of NAC in humans, especially in asthmatics, likely due to an anaphylactoid reaction [13].

The present work examines, for the first time, whether the oxidative burst in human eosinophils is inhibited by NAC. Eosinophils were stimulated with *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), a soluble inflammatory mediator that binds to specific membrane receptors [14]. Eotaxin and IL-5 were also used to activate eosinophils due to their importance as inflammatory mediators in asthma [15]. The effect of NAC on fMLP-induced release of ECP was also examined as a non-oxidant marker in allergic inflammation [16]. Part of this work was reported as an abstract [17].

Methods

Isolation of human eosinophils

Human blood from healthy donors was obtained and polymorphonuclear leucocytes were separated by standard laboratory procedures [18]. Eosinophils were then separated by depletion of neutrophils with anti-CD16-coated magnetic microbeads using the magnetic cell separation system (MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany) as outlined [19]; eosinophils of >95% purity (determined by May-Grünwald-Giemsa) and viability (Trypan blue exclusion) were used. This investigation was approved by the institutional ethics committee and informed consent was obtained from all donors.

Cytotoxicity assessment

The percentage of lactate dehydrogenase (LDH) release compared with values in cell lysates was taken as marker for cell damage by using a commercially available colorimetric assay as outlined [20].

Chemiluminescence assay

The formation of ROS was assessed by luminol-enhanced chemiluminescence essentially as described previously [21]. In brief, aliquots (100 µL) of the cell suspension (10^6 cells/mL) were pre-incubated for 10 min at 37 °C in Krebs solution with (mM) NaCl 140, KCl 5, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid] (HEPES) 10, $\text{CaCl}_2/\text{MgCl}_2$ 1, glucose 5.6 and bovine serum albumin (BSA) 0.05% (w/v), gelatine 0.2%, luminol 5 µM and microperoxidase 2 µM. Cells were pre-incubated with NAC (0.1–1 mM) for 60 min at 37 °C before cell activation with fMLP (100 nM). Chemiluminescence was recorded in a Wallac 1420 Victor² Multilabel counter (EG&G, Turku, Finland) for 3 min and the area under the curve (AUC) was calculated. Control experiments were carried out in parallel with drug vehicle. In additional experiments, eosinophils were pre-incubated with or without NAC (0.1–3 mM) in the presence of IL-5 (IL-5; 2 ng/mL) for 60 min at 37 °C,

before addition of eotaxin (100 ng/mL) and luminol as outlined previously [22], and chemiluminescence was measured as above for 50 min. In further experiments, the effect of NAC (1 mM; 60 min pre-incubation) on serum-opsonized zymosan (SOZ; 0.5 mg/mL)-induced chemiluminescence measured during 50 min was also studied. SOZ was prepared as previously outlined [19]. Drug effects are expressed as percent inhibition from control values. Pilot experiments showed that pre-incubation with NAC for 30 min produced similar inhibitory effects on fMLP-induced chemiluminescence responses as with 60 min of pre-incubation (data not shown); therefore, this shorter pre-incubation time was adopted in some further experiments, as indicated, to reduce their duration.

Flow cytometric analysis of intracellular reactive oxygen species generation

The intracellular oxidative burst was quantified by flow cytometry using 2',7'-dichlorofluorescein diacetate (DCFH-DA) to measure hydrogen peroxide as outlined [23]. Eosinophil suspensions (2×10^5 cells/mL) were incubated with NAC (0.1, 0.5 and 1 mM) for 45 min in Krebs-HEPES solution as above, and then loaded with 6 µM DCFH-DA for an additional 15 min at 37 °C, followed by activation with fMLP (100 nM). Control samples without drug treatment were run in parallel. The conversion of DCFH-DA to its fluorescent derivative (DCFH) was assessed in aliquots taken at the indicated time intervals after stimulation and stored on ice before analysis. Flow cytometric analysis was performed on 5000 cells using an Epics Profile II flow cytometer (Coulter Electronics, Hialeah, FL, USA).

Intracellular Ca^{2+} levels

Measurement of intracellular calcium concentrations ($[\text{Ca}^{2+}]_i$) was performed as outlined [24]. Cells (10^6 /mL) were suspended in Hanks balanced salt solution (HBSS) containing Ca^{2+} 1 mM, glucose 1 mM, BSA 0.5% and Fura2 AM 5 µM, and incubated for 45 min at 37 °C. After loading, eosinophils were washed in pre-warmed HBSS and resuspended as 5×10^5 cells/mL. Then, cells were incubated at 37 °C for 60 min with NAC (1 mM) or its vehicle before addition of fMLP (100 nM). The fluorescence intensity was monitored (excitation wavelengths 340 and 380 nm, emission wavelength 510 nm) using a spectrofluorometer (Perkin Elmer LS50, Waltham, MS, USA) with thermally controlled cuvette holder and a magnetic stirrer. The peak values of $[\text{Ca}^{2+}]_i$ and the AUC over 60 s after activation were calculated as outlined [24].

Measurement of glutathione levels

A flow cytometric assay based on the sulphydryl-reactive stain mercury orange was used to measure non-protein

thiols rather than a standard biochemical assay as mercury orange staining correlates closely with biochemical determinations of GSH [25]. Cells ($3 \times 10^5/\text{mL}$) were incubated in Krebs-HEPES for 30 min at 37°C in the absence and presence of NAC (1 mM) before activation with fMLP (100 nM). In separate experiments, the effect of *N*-ethylmaleimide (NEM), a thiol-alkylating agent, was studied [26]. Experiments were completed at the indicated times by introducing tubes into ice, followed by centrifugation (300 g, 5 min). Cell pellets were resuspended in an ice-cold mercury orange solution, and after 5 min, stained cells were centrifuged (300 g, 5 min), resuspended in phosphate-buffered saline (PBS) and analysed by flow cytometry (Epics Profile II, Coulter Electronics, Hialeah, FL, USA) as outlined [25].

Analysis of the translocation of p47^{phox} and p67^{phox} to eosinophil membrane

Eosinophils ($5 \times 10^5/\text{mL}$) were pre-incubated in HBSS containing 0.5 mM CaCl_2 , 5.6 mM glucose and 20 mM HEPES (pH 7.4) in the presence and absence of 1 mM NAC for 30 min at 37°C , before the addition of a stimulus (100 nM fMLP). The translocation to the cell membrane of p47^{phox} and p67^{phox} was performed as outlined [27]. After standing on ice for 10 min, eosinophils were spun at 1500 g for 5 min at 4°C and resuspended in relaxation buffer [100 mM KCl, 3 mM NaCl, 3.5 mM MgCl_2 , 1.25 mM ethyleneglycoltetraacetic (EGTA) and 10 mM 1,4-piperazinediethane sulphonic acid disodium salt monohydrate, pH 7.4] supplemented with 0.8 mM phenylmethyl sulphonyl fluoride, 10 $\mu\text{g}/\text{mL}$ pepstatin A and 50 $\mu\text{g}/\text{mL}$ leupeptin. Cells were then disrupted by sonication on ice and spun at 500 g for 5 min at 4°C . The resulting post-nuclear supernatants were further centrifuged at 200 000 g for 20 min at 4°C . The pellet, membrane fraction, was resuspended in 35 μL of 1 $\times$ sample buffer and maintained at -80°C until analysis. For immunoblot analysis, the membrane fraction, prepared as indicated above, was subjected to SDS-PAGE on 12% polyacrylamide gel, and the separated proteins were electrophoretically transferred onto nitrocellulose filters, blocked and probed with mouse anti-p47^{phox} and anti-p67^{phox} monoclonal antibodies (BD Biosciences, Franklin Lakes, NJ, USA). After washing, blots were further probed with horseradish peroxidase-conjugated rabbit anti-mouse IgG (DakoCytomation A/S, Glostrup, Denmark), and detected with the ECL Western Blotting Detection System (Amersham International plc, Little Chalfont, UK).

Eosinophil cationic protein release

All assays were performed on duplicate suspensions of 2.5×10^5 cells/mL in Dulbecco's PBS containing $\text{Ca}^{2+}/\text{Mg}^{2+}$ 1 mM, glucose 5 mM and BSA 0.1% (w/v) as outlined [21]. Cell suspensions were pre-incubated with NAC (1 mM)

for 30 min at 37°C before stimulation with fMLP (100 nM) for 30 min. Experiments were terminated by centrifugation, and the supernatants were stored at -20°C . Aliquots of the samples were taken for ECP measurements by an radioimmunoassay (RIA) method according to the manufacturer's instructions (anti-ECP- ^{125}I ; Pharmacia, Pharmacia Biotech, Uppsala, Sweden).

Drugs and solutions, statistical analysis of results

NAC was dissolved in deionized water and diluted in the appropriate buffer media as required (pH 7.4). A stock solution of fMLP was prepared in dimethylsulphoxide and diluted in the buffer media. DCFH-DA was purchased from Molecular Probes (Eugene, OR, USA), dissolved in ethanol at a concentration of 4 mM and stored in the dark at -40°C . Mercury orange [1(4-chloromercuriphenyl-azo-2-naphthol)] from Sigma (Madrid, Spain) was dissolved in acetone [25]. All the other drugs and chemicals used were from the sources stated previously [19, 24]. Water purified on a Milli-Q (Millipore Iberica, Madrid, Spain) system was used throughout.

Data are presented as mean $\pm$ SE mean of *n* experiments. The IC_{50} values were calculated from the concentration-inhibition curves by non-linear regression analysis (GraphPad Software Inc., San Diego, USA). Statistical analysis of results was carried out by ANOVA, followed by the Bonferroni's test or by Student's *t*-test as appropriate (GraphPad Software Inc.). Significance was accepted when $P < 0.05$.

Results

N-acetyl-L-cysteine produced no cytotoxicity in eosinophils

The cytotoxicity of NAC on human eosinophils has not been reported previously but this thiol compound may reduce the viability of blood cells at high concentrations [28, 29]. Therefore, to exclude that any inhibition of eosinophil functions observed with NAC was due to cell damage, we first examined the effect of NAC on LDH release as a marker of cell injury. In this respect, concentrations of NAC above those used in the experiments were studied. NAC, in concentrations up to 5 mM for 2-h incubation, showed no cytotoxicity assessed by LDH release ($2.67 \pm 0.82\%$ of total LDH for NAC 5 mM; $n = 5$). These values were not significantly different from those in control experiments in the absence of NAC (not shown).

N-acetyl-L-cysteine inhibited N-formyl-L-methionyl-L-leucyl-L-phenylalanine- and eotaxin-induced chemiluminescence

The chemotactic peptide fMLP activates eosinophils after binding to specific formyl-peptide receptors to produce an

oxidative burst [21]. Thus, stimulation of eosinophils with fMLP (100 nM) triggered a rapid chemiluminescence signal within seconds that declined towards baseline values in about 2–3 min. NAC decreased in a concentration-related manner the ROS generation by fMLP with a potency of $\sim 250 \mu\text{M}$ ($-\log \text{IC}_{50} = 3.61 \pm 0.03$) as shown in Fig. 1.

To test whether this inhibitory effect of NAC was specific to fMLP, the eosinophil responses to eotaxin in the presence of IL-5 were also examined [22]. These cytokines were selected as mediators involved in the pathogenesis of allergic asthma [15]. NAC inhibited in a concentration-related manner the ROS generation elicited by eotaxin in the presence of IL-5 with a potency of $\sim 400 \mu\text{M}$ ($-\log \text{IC}_{50} = 3.36 \pm 0.09$) as shown in Fig. 1.

Finally, to assure that this inhibitory effect of NAC does not represent a non-specific inhibitory effect, we studied its effect on SOZ-induced ROS generation, a phagocytic stimulus. SOZ elicited a rapid chemiluminescence response that peaked at ~ 10 min and declined to baseline in ~ 50 min. NAC (1 mM) failed to produce any

significant inhibition of SOZ-induced ROS generation ($\text{AUC}_{0-50 \text{ min}}$ amounted $1.55 \pm 0.07 \times 10^6 \text{ cps}$ in the absence and $1.61 \pm 0.06 \times 10^6$ in the presence of NAC; $n = 4$; $P > 0.05$).

N-acetyl-L-cysteine abolished intracellular reactive oxygen species generation

After assessing the inhibitory effect of NAC on the luminol-amplified chemiluminescence, we examined the intracellular release of hydrogen peroxide by fMLP (100 nM)-stimulated eosinophils as measured by flow cytometry using DCFH-DA. The production of ROS was measured at 0 (before stimulation) and at 50, 100 and 160 min post-stimulation of cells. The results expressed as mean fluorescence intensity values are shown in Fig. 2a, and a representative trace is shown in Fig. 2b. NAC produced a concentration-related decrease of fMLP-induced ROS generation that was almost complete at 1 mM NAC.

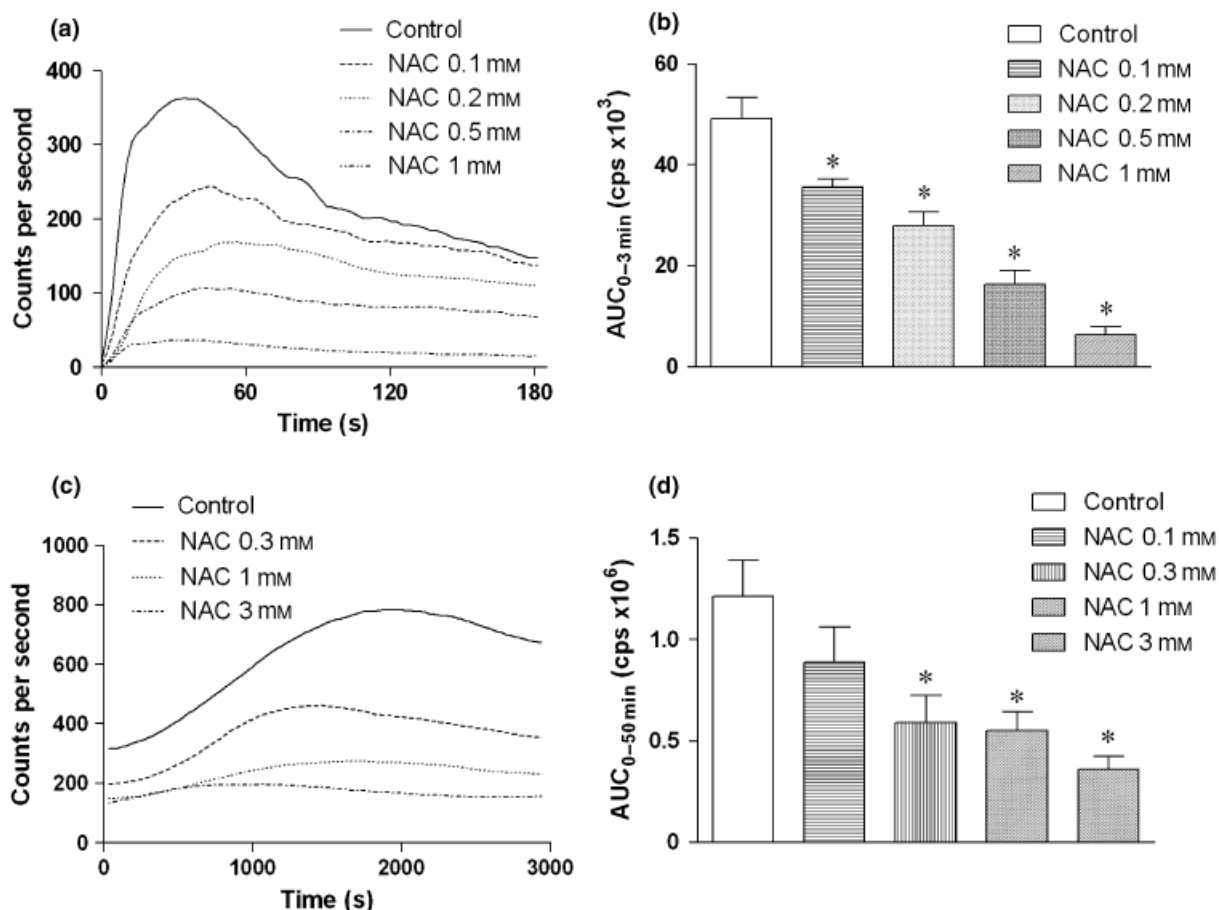


Fig. 1. Inhibitory effects of *N*-acetyl-L-cysteine (NAC) on the reactive oxygen species generation evoked by *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (100 nM) (a, b) or by eotaxin (100 ng/mL; in presence of IL-5) (c, d) in human eosinophils measured by luminol chemiluminescence. (a, c) Results from a representative experiment expressed as absolute values (counts per second). (b, d) Area under the curve (AUC) for the indicated times. Data are mean $\pm$ SEM of five independent experiments.

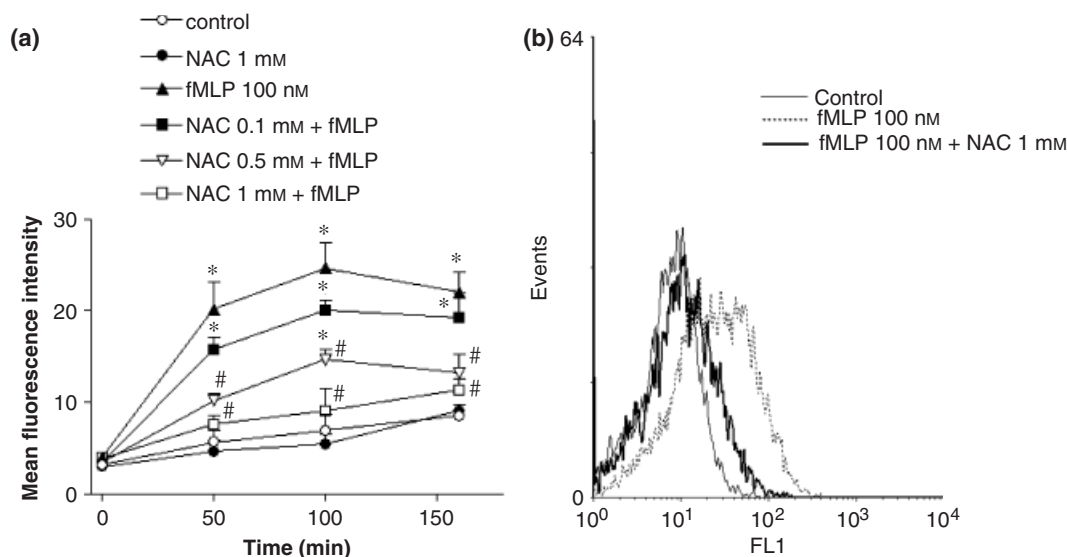


Fig. 2. Inhibitory effects of *N*-acetyl-L-cysteine (NAC) on the hydrogen peroxide generation evoked by *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (100 nM) in human eosinophils measured by flow cytometry with 2',7'-dichlorofluorescein diacetate (DCFH-DA). (a) Shows the fluorescence intensity values at different time-points. Data are mean $\pm$ SEM of four independent experiments. * P < 0.05 from control, # P < 0.05 from fMLP. (b) Shows a representative experiment at 5 min after fMLP addition to cells.

N-acetyl-L-cysteine produced no effect on the *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine-induced increase of intracellular Ca^{2+} levels

Activation of formyl-peptide receptors on eosinophils by fMLP resulted in the activation of phosphatidylinositol-specific phospholipase C and the immediate generation of an intracellular calcium signal [30]. Thus, addition of fMLP (100 nM) resulted in a rapid initial increase in intracellular Ca^{2+} concentration followed by a decline towards baseline levels as reported previously [30]. NAC (1 mM) failed to alter the peak and AUC_{0-60s} responses to fMLP in human eosinophils (Table 1).

N-acetyl-L-cysteine increased eosinophil glutathione levels

The oxidative burst triggered by eosinophil activation with fMLP is expected to result in the consumption of endogenous antioxidants such as cellular GSH as demonstrated previously for human neutrophils [31, 32]. We have extended this finding as we found that exposure of human eosinophils to fMLP transiently decreased GSH content (Fig. 3). In order to determine the specificity of the staining procedure, we used an agent that blocks SH groups such as NEM [26]. Incubation of eosinophils with this reagent markedly decreased the fluorescence emission (Fig. 3), thus confirming that thiol groups were assayed as shown previously [25].

NAC, in addition to scavenging oxidant species directly, serves as a GSH prodrug that increases GSH in human neutrophils [31–33]. Treatment with NAC (1 mM) did not significantly change control GSH levels but, in keeping

with the findings in neutrophils [32], increased GSH levels in fMLP-activated cells (Fig. 3).

N-acetyl-L-cysteine inhibited membrane translocation of $p47^{phox}$ and $p67^{phox}$

As in neutrophils, the ROS generation by eosinophils follows the assembly of NADPH oxidase on the plasma membrane [34]. Thus, after activation with phorbol of guinea-pig eosinophils, the cytosolic components $p47^{phox}$ and $p67^{phox}$ translocate to the plasma membrane to form the active NADPH oxidase [8]. In keeping with this report, we found that eosinophils activated with fMLP (100 nM) showed membrane translocation of $p47^{phox}$ and $p67^{phox}$ (Fig. 4). NAC has previously been shown to inhibit the translocation to the membrane of $p47^{phox}$ and $p67^{phox}$ in human neutrophils [35]. We extend this finding by showing that membrane translocation of $p47^{phox}$ and $p67^{phox}$ in fMLP (100 nM)-activated eosinophils was markedly inhibited by NAC (1 mM) (Fig. 4).

N-acetyl-L-cysteine inhibited eosinophil cationic protein release

In the previous experiments of this study, we have examined the effects of NAC on events related to the oxidative burst of activated eosinophils. Therefore, the effect of NAC on the release of non-oxidant material by degranulation was also examined for comparison. ECP was measured in the supernatant and in the cell pellet of stimulated and resting cells as outlined previously [21]. ECP in the cell pellet of unstimulated cells amounted to

$4463 \pm 208 \text{ ng}/10^6$ ($n = 5$) cells, which is in the range of values reported by others [21, 36]. The percentage of ECP measured in the supernatant of cells was $3.7 \pm 0.3\%$, which is also in agreement with the previously reported values [21]. The activation of eosinophils with fMLP (100 nM) resulted in an almost three-fold increase of ECP release, which was markedly inhibited by NAC (1 mM) (Fig. 5).

Discussion

The main finding of the present work is that NAC inhibits the oxidative burst of fMLP-activated human eosinophils. We are not aware of other studies examining the effect of NAC on the release of ROS from eosinophils either *in vitro*, or *ex vivo* following NAC administration. Although the

Table 1. Effect of NAC (1 mM) on the intracellular calcium ($[\text{Ca}^{2+}]_i$) response elicited by fMLP (100 nM) in human eosinophils

	<i>n</i>	Baseline value (nM)	Δ (nM)	AUC _{0–60s} (nM s)
Control	5	147 ± 14	227 ± 85	7646 ± 3742
NAC	6	134 ± 9	266 ± 45	$10\,041 \pm 1940$

The responses are assessed as the increase of $[\text{Ca}^{2+}]_i$ (Δ , peak value minus baseline), and the area under the curve (AUC) in the interval 0–60 s (AUC_{0–60s}).

Data are mean $\pm$ SEM of the number (*n*) of independent experiments indicated. Differences between groups were not significant by ANOVA ($P > 0.05$).

fMLP; *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; NAC, *N*-acetyl-L-cysteine.

activation of formyl-peptide receptors by chemotactic peptides may play a role in inflammatory lung diseases such as asthma [37], it is of interest to test whether the inhibitory effect of NAC extends to pro-inflammatory cytokines (IL-5) and chemokines (eotaxin) that are relevant to asthma pathogenesis [15]. In this study, we report for the first time that NAC also inhibited eotaxin (in the presence of IL-5)-induced ROS generation with a potency and efficacy similar to those displayed against fMLP as a stimulus. To assure that this inhibitory effect of NAC was not of a non-specific nature, we showed that NAC failed to reduce the SOZ-induced ROS production, a lack of inhibitory activity that is consistent with similar findings in human-isolated neutrophils [29]. Cytotoxicity is not involved in these inhibitory effects as we found no significant release of LDH, a gross marker of cell damage, for NAC concentrations up to 5 mM, which is consistent with reports in human neutrophils and monocytes [28, 29, 38]. The inhibitory effect observed in the present work extends to human eosinophils the findings from previous reports showing that NAC reduces the generation of ROS from human neutrophils activated *in vitro* [28, 31, 32, 35, 38–41].

The antioxidant effects of NAC may be mediated through direct scavenging of oxidant species and by furnishing cysteine to the intracellular synthesis of GSH [42]. The relative contribution of these two mechanisms is difficult to establish. NAC readily enters cells where deacetylation by cytosolic acylase is the primary step in its hydrolysis to yield cysteine [43, 44], but the level of acylase activity in eosinophils is unknown. Nevertheless, the inhibitory effects of NAC against extracellular and

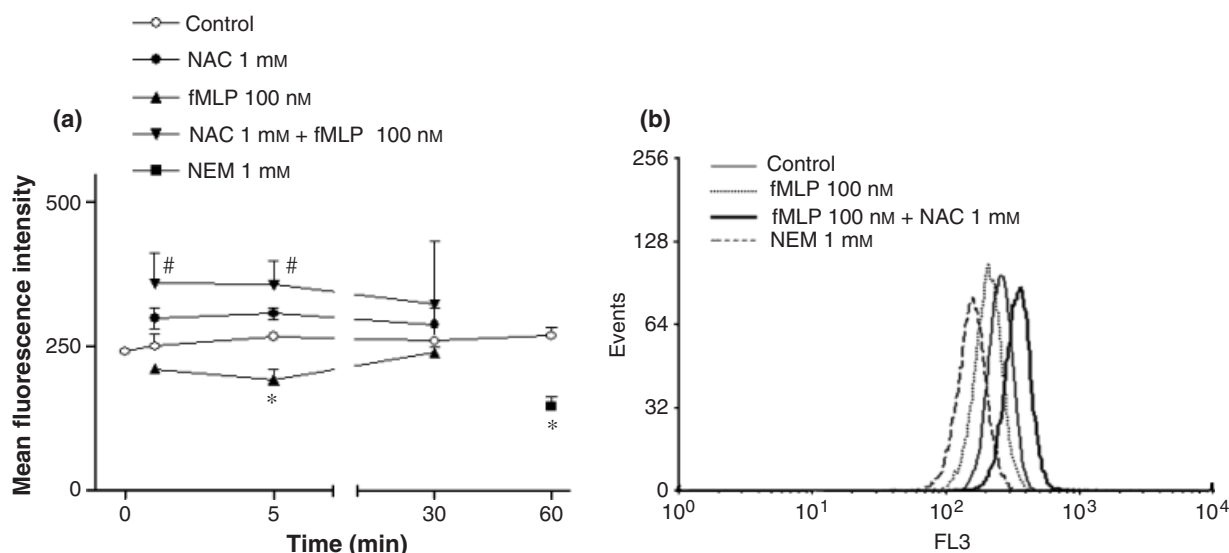


Fig. 3. Effects of *N*-acetyl-L-cysteine (NAC) on *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (100 nM)-induced changes of reduced glutathione levels in human eosinophils (a). *N*-ethylmaleimide (NEM), a thiol-alkylating agent, markedly reduced eosinophil glutathione. Glutathione was measured by flow cytometry with mercury orange. Data are mean $\pm$ SEM of three independent experiments. * $P < 0.05$ from control, # $P < 0.05$ from fMLP. (b) Shows a representative trace.

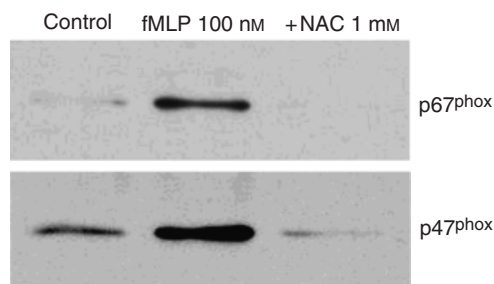


Fig. 4. Inhibitory effects of *N*-acetyl-L-cysteine (NAC) 1 mM on translocation to the membrane of p47^{phox} and p67^{phox} in *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (100 nM)-stimulated human eosinophils. The membrane fraction of eosinophils was prepared by differential centrifugation, and p47^{phox} and p67^{phox} components were analysed by SDS-PAGE and Western blotting. A representative blot of three different experiments is shown.

intracellular ROS generation in fMLP-activated human eosinophils are not likely explained by the sole contribution of the direct antioxidant effects of NAC. Therefore, additional studies were carried out to see whether NAC interferes with the intracellular calcium signal produced following cell activation and also whether the intracellular GSH concentration was altered by the oxidative burst.

The chemoattractant peptide fMLP promotes a rapid and transient increase of intracellular Ca²⁺ concentration in human eosinophils [30]. However, the generation of ROS is only marginally suppressed in Ca²⁺-depleted cells, suggesting that an increase in [Ca²⁺]_i does not play a major role in this process [30]. In keeping with this notion, we found that NAC (1 mM) did not alter the basal values of [Ca²⁺]_i or the fMLP-induced calcium signal, which is in agreement with a similar result in isolated human neutrophils [32].

The oxidative burst creates conditions of oxidative stress, and the stimulation of neutrophils by different stimuli including fMLP has been reported to decrease cellular GSH [31, 32, 45]. A cysteine precursor like NAC may therefore influence this process by replenishing the intracellular GSH as found in human neutrophils [31, 32] and eosinophils (this study), which is also consistent with the capacity of NAC to restore the GSH content of airway smooth muscle cells subjected to oxidant stress [9]. An increase of intracellular cysteine or GSH in neutrophils has not been demonstrated following oral NAC [46]; however, NAC is capable of increasing plasma cysteine or plasma GSH in these patients [46]. High-dose oral NAC has recently been reported to augment GSH in blood neutrophils of cystic fibrosis [33]. Therefore, the clinical relevance of the inhibitory effect of NAC on human isolated eosinophils is difficult to establish. Certainly, the concentrations of NAC required for inhibition of ROS generation from *in vitro* activated eosinophils are not attainable after the administration of NAC [42, 47].

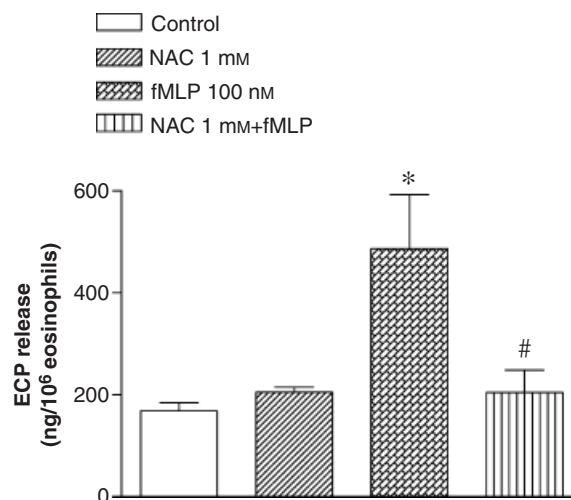


Fig. 5. Inhibitory effects of *N*-acetyl-L-cysteine (NAC) on *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (100 nM)-stimulated release of eosinophil cationic protein (ECP) into the supernatant of human eosinophils. Data are mean $\pm$ SEM of three independent experiments. **P* < 0.05 from the control group; #*P* < 0.05 from the fMLP group.

A critical step in the activation of the superoxide-generating NADPH oxidase is the assembly of membrane-bound cytochrome b₅₅₈ with cytosolic p47^{phox} and p67^{phox} as described in human eosinophils [8]. Upon activation, these cytosolic components translocate to the plasma membrane, where they associate with cytochrome b₅₅₈, forming the active NADPH oxidase [48]. Eosinophils display a higher superoxide production upon stimulation as compared with neutrophils [49], and the components of NADPH oxidase are expressed more abundantly in eosinophils than in neutrophils [8]. NAC has been recently shown to inhibit the translocation of p47^{phox} and p67^{phox} to the membrane of human neutrophils [35] and here we demonstrate a marked inhibition in eosinophils. The precise mechanism of this action of NAC is unknown, but it may be related to the inhibition of protein tyrosine kinase activity or p47^{phox}-p22^{phox} interaction as suggested previously [35].

To test whether NAC may also inhibit a non-oxidant product released by human eosinophils after activation with the same stimulus, we examined the effect of NAC on the fMLP-induced release of ECP, a marker of granular secretion and a cytotoxic product generated by eosinophils [16]. The release of ECP from fMLP-activated eosinophils was inhibited by NAC (~90% inhibition by 1 mM NAC). This finding is consistent with clinical reports showing that ECP in BALF [50] and sputum [51] was diminished after oral treatment with NAC. Interestingly, a cysteine prodrug also decreased ECP levels in bronchoalveolar lavage fluid in a murine model of allergic asthma [52]. While the decrease of ECP levels in *in vivo* studies may be interpreted as the consequence of the inhibitory effects of NAC on neutrophil function, our study

demonstrates that NAC also inhibits ECP secretion in isolated eosinophils. The intracellular signalling mechanism(s) of eosinophil degranulation and ECP secretion produced by fMLP are not precisely known but NAC may inhibit various protein kinases and phospholipases pathways, which appear to be critical for ECP secretion [53, 54].

In conclusion, NAC shows an *in vitro* inhibitory effect on the generation of oxidant species from human peripheral blood eosinophils activated with a chemoattractant peptide as well as by eotaxin and IL-5. The generation of the non-oxidant cytotoxic product ECP was also inhibited. Further experimental and clinical research will be necessary to find out whether these inhibitory actions may have some therapeutic potential in the treatment of asthma or other allergic conditions.

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