



Modulatory effects of *N*-acetyl-L-cysteine on human eosinophil apoptosis

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ABSTRACT: Eosinophils are oxidant-sensitive cells considered relevant in allergic inflammation. The present study aimed to examine the effects of the antioxidant *N*-acetyl-L-cysteine (NAC) on constitutive and cytokine-delayed apoptosis in human isolated eosinophils.

Human eosinophils were purified from the blood of healthy donors by a magnetic separation system. Apoptosis and cellular glutathione were assessed by cytofluorometric analysis and nuclear factor (NF)- κ B binding activity assessed by electrophoresis mobility shift assay.

The rate of spontaneous apoptosis of human eosinophils after 24 h culture, as assessed by annexin-V-positive staining, was mean \pm sem $48.2 \pm 1.4\%$, $n=5$. Granulocyte-macrophage colony-stimulating factor (GM-CSF; $10 \text{ ng}\cdot\text{mL}^{-1}$) decreased apoptosis to $19.4 \pm 1.8\%$, $n=5$. NAC (5 mM) inhibited spontaneous apoptosis ($33.6 \pm 2.7\%$, $n=5$) but augmented apoptosis in the presence of GM-CSF ($30.9 \pm 1.5\%$, $n=5$). NAC (5 mM) also increased the rate of apoptosis in the presence of tumour necrosis factor (TNF)- α ($10 \text{ ng}\cdot\text{mL}^{-1}$) and interleukin-5 ($5 \text{ ng}\cdot\text{mL}^{-1}$). NAC (5 mM) increased eosinophil glutathione content. The increase in eosinophil NF- κ B binding activity induced by GM-CSF and TNF- α was suppressed by NAC.

In conclusion, *N*-acetylcysteine modulates eosinophil apoptosis by inhibiting constitutive apoptosis but reversing the survival effect produced by inflammatory cytokines in human eosinophils.

KEYWORDS: Apoptosis, cytokines, human eosinophils, *N*-acetyl-L-cysteine

Activation and prolonged survival of human eosinophils are prominent features of allergic inflammation [1]. Activated eosinophils release a variety of pro-inflammatory mediators, including oxidant species [2], and thiol-sensitive redox regulation appears important in eosinophil function [3, 4]. Interestingly, oxidant stress is also implicated in promoting apoptosis on different cell types [5, 6]. Hence, antioxidant drugs may modulate eosinophil apoptosis and have a potential role in the pharmacological treatment of allergic inflammation [3, 4].

Although initially used as a mucolytic, *N*-acetyl-L-cysteine (NAC) is a thiol compound that acts directly as a free radical scavenger and a precursor in reduced glutathione (GSH) synthesis, thus protecting cells from oxidant damage [7]. NAC has proved beneficial in pulmonary diseases linked to oxidative stress [8]. Recent studies from the current authors' laboratory have also demonstrated the capacity of NAC to exert anti-inflammatory effects in an experimental model of allergic asthma [9] and to inhibit the generation of oxidant species from activated human eosinophils [10]. The present study addresses whether spontaneous and

cytokine-delayed eosinophil apoptosis may be modulated by a clinically used antioxidant like NAC. Granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF)- α and interleukin (IL)-5 were selected as inflammatory cytokines that prolong survival of human eosinophils [11].

METHODS

Isolation of human eosinophils

Human blood from healthy donors was obtained in heparin, and polymorphonuclear leukocytes were separated by standard laboratory procedures [12]. Eosinophils were then separated by depletion of neutrophils with anti-CD16 coated magnetic microbeads using the magnetic cell separation system (Miltenyi Biotec, Bergisch-Gladbach, Germany), as published previously [13]. Purity of eosinophils in all experiments, as determined by May-Grünwald Giemsa staining, was $>95\%$ and their viability, as measured by trypan blue exclusion, was $>95\%$. This investigation was approved by the institutional ethics committee (University Clinic Hospital, Valencia, Spain) and informed consent was obtained from all donors.

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Received:

June 05 2006

Accepted after revision:

May 04 2007

SUPPORT STATEMENT

This study was supported by grants SAF2005-00669, SAF2005-01649 and SAF2006-01002 from the Inter-ministerial Commission for Science and Technology (CICYT; Ministry of Education, Spain) and research grants (RG-03/166, CTIAE/C/03/116, GV04B72 and GV-04-B-229) from Regional Government (Generalitat Valenciana, Valencia, Spain).

STATEMENT OF INTEREST

None declared.

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

Cytotoxicity assessment

The percentage of lactate dehydrogenase (LDH) release compared with values in cell lysates was taken as a marker for cell damage by using a commercially available colorimetric assay [14].

Cytofluorometric analysis of eosinophil apoptosis

Apoptosis was determined by flow cytometry analysis of propidium iodide (PI) staining of DNA fragmentation. In brief, freshly isolated eosinophils were resuspended at a concentration of 2×10^6 cells·mL⁻¹ in L-glutamine (32 mM), penicillin (100 U·mL⁻¹)/streptomycin (100 µg·mL⁻¹), 25 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] and 10% foetal calf serum-supplemented RPMI. In total, 50 µL (~100,000 cells) of the cell suspension was cultured in a 96-well plate containing 150 µL supplemented RPMI in the absence and presence of 10 ng·mL⁻¹ recombinant human (rh)GM-CSF and treated with NAC (0.5 and 5 mM) or its vehicle. The cells were cultured for ≤48 h then permeabilised with ice-cold 70% ethanol. Thereafter, cells were incubated for 30 min at 37°C in phosphate buffered saline (PBS) before labelling with 50 µg·mL⁻¹ PI overnight at 4°C. The proportion of cells within the hypodiploid DNA region was assessed by flow cytometry (CyAn TM ADP flow cytometry analyser; Dako Denmark A/S, Glostrup, Denmark). Apoptosis was confirmed by light microscopy following Fast Panoptic DC staining (Panreac Quimica SA, Barcelona, Spain) on the basis of cells exhibiting apoptotic morphology, *i.e.* nuclear and cytoplasmic condensation.

In additional experiments, carried out after 24 h of incubation, assessment of apoptosis was performed by flow cytometry using annexin-V-fluorescein isothiocyanate and PI according to the manufacturer's instructions (Annexin-V-Fluos; Roche Applied Science, Barcelona, Spain). Cells (1×10^4 ·mL⁻¹) were analysed in a CyAn TM ADP flow cytometry analyser (Dako Denmark A/S) and differentiated as: early apoptotic (annexin-V-positive, PI-negative); late apoptotic and/or necrotic (annexin-V- and PI-positive); or viable nonapoptotic (annexin-V- and PI-negative) cells. In these experiments, apoptosis was measured in the absence and presence of rhGM-CSF (10 ng·mL⁻¹) in cells treated with NAC (5 mM), its isomer N-acetyl-D-cysteine (D-NAC; 5 mM) or their vehicle. In additional experiments, the survival effect of TNF-α (10 ng mL⁻¹) and IL-5 (5 ng·mL⁻¹) was examined in the absence and presence of NAC (5 mM). Concentrations of GM-CSF, TNF-α and IL-5 producing a significant eosinophil survival effect were selected as reported previously [15, 16]. Concentrations of NAC in the current study were derived from those inhibiting constitutive apoptosis in human eosinophils [3]. In additional experiments, the effects of pyrrolidine dithiocarbamate (PDTC; 300 µM), a nonthiol antioxidant, was examined [17, 18]. In experiments assessing the effects of antioxidants on cytokine-induced survival, antioxidants were present from 30 min prior to cytokine addition until the end of the experiment.

Measurement of glutathione levels

A flow cytometric assay, based on the sulfhydryl-reactive stain mercury orange, was used to measure nonprotein thiols rather than a standard biochemical assay, since mercury orange staining correlates closely with biochemical determinations of

GSH [19]. Cells (3×10^5 ·mL⁻¹) were incubated in supplemented RPMI for 30 min at 37°C in the absence and presence of NAC (5 mM), D-NAC (5 mM) or PDTC (300 µM). Experiments were terminated by placing the tubes into ice, followed by centrifugation (300×g, 5 min). Cell pellets were resuspended in ice-cold mercury orange solution and, after 5 min, stained cells were centrifuged (300×g, 5 min), resuspended in PBS and analysed by flow cytometry (Dako Denmark A/S). In additional experiments, the effect of N-ethylmaleimide (1 mM, 60 min incubation), a thiol alkylating agent, was studied for comparison [19].

Determination of nuclear factor-κB binding activity

Nuclear protein extracts were prepared from cells as described previously [9]. Aliquots of nuclear extracts with equal amount of protein (10 µg) were processed according to the manufacturer's instructions (DIG gel shift kit; Boehringer Mannheim and Enzo Diagnostics Inc., Mannheim, Germany). Electromobility shift assays were performed as previously outlined [20]. In brief, nuclear extracts were incubated in the presence of 3 µg polydeoxycytidylic acid together with a commercial double-stranded ³²P-labelled oligonucleotide (Promega, Madison, WI, USA) encoding the nuclear factor (NF)-κB consensus sequence (5'-AGTTGAGGGGACTTCCAGGC-3'). The specificity of binding was ascertained by competition with a 200-fold excess of unlabelled doubled-stranded oligonucleotide. For supershift experiments, anti-p65 antibodies (SantaCruz Biotechnology, Santa Cruz, CA, USA) were added to the binding reaction. DNA-protein complexes were electrophoretically separated and subjected to autoradiography.

Drugs and solutions

NAC and PDTC were obtained from Sigma-Aldrich (Madrid, Spain). The stereoisomer of NAC, D-NAC, was obtained from Research Organics Inc. (Cleveland, OH, USA). Drug concentrations are expressed in terms of the molar concentration of the active species. NAC was dissolved in deionised water and diluted in the appropriate buffer media as required (pH 7.4). Water purified on a Milli-Q (Millipore Iberica, Madrid, Spain) system was used throughout.

Statistical analysis of results

Data are presented as the mean ± SEM of n experiments. The median inhibitory concentration values were calculated from the concentration-inhibition curves by nonlinear regression analysis (GraphPad Software Inc., San Diego, CA, USA). Statistical analysis of the results was carried out by ANOVA followed by Bonferroni test or paired t-test as appropriate using pairwise comparison. Significance was accepted when $p < 0.05$.

RESULTS

Absence of cytotoxicity of NAC

NAC in concentrations ≤10 mM showed no significant cytotoxicity as assessed by LDH release (2.6 ± 0.4 and $2.8 \pm 0.6\%$ of total LDH for control and 10 mM NAC, respectively; $n=3$ for each group).

Influence of NAC on eosinophil apoptosis

The spontaneous survival of eosinophils in culture suffered a time-dependent decay during the 48-h observation period

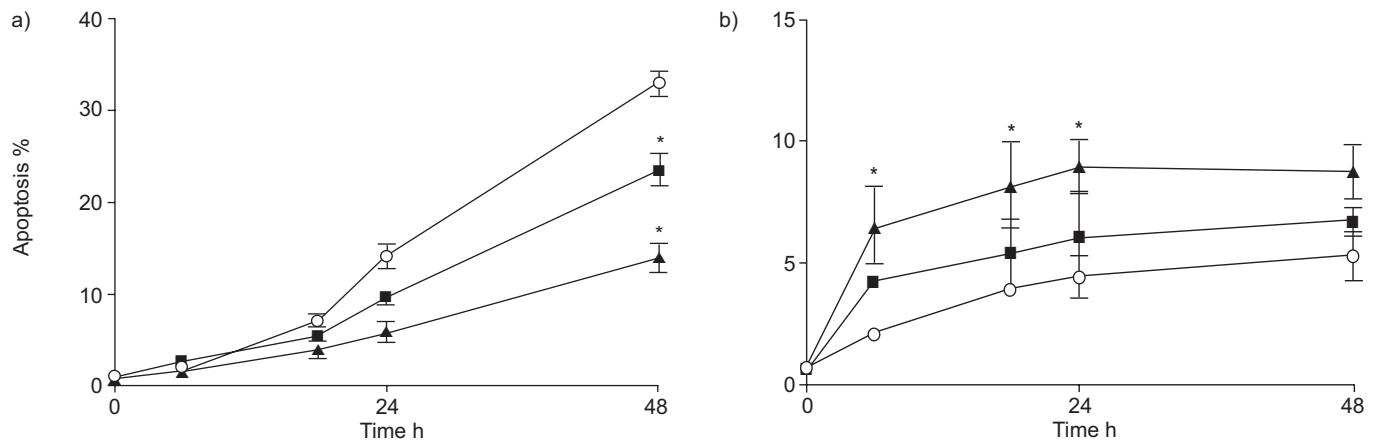


FIGURE 1. Effects of *N*-acetyl-L-cysteine (NAC) on the time-course of eosinophil viability *in vitro*. Eosinophil apoptosis was examined in a) the absence (spontaneous apoptosis) and b) the presence of granulocyte-macrophage colony-stimulating factor ($10 \text{ ng}\cdot\text{mL}^{-1}$). Eosinophils were incubated for 48 h in culture medium and apoptosis was determined by flow cytometry analysis of propidium iodide staining of DNA fragmentation. Data are presented as mean \pm SEM of five independent experiments. ○: vehicle control; ■: 0.5 mM NAC; ▲: 5 mM NAC. *: $p < 0.05$ versus the corresponding control.

(fig. 1a). NAC (0.5–5 mM) augmented the survival of eosinophils by inhibiting the constitutive eosinophil apoptosis assessed by DNA fragmentation in PI-stained cells. In the presence of GM-CSF, the viability of cultured eosinophils was significantly increased; however, NAC decreased survival of eosinophils in these experimental conditions (fig. 1b). These changes in apoptosis rate in the absence and presence of GM-CSF and NAC were confirmed by morphological criteria using Fast Panoptic staining of cultured eosinophils (not shown).

In additional experiments, apoptosis was measured using annexin-V and PI staining of eosinophils after culture for 24 h. NAC (5 mM) decreased the percentage of apoptotic (annexin-V-positive) eosinophils in the absence of GM-CSF but augmented these numbers in the presence of GM-CSF (fig. 2). Similar effects were obtained with the isomer D-NAC (fig. 2). A representative experiment for NAC is shown in figure 3. NAC decreased both early (annexin-V-positive, PI-negative cells: from 24.5 ± 1.5 to $17.2 \pm 2.6\%$) and late (annexin-V-positive, PI-positive cells: from 23.7 ± 0.4 to $16.3 \pm 1.8\%$) apoptotic cells in the absence of GM-CSF, and increased early (annexin-V-positive, PI-negative cells; from 12.7 ± 1.6 to $20.3 \pm 1.2\%$) and late (annexin-V-positive, PI-positive cells: from 6.8 ± 0.6 to $10.6 \pm 0.9\%$) apoptotic cells in the presence of GM-CSF ($n=5$ for each group, $p < 0.05$). The results obtained for cells stained positively for both PI and annexin-V are consistent with data shown in figure 1.

TNF- α ($10 \text{ ng}\cdot\text{mL}^{-1}$) also augmented survival of eosinophils at 24 h of culture (annexin-V-positive cells decreased from 43 ± 4 to $26 \pm 3\%$ in the absence and presence of TNF- α , respectively; $n=3$, $p < 0.05$). This survival effect of TNF- α was reversed in the presence of NAC ($36 \pm 5\%$ for TNF- α with 5 mM NAC; $n=3$, $p < 0.05$). Additional experiments in the presence of IL-5 ($5 \text{ ng}\cdot\text{mL}^{-1}$) showed augmented survival of eosinophils cultured for 24 h, and the survival effect of IL-5 was reversed in the presence of NAC and D-NAC (fig. 2). The nonthiol antioxidant PDTC ($300 \mu\text{M}$) also increased apoptosis in the presence of GM-CSF ($38 \pm 2\%$; $n=3$, $p < 0.05$) and IL-5 ($39 \pm 3\%$; $n=3$, $p < 0.05$).

NAC augmented eosinophil glutathione

NAC (5 mM) augmented the glutathione content of eosinophils as measured by flow cytometry (fig. 4). D-NAC, the metabolically inactive stereoisomer of NAC, increased eosinophil glutathione to a similar extent as NAC, whereas PDTC did not augment glutathione content in eosinophils (fig. 4).

NAC inhibited NF- κ B activity

Exposure of human eosinophils to GM-CSF ($10 \text{ ng}\cdot\text{mL}^{-1}$) or TNF- α ($10 \text{ ng}\cdot\text{mL}^{-1}$) resulted in augmentation of NF- κ B binding activity that was markedly decreased in NAC-treated eosinophils (fig. 5).

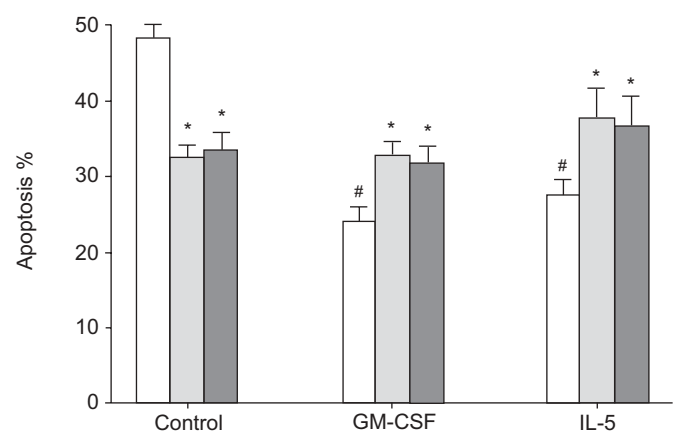


FIGURE 2. Effect of *N*-acetyl-L-cysteine (■; 5 mM) on apoptotic cells assessed as annexin-V-positive at 24 h of culture of human eosinophils. Eosinophil apoptosis was examined in the absence (control) and presence of granulocyte-macrophage colony-stimulating factor (GM-CSF; $10 \text{ ng}\cdot\text{mL}^{-1}$) or interleukin (IL)-5 ($5 \text{ ng}\cdot\text{mL}^{-1}$). The effect of the stereoisomer *N*-acetyl-D-cysteine (■; 5 mM) is shown for comparison. Data are presented as the mean \pm SEM of 6–8 independent experiments. *: $p < 0.05$ compared with the corresponding untreated group; #: $p < 0.05$ compared with the absence of cytokine.

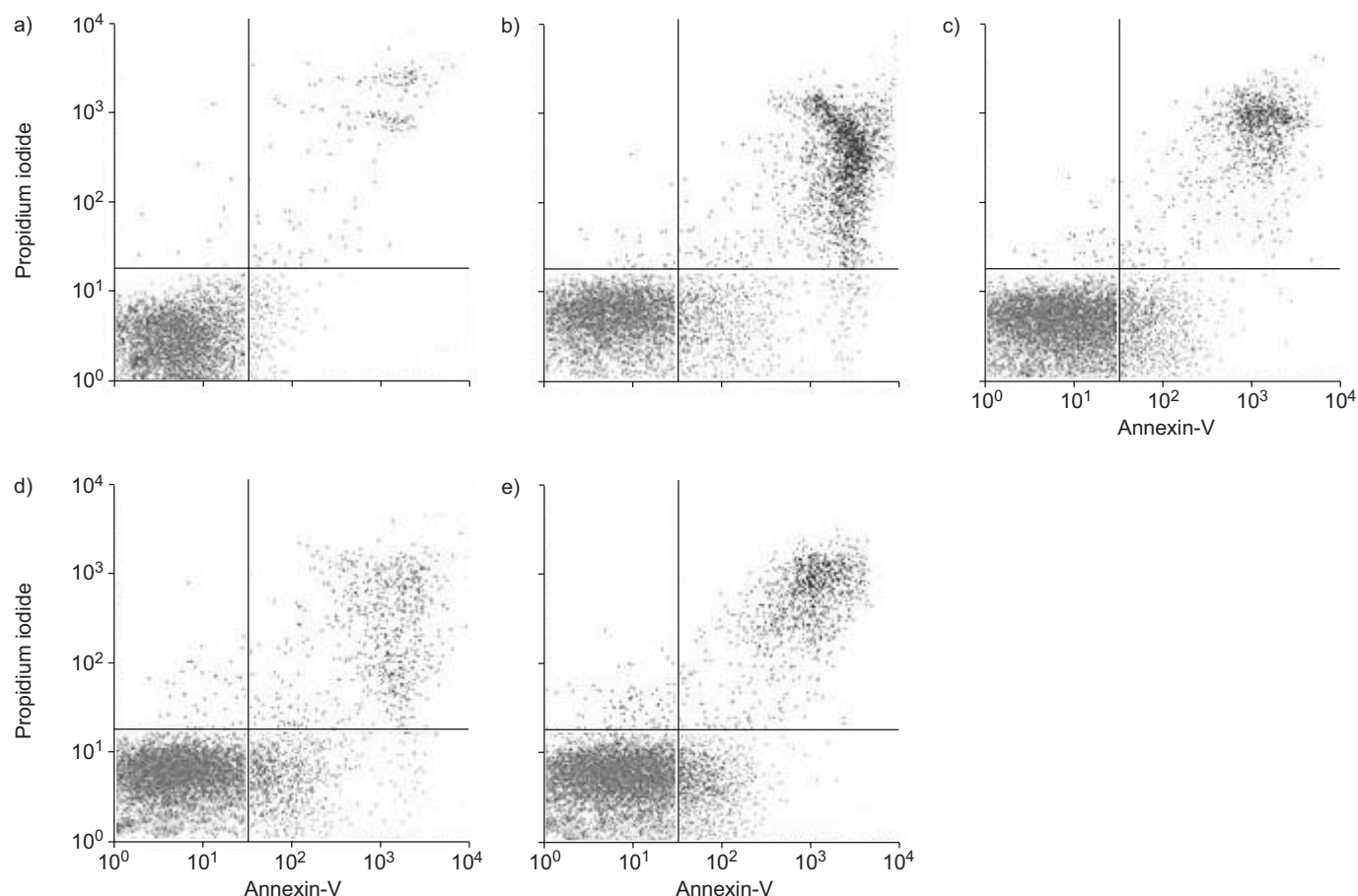


FIGURE 3. Representative flow cytometry showing apoptosis of human eosinophils at a) 0 h and b–e) 24 h of culture in the absence (b and c) and presence (d and e) of granulocyte-macrophage colony-stimulating factor (GM-CSF; $10 \text{ ng}\cdot\text{mL}^{-1}$), and in the absence (b and d) and presence (c and e) of *N*-acetyl-L-cysteine (NAC; 5 mM). Viable nonapoptotic eosinophils were quantified as the percentage of the total population of cells that were negative for both annexin-V and propidium iodide (PI). Early apoptotic cells were annexin-V-positive and PI-negative. Late apoptotic cells were positive for both annexin-V and PI. The percentage of cells in the bottom left, bottom right and top right quadrants, respectively, were as follows: a) 89.6, 8.32 and 1.73; b) 51.4, 24.7 and 23.5; c) 68.7, 21.4 and 9.44; d) 80.65, 13.6 and 5.31; and e) 71.6, 19.6 and 8.77. Note the decrease in spontaneous apoptosis (absence of GM-CSF) produced by NAC (c *versus* b) but the increase in apoptotic percentages obtained in the presence of GM-CSF (e *versus* d).

DISCUSSION

Apoptosis is involved in the resolution of inflammation, and many *in vitro* studies have shown that an array of inflammatory mediators present at inflamed sites augment the survival of human granulocytes [21]. Many agents which induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism, whereas many inhibitors of apoptosis show antioxidant properties [3, 22]. Thiol antioxidants such as NAC have been demonstrated to inhibit apoptosis *in vitro* in different cell types [23, 24]. In particular, NAC (1–10 mM) was reported to inhibit spontaneous apoptosis as well as Fas- and sodium arsenite-triggered apoptosis in human eosinophils [3]. These findings are confirmed in the present study, where NAC (0.5–5 mM) inhibited the spontaneous apoptosis of eosinophils cultured ≤ 48 h.

Interestingly, when NAC was added to eosinophils incubated with GM-CSF to extend their lifespan in culture, the opposite effect was found for NAC (0.5–5 mM), *i.e.* enhanced apoptosis. Cytotoxicity was not involved in this pro-apoptotic effect since

NAC (≤ 10 mM) did not damage eosinophils. Another inflammatory cytokine reported to extend eosinophil survival is TNF- α , which, in part, mediates its effect through the release of GM-CSF [25] and IL-5 using different signalling pathways [26]. Regarding GM-CSF, the current authors found that the survival effects of TNF- α and IL-5 were reversed to a pro-apoptotic effect in the presence of NAC.

Therefore, NAC appears to modulate apoptosis in human eosinophils by inhibiting constitutive and induced apoptosis but enhancing apoptosis in eosinophils subjected to the survival effects of different inflammatory cytokines, such as GM-CSF, TNF- α and IL-5. A possible explanation for the increased apoptosis by NAC may be the increased oxidative stress associated with its auto-oxidation, as reported for high concentrations of NAC (25 mM) in human neutrophils [27]. However, an increase in eosinophil glutathione content was found in the current study after incubation of eosinophils with NAC (5 mM); therefore, an increase in oxidant burden is not a likely explanation for the pro-apoptotic effect of NAC

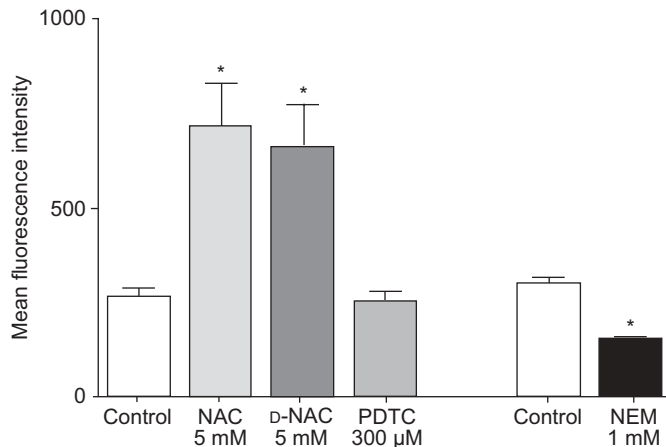


FIGURE 4. Eosinophil glutathione measured by mercury orange staining using flow cytometry. *N*-acetyl-L-cysteine (NAC) and *N*-acetyl-D-cysteine (D-NAC) markedly augmented the glutathione content, whereas pyrrolidine dithiocarbamate (PDTC) was without effect. *N*-ethylmaleimide (NEM) produced a significant decrease in glutathione as expected from its activity as an SH-blocking agent [19]. *: $p < 0.05$.

observed in cytokine-treated eosinophils. In additional experiments, the metabolically inactive stereoisomer of NAC, D-NAC, was found to produce similar effects to those observed for NAC, including an increase in eosinophil glutathione. Although unexpected, this result is consistent with similar findings for D-NAC in cultured neuronal [28] and vascular smooth muscle [29] cells, and is likely to be due to the reduction of extracellular cystine to cysteine, which is then efficiently transported into cells [28]. PDTC, a nonthiol antioxidant [18], which was shown not to increase eosinophil glutathione, also caused pro-apoptotic effects on cytokine-exposed eosinophils, similar to those elicited by NAC. These findings indicate that the direct antioxidant, radical scavenging effects and not the increase of antioxidant glutathione content are primarily responsible for the effects produced by NAC.

GM-CSF, TNF- α and IL-5 are inflammatory cytokines that activate the transcription factor NF- κ B, and may contribute to the production of other inflammatory cytokines in human eosinophils [30, 31]. Thus, NF- κ B activation is a critical step to prolong the survival of human eosinophils, probably by controlling the transcriptional activity of genes that induce the synthesis of survival proteins [15, 17]. The ability of NAC to inhibit NF- κ B activation has been demonstrated in different cells [9, 32, 33], and this observation has been extended to human isolated eosinophils in the present study. Therefore, it is possible that NAC, by inhibiting the activation of NF- κ B produced by cytokines, blocks the production of survival factors thus eliciting a pro-apoptotic effect. The nonthiol antioxidant, PDTC, which blocks cytokine-induced activation of NF- κ B [18], was also shown to produce pro-apoptotic effects on eosinophils, similar to NAC. These findings would be in keeping with the observation that incubation with Bay 11-7082, an inhibitor of phosphorylation of the inhibitory protein I κ B, also resulted in abrogation of pro-survival effects seen from exogenous GM-CSF and TNF- α in human eosinophils [25]. Inhibition of NF- κ B by a cell-permeable form of inhibitor

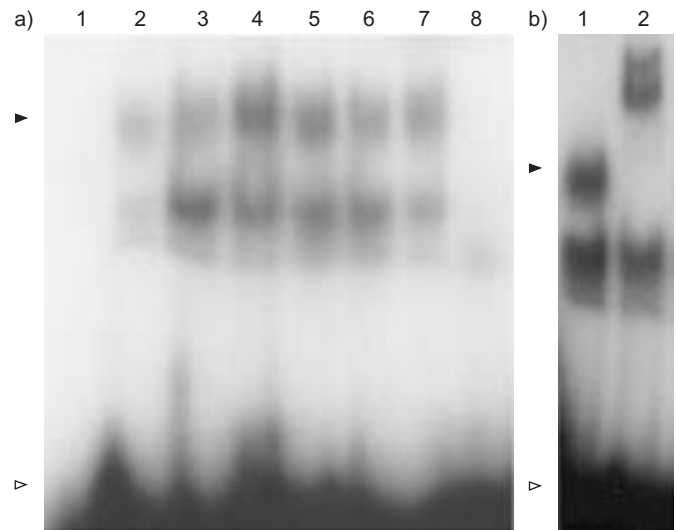


FIGURE 5. Electrophoresis mobility gel shift assay of nuclear factor (NF)- κ B activation in human eosinophils. Nuclear protein (10 μ g) was prepared from eosinophils. a) Eosinophils were subjected to the following treatment. Lane 1: probe without sample; lane 2: control; lane 3: *N*-acetyl-L-cysteine (NAC; 5 mM); lane 4: tumour necrosis factor- α (TNF- α ; 10 ng·mL⁻¹); lane 5: granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 ng·mL⁻¹); lane 6: GM-CSF and NAC; lane 7: TNF- α and NAC; and lane 8: competition with an excess of cold probe to determine specificity. Data presented are representative of three independent experiments. b) Eosinophils were stimulated with TNF- α and then incubated with specific radioactive probe alone (lane 1), or with an anti-p65 subunit antibody (lane 2) for 20 min. Data are representative of two separate experiments. The black arrowhead indicates the position of the NF- κ B complex; the white arrowhead indicates the position of free probe. The faster migrating complexes were not specific.

NF- κ B (I κ B) α also induces apoptosis in human eosinophils in the absence and presence of TNF- α [34]. Interestingly, increases of constitutive apoptosis have also been reported following inhibition of NF- κ B in human eosinophils by gliotoxin [15, 17] and by a synthetic peptide [35]. However, no increased apoptosis other than the known anti-apoptotic effect of NAC was observed in cultured eosinophils that were not incubated with inflammatory cytokines. In addition, although the presence of a constitutively active NF- κ B is required for eosinophil survival [36], other inhibitors of NF- κ B activation, such as PDTC (as reported currently) or Bay 11-7082 [25], also failed to influence constitutive apoptosis in human eosinophils, which suggests the existence of differences in the mechanisms of the constitutive *versus* cytokine-mediated activation of NF- κ B.

The present study did not explore NAC interference of other downstream steps of the signalling pathways of these cytokines but, in other cells, NAC has been shown to induce apoptosis by: increasing the pro-apoptotic *Bax* gene expression [37]; inhibiting NF- κ B-dependent expression of anti-apoptotic proteins [38]; or blocking the activation of c-jun N-terminal kinase and p38-mitogen-activated protein kinase [39].

Oxidative stress appears to be relevant to allergic inflammatory diseases such as asthma [40]. NAC has been reported to be beneficial in animal models of allergic asthma [9, 41] and also decreases eosinophil oxidant generation and the release of

eosinophil cationic protein [10]. Therefore, thiol antioxidant treatment strategies could potentially alleviate allergic inflammation, although the low oral bioavailability and potency of NAC represents an obvious limitation and convincing evidence from controlled clinical trials is currently lacking.

In conclusion, N-acetyl-L-cysteine has a modulatory *in vitro* effect on the apoptosis of human peripheral blood eosinophils. Further research is necessary to determine whether this modulatory action, and in particular the pro-apoptotic effect exerted on eosinophils exposed to inflammatory cytokines, might be of any therapeutic value in the treatment of chronic allergic inflammatory conditions.

ACKNOWLEDGEMENTS

The authors would like to thank C. Peiró (Dept of Pharmacology, Faculty of Medicine, Universidad Autónoma, Madrid, Spain) for help with the electromobility shift assay experiments. The technical assistance of P. Santamaría and D. Martí (Dept of Pharmacology, Faculty of Medicine, University of Valencia, Valencia, Spain) is also gratefully acknowledged.

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