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Modulatory effects of *N*-acetylcysteine on human eosinophil apoptosis

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ABSTRACT

Eosinophils are oxidant-sensitive cells considered relevant in allergic inflammation. We aimed to study the effects of the antioxidant *N*-acetylcysteine (NAC) on constitutive and cytokine-delayed apoptosis in human isolated eosinophils.

Human eosinophils were purified from blood of healthy donors by a magnetic separation system. Apoptosis and cellular glutathione were assessed by cytofluorometric analysis, and NF-κB binding activity by electrophoresis mobility shift assay.

The rate of spontaneous apoptosis of human eosinophils after 24 h of culture as assessed by annexin V positive staining was 48.2±1.4% (n=5). GM-CSF (10 ng ml^{-1}) decreased apoptosis to 19.4±1.8% (n=5; $P<0.05$). NAC (5mM) inhibited spontaneous apoptosis (33.6±2.7%, n=5, $P<0.05$) but augmented apoptosis in the presence of GM-CSF (30.9±1.5%; n=5; $P<0.05$). NAC (5mM) also increased apoptosis rate in presence of TNF-α (10 ng ml^{-1}) and interleukin-5 (5 ng ml^{-1}). NAC (5mM) increased eosinophil glutathione content. The increase in eosinophil NF-κB binding activity induced by GM-CSF and TNF-α was suppressed by NAC.

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

KEYWORDS: *N*-acetylcysteine; human eosinophils; apoptosis; cytokines.

Abbreviations: D-NAC, *N*-acetyl-D-cysteine; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage/monocyte-colony stimulating factor; GSH, reduced glutathione; HBSS, Hank's balanced salt solution; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]; IL-5, interleukin-5; NAC, *N*-acetyl-L-cysteine; NEM, N-ethylmaleimide; PBS, phosphate buffered saline; PDTC, pyrrolidine dithiocarbamate; PI, propidium iodide; rh, recombinant human; RPMI, Roswell Park Memorial Institute culture medium; TNF-α, tumor necrosis factor-α.

INTRODUCTION

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 mucolytic, *N*-acetyl-L-cysteine (NAC) is a thiol compound that acts directly as a free radical scavenger and as 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 our 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 work addresses the question whether spontaneous and cytokine-delayed eosinophil apoptosis may be modulated by a clinically used antioxidant like NAC. Granulocyte-macrophage/monocyte-colony stimulating factor (GM-CSF), tumour necrosis factor-α (TNF-α) and interleukin-5 (IL-5) were selected as inflammatory cytokines which 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 (MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany) as outlined [13]. Purity of eosinophils in all experiments was >95% (determined by May-Grünwald-Giemsa) and their viability as measured by trypan blue exclusion was >95%. This investigation was approved by the institutional ethics committee and informed consent was obtained from all donors.

Citotoxicity assessment

The percentage of lactate dehydrogenase (LDH) release compared to values in cell lysates was taken as marker for cell damage by using a commercially available colorimetric assay as outlined [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 $2x10^6$ cells ml^{-1} in L-glutamine (32mM), penicillin (100 U/ml) /streptomycin (100μg/ml) HEPES 25mM FCS 10% supplemented RPMI. Fifty μl (~100,000 cells) of the cell suspension were cultured in a 96-well plate containing 150 μl supplemented RPMI in the absence and presence of 10 ng ml^{-1} rhGM-CSF and

treated with NAC (0.5 and 5 mM) or its vehicle. The cells were cultured for up to 48 h. Cells were permeabilized with ice-cold ethanol 70%. Thereafter, cells were incubated 30 min at 37ºC in PBS before labeling (50µg/ml PI, 4ºC, overnight). The proportion of cells within the hypodiploid DNA region was assessed by flow cytometry (CyAn TM ADP flow cytometry analyzer; Dako Denmark A/S DK-2600, 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 at 24 h of incubation, assessment of apoptosis was performed by flow cytometry using annexin V-fluorescein isothiocyanate (FITC) and PI following the protocol indicated by the manufacturer (Annexin-V-Fluos; Roche Applied Science, Barcelona, Spain). Cells ($1x10^4$) were analysed in a CyAn TM ADP flow cytometry analyzer (Dako Denmark A/S DK-2600, Glostrup, Denmark) and differentiated as early apoptotic (annexin V^+, PI^-), late apoptotic and/or necrotic (annexin V^+ and PI^+), and viable nonapoptotic (annexin V^- and PI^-) cells. In these experiments, apoptosis was measured in the absence and presence of rhGM-CSF (10 ng ml^{-1}) in cells treated with NAC (i.e. N-acetyl-L-cysteine) or its isomer N-acetyl-D-cysteine (D-NAC) (each at 5mM) 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 (5mM). Concentrations of GM-CSF, TNF-α and IL-5 were selected to produce a significant eosinophil survival effect as reported [15,16]. Concentrations of NAC in this study were derived from those inhibiting constitutive apoptosis in human eosinophils [3]. In additional experiments, the effects of pyrrolidine dithiocarbamate (PDTC; 300 µM), a non-thiol antioxidant, was examined [17,18]. In

experiments assessing the effects of antioxidants on cytokine-induced survival, antioxidants were present from 30 min before cytokine addition until the end of the experiment.

Measurement of glutathione levels

A flow cytometric assay based on the sulfydryl-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 ($3x10^5$ ml^{-1}) were incubated in supplemented RPMI for 30 min at 37ºC in the absence and presence of NAC (5 mM), D-NAC (5 mM), or PDTC (300 μM). Experiments were finished by introducing 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 (CyAn TM ADP flow cytometry analyzer; Dako Denmark A/S DK-2600, Glostrup, Denmark). In additional experiments, the effect of N-ethylmaleimide (NEM; 1mM, 60 min incubation), a thiol alkylating agent, was studied for comparison [19].

Determination of NF-κB binding activity

Nuclear protein extracts were prepared from cells as outlined [9]. Aliquots of nuclear extracts with equal amount of protein (10 μg) were processed according to manufacturer instructions (DIG gel shift kit from Boehringer Mannheim and Enzo Diagnostics Inc., Mannheim, Germany). Electromobility shift assays were carried out as previously outlined [20]. In brief, nuclear extracts were incubated in the presence of 3 μg poly-dIdC together with commercial double-stranded ^{32}P-labelled oligonucleotide (Promega)

encoding the NF-κB consensus sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3'). Specificity of binding was ascertained by competition with a 200-fold excess of unlabeled doubled-stranded oligonucleotide. For supershift experiments, anti-p65 antibodies (SantaCruz Biotechnology) were added to the binding reaction. DNA-protein complexes were electrophoretically separated and subjected to autoradiography.

Drugs and solutions

N-Acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) were obtained from Sigma-Aldrich (Madrid, Spain). The stereoisomer of NAC, N-Acetyl-D-cysteine (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 deionized 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 mean±s.e.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 analysis of variance (ANOVA) followed by Bonferroni test or by Student's *t* test as appropriate using pairwise comparison (GraphPad Software Inc., San Diego, USA). Significance was accepted when $P<0.05$.

RESULTS

Absence of cytotoxicity of NAC

NAC, in concentrations up to 10 mM, showed no significant cytotoxicity assessed by LDH release (2.6±0.4% and 2.8±0.6% of total LDH for control and NAC 10 mM, 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 (Fig. 1A). NAC (0.5-5mM) 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 but NAC decreased survival of eosinophils in these experimental conditions (Fig. 1B). These changes in apoptosis rates in the absence and presence of GM-CSF and NAC were confirmed by morphologic criteria using Fast Panoptic staining of cultured eosinophils (not shown).

In additional experiments, we measured apoptosis by using annexin V and PI staining of eosinophils after 24 h of culture. NAC (5mM) decreased the percentages 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+ PI- cells; from 24.5±1.5% to 17.2±2.6%) and late (annexin V+, PI+ cells; from 23.7±0.4% to 16.3±1.8%) apoptotic cells in the absence of GM-CSF, and increased early (annexin V+ PI- cells; from 12.7±1.6% to 20.3±1.2%) and late (annexin

V+, PI+ cells; from 6.8±0.6% to 10.6±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 ng ml^{-1}) also augmented survival of eosinophils at 24 h of culture (annexin V positive cells decreased from 43±4% to 26±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±5% for TNF-α+NAC 5 mM; n=3, $P<0.05$). Additional experiments carried out in the presence of IL-5 (5 ng ml^{-1}) showed augmented survival of eosinophils cultured for 24 h, and this survival effect of IL-5 was reversed in the presence of NAC and D-NAC (Fig. 2). The non-thiol antioxidant PDTC (300 μM) also increased apoptosis in the presence of GM-CSF (38±2%; n=3, $P<0.05$) and IL-5 (39±3%; n=3, $P<0.05$).

NAC augmented eosinophil glutathione

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

NAC inhibited NF-κB activity

Exposure of human eosinophils to GM-CSF (10 ng ml^{-1}) or TNF-α (10 ng ml^{-1}) resulted in augmentation of NF-κB binding activity that was markedly decreased in NAC-treated eosinophils (Fig. 5).

DISCUSSION

Apoptosis is involved in resolution of inflammation, and many in vitro studies have shown that an array of inflammatory mediators present in inflammed 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 in vitro to inhibit apoptosis in different cell types [23,24]. In particular, NAC (1-10mM) 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 (0.5-5mM) inhibited the spontaneous apoptosis of eosinophils cultured up to 48 h.

Interestingly, when NAC was added to eosinophils incubated with GM-CSF to extend their life span in culture, we found the opposite effect for NAC (0.5-5 mM), i.e. enhanced apoptosis. Cytotoxicity is not involved in this pro-apoptotic effect since NAC (up to 10 mM) is not damaging eosinophils (this study). Another inflammatory cytokines which have been also reported to extend eosinophil survival are TNF-α which mediates in part its effect through the release of GM-CSF [25] and IL-5 that uses different signalling pathways [26]. As for GM-CSF, we 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 an increased oxidative stress associated to its autooxidation as reported for high concentrations of

NAC (25mM) in human neutrophils [27]. However, an increase of eosinophil glutathione content was found in this study after incubation of eosinophils with NAC (5mM), and therefore, an increase in oxidant burden is not a likely explanation for the pro-apoptotic effect of NAC observed in cytokine-treated eosinophils. In additional experiments, we found that the nonmetabolically active stereoisomer of NAC, D-NAC, was producing 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 likely due to reduction of extracellular cystine to cysteine which is efficiently transported into cells [28]. PDTC, a non-thiol antioxidant [18], that we show 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 which activate the transcription factor NF-κB, and may contribute to the production of other inflammatory cytokines in human eosinophils [30,31]. Thus, NF-κB activation is a critical step to prolong the survival of human eosinophils probably by controlling the transcriptional activity of genes which induce the synthesis of survival proteins [15,17]. The ability of NAC to inhibit NF-κB activation has been demonstrated in different cells [9,32,33], and we have extended this observation to human isolated eosinophils in the present study. Therefore, it is possible that NAC, by inhibiting the activation of NF-κB produced by cytokines, blocks the production of survival factors thus eliciting a pro-apoptotic effect. The non-thiol antioxidant, PDTC, which blocks cytokine-induced activation of NF-κB

[18], produces also pro-apoptotic effects on eosinophils similar to NAC (this study). These findings would be in keeping with the observation that incubation with Bay 11-7082, an inhibitor of IκB phosphorylation, also resulted in abrogation of pro-survival effect seen from exogenous GM-CSF and TNF-α in human eosinophils [25]. Inhibition of NF-κB by a cell permeable form of IκBα also induces apoptosis in human eosinophils in the absence and presence of TNF-α [34]. Interestingly, increases of constitutive apoptosis have been also reported following inhibition of NF-κB in human eosinophils by gliotoxin [15,17] and by a synthetic peptide [35]. However, no increased apoptosis but the known anti-apoptotic effect of NAC was observed in cultured eosinophils not incubated with inflammatory cytokines. In addition, although the presence of a constitutively active NF-κB is required for eosinophil survival [36], other inhibitors of NF-κB activation such as PDTC (this study) or Bay 11-7082 [25] failed also to influence constitutive apoptosis in human eosinophils which suggests the existence of differences in the mechanisms of the constitutive vs. the cytokine-mediated activation of NF-κB.

In the present study, we have not explored the interference by NAC of other downstream steps of the signaling pathways of these cytokines but in other cells, NAC has been shown to induce apoptosis by increasing the pro-apoptotic *Bax* gene expression [37], by inhibiting NF-κB-dependent expression of antiapoptotic proteins [38] or by blocking the activation of c-jun N-terminal kinase (JNK) and p38-mitogen-activated protein kinase [39].

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

cationic protein [10]. Therefore, thiol antioxidant treatment strategies have a potential value to 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, NAC has a modulatory in vitro effect on the apoptosis of human peripheral blood eosinophils. Further research will be necessary to find out 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.

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LEGEND OF FIGURES

Figure 1

Effects of N-acetyl-L-cysteine (NAC) on the time course of eosinophil viability in vitro. Eosinophil apoptosis was examined in the absence (spontaneous apoptosis; panel A) and in the presence of GM-CSF (10 ng ml^{-1}) (panel B). Eosinophils were incubated for 48 h in culture medium and apoptosis was determined by flow cytometry analysis of propidium iodide (PI) staining of DNA fragmentation. Data are mean±s.e.m. of 5 independent experiments. *P<0.05 vs. corresponding control.

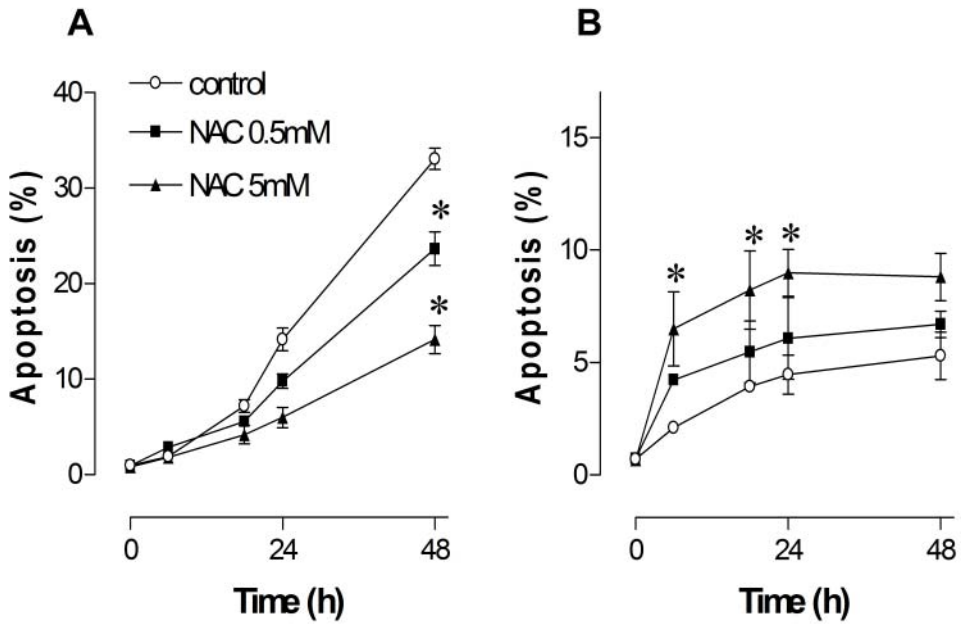

Figure 2

Effect of N-acetyl-L-cysteine (NAC; 5mM) 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 in the presence of GM-CSF (10 ng ml^{-1}) or IL-5 (5 ng ml^{-1}) as

indicated. The effect of the stereoisomer D-NAC (5 mM) is shown for comparison. Columns are mean±s.e.m. of 6-8 independent experiments. *P<0.05 compared to the corresponding untreated group; #P<0.05 compared to the absence of cytokine.

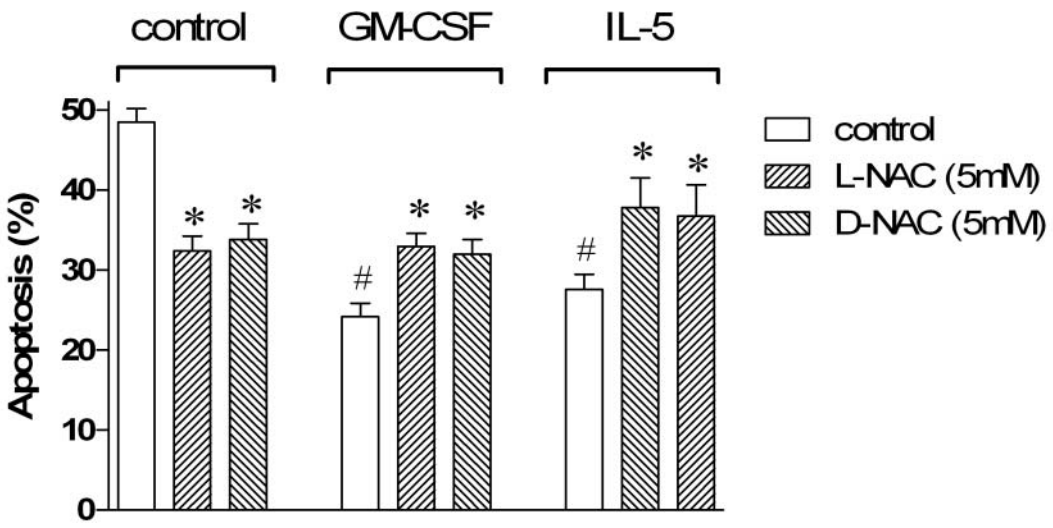

Figure 3

A representative flow cytometry showing apoptosis of human eosinophils at 0 h (panel A) and at 24 h of culture (panels B to E) in the absence (B,C) and presence (D,E) of GM-CSF (10 ng ml^{-1}), and in the absence (B,D) and presence (C,E) of NAC (5 mM). Viable nonapoptotic eosinophils were quantified as the percentage of 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 annexin V and PI positive. The numbers represent the percentage of cells in each quadrant. Note the decrease in spontaneous apoptosis (absence of GM-CSF) produced by NAC (C vs. B) but the increase in apoptotic percentages obtained in the presence of GM-CSF (E vs. D).

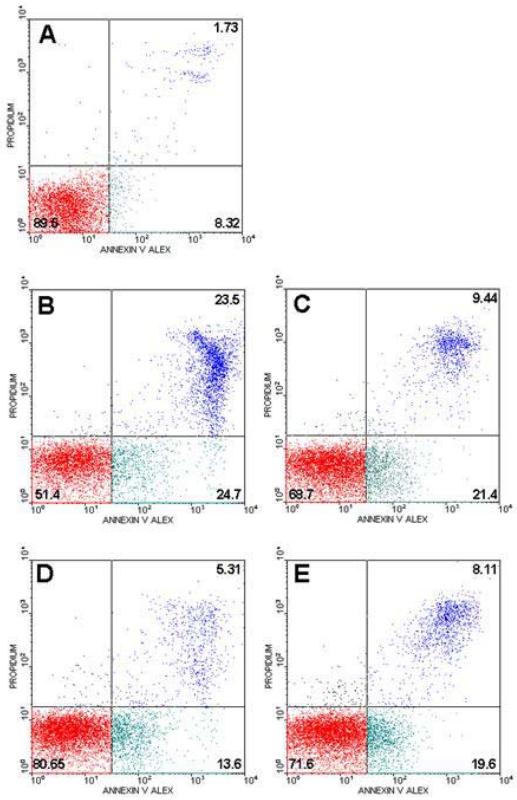

Figure 4

Eosinophil glutathione measured by orange mercury staining using flow cytometry. NAC and D-NAC (each at 5mM) 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 SH-blocking agent [19].

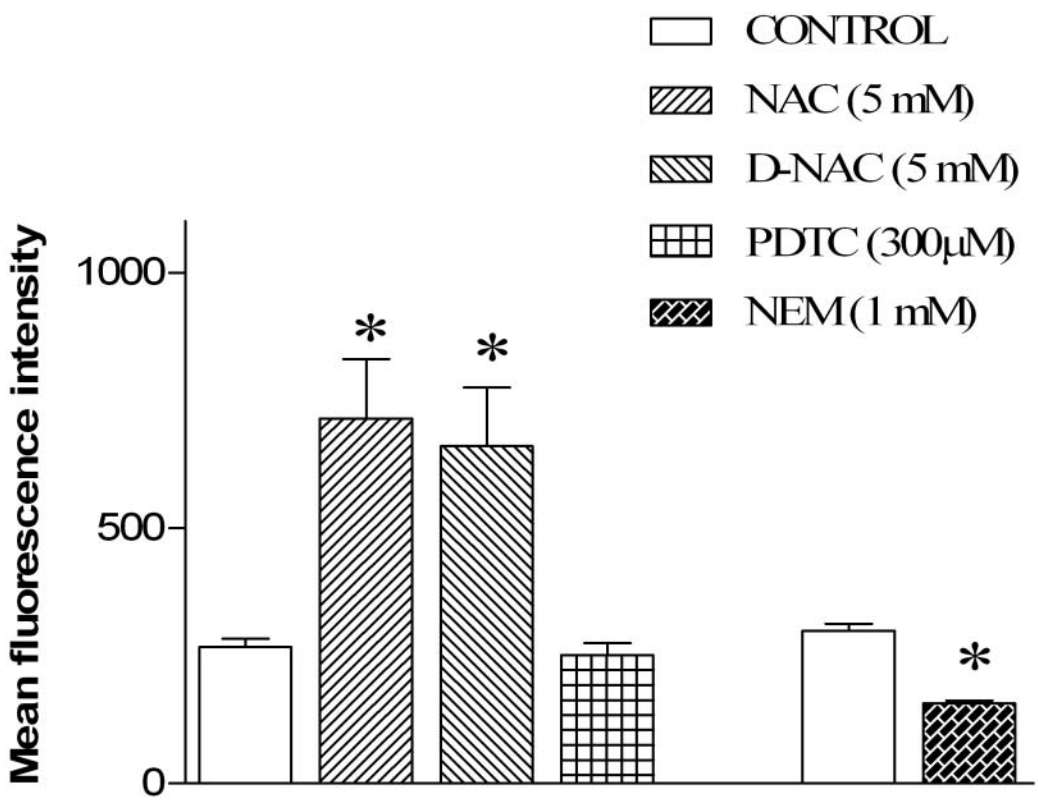

Figure 5

Electrophoresis mobility gel shift assay showing NF-κB activation in human eosinophils. Nuclear protein (10 μg) was prepared from eosinophils. Panel A: Eosinophils were subjected to the following treatment: lane 1, probe without sample; lane 2, control; lane 3, NAC (5 mM); lane 4, TNF-α (10 ng ml^{-1}); lane 5, GM-CSF (10 ng ml^{-1}); lane 6, GM-CSF + NAC; lane 7, TNF-α + NAC. Specificity was determined by competition with an excess of cold probe (lane 8). The filled arrowhead indicates the position of the NF-κB complex; the open arrowhead indicates the position of free probe. The faster migrating complexes were not specific. These results are representative of 3 independent experiments. Panel B: Eosinophils were stimulated with TNF-α and then incubated with specific radioactive

probe alone (lane 1), or with anti-p65 subunit antibody (lane 2) for 20 min. This result is representative of 2 separate experiments.