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Augmentation of human neutrophil and alveolar macrophage LTB_4 production by *N*-acetylcysteine: role of hydrogen peroxide

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1 The actions of *N*-acetylcysteine (NAC) on hydrogen peroxide (H_2O_2) and leukotriene B_4 (LTB_4) production by human resting and stimulated peripheral blood neutrophils and alveolar macrophages were investigated.

2 At a concentration of 100 μM, NAC significantly ($P<0.01$) suppressed the accumulation of H_2O_2 in the incubation medium of resting and opsonized zymosan (OZ; 0.5 mg ml^{-1})- or *N*-formylmethionyl-leucyl-phenylalanine (fMLP; 1 μM)-stimulated neutrophils and of resting and OZ-stimulated macrophages. At concentrations of 10 μM and above, NAC augmented significantly the level of LTB_4 in the supernatants of OZ- and fMLP-stimulated neutrophils ($P<0.01$ and $P<0.05$, respectively) and OZ-stimulated macrophages ($P<0.05$ at 10 μM, $P<0.01$ at 100 μM NAC).

3 NAC (100 μM) caused a significant ($P<0.01$) reduction in the quantity of measurable H_2O_2 when incubated with exogenous H_2O_2 concentrations equivalent to those released from OZ-stimulated neutrophils and macrophages. At no concentration did NAC affect quantitites of measurable LTB_4 when incubated with exogenous LTB_4.

4 Superoxide dismutase (SOD), which catalyzes the conversion of superoxide anion to H_2O_2 had no significant effect on LTB_4 production by human neutrophils. In contrast, catalase, which catalyzes the conversion of H_2O_2 to H_2O and O_2, caused a pronounced, statistically significant ($P<0.01$) increase in the levels of LTB_4 measured in the supernatants of OZ- and fMLP-stimulated neutrophils.

5 H_2O_2 (12.5 μM and 25 μM, concentrations equivalent to those measured in the supernatants of activated neutrophils and alveolar macrophages, respectively) caused a small (13%) decrease in the quantity of measurable LTB_4 ($P=0.051$ and $P<0.05$ at 12.5 μM and 25 μM, respectively) that was inhibited by NAC (100 μM) but not by catalase (400 u ml^{-1}).

6 In conclusion, the anti-oxidant drug, NAC, increases LTB_4 production by human neutrophils and alveolar macrophages, probably through the elimination of cell-derived H_2O_2. LTB_4 undergoes a H_2O_2-dependent oxidation that is inhibited by NAC but this is unlikely to account fully for the increased levels of LTB_4, suggesting that NAC may increase LTB_4 production by blocking the H_2O_2-dependent inhibition of a synthetic enzyme, such as 5-lipoxygenase.

Keywords: *N*-Acetylcysteine; neutrophils; macrophages, alveolar; antioxidants; hydrogen peroxide; catalase; leukotriene B_4

Introduction

Neutrophils (polymorphonuclear leukocytes) and macrophages (mononuclear phagocytes) are effector cells of general immunity. Through their capacity to produce toxic reactive oxygen metabolites and vasoactive lipid mediators they are involved in the inflammatory response leading to the clearing of foreign particles – including pathogenic microorganisms – from sites of invasion or infection. Since these cells participate in inflammatory reactions associated with the pathology of certain diseases, means have been sought to suppress the release of their inflammatory products in order to attenuate the tissue damage occurring at sites of inflammation in these conditions.

N-Acetylcysteine (NAC) is a derivative of the thiol-containing amino acid, cysteine, that has been demonstrated to exert anti-oxidant actions at high concentrations *in vitro* (Bernard *et al*., 1984; Moldeus *et al*., 1986; Kharazmi *et al*., 1988). It is unclear whether NAC also functions as an anti-oxidant *in vivo*, since its bioavailability after oral dosing has been shown to be less than 5% (Bernard *et al*., 1984), but various *in vivo* actions of NAC have been described, including the depression of serum levels of neutrophil granule enzymes and the reduction of neutrophil chemotactic activity in bronchoalveolar lavage (BAL) fluid (Eklund *et al*., 1988). Orally and intravenously administered NAC elevates levels of reduced glutathione (GSH) in serum and BAL of patients with idiopathic pulmonary fibrosis (Meyer *et al*., 1994; 1995); NAC is proposed to exert beneficial effects in this condition through the enhancement of the lung's anti-oxidant screen consequent to its actions on GSH levels.

In the present study, the direct actions of NAC upon neutrophil and alveolar macrophage function were investigated in order to determine whether NAC might exert additional, potentially beneficial, suppressive actions on inflammatory cells. The generation of hydrogen peroxide (H_2O_2) was measured as a marker of the capacity of the cells to produce reactive oxygen species; the release of leukotriene B_4 (LTB_4), a lipid mediator that is strongly chemotactic for neutrophils and other leukocytes, including monocytes and eosinophils, was measured as a marker of non-oxidant inflammatory activity of the cells. To determine whether the generation of reactive oxygen species and the production of the leukotriene are interdependent, the actions of the superoxide anion ($O_2^{\cdot-}$)-degrading and H_2O_2-degrading enzymes, superoxide dismutase (SOD) and catalase, upon LTB_4 generation were also investigated.

Parts of this work have been presented to the American Thoracic Society (Dent *et al*., 1994c; 1997).

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Methods

Cells

Neutrophils were isolated from the peripheral blood of normal volunteers (8 male, 7 female) by dextran sedimentation of erythrocytes followed by differential density centrifugation over Ficoll-Paque, as described by Böyum (1968). Cell preparations contained $96 \pm 1.2\%$ neutrophils (mean $\pm$ s.e.mean), as assessed by counting 200 cells on Wright's- stained cytocentrifuge preparations, which were $99 \pm 0.2\%$ viable at the time of experimentation, as assessed by Trypan blue exclusion.

Alveolar macrophages were obtained from hospital in-patients (7 male, 2 female) undergoing diagnostic bronchoscopy with BAL. Lavage was performed with five aliquots of 20 ml sterile normal saline, pre-warmed to 37°C. After routine processing, BAL fluid was filtered through two layers of nylon gauze and cells were recovered from the filtrate by centrifugation. The BAL samples used were all from patients exhibiting no acute airway inflammation and with no history of bronchial asthma; cells from patients receiving oral or inhaled corticosteroids were not studied. Cell preparations containing >90% macrophages were used for experiments. These preparations contained $94 \pm 1.3\%$ alveolar macrophages, which were $90 \pm 0.3\%$ viable.

To allow direct comparison of alveolar macrophages and neutrophils from the same subjects, blood was also donated by three of the patients from whom alveolar macrophages were obtained. These blood samples were taken on the day following BAL and the granulocyte preparations contained $98 \pm 0.6\%$ neutrophils of $99 \pm 0.3\%$ viability. Cells were washed twice in Hanks's balanced salts solution (HBSS) and resuspended in HBSS buffered to pH 7.4 at 37°C with 10 mM N - (2 - hydroxyethyl)piperazine - N′ - (2 - ethanesulphonic acid) (HEPES). All experiments were conducted in the same buffer solution (HEPES-HBSS).

Hydrogen peroxide measurement

Respiratory burst activity was assessed as the generation of H_2O_2, measured as the horseradish peroxidase (HRP)-catalyzed oxidation of phenol red, as described by Pick & Keisari (1980). Briefly, cells (10^6 ml^{-1}) were pre-incubated with HRP (final concentration 8.5 u ml^{-1}), in the absence or presence of NAC (100 nM – 1 mM), for 5 min at 37°C before the addition of phenol red (280 μM) and opsonized zymosan (OZ, 0.5 mg ml^{-1}) or an equal volume of HEPES-HBSS to a final volume of 1.2 ml (neutrophils) or 0.5 ml (alveolar macrophages). Mixtures were incubated for a further 45 min at 37°C, after which cells and OZ were precipitated by centrifugation at 12,000 *g* for 5 min. Supernatants were added to 1/1000 volume of 1 M NaOH and absorbance was measured at 620 nm and converted to H_2O_2 production by comparison with a standard curve constructed on the same day for known H_2O_2 concentrations. All experiments were conducted in duplicate and included a blank sample containing only HEPES-HBSS, HRP and phenol red, against which the other samples were read to correct for spontaneous oxidation of the indicator.

To determine whether the action of NAC was specific to particulate stimuli, a soluble stimulus - the chemotactic tripeptide, N-formylmethionyl-leucyl-phenylalanine (fMLP) was studied in some experiments. In view of the more rapid time-course of the response to fMLP, an incubation time of 30 min was used.

In order to determine the ability of NAC to react directly with the oxygen metabolites generated by activated cells, NAC (1 μM – 1 mM) or an equal volume of HEPES-HBSS was incubated with exogenous H_2O_2 (12.5 and 25 μM) for 45 min at 37°C, after which H_2O_2 was quantified spectrophotometrically, as described above.

Leukotriene B_4 measurement

LTB_4 generation was assessed under similar conditions to H_2O_2. Cells (10^6 ml^{-1}) were preincubated with or without NAC (final concentration 100 nM – 1 mM) for 5 min at 37°C before the addition of OZ (0.5 mg ml^{-1}) or fMLP (1 μM) or an equal volume of HEPES-HBSS to a final volume of 250 μl. Mixtures were incubated for a further 30 min at 37°C, after which cells and OZ were precipitated by centrifugation at 12,000 *g* for 5 min. Aliquots (200 μl) of cell supernatants were stored at −80°C until assayed for LTB_4 by competitive enzyme immunoassay (EIA), as described (Pradelles *et al*., 1985). In additional experiments, NAC was replaced in the incubation mixture by superoxide dismutase (SOD, 60 u ml^{-1} or 200 u ml^{-1}), catalase (200 u ml^{-1} or 400 u ml^{-1}) or a mixture of SOD (60 u ml^{-1}) and catalase (400 u ml^{-1}).

In order to determine the ability of NAC to react directly with LTB_4, NAC or an equal volume of HEPES-HBSS was incubated with exogenous LTB_4 (250 or 600 pg ml^{-1}) for 30 min at 37°C, after which LTB_4 was quantified by EIA, as described above. The effect of H_2O_2 on LTB_4 was assessed similarly, LTB_4 (600 pg ml^{-1}) being incubated with H_2O_2 (12.5 or 25 μM) in the absence or presence of NAC 100 μM or catalase (400 u ml^{-1}) for 30 min before quantification of LTB_4 by EIA.

Data analysis and statistics

Data from groups of untreated and NAC pretreated cells were compared by repeated measures analysis of variance (ANOVA; InStat, GraphPad Software, San Diego CA, U.S.A.). Data from experiments in cell-free systems were assessed by ordinary ANOVA. Where ANOVA returned a probability <0.05, comparisons between responses in cells treated with different NAC or enzyme concentrations and control (untreated) cells - or between analytes incubated in medium alone and those incubated in the presence of NAC or enzymes - were performed by means of Dunnett's test for multiple comparisons (InStat). Where comparisons between several groups were required, tests for significance were performed by Newman-Keuls Student's range procedure (InStat). A probability <0.05 was defined as significant.

Materials

Catalase (EC 1.11.1.6, from bovine liver), Ficoll-Paque (1.077 g ml^{-1}), fMLP, HEPES, HRP (EC 1.11.1.7, type II), *N*-acetyl-L-cysteine (NAC), phenol red, SOD (EC 1.15.1.1, from bovine erythrocytes) and zymosan A were purchased from Sigma Chemie GmbH (Deisenhofen, Germany). HBSS was obtained from Gibco-BRL Life Technologies GmbH (Eggenstein, Germany). LTB_4 EIA kits were supplied by Cayman Chemical Co. (Ann Arbor MI, U.S.A.).

Catalase, HRP, SOD and phenol red were dissolved in HEPES-HBSS. OZ was prepared immediately before use by boiling zymosan A particles for 10 min, opsonizing with 20% pooled fresh-frozen human serum at 37°C for 30 min and washing once with HBSS. fMLP was dissolved at 10 mM in 100% dimethyl sulphoxide and diluted in HEPES-HBSS. NAC was dissolved at 1 M in deionized water and diluted in HEPES-HBSS. Where indicated, 100 mM NAC solutions were neutralized by titration with NaOH before further dilution.

Results

N-Acetylcysteine

Neutrophils In initial experiments, 0.5 mg ml^{-1} was identified as a concentration of OZ inducing a reproducible, submaximal generation of both H_2O_2 and LTB_4 from human neutrophils. The incubation times selected for experiments were 45 min for H_2O_2 and 30 min for LTB_4 release: no further

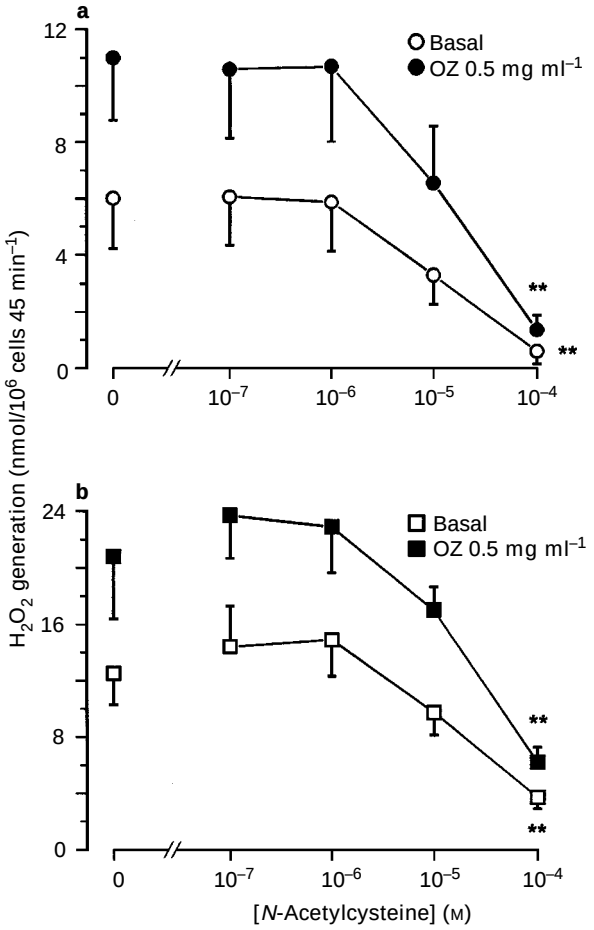

Figure 1 Effect of NAC pre-incubation on basal and OZ-stimulated generation of H_2O_2 by (a) human neutrophils ($n=6$) and (b) alveolar macrophages ($n=9$). Data are mean and vertical lines show s.e.mean from the indicated number of experiments, conducted in duplicate. $^{**}P<0.01$, compared to control cells pre-incubated with buffer only.

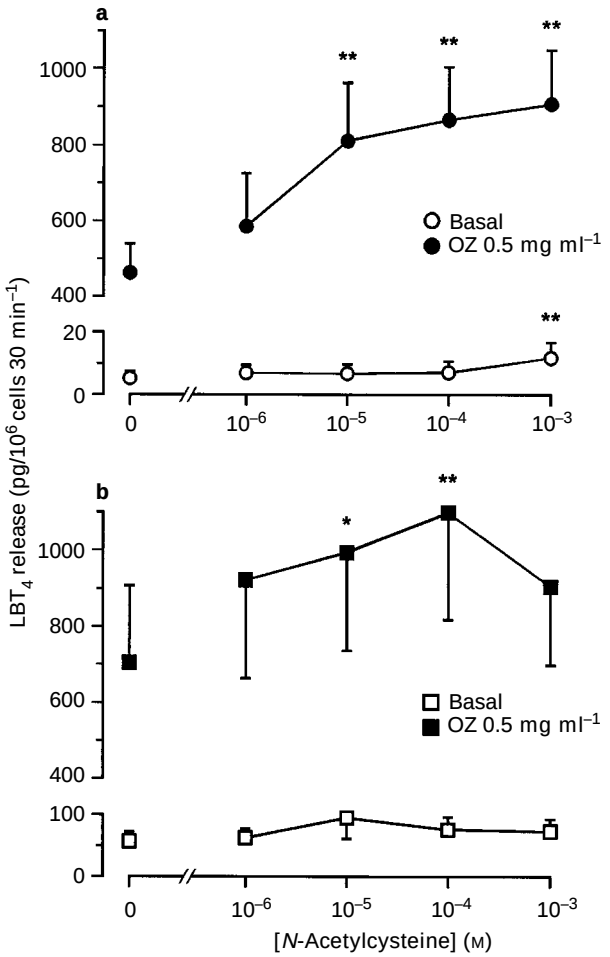

Figure 2 Effect of NAC pre-incubation on basal and OZ-stimulated release of LTB_4 by (a) human neutrophils ($n=6$) and (b) alveolar macrophages ($n=9$). Data are mean and vertical lines show s.e.mean from the indicated number of experiments, conducted in duplicate. $^{*}P<0.05$, $^{**}P<0.01$, compared to control cells pre-incubated with buffer only.

release of the products was observed at longer incubation times and the quantity of LTB_4 measured declined slightly at times >45 min (data not shown), possibly reflecting oxidation of LTB_4 occurring extracellularly.

Both basal and OZ-stimulated H_2O_2 generation were inhibited significantly by 100 μM NAC ($P<0.01$; Figure 1a). At a concentration of 1 mM, NAC alone caused significant colour change of phenol red in the absence of H_2O_2 ($A_{620}=0.023\pm0.001$ cm^{-1}, equivalent to 1.13 ± 0.05 nmol H_2O_2 ml^{-1}; $n=3$; $P=0.002$ by one-sample t test) and the effects of this concentration on H_2O_2 generation were, therefore, not included in the analysis.

Since the colour change of phenol red may occur as a result of the low pH of concentrated NAC solutions, further experiments were conducted with neutralized NAC solutions, produced as described in Methods. The inhibition of OZ-stimulated H_2O_2 production by neutralized NAC in the concentration range 100 nM – 100 μM was identical to that observed with non-neutralized solutions. At the high concentration of 1 mM, there was a small increase in absorbance at 620 nm, which may indicate the direct reduction of phenol red by NAC.

LTB_4 release from unstimulated neutrophils was low (5.3 ± 2.3 pg 10^6 cells^{-1}, $n=6$), with a small but significant increase above control at 1 mM NAC (Figure 2a). This concentration of NAC did not affect the detection by EIA of exogenous LTB_4 (see below). OZ induced a LTB_4 production of 460 ± 77 pg/10^6 cells that was significantly increased in the presence of NAC at concentrations of 10 μM – 1 mM (Figure 2a). When neutralized solutions were used, a slightly greater enhancement of OZ-induced LTB_4 production was observed at high NAC concentrations, although stimulated release in control cells was higher in these experiments than in those conducted with non-neutralized NAC (data not shown).

To assess the stimulus-specificity of these actions of NAC, H_2O_2 and LTB_4 production stimulated by fMLP was also studied. At a concentration of 1 μM (selected as a submaximally effective concentration from concentration-response curves for fMLP-induced H_2O_2 production; not shown), fMLP was a less effective stimulus than OZ for both responses. NAC exhibited similar actions against both stimuli for both responses, causing both significant inhibition of fMLP-induced H_2O_2 production (0.46 ± 0.43 nmol/10^6 cells 30 min^{-1} at 100 μM and 3.2 ± 0.81 nmol/10^6 cells 30 min^{-1} at 10 μM NAC vs 7.8 ± 1.2 nmol/10^6 cells 30 min^{-1} in the absence of NAC; $n=3$; $P<0.01$ and $P<0.05$, respectively) and significant augmentation of fMLP-induced LTB_4 release at concentrations of 10 μM and above (Figure 3).

Alveolar macrophages In order to allow direct comparison of the effects of NAC on neutrophil and macrophage functions, experiments with alveolar macrophages were conducted with the same OZ concentration and incubation times as those with neutrophils.

The action of NAC upon basal and OZ-stimulated alveolar macrophage H_2O_2 release was similar to the effect of the drug on this response in neutrophils (Figure 1b). H_2O_2 release was inhibited significantly by 100 μM NAC. While NAC caused a noticeable, but statistically non-significant, suppression of neutrophil H_2O_2 production at a concentration of 10 μM, this

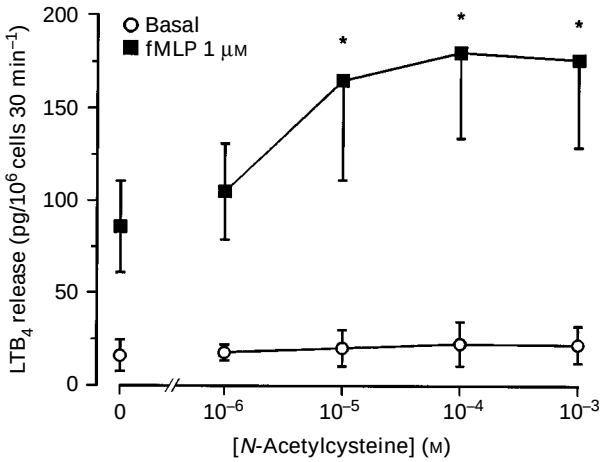

Figure 3 Effect of pre-incubation with neutralized NAC on basal and fMLP-stimulated release of LTB_4 by human neutrophils. Data are mean ± s.e.mean from five experiments, conducted in duplicate. $^*P<0.05$, $^{**}P<0.01$, compared to control cells pre-incubated with buffer only.

suppression was much less pronounced in alveolar macrophages, which produced larger quantities of H_2O_2.

Basal LTB_4 release from alveolar macrophages was higher than that from neutrophils (57 ± 15 pg/10^6 cells; $n=9$) and was unaffected by NAC (Figure 2b). OZ-stimulated LTB_4 release was highly variable (700 ± 200 pg/10^6 cells) but, like the neutrophil response, exhibited significant enhancement at NAC concentrations of 10 and 100 μM (Figure 2b).

Neutrophils and alveolar macrophages from the same donors Since there was some quantitative difference between the responses measured in neutrophils from normal donors and alveolar macrophages from hospital in-patients, neutrophils were isolated from three of the macrophage donors on the day after BAL (before the commencement of any drug therapy) to allow direct comparison of cells from the same donors.

The patterns for the effects of NAC in neutrophils and alveolar macrophages from the same donors were similar. The inhibition of H_2O_2 generation by NAC and augmentation of LTB_4 levels in supernatants of NAC-treated cells were essentially the same for neutrophils and alveolar macrophages from these donors (Table 1).

Cell-free system Incubation of 12.5 μM or 25 μM H_2O_2 (i.e. 12.5 and 25 nmol ml^{-1}, concentrations in the range of those produced by OZ-stimulated neutrophils and alveolar macrophages, respectively) with 100 μM NAC led to significant inhibition of the measured oxidation of phenol red (Figure 4), while NAC at this concentration had no effect on the absorbance of reaction mixtures to which no H_2O_2 was added.

Incubation of LTB_4 (250 pg ml^{-1} or 600 pg ml^{-1}, the latter concentration being in the range of those measured in the supernatants of activated neutrophils and alveolar macrophages) at 37°C for 30 min reduced the measurable LTB_4 content of the samples to 200 ± 16 pg ml^{-1} ($P<0.05$; $n=3$) and 571 ± 22 pg ml^{-1} (NS; $n=6$), respectively, possibly indicating non-enzymatic oxidation of LTB_4 at physiological temperatures. Measurable LTB_4 content was not affected significantly by NAC at any concentration in the range 100 nM – 1 mM.

Superoxide dismutase and catalase

Since NAC inhibited H_2O_2 accumulation in the supernatants of neutrophils and alveolar macrophages, apparently via a direct chemical interaction with H_2O_2, experiments were undertaken to assess whether H_2O_2 itself may participate in the regulation of LTB_4 production or degradation. To address this question, LTB_4 accumulation was measured in the supernatants of neutrophils treated with the enzymes SOD, which reduces superoxide anion radical ($O_2^{\cdot-}$) to H_2O_2, and catalase, which catalyzes the reduction of H_2O_2 to H_2O and oxidation of H_2O_2 to O_2. As shown in Figure 5a, while SOD (60 u ml^{-1}, a concentration that abolished $O_2^{\cdot-}$-dependent reduction of cytochrome c by OZ-stimulated neutrophils and eosinophils in previous experiments (Dent *et al.*, 1994a, and unpublished observations)) caused a marginal, non significant decrease in LTB_4 levels in the supernatants of OZ-stimulated neutrophils, catalase (400 u ml^{-1}, a concentration that abolishes H_2O_2/HRP-dependent oxidation of scopoletin by alveolar macrophages (Dent *et al.*, 1994b)) caused a substantial and significant increase in LTB_4 levels ($P<0.01$; $n=6$), which was not affected by the co-administration of SOD. Catalase caused a similar augmentation of LTB_4 release by fMLP (Figure 5b).

Since the lack of effect of SOD might have been attributable to its lower concentration, relative to the concentration of catalase used, a subset of 3 experiments used SOD and catalase at an additional concentration of 200 u ml^{-1}. There was no difference in effect between 60 and 200 u ml^{-1} SOD and no significant difference between 200 and 400 u ml^{-1} catalase (data not shown), indicating that the lack of effect of SOD is unlikely to be due to its being present at excessively low activity.

Table 1 Comparative effects of NAC on neutrophils and alveolar macrophages from the same donors

	Neutrophils	*Alveolar macrophages*
Hydrogen peroxide (nmol/10^6 cells 45 min^{-1})		
Control	22.7 ± 4.19	33.4 ± 9.69
NAC 100 μM	7.76 ± 2.56	19.6 ± 4.51
Leukotriene B_4 (pg/10^6 cells 30 min^{-1})		
Control	483 ± 144	604 ± 86.2
NAC 100 μM	805 ± 168	1240 ± 395

Opsonized zymosan (0.5 mg ml^{-1})-induced generation of H_2O_2 and LTB_4 by neutrophils and alveolar macrophages obtained on subsequent days from three patients. Data are mean ± s.e.mean.

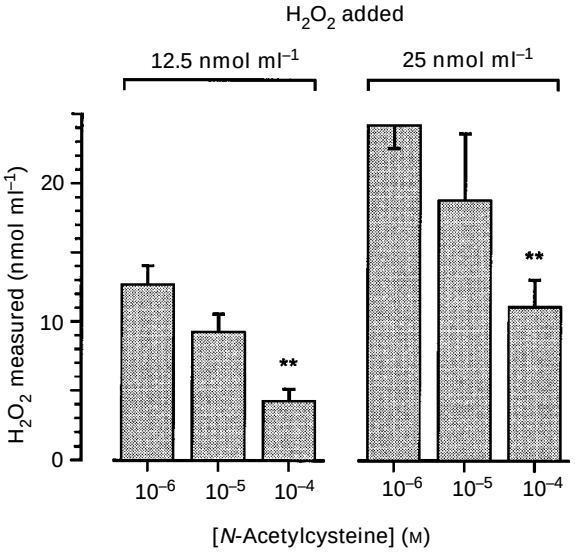

Figure 4 Effect of NAC upon measurable quantity of H_2O_2 in a cell-free system. The indicated concentrations of NAC were included in the assay medium with the indicated concentrations of exogenous H_2O_2. Data are mean ± s.e.mean from three experiments, conducted in duplicate. $^{**}P<0.01$, compared to H_2O_2 measured in the absence of NAC.

In order to determine whether NAC and catalase increase LTB_4 levels in cell supernatants by promoting its production or retarding its degradation, experiments were conducted in which LTB_4 (600 pg ml^{-1}) was incubated in the absence or presence of H_2O_2 with or without NAC or catalase. As shown in Figure 6, H_2O_2 caused a small decrease in the measurable quantity of LTB_4 amounting to approximately 13%, which was of marginal significance ($P=0.051$ at 12.5 μM H_2O_2, $P<0.050$ at 25 μM H_2O_2; $n=6$). This decrease was inhibited by NAC (100 μM) but was unaffected by the inclusion of catalase (400 u ml^{-1}).

Discussion

NAC, at a concentration of 100 μM, suppressed the levels of H_2O_2 measured in supernatants of unstimulated and OZ- or fMLP-stimulated human neutrophils and of unstimulated or OZ-stimulated alveolar macrophages. At concentrations of 10 μM and above, NAC significantly augmented the levels of LTB_4 measured in the supernatants of both cell types after OZ stimulation and of neutrophils after fMLP-stimulation. NAC appears, therefore, to exert actions on human immune effector cells that are potentially both anti-inflammatory and pro-inflammatory.

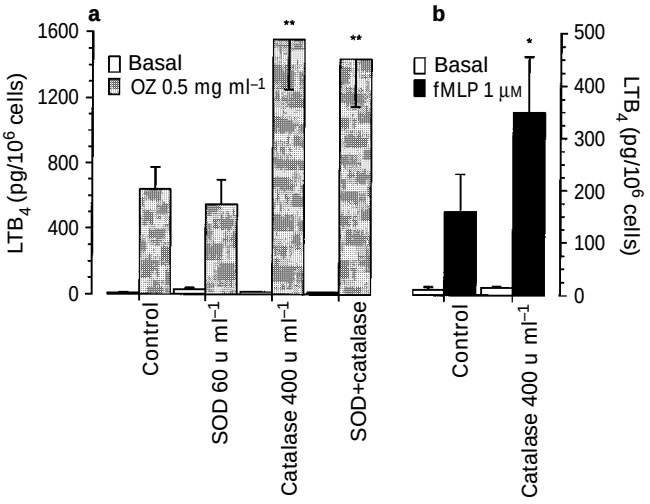

Figure 5 (a) Effects of SOD, catalase and a combination of the two enzymes on basal and OZ-stimulated LTB_4 release by human neutrophils. Data are mean ± s.e.mean from six experiments, conducted in duplicate. (b) Effect of catalase on basal and fMLP-stimulated LTB_4 release by human neutrophils. Data are mean ± s.e.mean from five experiments. $*P<0.05$, $**P<0.01$, compared to cells incubated with buffer only.

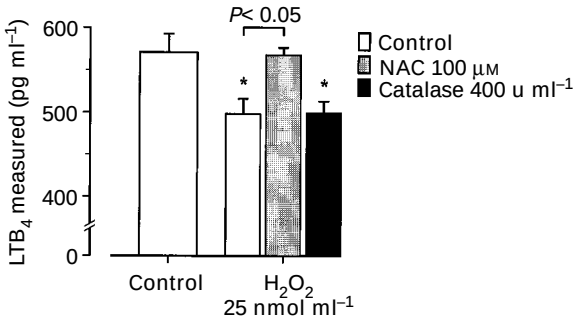

Figure 6 Effect of H_2O_2 upon measurable quantity of LTB_4 in a cell-free system. LTB_4 (600 pg ml^{-1}) was incubated for 30 min at 37°C with 25 μM H_2O_2 alone or in the presence of 100 μM NAC or 400 u ml^{-1} catalase. Data are mean ± s.e.mean from six experiments.

Conflicting evidence exists with regard to the ability of NAC to affect the generation of reactive oxygen species by phagocytic cells. Although very high concentrations of NAC (>15 mM) have been demonstrated to inhibit neutrophil and monocyte luminol-enhanced chemiluminescence (CL), this action appears to be due largely to the acidification of the medium by such high NAC concentrations (Kharazmi *et al*., 1988). Other workers have also described the suppression of fMLP- and OZ-induced neutrophil CL by high concentrations of NAC (150 μM – 3 mM) (Paulsen & Forsgren, 1989); no interference with either phagocytosis or intracellular killing was observed in that study but a more recent investigation has shown an enhancement of both phagocytosis and intracellular killing in alveolar macrophages and peripheral blood neutrophils by NAC at the highest concentration used (approximately 600 μM) (Oddera *et al*., 1994). In the latter study, enhanced phagocytosis and killing were not associated with increased self-killing of macrophages or neutrophils, implying a protective effect of NAC against damage of the phagocytic cells mediated by extracellular reactive oxygen species. Administration of NAC (400 mg day^{-1}, orally) to healthy volunteers leads to a significant reduction of neutrophil CL following OZ stimulation *ex vivo*, with no effect observed on monocyte CL or chemotaxis of either cell type (Jensen *et al*., 1988). However, more recently, oral administration of NAC (600 mg day^{-1} for 5 days) has been demonstrated to be ineffective in suppressing the generation of $O_2^{\cdot-}$ and H_2O_2 from human blood neutrophils and rat alveolar macrophages *ex vivo* (Drost *et al*., 1991).

In the present study, NAC was shown to suppress H_2O_2 levels in the supernatants of both resting and stimulated peripheral blood neutrophils and alveolar macrophages. In order to determine whether this suppression represented a 'pharmacological' action of NAC on the cells or a chemical interaction of NAC (a known anti-oxidant) with the cell-derived reactive oxygen species, the effect of NAC upon exogenous H_2O_2 was also assessed. At 100 μM, NAC was capable of significantly suppressing the HRP-catalyzed oxidation of phenol red by both 12.5 μM and 25 μM H_2O_2. These data suggest that the decrease in H_2O_2 generation measured in neutrophils and macrophages pretreated with NAC is likely to reflect the anti-oxidant action of NAC, rather than an action upon the cells themselves. NAC has been shown to react slowly with H_2O_2, but not with its precursor $O_2^{\cdot-}$ (Aruoma *et al*., 1989), and it is thus conceivable that cell-derived H_2O_2 might be reduced more rapidly by high concentrations of NAC than by the enzyme (HRP)/electron donor (phenol red) system that forms our assay.

Stimulant actions of NAC in the immune system are well documented. NAC enhances the phagocytosis of IgG-opsonized yeast particles by human neutrophils while also inhibiting the CL response at high concentrations (3 – 6 mM); no enhancement of phagocytosis of C3b-opsonized particles (analogous to OZ) was demonstrated (Ohman *et al*., 1992). NAC (approximately 600 μM) enhances the *in vitro* anti-fungal activity of peripheral blood monocytes, but not alveolar macrophages, from patients with chronic obstructive pulmonary disease, though exhibiting no effect on this activity *ex vivo* after treatment of patients with oral NAC (2×600 mg day^{-1} for 15 days) (Vecchiarelli *et al*., 1994). NAC has also been shown to augment both basal and anti-IgE-stimulated histamine release from human peripheral blood leukocytes, while inhaled NAC also suppresses 'allergen tachyphylaxis' in guinea-pigs and man, leading to exacerbated bronchial reactions after repeated allergen inhalation (Dorsch *et al*., 1987). Furthermore, NAC enhances the release of histamine from rat mast cells stimulated with F^- and Ca^{2+} or with compound 48/80 *in vitro* and amplifies the enhancement of lipopolysaccharide-stimulated release of tumour necrosis

factor from human neutrophils by nitric oxide (NO) donors, such as sodium nitroprusside (Hong *et al*., 1991; Van Dervort *et al*., 1994). The latter action is presumed to result from the ability of NAC to increase the bioavailability of NO.

We have demonstrated here the enhancement of stimulated LTB_4 release from human neutrophils and alveolar macrophages *in vitro* by NAC at concentrations of 10 μM and higher. This finding is novel but has a parallel in the action of NAC upon human alveolar macrophages *ex vivo*: macrophages of smokers have been found to exhibit markedly reduced LTB_4 production in response to OZ *in vitro* but cells obtained from smokers after eight weeks of treatment with oral NAC (2×200 mg day^{-1}) exhibited a partial, statistically significant reversal of this reduction (Wieslander *et al*., 1987; Linden *et al*., 1988). The elevation by NAC of LTB_4 levels in cell supernatants represents either a pharmacological action on the cells or an action on the metabolism of the leukotriene molecule, since NAC had no effect on exogenous LTB_4.

In order to determine whether the anti-oxidant property of NAC may account for the augmentation of LTB_4, the actions of two 'anti-oxidant' enzymes, SOD and catalase, were investigated. SOD, which converts O_2^- to H_2O_2, had no appreciable effect on LTB_4 levels, although it caused a slight, non-significant decrease (Figure 5). Catalase, which degrades H_2O_2, in contrast caused a substantial increase in measurable LTB_4 in the supernatants of OZ-activated neutrophils, amounting to an approximate 125% increase over control values compared to an increase of approximately 95% achieved with 1 mM NAC; the corresponding figures for fMLP-stimulated cells were 115% and 105%. These findings suggest either (i) that H_2O_2 may inhibit the generation of LTB_4 by neutrophils and alveolar macrophages or (ii) that H_2O_2 may effect an oxidative breakdown of LTB_4 that is blocked by catalase and by NAC through their inactivation of H_2O_2. The latter possibility was tested by incubating LTB_4 with H_2O_2 in a cell-free system. Although H_2O_2 did cause some breakdown of LTB_4, this effect was slight (approximately 13% decrease in measured LTB_4) and unlikely to account for the observed increase in LTB_4 in the supernatants of stimulated neutrophils or alveolar macrophages in the presence of NAC or catalase. The difference between cell-free LTB_4 levels after 30 min incubation with H_2O_2 and with buffer amounted to 78 pg ml^{-1} (Figure 6), compared to increases of >400 pg ml^{-1} in cell-derived LTB_4 in the presence of NAC (Figure 2). Although NAC blocked the degradation of LTB_4 by H_2O_2, catalase, surprisingly, did not. Since H_2O_2 was the last reagent added to these mixtures, it is possible that H_2O_2 reacts with LTB_4 and NAC more rapidly than it is broken down by catalase.

It appears, therefore, that cell-derived H_2O_2 may inhibit the generation of LTB_4 by neutrophils and alveolar macrophages and that NAC and catalase augment LTB_4 production by removing the H_2O_2, thus preventing autoinhibition of LTB_4 synthesis. H_2O_2 (10 μM) inactivates purified 5-lipoxygenase in the absence of reducing agents (Percival *et al*., 1992) and exogenous H_2O_2 has been demonstrated to inhibit 5-lipoxygenase activity in rat alveolar macrophages, apparently by depleting the cells of ATP, upon which the enzyme is dependent (Sporn & Peters-Golden, 1988), although this effect was observed with higher concentrations of H_2O_2 than were measured in the present study. However, it is probable that local concentrations around and within leukocytes undergoing respiratory burst are higher than those measured thoughout the supernatant medium.

In vivo, serum anti-oxidants, such as reduced glutathione, serve to inactivate reactive oxygen species derived from inflammatory cells. In interstitial lung diseases the increased oxidant production by macrophages and neutrophils in the alveolar walls places a large burden on these antioxidant defences and the concentration of reduced glutathione in the epithelial lining fluid is consequently reduced (Behr *et al*., 1995). Since glutathione is oxidized by inflammatory cell-derived H_2O_2 (Behr *et al*., 1995), NAC may serve to protect the anti-oxidant screen *in vivo* by acting as an alternative target for H_2O_2 oxidation. However, this remains to be demonstrated experimentally and some evidence suggests that NAC can, in contrast to glutathione, enhance H_2O_2-dependent oxidation of oxidative stress-sensitive proteins (Maier *et al*., 1996). Caution must, therefore, be exercised in extrapolating from the *in vitro* to the *in vivo* situation.

We conclude that, *in vitro*, NAC reduces levels of H_2O_2 in the supernatants of human neutrophils and alveolar macrophages – through what appears to be a direct anti-oxidant mechanism – and, by removing a H_2O_2-mediated inhibition of leukotriene biosynthesis, causes a significant augmentation of LTB_4 levels in the cell supernatants. Thus, while supression of oxidant accumulation at sites of inflammation may represent a useful action of potential anti-inflammatory drugs, this action may lead to increased generation of an important inflammatory mediator.

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