Lack of effect of N-acetylcysteine on the release of oxygen radicals from neutrophils and alveolar macrophages

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ABSTRACT: N-acetylcysteine (NAC) is rapidly de-acetylated *in vivo* to cysteine (CYSH), a precursor of glutathione (GSH) which is an antioxidant in cells and body fluids.

We investigated the effect of oral administration of N-acetyl cysteine for 5 days on the spontaneous and stimulated generation of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) from human and rat phagocytic leucocytes. Alveolar macrophages (AM) were obtained by bronchoalveolar lavage (BAL) in control rats and rats given NAC in their drinking water. Neutrophils (PMNL) were harvested from whole blood in normal nonsmoking volunteers before and after NAC was given by mouth.

The stimulated release of H_2O_2 and O_2^- from both rat AM and human PMN was not changed by administration of NAC. However, a small but significant increase was observed in both the spontaneous generation of O_2^- from rat AM and the spontaneous generation of H_2O_2 from human PMNL. Administration of NAC significantly increased cysteine levels in human plasma and rat BAL, but the levels in human PMNL and rat AM after NAC did not differ from control levels. GSH levels were not altered significantly by NAC.
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Phagocytic cells, alveolar macrophages and neutrophils, are present in increased numbers in the lungs of cigarette smokers [1–3] and patients with inflammatory lung diseases [4–6]. Stimulation of these inflammatory cells results in the release of proteolytic enzymes and an increase in the respiratory burst, causing the release of reactive oxygen intermediates [7–9]. Inappropriate or excessive release of these oxygen radicals may cause damage to the lung tissue, either directly, by peroxidation of membrane lipids and proteins, or indirectly by inactivating protease inhibitors, thus favouring proteolysis. The balance between the release of oxidants, and their inactivation by antioxidants could therefore be critical to the prevention of lung damage during the inflammatory response to bacteria and irritant inhalants such as cigarette smoke. Agents capable of reducing the release of oxidants, or inactivating them once released could therefore have a therapeutic value.

Excess reactive oxygen intermediates can be efficiently removed by the actions of superoxide dismutase on the superoxide anion (O_2^-), and by catalase and peroxidases on hydrogen peroxide (H_2O_2). Moreover, reducing the concentrations of H_2O_2 and O_2^- in the extracellular fluid limits the generation of the highly reactive hydroxyl radical (OH), produced by the Fenton reaction, and hypochlorous acid (HOCl), mediated by myeloperoxidase in the presence of halide [10].

Previous work has demonstrated the effectiveness of N-acetylcysteine (NAC) as an antioxidant *in vitro* [11–14]. However, *in vivo*, NAC needs to be present in high concentrations in extracellular fluid to scavenge oxygen radicals. Adequate concentrations of NAC may be difficult to achieve in plasma, since the bioavailability of the drug, following oral dosing, is reported to be less than 5% [12]. However, NAC has been observed to attenuate the injurious effects of endotoxin in the lungs of sheep [14] and to normalize cigarette smoke induced dysfunction in macrophages [2, 15, 16], lung fibroblasts and epithelial cells [12], in both rats and humans. Further *in vitro* work has suggested that NAC can prevent the inactivation of alpha$_1$-proteinase inhibitor (α_1-PI) by scavenging the neutrophil myeloperoxidase derived oxidant, hypochlorous acid (HOCl) [17, 18]. NAC is rapidly de-acetylated to cysteine during its first pass through the liver. Moreover, cysteine is a precursor in the biosynthesis of glutathione, itself and antioxidant [13, 15].

The aims of this study were to measure the effect of NAC, given by mouth, on NAC, cysteine and glutathione levels in human plasma and neutrophils, and in bronchoalveolar lavage fluids and alveolar macrophages from rats. Secondly, we wished to determine if the administration of NAC affected the subsequent generation of H_2O_2 and O_2^- from rat leucocytes.

Subjects and methods

N-acetylcysteine in the form of a water soluble powder containing 200 mg NAC per sachet was provided by Zambon Pharmaceuticals, Milan, Italy.

Rat bronchoalveolar lavage studies

Syngeneic specific pathogen free (SPF) rats (200 g) of the PVG strain were given NAC in their drinking water such that they ingested 10 mg·kg^{-1}·day^{-1} (twice the human dose). After treatment for 5 days the rats were killed, their lungs removed and lavaged with 4×8 ml aliquots of saline as described previously [19]. The resulting bronchoalveolar lavage fluid was spun at 1,600 rpm, at 4°C for 10 min. Bronchoalveolar leucocytes (97% alveolar macrophages and 3% lymphocytes) were resuspended in phosphate buffered saline (PBS) to a concentration of 1×10^7 cells·ml^{-1}. Comparisons were made with alveolar macrophages from PVG rats which were not given NAC in their drinking water.

Human neutrophil studies

Eleven normal, nonsmoking volunteers (aged 24–37 yrs) were given 600 mg of NAC by mouth for 5 days. Thirty ml of citrated venous blood were withdrawn from each subject on Day 1, prior to the first dose of NAC, and on Day 5, one and a half hours after the final dose. The samples of whole blood were mixed with Dextran (MW 70,000) (Travenol, UK) and allowed to sediment for one hour. The resulting leucocyte-rich plasma was overlaid on a Plasma/Percoll density gradient (Pharmacia, Sweden) and centrifuged to obtain a leucocyte band which was washed and lysed to remove contaminating erythrocytes, using a technique described previously [1]. The remaining neutrophil population (>95% pure) was resuspended in PBS at a concentration of 1×10^{7}cells·ml^{-1}. The viability of neutrophils harvested using this technique was >98% by trypan blue exclusion.

Hydrogen peroxide assay

H_2O_2 generation was measured by the method of PICK and KEISARI [20]. The reaction mixture consisted of 100 ml 10 mM potassium phosphate buffer (10 mM KH_2PO_4, 7.6 mM K_2HPO_4), containing 1 ml of stock Type II, salt-free horseradish peroxidase 5 mg·ml^{-1} in 0.05 M potassium phosphate buffer; 1 ml of 0.028 M phenol red; 140 mM sodium chloride and 5.5 mM dextrose (Sigma, UK). Alveolar macrophages or neutrophils (2.5×10^5 cells) were added to 1 ml of the reaction mixture. Both the spontaneous and phorbol myristate acetate (PMA, 1 µg·ml^{-1}) (Sigma, UK) stimulated release of H_2O_2 were measured after 2 h of incubation at 37°C, in 5% CO_2. The reaction was terminated by centrifugation at 2,000 rpm, at 4°C for 10 min and the colour reaction was completed by alkalization with 1 M NaOH. The absorbance of the supernatant fluid was measured on a Pye Unicam SP8-400 spectro photometer at 610 nm and compared with a standard curve derived from dilutions of a reference solution of H_2O_2.

Superoxide anion assay

O_2^- generation was measured by the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c [21]. Inclusion of the enzyme SOD eliminates all cytochrome c reduction by agents other than O_2^-. The reaction mixture contained 80 M cytochrome c and 0.65 mM dextrose (Sigma, UK) in 50 ml of PBS. Tubes containing 1 ml of the reaction mixture and 2.5×10^5 alveolar macrophages or neutrophils were set up with identical tubes which, in addition, contained 75,000 units SOD (Sigma, UK). Release of O_2^- either spontaneously, or when stimulated with PMA 1 µg·ml^{-1}, was measured after incubation for 2 h at 37°C in 5% CO_2. O_2^- generation is linear for up to 2 h incubation [21–23]. The reactions were terminated by centrifugation at 2,000 rpm, at 4°C for 10 min. The difference in absorbance of the supernatant fluids, in the presence or absence of SOD, was determined in a Pye Unicam SP8-400 spectrophotometer at 550 nm, and the amount of reduced ferricytochrome c was calculated based on an extinction coefficient of 21.0 mM^{-1}·cm^{-1} for cytochrome c.

NAC, cysteine and glutathione assays

NAC, cysteine and reduced glutathione concentrations were determined by derivatization with monobromobimane (Thiolyte, Calbiochem) to form fluorescent adducts, which were then separated and quantified using reverse phase high performance liquid chromatography [24, 25].

Catalase assay

Catalase activity was determined using a spectrophotometer assay with titanium sulphate [25].

Statistical analysis

Statistical analyses were performed on the mean values and standard deviations using the Student's t-test for paired samples.

Results

Reduced or free thiol levels measured in human plasma or neutrophils, and rat bronchoalveolar lavage fluid (BALF) or alveolar macrophages are shown in tables 1 and 2. In normal subjects following treatment with NAC, plasma cysteine increased significantly ($p<0.01$) with a trend for plasma GSH to increase, occurring in 5 of the 8 subjects studied. However, neither the level of intracellular cysteine nor free glutathione (GSH) in neutrophils changed significantly. In the rat, cysteine levels increased in BALF without significant change in the GSH levels after treatment with NAC (table 1). NAC was not detectable in human plasma and neutrophils nor in rat macrophages and BALF (data not shown).

In separate experiments, intracellular cysteine and GSH in rat macrophages did not alter following treatment with NAC. However, catalase activity measured in rat macrophages was observed to increase following NAC (control 63.6 ± 16.3, NAC 84.0 ± 13.8 U·mg^{-1} protein; n=6: $p<0.05$), but was not detectable in the extracellular fluid. In these experiments NAC produced a significant increase in BALF GSH but no change in cysteine levels (table 2).

The spontaneous or stimulated generation of H_2O_2 by rat alveolar macrophages did not change following treatment with NAC (spontaneous release H_2O_2: control 6.36 ± 2.4, NAC 6.9 ± 2.8 mM·2.5×10^{-5} cells; stimulated release H_2O_2: control 15.3 ± 3.8, NAC 14.4 ± 4.2 nM·2.5×10^{-3} cells; n=9, $p>0.05$) (fig. 1A).

There was a small, but statistically significant ($p<0.001$) increase in the spontaneous generation of O_2^- from rat alveolar macrophage after treatment with NAC, with no change in the stimulated O_2^- release (spontaneous release O_2^-: control 1.17 ± 0.3, NAC 2.14 ± 0.3 nM·2.5×10^{-5} cells; n=9, $p<0.001$; stimulated release O_2^-: control 9.23 ± 1.1, NAC 9.2 ± 0.6 nM·2.5×10^{-5} cells; n=9 $p>0.05$) (fig. 1B).

Table 1. – Cysteine (CYSH) and glutathione (GSH) levels in human plasma and neutrophils (PMNL) and rat bronchoalveolar lavage (BAL) before and after N-acetylcysteine (NAC)

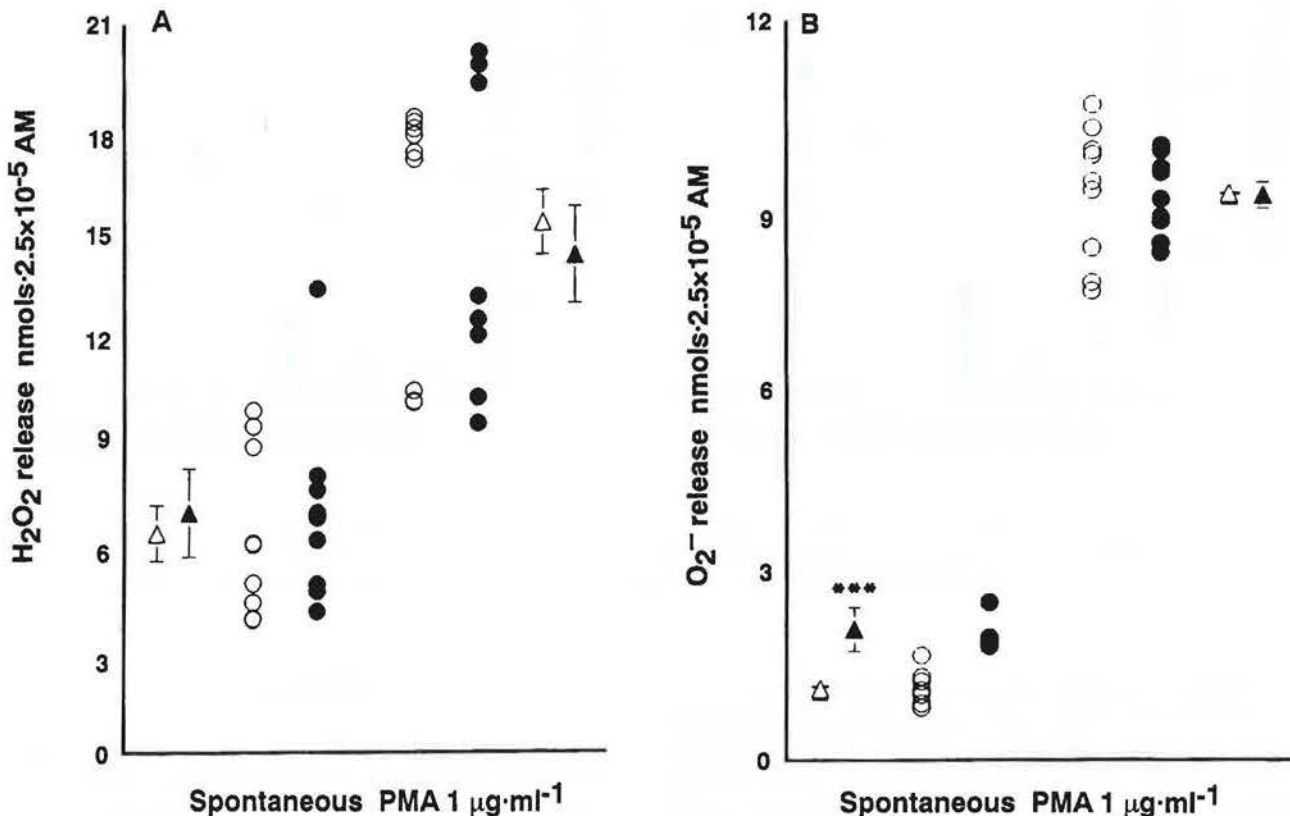| | CYSH | | GSH | |
	Control	NAC 5 days	Control	NAC 5 days
Normal subjects				
Plasma nM·ml^{-1}	6.7 ± 1.9 (n=11)	$9.8\pm3.5**$	2.9 ± 1.1 (n=8)	4.3 ± 2.4
PMNL nM·10^{-6} cells	0.1 ± 0.1 (n=11)	0.1 ± 0.0	1.7 ± 0.6 (n=10)	1.9 ± 0.4
PVG rats BAL nM·ml^{-1}	0.7 ± 0.6 (n=9)	$1.1\pm0.9*$	2.7 ± 2.7 (n=9)	2.5 ± 1.4

$**p<0.01$; $*p<0.05$.

Fig. 1. – (A) Hydrogen peroxide (H_2O_2) and (B) superoxide anion (O_2^-) production by alveolar macrophages obtained from 9 rats after 5 days' treatment with N-acetylcysteine (NAC) (●), and 9 control animals not given NAC (○). Both spontaneous levels and those on stimulation with phorbol-myristate-acetate (PMA) and the mean values (▲) are shown. The bars represent the standard errors of the mean. (***: $p<0.001$).

Table 2. – Cysteine (CYSH) and glutathione (GSH) levels in rat bronchoalveolar lavage (BAL) and alveolar macrophages (AM) before and after N-acetylcysteine (NAC)

	CYSH		GSH	
	Control	NAC 5 days	Control	NAC 5 days
PVG Rats BAL nM·ml⁻¹	0.3±0.2	0.3±0.1 (n=6)	3.2±0.9	4.5±1.1* (n=6)
AM nM·10⁻⁶ cells	0.7±0.2	0.6±0.1 (n=6)	3.4±0.8	3.2±0.9 (n=6)

*$p<0.05$.

statistical significance (stimulated release H_2O_2: control 27.1±10.0 NAC 45.7±27.1 $nM·2.5×10^{-5}$ cells; n=11, $p>0.05$).

The spontaneous generation of O_2^- from human neutrophils tended to increase following NAC but this change was not significant (spontaneous release O_2^-: control 1.5±0.4, NAC 2.2±1.4 $nM·2.5×10^{-5}$ cells; n=11, $p>0.05$). The PMA stimulated release of O_2^- by contrast tended to fall following treatment with NAC, but again this change was not significant (stimulated release O_2^-: control 18.6±1.6, NAC 9.9±5.2 $nM·2.5×10^{-5}$ cells; n=11, $p>0.05$) (fig. 2B).
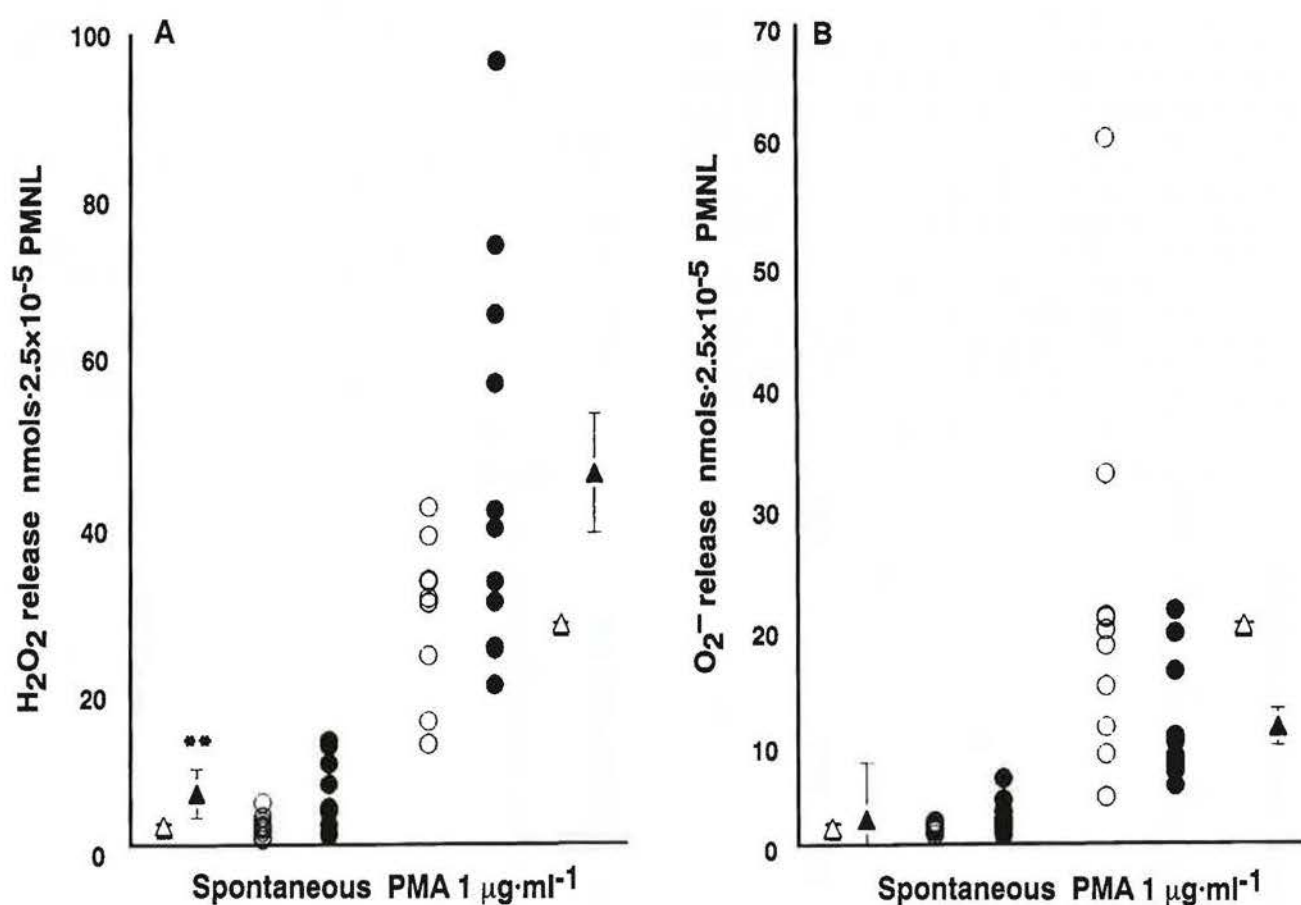

Fig. 2. – (A) Hydrogen peroxide (H_2O_2) and (B) superoxide anion (O_2^-) production by human neutrophils from 11 normal subjects before (O) and after (●) 5 days' treatment with N-acetylcysteine (NAC). Both the spontaneous levels and those on stimulation with phorbol-myristate-acetate (PMA) and the mean values (▲) are shown. The bars represent the standard errors of the mean. **: $p<0.02$.

Human neutrophils from 11 normal subjects treated with NAC for 5 days showed a small but significant increase in the spontaneous release of H_2O_2 compared with pretreatment values (spontaneous release H_2O_2: control 2.5±1.3, NAC 6.4±4.3 $nM·2.5×10^{-5}$ cells; n=11, $p<0.02$) (fig. 2A). Although neutrophils from 7 of the 11 subjects showed an increase in the PMA stimulated generation of H_2O_2, this trend did not achieve

Discussion

This study failed to show any significant decrease in the release of H_2O_2 or O_2^- from human peripheral blood neutrophils and rat alveolar macrophages. On the contrary, small increases in the spontaneous release of O_2^- from rat macrophages and H_2O_2 from human neutrophils was demonstrated.

The thiol compound N-acetylcysteine (NAC) is known to scavenge free electrolytes such as hydrogen peroxide (H_2O_2), and reactive oxygen species such as superoxide anion (O_2^-). *In vitro* studies have previously demonstrated a reduction in the chemiluminescence of stimulated human neutrophils following incubation with NAC [11]. Similarly BERNARD *et al.* [14], employed the chemiluminescence assay to demonstrate the reduced ability of zymosan or PMA stimulated sheep neutrophils to scavenge oxygen metabolites when incubated with NAC. However, the bioavailability of NAC is only 5%, because of its extensive first pass metabolism by the liver [12]. Indeed NAC was not detectable in plasma or BALF in this study, or in our previous study [26]. Thus, it is unlikely that concentrations of NAC in body fluids are high enough to achieve such a scavenging action.
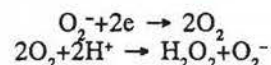
Nevertheless, recent *in vivo* work has shown that NAC protects against smoke induced cellular damage to both the rat [12] and human lung [2, 12, 18], possibly by enhancing cysteine levels and thus GSH biosynthesis [13]. Further *in vivo* studies, such as that by BERGSTRAND *et al.* [15] have also demonstrated a reduction in O_2^- generation by smokers' macrophages after treatment with NAC for 8 weeks. Similarly, treatment with NAC was observed to reduce the chemiluminescence of zymosan stimulated human neutrophils [27]. Furthermore, DE FLORA *et al.* [28] observed increased hexose monophosphate shunt (HMPS) activity in rat macrophage following NAC, and KHARAZMI *et al.* [11] observed an increased oxygen consumption, also suggesting increased HMPS activity, in the face of a reduction in chemiluminescence in neutrophils treated with NAC. The authors of these studies suggest that the increased activity of the HMPS following NAC may be explained by an increase in H_2O_2 catabolism by the glutathione system.

The aim of this study was to determine if NAC given *in vivo* resulted in increased glutathione biosynthesis, thereby preventing free radical generation as suggested in recent reports [29, 30]. However, in this study we have employed specific assays to detect nanomole amounts of H_2O_2 and O_2^-, whereas many of the previously published studies which have investigated the role of NAC as an antioxidant have used the less specific chemiluminescence assay [11, 14, 27].

Our results from human studies indicate that although there was a small increase in plasma cysteine, intracellular thiol levels were not significantly altered by NAC. Although MOLDEUS *et al.* [12] have shown a significant increase in plasma GSH in healthy volunteers following NAC 200 mg twice daily for two weeks, we have previously shown that following 5 days' treatment with a single dose of NAC the GSH levels peak at variable intervals between 2 and 8 h [26]. Plasma cysteine levels, however, peak 90 min after the last dose of NAC [26]. The fact that we sampled blood 90 min after the last dose of NAC may therefore explain the significant increase in plasma cysteine but not plasma GSH levels. It is also possible that any increase in GSH may have been lost as a result of the two hour period required to harvest a pure population of neutrophils. However, intracellular thiol levels which did not increase following NAC, are likely to be more stable than plasma and BALF levels (M. Bridgemen, unpublished observations). We assayed total *i.e.* both oxidised and reduced cysteine and GSH levels, in some of the human subjects (data not shown). These were only marginally higher than the reduced values alone, which is in agreement with other workers [31] and suggests that most of the thiols were present in the free, reduced form.

There was no significant reduction in the release of H_2O_2 or O_2^- from neutrophils following treatment with NAC. Indeed, the spontaneous production of H_2O_2 was significantly increased after NAC. A similar trend for the PMA stimulated release of H_2O_2 and the spontaneous release of O_2^- to increase was also observed. An increase in the spontaneous release of H_2O_2 from neutrophils, following NAC without a significant reciprocal change in O_2^- production, as observed in this study may simply be explained by the individual results in figure 2 which show a similar trend for spontaneous O_2^- to increase following NAC as with H_2O_2. However, the molar ratios observed in this study also suggest that significantly more H_2O_2 than O_2^- was generated from stimulated human neutrophils following treatment with NAC. This appears stoichometrically erroneous since the reduction of molecular oxygen:

$$O_2^-+2e \rightarrow 2O_2$$
$$2O_2+2H^+ \rightarrow H_2O_2+O_2^-$$

predicts a molar ratio of 2:1 for O_2^-/H_2O_2 production. This apparently contradictory result could be explained by the release of O_2^- from the cell membrane into phagosomes which would prevent its detection in our assay by the scavenger cytochrome *c*. Furthermore, within the phagosome the O_2^- would have more time, both spontaneously and in the presence of SOD, to dismutate to H_2O_2 before its secretion/diffusion out of the cell [32]. This mechanism may also explain the decrease observed in the stimulated release of O_2^- from human neutrophils following NAC occurring in 8 of the 11 subjects studied (fig. 2B). In addition, any catabolism of H_2O_2 by myeloperoxidase in the phagosome, or by catalase or the glutathione system in the cytosol could also reduce the levels of H_2O_2 which were detected. Indeed, plasma myeloperoxidase was observed by EKLUND *et al.* [33] to fall following treatment over 8 weeks with NAC. Yet, despite the possible catabolism of H_2O_2 by myeloperoxidase, an increased H_2O_2 production was evident in most individuals following NAC in this study. Reduced chemiluminescence observed by many workers following treatment with NAC [11, 14, 27] may reflect a reduction in myeloperoxidase activity as a result of myeloperoxidase entrapment in phagosomes and/or its catabolism of H_2O_2, as the chemiluminescence assay is both myeloperoxidase and O_2^- dependent [23]. Intracellular thiol levels in human neutrophils, however, did not increase following NAC.

In the animal study, our results indicate a small increase in the spontaneous O_2^- production from rat alveolar macrophage following treatment with NAC. The animals did ingest NAC, as shown by the significant increase in BAL cysteine, although the changes were small (table 1) and were not sufficient to have a significant effect since intracellular thiols did not alter (table 2). Recent human studies have demonstrated that thiol levels in BAL fluid increase in a dose-dependent fashion [26]. However, in separate experiments in rats, we showed a significant increase in intracellular catalase activity in alveolar macrophages but not in BAL, which may also explain the lack of any increase in spontaneous H_2O_2 generation from alveolar macrophages in spite of a small, but significant, increase in spontaneous O_2^- release following NAC. Interference of the O_2^- assay by catalase is unlikely as cytochrome c reduction is not affected by catalase. In this case, in contrast to the data in table 1, GSH but not cysteine levels increased in BALF (table 2). This apparent contradictory result, we believe, may result from a later sampling time of BALF following NAC ingestion in these experiments since our previous work suggests that cysteine peaks earlier than GSH in BALF in man following ingestion of NAC [26]. However, because of the difficulty in accurately determining the time from last dosage of NAC to the time when lavage was carried out in the rat, this hypothesis remains unproven.

In summary, although N-acetylcysteine may act as an antioxidant *in vitro* as a precursor of cysteine and thus glutathione biosynthesis, we found no evidence to suggest that administration of NAC resulted in reduced H_2O_2 and O_2^- generation from lung phagocytes. Indeed, NAC significantly increased the spontaneous oxygen radical production from these cells. This increase in oxygen radical release was small and, therefore, is unlikely to have any clinical significance. An increase in plasma cysteine, however, in the absence of free iron, may scavenge free radicals released into the micro-environment surrounding phagocytic cells, and may help to explain the effect of NAC in protecting the lungs against injury induced by inhaled oxidants such as cigarette smoke, or released from activated phagocytes during inflammation or infection.

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Absence d'effet de la N-acétyl cystéine sur la libération de radicaux d'oxygène à partir des neutrophiles et des macrophages alvéolaires. E. Drost, S. Lannan, M.M.E. Bridgeman, D. Brown, C. Selby, K. Donaldson, W. MacNee.

RÉSUMÉ: La N-acétyl cystéine (NAC) est rapidement désacétylée *in vivo* en cystéine (CYSH), un précurseur du glutathion (GSH) qui est un anti-oxydant dans les cellules et les liquides organiques.

Nous avons investigué les effets de l'administration orale de N-acétyl cystéine pendant 5 jours sur la production spontanée et stimulée de peroxyde d'hydrogène (H_2O_2) et d'anion superoxyde (O_2^-) par les leucocytes phagocytaires de l'homme et du rat. Des macrophages alvéolaires ont été obtenus par lavage broncho-alvéolaire chez les rats contrôle et les rats auxquels NAC avait été administrée dans leur eau de boisson. Les neutrophiles (PMNL) ont été prélevés dans le sang complet chez des volontaires normaux non fumeurs, avant et après administration de NAC par la bouche.

La libération stimulée de H_2O_2 et de O_2^- à partir des macrophages alvéolaires du rat et des polymorphonucléaires humains n'est pas modifiée par l'administration de NAC. Toutefois, une augmentation modeste mais significative a été observée dans la production spontanée de O_2^- par les macrophages alvéolaires du rat et la production spontanée de H_2O_2 par les polymorphonucléaires humains. L'administration de N-acétyl cystéine augmente significativement les niveaux de cystéine dans le plasma humain et dans le BAL du rat, mais les niveaux observés dans les PMNL humains et les AM du rat après NAC ne sont pas différents de ceux des contrôles. Les niveaux de GSH n'ont pas été affectés significativement par la NAC.
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