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Inhibitory Effects of *N*-acetylcysteine on Superoxide Anion Generation in Human Polymorphonuclear Leukocytes

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Abstract

It has been suggested that reactive oxygen species released by activated polymorphonuclear leukocytes (PMN) in man is one mechanism of tissue injury. Therapeutic action aimed at increasing antioxidant defence mechanisms is still a clinical challenge. This study examines the activity of *N*-acetylcysteine, a known antioxidant, in the protection of PMN exposed in-vitro to the chemoattractant peptide fMet-Leu-Phe (FMLP), the protein kinase C activator phorbol myristate acetate or the lipid peroxidation promoter *t*-butyl hydroperoxide.

FMLP (3–300 nM) and phorbol myristate acetate (160 pm–160 nM) induced concentration-related superoxide anion generation. Pre-treatment with *N*-acetylcysteine (33–333 μM) resulted in concentration-related inhibition of superoxide production induced by FMLP (30 nM) or phorbol myristate acetate (16 nM); $-\log$ IC50 values were $3{\cdot}97 \pm 0{\cdot}07$ and $3{\cdot}91 \pm 0{\cdot}10$, respectively. Changes in intracellular calcium ion concentration ($[Ca^{2+}]_i$) induced by FMLP (30 nM) were studied in fura-2-loaded human PMN. FMLP produced a transient calcium response, i.e. a peak followed by decay to a residual value above baseline. *N*-Acetylcysteine (333 μM) did not affect either basal $[Ca^{2+}]_i$ values or changes in $[Ca^{2+}]_i$ values after treatment with FMLP. Activation by phorbol myristate acetate caused a reduction in glutathione levels from $5{\cdot}94 \pm 0{\cdot}86$ (control) to $1{\cdot}84 \pm 0{\cdot}51$ nmol/3×10^6 cells ($P < 0{\cdot}05$ compared with control). Pre-treatment with *N*-acetylcysteine (333 μM) fully reversed the reduction in glutathione levels induced by phorbol myristate acetate ($4{\cdot}83 \pm 0{\cdot}68$ nmol/3×10^6 cells; $P > 0{\cdot}05$ compared with control). Exposure to *t*-butyl hydroperoxide (0·5 mM, 30 min) markedly increased malondialdehyde levels (from $0{\cdot}03 \pm 0{\cdot}02$ to $0{\cdot}73 \pm 0{\cdot}07$ nmol/10^6 cells), and index of lipid peroxidation. Malondialdehyde levels were significantly reduced in PMN treated with *N*-acetylcysteine (333 μM; $0{\cdot}55 \pm 0{\cdot}04$ nmol/10^6 cells; $P < 0{\cdot}05$ compared with untreated cells exposed to *t*-butyl hydroperoxide).

In conclusion, *N*-acetylcysteine reduces superoxide generation in response to FMLP and phorbol myristate acetate and partially protects against lipid peroxidation in PMN from man. The protection afforded by *N*-acetylcysteine is not related to alteration of the intracellular calcium signal but might be effected by replenishment of the intracellular glutathione levels.

In man polymorphonuclear leukocytes (PMN) are crucial in host defence mechanisms including the inflammatory reaction. A 'respiratory burst' with oxygen-radical formation usually accompanies PMN activation. Upon inappropriate activation, however, reactive oxygen radicals released by PMN might result in tissue injury. Thus, in the adult respiratory distress syndrome neutrophils were found to release increased amounts of oxygen radicals spontaneously (Laurent et al 1996). A defective antioxidant status in addition to reactive oxygen species produced by neutrophils and other recruited cells might contribute to the pathogenesis of various chronic inflammatory lung disorders, including asthma (Sanders et al 1995) and to other chronic inflammatory conditions.

The key role of the oxidant-antioxidant balance in the pathogenesis of tissue injury is supported by the protective effect of *N*-acetyl-L-cysteine (NAC), a known antioxidant, in some animal models and studies in man (Bernard 1991; Laurent et al 1996). Several mechanisms could underlie these beneficial effects of NAC. In particular, NAC could increase the intracellular stores of glutathione in PMN and other cells thereby enhancing endogenous anti-oxidative defence mechanisms (Reed 1990; Morris & Bernard 1994). Alternatively or in addition, NAC could directly scavenge radicals produced by inflamed cells (Gressier et al 1994).

This study was undertaken to examine the activity of NAC in human PMN stimulated by the synthetic chemotactic peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) or the protein kinase C activator phorbol 12-myristate 13-acetate to generate the superoxide anion ($O_2^{\cdot -}$). The effects of NAC on FMLP-induced changes in intracellular Ca^{2+} concentration, on phorbol myristate acetate-induced decrease of glutathione levels, and against lipid peroxidation promoted by *t*-butyl hydroperoxide were also determined in human PMN.

Materials and Methods

N-Acetyl-L-cysteine was provided by Zambon Laboratory (Barcelona, Spain). Fura-2/AM was obtained from Boehringer Mannheim (Germany). All other chemicals were from standard commercial sources. Water was purified on a Milli-Q (Millipore Iberica, Madrid, Spain) system.

Drug concentrations are expressed as the molar concentration of the active species.

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Isolation of human polymorphonuclear leukocytes

Human peripheral venous blood was obtained from healthy donors. Polymorphonuclear leukocytes (PMN) were separated by standard laboratory procedures including dextran-sedimentation, subsequent centrifugation on a Ficoll-Paque (Histopaque 1077; Sigma) density gradient and hypotonic lysis of contaminating red blood cells (Böyum 1968). The purity of PMN was approximately 95% and the viability as measured by trypan blue exclusion was >95%. After isolation, cells were stored at 4°C in Hanks' balanced salt solution.

Measurement of superoxide anion generation from human PMN

Superoxide anion generation by PMN was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome c with a modified micro-assay (Sedgwick et al 1988). With 96-well microtitre plates and a 200-μL reaction volume, 1×10^5 cells were added to cytochrome c (100 μM) in Hanks' balanced salt solution. To initiate the reaction, the cells were incubated with FMLP or phorbol myristate acetate. Immediately after the addition of activator, the absorbance of the reaction wells was measured at 550 nm in a Microplate Autoreader (EL309, Bio-Tek Instruments); these readings were repeated for 60 min. Between absorbance measurements, the plates were placed in a 5% CO_2 incubator at 37°C. Each reaction was performed in duplicate and against an identical control reaction, which contained 20 μg mL^{-1} superoxide dismutase. Results were adjusted to represent a 1-mL reaction volume, and $O_2^{\cdot -}$ generation was calculated as nmol cytochrome c reduced/5×10^5 cells. $O_2^{\cdot -}$ generation was measured in the absence (control) or presence of NAC (33, 100, or 333 μM). NAC or its vehicle was added 5 min before the addition of activator.

Measurement of intracellular Ca^{2+} levels

Intracellular Ca^{2+} levels were measured with the fura-2 method described by Schudt et al (1991) with modifications (Cortijo et al 1996). Cells 10^7 mL^{-1} were suspended in Hanks' balanced salt solution containing Ca^{2+}, 1 mM, and Fura-2/AM, 2 μM, and incubated for 45 min at 37°C. After loading, PMN were washed in pre-warmed Hanks' balanced salt solution (containing 1 mM Ca^{2+}), re-suspended as 10^6 cells mL^{-1} and equilibrated in a cuvette for 5 min at 37°C. After equilibration, PMN were pre-incubated at 37°C for 5 min with NAC (333 μM) or its vehicle and then cells were stimulated with FMLP (30 nM) for 3 min. The fluorescence intensity of PMN was monitored (excitation wavelengths 340 and 380 nm, emission wavelength 510 nm) by use of a spectrofluorimeter (Perkin-Elmer LS50) with a thermally controlled cuvette holder and a magnetic stirrer. Intracellular Ca^{2+}-concentrations ($[Ca^{2+}]_i$) were calculated according to Grynkiewicz et al (1985). Maximum fluorescence was achieved by completely lysing the cells with 0·1% Triton X-100 and minimum fluorescence by complexing calcium with 10 mM EGTA. FMLP induced a transient fluorescence signal which consisted in an initial rapid increase to a peak followed by a rapid decay to a plateau above the baseline values. To evaluate the effects of NAC we measured the value of $[Ca^{2+}]_i$ at the peak and 3 min after addition of FMLP, and the area under the curve ($AUC_{0-3\ min}$ expressed in nmol s mL^{-1}).

Measurement of glutathione levels, lipid peroxidation, and lactate dehydrogenase

Glutathione was measured according to the glutathione-transferase assay (Brigelius et al 1983) in control PMN and in PMN exposed to phorbol myristate acetate (16 nM, 30 min) in the absence and presence of NAC (333 μM). NAC was present for 5 min before and during exposure to phorbol myristate acetate. Lipid peroxidation was evaluated by measuring levels of malondialdehyde by reaction with 2-thiobarbituric acid according to procedures outlined in the colorimetric assay available commercially (LPO-586; Bioxytech S.A. of Boehringer Ingelheim; Esterbauer & Cheeseman 1990). Malondialdehyde levels were measured in control PMN and in PMN exposed to *t*-butyl hydroperoxide 0·5 mM, 30 min) in the absence and presence of NAC (333 μM). NAC was present for 5 min before and during exposure to *t*-butyl hydroperoxide. The activity of released lactate dehydrogenase was taken as a crude marker for severe cell damage and was measured by determining the first-order rate of NADH oxidation spectrophotometrically at 340 nm using pyruvate and NADH (Kornberg 1955). The activity was expressed in units (1 unit oxidized 1 μmol of NADH min^{-1}). The cells were treated with 0·1% triton X-100 for 15 min to measure the total lactate dehydrogenase activity; this was 1558 ± 29 units L^{-1} ($n = 6$). The activity of released lactate dehydrogenase was given as a percentage of the activity in totally disrupted cells.

Statistical analysis

Data are presented as mean ± s.e.m. Statistical analysis of results was carried out by analysis of variance followed either by the Bonferroni test or by Student's *t*-test, as appropriate (InStat, GraphPad software). Results were accepted as significant when $P < 0.05$.

Results

Effect of N*-acetylcysteine on superoxide release induced by FMLP and phorbol myristate acetate*

Stimulation of PMN with FMLP (3–300 nM) elicited a concentration-related increase of superoxide generation (Fig. 1a). FMLP (30 nM) was selected for subsequent experiments. NAC (33–333 μM) reduced the superoxide anion generation induced by FMLP in a concentration-dependent manner (Fig. 1b) with a $-\log$ IC50 value of 3.97 ± 0.07. Stimulation of PMN with phorbol myristate acetate (160 pM–160 nM) resulted in a concentration-related increase of superoxide generation (Fig. 1c). Phorbol myristate acetate (16 nM) was selected for further experiments. NAC (33–333 μM) reduced superoxide generation in a concentration-related fashion (Fig. 1d) with $-\log$ IC50 value of 3.91 ± 0.10.

Influence of N*-acetylcysteine on FMLP-induced increase of intracellular Ca^{2+} levels*

Changes in intracellular Ca^{2+} levels were monitored in fura-2 loaded PMN. Baseline values of $[Ca^{2+}]_i$ were 83 ± 6 nM ($n = 4$). Addition of FMLP (30 nM) caused a transient change in $[Ca^{2+}]_i$, i.e. a rapid increase to a peak followed by decay to a residual value slightly above baseline. Neither the baseline values nor the FMLP-induced changes in $[Ca^{2+}]_i$ were altered by *N*-acetylcysteine (333 μM), as is shown in Table 1.

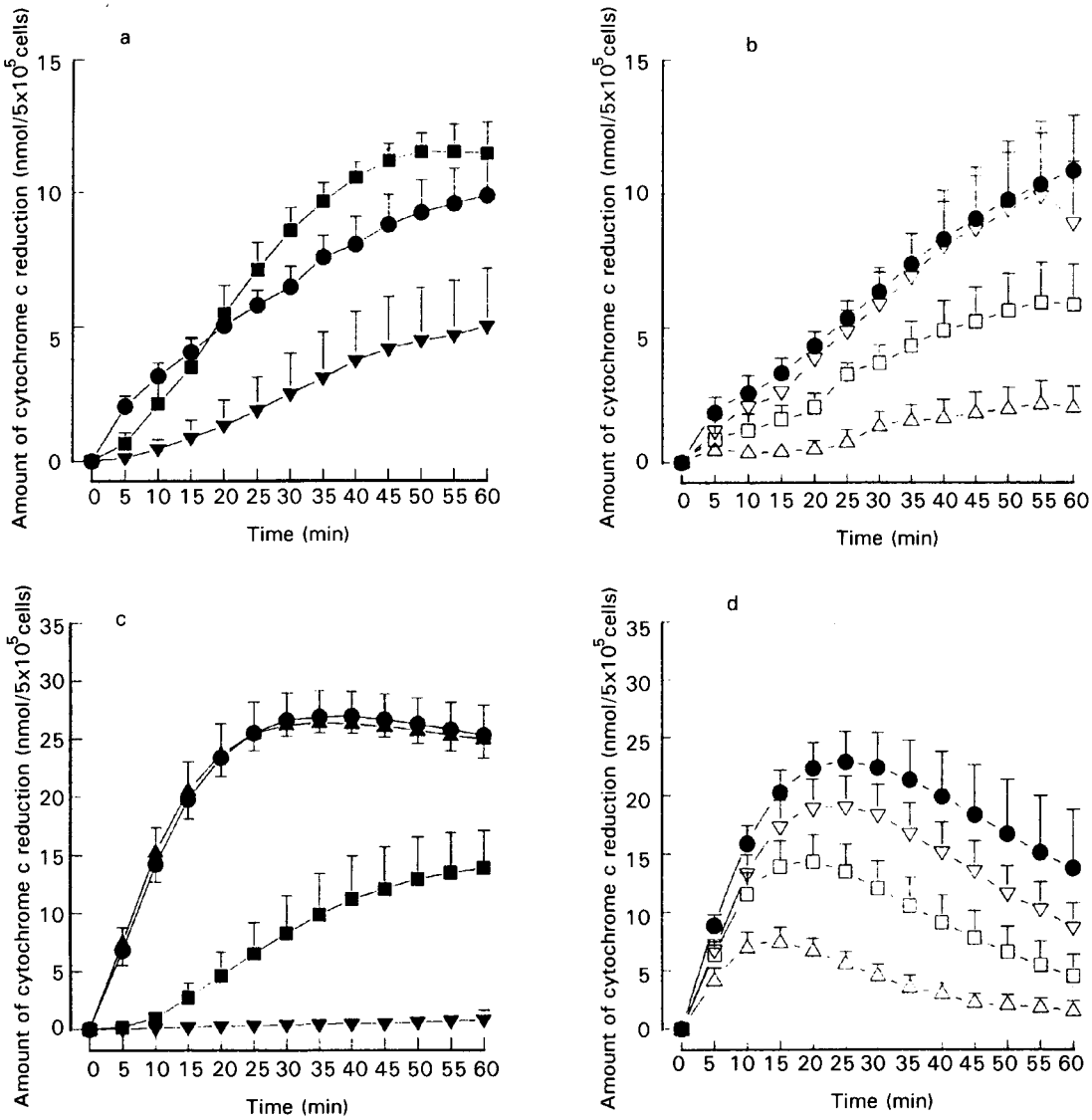

FIG. 1. Effect of *N*-acetylcysteine on superoxide anion generation induced by FMLP and phorbol myristate acetate in human polymorphonuclear leukocytes. (a) Superoxide production by FMLP (▼, 3 nM, n = 7; ●, 30 nM, n = 15; ■, 300 nM, n = 4). (b) Inhibition by *N*-acetylcysteine of FMLP (30 nM)-induced superoxide production (●, control, n = 9; ▽, *N*-acetylcysteine 33 μM, n = 5; □, *N*-acetylcysteine 100 μM, n = 5; △, *N*-acetylcysteine 333 μM, n = 5). (c) Superoxide production by phorbol myristate acetate (▼, 160 pM; ■, 1·6 nM; ●, 16 nM, ▲, 160 nM n = 5 for each group). (d) Inhibition by *N*-acetylcysteine of superoxide production induced by phorbol myristate acetate (16 nM) (●, control n = 9; ▽, *N*-acetylcysteine 33 μM, n = 5; □, *N*-acetylcysteine 100 μM, n = 5; △, *N*-acetylcysteine 333 μM, n = 5). Points are mean ± s.e.m. of n experiments.

Influence of N-*acetylcysteine on depletion of glutathione induced by phorbol myristate acetate*

Exposure of human PMN to phorbol myristate acetate induced a marked decrease in glutathione content (from control values of $5{\cdot}94 \pm 0{\cdot}86$ to $1{\cdot}84 \pm 0{\cdot}51$ nmol/3 × 10^6 cells; n = 5; $P < 0{\cdot}05$). The reduction in glutathione levels was not accompanied by severe damage to the cells as assessed by the activity of extracellularly released lactate dehydrogenase ($0{\cdot}08 \pm 0{\cdot}02$% of lactate dehydrogenase total, n = 3). Treatment with NAC (333 μM) resulted in replenishment of glutathione in human PMN exposed to phorbol myristate acetate ($4{\cdot}83 \pm 0{\cdot}68$ nmol/3 × 10^6 cells; n = 5; $P < 0{\cdot}05$ compared with values from exposed untreated cells, $P > 0{\cdot}05$ compared with control values).

Influence of N-*acetylcysteine on lipid peroxidation induced by* t-*butyl hydroperoxide*

Exposure of human PMN to *t*-butyl hydroperoxide (0·5 mM, 30 min) led to a marked increase in malondialdehyde levels (from control values of $0{\cdot}03 \pm 0{\cdot}02$ to $0{\cdot}73 \pm 0{\cdot}07$ nmol/10^6 cells; n = 6; $P < 0{\cdot}05$). Release of lactate dehydrogenase was maintained at low levels at the end of the exposure to *t*-butyl hydroperoxide ($0{\cdot}09 \pm 0{\cdot}02$% of total lactate dehydrogenase; n = 3). Treatment with NAC (333 μM) significantly reduced malondialdehyde levels in human PMN exposed to phorbol myristate acetate ($0{\cdot}55 \pm 0{\cdot}04$ nmol/10^6 cells; n = 6; $P < 0{\cdot}05$ compared with values in exposed untreated cells and compared with control values).

Table 1. Effect of *N*-acetylcysteine (333 μM) on the intracellular calcium response elicited by FMLP (30 nM).

	Baseline value (nM)	Increase in $[Ca^{2+}]_i$ at peak*	Values for $AUC_{0-3\ min}$ (%)	Increase in $[Ca^{2+}]_i$ at 3 min†
Control	83 ± 6	124·0 ± 8·2	100‡	65·3 ± 6·4
NAC	79 ± 7	113·1 ± 5·0	95·8 ± 0·5	61·3 ± 5·5

$[Ca^{2+}]_i$ was measured before addition of FMLP (baseline values), at the peak response to FMLP, and 3 min after FMLP addition. The $AUC_{0-3\ min}$ was also calculated (nmol s mL^{-1}). Data are mean ± s.e.m. of results from four experiments in each group. *Value at peak minus value at baseline (nM). † Value at 3 min after FMLP addition minus baseline value (nM). ‡This value corresponds to 17·20 ± 2·70 nmol s mL^{-1}.

Discussion

Human PMN are activated in-vitro when exposed to chemotactic factors such as fMet-Leu-Phe (FMLP) or after activation of protein kinase C by phorbol myristate acetate. In this study we found that *N*-acetylcysteine (NAC; 33–333 μM) inhibited, in a concentration-dependent manner, the superoxide generation induced by FMLP (30 nM) and phorbol myristate acetate (16 nM). The IC50 value for NAC against both activators was ~ 100 μM, and near maximum inhibition was attained with the highest concentration of NAC (333 μM) tested in this study. This concentration of NAC is well below the concentration found to be cytotoxic to human neutrophils (30 mM; Kharazmi et al 1988). The inhibitory effect of NAC found in this study confirms and extends the observations made in other studies. Thus, Gressier et al (1993, 1994) did not determine superoxide generation but showed that NAC inhibits hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and hydroxyl radical ($OH^{\cdot}$) production by human PMN stimulated with phorbol myristate acetate (160 nM) with IC50 values (30, 77 and 480 μM, respectively) in the range of those found in the current study. This in-vitro effect of NAC has been also observed in-vivo because Jensen et al (1988) reported that oral NAC administered to normal volunteers reduces neutrophil chemiluminescence, a measure of superoxide production, resulting from administration of opsonized zymosan.

The inhibitory effect of NAC on hydrogen peroxide, hypochlorous acid and hydroxyl radical production by human PMN might result from its direct scavenging effects because NAC is an avid scavenger of HOCl and $OH^{\cdot}$, and also reacts, although more slowly, with H_2O_2 (Aruoma et al 1989). The inhibitory effect of NAC on superoxide generation is, however, probably not a result of direct scavenging effect because NAC does not, apparently, reacting directly with $O_2^{\cdot -}$ (Aruoma et al 1989). NAC protection might, therefore, be acting as an intracellular antioxidant mechanism.

FMLP-receptor activation in human PMN results in a transient calcium signal as a result of inositol trisphosphate-mediated mobilization of intracellular Ca^{2+} stores and promotion of extracellular Ca^{2+} entry (Foder et al 1989). Exposure of cultured cells to oxidant reduces the generation of second messengers such as inositol trisphosphate (Brown 1994). Kimura et al (1992), on the other hand, reported that $[Ca^{2+}]_i$ is increased in cells exposed to reactive oxygen species as a result of facilitation of Ca^{2+} transport across the membrane. We therefore examined the effect of NAC on FMLP-induced $[Ca^{2+}]_i$ changes in fura-2-loaded human PMN. NAC (333 μM) did not alter either baseline, peak or residual values of $[Ca^{2+}]_i$ or the $AUC_{0-3\ min}$ after FMLP (30 nM). The possibility that NAC inhibits FMLP-induced superoxide generation by any alteration of intracellular calcium signal has, therefore, been ruled out.

NAC protection might be effected by replenishment of intracellular glutathione (Bernard 1991). Stimulation of PMN during phagocytosis or experimentally by opsonized zymosan has been reported to reduce cellular glutathione levels (Hatzelmann & Ullrich 1987). We found that activation by phorbol myristate acetate markedly reduced intracellular glutathione content thus confirming with a different stimulus that the 'respiratory burst' creates conditions of oxidative stress in human PMN. Superoxide generation results from the activity of NADPH oxidase, and the NADPH level is linked to the glutathione status by NADPH:GSSG oxidoreductase, thus stimulation of NADPH oxidase to generate superoxide leads to reduction of intracellular glutathione levels. In addition, reactive oxygen species directly oxidize glutathione thereby reducing cellular glutathione stores (Reed 1990). Treatment with NAC restores the normal glutathione concentration in human PMN stimulated by phorbol myristate acetate (this study), and $O_2^{\cdot -}$ scavenging might, therefore, be effected by NAC replenishment of the intracellular glutathione (Bernard 1991). Laurent et al (1996) recently demonstrated that intravenous NAC given to patients suffering from adult respiratory distress syndrome increased the glutathione content of their granulocytes.

Superoxide anion has been shown to cause lipid peroxidation but glutathione was ineffective at preventing it (Chakraborty et al 1994). We therefore tested lipid peroxidation elicited by a different stimulus, in-vitro exposure to *t*-butyl hydroperoxide (0·5 mM; 30 min). The concentration of *t*-butyl hydroperoxide and the time of exposure did not alter crude indicators of cellular integrity as assessed by release of lactate dehydrogenase into the medium (this study). We have used malondialdehyde, estimated by the 2-thiobarbituric acid test, as an index to determine the extent of lipid peroxidation, and found that NAC prevents *t*-butyl hydroperoxide-induced lipid peroxidation. This finding is consistent with results from studies in other cell systems, which showed that glutathione inhibited the lipid peroxidation mediated by sub-lethal exposure to *t*-butyl hydroperoxide (Bernard 1991; Chakraborty et al 1994).

To assess the clinical relevance of the in-vitro effects of NAC it would be necessary to compare the concentrations explored in this study with those achievable in the clinical setting. This comparison is, however, complicated by the rapid metabolism of NAC to cysteine by deacetylation. Peak plasma

levels of cysteine reached 50 μM after intravenous administration of 150 mg kg^{-1} of NAC (Bernard 1991), a concentration somewhat below but close to the IC50 values found for NAC in this and other studies (Gressier et al 1993, 1994). Cysteine is a precursor in the biosynthesis of glutathione, and although plasma-cysteine levels decay after the peak, there is a sustained increase in plasma glutathione levels during NAC therapy (Bernard 1991). Although the concentrations of NAC examined in the current study might not be reached with standard therapeutic regimes, it might, therefore, be possible that when increased demands are placed on glutathione pools, cysteine replacement by NAC administration restores normal glutathione concentrations (Laurent et al 1996) thereby mediating the beneficial effects observed in the clinics.

The conclusion from this in-vitro study is that *N*-acetylcysteine reduces superoxide generation in response to a chemoattractant peptide (FMLP) and to a protein kinase C activator (phorbol myristate acetate) without altering the intracellular calcium signal; it also replenishes cellular glutathione levels and partially protects against lipid peroxidation in human PMN. By reducing these particular aspects of PMN function, NAC might be endowed with clinically useful anti-inflammatory effects.

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