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Original Contribution

THE ANTIOXIDANT ACTION OF N-ACETYLCYSTEINE: ITS REACTION WITH HYDROGEN PEROXIDE, HYDROXYL RADICAL, SUPEROXIDE, AND HYPOCHLOROUS ACID

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Abstract—N-acetylcysteine has been widely used as an antioxidant in vivo and in vitro. Its reaction with four oxidant species has therefore been examined. N-acetylcysteine is a powerful scavenger of hypochlorous acid (H—OCl); low concentrations are able to protect α_1-antiproteinase against inactivation by HOCl. N-acetylcysteine also reacts with hydroxyl radical with a rate constant of 1.36×10^{10} $M^{-1}s^{-1}$, as determined by pulse radiolysis. It also reacts slowly with H_2O_2, but no reaction of N-acetylcysteine with superoxide (O_2^-) could be detected within the limits of our assay procedures.

Keywords—Free radicals, N-acetylcysteine, Hydroxyl radical, Hypochlorous acid, Superoxide, Hydrogen peroxide

INTRODUCTION

N-acetylcysteine has been used as an antioxidant in a wide variety of experiments. For example, it decreases membrane damage by superoxide-generating systems in porcine aortic endothelial cells,[1] diminishes endotoxin-induced lung damage in sheep,[2] decreases the toxicity of diquat to hepatocytes,[3] protects animals against paracetamol hepatotoxicity (e.g., [4]), prevents damage to human bronchial fibroblasts by tobacco smoke condensates,[5] and has been used in humans for treatment of various respiratory diseases.[6] N-acetylcysteine has been suggested to act by raising intracellular concentrations of cysteine, and hence of GSH, and/or by the scavenging of oxidant species (reviewed in [5]). Oxidants produced by activated phagocytic cells in lung include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl).[7] HOCl is produced by the oxidation of Cl^- ions catalyzed by neutrophil-derived myeloperoxidase in the presence of H_2O_2.[8] A major extracellular target of attack by HOCl is α_1-antiproteinase, the major circulating inhibitor of serine proteases such as elastase.[8] Inactivation of α_1-antiproteinase in lung is especially serious because it can lead to elastase-dependent hydrolysis of elastin and eventually to emphysema.[8,9] H_2O_2 and O_2^- can interact in the presence of certain transition metal ions to yield a highly-reactive oxidizing species, the hydroxyl radical, ·OH,[10] which can attack and inactivate many biomolecules, including α_1-antiproteinase.[9] Several experiments suggest that iron ion-dependent formation of ·OH from H_2O_2 and O_2^- can occur in lung in vivo and contribute to phagocyte-induced lung damage.[7]

What oxidants could N-acetylcysteine scavenge? It has been shown to react slowly with H_2O_2,[5] but a rate constant for this reaction was not determined. The rate of reaction of thiols with O_2^- is uncertain; Asada et al.[11] quoted a rate constant for reaction of cysteine with O_2^- as 2.7×10^6 $M^{-1}s^{-1}$, but Bielski[12] claimed that the reaction rate is insignificant. Many thiols are excellent scavengers of ·OH radical (e.g., for GSH,[13] k_2 is 8.8×10^9 at pH 1), but a rate constant for reaction of N-acetylcysteine with ·OH does not appear to have been measured. Many sulphur-containing compounds also react with HOCl; a recent survey of the reaction of anti-inflammatory drugs with HOCl concluded that most *could* react, but only in a few cases was the reaction fast enough for scavenging of HOCl by the drug to be feasible in vivo at the drug concentrations present in body fluids and tissues during normal therapeutic regimens.[14,15] Those few drugs for which scavenging in vivo is feasible include two thiol compounds, penicillamine and gold sodium thiomalate.[14]

In order to understand better the mechanisms by

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which N-acetylcysteine might be able to act as an oxidant scavenger in vivo, we have carried out a detailed study of the rate of its reaction with four oxidants; ·OH, O_2^-, H_2O_2, and HOCl.

MATERIALS AND METHODS

Reagents were of the highest quality available from Sigma or from BDH Chemicals Ltd, U.K. Potassium thiocyanate (KSCN) was carefully dried in an oven overnight before solutions were made up. α_1-Antiproteinase was Sigma type A9024. Elastase and α_1-antiproteinase were assayed essentially as described in [16]; full details are given in the legend to Table 1. HOCl was produced immediately before use by adjusting sodium hypochlorite (Na^+OCl^-) to pH 6.2 with dil H_2SO_4.[16] Pulse radiolysis was performed using the Paterson laboratories linear accelerator facility, using 10 mM KH_2PO_4—KOH solutions at pH 7.0. Solutions of N-acetylcysteine were made up immediately before use and, where necessary, the pH was adjusted to 7.4. Generation of O_2^- by the hypoxanthine-xanthine oxidase system was carried out essentially as described in reference 18. Reaction mixtures contained, in a final volume of 3 ml, 0.1 ml 30 mM EDTA, 10 μl 30 mM hypoxanthine in 50 mM KOH, 100 μl of 3 mM cytochrome *c* or 3 mM nitro-blue tetrazolium, and 50 mM (final concentration) KH_2PO_4—KOH buffer pH 7.4. Reaction was started by adding 0.2 ml of xanthine oxidase (freshly diluted in the above phosphate buffer to give one unit of enzyme activity per ml) and the rate of NBT or cytochrome *c* reduction measured at 560 or 550 nm respectively in a recording spectrophotometer at 25°C.

In early experiments, H_2O_2 was measured by the formation of a brown colour (recorded at 436 nm) in reaction mixtures containing, in a final volume of 1 ml, 0.15 M KH_2PO_4—KOH buffer pH 7.4, 50 μl guaiacol solution (made by adding 100 μl of pure liquid to 50 ml water) and 10 μl of Sigma type IV horseradish peroxidase (5 mg/ml in the same phosphate buffer). The rate of absorbance change at 436 nm is proportional to the concentration of H_2O_2 added. Substances to be tested for their reaction with H_2O_2 were incubated at concentrations up to 14.40 mM with 3.53–10.00 mM H_2O_2 for 30 min at 25°C. Aliquots were then taken and assayed for remaining H_2O_2 by using the peroxidase system. For later studies of the reaction of N-acetylcysteine with H_2O_2, both reagents were incubated in a 5 ml reaction mixture containing 1.5 ml of 100 mM KH_2PO_4—KOH buffer pH 7.4, for various times at 25°C. At intervals, 0.25 ml aliquots were added to another reaction mixture containing 2.5 ml of buffer and 0.25 ml of 6 mM DTNB (Ellman's reagent). The absorbance at 412 nm was measured after 5 min and the concentration of thiol determined from a calibration curve. The A_{412} was proportional to N-acetylcysteine concentrations up to 3 mM in the DTNB assay mixture.

RESULTS

Scavenging of hydroxyl radicals generated by pulse radiolysis

Radiolysis of a dilute (10 mM) aqueous phosphate-buffered (pH 7.0) solution saturated with nitrous oxide (N_2O) produces hydroxyl radical, ·OH

$$H_2O \xrightarrow{\sim\sim} \cdot OH,\ e^-_{(aq)},\ H\cdot,\ H_2O_2,\ H_2 \qquad (1)$$

$$e^-_{(aq)} + N_2O + H_2O \longrightarrow \cdot OH + OH^- + N_2 \qquad (2)$$

Table 1. Inactivation of α_1-antiproteinase by hypochlorous acid: Effect of N-acetylcysteine

Addition to First Reaction Mixture	Elastase Activity in Final Reaction Mixture ΔA_{410}/sec	Comment
Buffer only	13.4×10^{-3}	Activity of uninhibited elastase
α_1-antiproteinase	0	α_1AP inhibits elastase: no activity detected
α_1-antiproteinase + HOCl	12.3×10^{-3}	α_1AP inactivated by HOCl; no longer inhibits elastase
α_1AP + 24 μM N-Acetylcysteine	7.1×10^{-3}	N-Acetylcysteine scavenges HOCl rapidly enough to protect α_1AP
α_1AP + 48 μM N-Acetylcysteine	4.1×10^{-3}	
α_1AP + 72 μM N-Acetylcysteine	3.0×10^{-3}	
α_1AP + 96 μM N-Acetylcysteine	0	
α_1AP + 120 μM N-Acetylcysteine	0	
α_1AP + 180 μM N-Acetylcysteine	0	
α_1AP + 240 μM N-Acetylcysteine	0	

α_1-antiproteinase (0.2 mg/ml), HOCl (60 μM) and scavenger (if any) were incubated in a final volume of 1.0 ml in phosphate-buffered saline pH 7.4 (full details in [14]) at 25°C for 30 min. Then 2 ml of phosphate-buffered saline and 0.05 ml of elastase were added, followed by further incubation at 25°C for 20 min. This allows any α_1-antiproteinase still active to inhibit elastase. (Any HOCl remaining is diluted out to the point at which it cannot affect elastase itself.) The remaining elastase activity was then measured by adding elastase substrate,[14] which is hydrolysed by elastase, resulting in an increase in A_{410}.[14] Concentrations of scavengers added were those present in the first (1.0 ml) reaction mixture; scavengers and α_1-antiproteinase were mixed together before adding HOCl. Control experiments showed that none of the substances tested themselves affected elastase activity or interfered with the ability of α_1-antiproteinase to inhibit it.

If potassium thiocyanate is added to the solution, the ·OH radical reacts with thiocyanate ion (SCN^-) to give the radical anion $\cdot(SCN)_2^-$

$$\cdot OH + SCN^- \longrightarrow HOSCN^- \quad (3)$$

$$HOSCN^- \longrightarrow \cdot SCN + OH^- \quad (4)$$

$$\cdot SCN + SCN^- \longrightarrow \cdot(SCN)_2^- \quad (5)$$

At pH 7 reactions (4) and (5) are non-rate-determining and so the production of $\cdot(SCN)_2^-$ from KCNS can be considered as a single oxidation step with a second-order rate constant[17] of 1.1×10^{10} $M^{-1}s^{-1}$. The $\cdot(SCN)_2^-$ radical ion absorbs strongly in the visible region ($\epsilon = 7.1 \times 10^3$ $M^{-1}cm^{-1}$ at 500 nm).

By observing the ability of N-acetylcysteine to compete with SCN^- for ·OH and so decrease the absorbance changes observed, a rate constant for reaction of N-acetylcysteine with ·OH can be calculated. Figure 1 shows that it is an excellent scavenger of ·OH; a rate constant (as the mean of three values that differed by no more than 10%) of 1.36×10^{10} $M^{-1}s^{-1}$ was calculated.

Scavenging of superoxide

A mixture of hypoxanthine and xanthine oxidase at pH 7.4 generates O_2^-, which can be measured by its ability to reduce ferricytochrome *c* to ferrocytochrome *c*, measured as a rise in absorbance at 550 nm.[19] O_2^- can also reduce nitro-blue tetrazolium (NBT), measured as a rise in absorbance at 560 nm.[20] Any added substance that is itself able to react with O_2^- ion should decrease the rate of these absorbance changes. It was found that 1.5 mM final concentrations of N-acetylcysteine did not decrease the rate of reduction of 100

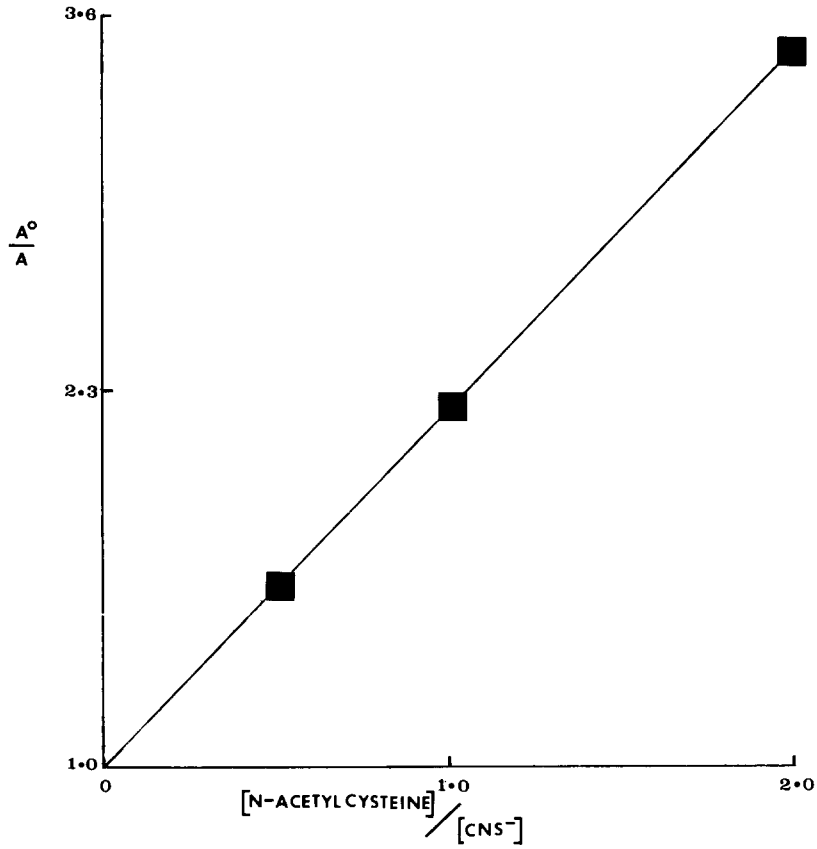

Fig. 1. Determination of a rate constant for the reaction of N-acetylcysteine (NAc) with hydroxyl radical. The absorbance at 500 nm of an irradiated solution of KSCN (100 μM) in 10 mM KH_2PO_4—KOH buffer pH 7.0, saturated with N_2O, was determined in the presence (A) or in the absence (A°) of NAc. Absorbances are related by the equation

$$\frac{A^\circ}{A} = 1 + \frac{K_c\,[NAc]}{K_1\,[KSCN]}$$

Thus plotting A°/A against [NAc]/[KSCN] allows calculation of K_c, the rate constant for reaction of NAc with ·OH. K_1 is taken as 1.1×10^{10} $M^{-1}s^{-1}$ (see text). Results of a typical experiment are shown.

μM cytochrome *c* by the hypoxanthine—xanthine oxidase system (N-acetylcysteine at this concentration did not reduce cytochrome *c* directly under our experimental conditions, although higher concentrations did). 3 mM N-acetylcysteine decreased the rate of NBT reduction by only 13–15%; there was no observable direct reduction of NBT by N-acetylcysteine.

Under our reaction conditions, cytochrome *c* reacts with O_2^- with a second-order rate constant[21] of approximately $2.6 \times 10^5\ M^{-1}s^{-1}$, and NBT reacts with a rate constant of about[22] $6 \times 10^4\ M^{-1}s^{-1}$. The inability of N-acetylcysteine, at concentrations fifteen times greater than those of cytochrome *c* or 30 times greater than those of NBT, to decrease significantly the rates of cytochrome *c* or NBT reduction, suggests that its reaction with O_2^-, if any, proceeds with a rate constant of less than $10^3\ M^{-1}s^{-1}$.

Scavenging of hydrogen peroxide

Initial attempts to investigate the reaction of N-acetylcysteine with H_2O_2 used a peroxidase-based assay system, as described in the Materials and Methods section. However, N-acetylcysteine was found to interfere in the assay system, probably because thiols are substrates for horseradish peroxidase.[23] The rate of reaction of N-acetylcysteine with H_2O_2 was therefore followed as loss of the $-SH$ group, using dithio*bis*(2-nitrobenzoic acid), DTNB.[24] Initial experiments showed that when N-acetylcysteine (final concentration 0.24–2.4 mM) was incubated with H_2O_2 (10.6 mM final concentration) at 25°C, the initial rate of loss of thiol was proportional to the concentration of N-acetylcysteine. For example, incubation of 0.24 mM N-acetylcysteine with 10 mM H_2O_2 at 25°C and pH 7.4 caused loss of approximately 25% of the thiol group in 30 seconds. A second-order rate constant of approximately $0.85\ M^{-1}s^{-1}$ ($\pm 10\%$) was calculated from the combined data.

Hence N-acetylcysteine does react slowly with H_2O_2, in agreement with reference 5, but the small rate constant for this reaction suggests that it may not be significant at the very low concentrations of H_2O_2 likely to be present in vivo.

Scavenging of hypochlorous acid; protection of α_1-antiproteinase

Hypochlorous acid (HOCl) is produced by oxidation of Cl^- ions at sites of inflammation by the neutrophil enzyme myeloperoxidase.[8] One of the major extracellular targets of HOCl attack is α_1-antiproteinase, the major circulating inhibitor of serine proteases such as elastase.[8,25] α_1-Antiproteinase is rapidly inactivated by HOCl, losing its elastase-inhibitory capacity.[8,16,25] A good scavenger of HOCl should therefore be able to protect α_1-antiproteinase against inactivation. In the experiments described below, HOCl was added directly rather than being generated by the myeloperoxidase-H_2O_2-Cl^- system, in order to avoid any confusion arising from the reaction of N-acetylcysteine with H_2O_2.

Table 1 (second line) shows that α_1-antiproteinase inhibited the activity of elastase in vitro; a concentration of α_1-antiproteinase able to inhibit elastase completely was used. Incubation of α_1-antiproteinase with 60 μM HOCl (a physiologically-relevant concentration[14–16]) led to loss of elastase-inhibitory capacity (Table 1, line three). However, inclusion of N-acetylcysteine in the reaction mixture was able to protect α_1-antiproteinase completely against the effects of 60 μM HOCl at only 96 μM concentrations (control experiments showed that N-acetylcysteine does not inhibit elastase directly, nor does it interfere with the ability of α_1-antiproteinase to inhibit elastase). It thus seems that N-acetylcysteine is a powerful scavenger of HOCl.

DISCUSSION

The ability of N-acetylcysteine to protect isolated cells[1,3,5] and animal lung in vivo[2] against oxidant damage could be explained both by its ability to maintain intracellular GSH concentrations[5] and by oxidant scavenging. N-acetylcysteine is a fairly poor scavenger of O_2^-; our rate constant of $<10^3\ M^{-1}s^{-1}$ at pH 7.4 for reaction of N-acetylcysteine with O_2^- is consistent with the report of Bielski[12] that reaction of cysteine with O_2^- was not detectable in his experiments.

N-acetylcysteine at low concentrations is a powerful scavenger of the myeloperoxidase-derived oxidant HOCl, being able to protect α_1-antiproteinase against damage by this oxidant (Table 1). This HOCl-scavenging action may be of special importance in lung, which is susceptible to proteolytic damage if α_1-antiproteinase is inactivated.[7–9] N-acetylcysteine, like other thiols,[13] is a powerful scavenger of ·OH radical. However, it must be realized that thiyl and other sulphur-containing radicals produced by hydrogen atom abstraction from N-acetylcysteine might themselves exert deleterious biological effects under certain circumstances (e.g., reference 26). However, the rate constants calculated in the present paper will enable more precise evaluation of the possibility that N-acetylcysteine might scavenge ·OH in different biological situations.

Reaction of N-acetylcysteine with H_2O_2 is slow. It is possible that some or all of the observed reaction might depend on the presence of transition metal ions

contaminating our reaction system. Care was taken to minimize metal ion contamination in our studies, but it is impossible to remove it completely.

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