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The specificity of thiourea, dimethylthiourea and dimethyl sulphoxide as scavengers of hydroxyl radicals

Their protection of α_1-antiproteinase against inactivation by hypochlorous acid

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Thiourea and dimethylthiourea are powerful scavengers of hydroxyl radicals ('OH), and dimethylthiourea has been used to test the involvement of 'OH in several animal models of human disease. It is shown that both thiourea and dimethylthiourea are scavengers of HOCl, a powerful oxidant produced by neutrophil myeloperoxidase. Hence the ability of dimethylthiourea to protect against neutrophil-mediated tissue damage cannot be used as evidence for a role of 'OH in causing such damage. Dimethyl sulphoxide also reacts with HOCl, but at a rate that is probably too low to be biologically significant at dimethyl sulphoxide concentrations up to 10 mM. Neither mannitol nor desferrioxamine, at the concentrations normally used in radical-generating systems, appears to react with HOCl.

INTRODUCTION

The O_2-derived species superoxide ($O_2^{\cdot -}$) and H_2O_2 have often been implicated in the pathogenesis of acute and chronic tissue injury by activated phagocytes [1–3]. Neutrophil involvement may be particularly important in some forms of lung injury, e.g. in the adult-respiratory-distress syndrome, and it has been proposed that O_2-derived species may be of importance in producing the lung damage in these cases [4]. Much of the tissue damage done by $O_2^{\cdot -}$ and H_2O_2 appears to arise by their metal-ion-dependent conversion into a highly reactive oxygen radical, which is probably hydroxyl radical ('OH) [5,6]. Direct detection of 'OH *in vivo* is extremely difficult because of its high reactivity, so attempts to gain evidence for a role of this radical as a toxic agent in animal studies have involved the use of 'OH 'scavengers' and of the iron-chelating agent desferrioxamine, which suppresses iron-dependent generation of 'OH [5,6].

Thiourea is a powerful 'OH scavenger (k_2 4.7×10^9 $M^{-1} \cdot s^{-1}$ [7]) that has frequently been used in experiments performed *in vitro* [8]. However, it is not specific as a scavenger of 'OH, in that it is a weak inhibitor of xanthine oxidase [8], reacts directly with H_2O_2 [9], binds to membrane proteins [10] and may bind metal ions in ways that affect their reactivity in generating 'OH [11]. Thiourea has also proved to be toxic to animals [12]. Hence the non-toxic dimethylthiourea was introduced as an allegedly specific scavenger of 'OH [13]. It has since become widely used in studies of neutrophil-mediated tissue damage, as a 'probe' for the role of 'OH [14–16], i.e. inhibition of damage by administration of dimethylthiourea has been taken as evidence that 'OH is responsible for the damage [13–16].

Activated neutrophils release not only $O_2^{\cdot -}$ and H_2O_2, leading to formation of 'OH, but also the enzyme myeloperoxidase. This enzyme uses H_2O_2 to oxidize Cl^- ions into a powerful oxidant that has been identified as HOCl [17–19]. The myeloperoxidase system can itself cause tissue damage. For example, HOCl rapidly inactivates α_1-antiproteinase, permitting uncontrolled proteinase activity and allowing neutrophil elastase to digest lung elastin (reviewed in [19]). In the present paper we have examined the ability of thiourea, dimethylthiourea and some other 'radical scavengers' to protect α_1-antiproteinase against attack by HOCl.

MATERIALS AND METHODS

Reagents

Dimethylthiourea was from Aldrich Chemical Co. and desferrioxamine (Desferal) from CIBA–Geigy. Thiourea and α_1-antiproteinase (type A9024) were from Sigma Chemical Co. Pig pancreatic elastase, NaOCl and other reagents used were from BDH Chemicals.

Assays

Elastase and α_1-antiproteinase were assayed at pH 7.4 essentially as described in [20] and [21]; full details are given in Table legends. HOCl was obtained immediately before use by adjusting NaOCl (BDH Chemicals) to pH 6.2 with dil. H_2SO_4, and its concentration was determined as described in [22]. Addition of the concentrations of HOCl used did not alter the pH of reaction mixtures. The h.p.l.c. equipment used was described in [21], [23] and [24].

RESULTS

Thiourea and dimethylthiourea

Table 1 (column A) shows that neither thiourea nor dimethylthiourea had any effect on the activity of

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Table 1. Effects of thiourea and dimethylthiourea on elastase, its inhibition by α_1-antiproteinase and inactivation of α_1-antiproteinase by HOCl

α_1-Antiproteinase (final concn. 0.5 mg/ml) was mixed with the final concentration of thiourea or dimethylthiourea stated plus HOCl (final concn. 30 μM) in 35.1 μl of buffer (140 mM-NaCl/2.7 mM-KCl/16 mM-Na_2HPO_4/2.9 mM-KH_2PO_4, pH 7.4) [20,21] and incubated at 25 °C for 60 min (reaction system 1). Then 3 ml of the same buffer [20,21] was added, followed by elastase. After further incubation for 30 min, the elastase activity remaining was assayed as a rise in absorbance at 410 nm [20,21]. Results are expressed as percentages of maximum elastase activity (100% is $\Delta A_{410} = 0.06$ unit·min^{-1}) and are the averages of duplicate determinations that varied by less than 5% (column C). Column D is as for column C, but HOCl and thiourea or dimethylthiourea were preincubated for 5 min before the addition of α_1-antiproteinase, then incubation was continued for a further 60 min before the addition of elastase and buffer, as above. For column A both HOCl and α_1-antiproteinase were omitted from reaction system 1, so that any effect of thiourea or dimethylthiourea on the elastase activity can be tested. For column B HOCl was omitted from reaction system 1, so that any effect of thiourea or dimethylthiourea on the ability of α_1-antiproteinase to inhibit elastase can be tested.

Reagent added	Final concn. in reaction system 1 (μM)	Elastase activity (% of maximum rate)			
		A	B	C	D
None	–	100	3	97	100
Thiourea	5	100	2	102	87
	7	104	2	99	16
	10	103	2	54	1
	15	101	3	23	2
	20	100	2	12	2
	25	98	2	3	2
Dimethylthiourea	5	97	3	100	73
	7	102	4	102	38
	10	105	3	69	9
	15	100	3	50	1
	20	99	2	14	1
	25	99	4	2	2

elastase over the concentration range tested. α_1-Antiproteinase inhibits elastase, and a concentration sufficient to inhibit elastase by approx. 97% was used for the experiment shown in Table 1. Column B shows that neither thiourea nor dimethylthiourea affected elastase inhibition by α_1-antiproteinase. If the antiproteinase is treated with HOCl, its elastase-inhibitory capacity is lost (Table 1, column C, first line). The concentration of HOCl used (30 μM) is within the range of concentrations likely to be produced adjacent to myeloperoxidase *in vivo* [17,18,25]. Column C also shows the effect of including thiourea or dimethylthiourea in the reaction mixture with HOCl and α_1-antiproteinase. At micromolar concentrations both were able to protect α_1-antiproteinase against HOCl, so that the α_1-antiproteinase could still inhibit elastase activity. Approx. 25 μM-thiourea or -dimethylthiourea offered almost complete protection against the effects of 30 μM-HOCl. Comparable protective effects were observed over a wide range of other elastase/α_1-antiproteinase concentration ratios. Both thiourea and dimethylthiourea could also protect α_1-antiproteinase against inactivation by a myeloperoxidase/H_2O_2/Cl^- system, as described in [18] (results not shown).

Table 1 (column D) shows that the protective effects of thiourea and dimethylthiourea are greatly increased if the HOCl is preincubated with them for 5 min before the addition of α_1-antiproteinase, i.e. 10 μM-thiourea or -dimethylthiourea could protect completely against 30 μM-HOCl. By contrast, if α_1-antiproteinase is incubated for 5 min with HOCl before the addition of thiourea or dimethylthiourea (with subsequent incubation for 60 min), no elastase-inhibitory capacity is observed. Hence, at the concentrations used in Table 1, neither thiourea nor dimethylthiourea is acting by restoring the activity of α_1-antiproteinase after attack by HOCl. In other experiments, incubation of inactivated α_1-antiproteinase with 10 mM concentrations of thiourea or dimethylthiourea (far greater than the concentrations used in the experiments in Table 1) could restore no more than 15% of its elastase-inhibitory capacity.

These studies suggest that thiourea and dimethylthiourea are protecting α_1-antiproteinase by reacting with HOCl. Direct evidence for this was provided by h.p.l.c. Fig. 1 shows a representative experiment, in which incubation of 200 μM-dimethylthiourea with 200 μM-HOCl caused almost complete oxidation of the dimethylthiourea; similar results were obtained with thiourea. The data in Table 1 (column D) suggest that, on preincubation, 1 mol of thiourea or dimethylthiourea can remove about 3 mol of HOCl (see above). The h.p.l.c. experiments provided an explanation for this. It was found that some of the products of oxidation of dimethylthiourea by HOCl would apparently themselves react with HOCl. For example, if the HOCl concentration used in the experiment reported in Fig. 1 was raised to above 200 μM, peak 4 (an oxidation product of dimethylthiourea) decreased sharply in intensity.

Dimethyl sulphoxide, mannitol and desferrioxamine

Dimethyl sulphoxide, another compound that has been used as an ''OH scavenger' in animal experiments,

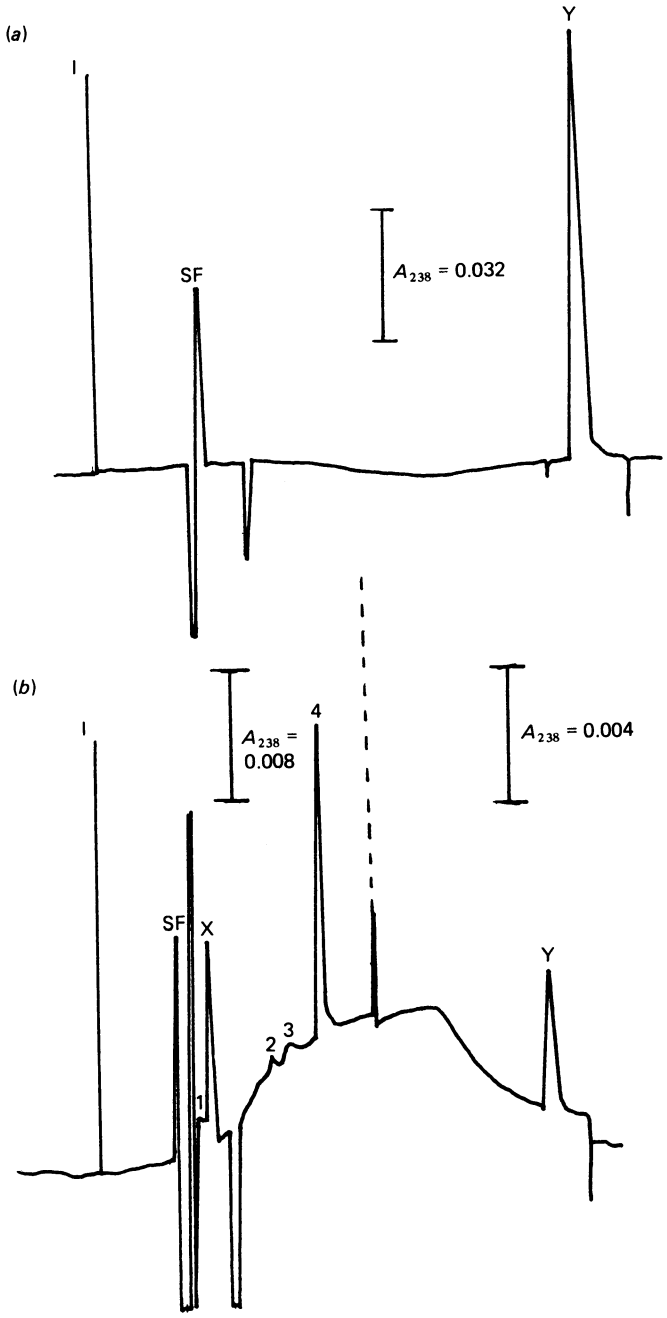

Fig. 1. Loss of dimethylthiourea on incubation with HOCl

Reaction mixtures containing 20 mM-KH_2PO_4/KOH buffer, pH 7.4, 200 μM-dimethylthiourea, 1 mM-EDTA and HOCl at the final concentration stated were incubated at 37 °C for 5 min and then subjected to h.p.l.c. in the apparatus described in [23] and [24] with a mobile phase consisting of 30 mM-sodium citrate/27.7 mM-sodium acetate buffer, pH 4.75, at a flow rate of 1.0 ml/min. Detection was by A_{238}. Peak Y is dimethylthiourea (retention time 17.18 min) and peak X (4.24 min) is a complex of EDTA with iron ions contaminating the reagents and solvents. (*a*) A 200 μM-dimethylthiourea standard. (*b*) HOCl (200 μM) added. Peak Y has decreased sharply and several new u.v.-absorbing peaks (marked 1, 2, 3 and 4 with retention times of 3.86, 6.56, 7.22 and 8.44 min respectively) have appeared. I indicates the injection spike, and SF the solvent front. The broken vertical line shows the position of the change of absorbance scale.

can also react with HOCl [18,22]. In agreement with this, preincubation of HOCl (30 μM) with millimolar concentrations of dimethyl sulphoxide for 5 min before addition of α_1-antiproteinase prevented inactivation of α_1-antiproteinase added subsequently (Table 2, column D). However, if 10 mM-dimethyl sulphoxide was included inthe reaction mixture together with HOCl and α_1-antiproteinase, it had little protective action. This suggests that, even at 10 mM concentrations, dimethyl sulphoxide cannot compete with α_1-antiproteinase for HOCl at a physiologically relevant [17,18,25] concentration.

Mannitol, tested at concentrations up to 100 mM, could not protect α_1-antiproteinase against inactivation by HOCl under any conditions; presumably mannitol does not react with HOCl because mannitol cannot be easily oxidized. Desferrioxamine, tested at the maximum concentration (1 mM) recommended [26,27] for use in studies of iron-dependent $^{\cdot}OH$ generation, also had no protective effect (Table 2).

DISCUSSION

HOCl reacts with a wide range of oxidizable bimolecules [17–22]. However, the ability of a given molecule to scavenge HOCl will only contribute to the biological effects of that molecule if, at the concentrations of scavenger that can be achieved *in vivo*, its reaction with HOCl is fast enough to protect important targets, such as α_1-antiproteinase, from attack by HOCl. Thus, if dimethyl sulphoxide is preincubated with HOCl before addition of α_1-antiproteinase, protection is seen (Table 2). This agrees with the reports that dimethyl sulphoxide reacts with HOCl [18,22]. However, if both dimethyl sulphoxide and α_1-antiproteinase are present together when HOCl is added, no protection of the protein is seen, i.e. under our reaction conditions α_1-antiproteinase reacts faster with HOCl than does dimethyl sulphoxide. When HOCl is generated by myeloperoxidase *in vivo*, both α_1-antiproteinase and any injected dimethyl sulphoxide should be present together. This suggests that scavenging of HOCl by dimethyl sulphoxide is probably not an explanation of its biological effects at the concentrations used here.

On the other hand, micromolar concentrations of thiourea and dimethylthiourea were able to protect α_1-antiproteinase against inactivation by HOCl, even if they were present simultaneously in the reaction mixture with α_1-antiproteinase when HOCl was added. They could not, at the concentrations used in Table 1, re-activate α_1-antiproteinase after it had been inactivated by HOCl (although much higher concentrations could produce a slight restoration of activity). Dimethylthiourea has been used at approx. 10 mM concentrations in studies of neutrophil damage to endothelial cells [15], in lung injury studies *in vivo* [14] and with isolated lungs [13], and at 45 mM in a study of neutrophil damage to hear [16]. An inhibitory effect has been proposed, in all these studies, as evidence for the involvement of $^{\cdot}OH$ in causing the damage. However, our results mean that scavenging of HOCl could be an equally valid explanation of the protective effects of dimethylthiourea against neutrophil-mediated damage. Thus inhibition of biological damage by millimolar concentrations of dimethylthiourea or thiourea cannot be regarded as evidence for a role of $^{\cdot}OH$ in causing such damage.

Table 2. Effects of dimethyl sulphoxide, mannitol and desferrioxamine on elastase, its inhibition by α_1-antiproteinase and inactivation of α_1-antiproteinase by HOCl

Assays were carried out exactly as described in the legend to Table 1. 100% elastase activity is $A_{410} = 0.059$ unit·min^{-1}. Results are the means for duplicate determinations that varied by less than 5%.

Reagent added	Final concn. in reaction system 1 (mM)	Elastase activity (% of maximum rate)			
		A	B	C	D
None	–	101	3	102	100
Mannitol	10	100	3	104	100
	100	100	4	98	101
Desferrioxamine	1.0	101	6	102	100
Dimethyl sulphoxide	0.8	100	6	98	8
	2	100	3	85	1
	4	100	2	75	3
	6	98	4	77	2
	8	99	3	73	2
	10	103	4	73	4

At concentrations that have been used experimentally, neither mannitol nor desferrioxamine appears to react with HOCl. Hence the protective effects of desferrioxamine against neutrophil-mediated lung damage observed *in vivo* [28] cannot be explained by scavenging of HOCl, although desferrioxamine itself may have several mechanisms of action [6,26].

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