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APPARENT INACTIVATION OF α_1-ANTIPROTEINASE BY SULPHUR-CONTAINING RADICALS DERIVED FROM PENICILLAMINE

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Abstract—α_1-Antiproteinase is the major inhibitor of proteolytic enzymes, such as elastase, in human plasma. Its elastase-inhibitory capacity can be inactivated by exposure to hydroxyl radicals (·OH) generated either by pulse radiolysis or by an Fe^{3+}–EDTA/H_2O_2/ascorbic acid system. Inactivation of α_1-antiproteinase by radiolytically-generated ·OH under anoxic conditions was decreased by adding a range of anti-inflammatory drugs to the reaction mixtures, including the thiol compound penicillamine. However, under conditions favouring formation of oxysulphur radicals, protection by thiols such as penicillamine was much decreased. It is proposed that sulphur-containing radicals resulting from attack of biologically-produced oxidants upon penicillamine in the presence of O_2 can themselves inactivate α_1-antiproteinase, and that such radicals might contribute to the side-effects produced by penicillamine or gold thiol therapy in rheumatoid arthritis.

α_1-Antiproteinase is the major circulating inhibitor of serine proteases, such as elastase, in human plasma [1, 2]. Inactivation of this protein can apparently exacerbate tissue damage in several conditions, including rheumatoid arthritis, adult respiratory distress syndrome and lung injury resulting from cigarette smoking [2–6]. Emphysema may result from failure to inhibit elastase adequately in the lung [1–6].

Unfortunately, α_1-antiproteinase is very sensitive to inactivation by oxidants, because a methionine residue essential for its activity is accessible to oxidative attack [1, 3, 4–6]. Thus the elastase-inhibitory activity of α_1-antiproteinase is quickly destroyed when this protein is exposed to hypochlorous acid (HOCl), an oxidant produced by the enzyme myeloperoxidase, released from activated neutrophils [1, 3, 7, 8]. Other oxidants reported to attack α_1-antiproteinase include peroxynitrates [9] and hydroxyl radicals, ·OH [4, 5, 9]. Hydroxyl radicals can be formed when O_2^- and H_2O_2, released by activated neutrophils, interact with "catalytic" iron ions at sites of tissue injury [10]. Neutrophils do not themselves release iron catalysts of ·OH generation [11, 12] but evidence exists for the presence of such catalysts at sites of neutrophil-mediated tissue injury *in vivo* (reviewed in Refs 10, 13 and 14). However, the ability of ·OH to inactivate α_1-antiproteinase has been inferred from studies using scavengers [4, 5] rather than demonstrated directly. Neither Wasil *et al.* [15] nor Pryor *et al.* [9, 16] were able to demonstrate significant inactivation of α_1-antiproteinase *in vitro* by mixtures of iron salts and H_2O_2, a source of ·OH [10]. This seems odd in view of the well-established ability of ·OH to damage proteins (reviewed in Ref. 17) and we thought that the point merited further investigation.

There has been considerable interest in the possibility that several of the drugs used in the treatment of inflammatory disease might act, in part, by scavenging oxidants *in vivo* (reviewed in Ref. 13). For example, all non-steroidal anti-inflammatory drugs examined have been found to react with ·OH at almost diffusion-controlled rates [18, 19], although in few cases are the concentrations of the drugs that are present at sites of inflammation sufficiently-high for scavenging of ·OH to be feasible *in vivo* (discussed in Ref. 13). In addition, it must not be forgotten that any reaction of drugs with ·OH that does occur will lead to formation of drug-derived radicals that, in the presence of oxygen, might be able to form peroxyl radicals. Both peroxyl radicals and the radicals formed by initial attack of ·OH on drugs might themselves be able to do biological damage [18]. For example, Willson [20] has referred to peroxyl radicals as "ultimate agents in oxygen toxicity".

In the present paper, the technique of pulse radiolysis has been used to investigate whether ·OH does indeed inactivate α_1-antiproteinase and whether radicals derived from various anti-inflammatory drugs might themselves be able to inactivate this protein.

MATERIALS AND METHODS

Reagents. Sodium hypochlorite (NaOCl) and procine pancreatic elastase were from BDH Chemicals Ltd (Poole, U.K.). α_1-Antiproteinase (type A9024), drugs and other reagents were from Sigma Chemical Co. (Poole, U.K.).

Assays. Pulse radiolysis was performed using the Paterson Laboratories linear accelerator facility, with phosphate-buffered solutions (10 mM KH_2PO_4–

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Table 1. Inactivation of α_1-antiproteinase by hydroxyl radicals

Radiation dose Gy	Hydroxyl radical μmol/dm^3	Elastase activity ($\Delta A \times 10^{-3}$/sec)	% inhibition of α_1AP
0	1	0	0
5	3	0.34	2
10	6	0.98	6
25	15	1.70	11
50	30	5.00	33
75	45	6.30	41
100	60	9.3	61
150	90	11.3	74
200	120	12.0	78
250	150	12.0	78
300	180	13.3	87

α_1-Antiproteinase (1 mg/ml) was dissolved in N_2O-saturated 10 mM KH_2PO_4–KOH pH 7.4 and subjected to radiolysis over a 6 min period to generate the total amounts of ·OH indicated. Immediately after radiolysis, a sample (0.1 mg) of the α_1-antiproteinase was incubated with porcine pancreatic elastase for 30 min at 25°. The residual elastase activity was then measured by adding [*N*-(3-carboxypropionyl)-trialanyl *p*-nitroanilide), which is hydrolysed by elastase with a rise in A_{410}. Elastase alone gave a ΔA_{410} of 1.53×10^{-2}/sec: a concentration of α_1-antiproteinase just sufficient to inhibit elastase completely was used in the control experiment.

KOH) at pH 7.4. For the steady-state studies on the inactivation of the protein, the irradiations were carried out using the accelerator in the continuous pulsing mode (50 pulses/sec) at a dose rate equivalent to 0.45 μM ·OH/sec for the time needed to give 180 μM ·OH overall (approximately 6 min). The yield of ·OH was calibrated daily using a Fricke dosimeter. Drug solutions were made up immediately before use in water or, where necessary, in alkaline solutions, and the pH of solutions adjusted to 7.4 immediately before use. After radiolysis, the elastase-inhibitory capacity of α_1-antiproteinase was assayed as in Ref. 15. Full details are given in the legend to Table 1. Deoxyribose degradation in iron/ascorbate/H_2O_2 systems was measured by the thiobarbituric acid (TBA) test [21].

RESULTS

Inactivation of $\alpha_1$antiproteinase by hydroxyl radicals

Radiolysis of a dilute (10 mM) aqueous phosphate-buffered solution saturated with nitrous oxide (N_2O) produces ·OH:

$$H_2O \longrightarrow \cdot OH, e^-_{(aq)}, H^\cdot, H_2O_2, H_2 \quad (1)$$

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

If α_1-antiproteinase (1 mg/ml, approximately 19 μM if M_r is taken as 53,000 [1]) was included in the radiolysis solution, its elastase-inhibitory capacity was inactivated (Table 1). About ten ·OH radicals per molecule of protein produced an almost-complete inactivation, suggesting that some "hits" by ·OH on the protein do not produce inactivation, and/or that not all the radicals generated attack the protein.

Protection by anti-inflammatory drugs

Table 2 shows a typical set of results obtained when nonsteroidal anti-inflammatory drugs were included, at concentrations up to 0.24 mM, in the reaction mixtures with α_1-antiproteinase during irradiation. A total dose of 180 μM ·OH was used so as to give substantial inactivation of the α_1-antiproteinase, so making it easier to detect protective effects. In these experiments, the irradiations were carried out using the accelerator in the continuous pulsing mode (see Materials and Methods), so that the total dose of 180 μM ·OH was spread out over approximately 6 min. This was done to prevent excessive build-up of drug-derived radicals (that might interact rapidly with each other), so creating conditions more favourable for any radical-protein interactions to occur (see below). Drug concentrations higher than 240 μM were difficult to test because of solubility problems.

Since all the anti-inflammatory drugs are able to react with ·OH [18, 19], it was expected that all the drugs would offer some protection to α_1-antiproteinase against inactivation by ·OH and this was indeed observed (Table 2). Indomethacin was slightly more effective than the others (reproducible in three experiments). Irradiation of the drug-containing solutions produced characteristic spectra of drug-derived radicals, which decayed by radical–radical interactions with second-order rate constants between 3.5×10^8 and 6.2×10^9 M^{-1}sec^{-1} (data not shown). This decay was slowed by using the accelerator in the continuous pulsing mode, which effectively converts it into an approximate continuous irradiation source.

If the solutions were bubbled with an 80% (v/v) N_2O/20% (v/v) O_2 mixture instead of with pure N_2O, some of the drug-derived radicals will be able to form peroxyl radicals. Under these conditions, most drugs tested were still able to protect α_1-antiproteinase to about the same extent (Table 3 shows

Table 2. Protection of α_1-antiproteinase by anti-inflammatory drugs

Drug added to reaction mixture	Elastase activity ($\Delta A \times 10^{-3}$/sec)	% protection of α_1AP by drug (as % decrease in elastase activity)
None	13.1	—
None (unirradiated α_1-antiproteinase)	0	—
5-Aminosalicylate 120 μM	11.9	9
240 μM	8.7	34
Piroxicam 120 μM	10.8	18
240 μM	7.6	42
DL-Penicillamine disulphide 120 μM	12.2	7
240 μM	8.8	33
DL-Penicillamine 120 μM	11.7	11
240 μM	9.2	30
Diclofenac sodium 120 μM	11.0	16
240 μM	7.8	40
Chloroquine 120 μM	10.3	21
240 μM	8.0	39
Sulphapyridine 120 μM	10.4	21
240 μM	7.6	42
Hydroxychloroquine 120 μM	8.7	34
240 μM	7.5	43
Indomethacin 120 μM	6.9	47
240 μM	6.6	50

α_1-Antiproteinase (1 mg/ml) was pulse-irradiated in N_2O-saturated buffer over a period of approximately 6 min, to generate 180 μmol ·OH/dm^3. Drugs were included in the reaction mixture at the final concentration stated. Elastase alone gave a ΔA_{410} of 1.84×10^{-2}/sec in this experiment. Assays were performed upon small aliquots as described in the legend to Table 1. Similar results were obtained in two other experiments. None of the drugs tested themselves affected elastase or α_1-antiproteinase. Solutions of drugs were made up immediately before use and the pH adjusted to 7.4 where necessary.

Table 3. Protection of α_1-antiproteinase by anti-inflammatory drugs

Drug added	% protection of α_1AP A	B
5-Aminosalicylate	34	49
Piroxicam	42	40
DL-Penicillamine disulphide	33	31
DL-Penicillamine	30	5*
Diclofenac sodium	40	44
Chloroquine	39	37
Sulphapyridine	42	39
Hydroxychloroquine	43	46
Indomethacin	50	40
N-Acetylcysteine	47	33
Mercaptopropionylglycine	43	14*

Experiments were carried out as described in the legend to Table 2. Drugs were used at 240 μM final concentration. Column A: solutions bubbled with N_2O; Column B: solutions bubbled with an 80% (v/v) N_2O/20% O_2 mixture. Percentage protection of α_1AP by drugs is calculated as shown in the last column of Table 2.

* Significant ($P < 0.05$) difference in protective ability observed ($N \geq 3$) between anoxic and oxic conditions.

a typical result). However, penicilliamine was much less protective under these conditions. The same was true of another thiol compound, mercaptopropionlglycine and, to a much smaller but reproducible extent, for *N*-acetylcysteine (Table 3).

Inactivation of α_1-antiproteinase by Fenton systems

A mixture of Fe^{3+}, H_2O_2 and ascorbate generates ·OH at pH 7.4, especially if the Fe^{3+} is chelated to EDTA [21]. The $FeCl_3$–EDTA/H_2O_2/ascorbate system did produce an inactivation of α_1-antiproteinase (Table 4). However, the $FeCl_3/H_2O_2$/ascorbate system produced much less inactivation of α_1-antiproteinase, even when comparable amounts of ·OH were formed, as detected by the ability of this radical to degrade the sugar deoxyribose [21]. Thus, in the experiment shown in Table 4, a reaction mixture containing 100 μM $FeCl_3$ produced sufficient ·OH to generate TBA-reactive material from deoxyribose corresponding to an A_{532} of 0.778. However, incubation of α_1-antiproteinase with this $FeCl_3$/ascorbate/H_2O_2 system caused only a 15% loss of its elastase-inhibitory capacity. If 10 μM $FeCl_3$–EDTA was used instead, 32% loss of activity was observed, even though this system produced only sufficient ·OH to degrade deoxyribose to an A_{532} of 0.532.

DISCUSSION

The results in the present paper show that, contrary to recent suggestions [15, 16], ·OH does inactivate α_1-antiproteinase. Exposure of this protein to an excess of ·OH (Table 1) causes almost complete loss of its elastase-inhibitory capacity, although about 10 radicals per mole of protein are necessary to produce complete inactivation. Hydroxyl radical can react with multiple sites on proteins, and only some of the "hits" may lead to inactivation [17].

The question as to why some authors have reported that Fenton (Fe^{2+}/H_2O_2) systems do not inactivate α_1-antiproteinase [15, 16] has to be addres-

Table 4. Inactivation of α_1-antiproteinase by hydroxyl radicals generated by iron-dependent systems

Reaction mixture	Elastase activity $\Delta A_{410} \times 10^{-3}$	% inactivation of elastase-inhibitory capacity of α_1-antiproteinase	Amount of ·OH formation, as deoxyribose degradation A_{532}
α_1-Antiproteinase alone	0	0	—
As above, 10 μM $FeCl_3$	0.7	6	0.622
As above, 10 μM $FeCl_3$–EDTA	4.0	32	0.532
As above, 100 μM $FeCl_3$	1.8	15	0.778
As above, 20 μM $FeCl_3$–EDTA	4.6	37	0.981
As above, 400 μM $FeCl_3$–EDTA	8.3	67	1.92

Reaction mixtures contained, in a final volume of 1.2 ml, α_1-antiproteinase (1 mg/ml), 10 mM KH_2PO_4–KOH buffer, pH 7.4, H_2O_2 (2.8 mM), ascorbate (200 μM), $FeCl_3$ at the concentration stated and, where indicated, EDTA. The elastase-inhibitory capacity of the α_1-antiproteinase was measured in aliquots of the reaction mixture as described in the legend to Table 1. In some experiments, α_1-antiproteinase was replaced by deoxyribose (2.8 mM) whose degradation was measured by the thiobarbituric acid method [21]. The elastase alone gave ΔA_{410} of 1.24×10^{-2}/sec.

sed. Table 4 shows that an $FeCl_3/H_2O_2$/ascorbate system is poorly effective in inactivating α_1-antiproteinase. However, an $FeCl_3$–EDTA/H_2O_2/ascorbate system was much more effective, even when rates of ·OH generation were comparable (as measured by the ability of ·OH to degrade the sugar deoxyribose). It is thought that both these systems do generate ·OH (reviewed in Refs 10 and 14). When ·OH is generated by the $FeCl_3$–EDTA/H_2O_2/ascorbate system, the radicals are accessible to scavenging by any added molecule and they appear to enter "free solution" [19, 21–23]. However, in the $FeCl_3/H_2O_2$/ascorbate system, the site of ·OH generation depends upon what the added Fe^{3+} ions bind to in the reaction mixture [19, 21–23]. It may be that iron attaches to the carbohydrate side-chains of the α_1-antiproteinase (a glycoprotein) or to other components of the reaction mixture, so that the ·OH generated does not efficiently reach the critical methionine residue on α_1-antiproteinase, and simple Fenton systems are inefficient at inactivating this protein [15, 16]. EDTA, by solubilizing the iron and permitting ·OH to be formed in "free solution", allows an inactivation of α_1-antiproteinase (Table 4) similar to that achieved by ·OH generated "free" by radiolysis of aqueous solutions (Table 1).

Various non-steroidal anti-inflammatory drugs were able to protect α_1-antiproteinase against inactivation by ·OH (Table 2). This is not surprising, since these drugs are known to react rapidly with ·OH [18, 19]. (Their ability to do this *in vivo* is limited or insignificant in most cases, since they do not accumulate to sufficient concentrations at sites of oxidant injury; discussed in (Refs 13 and 19.) In any case, we can conclude that any radicals that were produced by attack of ·OH upon these drugs *in vivo* would be less damaging to α_1-antiproteinase than is ·OH. It follows that radical formation from such drugs *in vivo* is probably not a *general* [20] mechanism of damage to proteins. There was no evidence from the pulse radiolysis experiments that any of the drug-derived radicals can react directly with the protein (rate constants $< 5 \times 10^6$ $M^{-1}sec^{-1}$).

If solutions were bubbled with 80% N_2O/20% O_2 instead of with pure N_2O, some of the drug-derived radicals would react with O_2 to give peroxyl radicals [18, 20]. This had little, if any effect on the ability of most drugs to protect the α_1-antiproteinase (Table 3), i.e. there was no evidence that drug-derived peroxyl radicals could damage this protein. However, the protective effects of penicillamine were significantly decreased. Similar effects were also observed with other thiols, such as mercaptopropionylglycine, and, to a much smaller extent, with *N*-acetylcysteine.

Attack of ·OH upon thiols produces thiyl (RS·) radicals. In the presence of O_2, these are thought to react and form oxysulphur radicals such as thiyl peroxyl (RSO_2·) and sulphenyl (RSO·) (reviewed in Refs 17, 24 and 25; also see Refs 26 and 27). Unfortunately, current understanding of the origin and fate of sulphur-centred radicals in the presence of O_2 is rather limited [17]. Our studies strongly suggest that some of these oxysulphur radicals are themselves sufficiently oxidizing to inactivate α_1-antiproteinase, accounting for the impaired protection under these conditions. It has already been suggested that thiyl peroxyl radicals might be able to inactivate enzymes [28]. RSO_2· and RSO· radicals might be able to react further with RSH under our reaction conditions, so it is impossible to assess their concentrations in our reaction mixtures and hence difficult to assess how effective such radicals might be in inactivating α_1-antiproteinase. Scavenging of some oxidants (e.g. HOCl) *in vivo* by penicillamine is feasible under certain circumstances, e.g. in rheumatoid arthritis [13, 29] and it is conceivable that some of the side-effects of this drug could be mediated by the resulting sulphur-containing radicals. Penicillamine also complexes copper ions *in vivo*, and the resulting complexes may lead to generation of sulphur-centred radicals by −SH reduction of Cu^{2+} ions. It is possible that the autoimmunity often caused by penicillamine treatment of rheumatoid patients could be due to the binding of sulphur-containing radicals to proteins, modifying their antigenicity.

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