

Inactivation of α_1 -antiproteinase by hydroxyl radicals

The effect of uric acid

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The elastase-inhibitory activity of α_1 -antiproteinase is inactivated by hydroxyl radicals ($\cdot\text{OH}$) generated by pulse radiolysis or by reaction of iron ions with H_2O_2 in the presence of superoxide or ascorbate. Uric acid did not protect α_1 -antiproteinase against inactivation by $\cdot\text{OH}$ in pulse radiolysis experiments or in the superoxide/iron/ H_2O_2 system, whereas it did in systems containing ascorbic acid. We propose that radicals formed by attack of $\cdot\text{OH}$ on uric acid are themselves able to inactivate α_1 -antiproteinase, but that these uric acid radicals can be 'repaired' by ascorbic acid.

Uric acid; Hydroxyl radical; Peroxy radical; Antiproteinase, α_1 -

1. INTRODUCTION

There has been much interest recently in the possible role played by oxidants in human disease, and in the protective effects of endogenous antioxidants [1–3]. It has been proposed that uric acid, an end-product of purine metabolism in man, may function as an antioxidant in vivo [4,5]. Indeed, measurement of oxidation products of uric acid may be a marker of oxidant generation in vivo [6,7]. Experiments in vitro have shown that uric acid protects erythrocytes against damage by singlet O_2 or *t*-butyl hydroperoxide [4], inhibits lipid peroxidation [8–10], decreases oxidation of haemoglobin by nitrite [11], inhibits oxidative degradation of hyaluronic acid [12], scavenges the myeloperoxidase-derived oxidant hypochlorous acid [13,14], protects against oxidant damage to DNA [15], inhibits ozone-induced degradation of nucleic acid bases [16] and binds iron and copper

ions in complexes that are poorly active in promoting free radical reactions [17,18]. Uric acid also scavenges (k_2 $7.2 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$) hydroxyl radicals [19], and can protect certain enzymes against inactivation by this reactive radical species. However, reaction of uric acid with $\cdot\text{OH}$ yields a uric acid radical, that can probably combine with O_2 to give a peroxy radical [19]. Kittridge and Willson [19] reported that uric acid-derived radicals inactivate yeast alcohol dehydrogenase, an enzyme that is very sensitive to oxidants, more rapidly than does $\cdot\text{OH}$, and they therefore cast doubt upon the proposed antioxidant role of uric acid [4,5].

However, uric acid is thought to act as an antioxidant in humans [4,5], who do not possess a yeast-type alcohol dehydrogenase. One protein in humans that is very sensitive to oxidants is α_1 -antiproteinase, the major circulating inhibitor of proteolytic enzymes such as elastase [20,21]. Inactivation of α_1 -antiproteinase can apparently exacerbate tissue damage in several diseases, including rheumatoid arthritis and emphysema [20–24]. The protein can be inactivated by hypochlorous acid [22–25,27], oxidizing species (probably peroxynitrates) in cigarette smoke [26] and

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hydroxyl radical [23,24,26]. We have therefore examined the ability of uric acid to protect α_1 -antiproteinase against inactivation by hydroxyl radicals ($\cdot\text{OH}$) generated either by radiolysis of aqueous solutions, or from H_2O_2 in the presence of iron ions and either ascorbic acid or a system generating the superoxide radical, O_2^- .

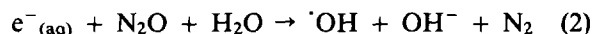
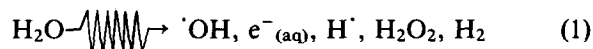
2. MATERIALS AND METHODS

Dimethyl sulphoxide and pig pancreatic elastase were from BDH; all other reagents (including uric acid) were of the highest quality available from Sigma. The ability of purified α_1 -antiproteinase (Sigma type A9024) to inhibit elastase was assayed essentially as in [28]: full details are given in the legend to table 1. Generation of $\cdot\text{OH}$ by the hypoxanthine/xanthine oxidase system was carried out essentially as described [38]; full details are given in the legend to table 3. Generation of $\cdot\text{OH}$ by the iron-EDTA/ H_2O_2 /ascorbic acid system was carried out essentially as in [31]; details are given in the legend to table 2. Pulse radiolysis was carried out using the Paterson Laboratories linear accelerator facility in a continuous pulsing mode (50 pulses/s [29]). Solutions of α_1 -antiproteinase (1 mg/ml) in N_2O -saturated 10 mM KH_2PO_4 -KOH buffer (pH 7.4) were subjected to 30 krad over approx. 6 min to generate $180 \mu\text{mol}/\text{dm}^3$ of $\cdot\text{OH}$ overall. The yield of $\cdot\text{OH}$ was calibrated daily using a Fricke dosimeter.

3. RESULTS

3.1. Generation of hydroxyl radicals by radiolysis

Irradiation of a dilute aqueous (10 mM KH_2PO_4 -KOH buffer, pH 7.4) solution saturated with nitrous oxide produces $\cdot\text{OH}$



If α_1 -antiproteinase (1 mg/ml, approx. $19 \mu\text{M}$) was included in the radiolysis solution, it was inactivated (table 1). Generation of $180 \mu\text{mol}/\text{dm}^3$ of $\cdot\text{OH}$ over about a 6-min period produced a substantial loss of activity of α_1 -antiproteinase, measured as its ability to inhibit elastase (table 1). Several compounds able to scavenge $\cdot\text{OH}$ protected the α_1 -antiproteinase, so that its elastase-inhibitory capacity was retained. Table 1 shows an example: protection by indomethacin and piroxicam, which scavenge $\cdot\text{OH}$ with rate constants close to $10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$ [30]. Despite its ability to scavenge $\cdot\text{OH}$,

Table 1
Inactivation of α_1 -antiproteinase by hydroxyl radicals: radiolysis

Expt	Elastase activity ($\times 10^{-2} \text{ s}^{-1}$) (ΔA_{410})	α_1 -Antiproteinase activity (as % inhibition of elastase)
(A)		
Unirradiated $\alpha_1\text{AP}$	0	100
Irradiated $\alpha_1\text{AP}$	1.33	13
+ 240 μM indomethacin	0.66	57
+ 240 μM piroxicam	0.76	50
+ 120 μM uric acid	1.29	16
+ 240 μM uric acid	1.48	3
(B)		
Irradiated $\alpha_1\text{AP}$		
+ 240 μM indomethacin	0.69	55
+ 240 μM piroxicam	0.76	50
+ 120 μM uric acid	1.58	0
+ 240 μM uric acid	1.50	2

All concentrations listed are final concentrations in the reaction mixtures. α_1 -Antiproteinase ($\alpha_1\text{AP}$, 1 mg/ml) in phosphate buffer was subjected to radiolysis over about a 6 min period with 30 krad to generate $180 \mu\text{mol} \cdot \text{OH}/\text{dm}^3$. Immediately after radiolysis, a sample (0.1 mg) of $\alpha_1\text{AP}$ was incubated with porcine pancreatic elastase for 30 min at 25°C . The residual elastase activity was then measured by adding elastase substrate [28], which is hydrolysed by elastase with a rise in A_{410} . Elastase alone (not exposed to $\alpha_1\text{AP}$) gave a ΔA_{410} of $1.53 \times 10^{-2} \text{ s}^{-1}$. A concentration of $\alpha_1\text{AP}$ just sufficient to inhibit elastase activity completely was used in the control experiment. Experiments were performed in buffer saturated with N_2O (A) or with 80% (v/v) $\text{N}_2\text{O}/20\%$ (v/v) O_2 (B)

Table 2
Inactivation of α_1 -antiproteinase by hydroxyl radicals: ascorbate/iron/ H_2O_2 system

Expt	Elastase activity (ΔA_{410}) ($\times 10^{-2} \text{ s}^{-1}$)	α_1 -Antiproteinase activity (1% inhibition of elastase)
α_1 -Antiproteinase alone	0	100
Complete reaction mixture (omit Fe^{3+} -EDTA)	1.17	3
+ 400 μM dimethyl sulphoxide	0.3	75
+ 200 μM uric acid	0.65	46
+ 200 μM uric acid	0.52	57
+ 400 μM uric acid	0.37	69

Reaction mixtures contained, in a final volume of 1 ml, the following reagents at the final concentrations stated: 10 mM KH_2PO_4 -KOH buffer (pH 7.4), 0.2 mM ascorbic acid, 0.1 mM Fe^{3+} -EDTA, 2.8 mM H_2O_2 and 1 mg/ml α_1 -antiproteinase. Solutions of ascorbic acid and FeCl_3 were made up fresh immediately before use. After incubation at 37°C for 1 h, an aliquot of the assay mixture was added to a cuvette containing 0.05 ml elastase (BDH, 1 mg/ml, freshly diluted). After standing for 20 min, the residual elastase activity was measured (see [28] and legend to table 1). The elastase alone gave a ΔA_{410} of $1.2 \times 10^{-2} \text{ s}^{-1}$

uric acid had almost no protective effect in this system (table 1).

If the solution is bubbled with an 80% N_2O /20% O_2 mixture instead of pure N_2O , the radicals produced by attack of $\cdot\text{OH}$ on added 'scavengers' will be able to form peroxy radicals, i.e. if X is the scavenger

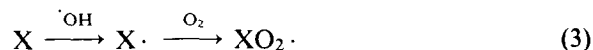


Table 1 shows that indomethacin and piroxicam (as

well as most other $\cdot\text{OH}$ scavengers tested) were still protective under these conditions. However, uric acid accelerated the inactivation of α_1 -antiproteinase: more of its elastase-inhibitory capacity was lost upon irradiation in the presence of uric acid.

3.2. Generation of hydroxyl radicals by biochemical systems

A mixture of Fe^{3+} -EDTA, H_2O_2 and ascorbic acid at pH 7.4 generates $\cdot\text{OH}$ [31,38]. If α_1 -anti-

Table 3
Inactivation of α_1 -antiproteinase by hydroxyl radicals: hypoxanthine/xanthine oxidase/iron system

Expt	Elastase activity (ΔA_{410}) ($\times 10^{-3} \text{ s}^{-1}$)	α_1 -Antiproteinase activity (% inhibition of elastase)
α_1 -Antiproteinase alone	0	100
Complete reaction mixture (omit FeCl_3 -EDTA)	7.7	6
+ 1 mM DMSO	0	100
+ 1 mM uric acid	4.5	45
+ 100 μM ascorbic acid	8.2	0
+ 100 μM ascorbic acid and 1 mM DMSO	5.9	28
+ 100 μM ascorbic acid and 1 mM uric acid	2.5	70
+ 100 μM ascorbic acid and 1 mM uric acid	4.3	48

Reaction mixtures contained, in a final volume of 1 ml, the following reagents at the final concentrations stated: 10 mM KH_2PO_4 -KOH buffer (pH 7.4), 1 mg/ml α_1 -antiproteinase, 100 μM Fe^{3+} -EDTA, 333 μM hypoxanthine and 0.033 U/ml of Sigma xanthine oxidase. Incubation was at 37°C for 1 h. An aliquot of the assay mixture was added to a cuvette containing elastase as described in the legend to table 2. The elastase alone gave a ΔA_{410} of $8.2 \times 10^{-3} \text{ s}^{-1}$. Solutions of ascorbate and FeCl_3 were made up fresh before use

proteinase was incubated with this reaction mixture, its elastase-inhibitory capacity was diminished. Several scavengers of $\cdot\text{OH}$ offered protection: table 2 shows an example for dimethyl sulphoxide (k_2 for reaction with $\cdot\text{OH}$ about $7 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ [31]). Uric acid was reproducibly protective in this system (table 2 shows a typical result): there was less inactivation of α_1 -antiproteinase in the presence of uric acid.

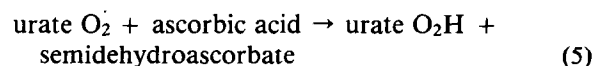
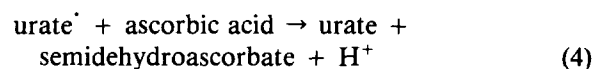
A mixture of hypoxanthine and xanthine oxidase generates O_2^- and H_2O_2 [32] which can form $\cdot\text{OH}$ if iron ions are present [38]. Such a reaction mixture also inactivated the elastase-inhibitory capacity of α_1 -antiproteinase (table 3). Protection was afforded by several scavengers of $\cdot\text{OH}$, such as dimethyl sulphoxide (table 3), or by omission of iron from the reaction mixture. Uric acid did not protect α_1 -antiproteinase: in fact, it slightly but reproducibly increased the inactivation. Addition of ascorbic acid to the reaction mixture slightly protected the α_1 -antiproteinase: it has been observed previously that addition of ascorbic acid to the hypoxanthine/xanthine oxidase/ FeCl_3 -EDTA system does not significantly increase the total amount of $\cdot\text{OH}$ generated [38]. However, in the presence of ascorbate, further addition of uric acid reproducibly decreased inactivation of α_1 -antiproteinase (table 3 shows a typical experimental result). Control experiments showed that none of these reagents affected the activity of xanthine oxidase under our reaction conditions.

4. DISCUSSION

Kittridge and Willson [19] reported that various radicals produced by attack of $\cdot\text{OH}$, generated by pulse radiolysis, upon uric acid are able to inactivate the enzyme yeast alcohol dehydrogenase, which is known to be very sensitive to oxidant damage [33]. Here, we have shown directly that $\cdot\text{OH}$ radicals are also able to inactivate α_1 -antiproteinase, in confirmation of previous conclusions based upon the use of scavengers [23,24,26]. α_1 -Antiproteinase is the major circulating inhibitor of serine proteases such as elastase and its inactivation, e.g. in the lung or at sites of inflammation, can have severe biological consequences [20-26]. Uric acid, especially in the presence of O_2 to allow formation of urate peroxy radicals, exacerbated the inactivation of α_1 -antiproteinase by $\cdot\text{OH}$. At

the high uric acid: α_1 -antiproteinase molar ratios used ($19 \mu\text{M}$ protein, 120 – $240 \mu\text{M}$ uric acid), almost all of the $\cdot\text{OH}$ generated would react with the uric acid. Hence, the inactivation is presumably mediated by uric acid-derived carbon-centred and peroxy radicals which appear, in the latter case especially, to be more effective at activating α_1 -antiproteinase than is $\cdot\text{OH}$ under our reaction conditions.

Inactivation of α_1 -antiproteinase by $\cdot\text{OH}$ generated in a hypoxanthine/xanthine oxidase/iron system, a biologically relevant source of $\cdot\text{OH}$ [34,38], has also been demonstrated. Again, uric acid enhanced the inactivation, whereas other $\cdot\text{OH}$ scavengers protected (table 3). By contrast, when $\cdot\text{OH}$ was generated by the ascorbate/ H_2O_2 /iron system, α_1 -antiproteinase was inactivated but uric acid offered protection (table 2). Similarly, when ascorbic acid was added to the hypoxanthine/xanthine oxidase/iron system, further addition of uric acid protected the α_1 -antiproteinase. Why should there be this difference? Maples and Mason [35] reported that a uric acid-derived radical could be reduced back to uric acid in a 'repair' reaction. Thus, by such reactions as



the urate and urate peroxy radicals can be removed and the ability of uric acid to scavenge $\cdot\text{OH}$ then allows it to protect α_1 -antiproteinase. It should be noted that iron was used chelated to EDTA in all these experiments, so that the ability of uric acid to bind metal ions [17,18] could not interfere with the $\cdot\text{OH}$ generation.

What is the physiological significance of these observations in humans? Uric acid is present at concentrations up to $500 \mu\text{M}$ in body fluids and appears to break down at an increased rate when oxidants are generated, e.g. at sites of inflammation [6,7]. On the other hand, ascorbic acid is also present in extracellular fluids, although the amounts may decrease under conditions of oxidant stress [36,37]. In view of the many other oxidant-scavenging properties of uric acid (section 1), it seems likely that its overall effect will be protective.

However, the results obtained in [19] and in the present paper remind us that no antioxidant is perfect [1].

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REFERENCES

- [1] Halliwell, B. and Gutteridge, J.M.C. (1985) *Free Radicals in Biology and Medicine*, Clarendon, Oxford.
- [2] Sies, H. (1985) *Oxidative Stress*, Academic Press, London.
- [3] Cross, C.E., Halliwell, B., Borish, E.T., Pryor, W.A., Ames, B.N., Saul, R.L., McCord, J.M. and Harman, D. (1987) *Ann. Int. Med.* 107, 526-545.
- [4] Ames, B.N., Cathcart, R., Schwiers, E. and Hochstein, P. (1981) *Proc. Natl. Acad. Sci. USA* 78, 6858-6862.
- [5] Cutler, R.G. (1984) *Arch. Gerontol. Geriatr.* 3, 321-348.
- [6] Grootveld, M. and Halliwell, B. (1987) *Biochem. J.* 243, 803-808.
- [7] Halliwell, B., Grootveld, M., Kaur, H. and Fagerheim, I. (1988) in: *Free Radicals: Methodology and Concepts* (Rice-Evans, C. and Halliwell, B. eds) pp. 33-59.
- [8] Matsushita, S., Ibuki, F. and Aoki, A. (1963) *Arch. Biochem. Biophys.* 102, 446-451.
- [9] Niki, E., Saito, M., Yoshikawa, Y., Yamamoto, Y. and Kamiya, Y. (1986) *Bull. Chem. Soc. Jap.* 59, 471-477.
- [10] Smith, R.C. and Lawing, L. (1983) *Arch. Biochem. Biophys.* 223, 166-172.
- [11] Smith, R.C. and Nunn, V. (1984) *Arch. Biochem. Biophys.* 232, 348-353.
- [12] Liu, K.M., Swann, D., Lee, P.F. and Lam, K.W. (1984) *Curr. Eye Res.* 3, 1049-1053.
- [13] Winterbourn, C.C. (1985) *Biochim. Biophys. Acta* 840, 204-210.
- [14] Grootveld, M., Halliwell, B. and Moorhouse, C.P. (1987) *Free Radical Res. Commun.* 4, 69-76.
- [15] Cohen, A.M., Aberdroth, R.E. and Hochstein, P. (1984) *FEBS Lett.* 174, 147-150.
- [16] Meadows, J. and Smith, R.C. (1986) *Arch. Biochem. Biophys.* 246, 838-845.
- [17] Davies, K.J.A., Sevanian, A., Muakkassah-Kelly, S.F. and Hochstein, P. (1986) *Biochem. J.* 235, 747-754.
- [18] Rowley, D.A. and Halliwell, B. (1985) *J. Inorg. Biochem.* 23, 103-108.
- [19] Kittridge, K.J. and Willson, R.L. (1984) *FEBS Lett.* 170, 162-164.
- [20] Kamboh, M.I. (1985) *Dis. Markers* 3, 135-154.
- [21] Travis, J. and Salvesen, G.S. (1983) *Annu. Rev. Biochem.* 52, 655-709.
- [22] Weiss, S.J. (1986) *Acta Physiol. Scand. suppl.* 548, 9-37.
- [23] Carp, H. and Janoff, A. (1979) *J. Clin. Invest.* 63, 793-797.
- [24] Carp, H. and Janoff, A. (1980) *J. Clin. Invest.* 66, 987-995.
- [25] Clark, R.A., Stone, P.J., El Hag, A., Calore, J.D. and Franzblau, C. (1981) *J. Biol. Chem.* 256, 3348-3353.
- [26] Pryor, W.A., Dooley, M.M. and Church, D.F. (1986) *Adv. Free Radical Biol. Med.* 2, 161-188.
- [27] Green, D.R., Fellman, J.H. and Eicher, A.L. (1985) *FEBS Lett.* 192, 33-36.
- [28] Wasil, M., Halliwell, B., Hutchison, D.C.S. and Baum, H. (1987) *Biochem. J.* 243, 219-223.
- [29] Keene, J.P. (1964) *J. Sci. Inst.* 41, 493-496.
- [30] Aruoma, O.I. and Halliwell, B. (1988) *Xenobiotica* 18, 459-470.
- [31] Halliwell, B., Gutteridge, J.M.C. and Aruoma, O.I. (1987) *Anal. Biochem.* 165, 215-219.
- [32] McCord, J.M. and Fridovich, I. (1969) *J. Biol. Chem.* 244, 6049-6055.
- [33] Vonn Sonntag, C. (1987) *The Chemical Basis of Radiation Biology*, pp. 434-435, Taylor & Francis, London.
- [34] McCord, J.M. (1985) *New Engl. J. Med.* 312, 159-163.
- [35] Maples, K.R. and Mason, R.P. (1988) *J. Biol. Chem.* 263, 1709-1712.
- [36] Lunec, J. and Blake, D.R. (1985) *Free Radical Res. Commun.* 1, 31-39.
- [37] Halliwell, B., Wasil, M. and Grootveld, M. (1987) *FEBS Lett.* 213, 15-18.
- [38] Rowley, D.A. and Halliwell, B. (1983) *Clin. Sci.* 64, 649-653.