Volume 244, number 1, 76–80 FEB 06789 February 1989

Inactivation of α_1-antiproteinase by hydroxyl radicals

The effect of uric acid

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Received 1 December 1988

The elastase-inhibitory activity of α_1-antiproteinase is inactivated by hydroxyl radicals ($^{\cdot}OH$) 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 $^{\cdot}OH$ 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 $^{\cdot}OH$ 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 7.2×10^9 $M^{-1} \cdot s^{-1}$) hydroxyl radicals [19], and can protect certain enzymes against inactivation by this reactive radical species. However, reaction of uric acid with $^{\cdot}OH$ 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 $^{\cdot}OH$, 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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Published by Elsevier Science Publishers B.V. (Biomedical Division)

hydroxyl radical [23,24,26]. We have therefore examined the ability of uric acid to protect α_1-antiproteinase against inactivation by hydroxyl radicals ($^{\cdot}OH$) 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, $O_2^{\cdot-}$.

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 $^{\cdot}OH$ 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 $^{\cdot}OH$ 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 μmol/dm^3 of $^{\cdot}OH$ overall. The yield of $^{\cdot}OH$ 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 $^{\cdot}OH$

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

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

If α_1-antiproteinase (1 mg/ml, approx. 19 μM) was included in the radiolysis solution, it was inactivated (table 1). Generation of 180 μmol/dm^3 of $^{\cdot}OH$ 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 $^{\cdot}OH$ 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 $^{\cdot}OH$ with rate constants close to 10^{10} $M^{-1} \cdot s^{-1}$ [30]. Despite its ability to scavenge $^{\cdot}OH$,

Table 1

Inactivation of α_1-antiproteinase by hydroxyl radicals: radiolysis

Expt	Elastase activity ($\times 10^{-2}$ s^{-1}) (ΔA_{410})	α_1-Antiproteinase activity (as % inhibition of elastase)
(A)		
Unirradiated α_1AP	0	100
Irradiated α_1AP	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 α_1 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 (α_1AP, 1 mg/ml) in phosphate buffer was subjected to radiolysis over about a 6 min period with 30 krad to generate 180 μmol $^{\cdot}OH$/dm^3. Immediate after radiolysis, a sample (0.1 mg) of α_1AP 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 α_1AP) gave a ΔA_{410} of 1.53 $\times$ 10^{-2} s^{-1}. A concentration of α_1AP 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) N_2O/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}) ($\times 10^{-2}$ s^{-1})	α_1-Antiproteinase activity (1% inhibition of elastase)
α_1-Antiproteinase alone	0	100
Complete reaction mixture	1.17	3
(omit Fe^{3+}-EDTA)	0.3	75
+ 400 μM dimethyl sulphoxide	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 1.2×10^{-2} 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 $^{\cdot}OH$ on added 'scavengers' will be able to form peroxy radicals, i.e. if X is the scavenger

$$X \xrightarrow{^{\cdot}OH} X\cdot \xrightarrow{O_2} XO_2\cdot \qquad (3)$$

Table 1 shows that indomethacin and piroxicam (as well as most other $^{\cdot}OH$ 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 $^{\cdot}OH$ [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}) ($\times 10^{-3}$ s^{-1})	α_1-Antiproteinase activity (% inhibition of elastase)
α_1-Antiproteinase alone	0	100
Complete reaction mixture	7.7	6
(omit $FeCl_3$-EDTA)	0	100
+ 1 mM DMSO	4.5	45
+ 1 mM uric acid	8.2	0
+ 100 μM ascorbic acid	5.9	28
+ 100 μM ascorbic acid and 1 mM DMSO	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 8.2×10^{-3} 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 $^{\cdot}OH$ offered protection: table 2 shows an example for dimethyl sulphoxide (k_2 for reaction with $^{\cdot}OH$ about $7 \times 10^9\ M^{-1} \cdot s^{-1}$ [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 $^{\cdot}OH$ 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 $^{\cdot}OH$, 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 $^{\cdot}OH$ 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 $^{\cdot}OH$, 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 $^{\cdot}OH$ 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 $^{\cdot}OH$. At the high uric acid: α_1-antiproteinase molar ratios used (19 μM protein, 120–240 μM uric acid), almost all of the $^{\cdot}OH$ 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 $^{\cdot}OH$ under our reaction conditions.

Inactivation of α_1-antiproteinase by $^{\cdot}OH$ generated in a hypoxanthine/xanthine oxidase/iron system, a biologically relevant source of $^{\cdot}OH$ [34,38], has also been demonstrated. Again, uric acid enhanced the inactivation, whereas other $^{\cdot}OH$ scavengers protected (table 3). By contrast, when $^{\cdot}OH$ 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

$$\text{urate}^{\cdot} + \text{ascorbic acid} \rightarrow \text{urate} + \text{semidehydroascorbate} + H^+ \quad (4)$$

$$\text{urate } O_2^{\cdot} + \text{ascorbic acid} \rightarrow \text{urate } O_2H + \text{semidehydroascorbate} \quad (5)$$

the urate and urate peroxy radicals can be removed and the ability of uric acid to scavenge $^{\cdot}OH$ 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 $^{\cdot}OH$ generation.

What is the physiological significance of these observations in humans? Uric acid is present at concentrations up to 500 μM 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].

Acknowledgements: We are grateful to Drs John Butler and Brigid Hoey for helping us to use pulse radiolysis facilities at the Paterson Institute for Cancer Research, Christie Hospital, Manchester. We thank Perstorp Carbotec for research support.

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