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The antioxidant action of human extracellular fluids

Effect of human serum and its protein components on the inactivation of α_1-antiproteinase by hypochlorous acid and by hydrogen peroxide

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The elastase-inhibitory capacity of purified human α_1-antiproteinase is inactivated by low concentrations of the myeloperoxidase-derived oxidant hypochlorous acid, but much higher concentrations are required to inhibit the elastase-inhibitory capacity of serum samples. The protective effect of serum appears to be largely due to albumin. High concentrations of H_2O_2 also inactivate the elastase-inhibitory capacity of α_1-antiproteinase, by a mechanism not involving formation of hydroxyl radicals. Serum offers protection against H_2O_2 inactivation of α_1-antiproteinase. The relevance of these results to the tissue damage produced by activated phagocytes is discussed.

INTRODUCTION

Activated neutrophils release O_2-derived species such as H_2O_2, superoxide radicals ($O_2^{\cdot-}$) and, possibly, hydroxyl radicals ('OH) [1–3]. They also release the enzyme myeloperoxidase, which uses H_2O_2 to oxidize Cl^- ions into a powerful oxidant that has been identified as hypochlorous acid (HOCl) [1,3–6]. Both the O_2-derived species and HOCl generated by myeloperoxidase activity play some role in bacterial killing by neutrophils [1–4, 7–10], and they have also been implicated in producing tissue damage by activated phagocytes. For example, 'OH radicals produced by metal-ion-dependent reactions of $O_2^{\cdot-}$ and H_2O_2 have been proposed to inactivate serum α_1-antiproteinase [11–13]. α_1-Antiproteinase accounts for about 90% of the elastase-inhibitory capacity of human serum [14,15], and its inactivation might greatly potentiate tissue damage [11,12,16], since elastase is also released from activated neutrophils. Emphysema may result from failure to inhibit elastase adequately in the lung [11,12].

Purified α_1-antiproteinase has also been shown to be rapidly inactivated by HOCl produced by myeloperoxidase [14,16,17]. Reaction of HOCl with nitrogen-containing compounds in serum, such as taurine, has been claimed to produce 'long-lived oxidants' that might also inhibit antiproteinase activity [18,19]. However, there has been considerable controversy in the literature concerning the actual amount of α_1-antiproteinase inactivation that might occur *in vivo* by oxidative mechanisms, e.g. during cigarette smoking [20–24]. Some scientists have been unable to find evidence for significant inactivation of α_1-antiproteinase *in vivo* [21,23].

Human extracellular fluids, such as plasma or synovial fluid, contain little of the antioxidant defence enzymes glutathione peroxidase (selenium enzymes), superoxide dismutase or catalase [25]. However, they do have powerful antioxidant activities, which minimize the toxicity of $O_2^{\cdot-}$ and H_2O_2 by preventing their metal-ion-dependent conversion into the highly reactive 'OH radical [25]. In the present paper we extend knowledge of the antioxidant activity of human serum by reporting its ability to protect α_1-antiproteinase against inactivation by HOCl, 'long-lived oxidants' derived from HOCl, and H_2O_2.

MATERIALS AND METHODS

Reagents and blood samples

All reagents were obtained from Sigma Chemical Co. unless otherwise stated. Peripheral-vein blood samples were obtained from healthy control subjects or from patients with seropositive rheumatoid arthritis, and were collected in vacuum containers. All subjects had given informed consent for samples to be taken. After centrifugation at 1000 *g* for 10 min, the serum was separated and either used at once or stored in small portions at −20 °C. Storage for up to 4 weeks had no significant effect on the results. 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 ref. [26]. Catalase was assayed by the method described in the Sigma Catalogue, based on the loss of H_2O_2 monitored at 240 nm.

Assay of α_1-antiproteinase

Purified α_1-antiproteinase (Sigma type A9024) was dissolved in phosphate-buffered saline (140 mM-NaCl/2.7 mM-KCl/16 mM-Na_2HPO_4/2.9 mM-KH_2PO_4, pH 7.4) to a concentration of 3.75 mg/ml. Other reagents were also added dissolved in phosphate-buffered saline usually to give a final reaction volume of 35.1 μl, containing 1.25 mg of α_1-antiproteinase/ml (final concentration). After incubation for 1–2 h at room temperature, 3 ml of phosphate-buffered saline was added, followed by 50 μl of a 0.1% (w/v) solution of pig

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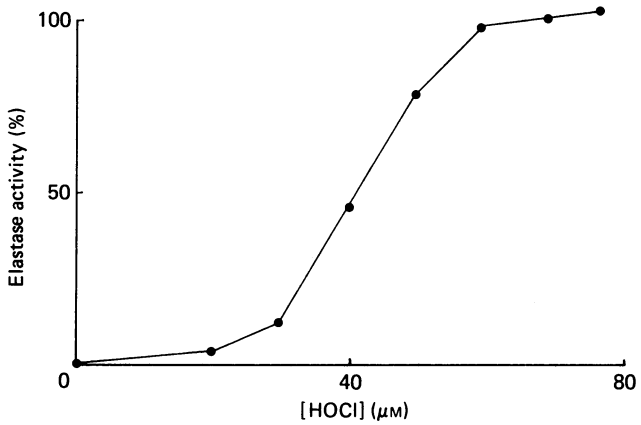

Fig. 1. Inhibition of α_1-antiproteinase by HOCl

Reaction mixtures contained, in a final volume of 23.4 μl, 1.25 mg of α_1-antiproteinase/ml (final concentration) in phosphate-buffered saline, and HOCl in phosphate-buffered saline at the final concentration stated. They were incubated for 1 h at room temperature (25±2 °C), and residual elastase-inhibitory capacity was determined (after addition of 3 ml of phosphate-buffered saline and elastase) as described in the Materials and methods section. Elastase activity is shown as a percentage of that in the absence of α_1-antiproteinase. As α_1-antiproteinase is inhibited, more elastase activity appears.

pancreatic elastase, prepared by diluting elastase (from BDH Chemicals) into phosphate-buffered saline. After incubation for 30 min, 100 μl of 25 mM-elastase substrate [*N*-(3-carboxypropionyl)trialanyl *p*-nitroanilide] in phosphate-buffered saline was added, and elastase activity was measured as a linear increase in absorbance at 410 nm. Neither H_2O_2 nor HOCl at the maximum concentrations used in these studies had any effect on elastase activity. Elastase assays were reproducible to within 5%.

RESULTS

Action of HOCl on α_1-antiproteinase

The myeloperoxidase/H_2O_2/Cl^- system has been reported to inactivate α_1-antiproteinase by producing HOCl [14,16,17,27]. In agreement with these results, we found that incubation of a quantity of human plasma α_1-antiproteinase preparation, normally sufficient to inhibit elastase completely, with HOCl at pH 7.4 caused rapid inactivation of its elastase-inhibitory capacity (Fig. 1). Concentrations of HOCl above 60 μM inhibited the α_1-antiproteinase by 95–100% and allowed expression of elastase activity (e.g. 100% in Fig. 1 and 96.7% in Table 1). If the HOCl was pre-mixed with an excess of taurine before addition to the reaction mixture, to form the 'long-lived oxidant' *N*-chlorotaurine [6,29], almost complete inhibition of the α_1-antiproteinase was still observed, in agreement with previous results [17,18,29]. Taurine itself did not affect the system (Table 1).

When HOCl is generated from myeloperoxidase released by activated neutrophils *in vivo*, many other proteins will be present in addition to α_1-antiproteinase. Table 1 shows the effect of including some other serum proteins at physiological concentrations on the inactivation of α_1-antiproteinase by HOCl. Transferrin offered some protection, as did caeruloplasmin at physiological concentrations. However, albumin had a striking protective effect. Concentrations of albumin less than those normally present in human plasma were able completely to inhibit inactivation of α_1-antiproteinase by HOCl under our reaction conditions.

In support of this conclusion, it was found that HOCl

Table 1. Protection by serum proteins against inactivation of α_1-antiproteinase by HOCl

Assays were performed as described in the legend to Fig. 1, except that the final reaction volume, before addition of 3 ml of phosphate-buffered saline and elastase, was 35.1 μl, containing a final concentration of 100 μM-HOCl. The concentrations given below are the final concentrations present in each reaction mixture. Normal human serum concentrations are α_1-antiproteinase 1.2–1.3 mg/ml [15], transferrin 2.92±0.38 mg/ml, caeruloplasmin 0.259±0.079 mg/ml and albumin 50.3±4.4 mg/ml [28]. None of the proteins had any action on elastase itself. Albumin preparations were fatty acid-free. In the taurine experiment the HOCl was premixed with taurine before addition to the reaction mixture. Elastase activity is shown as a percentage of that in the absence of α_1-antiproteinase.

Addition to reaction mixture	Concn.	Elastase activity in assay (% of maximum)	Activity of α_1-antiproteinase (%) (as 100−elastase activity)
None	–	96.9	3.1
None (HOCl omitted)	–	0	100
Taurine	0.5 mM	99.3	0.7
Taurine (HOCl omitted)	0.5 mM	0	100
Transferrin, human	1 mg/ml	89.6	10.4
	3 mg/ml	69.8	30.2
Caeruloplasmin, human	0.3 mg/ml	87.5	12.5
	1 mg/ml	65.8	34.2
Albumin, bovine	16.7 mg/ml	18.9	81.1
Albumin, human	3.3 mg/ml	16.0	84.0
	10 mg/ml	2.0	98.0
	16.7 mg/ml	0.5	99.5

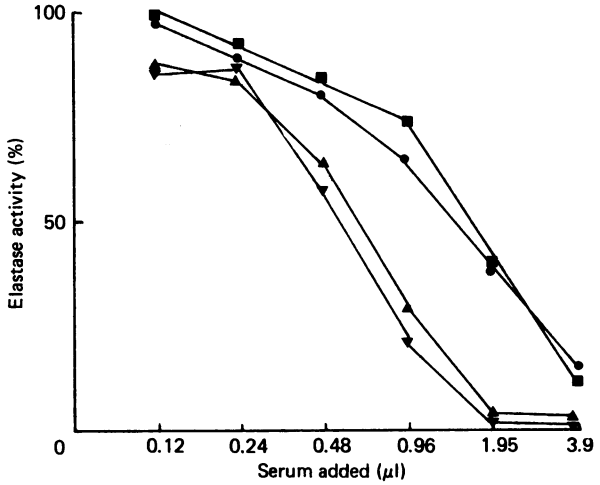

Fig. 2. Action of HOCl on elastase-inhibitory capacity of serum

Reaction mixtures contained, in a final volume of 35.1 μl, the indicated volume of normal serum or serum from a rheumatoid patient and, where indicated, 200 μM-HOCl (final concentration) in phosphate-buffered saline. After incubation for 1 h at room temperature, elastase-inhibitory capacity was determined as described in the Materials and methods section. ●, Normal serum; ■, normal serum plus HOCl; ▼, serum from a rheumatoid patient; ▲, serum from a rheumatoid patient plus HOCl. Elastase activity is shown as a percentage of that in the absence of added serum.

Table 2. Inactivation of the elastase-inhibitory capacity of serum by HOCl

Assays were performed on serum samples (11.7 μl, in a final reaction volume of 35.1 μl, before addition of phosphate-buffered saline and elastase) from a normal volunteer (serum A) and from a rheumatoid patient (serum B) as described in the legend to Fig. 2. The concentrations of HOCl stated are the final concentrations in the reaction mixture before addition of 3 ml of phosphate-buffered saline and elastase.

Concn. of HOCl (mM)	Elastase activity in assay (% of maximum)		Elastase-inhibitory capacity of serum (% of maximum)	
	Serum A	Serum B	Serum A	Serum B
0	0	0	100	100
0.05	0	0	100	100
0.2	0	0	100	100
1.0	31	17	69	83
2.0	37	13	63	87
3.0	41	22	59	78
4.0	44	32	56	68
5.0	46	45	54	55
6.0	50	55	50	45
7.0	53	70	47	30

at a final concentration of 200 μM, sufficient to inhibit purified α_1-antiproteinase completely (Fig. 1), had only a slight inhibitory effect on the elastase-inhibitory capacity of serum samples, which is known to be largely due to

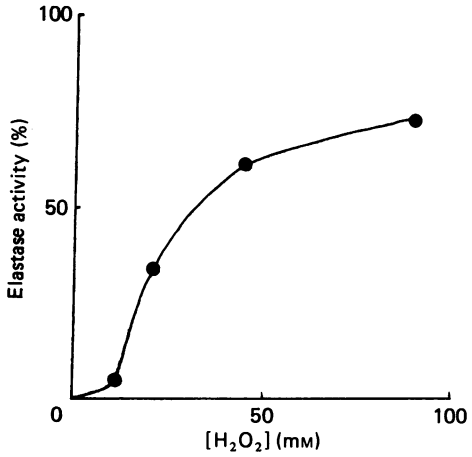

Fig. 3. Inhibition of α_1-antiproteinase by H_2O_2

Reaction mixtures contained, in a final volume of 35.1 μl, 1.25 mg of α_1-antiproteinase/ml in phosphate-buffered saline, and H_2O_2 in phosphate-buffered saline at the final concentration stated. They were incubated for 2 h at room temperature, and residual elastase-inhibitory capacity was determined (after addition of phosphate-buffered saline and elastase) as described in the Materials and methods section. Elastase activity is shown as a percentage of that in the absence of α_1-antiproteinase, i.e. 100% corresponds to full elastase activity.

α_1-antiproteinase [14,15]. This was shown both in normal serum and in serum from rheumatoid patients, which usually contains higher concentrations of α_1-antiproteinase than normal, since it is an acute-phase protein. Fig. 2 shows two experimental results, one for a normal serum sample and one for a sample from a rheumatoid patient. Indeed, it was necessary to raise the HOCl concentration to millimolar values before any marked loss of elastase-inhibitory capacity was seen in serum samples. Table 2 shows two representative experiments, one with a normal serum sample and one with a serum sample from a rheumatoid patient. Analysis of a number of experiments of this type showed no apparent difference between the normal serum sample and that from the rheumatoid patient.

Action of H_2O_2 on α_1-antiproteinase

Some authors have reported no [12] or only slight [30,31] effects of H_2O_2 at millimolar concentrations on α_1-antiproteinase activity, whereas others have reported H_2O_2 as a powerful inactivator of this protein [32]. In support of the studies reported in refs. [30] and [31], we found that high concentrations of H_2O_2 inactivated an α_1-antiproteinase preparation (Fig. 3). This inactivation did not appear to be mediated by formation of $^{\cdot}OH$ radicals from the H_2O_2 [25], in that it was not prevented by the $^{\cdot}OH$ scavenger mannitol (final concn. 100 mM). The iron-chelating agents desferrioxamine (final concn. 1 mM), human transferrin (1 mg/ml), human lactoferrin (1 mg/ml) or rabbit transferrin (1 mg/ml) did not inhibit the inactivation of α_1-antiproteinase by H_2O_2; all these agents will prevent iron-dependent decomposition of H_2O_2 to give $^{\cdot}OH$ [25,33,34]. EDTA (final concn. 1 mM) also had no protective effect. Addition of 1 mM-$FeSO_4$ or 1 mM-$CuSO_4$ to H_2O_2 (concentration range 1–44 mM) to

Table 3. Inactivation of α_1-antiproteinase by H_2O_2

Assays were performed as described in the legend to Fig. 3, with a final H_2O_2 concentration of 44 mM before addition of 3 ml of phosphate-buffered saline and elastase. The concentrations given below are the final concentrations present in each reaction mixture before addition of phosphate-buffered saline and elastase. None of the proteins had any action on elastase itself, or was contaminated with catalase activity, as measured by the spectrophotometric assay described in the Sigma catalogue. Albumin preparations were fatty acid-free.

Addition to reaction mixture	Concn.	Elastase activity in assay (% of maximum)	Activity of α_1-antiproteinase (%) (as 100−elastase activity)
None	–	78	22
None (H_2O_2 omitted)	–	0	100
Human caeruloplasmin	0.46 mg/ml	49	51
Human apotransferrin	1 mg/ml	78	22
Rabbit apotransferrin	1 mg/ml	79	21
	6 mg/ml	78	22
Human albumin	60 mg/ml	80	20
Human lactoferrin	1 mg/ml	80	20

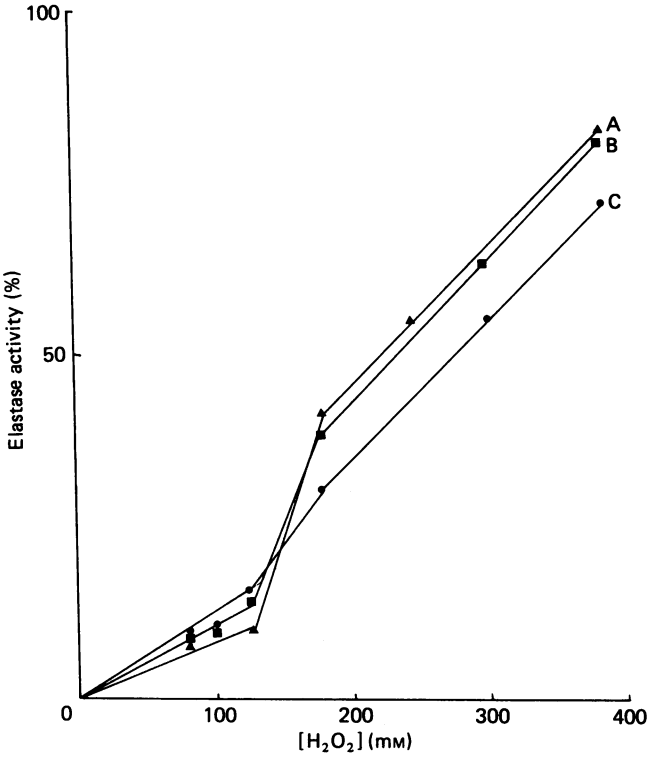

Fig. 4. Action of H_2O_2 on elastase-inhibitory capacity of serum

Reaction mixtures contained, in a final volume of 35.1 μl, 11.7 μl of serum and the final concentration of H_2O_2 stated in phosphate-buffered saline. After incubation for 2 h at room temperature, elastase-inhibitory capacity was determined as described in the Materials and methods section. Sera A, B and C are samples obtained from three healthy adult human volunteers. Elastase activity is shown as a percentage of that in the absence of added serum (100%).

accelerate $^{\cdot}OH$ formation [35] did not increase the observed inactivation of α_1-antiproteinase.

In contrast with the results obtained with HOCl (Table 1), serum albumin offered little protection to α_1-antiproteinase against inactivation by H_2O_2, although caeruloplasmin was slightly protective (Table 3). Despite this, we found that the elastase-inhibitory capacity of human serum required much greater concentrations of H_2O_2 (200–400 mM) for inactivation than did the isolated α_1-antiproteinase protein (Fig. 4). The serum samples did not contain measurable catalase activity, determined by a sensitive spectrophotometric assay based on the loss of absorbance at 240 nm as H_2O_2 is destroyed. Incubation of serum with H_2O_2 for up to 60 min showed no significant loss of H_2O_2.

DISCUSSION

The active reagent produced in the myeloperoxidase/H_2O_2/Cl^- system is known to be HOCl [4,5,27]. HOCl reacts with a wide range of proteins and small molecules [4,5,26,27]. When it is mixed with pure α_1-antiproteinase, the elastase-inhibitory capacity is destroyed, in agreement with previous results [14,16,17]. However, addition of physiological concentrations of serum proteins gave substantial protection. Caeruloplasmin has been reported to inhibit inactivation of α_1-antiproteinase by the myeloperoxidase system [36], but our studies show that, at physiological protein concentrations, albumin is much more effective. Presumably albumin reacts with HOCl [27,37] and preferentially scavenges this molecule.

It has been suggested that HOCl reacts with nitrogen-containing compounds, such as taurine, in body fluids to form 'long-lived oxidants' that inactivate α_1-antiproteinase [6,18,19,29]. Our experiments confirm this possibility in simple systems *in vitro* (Table 1). However, the high concentrations of HOCl needed to inhibit α_1-antiproteinase in serum (Fig. 2) suggest that the scavenging effects of serum proteins more than outweigh any ability of serum components to potentiate the toxicity of HOCl *in vivo*.

Hence the extent of inactivation of α_1-antiproteinase by HOCl *in vivo* would seem to be much more limited than has been supposed from studies of the action of HOCl or the myeloperoxidase system on pure α_1-antiproteinase. Our results might explain the observation that the α_1-antiproteinase inactivation produced in hamsters by intratracheal injection of H_2O_2 and

myeloperoxidase was much less than expected from experiments performed *in vitro* [24]. Our results do not mean that HOCl production *in vivo* is irrelevant in causing biological damage, but they suggest that important HOCl damage *in vivo* may, like damage by ·OH, occur at specific localized sites rather than in bulk solution [25,38–40]. It must also be noted that albumin and other protein concentrations are lower in synovial fluid [28], which might predispose to HOCl damage in the inflamed rheumatoid joint.

There is a controversy in the literature concerning the effect of H_2O_2 itself on α_1-antiproteinase [12,30–32]. Our results confirm the majority view [12,30,31] that this protein is fairly insensitive to inactivation of its elastase-inhibitory capacity by H_2O_2. The inactivation that is produced by high concentrations of H_2O_2 does not seem to be mediated by ·OH formation, and is perhaps a direct effect of the H_2O_2. Serum, shown to be free of catalase activity, offered a protective effect against α_1-antiproteinase inactivation by H_2O_2. This is another demonstration of the powerful antioxidant action of extracellular fluids [25,28,41], although the components responsible for the protective actions have not been identified (Table 3).

·OH radicals have been claimed to be involved in α_1-antiproteinase inactivation in some systems *in vitro* [11–13]. We were unable to demonstrate any inactivation of the protein by metal ion/H_2O_2 mixtures, a biologically relevant source of ·OH [35]. Pryor's group in the U.S.A. (W. A. Pryor, personal communication) has informed one of us (B. H.) of similar findings (also see ref. [42]). The ability of ·OH to attack proteins is well established [43], and our studies perhaps suggest that the sites of attack of ·OH on α_1-antiproteinase are not crucial to its elastase-inhibitory capacity [38,42].

The results in the present paper show that components of extracellular fluids have substantial antioxidant activity, not only by metal binding [25,41] and scavenging of peroxy radicals [44], but also by scavenging of HOCl and by protection against direct inactivation of α_1-antiproteinase by H_2O_2. The nature of the latter protection is worthy of further investigation, especially as it has been demonstrated that this protection is diminished in serum from rheumatoid patients [31].

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