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# Action of hypochlorous acid on the antioxidant protective enzymes superoxide dismutase, catalase and glutathione peroxidase

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The neutrophil enzyme myeloperoxidase generates hypochlorous acid (HOCl) at sites of inflammation. Glutathione peroxidase is very quickly inactivated by low concentration of HOCl. Inactivation of catalase is also rapid, but requires higher HOCl concentrations and the haem appears to be degraded. Inactivation of bovine CuZn superoxide dismutase is slower. Hence superoxide dismutase should not be easily inactivated by HOCl at sites of inflammation, which may contribute to its effectiveness as an anti-inflammatory agent and in minimizing reperfusion injury.

## INTRODUCTION

Activated neutrophils contain the enzyme myeloperoxidase, which uses $H_2O_2$ to oxidize $Cl^-$ ions into a powerful oxidant that has been identified [1] as HOCl:

$$H_2O_2 + Cl^- + H^+ \rightarrow H_2O + HOCl \quad (1)$$

HOCl generated at sites of inflammation can have multiple effects. For example, it can aggravate tissue damage by inactivating $\alpha_1$-antiproteinase and permitting uncontrolled proteinase activity [1–3], by attacking elastin [4], by depleting ascorbic acid [5] and by activating collagenase and gelatinase [6]. On the other hand, HOCl can diminish inflammation by killing bacteria and fungi [7,8], inactivating neutrophil chemo-attractants [9] and attacking other neutrophil-derived products [10]. Some anti-inflammatory drugs may exert part of their action by scavenging HOCl [11,12].

Activated neutrophils also release superoxide radical ion ($O_2^{\cdot -}$) and $H_2O_2$: these may, under certain circumstances, interact to form the highly reactive hydroxyl radical, $^{\cdot}OH$ [13]. $H_2O_2$, $O_2^{\cdot -}$ and $^{\cdot}OH$, as well as HOCl, are all thought to contribute to tissue damage at sites of active inflammation [13–15]. This has led to proposals for the use of superoxide dismutase [16,17], catalase [18–20] and glutathione peroxidase, or other GSH-dependent reagents that remove $H_2O_2$ [21], as anti-inflammatory agents. Although promising results have been obtained in a number of animal systems [16–21], no well-controlled clinical trials of superoxide dismutase, catalase or glutathione peroxidase have yet been reported in human inflammatory diseases. Hirschelmann & Bekemeier [22] reported that heat-inactivated catalase and superoxide dismutase were as effective as the native enzymes in their animal model of inflammation, suggesting that non-specific protein effects can sometimes occur.

Any antioxidant enzyme introduced to a site of inflammation will be susceptible to attack by the oxidants present. This is especially true for HOCl, which rapidly attacks many proteins [1,2,23]. In the present paper, we have therefore examined the action of HOCl on the activity of catalase, glutathione peroxidase and bovine CuZn superoxide dismutase. HOCl itself was used rather than the myeloperoxidase system, to avoid complications arising from inhibition of myeloperoxidase by the scavenging of $H_2O_2$ (see the Discussion section). The possibility that these enzymes could scavenge HOCl *in vivo* at a biologically significant rate was examined by testing their ability to protect $\alpha_1$-antiproteinase, a major physiological target of HOCl attack [1,2], against inactivation by HOCl.

## MATERIALS AND METHODS

### Reagents

Bovine superoxide dismutase, GSH, NADPH, elastase substrate, t-butyl hydroperoxide, yeast glutathione reductase (type III), $\alpha$-$_1$-antiproteinase and catalase were of the highest quality available from Sigma Chemical Co. NaOCl and pig pancreatic elastase were from BDH Chemicals. Glutatione peroxidase (bovine) was kindly given by Dr. J. M. C. Gutteridge. HOCl was obtained immediately before use by adjusting NaOCl to pH 6.2 [23].

### Enzyme assays

Superoxide dismutase was assayed by the cytochrome *c* method; 1 unit inhibits cytochrome *c* reduction by 50% under the assay conditions given in ref. [24]. Catalase was assayed by the fall in absorbance at 240 nm as $H_2O_2$ is degraded: 1 unit decomposes 1 $\mu$mol/min under the assay conditions described in the Sigma catalogue. Glutathione peroxidase was assayed by the fall in absorbance at 340 nm as NADPH is oxidized in the presence of t-butyl hydroperoxide and excess glutathione reductase [25]. Reaction mixtures contained, in a final volume of 3 ml, 0.25 mM-GSH, 0.15 mM-t-butyl hydroperoxide, 50 $\mu$M-NADPH, 0.1 M-Tris/HCl buffer, pH 7.8, and 0.5 unit of yeast glutathione reductase/ml. One unit of glutathione peroxidase oxidizes 1 $\mu$mol of NADPH/min under these reaction conditions at 25 °C. Assays of elastase and $\alpha_1$-antiproteinase were carried out essentially as described in ref. [23]; full details are given in the legend to Table 1.

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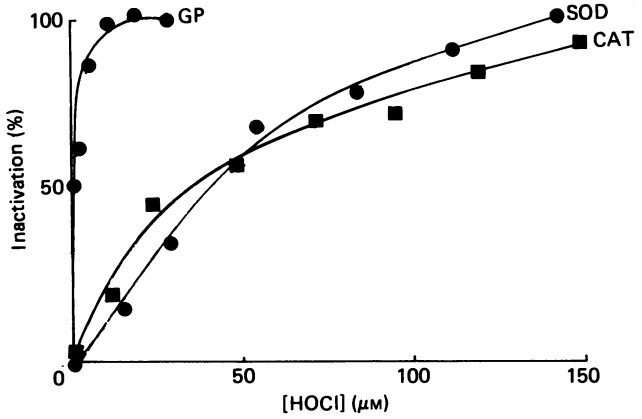


**Fig. 1. Action of HOCl on antioxidant defence enzymes**

Superoxide dismutase (SOD). A solution containing 0.2 μM-superoxide dismutase in 1 ml of phosphate-buffered saline (140 mM-NaCl/2.7 mM-KCl/16 mM-$Na_2HPO_4$/2.9 mM-$KH_2PO_4$, pH 7.4) was incubated at 37 °C for 30 min with the final concentration of HOCl stated. Comparable results were obtained with other superoxide dismutase concentrations (tested up to 0.83 μM). After incubation, a sample of the solution was tested for superoxide dismutase activity in the cytochrome *c* assay [24]. Care was taken to select sample volumes to give inhibitions lying on the approximately linear part (< 60% inhibition) of a plot of percentage inhibition of cytochrome *c* reduction against superoxide dismutase activity. Catalase (CAT). Bovine liver catalase (thymol-free; Sigma) was incubated at 37 °C for 30 min as described for superoxide dismutase. The concentration of catalase was approx. 0.83 μM. After incubation, residual catalase activity was assayed by the fall in absorbance at 240 nm as $H_2O_2$ is destroyed, by using the reaction conditions described in the Sigma catalogue. Glutathione peroxidase (GP). Bovine enzyme (approx. 0.8 μM) was incubated at 37 °C for 30 min with HOCl, and then assayed with t-butyl hydroperoxide as substrate. For all experiments, the maximum amount of HOCl that could have been carried over into the final assay mixtures was found to have no effect on the assay itself.

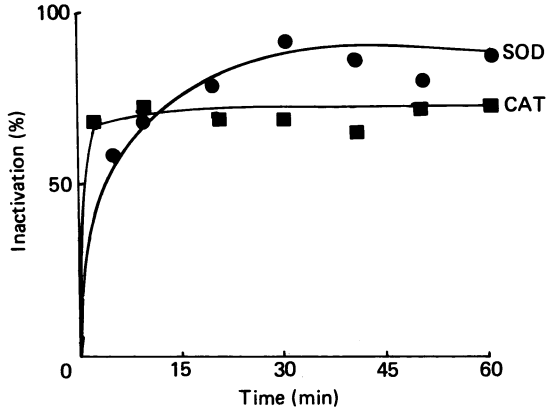


**Fig. 2. Action of HOCl on superoxide dismutase and catalase: effect of time of incubation**

Experiments were carried out as described in the legend to Table 1, except that [HOCl] was initially 70 μM. Samples of the incubation mixture were withdrawn for assay at the times stated. Data for glutathione peroxidase are not shown because inactivation was complete before the first sample could be assayed (2–3 min).

**Table 1. Inhibition of elastase by $\alpha_1$-antiproteinase: prevention by HOCl and protection by antioxidant defence enzymes**

A 0.3 ml portion of phosphate-buffered saline (for composition see the legend to Fig. 1) containing 0.67 mg of $\alpha_1$-antiproteinase/ml, antioxidant defence enzyme at the final concentration stated and 60 μM-HOCl was incubated at 37 °C for 30 min (reaction system A). Then 2.7 ml of phosphate-buffered saline was added, plus 50 μl of stock elastase (a 1:25 dilution of BDH pig pancreatic elastase). After 30 min at 25 °C, elastase activity remaining was measured as described in ref. [23], and expressed as $\Delta A_{410}$/s. All quoted concentrations are final concentrations in the appropriate reaction mixture. Control experiments showed that none of the agents tested (superoxide dismutase, catalase or catalase plus azide) interfered with the assay system by directly inhibiting elastase, or by preventing $\alpha_1$-antiproteinase from inhibiting elastase.

| Addition to reaction system A | $10^{-3}$ × Elastase activity ($\Delta A_{410}$/s) |
|---|---|
| None (HOCl omitted) | 1.3 |
| None | 8.2 |
| Superoxide dismutase (0.63 mg/ml) | 8.2 |
| Superoxide dismutase (2.0 mg/ml) | 8.1 |
| Catalase (1.0 mg/ml) | 5.4 |
| Catalase (2.0 mg/ml) | 2.0 |
| Catalase (1.0 mg/ml) + 1 mM-azide | 4.8 |
| Catalase (2.0 mg/ml) + 1 mM-azide | 2.0 |

## RESULTS

### Glutathione peroxidase

Treatment of bovine glutathione peroxidase with HOCl at 37 °C inactivated the enzyme. Loss of activity was complete within 2–3 min. Concentrations of HOCl as low as 14 μM produced complete loss of activity (Fig. 1).

### Superoxide dismutase

Incubation of bovine CuZn superoxide dismutase with HOCl at 37 °C led to a loss of activity, but much higher concentrations of HOCl were required than in the case of glutathione peroxidase (Fig. 1). Inactivation was also slower, needing 20–30 min for completion (Fig. 2).

If $\alpha_1$-antiproteinase, a major target of HOCl attack *in vivo* [1,2], is incubated with elastase, the elastase is inhibited (Table 1, first line). However, if $\alpha_1$-antiproteinase is first preincubated with HOCl (reaction system A, Table 1), the protein is inactivated and cannot inhibit elastase (Table 1, line 2). Adding superoxide dismutase to the preincubation mixture did not protect the $\alpha_1$-antiproteinase against inactivation by HOCl, even when 2 mg of superoxide dismutase/ml was present together with only 0.67 mg of $\alpha_1$-antiproteinase/ml. It was not possible to test glutathione peroxidase in this system because all the enzyme preparations available to us contain traces of thiols, which can themselves scavenge HOCl [26]. Attempts to remove these thiols diminished the enzyme activity.

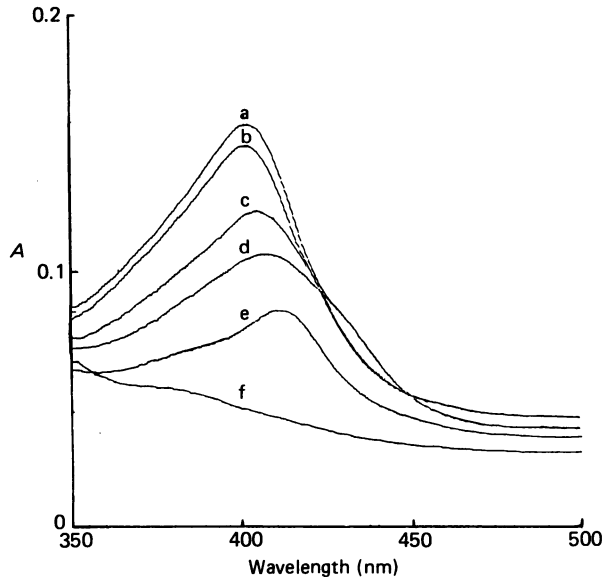


**Fig. 3. Action of HOCl on catalase**

A solution of 75 $\mu$M-catalase in phosphate-buffered saline was incubated with HOCl at the final concentration stated for 15 min at 37 °C. The absorption spectra are shown: a, no HOCl; b, 11.3 $\mu$M; c, 22.5 $\mu$M; d, 90 $\mu$M; e, 180 $\mu$M; f, 360 $\mu$M.

### Catalase

Catalase was also inactivated on incubation with HOCl at 37 °C. The concentration-dependence of the inactivation was comparable with that seen with superoxide dismutase (Fig. 1), but the rate of inactivation was greater, it being complete in 5 min (Fig. 2). Incubation of catalase with HOCl caused striking changes in the spectrum of the protein, suggestive of haem degradation (Fig. 3).

High concentrations of catalase (1–2 mg/ml) were able to protect partially $\alpha_1$-antiproteinase against inactivation by HOCl (Table 1). This was not due to an enzymic action of catalase, since complete inhibition of the catalase by including 1 mM-azide in the reaction mixture did not prevent it from protecting $\alpha_1$-antiproteinase. Control experiments showed that azide itself did not interfere with the assay system, i.e. it did not inhibit elastase or prevent $\alpha_1$-antiproteinase for inhibiting elastase.

## DISCUSSION

Activated neutrophils release HOCl by the action of the enzyme myeloperoxidase. A total of $5 \times 10^6$ activated human neutrophils in 1 ml were reported to produce $88.3 \pm 23.6$ nmol of HOCl in 2 h at 22 °C, which is approximately an 88 $\mu$M concentration [27]. At sites of inflammation *in vivo*, concentrations will be less than this because of scavenging by HOCl by various biomolecules [23]. If an $H_2O_2$-scavenging enzyme (catalase, or glutathione peroxidase plus GSH) is already present when the cells are activated, then HOCl generation will be prevented by depriving myeloperoxidase of $H_2O_2$ [28]. However, if catalase, glutathione peroxidase or superoxide dismutase is injected into a site of inflammation, some HOCl should already be present and might attack these molecules.

Inactivation of glutathione peroxidase by HOCl is almost instantaneous and is achieved at very low HOCl concentrations (Fig. 1). Since this enzyme is also inhibited by $O_2^{\cdot -}$ radicals [29], it seems that it would not survive long at sites of inflammation. The seleno-organic compound Ebselen (PZ51) has been reported to function as a glutathione peroxidase mimic *in vivo* [21], and it might prove more useful as an anti-inflammatory agent than the glutathione peroxidase enzyme itself. Catalase is also quickly (Fig. 2) attached by HOCl at physiologically feasible concentrations (Fig. 1). The haem is degraded (Fig. 3), which raises the possibility that iron might be lost from the protein. Since iron ions are pro-inflammatory [13,30], this possibility must be taken seriously in proposing the use of catalase as an anti-inflammatory agent. The ability of HOCl to release iron ions from some other iron proteins has been described [31].

Scavenging of HOCl by catalase was sufficiently rapid for high concentrations of this protein (1–2 mg/ml) to be able to protect $\alpha_1$-antiproteinase against inactivation (Table 1). Azide-inhibited catalase was equally protective. Hence the protection is unrelated to removal of $H_2O_2$ and is presumably due to a chemical reaction of HOCl with the protein, including the haem ring (Fig. 3). It is possible that this non-enzymic scavenging of HOCl by catalase could explain why Hirschelmann & Bekemeier [22] found that heat-inactivated catalase was as anti-inflammatory as the native enzyme in the animal model system that they studied.

By contrast, although CuZn superoxide dismutase can be inactivated by HOCl, the reaction is slower (Fig. 2), and superoxide dismutase cannot protect $\alpha_1$-antiproteinase against inactivation (Table 1). Hence the anti-inflammatory effect of inactivated superoxide dismutase reported in ref. [22] cannot be explained by HOCl scavenging. Our results suggest that superoxide dismutase is less likely than glutathione peroxidase or catalase to be destroyed by HOCl at sites of inflammation, and would thus seem to be more promising as an anti-inflammatory agent, a conclusion that is consistent with published experimental data [16,17,32,33]. These arguments support the proposed use of superoxide dismutase to minimize reperfusion injury [34], in which neutrophil-derived oxidants such as HOCl contribute to tissue damage [34,35].

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