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ACTION OF URIC ACID, ALLOPURINOL AND OXYPURINOL ON THE MYELOPEROXIDASE-DERIVED OXIDANT HYPOCHLOROUS ACID

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Both oxypurinol and uric acid react with the myeloperoxidase-derived oxidant hypochlorous acid at physiological pH, and they can protect the elastase-inhibitory capacity of human α_1-antiprotease against inactivation by hypochlorous acid. Allopurinol does not protect α_1-antiprotease, possibly because the redox potential of allopurinol at physiological pH is too positive to permit oxidation by hypochlorous acid.

KEY WORDS: Uric acid, allopurinol, oxypurinol, hypochlorous acid, reperfusion injury, xanthine oxidase.

INTRODUCTION

Oxygen radicals play some part in mediating reperfusion damage after hypoxia in animal tissues[1,2] and the enzyme superoxide dismutase has significant protective effects in intestinal, renal and skin-flap animal model systems.[2,3] The damage produced by O_2^- radical may be largely due to O_2^--dependent formation of the highly-reactive hydroxyl radical ($\cdot OH$) in the presence of certain transition metal complexes.[3,4] An important source of O_2^- in ischaemia/reperfusion injury is probably the enzyme xanthine oxidase,[2] produced from xanthine dehydrogenase during the hypoxic phase. It is also possible that oxidants generated by activated phagocytes invading a reperfused tissue are important mediators of damage.[5,6]

Often, the only evidence presented for the importance of xanthine oxidase as a radical generator in reperfused tissues is the partial protection observed with allopurinol, an inhibitor of this enzyme.[7,8] Allopurinol is often given to animals in large intravenous bolus dose on occlusion of the blood supply, and frequently the animals are also pretreated with it. For example, 50 mg (0.37 mmoles) per kg of intravenous allopurinol decreased reperfusion damage in cat intestine to about the same extent as 20 mg (0.26 mmoles) per kg of intravenous dimethylsulphoxide, used as a scavenger of $\cdot OH$ radicals.[9] Allopurinol is oxidized by xanthine oxidase to give oxypurinol,[7,8] which is a major metabolite of allopurinol.[8,10] Recent work has questioned the specificity of allopurinol as an inhibitor of xanthine oxidase, since both allopurinol and oxypurinol have been shown to be powerful scavengers of $\cdot OH$ radical.[4,11] In the present paper we show that oxypurinol is able to scavenge hypochlorous acid, an oxidant generated by myeloperoxidase released from activated phagocytic cells.[12,13]

MATERIALS AND METHODS

All reagents were obtained from Sigma Chemical Co. unless otherwise stated. HOCl was obtained immediately before use as described in 14. Purified α_1-antiprotease was dissolved in phosphate buffered saline[14] (PBS) at pH 7.4 to a concentration of 2.5 mg/ml. The required volume of HOCl to give a final concentration of 77 μM was mixed with scavenger (if any) dissolved in PBS, and after 5 minutes 90 μl of α_1-antiprotease solution was added giving a final volume of 300 μl. After standing for 5 min at room temperature, 10 μl of porcine pancreatic elastase (BDH Chemicals Ltd) was added. After a further 30 min, 100 μl of 25 mM elastase substrate (N-succinyltrialanyl-p-nitroanilide) was added and sufficient PBS to raise the volume to 3 ml. Elastase activity was measured as the initial linear rate of increase in absorbance at 410 nm.

The HPLC and associated uv and electrochemical detectors were as described in 15 and 16. Solutions of allopurinol and oxypurinol were made up as described in 11.

RESULTS

Protection of α_1-antiprotease against inactivation by HOCl

Activated phagocytic cells release the enzyme myeloperoxidase,[17] which uses H_2O_2 to perform a two-electron oxidation of Cl^- ions into a powerful oxidant that has been identified as hypochlorous acid,[12,13] HOCl. HOCl attacks a wide range of biomolecules,[12–14] but its most important biological target is probably α_1-antiprotease. α_1-Antiprotease is responsible for most of the elastase-inhibitory capacity of human extracellular fluids, and it protects tissue elastin from hydrolysis by elastase released from phagocytic cells.[18] Purified α_1-antiprotease is very quickly inactivated by HOCl produced by myeloperoxidase,[18–20] whereas elastase is not. Hence HOCl generation can lead to elastase-dependent tissue damage, which might contribute to reperfusion injury[17] since neutrophils accumulate in post-ischaemic tissues.[5,6] Uric acid, a biological antioxidant,[21] has already been shown to scavenge HOCl.[13]

Table I (column A) shows the effect of uric acid, allopurinol and oxypurinol on the activity of a pancreatic elastase preparation. None of these compounds had any significant inhibitory effect. The ability of α_1-antiprotease to inhibit elastase (column B) was similarly unaffected by any of these reagents. If the α_1-antiprotease was preincubated with HOCl, its elastase-inhibitory capacity was lost (column C, first line). Addition of uric acid to the HOCl before adding the α_1-antiprotease prevented this inactivation, so that the α_1-antiprotease still inhibited elastase. Allopurinol, tested at concentrations up to 500 μM, had no protective effect. However, oxypurinol offered significant protection to the antiprotease (Table 1, columns C and D). Allantoin, an oxidation product of uric acid,[21] did not protect.

HPLC analysis of the reaction of oxypurinol with HOCl

Allopurinol (100 μM) was incubated with varying concentrations of HOCl for 5 min at 37°C and then subjected to HPLC. No significant drop in its concentration, as measured by absorbance at 235 nm, was observed (Fig. 1A), nor did any other peaks appear on the chromatogram. However, oxypurinol was readily oxidised under the same conditions (Fig. 1A). Fig. 2 shows that five products could be detected by

TABLE I
Effect of reagents on elastase and its inhibition by α_1-antiprotease

Addition to reaction mixture	Concentration (μM)	$A_{410}\,sec^{-1}$			
		COLUMN A Elastase only	COLUMN B Elastase + α_1-antiprotease	COLUMN C Elastase + α_1-antiprotease + HOCl	COLUMN D % protection of α_1-antiprotease against inactivation by HOCl
None	–	0.0116	0.000	0.0125	0
Uric acid	100	0.0104	0.000	0.000	100
	200	0.0108	0.000	0.000	100
Allopurinol	100	0.0139	0.000	0.0125	0
	200	0.0122	0.000	0.0128	0
	500	0.0114	0.000	0.0118	0
Oxypurinol	50	0.0122	0.000	0.006	52
	100	0.0115	0.000	0.004	68
	200	0.0132	0.000	0.001	92
	500	0.0116	0.000	< 0.001	> 99
Allantoin	100	0.0109	0.000	0.0139	0
	200	0.0135	0.000	0.0119	0

Concentrations stated are the final concentrations in the reaction mixture (column A) or in the 300 μl preincubation mixture (columns B and C).

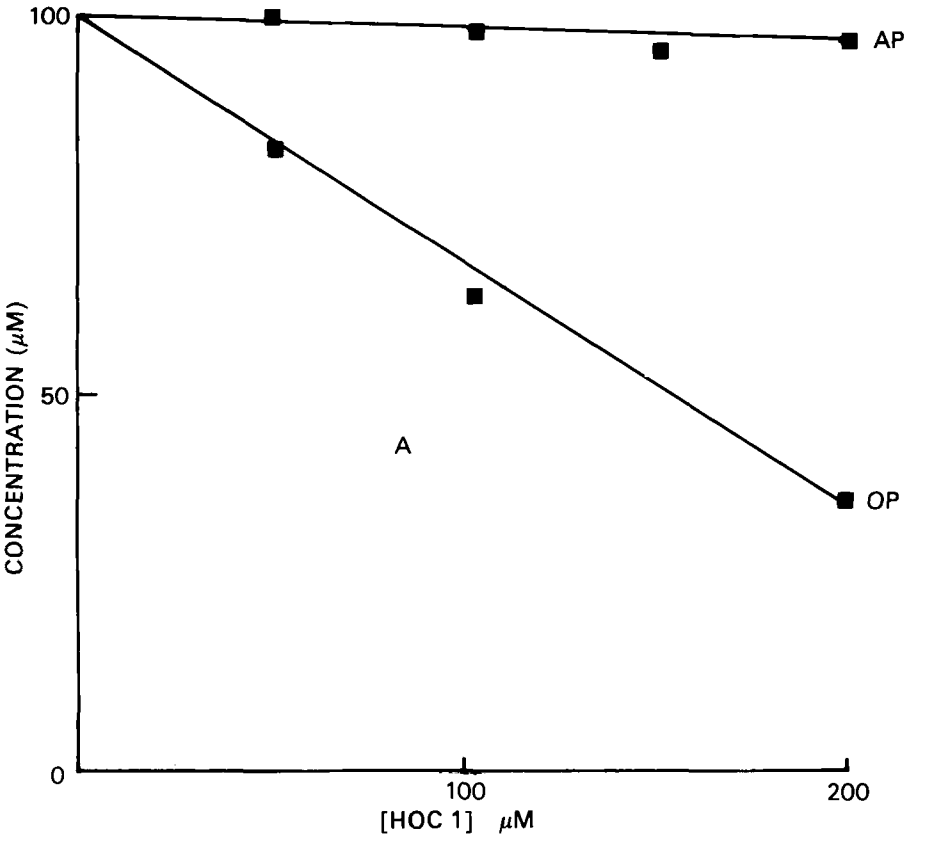

FIGURE 1 *Oxidation of oxypurinol by HOCl.* HPLC was carried out as described in the legend to Table 2 and compounds detected by A_{235}. *Part A* 100 μM allopurinol (AP) or oxypurinol (OP) was incubated for 5 min at 37°C with increasing concentrations of HOCl. *Part B* The peak height of compounds 4 and 5 (see Fig. 2) is plotted as a function of [HOCl] in the incubation mixture. All concentrations stated are final concentrations in the reaction mixture.

absorbance at 235 nm after oxidation of oxypurinol by HOCl; retention times are given in Table II. Three of the products (numbered 1–3 in Fig. 2) ran with the solvent front and could not be accurately quantitated, but two others (numbered 4 and 5) were clearly separated. The amounts of the latter two products in the reaction mixture increased with HOCl concentration (Fig. 1B).

Fig. 3 shows the uv absorbance spectrum of products 4 and 5. The shape of the spectrum suggests that 4 and 5 are N-monochloroamine derivatives of oxypurinol,[25,26] although we found that they were unable to oxidise excess (7.7 mM) I^- ions to I_2 at a measurable rate when incubated at 45°C for 40 min.

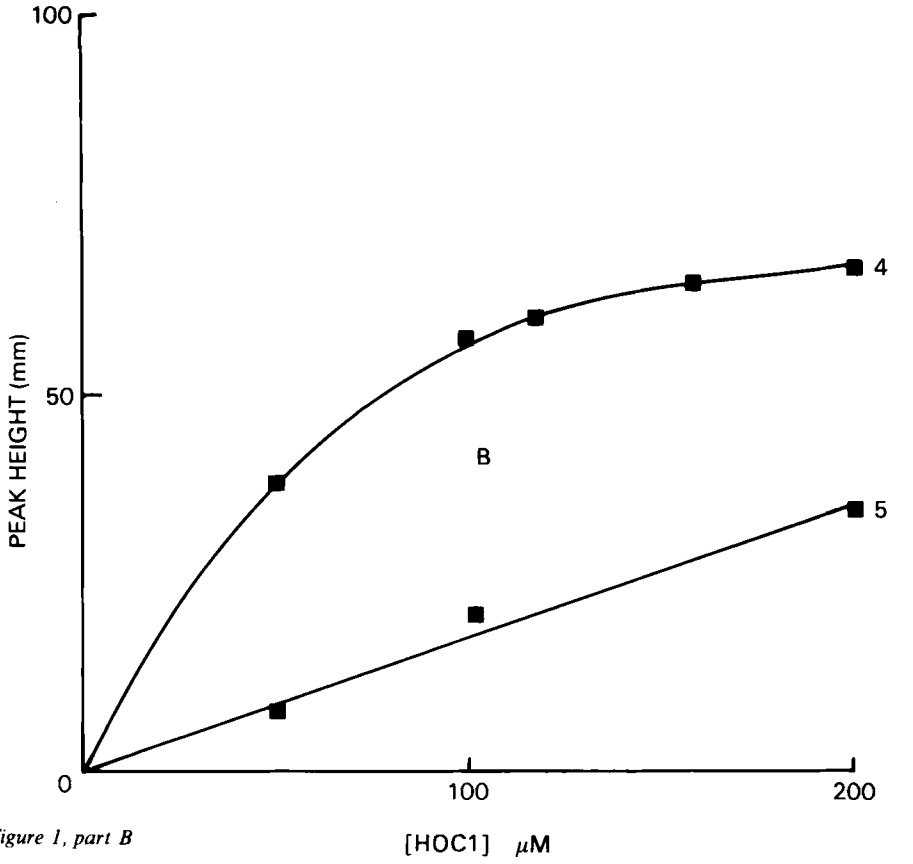

Figure 1, part B

TABLE 2
HPLC separation of allopurinol, oxypurinol and its oxidation products

Peak studied	Retention time	
	In minutes	Relative to Oxypurinol
Allopurinol	12.34	1.16
Oxypurinol	10.68	1.00
Peak 1	2.56	0.24
Peak 2	2.68	0.25
Peak 3	3.06	0.29
Peak 4	5.74	0.54
Peak 5	33.64	3.15

HPLC was carried out on a pre-packed 4.6 mm × 25 cm Anachem S5 ODS-2 column with an Anachem S5 ODS-2 guard column. The mobile phase was 98% (v/v) 30 mM sodium citrate/27.7 mM sodium acetate buffer pH 4.75 plus 2% (v/v) methanol at a flow rate of 1.40 ml min^{-1}. Compounds were detected by their absorbance at 235 nm.

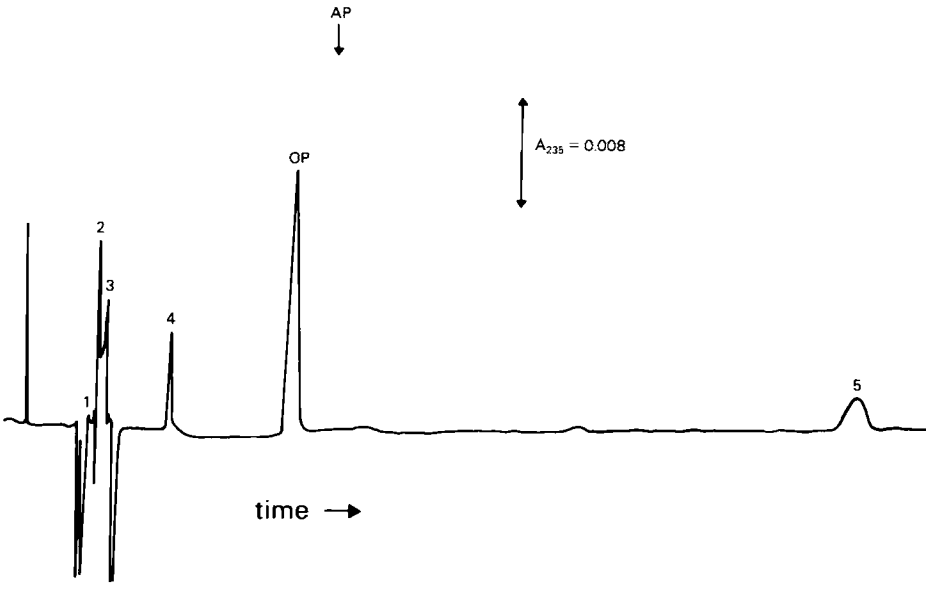

FIGURE 2 HPLC separation of oxypurinol and its oxidation products. HPLC was carried out as described in the legend to Table 2 and compounds detected by absorbance at 235 nm. A typical chromatogram is shown. OP – peak of unoxidized oxypurinol; 1–5 are oxidation products. The arrow marked AP indicates the point where allopurinol would run under the same conditions.

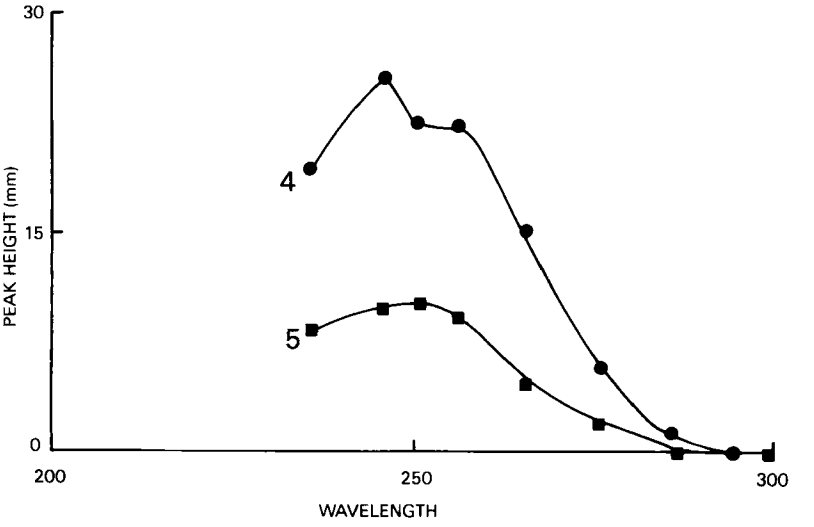

FIGURE 3 UV-absorbance spectrum of two products of oxypurinol oxidation by HOCl. The peak height of compounds 4 and 5 (Fig. 2) is plotted against the wavelength setting on the uv detector.

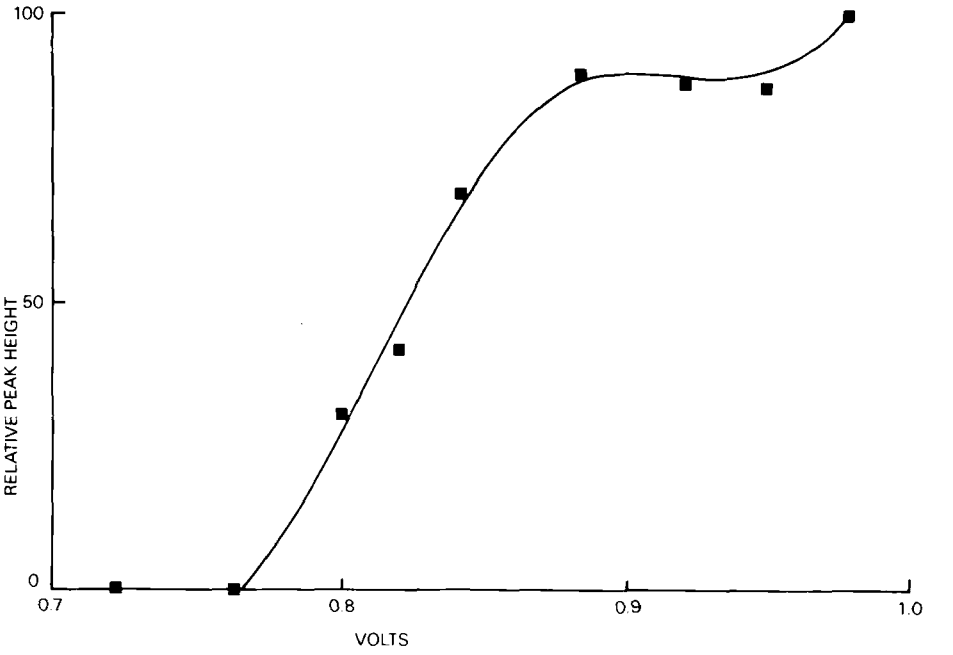

FIGURE 4 Hydrodynamic voltammogram of oxypurinol. HPLC analysis was carried out using an EDT LCA15 electrochemical detector equipped with a glassy carbon working electrode. An eluent of 98% (v/v) 30 mM sodium citrate/27.7 mM sodium acetate buffer pH 4.75, 2% (v/v) methanol was employed. The peak height of oxypurinol (relative to that at 0.98 V, taken as 100) is plotted as a function of oxidation potential (E^{01}). There is an indication of a second oxidation stage at 1.0 V. Allopurinol could not be detected even at a potential of + 1.1 V (using 22% (v/v) methanol to facilitate the use of oxidation potentials > 1.0 V).

Oxypurinol could also be measured using the electrochemical detector, as shown in Fig. 4. However, allopurinol could not be detected electrochemically at oxidation potentials up to 1.1 V, the maximum that could be achieved with the eluent used.

DISCUSSION

Uric acid at physiological concentrations and pH 7.4 is able to scavenge HOCl sufficiently rapidly to protect α_1-antiprotease against inactivation by this molecule. This is another example of uric acid's antioxidant properties.[21] Allopurinol at the concentrations likely to be achieved *in vivo* (up to 500 μM) had no effect on HOCl, which explains why Jones *et al.*[22] found that 1 mM allopurinol did not inhibit the chlorination of taurine by activated neutrophils. The redox potential (E^{01}) for the $HOCl/Cl^-$ half-cell at pH 7 has been quoted as 1.07 V.[23] The fact that an electrochemical detector setting of + 1.10 V was insufficiently oxidizing to detect allopurinol in our HPLC system (Fig. 4) suggests that oxidation of allopurinol by HOCl may not be thermodynamically feasible. It certainly does not occur at significant rates (Fig. 1).

However, our data show that it is *not* possible to dismiss HOCl scavenging as a mechanism of action of allopurinol *in vivo*, in that even low concentrations of

oxypurinol were able to react with HOCl and protect α_1-antiprotease against inactivation by this molecule. The HPLC experiments showed formation of multiple products from the oxypurinol (Fig. 2) and the electrochemical studies confirm that this oxidation is thermodynamically feasible.

The results in the present paper, combined with our previous demonstration[4,11] that both allopurinol and oxypurinol scavenge hydroxyl radicals, means that allopurinol can no longer be regarded for *in vivo* use as a "specific" inhibitor of xanthine oxidase. This in no way negates its potential therapeutic use in minimizing cardiac reperfusion damage[2] or the effects of haemorrhagic shock.[24] The therapeutic action of oxypurinol in these systems might be worth investigation.

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