Volume 213, number 1, 23–28 FEB 04481 March 1987

Allopurinol and oxypurinol are hydroxyl radical scavengers

P. Christopher Moorhouse, Martin Grootveld, Barry Halliwell, J. Gregory Quinlan* and John M.C. Gutteridge*

Department of Biochemistry, King's College, Strand Campus, London WC2R 2LS and **National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, England*

Received 4 December 1986

Allopurinol is a scavenger of the highly reactive hydroxyl radical (k_2 approx. 10^9 $M^{-1}\cdot s^{-1}$). One product of attack of hydroxyl radical upon allopurinol is oxypurinol, which is a major metabolite of allopurinol. Oxypurinol is a better hydroxyl radical scavenger than is allopurinol (k_2 approx. 4×10^9 $M^{-1}\cdot s^{-1}$) and it also reacts with the myeloperoxidase-derived oxidant hypochlorous acid. Hence the protective actions of allopurinol against reperfusion damage after hypoxia need not be entirely due to xanthine oxidase inhibition.

Ischemia; Reperfusion damage; Xanthine oxidase; Allopurinol; Oxypurinol; hydroxyl radical scavenger

1. INTRODUCTION

Oxygen radicals play some part in mediating reperfusion damage after ischaemia in animal tissues [1] and the enzyme superoxide dismutase (SOD) has significant protective effects in intestinal, renal, cardiac and skin-flap animal model systems [2,3]. Superoxide dismutase is a specific scavenger of superoxide radical, O_2^-. Superoxide may have some direct cytotoxic effect [4], but it also accelerates the formation of hydroxyl radical from hydrogen peroxide in the presence of suitable transition metal catalysts [5,6]. Hydroxyl radical is much more reactive than O_2^-; it can attack almost all cell constituents and it initiates the process of lipid peroxidation [6]. Indeed, scavengers of hydroxyl radical (e.g. mannitol, dimethyl sulphoxide) and inhibitors of lipid peroxidation (e.g. α-tocopherol) often show partial protective effects against reperfusion injury in animal model or isolated organ systems (e.g. [7–11]).

An important source of O_2^- in ischaemic/reperfusion injury in intestine is probably the enzyme xanthine oxidase, produced from xanthine dehydrogenase during the ischaemic phase [2]. Xanthine oxidase has been detected in rat [10] and dog [11] heart, but its activity in human heart is unclear [12]. It is possible that O_2^- radicals generated by activated phagocytes invading a reperfused myocardial infarct are also important in vivo [13,14].

Often (e.g. [3,11,15,16]) the only evidence presented for the importance of xanthine oxidase as a radical generator in ischaemic/reperfused tissue is the partial protection observed with allopurinol, an inhibitor of xanthine oxidase [17,18]. Allopurinol is usually given to animals in large intravenous bolus dose (e.g. 30–50 mg/kg) on occlusion of the blood supply, and often additionally the animals are pre-treated with it. For example, 50 mg (0.37 mmol) per kg intravenous allopurinol decreased reperfusion damage in cat intestine to about the same extent as 20 mg (0.26 mmol) per kg intravenous dimethyl sulphoxide, used as a scavenger of hydroxyl radicals [19]. Allopurinol has also been included in the reperfusion media of isolated organs at millimolar concentrations [16].

Allopurinol has a structure related to purines, and it is well known that aromatic compounds are

Correspondence address: B. Halliwell, Department of Biochemistry, King's College, Strand, London WC2R 2LS, England

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

powerful hydroxyl radical scavengers [20]. Indeed, serum uric acid has been proposed to be an important antioxidant in humans [21]. This led us to examine the radical-scavenging activity of allopurinol.

When animals are pre-treated with allopurinol, they will form oxypurinol (4,6-dihydroxypyrazolo-[3,4-*d*]pyrimidine), which is a major metabolite of allopurinol [18,23]. It was thus of interest to compare the radical-scavenging activity of allopurinol with that of oxypurinol.

2. MATERIALS AND METHODS

2.1. *Reagents*

Deoxyribose, $FeCl_3$, allopurinol and oxypurinol were from Sigma. Phenol, catechol, resorcinol and hydroquinone were from Aldrich. HPLC solvents and all other reagents were of the highest quality available from BDH Chemicals.

2.2. *Assay of hydroxyl radical production*

Aromatic hydroxylation with phenol as substrate was carried out as in [25]. The deoxyribose assay [24] was carried out by incubating, in a final volume of 1.0 ml, the following reagents at the final concentrations stated: deoxyribose (variable), KH_2PO_4-KOH buffer, pH 7.4 (20 mM), $FeCl_3$ (100 μM), EDTA (104 μM), H_2O_2 (1 mM), ascorbate (100 μM). After incubation with gentle shaking at 37°C for 1 h, colour was developed as in [24]. Preliminary HPLC experiments (for details of methodology see [32]) showed that exposure of allopurinol and oxypurinol to strongly alkaline solutions could cause their degradation, so solutions were made up at pH $\leqslant$ 10 and their exact concentrations determined using a molar absorption coefficient of 7400 $M^{-1} \cdot cm^{-1}$ for allopurinol (253 nm) and 9200 $M^{-1} \cdot cm^{-1}$ (242 nm at pH 10.5) for oxypurinol [26].

3. RESULTS

3.1. *Deoxyribose assay*

Deoxyribose is attacked by hydroxyl radicals ($^{\cdot}OH$) to form a product that reacts, on heating with thiobarbituric acid (TBA), to form a pink chromogen (λ_{max} 532 nm) [24]. Hydroxyl radicals were generated by a mixture of Fe^{3+}, ascorbic acid and H_2O_2 in the presence of a slight excess of EDTA over the iron salt [27], according to the reactions [24,28,29],

$$Fe^{3+}\text{-EDTA} + \text{ascorbate} \longrightarrow Fe^{2+}\text{-EDTA} + \text{oxidised ascorbate} \quad (1)$$

$$Fe^{2+}\text{-EDTA} + H_2O_2 \longrightarrow Fe^{3+}\text{-EDTA} + {}^{\cdot}OH + OH^- \quad (2)$$

$$^{\cdot}OH + \text{deoxyribose} \longrightarrow \text{product} \xrightarrow[\text{TBA}]{\text{heat with}} \text{chromogen} \quad (3)$$

Any $^{\cdot}OH$ radicals that escape scavenging by the EDTA, which is present in slight excess over iron ions, will be equally accessible to deoxyribose and

Table 1

Inhibition of deoxyribose degradation by allopurinol, oxypurinol, dimethyl sulphoxide and mannitol

Scavenger added	Concentration (mM)	% inhibition of deoxyribose degradation	
		At 0.336 mM deoxyribose	At 2.68 mM deoxyribose
None	–	0	0
Allopurinol	0.5	57	17
	1.0	62	22
	1.5	73	32
Dimethyl sulphoxide	0.5	86	71
	1.0	93	78
	1.5	94	80
Mannitol	0.5	46	7
	1.0	58	18
	1.5	66	26
Oxypurinol	0.5	70	25
	1.0	76	55
	1.5	83	67

A typical experiment is shown. The extents of deoxyribose degradation corresponding to 0% inhibition (no scavenger added) were, as A_{532} values, 0.887 (0.336 mM deoxyribose) and 2.209 (2.68 mM deoxyribose). All concentrations stated were final concentrations in reaction mixtures. In a series of experiments, the inhibition by allopurinol was generally similar to that produced by mannitol but the inhibition by oxypurinol was greater

to any other added molecule that reacts with 'OH [28]. Table 1 shows that mannitol and dimethyl sulphoxide, two established scavengers of 'OH, inhibited deoxyribose degradation, presumably by competing with it for the 'OH generated in the reaction mixture. Indeed, raising the deoxyribose concentration lessened the inhibition by fixed concentrations of mannitol or dimethyl sulphoxide, as expected (table 1). Both allopurinol and oxypurinol also inhibited the deoxyribose degradation (table 1). In a range of experiments, the inhibitory effect of allopurinol was comparable to that of mannitol, but that of oxypurinol was greater. Neither compound was as inhibitory as dimethyl sulphoxide.

Fig.1 shows that the inhibition by allopurinol and oxypurinol followed simple competition kinetics. From the slopes of the lines approximate second-order rate constants for the reactions between these substances and 'OH were obtained. Values for allopurinol, in a series of six experiments, ranged from 0.84×10^9 to 2.86×10^9, mean value 1.81×10^9 $M^{-1} \cdot s^{-1}$. Values for ox-

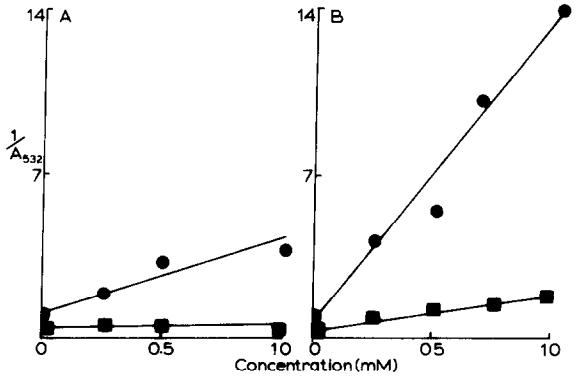

Fig.1. Inhibition of deoxyribose degradation by allopurinol and oxypurinol. A typical experiment is shown. Both deoxyribose and scavenger concentrations were varied and the extent of deoxyribose degradation determined as A_{532}. Plots of $1/A_{532}$ versus [allopurinol] or [oxypurinol] are linear, confirming a competition between these molecules and deoxyribose for 'OH. The rate constants were calculated as k = slope $\cdot k_{DR}[DR]A^0$ where the rate constant for reaction of deoxyribose with 'OH (k_{DR}) is taken as 1.9×10^9 $M^{-1} \cdot s^{-1}$ (●) 0.336 mM deoxyribose, variable allopurinol (A) or oxypurinol (B) concentrations, (■) 2 mM deoxyribose, variable oxypurinol (B) or allopurinol (A) concentrations.

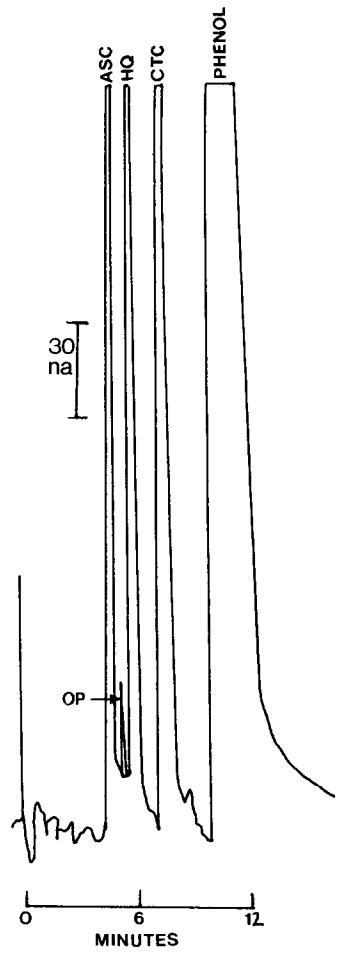

Fig.2. Formation of oxypurinol during attack of hydroxyl radical upon allopurinol. HPLC was carried out on a pre-packed 4.6 mm × 25 cm Anachem S5 ODS-2 column with an Anachem S5 ODS-2 guard column. The mobile phase was 54.5% (v/v) 30 mM sodium citrate/27.7 mM sodium acetate buffer, pH 4.75, and 45.5% methanol at a flow rate of 1.10 ml/min continuously sparged with helium. Detection of hydroquinone (HQ), catechol (CTC) and oxypurinol (OP) by HPLC was by electrochemical detection at 0.78 V. Typical retention times for hydroquinone, oxypurinol, catechol and phenol using the above eluent were 5.42, 5.40, 7.36 and 10.34 min respectively. Oxypurinol was a minor product of the reaction of allopurinol with 'OH, e.g. only 4.5 μM oxypurinol was produced in reaction mixtures containing 2 mM allopurinol and 1 mM phenol. na, nanoamps; ASC, ascorbic acid.

Table 2

Inhibition of phenol hydroxylation by allopurinol, oxypurinol and mannitol

Scavenger added	Concentration (mM)	Amount of hydroxylated product			% inhibition
		Catechol	Hydroquinone	Total	
None	–	41.6	28.8	70.4	0
Mannitol	1	31.4	23.2	54.6	22
	2	23.7	18.7	42.4	40
Allopurinol	1	29.7	21.7	51.4	27
	2	25.2	17.3	42.5	40
Oxypurinol	1	22.4	16.8	39.2	44
	2	13.4	10.2	23.6	66

Reaction mixtures contained, in a final volume of 1.0 ml, the following reagents added in the order stated to give the final concentrations in brackets; KH_2PO_4-KOH buffer, pH 7.4 (20 mM); EDTA (100 μM); phenol (1 mM); allopurinol, oxypurinol or mannitol (see below); H_2O_2 (100 μM); $FeCl_3$ (100 μM); ascorbate made up immediately before use in deaerated water (100 μM). Reaction mixtures were incubated at 37°C for 30 min and a 20 μl sample injected into the HPLC system [20,25]. Results are means of duplicates that differed by 12% or less. % inhibitions are calculated using the figure for total hydroxylated product formed

ypurinol, in four separate experiments, ranged from 3.05×10^9 to 7.14×10^9, mean value 4.27×10^9 $M^{-1} \cdot s^{-1}$. Similar competition plots for mannitol (not shown) gave values in the range 1×10^9–2×10^9 $M^{-1} \cdot s^{-1}$ and for dimethyl sulphoxide values in the range 7×10^9–1×10^{10} $M^{-1} \cdot s^{-1}$. These values compare well with published rate constants, determined by pulse radiolysis, for mannitol (10^9–1.8×10^9 [30]) and dimethyl sulphoxide (7×10^9 [31]).

3.2. *Aromatic hydroxylation assay*

In order to confirm the scavenging ability of allopurinol and oxypurinol, a completely different detector molecule for ·OH was used. Phenol is attacked by ·OH to give a mixture of catechol and hydroquinone, which can easily be separated by HPLC [25]. Table 2 shows a typical experiment. It may be seen that mannitol inhibits phenol hydroxylation, presumably by competing for the available ·OH radicals [25]. The inhibition by allopurinol was comparable to that produced by mannitol, but the inhibition by oxypurinol was greater.

Fig.2 shows that HPLC analysis of a reaction mixture containing allopurinol and an ·OH-generating system (legend to table 2) gave a peak running at the retention time for oxypurinol, Fig.3 confirms the identity of this peak as oxypurinol by comparing its electrochemical behaviour with that of an authentic sample of oxypurinol. Hence attack of ·OH radical upon allopurinol produces, among other products, oxypurinol.

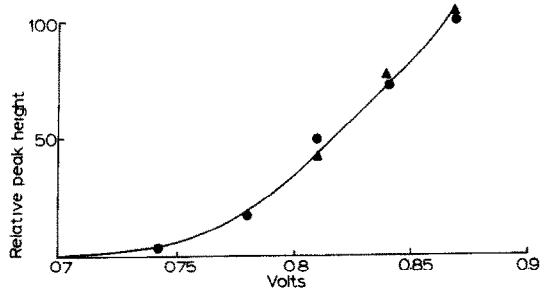

Fig.3. Electrochemical evidence for the identity of the putative oxypurinol peak formed on exposing allopurinol to a system generating hydroxyl radicals. The oxidation potential of the electrochemical detector was varied and the height of the putative oxypurinol peak (fig.2) was measured. The relative peak height is plotted as a function of oxidation potential (●). A similar experiment was conducted with authentic oxypurinol (▲). It may be seen that the two curves match closely.

4. DISCUSSION

Allopurinol is a powerful scavenger of ·OH radicals, with an effectiveness comparable to that of mannitol in both deoxyribose and aromatic hydroxylation assays, suggesting that its second-order rate constant for reaction with ·OH is about $10^9\ M^{-1} \cdot s^{-1}$. Considering the large amounts of allopurinol that have been used with isolated organs and animal model systems of ischaemia/reperfusion damage (see section 1), together with the protective effects of ·OH scavengers (such as mannitol and dimethyl sulphoxide) frequently reported in such systems, it may be seen that protection by allopurinol cannot be taken as evidence that xanthine oxidase is important in mediating the reperfusion damage. It follows that an inhibitory effect of allopurinol cannot be used as the sole evidence that xanthine oxidase is present in a system. Oxypurinol, a major metabolite of allopurinol, is an even better ·OH radical scavenger than is allopurinol, which further complicates in vivo studies in which animals are pre-treated with allopurinol. Although in the feline intestine [2,22] and dog heart [11] model systems, other evidence supports a role for xanthine oxidase, this need not necessarily be true for other systems. It must also be noted that oxypurinol is a scavenger of the myeloperoxidase-derived oxidant hypochlorous acid [32], which may cause some tissue damage during reperfusion in vivo [33].

ACKNOWLEDGEMENTS

We thank the Arthritis and Rheumatism Council for research support. B.H. is a Lister Institute Research Fellow.

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