Free Rad. Res. Comms., Vol. 4, No. 4, pp. 259–263
Reprints available directly from the publisher
Photocopying permitted by license only

Printed in Great Britain

ON THE SPECIFICITY OF ALLOPURINOL AND OXYPURINOL AS INHIBITORS OF XANTHINE OXIDASE. A PULSE RADIOLYSIS DETERMINATION OF RATE CONSTANTS FOR REACTION OF ALLOPURINOL AND OXYPURINOL WITH HYDROXYL RADICALS

B.M. HOEY,[1] J. BUTLER[1] and B. HALLIWELL[2,‡]

[1]*Paterson Institute for Cancer Research, Christie Hospital and Holt Radium Institute, Wilmslow Road, Manchester, M20 9BX, UK*
[2]*Department of Biochemistry, King's College (KQC), Strand Campus, London WC2R 2LS, UK*

(Received May 16, 1987)

Allopurinol has been employed as a "specific" inhibitor of xanthine oxidase in studies of hypoxic/reoxygenation injury. Pulse radiolysis was used to establish rate constants for the reactions of allopurinol and its major metabolite oxypurinol with hydroxyl radicals: values were $(1.45 \pm 0.24) \times 10^9\,M^{-1}s^{-1}$ for allopurinol and $(4.95 \pm 0.84) \times 10^8\,M^{-1}s^{-1}$ for oxypurinol. These rate constants show that, in view of the amounts of allopurinol that have been used in animal studies, hydroxyl radical scavenging by this molecule could contribute to its biological actions, especially if animals are pre-treated with allopurinol, so allowing oxypurinol to form. The ability of allopurinol to protect tissues not containing xanthine oxidase against reoxygenation injury may be related to radical scavenging by allopurinol and oxypurinol.

KEY WORDS: Allopurinol, oxypurinol, hydroxyl radical, xanthine oxidase, reperfusion injury, pulse radiolysis.

INTRODUCTION

Oxygen free-radicals play some part in mediating reperfusion (reoxygenation) damage after hypoxia in several animal tissues, in that there is often significant protection against reperfusion injury by superoxide dismutase and by scavengers of hydroxyl radicals, such as mannitol and dimethylsulphoxide.[1-3] An important source of O_2^- in hypoxia/reoxygenation injury in intestine is probably the enzyme xanthine oxidase, generated from xanthine dehydrogenase during the ischaemic phase.[1] Another possible source of oxygen radicals is the activation of neutrophils invading a tissue upon reperfusion.[4,5]

Allopurinol has been used as an inhibitor of xanthine oxidase, and often (eg.[6-9]) the only evidence presented for the importance of xanthine oxidase as a radical generator in reperfused tissue is a partial inhibition of reoxygenation injury by allupurinol. However, it has recently been reported that allopurinol can protect against reo-

‡To whom correspondence should be addressed.

xygenation injury in tissues that do not contain xanthine oxidase,[16] which means that allopurinol must exert effects in addition to xanthine oxidase inhibition.

Most molecules can act as scavengers of the highly-reactive hydroxyl radical, provided that enough of the molecule is present. Evidence for .OH scavenging by allopurinol was obtained using simple test-tube assays,[15] but the use of such assays to determine rate constants for oxygen radical reactions has been criticized.[17] Allopurinol is usually given to animals in large intravenous bolus dose (30–50 mg/kg) on restriction of the blood supply, and often additionally the animals are pre-treated with it. For example, 20 mg/kg of dimethysulphoxide, used as a .OH scavenger, decreased reoxygenation damage in cat intestine to about the same extent as 50 mg/kg of intravenous allopurinol.[10] This corresponds to 0.26 mmoles/kg of dimethylsulphoxide, but 0.37 mmoles/kg of allopurinol! At these high doses, .OH radical scavenging by allopurinol might contribute to its protective action even if its rate constant for reaction with .OH is no higher than that of most other molecules.

When animals are pre-treated with allopurinol, they will form its major metabolite oxypurinol,[11,12] which may also be able to scavenge .OH radicals.[15] In evaluating the extent to which the high concentrations of allopurinol and oxypurinol that have been used in animal experiments might be able to scavenge .OH *in vivo*, it is helpful to know the rate-constants for their reactions with .OH. Rate constants for reactions of .OH are best determined by pulse radiolysis, and such measurements are the subject of this paper.

MATERIALS AND METHODS

Pulse radiolysis experiments were conducted using the Paterson Laboratories linear accelerator facility. The optical detection system consisted of a tungsten lamp and a Kratos uv/visible monochromator. Microcells of 2.5 cm path length were used throughout. All solutions were made up in double distilled water. Experiments were conducted in 10 mM KH_2PO_4-KOH buffer pH 7.0. Allopurinol and oxypurinol were obtained from Sigma Chemical Co. and solutions made up as described in.[15] All other reagents were of the highest quality available from BDH Chemicals Ltd.

RESULTS

Radiolysis of a dilute phosphate-buffered solution, pH 7.0, saturated with nitrous oxide, produces .OH radicals

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

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

If potassium thiocyanate is added to the solution, the .OH radical reacts with thiocyanate ion (SCN^-) to give the radical anion $.(SCN)_2^-$

$$.OH + SCN^- \longrightarrow HOSCN^- \qquad (3)$$

$$HOSCN^- \longrightarrow .SCN + OH^- \qquad (4)$$

$$.SCN + SCN \longrightarrow {}^{\cdot}(SCN)_2^- \qquad (5)$$

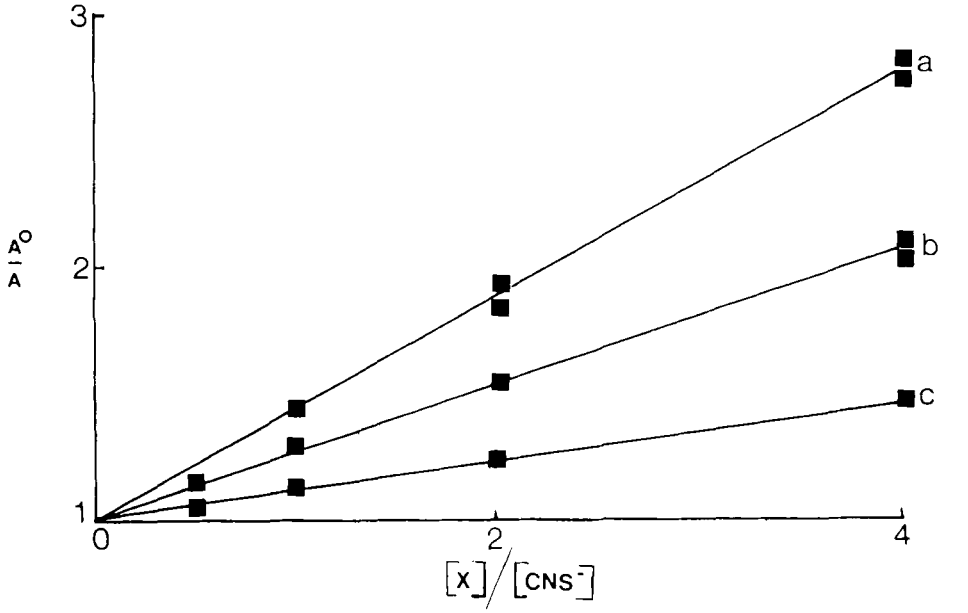

FIGURE 1 Determination of rate constants for the reactions of allopurinol, oxypurinol and mannitol with hydroxyl radical by competition with thiocyanate. The absorbance at 500 nm of an irradiated solution of KCNS (3.65×10^{-4} M) in 10 mM phosphate buffer saturated with N_2O was determined in the presence (A) or in the absence (A°) of allopurinol, oxypurinol or mannitol. Absorbances are related by the equation

$$\frac{A^\circ}{A} = 1 + \frac{k_x}{k_i} \frac{[\text{added scavenger}]}{[\text{thiocyanate}]}$$

Thus plotting A° A against the [scavenger]/[KCNS] concentration ratio allows calculation of k_x, the rate constant for reaction of scavenger with ·OH. k_i is taken as $1.10 \times 10^{10}\,M^{-1}s^{-1}$ (see text). Plot a shows data for oxypurinol, b for mannitol and c for allopurinol.

At pH 7 reactions (4) and (5) are non-rate-determining and so the production of $^{\cdot}(SCN)_2^-$ from KCNS can be considered as a single oxidation step with a second-order rate constant of $1.1 \times 10^{10}\,M^{-1}s^{-1}$.[18] The $^{\cdot}(SCN)_2^-$ radical ion absorbs strongly in the visible region ($\varepsilon = 7.1 \times 10^3\,M^{-1}cm^{-1}$ at 500 nm).

By observing the ability of allopurinol and oxypurinol to compete with SCN^- for .OH, and so decrease the absorbance changes observed, rate constants for their reaction with .OH can be calculated. Figure 1 shows the results of a typical experiment. It may be seen that allopurinol does indeed scavenge .OH radicals. Analysis of four experiments of this type gave k_c as $(1.45 \pm 0.24) \times 10^9\,M^{-1}s^{-1}$ (mean ± SD). Oxypurinol was a better scavenger of .OH (Figure 1), with k_c of $(4.95 \pm 0.84) \times 10^9\,M^{-1}s^{-1}$. Mannitol, a popular .OH scavenger in biochemical experiments,[16,19] was included for comparison; the value of k_c obtained, $(2.70 \pm 0.46) \times 10^9\,M^{-1}s^{-1}$, was comparable to that obtained in previous pulse radiolysis studies on this sugar.[20]

DISCUSSION

The results in the present paper establish rate constants for reactions of allopurinol and oxypurinol with .OH. They show that allopurinol scavenges .OH at rates comparable to those of mannitol, perhaps the most widely-used scavenger in biological systems.[20] Hence, at the high concentrations of allopurinol that have been used in animal experiments (see the Introduction), .OH scavenging could contribute to its biological effects. Oxypurinol is a more effective .OH scavenger than either mannitol or allopurinol, and its formation in allopurinol-treated animals will enhance scavenging activity. Oxypurinol also combines with the myeloperoxidase-derived oxidant hypochlorous acid.[13]

These studies confirm the proposal[15] that radical scavenging by allopurinol and oxypurinol could contribute to their biological effects, and they offer an explanation for the ability of allupurinol to protect against reoxygenation injury in rabbit heart, which does not contain xanthine oxidase.[16] It follows that an inhibitory effect of allopurinol cannot be used as the sole evidence that xanthine oxidase is present in a system. Although in the feline intestine[1,21] and dog heart[7] model systems, other evidence supports a role for xanthine oxidase, this is not true of some cardiac systems, e.g. rabbit hearts.[16]

Our results in no way negate the potential therapeutic use of allopurinol in minimizing reperfusion damage[1] or the effect of haemorrhagic shock,[22] but they do suggest that the therapeutic action of oxypurinol might be worth investigation, since this compound is a powerful radical scavenger as well as a xanthine oxidase inhibitor.

Acknowledgements

We thank the Arthritis and Rheumatism Council, Cancer Research Campaign and the Medical Research Council for support. BH is a Lister Institute Research Fellow.

References

1. McCord, J.M. Oxygen-derived free radicals in postischaemic tissue injury, *New Engl. J. Med.*, **312**, 159–163, (1985).
2. Guarnieri, C., Flamigni, F. and Caldarera, C.M. Role of oxygen in the cellular damage induced by re-oxygenation of hypoxic heart, *J. Mol. Cell. Cardiol.*, **12**, 797–808, (1980).
3. Gauduel, Y. and Duvelleroy, M.A. Role of oxygen radicals in cardiac injury due to reoxygenation, *J. Mol. Cell Cardiol.*, **16**, 459–470, (1984).
4. Mullane, K.M., Read, N., Salmon, J.A. and Moncada, S. Role of leukocytes in acute myocardial infarction in anesthetised dogs: relationship to myocardial salvage by anti-inflammatory drugs, *J. Pharm. Exp. Ther.*, **228**, 510–522, (1984).
5. Romson, J.L., Hook, B.G., Kunkel, S.L., Abrams, G.D., Schork, A. and Lucchesi, B.R. Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog, *Circulation*, **67**, 1016–1023, (1983).
6. Im, M.J, Manson, P.N., Bulkley, G.B. and Hoopes, J.E. Effects of superoxide dismutase and allopurinol on the survival of acute island skin flaps, *Ann. Surg.*, **201**, 357–360, (1985).
7. Chambers, D.E., Parks, D.A., Patterson, G., Roy, R., McCord, J.M., Yoshida, S., Parmley, L.F and Downey, J.M. Xanthine oxidase as a source of free radical damage in myocardial ischaemia, *J. Mol. Cell Cardiol.*, **17**, 145–152, (1985).
8. Itoh, M. and Guth, P.H. Role of oxygen-derived free radicals in hemorrhagic shock-induced gastric lesions in the rat, *Gastroenterol.*, **88**, 1162–1167, (1985).
9. Myers, C.L., Weiss, S.J., Kirsh, M.M. and Shlafer, M. Involvement of hydrogen peroxide and hydroxyl radical in the "oxygen paradox": reduction of creatine kinase release by catalase, allopurinol or deferoxamine, but not by superoxide dismutase, *J. Mol. Cell. Cardiol.*, **17**, 675–684, (1985).

10. Parks, D.A. and Granger, D.N. Ischaemia-induced vascular changes: role of xanthine oxidase and hydroxyl radicals, *Amer. J. Physiol.*, **245**, G285–G289, (1983).
11. Chalmers, R.A., Kromer, H., Scott, J.T. and Watts, R.W.F. A comparative study of the xanthine oxidase inhibitors allopurinol and oxypurinol in Man, *Clin. Sci.*, **35**, 353–362, (1968).
12. Reiter, S., Simmonds, H.A., Webster, D.R. and Watson, A.R. On the metabolism of allopurinol, *Biochem. Pharmacol.*, **32**, 2167–2174, (1983).
13. Grootveld, M., Halliwell, B. and Moorhouse, C.P. Action of uric acid, allopurinol and oxypurinol on the myeloperoxidase-derived oxidant hypochlorous acid, *Free Radical Res. Commun.* **4**, 69–76.
14. Weiss, S.J. Oxygen, ischaemia and inflammation, *Acta Physiol. Scand. suppl.*, **548**, 9–37, (1986).
15. Moorhouse, C.P., Grootveld, M., Halliwell, B., Quinlan, G.J. and Gutteridge, J.M.C. Allopurinol and oxypurinol are hydroxyl radical scavengers, *FEBS Lett.*, **213**, 23–28, (1987).
16. Grum, C.M., Ragsdale, R.A., Ketai, L.H. and Shlafer, M. Absence of xanthine oxidase or xanthine dehydrogenase in the rabbit myocardium. *Biochem. Biophys. Res. Commun.*, **141**, 1104–1108, (1986).
17. Bull, C., Fee, J.A., O'Neill, P. and Fielden, E.M. Iron-EDTA catalysed superoxide dismutation revisited: an explanation of why the dismutase activity of Fe-EDTA cannot be detected in the cytochrome c/xanthine oxidase system, *Arch. Biochem. Biophys.*, **215**, 551–555, (1982).
18. Farhataziz and Ross, A.B. National Standards Reference Data Series, National Bureau of Standards, Vol. 59, (1977).
19. Halliwell, B. Superoxide-dependent formation of hydroxyl radicals in the presence of iron chelates, *FEBS Lett.*, **92**, 321–326, (1978).
20. Goldstein, S. and Czapski, G. Mannitol as an OH· scavenger in aqueous solution and in biological systems, *Int. J. Radiat. Biol.*, **46**, 725–729, (1984).
21. Granger, D.N., McCord, J.M., Parks, D.A. and Hollworth, M.E. Xanthine oxidase inhibitors attenuate ischaemia-induced vascular permeability changes in the cat intestine, *Gastroenterol.*, **90**, 80–84, (1986).
22. Allan, G., Cambridge, D., Lee-Tsang-Tan, L., Van Way, C.W. and Whiting, M.V. The protective action of allopurinol in an experimental model of haemorrhagic shock and reperfusion, *Br. J. Pharmacol.*, **89**, 149–155, (1986).

Accepted by Dr. J.M.C. Gutteridge