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ALLOPURINOL INHIBITS LIPID PEROXIDATION IN WARM ISCHAEMIC AND REPERFUSED RABBIT KIDNEYS

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Rabbit kidneys were subjected to 120 min of warm ischaemia or to 120 min of warm ischaemia followed by 60 min reperfusion with blood *in vivo* before being removed, homogenised and incubated at 37°C for 90 min. Lipid extracts were obtained and monitored for Schiff base (fluorescence emission 400–450 nm, excited at 360 nm), thiobarbituric acid (TBA)-reactive material (emission 553 nm, excited at 515 nm) and diene conjugates (absorbance at 237 nm). Samples removed before incubation were assayed for reduced glutathione (GSH) and oxidised glutathione (GSSG) to provide an index of glutathione redox activity (GSH : GSSG). Allopurinol injected systemically i.v. (a) 15 mins before kidneys were clamped, (b) 15 mins before they were reperfused or (c) as two injections (before clamping *and* before reperfusion) significantly inhibited these biochemical markers of lipid peroxidation. Administration before reperfusion had a markedly more pronounced effect than when allopurinol was given before warm ischaemia only. It is concluded that allopurinol is probably effective because of its ability to inhibit xanthine oxidase and consequently lipid peroxidation during reperfusion rather than by preventing loss of purine nucleotides from hypoxic cells during ischaemia.

KEY WORDS: Allopurinol, lipid peroxidation, ischaemia, rabbit kidneys.

INTRODUCTION

The mechanisms causing ischaemic death in tissues and solid organs are still unidentified and therapeutic attempts at protection have, at best, merely extended the length of ischaemic time beyond which failure is irreversible. Whatever the basic cause, the end-result is that cells undergo hydropic degeneration and swelling, parenchymal oedema builds up, and attempts to re-establish the circulation are prevented by venous outflow block. The observation that reperfusion results in markedly deteriorating morphology has concentrated attention on the role of oxygen-derived free radicals during this period when the cells are re-exposed to molecular oxygen[1] and has lead to a search for different agents which might be beneficial if administered locally into the tissue or given to the animal systemically before reoxygenation.

The potent xanthine oxidase inhibitor allopurinol has been used in a variety of ischaemic models and protection claimed in some but not all cases. For example, whereas 2 hr of warm ischaemic injury was lethal to canine kidneys in 5/5 untreated dogs, 4/4 animals survived contralateral nephrectomy if they were treated with allopurinol on 3 successive days.[2] Similarly, donor treatment with allopurinol togeth-

er with hypoxanthine partially protected canine kidneys exposed to 24 hr of haemorrhagic hypotension and transplanted as allografts.[3] It also appeared to provide considerable protection after myocardial infarction in *in situ* sheep and dog hearts[4] and after haemorrhagic shock.[5]

In contrast, in other studies, neither in dogs[6] nor in rats[7] did allopurinol prove beneficial in renal ischaemia. More recently, this agent was shown to prevent erythrocyte accummulation otherwise observed after ischaemia[8] but had little effect on subsequent renal function.[9]

Until recently, the explanation proferred for any beneficial effect of allopurinol was that it prevented irreversible degradation of purine nucleotides and preserved intracellular purine pools.[10] It could do so either by inhibiting xanthine oxidase which catalyses the oxidation of hypoxanthine and xanthine to uric acid or by its ability to inhibit phosphomonoesterase activity.[11] Since the administration of the purine nucleosides adenosine and inosine has also been claimed useful in protecting kidney[7,12] and lung tissue[13] against normothermic ischaemia, this would be a logical assumption. However, an alternative explanation could be that allopurinol protects tissues from reperfusion injury after normothermic ischaemia. The ability of allopurinol to protect pedicled skin flaps in rabbits[14] and ischaemic skin flaps in rats in which it was also shown to prevent the increase in xanthine oxidase activity in the ischaemic tissue[15,16] provides rather tenuous evidence that it might be important in preventing generation of oxygen-derived free radicals, eg superoxide anions.[16] Similary, in another model, evidence that allopurinol protected ischaemic rat kidneys[17] if given after warm ischaemia but before reperfusion provides further support for the belief that it protects tissues from reperfusion injury.

In the present experiments, an attempt was made to identify more closely the possibility that allopurinol might inhibit free radical production and thence lipid peroxidation after warm ischaemia and reperfusion. A model of renal ischaemia and reperfusion already described[18,19] was used to assess the ability of allopurinol, injected systemically either before vascular occlusion, before reperfusion or in both circumstances, to prevent rises of *in vitro* markers of oxidative stress. The markers used were Schiff bases, thiobarbituric acid (TBA)-reactive material, diene conjugates and a fall in glutathione redox activity (the ratio of reduced (GSH) glutathione to oxidised (GSSG) glutathione − GSH:GSSG) in *in vitro* incubated (37°C) homogenates.

MATERIALS AND METHODS

Materials

All chemicals were Analar grade, except methanol and chloroform which were special spectral grade. Allopurinol was obtained from Sigma Chemical Company, Poole, England and dissolved in an equimolar amount of 1.0 M sodium hydroxide to produce the sodium salt which was then dissolved in 10 ml of isotonic saline solution. The final solution was administered intravenously (i.v.) to those rabbits scheduled for treatment.

Surgical technique

Eleven groups of adult New Zealand white rabbits were each weighed and anaes-

thetised by intramuscular injection of fentanyl-fluanisone (Hypnorm®) at 0.2 ml/kg initially, followed by slow i.v. injection of diazepam (1.0 mg/kg). Oxygen was supplied continuously at 2 l/min via an open face mask.

The kidneys were approached through a mid-line laparotomy and, after careful dissection, the renal artery and vein as well as the ureter were occluded with microsurgical clamps for 120 mins. The abdomen was temporarily closed and core temperature maintained at 37°C. At the end of the WI period, the kidneys were either harvested immediately (Gp 2–5) or the clamps were removed and the kidneys allowed to reperfuse with blood *in vivo* for 60 min before harvesting for biochemical assay (Gp 6–11).

Assays for lipid peroxidative markers

The kidneys were processed immediately after harvesting. Thin slices were weighed and added to phosphate buffered saline (PBS = 40 mM KH_2PO_4/K_2HPO_4 + 140 mM NaCl – pH 7.4) to give an approximate homogenate concentration of 100 mg/ml wet weight. These were then homogenised for 60 sec in a Silverson homogeniser and samples were incubated at 37°C in open vessels with mechanical shaking. One ml aliquots were removed at 0 and 90 min and added immediately to 4 ml 2:1(v/v) chloroform:methanol. Lipid extracts were obtained and monitored for Schiff base formation at a fluorescence maximum between 400 and 450 nm when excited at 360 nm.[20] The formation of TBA-reactive material was measured by removal of 2 ml aliquots at 0 and 90 min and assayed by a modified technique,[21] monitoring for a fluorescence maxima at 553 nm when excited at 515 nm. Diene conjugate formation was measured in 200 μl aliquots of homogenate taken before incubation: absorbance was read at 237 nm as previously described.[22] Further samples were taken before incubation and assayed for glutathione redox activity, using an indirect method in which the ratio of reduced glutathione (GSH) to oxidised glutathione (GSSG) is thought to provide a relative index of redox activity.[23] GSH levels were measured by utilising the reaction between GSH and o-phthalaldehyde to form a fluorescent product which can be detected fluorimetrically at an emission wavelength of 395 nm when excited at 350 nm. GSSG was assayed by first removing all GSH present in the sample by addition of n-ethyl maleimide, then converting the GSSG present to GSH by adding NaOH and assaying for GSH as above. All results were corrected for variability in homogenate protein concentration by a standard method.[24]

Experimental protocol

Group 1. Control kidneys were freshly harvested and not subjected to any warm ischaemic insult.

Group 2. Kidneys were clamped for 120 mins warm ischaemia.

Group 3. Kidneys were clamped for 120 mins warm ischaemia following i.v. administration of 100 mg/kg allopurinol 15 min before clamping.

Group 4. Kidneys were clamped for 120 mins warm ischaemia following i.v. administration of 50 mg/kg allopurinol 15 mins before clamping.

Group 5. Kidneys were clamped for 120 mins warm ischaemia following i.v. administration of 10 mg/kg allopurinol 15 mins before clamping.

Group 6. Kidneys were clamped for 120 mins warm ischaemia, then reperfused with blood *in vivo* for 60 mins.

Group 7. Kidneys were clamped for 120 mins warm ischaemia, then reperfused with blood *in vivo* for 60 mins following i.v. administration of 50 mg/kg allopurinol 15 mins before commencing reperfusion.

Group 8. Kidneys were clamped for 120 mins warm ischaemia following i.v. administration of 50 mg/kg allopurinol then reperfused with blood *in vivo* for 60 mins following i.v. administration of 50 mg/kg allopurinol 15 min before commencing reperfusion.

Group 9. Kidneys were clamped for 120 mins warm ischaemia following i.v. administration of 10 mg/kg allopurinol 15 min before clamping, then reperfused with blood *in vivo* for 60 mins.

Group 10. Kidneys were clamped for 120 mins warm ischaemia then reperfused with blood *in vivo* for 60 mins following i.v. administration of 10 mg/kg allopurinol 15 mins before commencing reperfusion.

Group 11. Kidneys were clamped for 120 mins warm ischaemia following i.v. administration of 10 mg/kg allopurinol then reperfused with blood *in vivo* for 60 mins following i.v. administration of 10 mg/kg allopurinol 15 mins before commencing reperfusion.

(n = 10 in all groups).

RESULTS

When the homogenised renal tissue was incubated *in vitro* at 37°C to assess its susceptibility to lipid peroxidation, the levels of all three markers (Schiff bases, TBA-reactive material and diene conjugates) were significantly elevated in homogenates from ischaemic kidneys (Group 2) as compared with fresh control kidneys (Group 1). This was even more marked in samples from kidneys which had been reperfused for 60 min after 2 hr of warm ischaemia (Table 1). However, in all groups in which allopurinol was administered, either 15 min before reflow or before clamping as well as before reflow (Groups 7–11) Schiff base, TBA-reactive material and diene conjugate levels were significantly reduced and in some groups were measured at or near control levels. In those groups in which allopurinol was administered before clamping only (Groups 3,5 and 9), Schiff base and diene conjugate levels were significantly reduced compared with those in untreated kidneys (Groups 2 or 6) but it was noticeable that the effect was much less pronounced: in these groups too, the TBA-reactive material levels were not significantly reduced and in Group 4 were even elevated, a result which we are unable to explain (Table 1). Overall then, from these data, it appears that allopurinol was most effective if administered 15 min before reflow; administration 15 min before vascular occlusion had only a marginal effect. In general, Schiff base, TBA-reactive material and diene conjugate formation in the homogenates correlated well except for the one TBA-reactive group already emphasised.

The levels of reduced glutathione (GSH) were significantly decreased in samples from warm ischaemic kidneys and this fall was even more marked in kidneys subjected to warm ischaemia and reperfusion (Table 2). Again allopurinol administration either prevented or inhibited this fall significantly and in some groups maintained the levels

TABLE 1

Changes in Schiff base (SB), TBA-reactive material (TBAR) and diene conjugate (DC) formation in rabbit kidneys subjected to 120 min warm ischaemia (WI) with/without 60 min reperfusion (RP) and treatment with allopurinol (ALP) (n = 10 kidneys)

Group	Period of WI:RP (hr)	ALP† (mg/kg) i.v.	SB (mean change in fluorescence intensity/ hr/mg protein ± SD)	TBAR (mean change in fluorescence intensity/ hr/mg protein ± SD)	DC (optical density/mg protein × 18^{-2}
1	0:0	–	1.05 ± 1.04	0.85 ± 0.96	9.06 ± 0.87
2	2:0	–	4.00 ± 0.83	4.32 ± 1.18	9.79 ± 2.01
3	2:0	100 bc	1.87 ± 1.19**	3.32 ± 1.51	7.45 ± 1.12*
4	2:0	50 bc	2.67 ± 1.27*	8.10 ± 3.69	7.09 ± 1.20*
5	2:0	10 bc	2.83 ± 1.45*	2.40 ± 1.99*	6.49 ± 1.55**
6	2:1	–	5.67 ± 0.75	6.28 ± 0.98	10.15 ± 1.11
7	2:1	50 br	1.27 ± 0.90***	2.48 ± 1.82**	6.06 ± 1.22***
8	2:1	50 bcbr	0.40 ± 0.39***	1.04 ± 1.13***	5.43 ± 1.17***
9	2:1	10 bc	4.33 ± 0.89*	4.64 ± 2.19*	6.32 ± 0.95***
10	2:1	10 br	3.53 ± 0.68*	2.78 ± 1.24**	5.39 ± 0.57***
11	2:1	10 bcbr	1.13 ± 0.85***	1.65 ± 1.34***	5.61 ± 1.76***

†ALP administered i.v. 15 min before clamping (bc), before reflow (br) or both (bcbr)
Significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) – differences
Gp. 3, 4 & 5 to Gp. 2 and Gp. 7, 8, 9, 10 & 11 to Gp. 6

TABLE 2
Reduced glutathione (GSH) levels and glutathione redox activity (GSH − GSSG) in homogenates of rabbit kidneys subjected to 120 min warm ischaemia (WI) with/without 60 min reperfusion (RP) and treatment with allopurinol (ALP) (n = 10 kidneys in all groups)

Group	Period of WI:RP	ALP† (mg/kg) i.v.	mg GSH/mg protein ($\times 10^{-5}$)	mg GSH/mg protein / mg GSSG/mg protein
1	0:0	–	3.40 ± 1.14	2.00 ± 1.28
2	2:0	–	2.01 ± 0.22	1.64 ± 0.26
3	2:0	100 bc	4.09 ± 0.73**	2.88 ± 0.65**
4	2:0	50 bc	3.37 ± 0.39**	2.13 ± 0.24*
5	2:0	10 bc	2.99 ± 0.88*	2.36 ± 0.50*
6	2:1	–	1.62 ± 0.41	1.19 ± 0.34
7	2:1	50 br	3.31 ± 1.10**	2.75 ± 0.30**
8	2:1	50 bcbr	3.07 ± 0.58**	2.62 ± 0.19**
9	2:1	10 bc	2.52 ± 0.22**	2.51 ± 0.27**
10	2:1	10 br	2.44 ± 0.24*	2.24 ± 0.50*
11	2:1	10 bcbr	3.63 ± 0.89**	2.81 ± 0.28**

†ALP administered i.v. 15 min before clamping (bc), before reflow (br) or both (bcbr)
Significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) – differences
Gp. 3, 4 & 5 to Gp. 2 and Gp. 7, 8, 9, 10 & 11 to Gp. 6

at or near control kidneys. Glutathione redox activity fell numerically after warm ischaemia alone and significantly after warm ischaemia and reperfusion in inverse proportion to Schiff base, TBA-reactive material and diene conjugate formation (Table 2). However, allopurinol administration prevented this fall in all treated groups, so that the measured levels were at or near those in controls (Group 1).

DISCUSSION

The aim of the present experiments was to extend previous findings in this warm ischaemia and reperfusion model in which *in vitro* markers of lipid peroxidation were altered broadly in line with the insult and in which an iron-chelating agent desferrioxamine was shown to be effective in inhibiting lipid peroxidation if administered i.v. to the animals in different dosage protocols.[19] It was hoped that by evaluating the effect of another putative free radical inhibitor (allopurinol) but one which is thought to act principally because it is a hypoxanthine analogue and potent inhibitor of xanthine oxidase, it might be possible to gain added insight into the mechanisms of lipid peroxidation in ischaemic tissues. In addition, by administering allopurinol either before vascular occlusion, hence limiting any observed effect to warm ischaemia, or just before reperfusion, it was hoped to address the question – in those situations where allopurinol is therapeutically beneficial, is this because it inhibits dephosphorylating enzymes and hence maintains purine pools within ischaemic tissues or is it preventing generation of superoxide anions ($O_2^{\cdot-}$) during reperfusion when the tissues are subjected to oxidative stress?

These data are consistent with our earlier findings that markers of lipid peroxidation measured as rates of change in homogenates during *in vitro* incubation at 37°C increase in line with the duration of warm ischaemia and further still after reperfusion *in vivo*. It must be stressed that changes in Schiff base and TBA-reactive markers are

not evident (or at least not measurable) at the beginning of incubation but rise over a 90 minute period. We are making the assumption that *in vivo* damage is reflected in *in vitro* changes in lipid peroxidation, in other words, the tissues have been rendered susceptible to change by the *in vivo* insult. Administration of the hypoxanthine analogue allopurinol to the animals was most effective if given just before the kidneys were reperfused with blood or both before vascular occlusion and before reperfusion. A single dose administered before clamping was considerably less beneficial. It could be argued that the observed *in vitro* inhibition of lipid peroxidative markers simply reflected carrying over of allopurinol into the homogenates as the kidneys were harvested only 60 mins after systemic administration of the agent. However, in other studies we have shown that very high *in vitro* concentrations of free-radical scavengers are needed to inhibit these markers to a similar degree[25] and it would be impossible for these levels to be obtained in a kidney after *in vivo* dilution and tissue equilibration has taken place. Another possible criticism of these studies is that no attempt has been made to show that xanthine oxidase levels rise in rabbit kidneys after warm ischaemia. However, other studies have demonstrated that renal parenchyma contains xanthine oxidase and that tissue levels of hypoxanthine, a substrate for xanthine oxidase rise rapidly to levels ten to 300 times greater than normal during ischaemia.[26] In hyperaemic island skin flaps it has been shown that xanthine oxidase activity increases after vascular occlusion and reperfusion but is prevented by systemic administration of allopurinol before warm ischaemia.[15] In recent studies, administration of allopurinol before vascular occlusion has had little effect on subsequent function of rabbit kidneys[9] or on induction of oedema in gerbil brains[27] whereas the agent given just before reperfusion was beneficial in rat kidneys.[17] These results, taken in conjunction with the findings of the present study that allopurinol administered just before reflow was markedly more effective at preventing lipid peroxidation after warm ischaemia and reperfusion in rabbit kidneys strongly suggest it is interfering with free radical production. As to the exact site of allopurinol activity within the kidneys, comparison of histological samples may provide clues (the subject of a separate report). However, ischaemic damage in untreated tissues or solid organs is associated with a venous outflow block and this appears to be improved by administration of allopurinol. This evidence, considered alongside the observations that xanthine oxidase appears to be located primarily in endothelial cells[28] and hence that endothelial cells will generate oxygen-derived free radicals *in vitro*[29] suggests that it may be primarily effective in the vascular bed. As neutrophils are also likely to contribute to injury[30,31] via xanthine oxidase-dependent mechanisms, it is also possible that allopurinol protects against neutrophil-endothelial cell-platelet interactions within the vasculature.

In conclusion, it is possible that allopurinol will prove a useful therapeutic agent for preventing reperfusion injury. It seems far more likely that it provides benefit by preventing xanthine oxidase-dependent $O_2^{\cdot-}$ generation on exposure to molecular oxygen during reperfusion rather than by preventing irreversible loss of purine nucleotides from hypoxic cells through inhibition of nucleotide dephosphorylating enzymes.

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