Failure of the xanthine oxidase inhibitor allopurinol to limit infarct size after ischemia and reperfusion in dogs

KEITH A. REIMER, M.D., PH.D., AND ROBERT B. JENNINGS, M.D.

ABSTRACT During the acute phase of myocardial ischemia, adenine nucleotides are degraded to nucleosides and bases, especially inosine and hypoxanthine. Simultaneously, xanthine dehydrogenase is converted to xanthine oxidase, an enzyme that converts hypoxanthine to xanthine, and xanthine to uric acid, producing a superoxide anion for each molecule of hypoxanthine or xanthine oxidized. To determine if free radicals via this enzymatic source contribute to cell death in myocardial ischemia, we determined whether allopurinol, an inhibitor of xanthine oxidase, could limit infarct size in a reperfusion preparation of myocardial infarction. The circumflex coronary artery of each of 34 dogs was occluded for 40 min, followed by reperfusion for 4 days. Infarct size then was measured by histologic methods and was related to major baseline predictors of infarct size, including anatomic area at risk and collateral blood flow. Infarct size was larger (NS) in the allopurinol (n = 8) than in the control (n = 11) group, a trend that was related to slightly higher (NS) collateral blood flow in the control group. We conclude that allopurinol has no beneficial effect in this preparation of experimental myocardial infarction. The results oppose the hypothesis that free radicals, produced via the xanthine oxidase reaction, are an important contributing factor in myocardial ischemic cell death.


FREE RADICALS, including the superoxide anion (O₂⁻) and the hydroxyl radical (-OH), have been implicated as causal or contributing factors in a variety of types of cell injury, including myocardial ischemia.⁴⁻⁸ Several metabolic pathways may lead to production of free radicals during ischemia,⁴⁻⁶ including the xanthine oxidase reaction in which hypoxanthine is oxidized to xanthine or xanthine is oxidized to uric acid.⁵ The xanthine oxidase reaction is of particular interest because large quantities of hypoxanthine, and its precursor inosine, accumulate rapidly in severely ischemic myocardium as a consequence of the degradation of the purine nucleotides.⁹⁻¹² Especially when reperfusion is established, the xanthine oxidase reaction, fueled by an initially large substrate supply plus abundant oxygen resulting from reactive hyperemia, might produce an excessive load of superoxide anion. The latter might cause death of myocytes that would otherwise have survived the temporary period of ischemia.

If this hypothesis is valid, inhibition of the xanthine oxidase reaction should prevent some or all of the cell death, and thereby limit infarct size in a temporary occlusion/reperfusion setting. Accordingly, the purpose of the present study was to test whether allopurinol, a xanthine oxidase inhibitor, alters myocardial infarct size in a coronary occlusion/reperfusion preparation of ischemic injury.

Methods

The experimental preparation used was similar to that described previously,¹³ in which methods of experimental design and end point analysis established in the NHLBI cooperative AMPIM study were used.¹⁴

Animal selection and exclusion criteria. Thirty-four adult mongrel dogs of both sexes and weighing 10 to 25 kg were used. Dogs were not accepted into the study if they had clinically evident infections or circulating heart worm filariae. No dogs that were entered into the study were later found to have heart worms.

Surgical preparation. Dogs were fasted overnight before the study and were anesthetized with 30 to 40 mg/kg of intravenous sodium pentobarbital. Additional anesthesia was administered during the experiment, as needed. The dogs were intubated and ventilated at 200 ml/kg/min of room air supplemented with a low flow of oxygen. An aseptic surgical technique was used, including the use of sterile towels, gowns, instruments, and gloves; each dog was given 600,000 units of penicillin intramuscularly. Catheters were placed in the femoral...
artery and vein. Arterial blood gases were checked and the ventilation rate or oxygen flow rate was adjusted, if necessary, to achieve a $P_{O_2}$ of 100 to 140 mm Hg, a $P_{CO_2}$ of 32 to 40 mm Hg, and pH 7.37 to 7.47. A 4 to 5 cm thoracotomy was performed in the fourth intercostal space and the heart was suspended in a temporary pericardial cradle. The proximal circumflex artery was isolated and a No. 1 silk suture was passed around it for later temporary occlusion. The occlusion site was always distal to the atrial branch, but proximal to the first large marginal branch of the artery. Two catheters were then placed into the left atrium for measurement of atrial pressure and for microsphere injection, respectively. The pericardial cradle was then relaxed. Lead II of the standard electrocardiogram and left atrial and peripheral blood pressures were monitored on a Brush 2400 recorder throughout the experiment. Temporary circumflex occlusion (see below) was accomplished by snaring the artery into a small glass tube. After release of the occlusion, any dog that developed ventricular fibrillation was cardioverted, if possible, with an MRL Model 560 defibrillator with internal paddles. Incisions were closed, air was evacuated from the chest, and dogs were allowed to survive for 4 days. All dogs were then reanesthetized and heparinized and their hearts were removed for postmortem analysis.

**Regional distribution of blood flow.** The regional distribution of myocardial blood flow was assessed with $9 \pm 1$ $\mu$m radioactive microspheres at three times: before drug administration, after initial drug therapy but before coronary occlusion, and 20 min after the onset of coronary occlusion. The spheres were obtained from stock solutions containing 0.10% dye or 0.05% Tween 80. Two or three million spheres labeled with scandium-46, strontium-85, or cerium-141 were injected via the left atrial catheter and this was followed by a 7 ml saline flush. A reference sample of arterial blood was collected from the femoral artery at 7.75 ml/min for 2.5 min after injection of spheres.

**Experimental groups and treatment protocol.** Infarcts were produced by 40 min of coronary occlusion followed by reperfusion. After the initial surgical preparations but approximately 35 min before occlusion, dogs were randomly assigned to either allopurinol treatment or a control group. The total dose of allopurinol each treated dog received was 100 mg/kg, administered in 150 ml of normal saline. Allopurinol was brought into solution as the salt with xylose (equimolar quantities of sodium hydroxide). Half of the dose (50 mg/kg) was administered slowly over 10 min, beginning 30 min before coronary occlusion; the remainder was administered continuously (0.5 mg/kg/min) over 100 min beginning 20 min before occlusion and ending 40 min after reperfusion. Control dogs received a similar infusion of normal saline.

**Postmortem studies. Area at risk.** To define the anatomic boundaries of the previously ischemic and nonischemic vascular beds, catheters were placed in the left main and the circumflex arteries at the site of previous occlusion; both vessels were perfused simultaneously with dye solutions at 120 to 140 mm Hg pressure. The perfusion fluid was sodium phosphate buffer (8.8 $\times$ 10$^{-2}$M dibasic and 0.18 $\times$ 10$^{-2}$M monobasic sodium phosphate/liter, pH 8.25 to 8.6) plus 6% dextran (82,000 mol wt; Sigma Chemical) to which either 1.0% triphenyltetrazolium chloride (TTC) (for the previously occluded circumflex artery) or 0.05% mona blue dye (left main artery) was added. The heart then was fixed by immersion in a large volume of phosphate-buffered formalin. The fixed left ventricle was then sectioned into eight transverse slices (figure 1) that were weighed and the apical surfaces were photographed. The previously occluded vascular bed (area at risk) was identified and traced from enlarged projections of the photographic slide of each ventricular slice. The area at risk was measured by "cut and weigh" techniques with use of copies of these tracings.

**Histologic analysis.** The method of sampling for histologic and blood flow analysis is shown in figure 1. The apical surfaces

![FIGURE 1. The postmortem sampling technique for calculating area at risk, histologic infarct size, and regional blood flow. The formalin-fixed left ventricle was isolated and sliced first into four slices and then into eight slices with a commercial meat slicer. These eight slices were used to estimate area at risk. Histologic sections were obtained from the apical surfaces of slices 1a, 2a, 3a, 4a, and 4b and were assumed to be representative of the four composite slices, respectively. The remaining myocardium of composite slices 1 through 3 was further subdivided for blood flow analysis. Nonischemic and central ischemic regions were divided into inner, middle, and outer thirds. Lateral and septal border zone regions were excluded from flow analysis to avoid measurements from samples containing admixtures of ischemic and nonischemic tissue.](image-url)
of slices 1a, 2a, 3a, 4a, and 4b were used for histologic analysis. Two sections from each tissue sample were cut and one was stained with hematoxylin and eosin and the other with Mallory's variant of Mallory's connective tissue stain. Infarct size was determined as a percentage of each of four composite slices of the left ventricle and as a percentage of the entire left ventricle. Cut and weigh techniques of infarct size analysis were used based on copies of tracings of the necrotic and viable areas, which were made from projections of the histologic sections.

**Regional blood flow.** Flow measurements were made in the remaining portions of the first three composite slices (figure 1). The slices were divided into nonischemic and central ischemic zones (central zone = 50% of the vascular bed at risk). The septal and lateral border zones were excluded from analysis to avoid misinterpretation of measurements from samples that might have contained nonischemic as well as ischemic myocardium. The nonischemic and central ischemic regions were further subdivided into subendocardial, midmyocardial, and subepicardial thirds. The calculation of regional myocardial blood flow in each sample was performed with the formula

\[ Q_m = \frac{(Q_r \times C_m) \times Cr}{\text{Cr}} \]

where \( Q_m \) = myocardial blood flow (ml/min); \( Q_r \) = reference blood flow (ml/min); \( C_m \) = counts/minute in each myocardial sample; \( Cr \) = counts/minute in the reference sample. The myocardial blood flow was expressed relative to sample weight (ml/g). Corrections were made for apparent microsphere loss \(^{16}\) in three dogs that had preocclusion ischemic region/nonischemic flow ratios of less than 0.9. Apparent sphere loss was calculated as

\[ \% \text{ apparent sphere loss} = \frac{(MBF_{NI} - MBF_{PO}) \times 100}{MBF_{NI}} \]

where \( MBF_{PO} \) = mean preocclusion blood flow in the ischemic region; \( MBF_{NI} \) = mean preocclusion blood flow in nonischemic samples. Ischemic blood flow measurements for each sample were then corrected with the following equation:

\[ MBF_c = \frac{MBF_u \times 100}{(100 - \% \text{ apparent sphere loss})} \]

where \( MBF_c \) and \( MBF_u \) = corrected and uncorrected blood flow values. Values for infarct size and area at risk were also corrected in these three dogs, based on the assumption that apparent microsphere loss is a reflection of edema, inflammation, or hemorrhage causing artifactual infarct swelling.\(^{16}\)

**Data analysis.** Data are expressed as group means ± SD and statistical comparisons of group means were by Student's two-tailed t test. Paired t tests were also used to detect any changes in hemodynamic parameters or regional blood flow induced by therapy or coronary occlusion within a treatment group. Area at risk and collateral blood flow have been identified as major determinants of myocardial infarct size.\(^{16, 17}\) To include these parameters in the analysis, infarct size has been expressed as a percentage of area at risk as well as a percentage of the left ventricle. The regression of infarct size (as a percentage of area at risk) vs collateral blood flow was also evaluated.

**Results**

The survival rate and the timing of death among nonsurviving dogs in the study are shown in table 1. Fourteen of 18 (78%) control dogs survived, a survival rate comparable to that reported previously with this preparation.\(^{13, 17}\) Of the four deaths, one occurred during the 40 min period of occlusion, two occurred at the time of reflow, and one occurred during the 4 day recovery period. Only eight of 16 (50%) dogs in the allopurinol-treated group survived. Of the eight deaths, four occurred during coronary occlusion and four occurred late during the 4 day recovery period. However, group sizes were too small to establish whether the high mortality in the allopurinol group was a real effect of treatment.

Among the surviving dogs, three controls were found to have had only mild ischemia. These dogs were considered atypical of most dogs with circumflex coronary occlusions\(^{13, 17}\) and their data have been excluded from the analysis to improve comparability of collateral flow, as a baseline predictor of infarct size, within the two groups.

The average size of the anatomic vascular area at risk was virtually identical in the two groups (table 2) and hemodynamic parameters, including heart rate, atrial and arterial blood pressures, and the rate-pressure product were also similar in the two groups. Despite exclusion of data from three control dogs with high collateral blood flow, mean collateral blood flow was still slightly higher in the control group as compared with the allopurinol-treated group.

Infarct size as a percentage of the left ventricle or when normalized as a percentage of the area at risk was nearly twice as large in the allopurinol group as in the control group. In figure 2, the relationship between infarct size and collateral blood flow is shown. This analysis shows, as reported previously\(^{13, 14, 17}\) an inverse relationship between infarct size and collateral blood flow. The relationship was not altered by allopurinol therapy; however, the relatively high collateral

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of dogs</th>
<th>Occlusion VF</th>
<th>Reperfusion VF</th>
<th>Defibrillated</th>
<th>Late deaths</th>
<th>Survival</th>
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<td>0</td>
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<tr>
<td>Allopurinol</td>
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<td>0</td>
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<td>8/50</td>
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</table>

VF = ventricular fibrillation observed during the 40 min period of coronary occlusion or in the first 2 min after reperfusion.
The table below shows the area at risk, infarct size, regional blood flow, and hemodynamic parameters in untreated and allopurinol-treated dogs.

<table>
<thead>
<tr>
<th>Group</th>
<th>LCC bed (% of LV)</th>
<th>% of LV</th>
<th>% of LCC bed</th>
<th>Control (ml/mm/g)</th>
<th>20 min collateral blood flow (ml/min/g)</th>
<th>Mean</th>
<th>HR</th>
<th>MAP</th>
<th>SP/DP</th>
<th>RPP( ^a )</th>
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LCC = left circumflex coronary (ischemic); LV = left ventricle; % = inner (subendocardial) third; M = middle third; O = outer (subepicardial) third; HR = heart rate (beats/min); MAP = mean left atrial pressure (mm Hg); SP/DP = systolic/diastolic blood pressure (mm Hg); RPP = rate-pressure product (heart rate × systolic blood pressure/1000); 20 min measurements refer to those obtained 20 min after the onset of coronary occlusion.

- Allopurinol

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*Allopurinol\( ^a \) divided by 1000.

blood flow in several of the control dogs is a likely cause for the small mean infarct size in this group.

**Discussion**

**Hypothesis.** The onset of acute myocardial ischemia results in the rapid utilization and depletion of intracellular high-energy phosphates, including ATP. The degradation of ATP to ADP and AMP is soon followed by further catabolism of AMP to the nucleosides and bases (adenosine, inosine, hypoxanthine, and xanthine). The major degradation products of the adenine nucleotide pool during the early phase of ischemia are inosine and hypoxanthine. Indeed, recent studies\(^ {12} \) have shown that by 15 min after the onset of ischemia, a time when myocyte injury is still reversible, nearly half of the adenine nucleotide pool has been converted to these two catabolites. Also, after the onset of myocardi al ischemia, xanthine dehydrogenase, which catalyzes the conversion of hypoxanthine to xanthine and xanthine to uric acid by hydrogen transfer to NAD, is converted to xanthine oxidase, an enzyme that also converts hypoxanthine to xanthine and then to uric acid using molecular oxygen as an electron acceptor.\(^ {18, 19} \)

The latter reaction results in production of one superoxide radical for each molecule of hypoxanthine oxidized. Thus, especially in the setting of transient ischemia followed by reperfusion, ischemic myocardium becomes primed for markedly increased production of the superoxide radical, particularly if reperfusion is established to provide an unlimited source of oxygen. On this basis, the hypothesis that production of free radicals via the xanthine oxidase reaction could contribute to irreversible myocardial ischemic injury is reasonable, as is the corollary hypothesis that protec-
tion of ischemic myocardium should be achieved by therapy with a xanthine oxidase inhibitor such as allopurinol.

The results of the present study do not support the aforementioned hypotheses. Allopurinol failed to limit infarct size when administered to dogs before a 40 min test period of myocardial ischemia.

Comparison with previous studies of allopurinol. Interest in allopurinol as a possible protective agent against ischemic injury first developed from the recognition that adenine nucleotide depletion occurs early in ischemia and that inhibition of xanthine oxidase might prevent this progressive loss of adenine nucleotides. Based on this hypothesis, several studies of the effects of allopurinol, particularly in myocardial and renal ischemia, were done with mixed results. For example, Cunningham et al. reported that allopurinol preserved adenine nucleotides during renal ischemia in rats, but Collins et al. found no such effect in rabbits. Chatterjee and Berne reported improved postischemic renal function, as determined from serum creatinine levels, in dogs treated with allopurinol, but no functional improvement could be demonstrated in similar studies by Murdock and Cho. DeWall et al. reported reduction in myocardial cyanosis and ST segment elevation in dogs with coronary occlusions treated with allopurinol. However, Furuse et al. also using dogs with coronary ligations, found no effect of allopurinol on myocardial PO₂ measured polarographically. Arnold et al. reported smaller areas of ischemia in dogs pretreated with allopurinol compared with those in control dogs with similarly placed ligations of the anterior descending coronary artery. In the first direct, quantitative study of myocardial infarct size, Shatney et al. found no effect of allopurinol administered 15 min after coronary ligation.

Interest in allopurinol for protection against ischemic injury subsequently waned but was recently rekindled with the advancement of the hypothesis that free radicals, and particularly the superoxide anion, might be important causes of cell injury in ischemia; this was coupled with the realization that the xanthine oxidase reaction might be an important source of superoxide. Testing this hypothesis with respect to renal ischemia, Hansson et al. reported improved postischemic renal blood flow. Recently, Akizuki et al. have reported limitation of myocardial infarct size after coronary embolization in dogs, and Chambers et al. have reported limitation of infarct size measured by gross staining techniques after 1 hr of ischemia followed by 4 hr of reperfusion in an open-chest dog preparation. The reasons for the dramatically positive results in the latter two studies vs the negative results observed in the present study are unknown. It may be important that infarct size was not analyzed in relation to collateral coronary blood flow in either of the aforementioned studies.

Importance of incorporating baseline variables in the analysis of therapeutic effect. The results of this study illustrate the importance of measuring the major variables that predict infarct size in animal preparations of myocardial ischemia. Even though the selection of therapy was randomized and survival rate was lower in the allopurinol-treated dogs, collateral blood flow was lower in the surviving allopurinol-treated dogs than in the controls. This difference most likely was not the result of a negative effect of allopurinol on collateral blood flow, but rather was probably an artifact of selection, which can occur despite randomization when relatively small experimental groups are used. This conclusion is supported by the observation that collateral blood flows reported in other groups of control dogs studied by similar experimental protocols were similar to those observed in our allopurinol group, and lower than those observed in the present control group. Because selection artifacts are possible with small groups of dogs, it is crucial in studies of this type to incorporate the major determinants of infarct size into the analysis. It is clear from our analysis (figure 2) that, for a given level of collateral blood
flow, infarct size was neither larger nor smaller in the treated group.

**Cause of negative results.** Allopurinol is a potent competitive inhibitor of xanthine oxidase, with reported K_1 values of 7.6 x 10^{-9} M to 7.0 x 10^{-9} M.32 It has a plasma half-life of 75 min in dogs due to part to renal excretion and in part to its conversion to oxypurinol, which is also an inhibitor of xanthine oxidase.32 Thus, the dose of allopurinol used in this study (100 mg/kg) was a substantial one; it is highly unlikely that an insufficient dose of drug was the cause of our negative results. Moreover, the dose of allopurinol used in the present study was in the same range as those used in the aforementioned studies of allopurinol in myocardial ischemic injury in dogs,25, 27, 30, 31 studies in which dramatic beneficial effects were found.

The hypothesis that xanthine oxidase activity contributes to myocardial ischemic injury may be ill-founded. One difficulty is that xanthine oxidase activity is quite low in myocardium compared with that in other organs.34, 35 In addition, the precise cellular location of xanthine oxidase in myocardium has not been elucidated. It is known that nucleoside phosphorylase, the enzyme that converts inosine to hypoxanthine, is absent from cardiac myocytes but is present in myocardial endothelial cells and pericytes.36 If xanthine oxidase is also confined to microvascular sites, superoxide anions produced by this reaction might be inaccessible to ischemic or reperfused myocytes. Endothelial xanthine oxidase activity might be detrimental to the microcirculation, but microvascular damage is most likely not a determinant of the extent of myocardial infarct size.37 Another difficulty with the hypothesis is that after reperfusion, when reactive hyperemia would provide excessive oxygen such that xanthine oxidase activity might be accelerated, large concentrations of hypoxanthine would persist only briefly before being washed into the systemic circulation. Finally, much of the inosine and hypoxanthine that accumulates in severely ischemic myocardium does so within the first 15 min after coronary occlusion,12 a period during which reperfusion prevents myocyte necrosis.38 Thus, the accumulation of substrate for xanthine oxidase is, by itself, insufficient to cause irreversible myocardial ischemic cell injury.

The results of the present study indicate no limitation of myocardial infarct size induced by allopurinol in a reperfusion preparation of myocardial ischemic injury. These results do not support the hypothesis that superoxide production, via the action of xanthine oxidase, contributes significantly to the development of irreversible myocardial ischemic cell injury. These results do not permit conclusions to be drawn with respect to the role free radicals generated in the reperfused myocardium by other enzymatic pathways may play in damaging ischemic myocytes. However, other sources of free radicals in this system remain poorly defined and largely theoretical.

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