Evaluation of the Role of Xanthine Oxidase in Myocardial Reperfusion Injury*

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The free radical-generating enzyme xanthine oxidase has been hypothesized to be a central mechanism of the injury which occurs in postischemic tissues; however, its importance remains controversial. Much attention has focused on the role of this enzyme in myocardial reperfusion injury. While xanthine oxidase has been observed in ischemic tissue homogenates, the presence and importance of radical generation by the enzyme in intact tissues are unknown. Therefore, we performed electron paramagnetic resonance, nuclear magnetic resonance and hemodynamic studies to measure the presence and significance of xanthine oxidase-mediated free radical generation in the isolated rat heart. When isolated perfused rat hearts were reperfused after 30 min of global ischemia, myocardial function and coronary flow were significantly improved in the presence of the definitive xanthine oxidase blocker oxypurinol. Free radical concentrations measured by spin-trapping with 5,5'-dimethyl-1-pyrroline-N-oxide were significantly decreased by oxypurinol and the energetic state of the heart was improved as reflected by an increased recovery of phosphocreatine and a higher phosphocreatine/Pi ratio. ATP recovery, however, was not altered, indicating that the improved functional and metabolic state of the heart was not due to ATP salvage. Spectrophotometric assays for the enzyme showed an increase in the amount of xanthine oxidase relative to dehydrogenase following ischemia, and a total available xanthine oxidase pool in the rat heart of approximately 150 milliunits/g of protein. Thus, xanthine oxidase is a significant source of the oxidative injury which occurs upon reperfusion of the ischemic rat heart.

The role of xanthine oxidase in the pathophysiology of myocardial reperfusion injury is controversial. In normal tissues, the native dehydrogenase form of the enzyme uses NAD* as its electron acceptor in the conversion of hypoxanthine to xanthine and uric acid (1). In ischemic tissues, McCord and Roy (2) have proposed that the xanthine dehydrogenase undergoes proteolytic conversion to the oxidase form which uses O2 as its electron acceptor. It is known that xanthine oxidase in the presence of the substrates hypoxanthine or xanthine reduces molecular oxygen to 'O2 and H2O2 (3). Recently it has been demonstrated that the enzyme can further reduce H2O2 to OH (4). During ischemia, substrate for this reaction accumulates due to the breakdown of ATP to AMP, adenosine, inosine, and finally to hypoxanthine (5). Upon reperfusion O2 is reintroduced. Thus it has been hypothesized that xanthine oxidase and its requisite substrates would be present in high concentrations in reperfused tissue and consequently would result in oxygen free radical generation upon reperfusion. The 'OH and 'O2 radicals generated by the enzyme could in turn react with cellular proteins and membranes causing cellular injury. Several studies have previously been performed to investigate whether inhibitors of xanthine oxidase prevent reperfusion injury. Unfortunately, there have been conflicting reports in the literature that either support (6-11) or deny (10, 12, 13) that this mechanism of injury occurs in the postischemic heart. Specific points of controversy include: (i) whether xanthine oxidase is present in heart tissue; (ii) whether xanthine oxidase causes reperfusion injury; and (iii) whether xanthine oxidase blockers prevent injury and, if so, by what mechanism. Various indices of injury including cardiac function and infarct size were evaluated in these studies, but no direct measurement of xanthine oxidase-mediated free radical generation has been performed. Thus, it remains unclear whether free radicals are generated by the xanthine oxidase pathway in reperfused hearts and whether these radicals are actually associated with impaired myocardial function.

In the present study, we performed experiments to determine whether xanthine oxidase is a source of free radical-mediated reperfusion injury. Experiments were performed in the presence and absence of oxypurinol (4,6-dihydropyrazolo[3,4-d]pyrimidine), a high affinity inhibitor of xanthine oxidase (Kf = 100 nM, bovine) (14). Measurements of enzyme activity, cardiac function, radical generation, and bioenergetics were performed. These studies demonstrate that xanthine oxidase is present in the postischemic rat heart and that free radical generation by the enzyme is a significant, although not exclusive, mechanism of reperfusion injury.

MATERIALS AND METHODS

Four separate groups of experiments were performed on a total of 65 isolated perfused rat hearts. In all experiments, female retired breeder Sprague-Dawley rats (250-300 g) were heparinized and anesthetized with sodium pentobarbital by intraperitoneal injection. Hearts were isolated and perfused at 37 °C by a modification of the Langendorff Constant pressure perfusion of 80 mm Hg

* This work was supported by the National Institutes of Health Grants HL-17655-13, HL-38324, and HL-07227 and Squibb American Heart Association Clinician Scientist Award. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations and trivial names used are: oxypurinol, 4,6-dihydropyrazolo[3,4-d]pyrimidine; DMPO, 5,5'-dimethyl-1-pyrroline-N-oxide; DTT, diithiothreitol; PMSF, phenylmethylsulfonylfluoride; PCr, phosphocreatine.
was used with Krebs bicarbonate buffer consisting of 120 mM NaCl, 17 mM glucose, 25 mM NaHCO₃, 5.9 mM KCl, 1.2 mM MgCl₂, and 2.5 mM CaCl₂, and bubbled with 95% O₂, 5% CO₂ gas. The perfusate solution was passed through a 0.8-μm Millipore filter prior to use. Oxypurinol was obtained from Sigma.

Preparation of Tissue Extracts—Xanthine dehydrogenase and xanthine oxidase activities were directly measured in 24 rat hearts using three procedures developed from several published methods (8, 15, 16). Two of the procedures involved a final Sephadex separation, whereas the third involved dialysis. We chose to do this assay in several different ways because preliminary experiments showed that the method significantly influenced the measured activity. In all cases, hearts were isolated and perfused as described above. Control hearts were not ischemic and then immediately frozen on dry ice. Ischemic hearts were perfused for 5 min, infused with vehicle for 30 s, made globally ischemic for 30 min, and then frozen in liquid N₂. Another group of hearts were perfused for 20 s following 30 min of ischemia, and then immediately frozen in liquid N₂. Frozen hearts for the two Sephadex assays were minced with scissors in 5 ml of ice-cold 0.05 M potassium phosphate buffer (pH 7.8) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (n = 6) or 1 mM PMSF + 10 μM diithirotetrahydrofurane (DTT) (n = 19). Frozen hearts for the dialysis assay (n = 6) were minced with scissors in 5 ml of ice-cold 0.1 M Tris buffer (pH 8.1) containing 1 mM PMSF and 10 mM DTT. The PMSF is a serine protease inhibitor and should eliminate proteolytic conversion of xanthine dehydrogenase during the homogenization procedure. The DTT should reverse any conversion to xanthine oxidase which occurred due to sulfhydryl oxidation and therefore should allow measurement of only irreversibly converted enzyme (2, 17).

Hearts were further minced with an Ultra-Turrax (SDT 1810, IKA Labortechnik) and homogenized with a Teflon pestle. A small amount of homogenate was removed and frozen for an assay of total protein (18). Homogenates were centrifuged at 600 × g for 20 min at −4 °C, and the supernatants centrifuged at 150,000 × g for 60 min at 4 °C. The lipid layer was removed, and the supernatants were either passed through a Sephadex G-25 column equilibrated with phosphate buffer or dialyzed against 200 volumes of 0.1 M Tris (once for 3 h, then again overnight).

Enzyme Assay—The processed supernatant was assayed spectrophoto metrically at 295 nm for uric acid production using a Hewlett-Packard 8452A diode array spectrophotometer. The reaction mixture for the Sephadex assays contained 90 μl of eluant, 50 mM phosphate buffer, and 60 μM xanthine in a 1-ml quartz cuvette for the determination of xanthine oxidase activity. The reaction mixture for the dialysis assay contained 200 μl of dialysates, 0.1 M Tris buffer, and 60 μM xanthine. In all cases, dehydrogenase plus oxidase activity was measured after the addition of 0.6 mM NAD⁺ to the reaction mixture. Enzyme activity is expressed in milliliters per gram protein, where 1 unit of activity equals 1 μmol of substrate converted to uric acid per min.

Hearts were further minced and perfused as described above. Coronary flow was measured by collecting the heart effluent. Heart rate and left ventricular developed pressure were measured using a fluid-filled balloon secured into the left ventricle. The balloon was connected to a Statham P23XL transducer via a hydraulic line, and the transducer output was amplified to a Gould Hydratronics transducer. The balloon was connected to a Statham P23XL transducer via a hydraulic line, and the transducer output was amplified to a Gould Hydratronics transducer. The balloon was connected to a Statham P23XL transducer via a hydraulic line, and the transducer output was amplified to a Gould Hydratronics transducer. The balloon was connected to a Statham P23XL transducer via a hydraulic line, and the transducer output was amplified to a Gould Hydratronics transducer. The balloon was connected to a Statham P23XL transducer via a hydraulic line, and the transducer output was amplified to a Gould Hydratronics transducer. The balloon was connected to a Statham P23XL transducer via a hydraulic line, and the transducer output was amplified to a Gould Hydratronics transducer.

Electron Paramagnetic Resonance—Rat hearts were isolated and perfused as described above. The experimental protocol was the same except that the spin-trap, 5,5′-dimethyl-1-pyrroline-N-oxide (DMPO) was infused through a second sidearm located just proximal to the top of the heart perfusion cannula with a final concentration of 40 mM. Spin-trap containing effluent was collected in 20-s aliquots prior to ischemia (control) and during the first 9 min of reflow. Aliquots were also collected after 5 and 10 min of reflow. Care was taken to keep the room dark and the DMPO solution covered to minimize light-induced degradation. The DMPO was purchased from Sigma and further purified by double distillation.

Electron paramagnetic resonance (EPR) spectra were recorded in flat cells at room temperature with a Bruker-IBM ER 300 spectrometer operating at X-band with a TM 110 cavity using a modulation frequency of 100 kHz, modulation amplitude of 0.5 G, microwave power of 20 milliwatts, microwave frequency of 9.77 GHz, and acquisition times of 1-10 min scans. The digitized Bruker spectral data were transferred to an AST 386 personal computer for analysis. Software capable of isotropic spectral simulation, developed in this laboratory, was used for component analysis of experimental spectra, as described previously (4, 19, 20). Spectral simulations consisting of linear combinations of the component signals were performed to match the observed spectra. From the weighted intensities of each component in these simulations, the relative amount of each component signal was determined. The total radical concentration was then determined from the ratio of the double integral of the observed spectra to that of a known concentration of the 2,2,6,6-tetramethylpiperidinoxyl free radical in aqueous solution as described previously (19, 20). The concentration of the 2,2,6,6-tetramethylpiperidinoxyl standard was further validated with respect to an aqueous standard of MnCl₂. P-31 Nuclear Magnetic Resonance—Eight rat hearts were isolated and perfused as described above, except that each was placed into a 25-mm NMR tube and lowered into a wide-bore 4.25 T superconducting magnet. Again, the same experimental protocol was followed, and bioenergetic status in the presence or absence of oxypurinol was determined. The total radical concentration was then determined from the ratio of the double integral of the observed spectra to that of a known concentration of the 2,2,6,6-tetramethylpiperidinoxyl free radical in aqueous solution as described previously (19, 20).
Role of Xanthine Oxidase in Reperfusion Injury

Because of the controversy regarding the presence of xanthine oxidase in normal and ischemic heart tissue, a series of spectrophotometric assays were performed in order to directly measure the presence and activity of the enzyme. Different methods have been described in the literature for assaying xanthine oxidase in biological tissues. The technique first described for assaying this cytosolic enzyme consisted of tissue homogenization followed by high speed ultracentrifugation and dialysis of the supernatant against the final buffer (15). Subsequent investigators recognized that proteolytic conversion of xanthine dehydrogenase to xanthine oxidase could occur in tissue homogenates and therefore introduced homogenization with the reducing agent DTT alone or together with the protease inhibitor PMSF to prevent this conversion (2, 17). A further refinement in the assay was introduced with the use of molecular exclusion chromatography (Sephadex columns) rather than dialysis (7, 8). Since the differences in these methods could explain some of the different results reported in the literature, we performed measurements of xanthine oxidase and xanthine dehydrogenase activity using both the dialysis and the more rapid Sephadex chromatography methods. The results of these assays for xanthine oxidase and xanthine dehydrogenase are presented in Table I, and a representative example of the measured absorbances is shown in Fig. 1. With the chromatography technique, regardless of whether or not DTT was present in the buffer solution, similar xanthine oxidase activities were measured in both control heart tissue and tissue from hearts subjected to 30 min of ischemia. The amount of xanthine oxidase present was not significantly increased by the presence of DTT suggesting that the enzyme measured was not reversibly oxidized to the oxidase form. In general, however, the enzyme activity (xanthine oxidase and xanthine dehydrogenase) was greater in the presence of DTT.

Table I

<table>
<thead>
<tr>
<th>Ischemic duration</th>
<th>Assay conditions</th>
<th>XO</th>
<th>XD</th>
<th>Ratio XD/XO</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Sephadex</td>
<td>17.2 ± 1.1</td>
<td>120.2 ± 3.3</td>
<td>7.2 ± 0.5</td>
</tr>
<tr>
<td>30</td>
<td>[DTT + PMSF]</td>
<td>17.2 ± 1.5</td>
<td>101.1 ± 4.7</td>
<td>6.1 ± 0.4</td>
</tr>
<tr>
<td>0</td>
<td>PMSF</td>
<td>16.0 ± 2.1</td>
<td>75.4 ± 2.7</td>
<td>5.3 ± 0.7</td>
</tr>
<tr>
<td>30</td>
<td>Dialysis</td>
<td>15.4 ± 0.7</td>
<td>75.3 ± 2.3</td>
<td>4.9 ± 0.2</td>
</tr>
</tbody>
</table>

Dehydrogenase to oxidase was decreased by 16% with ischemia from 7.2 ± 0.5 to 6.1 ± 0.4 in the presence of DTT, and by 6% from 5.3 ± 0.7 to 4.9 ± 0.2 in the absence of DTT. Thus, although the absolute amount of xanthine oxidase present in each group of hearts was not different, the amount relative to the xanthine dehydrogenase was increased with ischemia. Since maximal radical generation is thought to occur in the early seconds of reperfusion, additional studies were performed measuring xanthine oxidase and xanthine dehydrogenase activity in reperfused heart tissue (Sephadex, DTT + PMSF). In hearts reperfused for 20 s, the dehydrogenase/oxidase ratio was further decreased to a value of 4.5 ± 0.2, which was significantly different from the control and ischemic hearts (p < 0.01); however, xanthine oxidase, as well as total enzyme activity, was lower.

With the dialysis technique, all of the enzyme was in the oxidase form which suggests that proteinase occurred during the dialysis step (Table I). This technique thus measures only the total possible xanthine oxidase pool within the tissue. Therefore, the maximal amount of xanthine oxidase available in rat heart was approximately 150 milliunits/g protein.

Thus, xanthine oxidase is present in the perfused rat heart, although the measured activity is highly dependent on the method of tissue processing which is used. The amount of xanthine oxidase relative to dehydrogenase increased slightly following ischemia and further upon reperfusion; however, it is clear that the enzyme is present in both normally perfused as well as ischemic and reperfused hearts.

In order to determine if the xanthine oxidase present in heart tissue induces measurable reperfusion injury with altered contractile function and coronary flow, hearts were subjected to 30 min of global ischemia and reperfusion in the presence or absence of the xanthine oxidase blocker oxypurinol. Recovered coronary flow expressed as a percent of control is shown in Fig. 2 over the course of 45 min of reperfusion. Oxypurinol-treated hearts exhibited significantly (p < 0.01) improved recovery of coronary flow over the entire time course of reflow. Final (45 min) recovery of coronary flow was 50.2 ± 6.8% in the oxypurinol-treated hearts, and only 37.1 ± 5.7% in the control hearts.

Contractile function was also significantly improved with oxypurinol treatment. Higher recovered left ventricular developed pressures were observed in the hearts treated with the blocker. As shown in Fig. 3, the rate-pressure product (an index of cardiac contractile work) was the same for both
Role of Xanthine Oxidase in Reperfusion Injury

Infused with vehicle; closed circles, hearts infused with oxypurinol. Oxypurinol significantly improved the recovery of coronary flow over the entire duration of reflow (p < 0.01).

Oxypurinol significantly improved recovery of mechanical function during reflow.

The improved coronary flow observed with oxypurinol appears to be secondary to the diminution of ischemic or reperfusion injury, not a nonspecific vasodilatory effect of the drug. The recovery of cardiac contractile function may lag behind the coronary flow since it may require time for the muscle to replete substrate, reverse acidosis, and reestablish Ca2+ and electrolyte homeostasis. Thus, oxypurinol significantly decreased postischemic injury as evidenced by the increased recovery of contractile function and coronary flow following 30 min of global ischemia.

The predominant radical signal in this heart was the DMPO-OH adduct, though small amounts of DMPO-R adduct and ascorbate radical are also present. The predominant radical signal in this heart was the DMPO-OH adduct, though small amounts of DMPO-R adduct and ascorbate radical are also present. The top panel shows no signal in the preischemic heart, while the following panels show signals obtained upon reperfusion at the times indicated.

Fig. 2. Percent recovered coronary flow in hearts reperfused following 30 min of global ischemia. Open circles, hearts infused with vehicle; closed circles, hearts infused with oxypurinol. Oxypurinol significantly improved recovery of mechanical function during reflow (p < 0.05).

Fig. 3. The index of mechanical function (heart rate x developed pressure) prior to 30 min of global ischemia and during 45 min of reperfusion. Symbols are defined as in Fig. 2. Oxypurinol significantly improved recovery of mechanical function during reflow (p < 0.05).

Fig. 4. EPR spectra prior to ischemia and over the course of reflow in a control heart subjected to 30 min of global ischemia. The predominant radical signal in this heart was the DMPO-OH adduct, though small amounts of DMPO-R adduct and ascorbate radical are also present. The top panel shows no signal in the preischemic heart, while the following panels show signals obtained upon reperfusion at the times indicated.

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In order to determine whether xanthine oxidase-mediated free radical generation occurs in the reperfused heart and, if so, whether oxypurinol improved contractile function by preventing the generation of these radicals, we measured free radical generation using EPR spin trapping in the presence of the spin-trap DMPO. We have previously demonstrated that there is a burst of free radical generation upon reperfusion of the ischemic rabbit heart which can be detected with infusion of the spin-trap DMPO (19). Two other laboratories have also demonstrated that there is a similar EPR detectable burst of free radical generation upon reperfusion of the isolated rat heart (24, 25).

In the present study, we first sought to determine whether we could detect free radical generation in the reperfused rat heart. Therefore, rat hearts were subjected to 30 min of global ischemia at 37 °C followed by reperfusion in the presence of 40 mM DMPO. This low DMPO concentration was used because it has previously been shown that DMPO concentrations of greater than 50 mM can induce significant hemodynamic alterations (19). With a DMPO concentration of 40 mM, no hemodynamic alterations were seen. In the present study, control infusions of 40 mM DMPO produced no changes in coronary flow or developed pressure. Coronary flow was 14.8 ± 0.4 ml/min prior to DMPO infusion and 14.8 ± 0.4 ml/min immediately following the infusion. Similarly, developed pressure was 188 ± 13 mm Hg pre-DMPO and 182 ± 15 mm Hg post-DMPO infusion. The coronary effluent was collected in 30 s aliquots during both preischemic and postischemic infusion and EPR measurements were performed on each sample. No detectable spectrum was observed prior to ischemia; however, a prominent spectrum was seen immediately upon reflow (Fig. 4). This spectrum consists of a large 1:2:2:1 quartet signal with hyperfine coupling constants aQ = 14.9 G indicative of DMPO-OH, and a smaller 1:1:1:1:1 six-peaked spectrum with aQ = 16.0 and aN = 22.0 G indicative of DMPO-R. Maximum signal was observed in the first 20 s of reflow and then gradually declined over the next 10 min of reflow.

Experiments were performed in which either control or oxypurinol-treated hearts were subjected to 37 °C global ischemia followed by reperfusion in the presence of 40 mM DMPO. A marked decrease in the EPR signals observed during reflow was seen in the hearts treated with oxypurinol (Fig. 5). The time course of free radical generation upon reperfusion was measured, and a maximum free radical concentration was observed during the first 20 s of reflow with a peak concentration of 165 ± 48 nM in control hearts and 77 ± 22 nM with oxypurinol-treated hearts (Fig. 6). Oxypurinol thus decreased the peak radical concentration by approximately 54%, and a similar decrease was observed throughout
The Role of Xanthine Oxidase in Reperfusion Injury

CONTROL

OXYPURINOL

FIG. 5. EPR spectra prior to ischemia (top traces) and during the first 20 s of reflow (bottom traces) in a control and an oxypurinol-treated heart. As shown, the radical signals were markedly attenuated by oxypurinol.

FIG. 6. Total free radical concentration measured using 40 mM DMPO and EPR during the first 10 min of reperfusion. Symbols are defined as in Fig. 2. In control hearts an initial burst of radical generation was observed, with a gradual decline over 10 min. With oxypurinol, radical concentration was significantly reduced over the first 2 min of reflow (p = 0.01). Both groups showed equally low radical concentrations by 5 and 10 min of reflow.

FIG. 7. Component analysis of one spectrum obtained from a control heart. a–c are simulated spectra for DMPO-OH, DMPO-R, and DMPO-H, respectively. d shows the spectrum simulated from these individual components to match the experimental spectrum which appears in e.

Intracellular pH, PCr, and ATP levels before, during, and after 30 min of ischemia were measured in the isolated perfused rat heart using 31P NMR spectroscopy (Fig. 8). It was observed that the pH fell rapidly during ischemia from a baseline value of 7.1 to a minimum of approximately 5.9 within 20 min (Fig. 9). After reperfusion, the pH gradually returned to control values over a 20-min period. The PCr concentration also dropped rapidly during ischemia; however, only a partial recovery was observed upon reflow. After 45 min of reflow the PCr concentration in control hearts was only 16% of baseline values, while in the oxypurinol-treated hearts the PCr concentration returned to 29% of preischemic values (p < 0.005). The PCr to Pi ratio was even more dramatically improved in oxypurinol-treated hearts (Fig. 10) with a 5-fold increase in the PCr/Pi ratio (p < 0.005), although again only a partial recovery was observed with values of 1.6% in controls and 8.1% with oxypurinol. The ATP concentration fell during ischemia and showed no significant recovery after reperfusion. Oxypurinol had no effect on the extent to which ATP fell, nor on the recovery with reflow. The signal-to-noise ratio in the NMR spectra was approximately 23:1 for PCr and 16:1 for ATP, therefore even our minimal recovery values were within the limit of detection. Thus, hearts in which the...
enzyme xanthine oxidase was blocked exhibited identical alterations in high energy phosphates and pH during ischemia as in control hearts; however, a markedly improved bioenergetic state was observed in these hearts upon posts ischemic reflow as evidenced by the higher [PCr] and PCr/Pi ratio. The marked degradation of ATP which was observed during ischemia would give rise to substrate for the xanthine oxidase reaction.

**DISCUSSION**

We have shown that the inhibition of xanthine oxidase by oxypurinol improved recovery of myocardial function and coronary flow following 30 min of ischemia. Oxypurinol inhibited approximately 54% of radical generation, and also enhanced the recovery of phosphocreatine. Furthermore, the amount of xanthine oxidase relative to dehydrogenase was increased following ischemia as compared to control hearts. These results provide strong evidence that the enzyme xanthine oxidase significantly contributed to myocardial reperfusion injury in the isolated rat heart. The mechanism of benefit with xanthine oxidase blockade by oxypurinol seems to be primarily through quenching radical production, and secondarily through enhancing bioenergetic status. However, recovery of physiological function, even in the presence of oxypurinol, was only 31.5% of prereschmic values for contractile function and 50.2% for coronary flow. Therefore, xanthine oxidase mediated damage is a significant component of the overall injury but should not be construed as the sole source. It should also be noted that the present studies may not distinguish between ischemic injury and recovery following reperfusion, since the oxypurinol was administered both prior to ischemia and during the reflow period.

There has been considerable debate as to the presence and localization of xanthine oxidase in the hearts of different species. While it is fairly well accepted that the enzyme is present in rat heart, it is not clear whether it exists normally or whether it is proteolytically converted during ischemia. McCord et al. (8) reported a xanthine dehydrogenase/oxidase ratio of 0.82 in the blood-perfused ischemic rat heart with a half-time of 5 min. However, reports by Engerson et al. (26) and Chambers et al. (7) showed no significant conversion of dehydrogenase to oxidase in crystalloid-perfused isolated rat hearts following periods of global ischemia lasting 3 h in the former study and 30 min in the latter. Similarly, in the present study we showed no increase in the absolute amount of xanthine oxidase following 30 min of global ischemia. Like the Chambers et al. study (7), we measured approximately equal amounts of xanthine oxidase in control hearts and in ischemic hearts. We did, however, observe a decrease in the dehydrogenase/oxidase ratio with ischemia, and an even more marked decrease after 20 s of reflow, the time of peak radical generation, but this reflected a loss of the dehydrogenase rather than an increase in the oxidase form of the enzyme. It may be that rather than proteolytic conversion of the enzyme during ischemia, there is inactivation of the dehydrogenase form leading to a greater proportion of the superoxide-generating oxidase form. Alternatively, the reported differences could be due to differences between blood and crystalloid perfused tissue. Regardless of the mechanism, however, we have demonstrated that in the isolated perfused rat heart, xanthine oxidase can be measured under control, ischemic, or reflow conditions. The generation of radicals by the enzyme must therefore be dependent upon substrate availability. During ischemia, we observed a 6.5 mM drop in ATP which could potentially be metabolized to hypoxanthine and thus provide the necessary substrate for the xanthine oxidase reaction.

The present EPR results show that xanthine oxidase-mediated radical generation coincides with functional manifestations of myocardial reperfusion injury. Several other reports in the literature support the hypothesis that xanthine oxidase blockade enhances physiological recovery following reperfusion by preventing the generation of oxygen free radicals (7–9, 27). Chambers et al. (27) used both oxypurinol and allopurinol (a “suicide” substrate, (Ref. 28) in an isolated, perfused working rat heart model of ischemic cardiac arrest. They observed improvement in all indices of cardiac function with either xanthine oxidase inhibitor, and speculated that the mechanism was the prevention of the formation of reactive oxygen species. McCord et al. (8) observed significantly less release of creatine kinase (an index of cellular damage) with allopurinol in a similar model, and also concluded that the protection was correlated with an inhibition of radical generation. Charlat et al. (8) and Chambers et al. (7) drew the same conclusion in their studies of regionally ischemic dog hearts treated with allopurinol. In all of these studies it was, thus, inferred that allopurinol and/or its active metabolite, oxypurinol, entered into myocardial cells and blocked radical generation by the cytosolic enzyme xanthine oxidase.

It has previously been suggested that the xanthine oxidase...
Role of Xanthine Oxidase in Reperfusion Injury

The time course of intracellular pH, which recovers to 100% during reflow regardless of treatment, is shown in Fig. 9A. The time course of [PCr] also recovers significantly better with oxypurinol treatment; however, ATP showed no improvement. Das et al. (31) reported significantly higher levels of both phosphocreatine and ATP recovery with oxypurinol in pig hearts following 2 h of cardioplegic ischemia. DeWall et al. (6) reported significantly less uric acid production by guest on September 23, 2017 http://www.jbc.org/ Downloaded from blocker allopurinol may prevent free radical injury by scavenging 'OH rather than by specifically blocking 'O2 generation by xanthine oxidase (29). In studies of bovine aortic endothelial cells subjected to hypoxia and reoxygenation, it has been observed using EPR spin-trapping techniques that, indeed, allopurinol acted partially as a radical scavenger and partially as a blocker of 'OH generation, while oxypurinol simply blocked radical generation (30). It was observed that in the presence of allopurinol, additional alkyl radical adducts were formed, while with oxypurinol, there was simply a quenching of radical generation. In the present study, we observe that when oxypurinol, the active metabolite of allopurinol, is used to inhibit xanthine oxidase within the heart, a 54% decrease in the intensity of the radical signals generated upon reperfusion occurs. Both the hydroxyl and alkyl radical adducts were similarly decreased and no new alkyl or other radical adducts were observed. These results suggest that oxypurinol acts directly as an inhibitor of xanthine oxidase-mediated radical generation and not as a nonspecific radical scavenger.

Since the technique of spin-trapping measures the DMPO adduct signals in the heart effluent, it is important to note that both the DMPO and the free radicals equilibrate between the intra- and extracellular compartments. The propagation and/or transduction of radicals from intracellular sites to extracellular sites is likely to be a complicated process. Although it is likely that the radical adducts observed in the present study were derived from xanthine oxidase-mediated superoxide, it is possible that they were derived from other sources or perhaps were products of a series of radical propagation reactions. Furthermore, though we do measure a significant level of radical generation which is decreased by oxypurinol, it is not clear whether the initiating radical or the end products are the most important components in the damage to cellular macromolecules.

Another alternative hypothesis that oxypurinol reduces reperfusion injury by salvaging high energy phosphates was also considered in the present study. We showed that following 30 min of ischemia, the recovery of phosphocreatine was significantly better with oxypurinol treatment; however, ATP showed no improvement. Das et al. (31) reported significantly higher levels of both phosphocreatine and ATP recovery with oxypurinol in pig hearts following 2 h of cardioplegic ischemia. DeWall et al. (6) reported significantly less uric acid production accompanied by improved cardiac function in the presence of allopurinol following coronary ligation in dog and sheep hearts. They attributed the benefit of allopurinol to the prevention of the irreversible loss of purines from the cell during hypoxia. A similar conclusion was reached by Lasley et al. (32) who demonstrated improved functional recovery with higher levels of ATP and total adenine nucleotides following treatment with allopurinol in isolated-perfused rat hearts made globally ischemic for 10 min. Weiss et al. (33) described a protective effect of oxypurinol separate from its ability to block xanthine oxidase. They compared oxypurinol to amiflutilozole, another potent inhibitor of xanthine oxidase, and found that only oxypurinol showed protection in canine myocardium. Therefore, their results were also consistent with the possibility that oxypurinol works to attenuate reperfusion injury by some mechanism other than inhibition of xanthine oxidase, such as protection of high energy phosphates. Although the present study does not completely rule out the possibility of high energy phosphate salvage as a mechanism of action for oxypurinol, the fact that ATP concentrations were unaffected and radical concentrations were substantially decreased by this inhibitor suggests that radical

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**Fig. 9.** Data obtained from analysis of the NMR spectra from rat hearts during control, 30 min of global ischemia, and 45 min of reperfusion. Symbols are defined as in Fig. 2. A, time course of intracellular pH which recovers ~100% during reflow regardless of treatment. B, the time course of [PCr] which recovers significantly better in hearts treated with oxypurinol. C, the time course of [ATP] which decreases during ischemia and does not recover in either group during 45 min of reflow.

**Fig. 10.** Graph of the time course of the PCr/Pi ratio as a function of the time of reflow, obtained from analysis of the NMR spectra. A higher PCr/Pi ratio is observed in hearts treated with oxypurinol.
generation is the primary source of xanthine oxidase-mediated reperfusion injury in the rat heart.

Thus, xanthine oxidase is present in meaningful concentrations in rat hearts. When sufficient substrate becomes available to the enzyme following 30 min of ischemia and oxygen is reintroduced upon reperfusion, the enzyme gives rise to a burst of free radical generation as measured by the electron paramagnetic resonance studies. Associated with this radical generation or reperfusion injury in the isolated rat heart; however, it is clearly not the only source of radical generation or reperfusion injury in the postischemic heart.

**Acknowledgments**—We gratefully acknowledge Dr. Periannan Kuppusamy who made important contributions to this work regarding software development for transferring and analyzing the EPR spectra. We thank Stephanie Vathis for excellent secretarial assistance. We would also like to thank Drs. Joe McCord, jim Downey, Keith Reimer, and David J. Chambers for helpful discussions regarding this work.

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