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XANTHINE OXIDASE IS NOT RESPONSIBLE FOR REOXYGENATION INJURY IN ISOLATED-PERFUSED RAT HEART

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The massive leakage of intracellular enzymes which occurs during reoxygenation of heart tissue after hypoxic or ischemic episodes has been suggested to result from the formation of oxygen radicals. One purported source of such radicals is the xanthine oxidase-mediated metabolism of hypoxanthine and xanthine. Xanthine oxidase (O form) has been suggested to be formed *in vivo* by limited proteolysis of xanthine dehydrogenase (D form) during the hypoxic period (Granger *et al.*, *Gastroenterology*, **81**, 22 (1981)). We measured the activities of xanthine oxidase in both fresh and isolated-perfused (Langendorff) rat heart tissue. Approximately 32% of the total xanthine oxidase was in the O form in fresh and isolated-perfused rat heart. This value was unchanged following 60 min of hypoxia and 30 minutes of reoxygenation. The infusion of 250 μM allopurinol throughout the perfusion completely inhibited xanthine oxidase activity but had no effect on the massive release of lactate dehydrogenase (LDH) into the coronary effluent upon reoxygenation of heart tissue subjected to 30 or 60 min of hypoxia. Protection from 30 min of hypoxia was also not obtained when rats were pretreated for 48 h with allopurinol at a dose of 30 mg/kg/day and perfused with allopurinol containing medium. Superoxide dismutase (50 units/ml), catalase (200 units/ml), or the antioxidant cyanidanol (100 μM) also had no effect on LDH release upon reoxygenation after 60 min of hypoxia. Xanthine oxidase activity was detected in a preparation enriched in cardiac endothelial cells while no allupurinol-inhibitable activity could be measured in purified isolated cardiomyocytes. It is concluded that xanthine dehydrogenase is not converted to xanthine oxidase in hypoxic tissue of the isolated perfused rat heart, and that the release of intracellular enzymes upon reoxygenation in this experimental model is mediated by factors other than reactive oxygen generated by xanthine oxidase.

KEY WORDS: Heart, cardiomyocytes, hypoxia, xanthine oxidase, allopurinol, antioxidants.

INTRODUCTION

Reoxygenation injury in the heart, also known as the "oxygen paradox", was originally described by Hearse *et al.*[1] as the abrupt release of cytoplasmic constituents, including a variety of soluble enzymes, upon reoxygenation of the myocardium following a sustained period of hypoxic perfusion. Massive ultrastructural damage accompanies this release and a similar phenomenon occurs following ischemic episodes.[2].

Despite extensive investigation, the mechanism underlying the "oxygen paradox" remains unclear. The possibility that the sudden influx of oxygen results in the formation of oxygen radicals has received much attention. Oxygen radicals have been

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implicated in reperfusion injury in small intestine, stomach, liver, pancreas, kidney, heart, skin and brain. The source of such radicals is unknown and may vary with the tissue being studied. Recently, a mechanism by which oxygen radicals may arise following reoxygenation of ischemic tissue has been proposed.[3] There is a rapid breakdown of ATP to hypoxanthine during ischemia. At the same time, there is an influx of Ca^{++} which, this hypothesis suggests, may activate a latent protease. This protease would act on xanthine dehydrogenase (D form) converting it to xanthine oxidase (O form). Upon reoxygenation the formation of superoxide anions would then occur as hypoxanthine was metabolized to urate by the action of xanthine oxidase. Some evidence is available supporting this hypothesis. However, the postulated D to O conversion during hypoxia or ischemia appears not to have been well studied although the group which proposed this hypothesis has reported that the conversion occurs in various tissues of the rat[4] and in ischemic dog heart.[5]

The purpose of the present study was to assess these two enzymatic activities in freshly isolated and perfused rat heart tissue. The data demonstrated that, in contrast to these previous reports, this conversion did not occur in hypoxic-reoxygenated rat myocardium, although there was a substantial percentage of total xanthine oxidase activity present normally in the O form. In addition, allopurinol, an inhibitor of xanthine oxidase, and other antioxidants did not prevent the release of lactate dehydrogenase (LDH) during the reoxygenation of myocardial tissue subjected to 30 or 60 min of hypoxia.

MATERIALS AND METHODS

Dithioerythritol, phenylmethylsulfonyl fluoride (PMSF), NAD^+, allopurinol, xanthine and superoxide dismutase were obtained from Sigma (St. Louis, MO). Catalase, pyruvate, and NADH were obtained from Boehringer (Mannheim, FRG). Cyanidanol was obtained from Fluka (Buchs, Switzerland). All other chemicals used were of reagent grade.

Male Wistar rats, 170–250 g, fed on stock diet (Altromin), were used. The rats were anesthetized with pentobarbital and heparinized with 200 IU heparin through the inferior vena cava. Hearts were rapidly removed and placed into ice-cold Krebs-Henseleit bicarbonate solution containing 2.5 mM $CaCl_2$ and 10 mM glucose. Hearts were perfused with the same solution by the method of Langendorff[6] with a constant pressure of 80 cm water at 36°C as described previously.[7] The standard perfusion medium was equilibrated with 95% O_2, 5% CO_2 and all hearts were allowed to stabilize for 30 min with these media before initiating hypoxia. Hypoxic perfusions were performed for 30 or 60 min by equilibrating the same perfusion medium with 95% N_2, 5% CO_2.

Samples of the coronary effluent were collected continuously for determination of oxygen concentration and at selected intervals for the determination of coronary flow rates and LDH release. LDH activity, at 24°C, was determined spectrophotometrically at 340 nm in 100 mM triethanolamine HCl buffer, pH 7.6, containing 0.15 mM NADH, 1 mM EDTA, and 1.5 mM pyruvate. Enzyme activity is expressed as mU/g wet heart weight where 1 mU is that which oxidizes 1 nmol NADH per minute.

Xanthine oxidase activity was measured in the 27,000 × g supernatant fraction of tissues or cells homogenized in 0.05 M potassium phosphate buffer, pH 7.8, containing 1 mM PMSF and 10 mM dithioerythritol.[5,8] Whole hearts were homogenized

within 15 seconds of removal from the perfusion apparatus or the rat's body. These latter hearts (fresh controls) were briefly infused with oxygenated Krebs–Henseleit solution *in situ* to remove all blood. The reaction mixture contained 60 μM xanthine, and urate production was monitored at 293 nm in the absence of NAD^+ to determine the oxidase activity and in the presence of 0.67 mM NAD^+ to determine the oxidase plus the dehydrogenase activity. Reaction rates were corrected for nonspecific absorbance changes by subtracting any residual activity measured in the presence of 167 μM allopurinol. Enzyme activity was calculated as nmol urate formed/min/mg protein using an extinction coefficient of 12.6 $mM^{-1} cm^{-1}$ for urate. Protein was determined by the microbiuret method.[9]

Cardiomyocytes were isolated from ventricular myocardium of the rats described above by a modification of a previously described procedure.[10] This modification consisted of centrifuging the dispersed cells for 1 min at 25 × g through a 4% albumin solution two times: once before and once after the addition of 1 mM Ca^{++} to the cell suspension. Small cells and debris were effectively removed by this procedure and more than 80% of the remaining myocytes were rod-shaped. In a single experiment, these cells were cultured for 4 h in medium 199 (Boehringer, Mannheim, FRG) with 4% fetal calf serum. The dishes were washed with Krebs-Ringer solution, which leaves only attached, rod-shaped myocytes,[10] before the cells were tested for xanthine oxidase activity. A fraction enriched in endothelial cells was obtained by pelleting, at 200 × g the combined supernatants from all centrifugation steps performed for the selection of cardiomyocytes.

Data are expressed as mean ± S.E. Multiple groups were analyzed by one way analysis of variance, and comparisons between groups were done with Newman-Keuls or Students' t test.[11]

RESULTS

Isolated cardiac myocytes contained no allopurinol-inhibitable xanthine oxidase activity (Table I). The same was true in a preparation cultured for 4 h to allow recovery from the isolation procedure and to remove remaining debris. In contrast, a preparation enriched in cardiac endothelial cells contained significant xanthine oxidase activity which was inhibited by allopurinol (Table I). The percentage of the O form was the same as in whole heart tissue. Although at least a ten-fold increase in specific activity was expected if all xanthine oxidase activity had been recovered in

TABLE I
Xanthine oxidase activity in rat heart

Sample	O Form[1]	D + O Form[1]	% O Form
cardiomyocytes	< 0.03 (3)	< 0.03 (3)	–
cardioendothelial cells	0.34 ± 0.09 (2)	1.26 ± 0.50 (2)	26.8 ± 2.1 (2)
fresh heart	0.25 ± 0.02 (7)	0.79 ± 0.05 (7)	31.8 ± 1.7 (7)
oxygenated heart[2]	0.34 ± 0.06 (4)	0.93 ± 0.13 (4)	36.6 ± 2.8 (4)
hypoxic/reperfused heart[3]	0.36 ± 0.05 (4)	1.04 ± 0.09 (4)	34.8 ± 4.6 (4)

[1] Data are expressed as nmol urate formed/min/mg protein ± S.E. Values in parentheses = *n*.
[2] Hearts were perfused for 2 h with oxygenated medium.
[3] Hearts were perfused for 30 min with oxygenated, 60 min with hypoxic, and 30 min with oxygenated media.

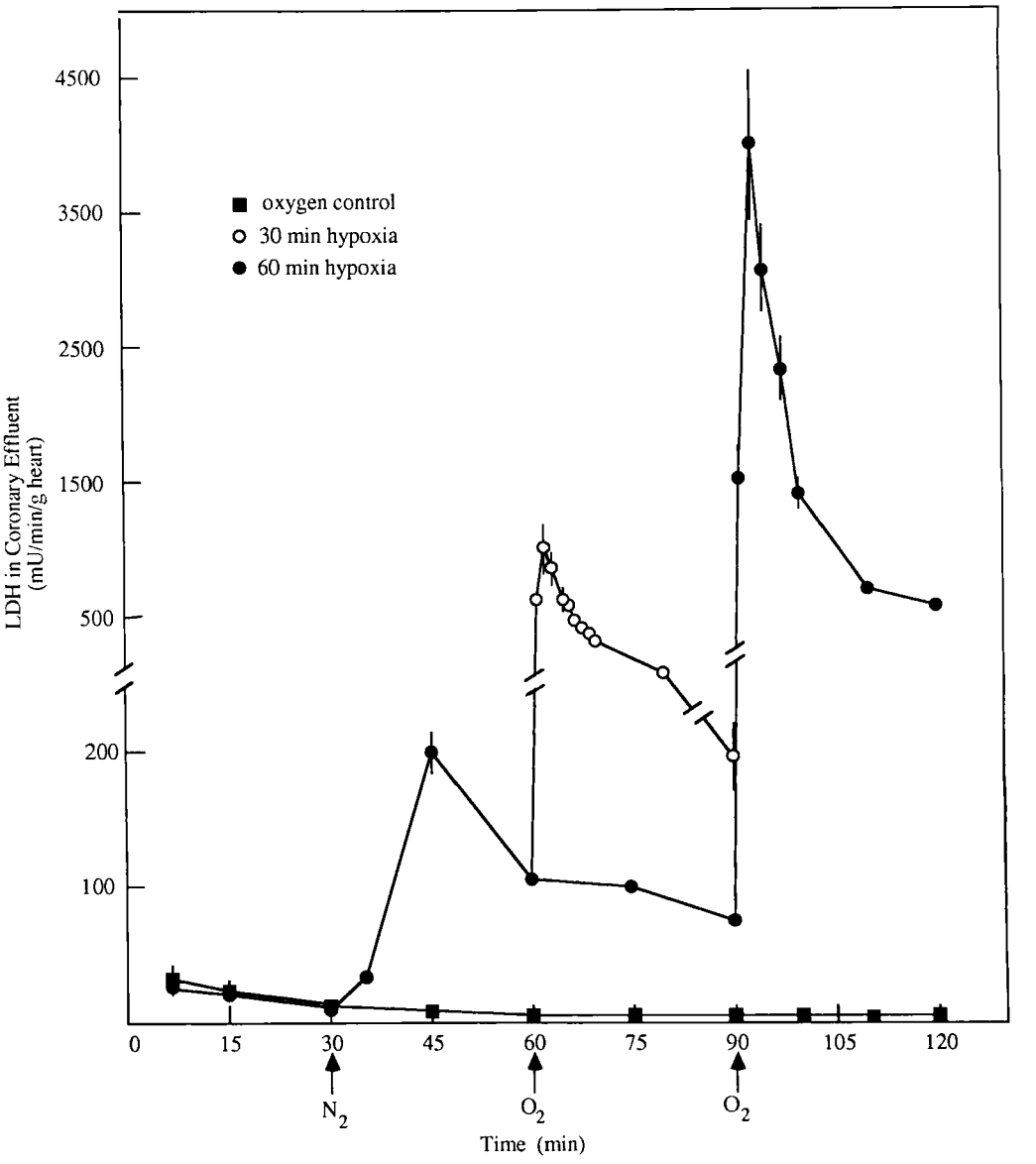

FIGURE 1 LDH released into the coronary effluent from isolated perfused hearts. Oxygen control hearts were perfused with oxygenated Krebs-Henseleit solution throughout. Hypoxia and reoxygenation were performed at the indicated times. *n* values ranged from 4–6 for each treatment. LDH release did not differ up to the time of reoxygenation and the data up to 60 min from all hypoxic hearts were, therefore, pooled. Data are expressed as mean ± S.E.

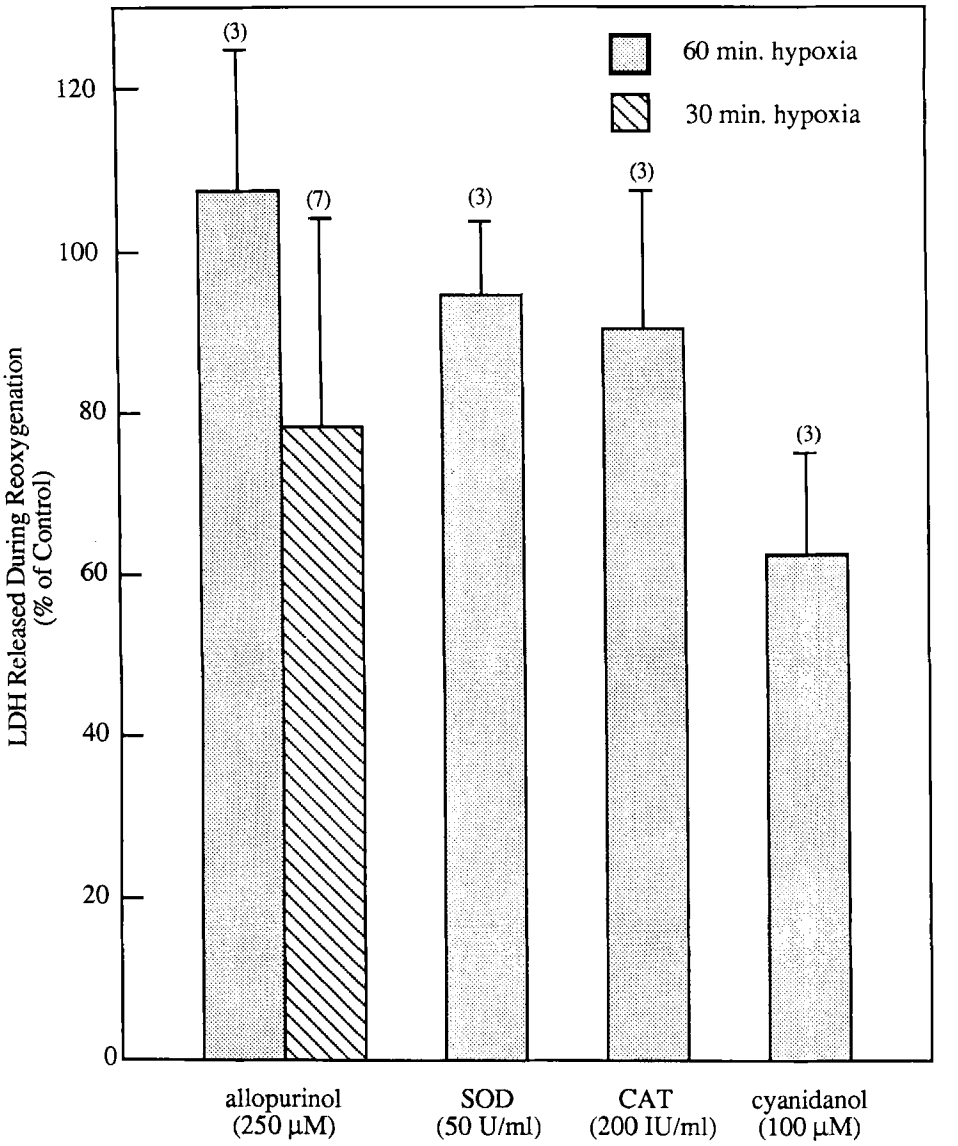

FIGURE 2 LDH released upon reoxygenation following treatment with various antioxidants. Allopurinol was present throughout the perfusion. Superoxide dismutase (SOD) and catalase (CAT) were added at the time hypoxia was initiated and cyanidanol was added 15 min before hypoxia. LDH release was measured during 10 min of reoxygenation. Control LDH release after 30 and 60 min of hypoxia averaged 611 ± 161 (n = 7) and 2474 ± 615 mU/min/g heart(n = 4), respectively. Data are expressed as mean ± S.E. Values in parentheses = n. There were no significant differences from hypoxic control values with any treatment ($p > 0.05$).

the endothelial cell fraction, there was only a slight increase, compared to fresh heart, perhaps due to losses during the isolation procedure.

Analysis of myocardial tissue following a 2 h perfusion with oxygenated Krebs-Henseleit solution did not reveal any significant changes in the percentage of xanthine oxidase activity in the O form, compared to fresh heart tissue. Perfusing the myocardium for 30 min with oxygenated medium followed by 60 min with hypoxic medium and a subsequent 30 min of reoxygenation also had no significant effect on this percentage (Table I).

All hypoxic perfused hearts released an increased amount of LDH at 45 min (15 min of hypoxia). This release gradually declined, but remained elevated above nonhypoxic hearts, to 90 min (Fig. 1). There was a much greater release of LDH activity into the coronary effluent within 2 min of reoxygenation after either 30 or 60 min of hypoxia (Fig. 1). Hearts perfused with oxygen-saturated Krebs medium for 2 h released less than 10 mU of LDH/min/g heart during this period (Fig. 1).

The addition of allopurinol (250 μM) to the perfusate for the entire perfusion period completely inhibited xanthine oxidase activity but had no effect on LDH release upon reoxygenation after 30 or 60 min of hypoxia (Fig. 2). Pretreatment with allopurinol at 30 mg/kg/day for 2 d before perfusing with allopurinol-containing medium was also unable to block LDH release upon reoxygenation after 30 min of hypoxia. These data were, therefore, combined with that from non-pretreated hearts perfused with allopurinol containing medium.

The addition of superoxide dismutase (50 U/ml) or catalase (200 IU/ml) to the perfusate, beginning at the time of hypoxia and continuing throughout the remainder of the perfusion, had no significant effect on LDH release upon reoxygenation after 60 min of hypoxia (Fig. 2). A similar lack of protection was seen following the addition of the antioxidant cyanidanol (100 μM) to the perfusate from 15 to 120 min (Fig. 2). LDH release was not increased in the presence of cyanidanol alone.

There were no treatment-related effects in coronary flow or oxygen uptake evident between the various groups (data not shown). Coronary flow increased in response to hypoxia in all experiments. Severe contracture and an obvious loss of myoglobin were evident upon reoxygenation after 60 min of hypoxia. There was also a decrease in oxygen uptake in these hearts suggestive of deterioration or a decreased demand due to the absence of normal mechanical activity in all treatment groups. All but one heart resumed normal beating upon reoxygenation following 30 min of hypoxia although fibrillation occurred in two of seven non-allopurinol treated hearts.

The specific activity (per mg protein) of the O form of xanthine oxidase was approximately the same in freshly isolated rat liver and heart tissue while that of the D form was five-fold higher in liver than in heart (Tables I and II). The percentage of xanthine oxidase activity in the O form was, therefore, 12% in liver and 32% in heart. This percentage was unaffected in liver homogenates frozen for 24 h before assay if dithioerythritol and PMSF were present in the buffer. In the absence of these agents, the percentage of xanthine oxidase in the O form increased to 41% (Table II).

DISCUSSION

D to O Conversion of Xanthine Oxidase

The localization of xanthine oxidase in the capillary endothelial cells has been well documented by immunologic techniques.[12,13] The data presented here demonstrate,

TABLE II
Xanthine oxidase activity in rat liver

Sample	O Form[1]	D Form[1]	% O Form
fresh liver[2]	0.37 ± 0.08 (3)	2.95 ± 0.47 (3)	12.3 ± 1.2 (3)
liver after 24 hr[3]	0.37 ± 0.01 (2)	2.77 ± 0.33 (2)	13.8 ± 1.8 (2)
liver after 24 hr without inhibitors[4]	1.06 ± 0.17 (2)	2.57 ± 0.47 (2)	41.5 ± 1.0 (2)

[1] Data are expressed as nmol urate formed/min/mg protein ± S.E. Values in parentheses = *n*.
[2] Liver was homogenized in buffer containing dithioerythritol and PMSF and assayed immediately.
[3] Liver was homogenized in buffer containing dithioerythritol and PMSF, frozen for 24 h, thawed and assayed.
[4] Liver was homogenized in buffer without dithioerythriol and PMSF, frozen for 24 h, thawed and assayed.

for the first time by an enzymatic assay, that isolated cardiomyocytes lack any measurable xanthine oxidase activity. Severe contraction band necrosis occurs during reoxygenation injury.[14] Despite the loss of 10–30% of tissue LDH, the specific activity of xanthine oxidase was actually slightly higher in reoxygenated heart tissue. Since xanthine oxidase activity is localized in endothelial cells, this may be due to the loss of myocyte, but not endothelial, cell protein suggesting that the cells which contain xanthine oxidase are less susceptible to reoxygenation injury.

The D to O conversion of xanthine oxidase was extensively studied by Stirpe and Della Corte in liver tissue.[8,15,16] This process can occur both reversibly by sulfhydryl oxidation and irreversibly by proteolysis. We prevented irreversible conversion after homogenization by including PMSF in the assay buffer. Dithioerythritol was also present thereby preventing the reversible conversion. When these agents were omitted, the D to O conversion could be observed in frozen and thawed liver homogenates as reported previously.[8,16] Xanthine oxidase does not undergo an irreversible D to O conversion in hypoxic/reoxygenated perfused rat heart tissue (Fig. 1, Table I). This is in contrast to observations with rat heart suggesting conversion to nearly 100% type O within 15 min[17] and to results in dog heart where the percentage in the O form increased from 10 to 33% following ischemia.[5]

Battelli *et al.*[15] originally noted that all measurable xanthine oxidase activity in rat heart tissue was in the D form. This finding conflicts with results from dog myocardium[5] and a more recent report on rats from Oei *et al.* which indicated that 24% is normally in the O form.[18] This latter percentage differs from an earlier value of 15% by some of the same workers,[19] but is virtually identical to our results suggesting that, at least in the rat, a significant level of oxidase activity is normally present. Importantly, the specific activity of the O and the D forms in heart and liver were also similar to our work and that of Oei *et al.* [18] All of these relative values for the O form are higher than the maximum of 10% previously reported in healthy tissue.[3,4]

Effects of Allopurinol

Xanthine oxidase activity could still be involved in reoxygenation injury even if the conversion did not occur, since it is present initially at appreciable levels. However, infusing allopurinol at a concentration sufficient to completely inhibit xanthine oxidase activity had no effect on the release of intracellular enzymes from reoxygenated heart tissue. This contrasts with reports in dogs, rabbits and rats,[20–23] but is in agreement with results that allopurinol has no beneficial effects against reperfusion injury in dogs.[24,25] Comparison among these studies must be done with caution,

however, since in addition to the variations discussed above, different mechanisms may be involved between global or partial ischemia and hypoxia *in vivo* or in a perfused organ. For example, although the hypothesized accumulation of hypoxanthine may occur during complete ischemia, collateral flow is sufficient to wash out purines from the ischemic area in partially occluded models of ischemia and reperfusion.[26] Accumulation is also unlikely during hypoxia where cardiac perfusion is maintained. Nevertheless, reoxygenation injury is evident in these models, suggesting that such an accumulation of hypoxanthine is unnecessary.

Interestingly, reports of allopurinol's effectiveness in ameliorating reperfusion injury have largely involved animals which were pretreated for 24–48 h before the ischemic episode[5,21] or were perfused at decreased temperatures.[20] The requirement for pretreatment has been related to the metabolism of allopurinol to oxypurinol, which is the actual inhibitory species against xanthine oxidase. However, since xanthine oxidase itself rapidly performs this metabolic step, the ability of allopurinol to protect under these conditions may involve some other mechanism such as enhancing electron transport.[27] This ability could also increase the reduction of dyes used to assess infarct size thereby producing misleading results regarding the protective ability of allopurinol.[27]

In the present study, allopurinol failed to prevent the reoxygenation-induced release of LDH even when animals were pretreated and the drug was present throughout the perfusion period. We did, however, observe a decrease in fibrillation upon reoxygenation after 30 min of hypoxia (data not shown). This effect of allopurinol has been reported previously,[26] and could explain the functional improvement seen in some model systems of reoxygenation injury.

Differences Between Models of Reoxygenation Injury

Studies of reoxygenation injury in the heart have utilized a variety of *in vivo* and *in vitro* model systems. Unfortunately, there has been no standard time of oxygen deprivation or of perfusion temperature, and the conflicting results obtained following treatment with various substances are difficult to explain. Both perfusion temperature and the period of hypoxia or ischemia are critical variables for the massive release of intracellular enzymes upon reoxygenation.[2,24] Studies examining reperfusion injury at lower temperatures or with shorter times of hypoxia may not, therefore, be examining the same molecular events as those at higher temperatures and longer times of hypoxia. Furthermore, *in vivo* studies permit normal physiological responses, such as the infiltration of leukocytes, which may play a prominent role in radical generation during reperfusion injury.[28]

Although the massive release of intracellular enzymes upon reoxygenation of hypoxic heart tissue may not be representative of the less severe injury obtained with regional ischemia, the absence of any D to O conversion in heart, and the inability of allopurinol to provide protection, indicate that neither this conversion nor xanthine oxidase activity is involved in the oxygen paradox in isolated perfused rat heart tissue. The hypothetical role of xanthine oxidase in the production of oxygen radicals during reoxygenation has been widely cited even though the production of oxygen radicals has not been established in reoxygenated tissues. The positive results within antioxidants in some, but not all, models of reoxygenation injury are certainly suggestive that oxygen radicals are involved in some aspects of this process. In particular, studies in intestinal models of ischemia suggest that this phenomena may occur in this tissue.[29]

The mechanisms underlying reoxygenation injury in blood-free isolated perfused

heart tissue after episodes of ischemia or hypoxia remain unclear. One study has reported that superoxide dismutase or catalase could block the oxygen paradox in isolated perfused rat heart[22] while another reported protection with catalase, allopurinol, or desferoxamine, but not superoxide dismutase, in rabbit hearts.[23] This latter report found no difference, however, whether these substances were present only during the hypoxic period or also during reoxygenation, and functional recovery did not occur. Thus, factors other than oxygen radicals may be involved. Additional studies have demonstrated protective effects on cardiac function, arrhythmias or infarct size with these substances in other models of reperfusion injury.[30–33] In contrast, our work failed to find beneficial effects with these substances in the perfused heart. We also obtained no significant protection with the naturally occurring flavonoid antioxidant cyanidanol which is water-soluble and should function as an effective radical scavenger in biologic systems.[34]

Some of these conflicting results could arise from differences in the model systems as described above, or they may relate to the different endpoints used to assess the status of the hearts. For example, evidence of functional improvement has been obtained following treatment with superoxide dismutase even though the release of intracellular enzymes was not altered.[35] Thus, reoxygenation may trigger the release of intracellular enzymes from cells damaged during hypoxia without the intervention of radicals. Oxygen radical-mediated damage, if it occurs, may be reflected by measuring a different endpoint such as cardiac function or capillary permeability. Another possibility is that the window in time when treatment with antioxidant substances can exert a protective effect is narrow. We studied both 30 and 60 min of hypoxia in an attempt to examine this question. The absence of any protection at the 30 min time period indicates that such a window, if it exists, is less than 30 min long. However, McCord and associates reported protecting perfused rat hearts from reoxygenation injury with allopurinol, superoxide dismutase and catalase following 40 min of hypoxia using glucose free medium.[32] Our data, under even less severe conditions, directly contradict these findings.

Together, these data suggest that neither xanthine oxidase nor oxygen radicals are involved in reoxygenation injury in this model system. The sudden release of intracellular enzymes upon reoxygenation may be related to the mechanical events of contracture.[36] This could be triggered by the reformation of ATP since cyanide completely prevents the release of intracellular enzymes.[37] Calcium is also involved since its removal from the perfusion medium prevents this effect.

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