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ABSENCE OF XANTHINE OXIDASE OR XANTHINE DEHYDROGENASE IN THE RABBIT MYOCARDIUM

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We directly measured the activity of the enzymes xanthine oxidase and xanthine dehydrogenase in rabbit and rat hearts, using a sensitive radiochemical assay. Neither xanthine oxidase activity nor xanthine dehydrogenase activity was detected in the rabbit heart. In the rat heart, xanthine oxidase activity was 9.1 ± 0.5 mIU per gram wet weight and xanthine dehydrogenase activity was 53.0 ± 1.9 mIU per gram wet weight. These results argue against the involvement of the xanthine oxidase/xanthine dehydrogenase system as a mechanism of tissue injury in the rabbit heart, and suggest that the ability of allopurinol to protect the rabbit heart against hypoxic or ischemic damage must be due to a mechanism other than inhibition of these enzymes.

Recent studies have emphasized the participation of toxic oxygen metabolites in acute cardiac injury caused by ischemia or hypoxia (1). One postulated source of these metabolites is the enzyme xanthine oxidase which is thought to be formed from xanthine dehydrogenase by calcium-activated proteases during ischemia (2). Ischemia and hypoxia also activate a cascade of purine degradation to increase the availability of substrates for xanthine oxidase and xanthine dehydrogenase, hypoxanthine and xanthine (3,4). Upon reperfusion or reoxygenation, oxidation of these substrates concomitantly forms the univalent reduction products of molecular oxygen, O_2^- and H_2O_2, which can react with transition metals (e.g., Fe^{3+}) to form highly toxic $\cdot OH$ (5). The ability of allopurinol to reduce acute ischemic or hypoxic damage to hearts of various species, including rabbit (6-8), may therefore have great applicability to human medicine. The major pharmacologic action of this drug, inhibition of xanthine oxidase, further supports the injury mechanism outlined above. Nevertheless there are data (9-11) which do not support this scheme, at least in rabbit myocardium. In particular, we have shown that purines washed out of reperfused ischemic rabbit heart do not include expected products of xanthine oxidase or xanthine dehydrogenase activity, xanthine and

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uric acid (11). This indirectly suggests an absence of the xanthine oxidase/dehydrogenase enzyme system. Xanthine oxidase and xanthine dehydrogenase activity have never directly been measured in the rabbit myocardium, but have been demonstrated in the rat myocardium (12). The absence of this enzyme system in nonischemic rabbit heart would certainly make it an unlikely participant in the ischemic/hypoxic damage process.

In this paper we report the absence of directly measured xanthine oxidase and xanthine dehydrogenase in the rabbit myocardium using a radiochemical assay. This novel finding implies: 1) a species variation of the myocardial xanthine oxidase/dehydrogenase enzyme system and consequently a variation in the mechanisms of ischemic injury, 2) that the mechanism of allopurinol cardioprotection cannot be universally ascribed to its inhibition of xanthine oxidase, 3) the extension of animal data and pharmacologic approaches to protect the myocardium from ischemic damage may or may not be applicable to the human situation.

METHODS

Animals: Six New Zealand rabbits and a parallel set of six Sprague-Dewey rats were used for these experiments. The rats were killed by decapitation, the rabbits by cervical dislocation and exsanguination. Hearts were immediately excised and perfused in a retrograde fashion (Langendorff) with oxygenated (95% oxygen and 5% carbon dioxide) physiologic saline solution for 5 min (7,8,13). The ventricles were removed and immediately immersed in liquid nitrogen. The frozen ventricles were weighed (wet weight) and immediately placed in an ice cold buffer containing 0.125 M tris(hydroxymethyl)aminomethane, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM EDTA, at a pH of 9.0. The dithiothreitol and phenylmethylsulfonyl fluoride prevent in vitro conversion of xanthine dehydrogenase to xanthine oxidase (14). The heart tissue was minced with scissors and homogenized in 9 ml of buffer for each gram of tissue. The homogenate was spun at 12,000 g for 20 min at 4°C. The supernatant was dialyzed at 4° C in a 200:1 dilution of the buffer overnight to remove enzyme inhibitors and endogenous purines. Aliquots were then assayed for enzyme activity.

Enzyme Assay: The radiochemical enzyme assay for xanthine oxidase/dehydrogenase has been described elsewhere (15,16). The buffer constituents and the pH were modified to optimize the enzyme activity according to the method of Mousson et al. (17). The reaction mixture contained 30 µl of buffer and 10 µl of [8-^{14}C]hypoxanthine (final concentration of 95 µM), warmed to 37°C in a shaking water bath before 10 µl of the dialyzed supernatant (sample) was added to start the reaction. Six aliquots of each sample were reacted with [8-^{14}C]hypoxanthine under different conditions. Aliquot #1: To assess the combined activities of xanthine oxidase and xanthine dehydrogenase, NAD^+ (final concentration 1 mM) was included in the reaction mixture (18). Aliquot #2: Identical samples run in the absence of added NAD^+ were used to assess xanthine oxidase activity. The difference between the two values, if any, was used to calculate enzyme activity due to xanthine dehydrogenase alone. Aliquots #3 and #4: Two other aliquots (with and without NAD^+) were run in the presence of allopurinol (1 mM final concentration) to assess its ability to pharmacologically inhibit the enzymes. All sample counts were adjusted by subtracting from the raw counts the counts obtained by controls (aliquots #5 and #6) to which perchloric acid (283 mM final concentration) was added to the reaction medium prior to the addition of dialyzed supernatant. All the mixtures were incubated in a shaking water bath at 37°C for 15 min, as Watts

et al. have shown nonlinearity of the reaction rate after incubation times in excess of 20 min (19). The reaction was stopped by adding 10 µl perchloric acid (280 mM final concentration) and immediately chilling the reaction mixture in ice. The reaction mixture was spotted on electrophoresis paper and the substrate and products of the reaction were separated with high voltage electrophoresis as previously described (16). The spots were identified with ultraviolet light, cut, and radioactivity counted by liquid scintillation spectrometry. Enzyme activity was computed and is expressed in mIU per gram wet tissue weight, which corresponds to 1 nmol of hypoxanthine oxidized per minute.

RESULTS

No xanthine oxidase or xanthine dehydrogenase activity was detected in the rabbit hearts. Under conditions used to assess xanthine oxidase and xanthine dehydrogenase activity of dialyzed rabbit heart supernatants, the radioactivity of electrophoresis spots containing xanthine plus uric acid minus the background counts (perchlorate-pretreated samples) was 2 ± 28 counts/min. This mean count was not different from the background count ($p > 0.9$; paired t-test). Furthermore, no individual radioactivity value in the enzyme assay mixture exceeded the background radioactivity by one standard deviation. The corresponding activity measured under conditions to assess xanthine oxidase activity minus background was -2 ± 20 counts/min. Again, this mean was not different from background ($p > 0.9$) and no individual count exceeded the background by one standard deviation. This indicates that there was no xanthine oxidase or xanthine dehydrogenase activity detected in rabbit

Table 1: Xanthine Oxidase and Xanthine Dehydrogenase Activities in Non-ischemic Rabbit and Rat Ventricular Myocardium*

	Enzyme Activity (mIU/g wet weight)	
	Xanthine Oxidase + Xanthine Dehydrogenase	Xanthine Oxidase
Rabbit (N = 6)	none detected	none detected
Rat (N = 6)	62.1 ± 2.2	9.1 ± 0.5
Inhibition by 1 mM allopurinol	99.9%	100%

*The reaction buffer constituents are described in the Methods section. Xanthine oxidase and xanthine dehydrogenase activities were obtained by assaying formation of xanthine and uric acid from labelled hypoxanthine in the presence of 1 mM NAD^+, for 15 min as described in the text. Xanthine oxidase activity was measured in the absence of NAD^+. Pharmacologic inhibition of xanthine oxidase or xanthine dehydrogenase activity was assessed by including 1 mM allopurinol in the reaction medium, with or without NAD^+. Nonenzymatic formation of xanthine and uric acid was measured by perchloric acid inactivation of dialyzed supernatant prior to incubation of the reactants, and the obtained value (counts/min) was subtracted from those of all other samples. One mIU of enzyme equals the oxidation of 1 nmol of hypoxanthine per minute.

myocardial samples. A second set of six rabbits were also assayed after 20 minutes of ischemia prior to immersion in liquid nitrogen. Again, no xanthine oxidase or xanthine dehydrogenase activity was detected. Finally, dialyzed supernatants from two additional rabbit hearts were reacted with [8-^{14}C]hypoxanthine for 60 minutes, yet no xanthine oxidase activity was measured even with this prolonged reaction time. In contrast, spots obtained with rat myocardial samples incubated with NAD^+ were 7,691 ± 283 counts/min above background ($p < 0.001$), and those incubated without NAD^+ were 1,258 ± 58 counts/min above background ($p < 0.001$). The computed enzyme activities are shown in Table 1 along with allopurinol inhibition data. The calculated value of xanthine dehydrogenase activity in the rat myocardium from this data is 53.0 ± 1.9 mIU/g wet weight. The counts/min for the allopurinol containing reaction mixtures in rat ventricular homogenate also were not different from the background counts ($p \geq 0.7$).

DISCUSSION

Allopurinol protection of the myocardium against ischemic injury has been postulated to be due to its inhibition of the enzyme xanthine oxidase (1,2). Although allopurinol has shown to be protective in both rabbit and rat ischemic/hypoxic heart models (6,8,13), recent data has raised the suspicion that rabbit heart may lack xanthine oxidase and xanthine dehydrogenase (9-11). This experiment shows by direct measurement the absence of xanthine oxidase or xanthine dehydrogenase in the rabbit myocardium.

Our values for both xanthine oxidase and xanthine dehydrogenase in the rat hearts are slightly higher than values previously reported (12). This most likely is due to the fact that we optimized a sensitive radiochemical assay for enzyme activity, whereas prior studies used a less sensitive spectrophotometric assay (12,18). Despite the optimized assay conditions, no activity was seen in the rabbit myocardium. This study therefore documents species variability of the xanthine oxidase and xanthine dehydrogenase enzyme system. Our findings suggest that the mechanism of ischemic or hypoxic myocardial injury is species specific, at least as it pertains to purine catabolism or oxygen radical generation by the enzyme that we studied. Although toxic oxygen metabolites have been implicated as participating in injury to the ischemic or hypoxic rabbit myocardium, they must be generated by a mechanism other than xanthine oxidase.

This study also implies that the mechanisms of allopurinol cardioprotection are more varied than previously thought. Although inhibition of xanthine oxidase may be important in the rat, this is clearly not the case in the rabbit. Allopurinol may have direct myocardial protective effects either by vasodilation (20), by enhancing purine salvage (21), or through other metabolic effects (22).

Since there is little data concerning the presence of activity of these enzymes in human myocardium, at least as assessed by current methods using fresh tissue and controlled experimental conditions, we suggest caution in extending the results of animal data to the human situation.

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REFERENCES

1. McCord, J.M., and Roy, R.S. (1982) Can J Physiol Pharmacol 60, 1346-1352.
2. McCord, J.M. (1985) N Engl J Med 312, 159-163.
3. Fox, I.H. (1981) Metabolism 30, 616-634.
4. Grum, C. M., Simon, R. H., Dantzker, D. R., and Fox, I. H. (1985) Chest 88, 763-767.
5. McCord, J.M., and Fridovich, I. (1968) J Biol Chem 243, 5753-5760.
6. Chambers, D.W., Parks, D.A., Patterson, G., Roy, R., McCord, J.M., Yoshida, S., Parmley, L.F. and Downey, J.M. (1985) J Mol Cell Cardiol 17, 145-152.
7. Myers, C.L., Weiss, S.J., Kirsh, M.M., Shepard, B.M., and Shlafer, M. (1986) J Thorac Cardiovasc Surg 91, 281-289.
8. Myers, C. L., Weiss, S. J., Kirsh, M. M., and Shlafer, M. (1985) J Mol Cell Card 17, 675-684.
9. Ketai, L., Grum, C., Myers, C., and Shlafer, M. (1985) Circulation 72(III), 357.
10. Yellon, D.M., Richard, V., Hearse, D.J., deLeiris, J., Maxwell, M., Kithas, P., Frank, S., Artman, M., and Downey, J.M. (1985) Fed Proc 44, 1480.
11. Grum, C.M., Ketai, L. H., Myers, C. L., Shlafer, M. Am J Physiol (in press).
12. Schoutsen, B., DeJong, J.W., Harmsen, E., DeTombe, P.P., and Achterberg, P.W. (1983) Biochim Biophys Acta 762, 519-524.
13. Shlafer, M., Kane, P.F., and Kirsh, M.M. (1982) J Thorac Cardiovasc Surg 83, 830-839.
14. Batelli, M. G., Della Corte, E., Stirpe, F. (1972) Biochem J 126, 747-749.
15. Boss, G. R., Ragsdale, R. A., Zettner, A., and Seegmiller, J. E. (1980) J Lab Clin Med 96, 783-789.
16. Sinclair, D.S., and Fox, I.H. (1975) J Rheumatol 2, 437-445.
17. Mousson, B., Desjacques, P., and Baltassat, P. (1983) Enzyme 29, 32-34.
18. Stirpe, F., and Della Corte, E. (1969) J Biol Chem 244, 3855-3863.
19. Watts, R. W. E, Watts, J. E. M., and Seegmiller, J. E. (1965) J Lab Clin Med 66, 688-697.
20. Dewall, R.A., Vasko, K.A., Stanley, E.L., and Kezdi P. (1971) Am Heart J 82, 362-370.
21. Edwards, N.L., Recker, D., Airozo, D., and Fox, I.H. (1981) J Lab Clin Med 98, 673-683.
22. Crowell, J.W., Jones, C.E., and Smith, E.E. (1969) Am J Physiol 216, 744-748.