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ROLE OF XANTHINE OXIDASE INHIBITOR AS FREE RADICAL SCAVENGER: A NOVEL MECHANISM OF ACTION OF ALLOPURINOL AND OXYPURINOL IN MYOCARDIAL SALVAGE

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Xanthine oxidase (XO) has been hypothesized to be a potential source of oxygen-derived free radicals during reperfusion of ischemic myocardium based on the fact that allopurinol, a XO-inhibitor, can reduce reperfusion injury. In this communication we report that both allopurinol and oxypurinol, the principle metabolite of allopurinol, prevent the reperfusion injury in isolated pig heart. However, we found that neither pig heart nor pig blood contain any XO activity. Our study showed a direct free radical scavenging action of these XO-inhibitors during ischemia and reperfusion, as judged by the reduction of free radical signals when compared using an Electron Paramagnetic Resonance Spectrometer. Using a Luminometer, we also confirmed that both allopurinol and oxypurinol can scavenge $ClO_2^{\cdot}$, HOCl, and significantly inhibit free radical signals generated by activated neutrophils. These XO-inhibitors, however, failed to scavenge $O_2^{-\cdot}$ and $OH^{\cdot}$ radicals. Our results suggest that these XO-inhibitors salvaged the ischemic-reperfused myocardium by scavenging free radicals, and not by inhibiting XO in the pig heart. © 1987 Academic Press, Inc.

It is well established that oxygen-derived free radicals play a crucial role in the pathogenesis of ischemic-reperfusion injury (1-3). However, the source of these radicals remains unknown. McCord has proposed that oxygen radicals are generated during reoxygenation of ischemic myocardium by the action of XO on hypoxanthine (4). A number of investigators supported this hypothesis based on their observations of allopurinol-mediated reduction of reperfusion injury (5-7). These authors believe that ATP is degraded during ischemia progressively to yield hypoxanthine, which then acts as substrate for XO, which is also being produced during ischemia from xanthine dehydrogenase by the action of a protease. Recently, the beneficial action of allopurinol to salvage ischemic-reperfused myocardium has been challenged by Reimer et al (8).

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Abbreviations: DTPA: diethylenetriamine-pentaacetic acid.
KTBA: 2-keto-4-thiomethylbutyric acid.
FMLP: n-formyl-methionyl-leucyl-phenylalanine.
ZAS: zymosan activated serum.

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In addition, most of the mammalian hearts, with the exception of rat heart, have been found to contain negligible or no XO activity at all (9).

The purpose of the present study was, therefore, to investigate the role of two XO inhibitors, allopurinol and oxypurinol, on the ischemic-reperfused heart and to explore their mechanisms of action. Our results suggest that these XO inhibitors salvage the ischemic-reperfused myocardium by scavenging the free radicals, and not by inhibiting XO enzyme.

METHODS

Yorkshire pigs of either sex, weighing between 20-25 kg, were properly anesthetized, placed on positive pressure artificial respiration, and median sternotomy was performed. The animals were then placed on cardiopulmonary bypass, and hearts were isolated from the systemic circulation as described elsewhere (10). Hearts were allowed to stabilize for 15 min and then pre-perfused for a further period of 15 min with either oxypurinol or allopurinol (1 μM each). Control experiments were simultaneously performed in the absence of any XO inhibitors. Global hypothermic cardioplegic arrest was then induced with potassium crystalloid cardioplegia for 2 hrs, followed by 1 hr of reperfusion. Functional parameters including left ventricular developed pressure (LVDP), left ventricular max. dp/dt (LV dp/dt), and left ventricular end-diastolic pressure (LVEDP) were measured as described previously (11). Biochemical assays included estimation of high-energy phosphate compounds, hypoxanthine and xanthine (12), as well as assay for xanthine oxidase (9). Left ventricular tissue biopsies were instantly frozen at liquid nitrogen temperature for subsequent free radical analysis by electron paramagnetic resonance spectrometer (EPR).

Various free radicals were generated in vitro using known radical-generating systems. Oxygen-free radicals and hydroxyl radicals were generated by the action of hypoxanthine on XO and (Fe^{2+} + DTPA + H_2O_2 + KTBA), respectively (13). Polymorphonuclear leukocytes were activated with FMLP or ZAS to generate free radicals. Chlorine radical ($ClO_2^{\cdot}$) and hypochlorous acid (HClO) were generated with Alcide† chemical. Scavenging actions of allopurinol and oxypurinol were examined using either EPR or Luminometer.

RESULTS AND DISCUSSION

Xanthine Oxidase-Hypoxanthine System In Pig Heart

Xanthine dehydrogenase, XO, hypoxanthine and xanthine were assayed from the biopsies obtained from control, ischemic, and reperfused hearts. Xanthine oxidase activity was not detected in any of the heart biopsies (Table 1). Although measurable quantities of hypoxanthine were found in the biopsies, only a negligible amount of xanthine was detected. Thus, our results contradict the hypothesis that free radicals may be produced via XO reactions in pig heart. To the contrary, these results support the observations by Reimer and Jennings that XO activity may not contribute to the myocardial ischemic-reperfusion injury (8). However, contrary to their results, we found significant protection of heart from reperfusion injury. This discrepancy may be explained due

† Gift from Dr. Robert Cross, Alcide Corporation, Farmingdale, NY.

Table 1. Hypoxanthine, Xanthine, and Xanthine Oxidase Concentrations in Pig Heart

	Hypoxanthine (nmol/gm)	Xanthine (nmol/gm)	Xanthine Oxidase (units)
Control	50 ± 15	< 1	0
2 hr Ischemia	85 ± 11	< 1	0
1 hr Reperfusion	77 ± 20	< 1	0

to the species variation, as well as protocol of the experiment such as duration of ischemia, mode of ischemic insult, and dose of allopurinol.

Myocardial Salvage by Xanthine Oxidase Inhibitors

We examined the effects of allopurinol and its primary metabolite oxypurinol on the salvage of myocardial ischemic-reperfusion injury when added to the coronary perfusate prior to ischemia. Both oxypurinol and allopurinol were able to preserve the ATP levels at significantly higher values compared to control (Table 2). Creatine phosphate levels were decreased significantly during ischemia, but their values reached supranormal levels during reperfusion in the treated groups. Cardiac contractility and compliance were also better restored by allopurinol and oxypurinol. LVDP and LV max dp/dt were significantly higher in the treated groups. Thus, these results support the previous reports of myocardial protection by XO inhibitors (4-7).

Table 2. Effects of Oxypurinol and Allopurinol on Myocardial High-Energy Phosphate Compounds, LVDP and LV dp/dt

	Control	Pre-Perfusion 15 min with XO-inhibitors	2 hrs of Ischemia	60 min Reperfusion[a]
ATP (mM)				
Non-treated	28.0±2.8	26.1±1.9	23.5±2.0	23.5±1.7*
Oxypurinol	27.6±3.1	29.8±2.3	26.3±2.5	29.6±1.5*
Allopurinol	26.9±4.0	28.0±2.7	27.7±1.8	28.9±1.4*
CP (mM)				
Non-treated	47.5±3.0	48.1±2.2	7.6±1.2	53.5±2.1*
Oxypurinol	50.7±2.7	56.8±3.1	8.0±2.2	69.9±1.7*
Allopurinol	51.6±1.9	52.5±1.7	6.8±2.7	70.0±1.8*
LVDP (% control)				
Non-treated	100	99.7±1.64		75.0±5.56*
Oxypurinol	100	99.3±3.84		94.4±6.91
Allopurinol	100	99.5±2.66		95.5±5.48
LV dp/dt (% control)				
Non-treated	100	105.0±4.52		80.9±3.76*
Oxypurinol	100	94.6±12.0		104.7±7.54*
Allopurinol	100	96.0±8.21		99.2±3.88

Results are expressed as Means ± SE of 6 animals in each group.
[a] ATP and CP values are given at 15 min of reperfusion. * $p < 0.05$.

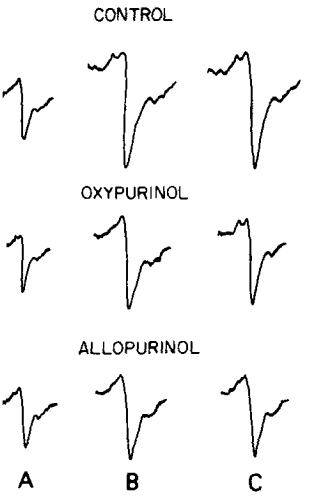

Figure 1. EPR spectra of (A) non-ischemic control, (B) ischemic, and (C) reperfused left ventricular tissue biopsies. Samples were analyzed at 77°K, microwave power 200 microwatts, microwave frequency of 9.5 GHz, and modulation amplitude 2.5 G.

Identification of Free Radicals in Heart and Their Inhibition by Oxypurinol and Allopurinol

The left ventricular tissue biopsies, when examined at 77°K using EPR, showed multiple EPR signals (Fig. 1) at g=1.985, 2.000, and 2.012. These EPR signals were significantly amplified in ischemic and reperfused heart, compared to those in pre-ischemic controls. Both oxypurinol and allopurinol were able to reduce these signals in ischemic and reperfused tissue, suggesting free radical scavenging abilities of these XO inhibitors. Our study, however, did not identify the EPR spectra generated in heart biopsies; it showed only enhancement of free radical signals during ischemia and reperfusion, and their reductions by oxypurinol and allopurinol.

In Vitro Scavenging of Free Radicals by Oxypurinol and Allopurinol

Oxygen-derived free radicals and hydroxyl radicals were generated as described in Methodology. Freshly isolated PMNs were activated with FMLP or ZAS to generate free radicals. Alcide has been found to generate $ClO_2^{-\cdot}$ and HClO simultaneously in our laboratory. Scavenging actions of oxypurinol and allopurinol were studied by placing these XO inhibitors with the free-radical generating systems. Both EPR and Luminometer were used for the detection of free radicals. Figure 2 and Table 3 show the results, which indicate that both oxypurinol and allopurinol (1 μM) can scavenge free radicals, but allopurinol seems to be a better scavenger. We found that at a lower concentration, these XO inhibitors do not function as free radical scavengers (data not shown).

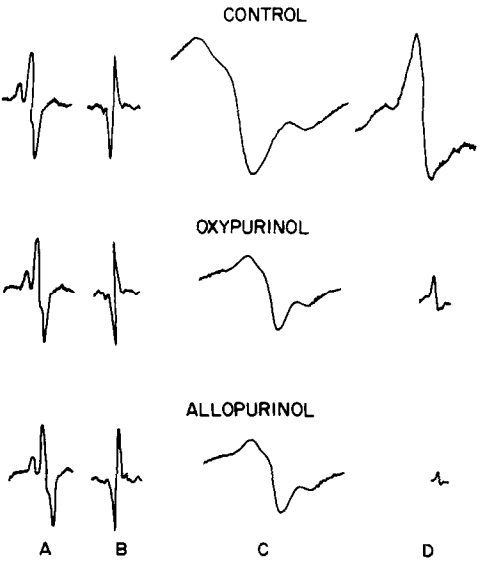

Figure 2. EPR spectra of (A) oxygen radical generated by the action of hypoxanthine on xanthine oxidase, (B) hydroxyl radical generated from the reaction of Fe^{+}, DTPA, H_2O_2 and KTBA, (C) PMN activated with FMLP, and (D) chlorine dioxide radical and HOCL generated from ALCIDE. Samples were analyzed at 77°K, microwave power 200 microwatts, microwave frequency 10 GHz, and modulation amplitude 2.5 G.

Recent work by Peterson and his coworkers also suggested that allopurinol might function as a free radical scavenger and act as an electron-transfer agent from ferrous iron to ferric cytochrome C (14). Our results support this earlier hypothesis and further confirm that at a higher concentration (> 1 μM) these XO inhibitors can scavenge free radicals.

Table 3. Effects of Oxypurinol and Allopurinol on Various Free-Radical Generating Systems

Free Radical Generating Systems	Radicals	% Inhibition of Chemiluminescent Response	
		Oxypurinol	Allopurinol
Hypoxanthine + Xanthine Oxidase	$O_2^{-\cdot}$	0	0
Fe^{+} + DTPA + H_2O_2 + KTBA	$OH^{\cdot}$	0	0
PMNs + FMLP	$O_2^{-\cdot}$ + HOCL	50	50-60
PMNs + ZAS	$O_2^{-\cdot}$ + HOCL	50-60	60-70
Alcide	$CLO_2^{-\cdot}$ + HOCL	> 80	> 90

The results of our study clearly indicate that both oxypurinol and allopurinol salvage myocardial functions during ischemia and reperfusion by scavenging free radicals, and not by inhibiting XO. Furthermore, a relatively higher concentration of these XO inhibitors must be used, because at lower doses they do not function as scavengers of free radicals.

REFERENCES

1. Das, D.K., Engelman, R.M., Rousou, J.A., Breyer, R.H., Otani, H., and Lemeshow, S. (1986) Basic Res. Cardiol. 81, 155-166.
2. Das, D.K., Flansaas, D., Engelman, R.M., Rousou, J.A., Breyer, R.H., Jones, R., Lemeshow, S., and Otani, H. (1987) Biol. Neonate 51, 156-169.
3. Otani, H., Engelman, R.M., Rousou, J.A., Breyer, R.H., and Das, D.K. (1986) J. Mol. Cell. Cardiol. 18, 953-961.
4. McCord, J.M. (1985) N. Engl. J. Med. 312, 159-162.
5. Werns, S.W., Shea, M.J., Mitsos, S.E., Dysko, R.C., Fantone, J.C., Schork, M.A., Abrams, G.D., Pitt, B., and Lucchesi, B.R. (1986) Circulation 73, 518-524.
6. Hearse, D.J., Manning, A.S., Downey, J.M., and Yellon, D.M. (1986) Acta Physiol. Scand. Suppl. 548, 65-78.
7. Stewart, J.R., Crute, S.L., Loughlin, V., Hess, M.L., and Greenfield, L.J. (1985) J. Thorac. Cardiovasc. Surg. 90, 68-72.
8. Reimer, K.A., and Jennings, R.B. (1985) Circulation 71, 1069-1075.
9. Parks, D.A., and Granger, D.N. (1986) Acta Physiol. Scand. (Suppl) 548, 87-99.
10. Das, D.K. (1986) In Enzymes of Lipid Metabolism (L. Freysz and S. Gatt, eds.) Vol. II, p. 93. Plenum, New York.
11. Otani, H., Engelman, R.M., Rousou, J.A., Breyer, R.H., Lemeshow, S., and Das, D.K. (1986) J. Thorac. Cardiovasc. Surg. 91, 290-295.
12. Das, D.K., Engelman, R.M., Otani, H., Rousou, J.A., Breyer, R.H., and Lemeshow, S. (1986) Clin. Physiol. Biochem. 4, 187-198.
13. Cederbaum, A.I., and Cohen, G. (1985) In Handbook for Methods in Oxygen Radical Research (R.A. Greenwald, ed.), pp. 81-87. CRC Press, Florida.
14. Peterson, D.A., Kelly, B., and Gerrard, J.M. (1986) Biochem. Biophys. Res. Commun. 137, 76-79.