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Allopurinol Enhances Adenine Nucleotide Levels and Improves Myocardial Function in Isolated Hypoxic Rat Heart

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Abstract—Allopurinol, a competitive inhibitor of xanthine oxidase, was found to have a protective effect on ischemic myocardium. Its mechanism of action is still controversial. We used Langendorff isolated rat heart preparation to test the hypothesis that allopurinol could maintain a level of the adenine nucleotide pool (ATP, ADP, and AMP) that would protect and improve the functional activity of the heart during a period of hypoxia. Hearts were initially perfused for 30 min until steady state was attained. This was followed by 20 min of experimental perfusion divided into 5 min of control perfusion followed by 15 min of hypoxic perfusion with or without allopurinol in the perfusate. Hearts were quick-frozen and enzymatically analyzed for adenine nucleotides and creatine phosphate at the end of the hypoxic period. Left ventricular pressure, heart rate, and coronary flow were measured in all preparations. Allopurinol (0.1 mM) treated hearts had greater levels of ATP (12.3 ± 0.8 vs. 9.3 ± 0.8 μmol/g dry weight; $p < 0.01$). This improvement occurred in the presence as well as the absence of glucose. Total adenine nucleotides improved from 17 ± 1 to 20.3 ± 2.4 μmol/g dry weight ($p < 0.01$). This improvement also occurred in the presence as well as in the absence of glucose in the perfusate. It also improved cell energy state significantly in the presence as well as the absence of glucose. There was insignificant change in creatine phosphate. Allopurinol improved left ventricular pressure from $38 \pm 7\%$ to $55 \pm 9\%$ ($p < 0.002$) in the presence of glucose and from $8 \pm 3\%$ to $27 \pm 6.3\%$ ($p < 0.001$) in the absence of glucose. Coronary flow improved from $110 \pm 5\%$ to $120 \pm 8\%$ ($p < 0.04$) in the presence of glucose. These results support the suggestion that allopurinol at 0.1 mM exerts its protective effect on rat heart during hypoxia by enhancing the adenine nucleotide pool.

Key words: allopurinol, adenine nucleotides, myocardial function, hypoxia

Acute myocardial ischemia has been established to cause rapid utilization and depletion of high-energy phosphates, i.e., the adenine nucleotides adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) and creatine phosphate (CP). The nucleotides are degraded to nucleosides and active bases, particularly inosine and hypoxanthine [1-6]. Hypoxanthine is converted to xanthine and uric acid by xanthine oxidase [7-9]. These apolar compounds can pass through the cell membrane and move out of myocytes. Because of this loss of purines from myocytes, ATP synthesis would be reduced. Also the conversion of hypoxanthine is a major potential source of oxygen free radicals (O_2^-), which have been proposed to play an important role in the genesis of ischemic injury and reperfusion-induced injury [10]. Since the breakdown reactions of ATP to xanthine are initially reversible, it could be hypothesized that by blocking the conversion of hypoxanthine to xanthine and to uric acid by the xanthine oxidase inhibitor allopurinol might preserve the active purine bases for subsequent rebuilding of nucleotides by salvage pathways. Hypoxanthine can be salvaged by phosphorylation with 5-phosphoribosyl-1-pyrophosphate (PRPP) by hypoxanthine-guanine phosphoribosyl transferase to inosine monophosphate (IMP), which is converted to AMP [11]. The production of IMP was reported to be enhanced by anoxia and ischemia [12]. In rat heart, the incorporation of IMP and hypoxanthine into ATP was found to be about 3 nmole/min per g dry weight [13]. Harmsen et al. [14] found hypoxanthine at concentration of 0.02 mM was incorporated into ATP at 14.6 mmole/15 min per g dry weight during reperfusion after ischemia.

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Allopurinol was reported to have a considerable protective effect following acute myocardial ischemia. Several studies have shown allopurinol to produce an increase in myocardial contractility and cardiac output and to prevent or reverse electrophysiological S-T changes of ischemic origin in rat, rabbit, dog, pig, and new born lambs as well as in human [15-21]. It was reported to reduce infarct size and the incidence of ventricular fibrillation following myocardial infarction [22-25].

The precise mechanism(s) of allopurinol protection on the ischemic myocardium is not clear. Two suggestions have been put forward. One of the suggestions implicated increased salvaging of hypoxanthine, by preventing its breakdown by allopurinol, thus increasing the production of ATP through salvage pathways [7, 12, 26-28]. The other suggestion attributed the protective effect to the inhibition of O_2^- production as a result of inhibiting xanthine oxidase during reperfusion [21-23]. In the above forgoing studies, the animals used were treated with allopurinol for several days before performing the experiment, or the hearts were perfused with the drug for the entire duration of the perfusion. Beside this, the effect of allopurinol was studied on reperfused heart after being exposed to a period of ischemia. Such perfusion conditions would be complicated by the production of O_2^- free radicals which are generated during reperfusion [18, 26, 28]. The aim of our study was to look at the effects of allopurinol on the myocardium at the end of 15-min hypoxia without reperfusion (i.e., re-oxygenation) and without any pretreatment with allopurinol. We used Langendorff perfused isolated rat heart to test the effect of allopurinol on left ventricular pressure (LVP), coronary flow (CF), and heart rate (HR) as well as its effect on the levels of ATP, ADP, AMP, and CP.

MATERIALS AND METHODS

Forty male Sprague Dawley rats weighing 250-300 g were divided into five groups (I-V). The animals were lightly anaesthetized with diethyl ether then killed by rapid decapitation. The chest was immediately opened and the heart rapidly excised and washed in ice-cold Krebs solution. The heart then was cannulated through the aorta on a Langendorff apparatus consisting of water-jacketed double coils (Aimer Ltd., England). Perfusion started immediately with control Krebs running in one of the coils. The testing Krebs containing allopurinol was placed in the other coil. The perfusing Krebs could be changed by turning a tap that is part of the double coil and placed at the bottom of the apparatus. The control Krebs was continuously gassed with 95% O_2 and 5% CO_2 (pH 7.2-7.4) and consisted of (mM): NaCl, 117; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 1.8; $NaHCO_3$, 25; and glucose, 10. The hypoxic Krebs running in the other coil was continuously gassed with 95% N_2 and 5% CO_2 and was identical to the control Krebs in its constituents except that which perfused groups III and IV did not contain glucose. Allopurinol (0.1 mM) was added to the perfusate in groups IV and V. All Krebs solutions were filtered by inline filters with 50-mm diameter and 8 μm pore size (Sartorius, Germany) and warmed to 37°C before perfusing the heart at 70 cm H_2O pressure. The heart was placed in jacketed glass chamber warmed to 37°C.

Experimental protocol. The animals were divided into five groups. Each group was assigned to one of the following perfusing conditions: group I (control), group II (hypoxia with glucose), group III (hypoxia without glucose), group IV (allopurinol treated hypoxia with glucose), and group V (allopurinol treated hypoxia without glucose). All hearts were perfused with normal Krebs until reaching steady state as indicated by constant heart rate, left ventricular pressure, and coronary flow. This equilibrating period was reached within about 30 min of starting the perfusion. This equilibrating period was followed by 20 min of experimental perfusion. In the first 5 min the hearts were perfused with control Krebs before switching to one of the above hypoxic conditions for the other 15 min. For the control (group I), the hearts were perfused with control Krebs for the entire 20-min duration. At the end of the 20-min experimental period, the hearts, while still beating, were quick-frozen in Wollenberger tongs pre-cooled in liquid nitrogen. The hearts were stored in liquid nitrogen until extracted on the following day.

Tissue extraction. The frozen heart tissues were freed of atrial muscle and the rest were pulverized to powder. Liquid nitrogen was added as needed to keep the tissue frozen. The tissue was then homogenized with an Ultra-Turraks homogenizer in 2 ml of cold salt–ice solution of 10% trichloroacetic acid (TCA) and 20% methanol. The slurry suspension was centrifuged at 0°C for 15 min at 3500*g*. The supernatant was decanted and the pellet re-homogenized in 2 ml of fresh TCA solution. The supernatants were added together and its pH adjusted to 7.0-8.5 and made up to 5 ml with distilled water. The extracts were stored for 12 h at −30°C before being enzymatically analyzed for ATP, ADP, AMP, and CP.

Measurements of cardiac function. CF (ml/min) was determined from timed collection of effluent dripping from the heart chambers. LVP (cm H_2O) was recorded by a glass cannula inserted into the left ventricle and connected to a channel recorder via a PT 400 pressure transducer (Bell and Howell). HR (beat/min) was calculated from the pressure trace.

Biochemical determinations. Combined enzyme assay was carried out for ATP and CP according to Trautschold et al. (see [29] for details).

Hexokinase plus ATP was used to convert glucose into glucose-6-P. Glucose-6-P is then converted into 6-

phosphoglucono-lactone by glucose-6-P dehydrogenase. In this reaction $NADP^+$ is converted to NADPH. For each mole of ATP, one mole of NADPH is produced. The concentration of NADPH was determined by measuring the absorbance at 339 nm. Similarly, CP was measured after adding creatine kinase to the reaction mixture and the absorbance of NADPH produced was measured at 339 nm. Another combined assay for ADP and AMP measurement according to the methods of Jaworek and Welsh as described in Bergmeyer [29] was used. Myokinase, pyruvate kinase, and lactate dehydrogenase were used in the reaction. The decrease in NADH as measured by the change in absorbance at 339 nm is proportional to the amount of AMP and ADP present (see [29] for details). Energy charge potential (E*) was calculated by the formula proposed by Atkinson [30]:

$$E^* = (ATP + 0.5ADP)/(ATP + ADP + AMP).$$

Statistical analysis. All data are expressed as mean ± SD. The analysis of variance (ANOVA) test was performed on all corresponding groups to test for significance. $p < 0.05$ was considered significant.

RESULTS

Table shows the effect of allopurinol (0.1 mM) treatment on ATP, ADP, AMP, and CP levels after 15 min of hypoxia. The table shows that hypoxia caused a significant decrease in the levels of ATP and CP, which was 2- and 3-fold, respectively ($p < 0.001$), and an increase in the levels of both ADP by 30% ($p < 0.01$) and AMP by 5-fold ($p < 0.002$). Allopurinol improved significantly the levels of ATP from 9.3 ± 0.8 to 12.3 ± 0.8 μmol/g dry weight ($p < 0.01$) in the presence of glucose and from 4.9 ± 0.8 to 9.1 ± 1.3 μmol/g dry weight ($p < 0.01$) in the absence of glucose. Total adenine nucleotides (TAN) improved from 17 ± 1 to 20.3 ± 2.4 μmol/g dry weight ($p < 0.01$) in the presence of glucose and from 11.6 ± 2.4 to 18.3 ± 2.7 μmol/g dry weight ($p < 0.01$) in the absence of glucose. Although glucose in the perfusate increased ATP and TAN, the increase due to treatment of allopurinol was added to the increase due to glucose alone. CP was not affected significantly by allopurinol. The E* ratio, which is an indicator of the myocardial cell energy state, was significantly improved by allopurinol in the presence as well as in the absence of glucose from 0.67 ± 0.02 to 0.73 ± 0.04 ($p < 0.04$) and from 0.61 ± 0.04 to 0.65 ± 0.02 ($p < 0.04$), respectively. Hypoxia decreased E* from 0.89 ± 0.02 in normoxia group I to 0.67 ± 0.02 in hypoxic group II ($p < 0.001$) and to 0.61 ± 0.04 ($p < 0.001$) in hypoxic group III. In groups receiving allopurinol, IV and V, E* was 0.73 ± 0.04 and 0.65 ± 0.02, respectively, which were significantly higher than those in group II (0.67 ± 0.02) and in group III (0.61 ± 0.04) without allopurinol treatment. These results suggested improved myocardial energy state in the allopurinol treated groups in the presence

Levels of ATP, ADP, AMP, total adenine nucleotides (TAN), and creatine phosphate (CP) in μmol/g dry weight in control and under different conditions of hypoxic perfusion

Group	Content of compounds, μmol/g dry weight of ventricular tissues					
	ATP	ADP	AMP	TAN	CP	energy charge, E*
Control, group I ($n = 8$)	18.7 ± 2.9	3.8 ± 0.4	0.62 ± 0.1	23.3 ± 2.9	19.2 ± 5.6	0.89 ± 0.02
Hypoxia plus glucose, group II ($n = 8$)	9.3 ± 0.08[a]	4.9 ± 0.7	3.3 ± 0.5[a]	17 ± 1[a]	5.9 ± 0.9[a]	0.67 ± 0.02[a]
Hypoxia without glucose, group III ($n = 8$)	4.9 ± 0.8[d]	4.3 ± 0.9	2.3 ± 0.8	11.6 ± 2.4[d]	4.1 ± 1.2	0.61 ± 0.04[d]
Hypoxia plus allopurinol plus glucose, group IV ($n = 8$)	12.3 ± 0.8[b]	4.9 ± 0.9	3.2 ± 1.4	20.3 ± 2.4[b]	4.9 ± 1	0.73 ± 0.04[c]
Hypoxia plus allopurinol without glucose, group V ($n = 8$)	9.1 ± 1.3[e]	4.6 ± 0.6	3.6 ± 0.8	18.3 ± 2.7[e]	4.7 ± 0.7	0.65 ± 0.02[f]

[a] $p < 0.01$ compared to group I (control).
[b] $p < 0.01$ compared to group II.
[c] $p < 0.04$ compared to group II.
[d] $p < 0.001$ compared to group I (control).
[e] $p < 0.01$ compared to group III.
[f] $p < 0.04$ compared to group III.

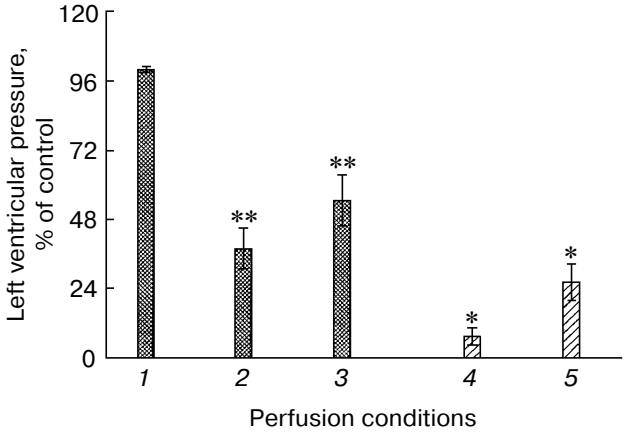

Fig. 1. Effect of 0.1 mM allopurinol on left ventricular pressure expressed as percent of the preceding steady state during control perfusion (ordinate axis); perfusion conditions (abscissa axis) (* $p < 0.002$; ** $p < 0.001$): *1*) control; *2*) hypoxia + glucose; *3*) hypoxia + allopurinol + glucose; *4*) hypoxia without glucose; *5*) hypoxia + allopurinol without glucose.

as well as in the absence of glucose. The effect of allopurinol on LVP is shown in Fig. 1. Allopurinol significantly improved LVP in group IV as compared to II ($55 \pm 9\%$ vs. $38 \pm 7\%$, $p < 0.002$) and in group V as compared to III ($27 \pm 6\%$ vs. $8 \pm 3\%$, $p < 0.001$). Allopurinol had no effect on HR (Fig. 2) and improved CF from $110 \pm 5\%$ to $120 \pm 8\%$ but only in the presence of glucose (Fig. 3).

DISCUSSION

Allopurinol was shown in several studies to have a beneficial effect on the ischemic myocardium in humans and in animals. Allopurinol caused a significant increase in myocardial contractility and improved cardiac output as well as left ventricular systolic pressure in rat, dog, rabbit, and pig [15-19, 21, 28, 31-33]. Castelli et al. [16] studied the effect of allopurinol on cardiac performance after coronary bypass in human. They found allopurinol treated patients had better recovery of cardiac output and left ventricular stroke work. Allopurinol also prevented electrophysiological S-T segment changes due to ischemia. It reduced the incidence of ventricular tachycardia and the number of premature beats, suggesting a protective effect from ischemia-induced arrhythmias [19, 21, 23, 32]. Some reports showed that allopurinol diminished the size of the infarcted area produced by coronary occlusion [24, 25, 34]. In line with these findings, our results suggest that allopurinol has a protective effect on hypoxic isolated Langendorff perfused rat heart as indicated by significant improvement in LVP, CF, and in adenine nucleotide levels. The precise mechanism of this protective action is still controversial.

It is well established that myocardial ischemia (or hypoxia) causes a rapid breakdown of myocardial ATP to ADP and AMP, which is catabolized to adenosine, inosine, hypoxanthine, and uric acid [1-6, 35, 36]. Bruvand et al. [2] showed rapid and parallel decrease in ATP and accumulation of adenosine, inosine, hypoxanthine and xanthine in both epicardial and endocardial layers of the ischemic myocardium within the first 20 min of coronary occlusion. The results of ATP, TAN, and E* in our study (table) suggested that allopurinol, by inhibiting the conversion of hypoxanthine to xanthine, caused an improvement in the total adenine nucleotide pool and improved the energy state of the hypoxic myocardium, which is in agreement with previous reports. Several reports have shown allopurinol significantly improves myocardial cell ATP and TAN levels. Beside the fact that the effect of

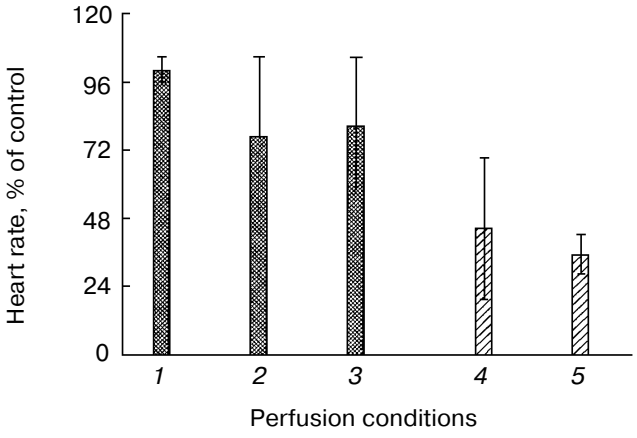

Fig. 2. Effect of 0.1 mM allopurinol on heart rate expressed as percent of the preceding steady state during control perfusion; perfusion conditions are the same as in Fig. 1.

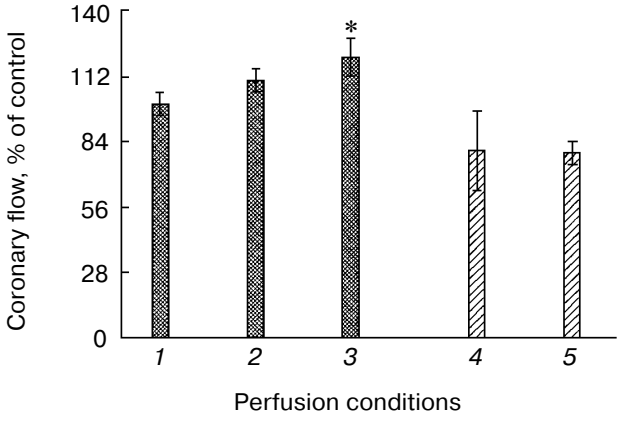

Fig. 3. Effect of 0.1 mM allopurinol on coronary flow at the end of 15 min of hypoxia expressed as percent of the preceding steady state during control perfusion; perfusion conditions are the same as in Fig. 1.

allopurinol was tested during reperfusion, the experimental animals in those studies were treated with allopurinol for some days before experimentation on their hearts, and in some studies allopurinol was included in the perfusate for the entire period of perfusion [18, 26, 28, 33]. The protective effect of allopurinol during reperfusion in those studies would be complicated by the prevention of oxygen free radicals that are generated during reperfusion. The present study showed allopurinol significantly increased ATP and TAN levels as well as significantly improved the myocardial energy state at the end of 15 min of hypoxia without re-oxygenation, thus in the absence of free radical production. These results, therefore, support the suggestion that allopurinol protects hypoxic myocardium by enhancing the adenine nucleotide pool. It is hypothesized that allopurinol, by preventing the conversion of hypoxanthine to xanthine and uric acid, preserves the active purine bases that can be salvaged for the production of adenine nucleotides. Hypoxanthine, produced under ischemic conditions, was demonstrated to be salvaged by phosphorylation with 5-phosphoribosyl-1-pyrophosphate (PRPP) by hypoxanthine-guanine phosphoribosyl transferase to inosine monophosphate (IMP), which could be converted to AMP and ATP [7, 9, 12-14, 37]. Labeled hypoxanthine has been shown to be incorporated into ATP, ADP, AMP, and IMP [13, 14]. The improvement in adenine nucleotide levels by allopurinol seen in our study can be explained by the protective effect of the drug on the structural and functional integrity of myocardial and liver mitochondria, which was demonstrated in several studies [18, 38, 39]. They reported a significant improvement in the rate of mitochondrial ATP generation in allopurinol treated hearts.

In contradiction to the above theory of adenine nucleotide enhancement, a few reports showed evidence to suggest that the protective effect of allopurinol to be due to its ability to prevent the generation of oxygen free radicals during reperfusion in ischemic myocardium [21-23, 34]. Chambers et al. [34] found both allopurinol and superoxide dismutase (a free radical scavenger) to diminish the region of infarction to the same extent. Similarly, Stewart et al. [21] found allopurinol and superoxide dismutase to improve left ventricular mechanical function to the same degree. Both Chambers et al. and Stewart et al. concluded that allopurinol and dismutase acted by same mechanism, i.e., by inhibiting free radical generation. Although these studies provided evidence to suggest that allopurinol prevented the production of superoxide free radicals that cause tissue damage during reperfusion. Our results showed allopurinol had a protective effect also during hypoxic perfusion, conditions under which hardly any oxygen free radicals are produced.

In summary, this study suggests that allopurinol has a protective effect on myocardium in terms of improving cardiac function as shown by improved LVP (by 17%) and CF (by 10%). Also, improved levels of ATP and TAN under hypoxic conditions were found. It is suggest that the protective effect of allopurinol may be due to increasing adenine nucleotides levels rather than to the inhibition of oxygen free radicals generation, though the latter cannot be fully ruled out.

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