

**IMPAIRMENT OF THE POST-ANOXIC RECOVERY
OF ISOLATED RAT HEARTS
BY INTRAVASCULAR HYPOXANTHINE AND XANTHINE**

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ABSTRACT

Hypoxanthine is the final product of the catabolism of ATP in the stored red cell. Upon transfusion, this purine may be uptaken by the endothelial cell and oxidized in a post-ischemic or post-anoxic environment with production of oxygen-derived free radicals. We have tested this hypothesis with a isolated perfused rat heart model monitoring the recovery of the heart function from 20 min anoxia in the presence of 0.1 mM hypoxanthine or xanthine. Addition of 0.1 mM guanine minimized the fraction of hypoxanthine to be salvaged. The presence of hypoxanthine in the vascular space impaired the recovery of the end-diastolic pressure,

left ventricular developed pressure, contraction rate, and coronary perfusion pressure. We conclude that intravascular hypoxanthine is oxidized by the endothelial cell xanthine oxidase contributing to the post-anoxic reoxygenation injury. Since the injury led by equimolar xanthine was nearly half of that observed for hypoxanthine, this injury appears to be correlated to the stoichiometry of the oxygen-derived free radical generating reaction.

INTRODUCTION

The mature red cell has lost its capacity to degrade or salvage hypoxanthine, final compound of the catabolism of ATP, due to the lack of the enzymes adenylo succinate synthetase [1], xanthine dehydrogenase and xanthine oxidase [2,3]. Hypoxanthine is therefore the main purine species produced by the red cell during storage in the most common blood preservatives [4]. In addition, hypoxanthine is readily transported across the red cell membrane along its concentration gradient by a passive carrier [5]. It can therefore be thought that upon transfusion of such hypoxanthine-rich banked red cell into a recipient that normally has little or no hypoxanthine in blood, hypoxanthine is readily released into the vascular space. On the other hand, it is well known that hypoxanthine formed in the tissue cell under energy depletion conditions such as ischemia or anoxia, may participate to the reaction of xanthine oxidase on cell reoxygenation or reperfusion, generating the oxygen-derived free radical and injuring the tissues [6,7].

In this work, we test the hypothesis that the intravascular hypoxanthine released by the stored RBC may participate to the oxygen radical generating reaction in the same way as the endogenous hypoxanthine formed from the catabolism of ATP in the tissue cell. The final result should be an exacerbation of the injury led by endogenous hypoxanthine. To test this hypothesis, we have monitored the functional response of the post-anoxic Langendorff isolated rat heart to the presence of hypoxanthine in the vascular space. Such model is particularly suitable for its high sensitivity to metabolic changes and the possibility to monitor functional and metabolic alterations without the masking effect of the other organs. We will show that the myocardial function is greatly impaired in the presence of hypoxanthine, and that different responses are evoked by hypoxanthine and xanthine, strongly suggesting the involvement of the oxygen-derived free radical.

MATERIALS AND METHODS.

Male Sprague Dawley rats weighting 250-270 g were anesthetized with heparin and urethan, the chest was opened, and the venae cavae were tightly ligated. The heart was rapidly excised, the aorta was cannulated onto a stainless-steel 1.8 mm o.d. cannula, and the retrograde perfusion started immediately with an oxygenated Krebs-Henseleit buffer. A drainage was inserted in the apex of the ventricle, and a Latex balloon in the left ventricle was filled with saline and connected to a pressure transducer (Harvard Apparatus mod.52-9966, Natick, MA) to monitor the mechanical functions. A teflon cannula inserted in the

pulmonary artery was used for the collection of the perfusate.

The Krebs-Henseleit buffer (115.6 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 0.5 mM EDTA, 1.2 mM Na_2SO_4 , 3 mM CaCl_2 , 28.5 mM NaHCO_3 , 1.2 mM MgCl_2 , 16.6 mM glucose) was flushed with either 94/6% O_2/CO_2 or 94/6% N_2/CO_2 and filtered through a 8 μm pore size, 47 mm diameter polycarbonate Nuclepore filter. The flow through the heart was kept at 15 ml/min by a roller pump (Watson Marlow mod.503-S, Falmouth, England). An additional pump (Razel Scientific Instrument mod.A-99, Stanford, CT) delivered hypoxanthine or guanine or xanthine at 15 $\mu\text{l}/\text{min}$ (0.1 mM final concentration of each of the purines). A pressure transducer placed above the aortic cannula monitored the coronary perfusion pressure. The measured parameters included the end-diastolic and the systolic pressures and the rate of pressure development (+dP/dt). Furthermore, pO_2 , pCO_2 and pH of the perfusate was monitored by a IL1304 gas-analyzer (Instrumentation Laboratory, Paderno Dugnano, Italy).

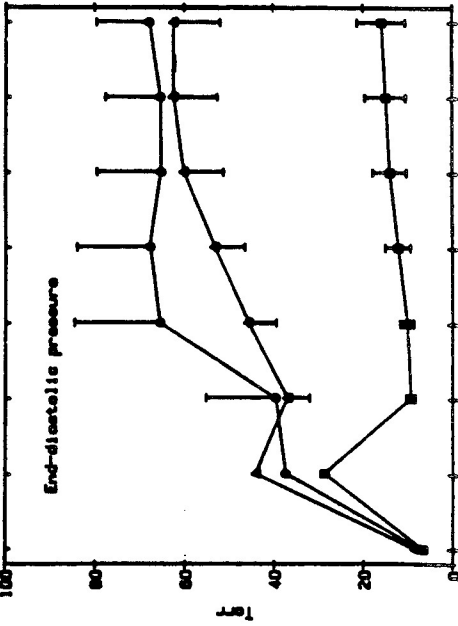
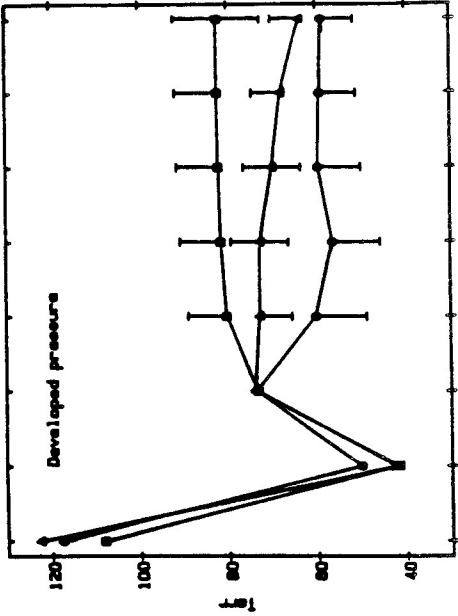
The spontaneously contracting hearts were allowed to stabilize with the oxygenated buffer for 30-45 min at 7.5 (0.8) [mean (SEM)] torr end-diastolic pressure. The infusion of guanine started shortly before the 20 min perfusion of the heart with the deoxygenated buffer (anoxia). The infusion pump was then loaded with either 0.1 M hypoxanthine plus 0.1 M guanine, or 0.1 M xanthine plus 0.1 M guanine just before the reoxygenation. In the control experiments, the syringe containing guanine was not replaced. When the oxygenated buffer was allowed again in the heart, all the mechanical and metabolic parameters were monitored for 30 min until the end of the experiment.

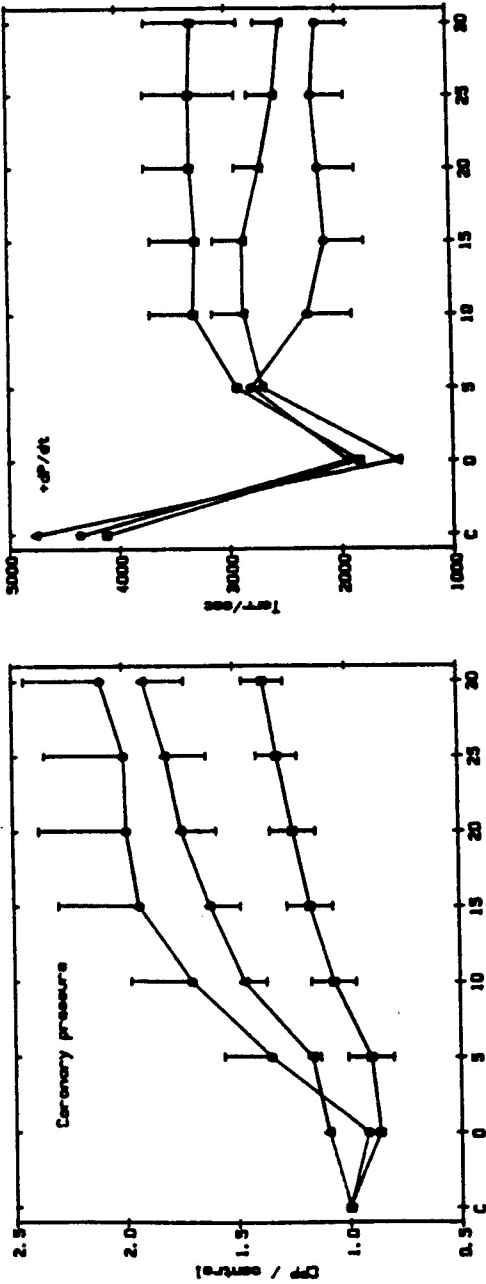
RESULTS.

The recovery of the heart rate was the same in all the groups and more than 96% complete in less than 10-15 min. The recovery of other functions is shown in figure 1. All the parameters recovered better in the controls (n=6) than in the presence of 0.1 mM hypoxanthine (n=6) or 0.1 mM xanthine (n=6). The difference between controls and the hypoxanthine-treated hearts at the end of the experiment was significant ($p < 0.001$, 0.05, 0.03, and 0.04 for the end-diastolic pressure, the left ventricular developed pressure, the contractility rate, and the coronary perfusion pressure, respectively). The difference between the hypoxanthine- and xanthine-treated hearts, however, was not significant at the $p = 0.1$ level. Nevertheless, the recovery of the xanthine-treated hearts in most cases is intermediate between the controls and the hypoxanthine-treated hearts, indicating that the injury led by xanthine is less (roughly half) of that led by hypoxanthine at the same concentration.

DISCUSSION.

The experimental protocol selected for this work has allowed to stress the effect of the intravascular hypoxanthine from that of the endogenous hypoxanthine derived from the catabolism of ATP following anoxia in the endothelial cell. Hypoxanthine in the buffer here mimicks the fraction of purine released by the banked red cell following transfusion into the hypoxanthin. free blood stream. The concentration of hypoxanthine in banked blood may be as high as 3 umoles/g hemoglobin [4]. Thus, if 1 liter of such blood is transfused into a 5-liter blood recipient, then the concentration





Time after reoxygenation, min

Fig. 1. Post-anoxic recovery of (clockwise) end-diastolic pressure, left ventricular developed pressure, myocardium contractility (+dP/dt), and coronary perfusion pressure (as the ratio of the actual value over the pre-anoxic control) in controls (squares), xanthine (triangles), and hypoxanthine-circles) treated isolated rat hearts. All data are mean(SEM) of 6 experiments.

of hypoxanthine in the recipient's blood may theoretically peak to 0.096 mM, fairly above the K_M value of xanthine oxidase for hypoxanthine (0.01 mM [7]). Even if this is a non-realistic case (transfusions generally take hours, and the uptake of hypoxanthine by the tissue cell for purine salvage is effective), the possibility that intravascular hypoxanthine is the limiting factor in the generation of oxygen-derived free radicals cannot be ruled out, at least in some circumstances involving post-ischemic human gut, liver, and kidneys, tissues with high xanthine oxidase activity [14].

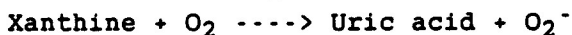
Hypoxanthine may also be formed endogenously in the endothelial cell from the catabolism of ATP under energy depletion conditions. It was shown that its concentration is up to 150 nM/g wet wt in the ischemic rat heart [8]. At such concentrations, endogenous hypoxanthine can possibly mask the effect of intravascular hypoxanthine. Thus, it was necessary to wash-out the endogenous hypoxanthine before the reoxygenation in order to stress the effect of intravascular hypoxanthine. This task was accomplished allowing the anoxic buffer to continuously flow through the heart during anoxia, and was facilitated by the postulated presence of a bidirectional carrier in the endothelial cell [9]. In this way, we have been able to attribute the observed responses to the intravascular hypoxanthine. Indeed, when the post-anoxic heart was reoxygenated in the absence of hypoxanthine, little injury of the myocardium was observed (figure 1), suggesting that all hypoxanthine has been removed before the reoxygenation.

Hypoxanthine, as other purine bases, is strongly salvaged by the post-ischemic or post-anoxic myocar-

dium endothelium by the enzyme hypoxanthine-guanine phospho-ribosyl transferase (HGPRT) [9,10]. Thus, it was mandatory to inhibit the salvage of hypoxanthine by the HGPRT route in order to get the maximal signal for the oxidative injury. For this purpose, we have used 0.1 mM guanine in the perfusion buffer, because the K_M value of HGPRT for guanine is smaller than that of hypoxanthine (0.003 vs 0.01 mM, respectively [11]). Therefore, the active site of the HGPRT reaction, that is the same for guanine and hypoxanthine [12], has a preference for guanine, and the uptake of hypoxanthine for the purine salvage pathway is decreased, thus increasing the availability of hypoxanthine for the oxidation by xanthine oxidase and the oxygen radical generation.

The oxygen deprivation triggers the conversion of xanthine dehydrogenase into active xanthine oxidase within minutes at 37C [7]. It was also shown that 20-min anoxia elicits the maximal response in terms of the free radical production by the xanthine oxidase reaction in the human umbilical vein endothelial cell [13]. The activity of xanthine oxidase in the rat heart (25-70 mU/mg wet wt) is not far from that observed in human liver and gut [14]. Thus, although xanthine oxidase activity is apparently absent in the human heart [15], the employment of the rat heart model would allow some extension to the human model.

Xanthine oxidase is a bifunctional enzyme catalyzing two reactions:



The stoichiometry of these reactions suggests that, if the product is expressed in terms of the oxidative injury and thus of the produced O_2^- , then the response

obtained using xanthine as substrate would be half of that obtained using equimolar hypoxanthine. This prediction is confirmed by the here reported data, although the significativity of the differences between the recovery of the hypoxanthine- and xanthine-treated hearts is low, perhaps indicating that the concentration of the purines is almost saturating with respect to the available enzyme. This feature strongly suggests that the oxygen-derived free radical is directly involved in the injury to the post-anoxic myocardium. This interpretation is furtherly stressed by the observation that the functional impairment observed in this work is very similar to the pattern observed in rabbit hearts subjected to the electrolytic generation of the oxygen-derived free radicals [16].

In conclusion, hypoxanthine appears to participate to the xanthine oxidase reaction in the post-anoxic heart even from the intravascular space, thus possibly exacerbating the damage led by endogenous hypoxanthine. Extension of these studies to the post-ischemic condition is presently under way. Nevertheless, these data suggest that hypoxanthine is a critical parameter when assessing the safety of blood or of red cell substitutes for transfusion purposes.

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