MYOCARDIAL ATP CATABOLISM AND ITS PHARMACOLOGICAL PREVENTION

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DE AFBRAAK VAN ATP IN HET HART EN HET FARMACOLOGISCH VOORKOMEN ERVAN

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CONTENTS

Chapter 1

INTRODUCTION

Myocardial ischemia

Essentially the heart is a pump which circulates blood through the body. Blood carries substrates to and products from organs. Impairment of cardiac blood supply alters myocardial contractility and may lead to a life-threatening situation. The consequences of myocardial ischemia can be divided into two groups representing its severity: reversible and irreversible alterations. The reversible effects of (transient) ischemia include 1) decreased energy stores as a result of reduced availability of oxygen and substrates, 2) metabolic toxification due to insufficient removal of metabolic breakdown products, and 3) probably damage caused by free radicals. The latter can be generated during ischemia and reperfusion. They cause damage when defence mechanisms against free radicals are impaired. The irreversible effects of ischemia include loss of mitochondrial respiratory control, loss of cellular integrity (leakage of enzymes and other macromolecules), and finally autolysis. Figure 1 shows a list made by Hearse[91] that describes the sequence of cellular events occurring after onset of myocardial ischemia.

Myocardial ischemia and energy metabolism

The balance between dephosphorylation and rephosphorylation of myocardial high-energy phosphates is essential for the heart's contractile behaviour. Dephosphorylation of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) supplies the energy needed for contractions. Under normal conditions ADP is rephosphorylated to ATP. The energy needed for this resynthesis is mainly derived from the oxidation of lipids and carbohydrates. Lack of oxygen stops the beta-oxidation of lipids while glycolysis is accelerated. Since only the anaerobic part of the carbohydrate breakdown pathway is active, phosphorylation of 1 mol ADP requires 0.5 mol glucose. This is only 5 % of the net ATP produced during aerobic glucose oxidation[162].

A diminished blood flow to the myocardium causes ischemia: insufficient supply of oxygen and substrates and insufficient removal of breakdown products. This may lead to imbalance between the dephosphorylation and rephosphorylation of ATP. During ischemia dephosphorylation will continue to provide the energy for contractions but the rate of rephosphorylation, for which oxygen and substrate are needed, will slow down to less than 10% of the level necessary to maintain cardiac function[116]. During the first seconds of ischemia, the ATP levels are maintained. Creatine phosphate stores provide the energy-rich phosphate bond through a creatine kinase catalyzed reaction.

During prolonged ischemia, myocardial high-energy phosphate content will decrease. The extent of this reduction depends on the duration of ischemia. Several studies show a good correlation between myocardial ATP content and cardiac function[40,109,180]. However, in some experimental models an acceptable myocardial performance at a seriously deprived ATP content has been found[100,153]. The myocardial ATP content is generally used as a measure for the severity of the ischemia. Until the early eighties, it was assumed that recovery from myocardial ischemia was complete within a short period because of the return of mechanical function. Reimer et al.[180] demonstrated that it takes at least a week to regenerate the myocardial nucleotides that were lost during ischemia.

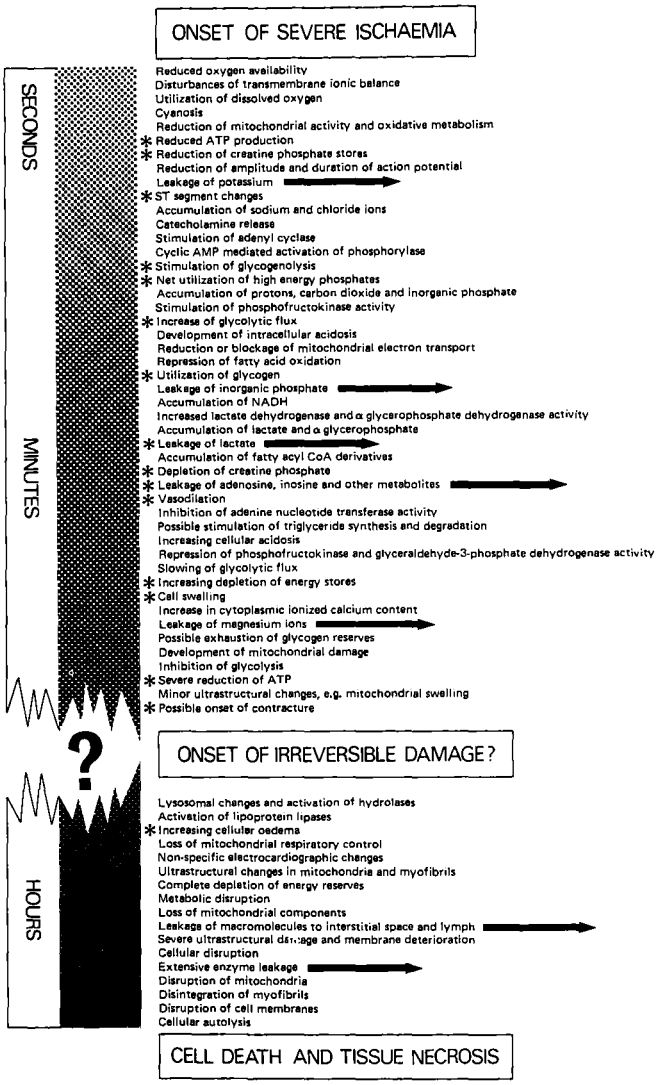

*Figure 1. Sequence of events occurring after onset of myocardial ischemia. The list is not intended to be exact. The progression of changes is a dynamic process; only the onset of some changes is listed. The separation between reversible and irreversible damage is also speculative. Arrows indicate efflux from the cell; * indicate matters of study in this thesis. (figure taken from reference 91, with permission).*

The phenomenon "myocardial stunning", the incomplete recovery of function in reperfused heart, seems to confirm the importance of metabolic recovery.

Protection of the myocardium against ATP breakdown

One contraction of the heart uses about 5 % of the myocardial intracellular ATP content. Therefore, it is of the utmost importance to detect and treat ischemia as quickly as possible to limit its deleterious effects.

Methods used in clinical practice to increase the functional reserve of the heart also improve myocardial energy balance. These methods are 1) coronary artery bypass surgery, 2) percutaneous transluminal coronary angioplasty (both methods increase oxygen supply to the myocardium) and 3) pharmacological treatment to either increase oxygen supply or reduce the oxygen demand.

Organization of the thesis

This thesis deals with high-energy phosphate metabolism during myocardial ischemia. describes the effects of some pharmacological interventions on the energy metabolism of ischemic myocardium. The chapters of this thesis are based on 11 published papers, added as appendices.

Chapter 2 is divided into two parts. The first part highlights three models that are used to study myocardial ischemia. We developed a model to study high-energy phosphate metabolism in cultured neonatal rat myocytes during normoxia and during simulated ischemia and anoxia. The results of this study are presented in appendix 1 (also see [166]). The second model described is the isolated perfused heart. We used this model to study the activity of the enzyme xanthine oxidoreductase in the heart of various species. Appendix 4 presents the results of this study. We also used the isolated rat heart in a number of pharmacological studies (appendices 6-9,11), as discussed below. The third model concerns myocardial ischemia in cardiological practice, i.e., during percutaneous transluminal coronary angioplasty and during an atrial pacing stress test (see also appendix 5 and [194]). The second part of Chapter 2 deals with the role of high-energy phosphate catabolites as markers for ischemia and discusses methods to quantitate them. The changes during development in activity of enzymes that regulate the catabolic and anabolic pathways of the adenine nucleotide metabolism (see also appendix 3). In addition Chapter 2 discusses the role of the enzyme xanthine oxidoreductase and the controversy in the literature on its presence and importance in various species (see also appendices 2 and 4).

Chapter 3 is dedicated to the pharmacological protection of the myocardium against ischemia. The use of calcium entry blockers, angiotensin-converting-enzyme inhibitors and the high-energy phosphate metabolites inosine and adenosine is discussed. Appendices 5-11 present studies carried out with the calcium antagonists diltiazem, nisoldipine and bepridil, with the angiotensin-converting-enzyme inhibitor captopril and with the purine inosine. In chapter 3 we also discuss the application of the purine adenosine in experimental and in clinical cardiology.

Chapter 4 summarizes the thesis.

Chapter 2

PATHOLOGY: MODELS AND MARKERS TO STUDY ATP CATABOLISM

Models

Ischemia of the heart and the consequences during recovery is a complex field of study. Many experimental models are used to answer partial questions concerning ischemia. None of the models suffices to study all aspects. Functional studies need a different approach and consequently a different model than studies on biochemistry or electrophysiology. In this chapter we will discuss three models for studying myocardial high-energy phosphate metabolism.

Heart-cell culture

The isolation and culture of cardiac tissue dates back to 1910, when Burrows[24] succeeded in cultivating embryonic chicken hearts. Fifty years later Harary and Farley[86] cultured cells of neonatal rat hearts after separation with proteolytic enzymes. These cells had a rounded shape after isolation but grew to a confluent layer of beating cells within two days. In 1977, Jacobson[104] described a model of adult rat heart-cell culture. In 1985, Piper et al.[167] described an improved method to isolate non-beating, rod-shaped adult myocytes. These cells were attached to the surface of a Petri-dish. These cultures could be kept stable for up to a week after which they dedifferentiated.

The mammalian myocardium consists for about 80% of the mass of myocytes, but in number those cells only represent 20% of the cardiac cells[15,56,105,140]. The other cells comprise fibroblasts, endothelial cells, neurons, pericytes, fat cells and smooth muscle cells. In this mixed cell population it is not possible to attribute properties to a single type of cells but, since it is possible to isolate a homogenous populations of cells, we can study the metabolism and enzymatic properties of one single cell type. Another point of consideration is that within one organ, the capillary wall provides a barrier which is not the case in isolated cells. Also arterio-venous gradients, probably varying over the organ, may exist.

A drawback of cell culture is that it is impossible to mimic ischemia completely. It is only possible to study a part of the changes associated with ischemia in organs. Cell-cell interactions, attraction of leucocytes, contracture and other phenomena cannot be studied. Moreover it is difficult to remove oxygen from a cell culture system.

For the study of energy metabolism, it is a serious limitation that adult myocytes do not contract spontaneously. Only about 20% of the ATP used in contracting cells is used by sarcolemmal ionic pumps and for biosynthetic processes. However this model is suitable for enzymatic studies as we show in appendix 3 and in reference 166.

Appendix 1 presents a study in neonatal heart-cell culture. We developed models for hypoxia and ischemia and compared the myocardial adenine nucleotide catabolism during anoxia and ischemia in the presence and absence of glucose. We induced anoxia by removal of oxygen from the incubation medium and simulated ischemia by simultaneous reduction in volume of the extracellular medium. This study shows that, when glucose was present, ischemia but not anoxia reduced the intracellular ATP

content. This maintenance of ATP levels could be due to an accelerated glycolytic flux. Without glucose, both anoxia and ischemia caused a rapid ATP breakdown. Under anoxic conditions, both the intracellular glycogen reserves and the large volume of (glucose containing) medium could contribute to the maintenance of high-energy phosphate levels.

Appendix 3 presents the age related alterations in activity of enzymes that catalyze ATP anabolism and catabolism. We measured the enzyme activities in cultured neonatal rat cardiomyocytes, in isolated adult rat cardiomyocytes, and in neonatal and adult rat heart homogenates. The age related changes observed will be discussed later. We conclude that rat cardiac myocytes have substantial activities of nucleoside phosphorylase, adenosine kinase and adenosine deaminase, in contrast to xanthine oxidoreductase.

Isolated rat heart

Many ways to perfuse isolated hearts have been described[186]. These models have the advantages of perfusing the myocardium through its own capillary bed. In a number of studies in this thesis we used the nonworking Langendorff heart, first described in 1895[126] (appendices 4,6,7,8,9,11). In this model the heart is perfused through the aorta. It is easy to mimic ischemia in isolated hearts by reducing coronary flow. Other advantages, compared with isolated cells or with the heart in situ, are: the heart is denervated, isolated from systemic influences and effects of other organs, but still functioning as the organ in situ. In this model it is possible to study the effects of external signals on coronary flow and contractility. In addition it is relatively easy to draw coronary arterial and venous samples to assess the arteriovenous differences in, e.g., lactate, purines, pH, pO_2 and enzymes. Furthermore it is possible to freeze-clamp the heart for the determination of unstable metabolites such as creatine phosphate and ATP.

Experiments in the Langendorff rat heart, carried out in our laboratory, showed that in unpaced hearts it is very difficult to induce severe ischemia by reduction of coronary flow. An 85% flow reduction caused a drop in heart rate from 250 to 125 beats per minute but the hearts didn't cease beating. This reduction in heart rate provided a good protection against ATP breakdown. After 30 min ischemic perfusion followed by 5 min reperfusion, only 2.3 μmol·g dry weight^{-1} (dwt^{-1}) ATP (about 12%) was lost as breakdown products and no ischemic or reperfusion arrhythmias were observed. Hearts paced at 300 beats per min, all stopped beating during ischemia, lost 7.5 μmol·g dwt^{-1} purines and oxypurines and all developed arrhythmias (unpublished observations). In the studies presented in appendices 6-9 and 11 we therefore decided to pace rat hearts at a constant rate of 300 beats per min using a pacemaker.

The set-up of the Langendorff heart model implies that it doesn't perform external work although it is beating. The work output is much lower than in the heart in situ or in the isolated "working" heart[50]. Several methods are used to define function in the Langendorff heart. Among them are displacement of the apex of the heart (see appendix 6), tension development measured with a force transducer (see appendices 8 and 9) and pressure development, measured with a fluid-filled balloon inserted into the left ventricle (see appendix 8). All three methods are suitable to provide an index of function but the rather high production of purines by the normoxic heart during measurement of function with an intraventricular balloon indicates that the endocardium could be damaged mechanically by the balloon[98].

Human heart in situ

An increased production of purines and oxypurines by the heart (e.g. during ischemia) reflects the breakdown of myocardial high-energy phosphates. In man this production can be assessed by measurement of the arteriovenous difference of these breakdown products.

Percutaneous transluminal coronary angioplasty (PTCA) for therapeutic applications, allowed the study of alterations in myocardial energy metabolism during and directly after the ischemic episodes[193,195,169]. These episodes are generated by transient interruptions of coronary flow during balloon inflation. The detection of persisting metabolic alterations may be very important as it is unclear whether intermittent brief periods of ischemia have a cumulative effect[4,76] or not[96,125,179,181]. We used this model of ischemia in the studies presented in appendices 2 and 5. In blood plasma of patients, catheterized for PTCA, we measured the coronary arterial and venous urate concentrations in the heart (appendix 2). Urate, the end-product of adenine nucleotide breakdown, rose significantly across the heart, indicating xanthine oxidoreductase activity in the heart. In another group of patients, urate release was maximal after the last of four consecutive occlusions and concomitant with increased hypoxanthine levels. We also showed that treatment of the patients with the Ca^{2+} antagonist diltiazem before and during angioplasty could reduce this hypoxanthine and urate release by 65 to 90 % (appendix 5).

Atrial pacing stress test (APST) provides another model of transient ischemia. This test is performed in patients, suspected of ischemic heart disease. The heart is electrically stimulated at increasing pacing rates until either chest pain appears or atrio-ventricular dissociation takes place. When the oxygen supply to the heart fails, ischemic markers like purine derivatives and lactate can be measured in the coronary effluent[85,121,182]. In addition reduced venous oxygen content and ECG changes are indices for ischemia in this model.

Purines as markers for ischemia

During and after ischemia of the heart, an excessive breakdown of ATP takes place. This process is described in Chapter 1. The ATP breakdown products as well as the high-energy phosphates themselves can be quantitated using high-performance liquid chromatography (HPLC) and UV detection.

Some of the ATP degradation products adenosine, inosine, hypoxanthine, xanthine and urate can be used as early indicators of myocardial ischemia because they can pass through the intact cell membrane. At a much later stage of ischemia, enzymes appear in the circulation, indicating an extensive cell damage.

In the Tyrode buffer, used for Langendorff perfusions, AMP-catabolites in the coronary effluent are relatively stable, provided that the effluent is collected on ice and HPLC is performed within hours. In blood, adenosine is very unstable; it has a half life of a few seconds[141]. Without precautions it is not possible to measure levels of adenosine (and its breakdown products) in blood plasma or in whole blood accurately. On the one hand, there is rapid adenosine uptake by erythrocytes (as also by capillary endothelium and by cardiac myocytes) where it is phosphorylated by adenosine kinase, on the other hand it is rapidly deaminated to inosine by adenosine deaminase which has a much

higher activity than adenosine kinase (appendix 3).

In our patient studies (appendices 2 and 5) we mixed the blood with 2,6-bis-[diethanolamino]-4,8-dipiperidinopyrimido-[5,4-d]pyrimidine (dipyridamole) and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA). These drugs inhibit the uptake and breakdown of adenosine in blood[62,160] and thereby stabilize the blood nucleoside pool. Other drugs that can be used to inhibit adenosine metabolism are the adenosine deaminase inhibitor 2'-deoxycoformycin and the adenosine kinase inhibitor 5-iodotubercidin[39]. Also the nucleoside transport inhibitors dilazep and nitrobenzylthioinosine inhibit cellular adenosine metabolism[39]. For rapid stabilization of adenosine, a double syringe to mix blood and a stop solution during sampling[160] and even a double-lumen catheter with one lumen for sampling, and the other for injecting a stop solution into the sampling catheter have been developed[196]. In our experiments we mixed the blood immediately after sampling with the stop solution. This method proved to be suitable to measure the adenine nucleotide catabolites hypoxanthine and urate, indicators of myocardial ischemia. Plasma hypoxanthine and urate levels remained constant during 30 min storage on ice when the blood was mixed with EHNA + dipyridamole (unpublished observations).

Inosine is a sensitive marker of ischemia. In myocardial cells it is rapidly broken down by nucleoside phosphorylase (appendix 11). We found that, in adult rat heart, the enzyme is equally distributed between myocytes and non-cardiac cells. These observations contrast with data for guinea-pig heart where the enzyme is low in myocytes but is high in endothelial cells[187]. Studies in the Langendorff rat heart revealed that after ischemia the rise in ATP breakdown products is most pronounced for inosine[43]. This is clearly shown in figures 5 of appendices 6 and 8). It makes inosine very suitable as an indicator for myocardial ischemia. Although the rise in hypoxanthine efflux during and after ischemia is less pronounced than that of inosine, there are two reasons to consider hypoxanthine as the best ischemic parameter in the blood of patients. The first reason is that hypoxanthine release lasts longer than that of the other compounds; the second is of practical origin. The HPLC method, used to quantitate the purines in blood plasma as described in appendix 5, is hampered by high concentrations of X-ray contrast which is necessary to assess the orientation of the catheters in the heart. Figure 2 gives a series of chromatograms of a coronary venous plasma sample obtained by measurement of UV absorbance at various wavelengths. The figure shows that the absorbance of the contrast agent is many times higher than that of the hypoxanthine peak, the subject of interest. In addition it is difficult to find the optimal chromatographic conditions (pH, buffer composition, flow) to separate of the hypoxanthine and contrast peaks sufficiently.

Xanthine and uric acid seem less suitable to quantify myocardial ischemia because of the activity of xanthine oxidoreductase. The activity of the enzyme, that converts hypoxanthine to xanthine and xanthine to uric acid, differs enormously in various species. Its activity in the human heart is still debated. Data vary from very high to virtually absent (see Table 1). The next part of this chapter will highlight some aspects of the enzyme xanthine oxidoreductase.

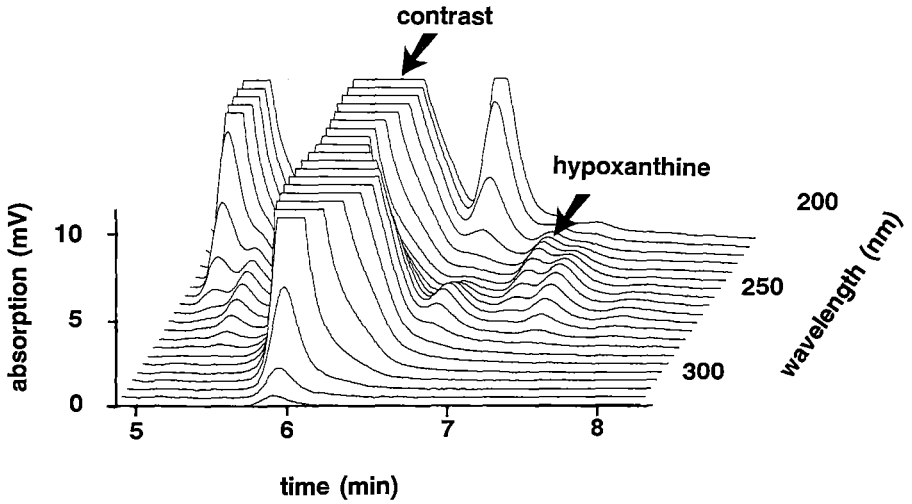

Figure 2. A part of a series of chromatograms obtained by measurement of the absorption of ultraviolet light with a wavelength between 220 and 320 nm. The Y-axis shows the mV signal which has a linear relationship with the amount of each compound. The height of a peak at each wavelength is determined by 1) the amount of the substance, 2) the molar absorption coefficient at that wavelength. Chromatograms were obtained using a diode array detector, on loan from Spectraphysics.

Xanthine oxidoreductase

The production of urate indicates that active xanthine oxidoreductase is catalyzing the catabolic reactions hypoxanthine → xanthine and xanthine → urate. Histochemical techniques showed that in bovine and in human heart xanthine oxidoreductase is localized in the cytoplasm of capillary endothelial cells[106]. This is in line with our observations in cultured rat-heart cells[191]: a high xanthine oxidoreductase activity in non-muscular cells and minimal activity in myocytes. In mammals the enzyme xanthine oxidoreductase can exist in two forms: xanthine dehydrogenase and xanthine oxidase. This enzyme, which usually occurs in the dehydrogenase configuration, is likely to be converted to xanthine oxidase during ischemia.

A consequence of this conversion is the production of superoxide radicals during the oxidation of (hypo)xanthine to urate. These are potentially toxic. The oxidase configuration of xanthine oxidoreductase can be converted back to dehydrogenase. After proteolysis, however, this is not possible[221]. Myocardial xanthine oxidase could play a role in the induction of atherosclerosis[164]. It cannot be an essential step in atherogenesis, since pigs can develop coronary atherosclerosis[202] in spite of the very low xanthine oxidoreductase activity in the heart of that species[45]. Xanthine oxidase, however, could play a role in myocardial reperfusion injury, a phenomenon first described by Hearse and colleagues in 1973[89,92]. In rat hearts the outcome of reoxygenation after ischemia seems

to be related with the glutathione status[163]. Oxidized glutathione will be released, indicative of the anti(per)oxidative action of reduced glutathione. The release of malondialdehyde, an end product of lipid peroxidation during, reperfusion[83] also indicates the operation of oxygen free radicals. The protective effect of the xanthine oxidase inhibitors allopurinol and oxypurinol[7,27,144] supports the idea of a role of xanthine oxidase in the generation of free radicals (Fig. 3), although those compounds may act as free radical scavengers[9,38].

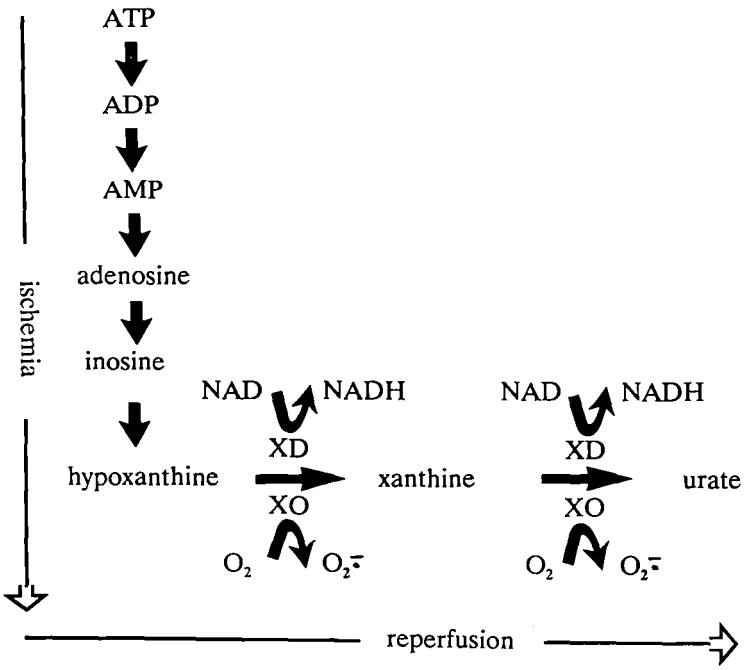

Figure 3. Catabolism of ATP during ischemia, which also causes conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO). The latter generates free radicals during reintroduction of oxygen (after McCord[137]).

Age

The age of the animal under study can affect the extent of reperfusion injury. The neonatal rat and rabbit heart are less susceptible to ischemia and hypoxia than adult heart[1,8,80,108,134,155]. The protective mechanism appears to be related to a better maintenance of myocardial ATP content which could be the result of more efficient glycolysis or of reduced purine loss[1]. The latter may be due to age-related changes in the activities of enzymes involved in adenine nucleotide metabolism[134].

We checked in rat heart whether xanthine oxidoreductase and other enzymes involved in myocardial energy metabolism varied with age (appendix 3), and we found an age-

related increase in xanthine oxidoreductase activity in rat hearts. A rise in the number of capillaries in heart[174] can explain most of the rise in activity during development. It could support the higher sensitivity to reoxygenation injury of adult rat heart[1]. We reported less urate production and better ATP preservation in neonatal than in adult reperfused rat heart[1,42]. The activities of adenosine kinase and xanthine oxidase were more than 10 times lower than those of adenosine deaminase and nucleoside phosphorylase. During development the ratio of the rate-limiting enzymes for anabolism (adenosine kinase) and catabolism (xanthine oxidoreductase) dropped 100-fold in ventricles. In myocytes the ratio remained unchanged. The large difference, observed in heart homogenates, explains why the rate of ATP catabolism due to hypoxia is less in neonatal than in adult heart. Because this change is absent in myocytes, we speculate that, during development, endothelial activities of the rate-limiting enzymes adenosine kinase and xanthine oxidoreductase are responsible for the shift in purine metabolism from anabolism to catabolism (appendix 3).

Species differences

Literature data on xanthine oxidoreductase activity in the heart of a number of species vary strongly (Table 1); especially the data on the human heart are controversial. Ronca-Testoni and Borghini[184] were the first to show urate production in the isolated, perfused rat heart. We confirmed their observation that urate is the major purine released from rat heart under basal conditions[42,190]. Gerlach et al.[77] did similar observations in guinea-pig heart. We reported blockade of hypoxanthine breakdown during anoxia by allopurinol[190]. The observations strongly suggested that rat and guinea-pig heart contain xanthine oxidoreductase. Enzymatic measurements confirmed this hypothesis[112,136,143,190].

Enzymatically active xanthine oxidase has been demonstrated in the heart of a number of species. Literature data on xanthine oxidoreductase activity in human heart vary from high[106,120,217] to very low [61,81,143,175,199,220]. The number of samples assayed was often very small. Muxfeldt and Schaper[143] found very low amounts of xanthine oxidoreductase in the two human-heart biopsies studied. Krenitsky et al.[120] report data on one autopsy sample. These authors observed enzyme activity with ferricyanide as the electron acceptor, but did not use NAD^+ or oxygen as the cosubstrate. Allopurinol inhibited the activity. Eddy et al.[61], Grum et al.[81] and Smoleński et al.[199] could not demonstrate xanthine oxidoreductase in human myocardial tissue. In sharp contrast is the high activity in nine biopsies, reported by Wajner and Harkness[217].

Data obtained in catheterized patients[154] suggested that the human heart can release urate, an indication of cardiac xanthine oxidoreductase activity. These observations initiated the study in patients undergoing coronary angioplasty (appendix 2). In this study we showed that in catheterized patients the arterio-venous differences in urate were significant. It has been suggested that patients with a more severe ischemic heart disease produced the highest amounts of urate. Patients experiencing pain during a pacing stress test released lactate and showed the highest urate production[154]. Czarnecki observed that patients with a history of subendocardial infarction produced high amounts of urate whereas patients with normal myocardium or extensive myocardial damage produced less[35]. Our study supports this idea. Patients, hospitalized to undergo coronary angioplasty, with a high grade of ischemia, showed significant urate production before angioplasty while less severe ischemic patients only started to produce significant amounts of urate after several dilations. In these patients the venous plasma

hypoxanthine concentration (the substrate for xanthine oxidoreductase) rose 4x after each dilation. Supposedly ischemic myocardium at risk of infarction produces urate.

On the other hand, based on our isolated heart perfusions (appendix 4), we must conclude that hearts from patients with cardiomyopathy or end-stage ischemic heart disease are almost devoid of xanthine oxidoreductase activity. We cannot exclude the possibility that the diseased human heart contains inactive xanthine oxidoreductase. In 1972, Edmondson et al. already mentioned the existence of active and inactive xanthine oxidase, and developed a method to separate these[64]. Recently Terada et al. described that xanthine oxidase can destroy itself by self-generated O_2 metabolites[206]. At present we assume that extracardiac factors, such as neutrophils or other blood components, are responsible for the apparent xanthine oxidoreductase activity observed in catheterized patients or autopsies[217,120]. It is likely that the urate production measured in patients during angioplasty originated from xanthine oxidoreductase activity in polymorphonuclear neutrophils, adhering to areas of the coronary endothelium that are injured by the balloon during inflation. This would explain the discrepancy between the high xanthine oxidoreductase activity measured in catheterized patients and the very low activity in the isolated, salt perfused human heart.

In a study in isolated perfused hearts (appendix 4) we compared the apparent activity of xanthine oxidoreductase in isolated mouse, rat, guinea pig, rabbit, pig, cow and human hearts. For this purpose we measured the conversion of exogenous hypoxanthine to xanthine and urate. We assayed these purines with high-performance liquid chromatography. The apparent xanthine oxidoreductase activities were (mU/g wet weight): mouse, 33 ± 3 (n=5); rat, 28.5 ± 1.4 (n=9); guinea pig, 14.4 ± 1.0 (n=5); rabbit, 0.59 ± 0.09 (n=7); pig, <0.1 (n=6); man, 0.31 ± 0.04 (n=7); and cow, 3.7 ± 0.8 (n=4). In rabbit heart the conversion of hypoxanthine to xanthine was slow, that of xanthine to urate even slower. On the other hand, guinea-pig and human heart released little xanthine, indicating that xanthine breakdown exceeds its formation. We concluded that isolated perfused mouse, rat, guinea-pig, and bovine hearts show considerable xanthine oxidoreductase activity, contrasting rabbit, porcine and human hearts. We are unable to understand the evolutionary reasons for the differences in activity found.

Author(s), year	Reference	Human	Dog	Rat	Mouse	Cat	Cow	Sheep	Rabbit	Guinea pig	Pig	Monkey
Al-Khalidi & Chaglassian '65	(2)		0.3			0.01	0.22	0.007				
Watts et al. '65	(220)	0.01&0.02*										
Ramboer '69	(175)	0	0	12			6					
Battelli et al. '72	(10)			60								
Maguire et al. '72	(133)			33								
Brunschede & Krooth '73	(23)			12*								
Lee '73	(129)				3.4							
Krenitsky et al. '74	(120)	74										<30
Prajda et al. '76	(171)			$2{\cdot}10^5$*								
Ho & Clifford '77	(94)								0			
Amory et al. '78	(3)			0								
Gandhi & Ahuja '79	(75)			0					0			
Kela et al. '80	(115)			73								
Jarasch et al. '81	(107)						+					
Oei et al. '82	(157)			0.05*								
Ronca-Testoni & Borghini '82	(185)			15								
Bruder et al. '83	(22)						7.3*					
Schoutsen et al. '83	(190)			31								
Chambers et al. '85	(26)		14*									
Gerlach et al. '85	(77)									3		
Grum et al. '86	(82)			53					0			
Jarasch et al. '86	(106)	+					+					
Kaminski et al. '86	(112)			28								
Downey et al. '87	(57)			25					<0.5			
Das et al. '87	(38)									0		
Eddy et al '87	(61)	<0.002		47								
Engerson et al. '87	(65)			61*								
Matucci et al. '87	(136)									8		
Muxfeldt & Schaper '87	(143)	1.3	0.95	19.5					0.05	7.8	0	
Podzuweit et al. '87	(168)										0	
Schoutsen et al. '87	(191)			25					0.003			
Achterberg et al. '88	(1)			28								
Grum et al. '89	(81)	0										
Smolenski et al. '89	(199)	<0.1										
Wajner & Harkness '89	(217)	67*							54[4]			
De Jong et al. '90	(46)	0.3		28.5	33		3.7		0.59	14.4	<0.1	

Table 1. *Xanthine oxidoreductase activity in the heart of several species. Activity in mU g/wet weight (1 U = 1 μmol/min); * Data based on supernatant protein content have been recalculated to wet weight, using a factor of 18 (ref. 143); + XOD demonstrated histochemically.*

Chapter 3

THERAPY: PHARMACOLOGICAL INTERVENTIONS

In this chapter the effects of several chemical compounds on myocardial energy metabolism are described, focusing mainly on their potency to protect the heart against the deleterious effects of ischemia.
The selection of compounds described in this chapter is based on the studies in appendices 5,6,7,8,9 and on the general interest of our laboratory in high-energy phosphates and related compounds as described in appendices 10 and 11. It is not intended to provide a complete list of possible cardioprotective agents.

Calcium antagonism

Calcium

The myocardial extracellular Ca^{2+} content in mammals is about 2 mM; intracellular Ca^{2+} is mainly located in the endoplasmic reticulum. The cytosolic Ca^{2+} concentration is 200 to more than 20,000 times lower than the extracellular concentration during systole and diastole, respectively. The changes in cytosolic concentration can occur via mobilisation of calcium from subcellular organelles or from outside the cell. Calcium can enter the cell by passive diffusion, voltage-activated transport, and in exchange with sodium or with potassium. Calcium influx and extracellular release from organelles on the one hand and removal on the other hand during one contractile cycle vary the cytosolic calcium concentration between 0.1 and 10 μM[127]. This increase, caused by a voltage-dependent Ca^{2+} influx triggers an intracellular calcium pool from which additional Ca^{2+} necessary for contraction is released. During the relaxation phase the Ca^{2+} is removed from the cell by calcium pumps and exchange mechanisms. During oxygen deprivation, the protons, ammonium ions and phosphates produced by degradation of creatine phosphate and adenine nucleotides, as well as the intermediates produced by the anaerobic catabolism of glucose and glycogen increase the intracellular osmolarity[203]. Because ATP-ase activities (which regulate the maintenance of sodium, potassium and calcium homeostasis) decrease, intracellular potassium decreases, intracellular sodium increases and cytosolic calcium rises. This calcium does activate endogenous proteases and phospholipases and thereby cause further damage to the cell membrane which finally results in cell death and necrosis.

Calcium entry blockade

The cytosolic alteration in calcium content is not an early event in myocardial ischemia. Normal cytosolic calcium concentrations can be measured despite serious declines in ATP and creatine phosphate[204]. Nevertheless drugs that prevent Ca^{2+} influx into the cell proved to protect the myocardium against ischemic damage.

The discovery of Ca^{2+} antagonism dates back to 1964 when Fleckenstein reported two compounds that mimicked Ca^{2+} withdrawal[72]. In the definition of Fleckenstein[70,71], a calcium antagonist specifically blocks excitation-contraction coupling by mitigating the effects of Ca^{2+}. Nayler states that a substance can be labelled a calcium antagonist when

it 1) exerts a dose-dependent stereoselective inhibitory effect on the slow Ca^{2+} current; and 2) exhibits stereoselective high-affinity binding for its receptor. Other names used are "calcium entry blocker", "calcium channel inhibitor" or "slow-channel inhibitor". These names refer to the common property of these compounds to inhibit the slow inward calcium flux which causes the plateau phase of the action potential.

During myocardial ischemia calcium antagonists can diminish the reduction of myocardial ATP (thereby decreasing the release of purines from the heart) by increasing ATP formation and/or reducing energy expenditure. Increased capillary perfusion may improve the availability of oxygen and substrates, and accelerate the removal of metabolic products[148,215,218,219]. Depressed myocardial contractility reduces energy expenditure[148,218]. In addition some Ca^{2+} antagonists decrease the heart rate. The preservation of myocardial ATP content seems to be due to decreased ATP use, not an increased rate of ATP synthesis as we show in appendix 7 that, during low flow ischemia in isolated rat heart, glycolysis is diminished by nisoldipine treatment. Related cardioprotective effects of Ca^{2+} antagonists are: protection of vascular endothelium[138], sarcolemma[37] and mitochondria[146]; slower release of lysosomal enzyme[99]; inhibition of platelet aggregation[101]; reduction of reperfusion arrhythmias[212]; prevention of noradrenaline displacement[147] and slower Ca^{2+} accumulation during reperfusion.

Recently, Nayler reported that nifedipine could reduce the deleterious effect of endothelin. This polypeptide secreted by vascular endothelial cells[228] causes vasoconstriction[228], is positive inotropic[102], causes an intracellular Ca^{2+} rise and induces a depolarization, sufficient to activate the voltage-sensitive calcium channels[102] (Fig. 4). These events ultimately result in irreversible tissue injury. Ischemia and reperfusion increase endothelin binding-site density[150]. Pretreatment of hearts with nifedipine reduced the increase in binding-site density while the endothelin-receptor affinity was unaltered[150]. Thus nifedipine, and probably other calcium antagonistic drugs, reduces the endothelin-related rise in Ca^{2+}.

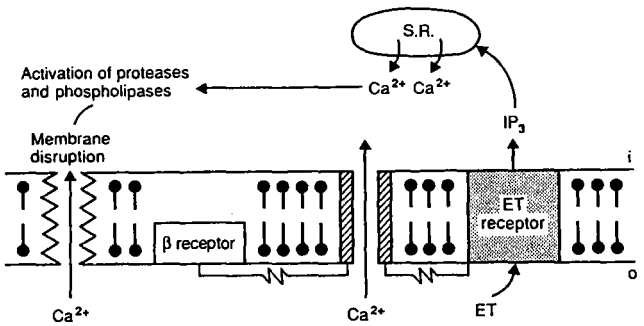

Figure 4. Schematic representation of the possible involvements of endothelin receptor activation during ischemia and reperfusion. (figure taken from reference 150, with permission). S.R. = sarcoplasmic reticulum; ET = endothelin; IP_3 = inositol triphosphate; i = inner membrane; o = outer membrane.

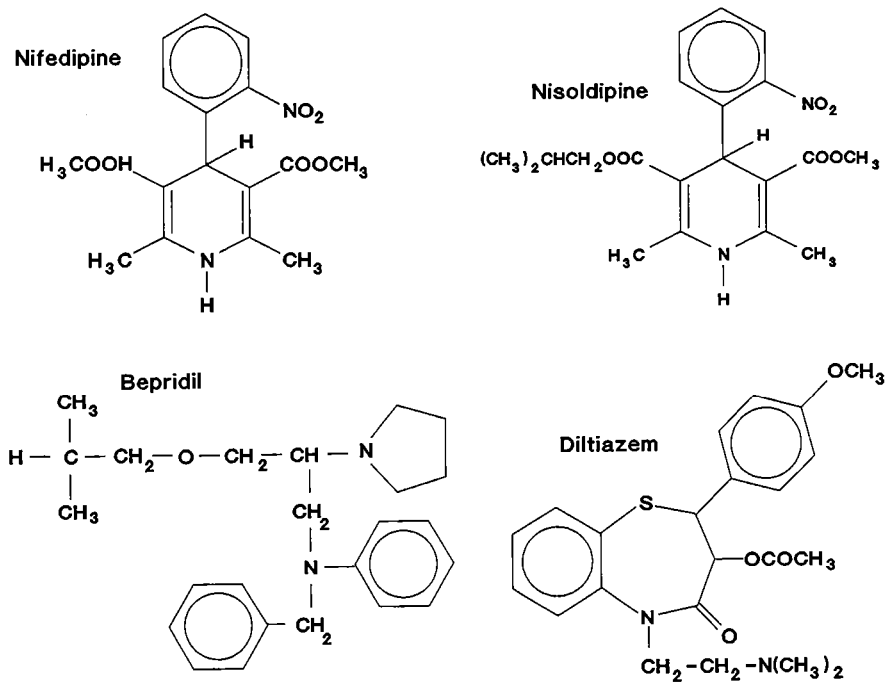

Figure 5. Chemical structure of the calcium antagonists used in our studies.

Classification of calcium antagonists

The heterogeneity of calcium antagonists calls for classification. Several criteria are used as a basis for classification (for a review, see ref. 149). Recently two descriptive classifications were developed, one based on comparable pharmacology, in relation to recognized prototypes[216], the other on the combination of pharmacology and specific binding site interaction[161]. In this thesis we use a third classification, based on the chemical structure of the compounds[149]. It has as a drawback that tissue- and organ specificity is not taken into account but it is a simple approach.

Basically calcium antagonists can be divided into an organic and an inorganic group. Co^{2+}, Ni^{2+}, La^{3+} and Mn^{2+} belong to the inorganic Ca^{2+} antagonists[149]. Clinically they are useless because of their toxicity. A wide variety of natural and synthetic compounds form the organic Ca^{2+} antagonists. Some naturally occurring calcium antagonists originate from plants, others are exogenous or endogenous in animals, including man (for a review, see ref. 149). The adenine nucleotide metabolite adenosine has Ca^{2+}-antagonistic properties but they are too weak to account for the strong vasodilator potency[142]. The calcium antagonistic action of adenosine is restricted to neuronal calcium channels[142]. On the basis of their chemical structure the synthetic Ca^{2+} antagonists can be grouped. Some of these groups are:

Phenylalkylamines

Phenylalkylamine-based calcium antagonists are water-soluble, light-stable compounds. It seems likely that their calcium antagonistic properties depend on the presence of two benzene rings and a tertiary-amino group linking these rings. As a group, they lack selectivity in the cardiovascular system. They are equipotent in blocking Ca^{2+} channels in the myocardium and the vasculature, they also affect atrioventricular conducting tissue. Some of them are long-acting drugs, probably useful in the treatment of hypertension[149]. A few examples, in rank order of their potency as Ca^{2+} antagonists, are: anipamil, gallopamil, verapamil, prenylamine, fendiline, terodiline and tiapamil.

Dihydropyridines

Nifedipine is the prototype in this group. It is insoluble in water, photolabile and acts preferentially on the vasculature. The dihydropyridine structure and the NH-group are essential. Other substituents in the basic structure of this group determine the potency, vascular selectivity and duration of action.

Nisoldipine differs from nifedipine in an isobutyl instead of a methyl substitute at one of the two 3,5-ester groups of the heterocyclic ring. This results in a higher vascular selectivity and a longer duration of action when compared with nifedipine[114]. Newer, more potent and longer acting dihydropyridine derivatives include: nimodipine, niludipine, niguldipine, isradipine, PY 108-068, 8363-5 and (-)R-202-791, KW-3049.

Benzothiazepines

The benzothiazepines are photostable and soluble in water where they become ionized. They are powerful coronary vasodilators without vascular selectivity. Diltiazem is the first developed representative of this group. In contrast with the dihydropyridines this group is equipotent for the myocardium, vascularity and atrioventricular conductive tissue.

Other calcium antagonists

Many other compounds exert Ca^{2+} antagonistic properties. Examples include quinoxaline and quinazoline derivatives, molsidomine, perhexiline and some drugs with Na^{+}-channel blocking properties, such as bepridil and the piperazine derivatives lidoflazine, cinnarizine and flunarizine.

Ca^{2+} antagonists in isolated rat heart

The papers presented in this thesis, in which the isolated rat heart preparation was used include two studies with the calcium antagonist nisoldipine (appendices 6 and 7) and one study with bepridil (appendix 8). Nisoldipine as well as bepridil increased coronary flow rate and decreased contractility in a dose-dependent manner. For nisoldipine the vasoactive concentration was about 30 times lower than that needed to induce negative inotropy. For bepridil this difference was about 3-fold but, nisoldipine showed to be a 1000 times more powerful vasodilator and a 100 times more potent negative inotropic agent than bepridil (Table 2). Both compounds dose-dependently reduced high-energy phosphate breakdown. Again nisoldipine was 100 times more potent than bepridil.

We observed a striking correlation between negative inotropy before ischemia and purine efflux during ischemia (Fig. 6). On the other hand, these studies with nisoldipine and bepridil show that concentrations which are too low to induce negative inotropy

could reduce purine efflux to some extent. This indicates that factors other than negative inotropy protect the isolated heart against ischemic damage.

We calculated whether a decreased purine efflux from the heart reflected a better preservation of myocardial ATP or resulted from a reduced membrane permeability. The close correlation between ATP loss and purine efflux (appendices 6,8) confirmed a lower ATP breakdown in both studies.

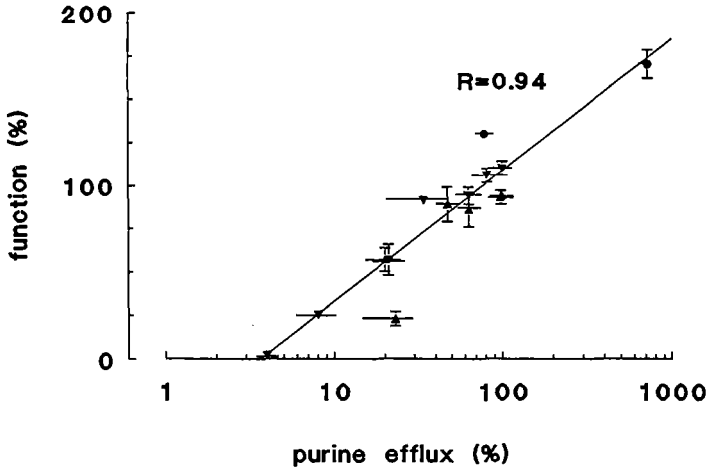

Figure 6. Correlation between normoxic function and ischemic energy metabolism. Data were obtained from appendices 6 and 8. Closed dots: 5.0 and 1.4 mM $CaCl_2$ in perfusion medium. ▲ and ▼: various concentrations of bepridil and nisoldipine in the perfusion medium, respectively. Each point shows the mean of 4-6 observations; vertical and horizontal lines indicate the s.e.; function 100% = function of untreated hearts; purine efflux 100% = efflux of untreated hearts.

Administration of nisoldipine only during ischemia and/or reperfusion provided no protection against ATP breakdown[49]. Diltiazem, on the other hand, gave some protection, when administered during ischemia, However, better protection was obtained when it was given before ischemia[44]. Even when it was only given during reperfusion, diltiazem reduced reperfusion-induced ventricular fibrillation[212]. The latter effect may result from α-adrenergic-antagonist activity that have been described for verapamil and diltiazem[151]. Nifedipine does not possess such properties[151].

Table 2 shows that in the Langendorff preparation, various Ca^{2+} antagonists show differences in their vasodilator and negative inotropic efficacy and vary in their potency to diminish high-energy phosphate breakdown, suggesting different sensitivities of vascular smooth- and striated (parenchymal) muscle cells for the drugs.

Ca^{2+} antagonist	coronary flow plus 50 %	contractility minus 50 %	purine efflux minus 50 %
bepridil	1 μM	3 μM	0.3 - 1 μM
diltiazem	10 μM	5 μM	10 μM
nifedipine	30 nM	100 nM	100 nM
nisoldipine	1 nM	30 nM	3 - 10 nM

Table 2. Concentration of Ca^{2+} antagonist that caused a 50 % change compared with control values. Data are obtained from studies in our laboratory (refs. 43,44, appendices 6 & 8), carried out in the Langendorff rat heart.

Ca^{2+} antagonists during angioplasty

The effects of calcium antagonists in the intact circulation is quite distinct from the effects in isolated preparation. The negative inotropy can be concealed by accompanying changes in pre- and afterload and by reflex changes caused by the drop in blood pressure. It is only when contractility has already been impaired or the reflex compensatory mechanisms are inhibited that the negative inotropy plays an important role.

Appendix 5 describes a study in humans, catheterized for percutaneous transluminal coronary angioplasty. In this study the Ca^{2+} antagonist diltiazem (intravenously administered) was used in an attempt to protect the myocardium against ischemia during balloon inflations. The efflux of ATP catabolites was significantly lower in diltiazem-treated patients than in controls. The drug tended to reduce the amount of lactate produced. However, this effect was not statistically significant. Supposedly this lack of statistical significance is due to the large individual variation in lactate. Diltiazem could not diminish ECG changes. Nifedipine, administered intracoronarily provided some protection in a similar model[169]. In this study less lactate was produced by hearts of treated patients during the ischemic episodes indicating a less severe ischemia. This was confirmed by less elevation of the ST-segment in the ECG. It is puzzling that diltiazem did not reduce ECG changes.

The renin-angiotensin system

Until the mid-seventies the renin-angiotensin system was considered to be a system in the blood circulation, dependent on the renin concentration in plasma and on the angiotensinogen produced in the liver. At first the end-product angiotensin II seemed to be important only in maintaining blood pressure after sodium depletion. However, in later years it became clear that angiotensin II could also be generated by other organs. These independently operating renin-angiotensin systems may be involved in (ischemic) heart disease[25,59,60,132,177]. The existence of locally operating renin-angiotensin systems has been established biochemically, functionally and genetically in a number of tissues. Conversion of angiotensin I to angiotensin II has been demonstrated in the heart of several species[34,145,152,230]. Among them is the isolated perfused rat heart[78,227]. In the isolated rat heart perfusion with angiotensin I increased angiotensin II levels ([132,226],

appendix 9). Angiotensin II exerts a vasoconstrictor effect on coronary arteries and generally it is positive inotropic in a direct and indirect way. In isolated heart the effects on contractility are controversial. Many studies reveal a positive inotropic effect of angiotensin II[117,118,131] but some studies report no effect ([74], appendix 9) or even negative inotropism[189]. It took a lot of effort to separate the indirect and direct positive inotropy of angiotensin II. Its direct effect is mediated through an increased calcium current[208], the indirect inotropy is caused by activation of cardiac sympathetic neurotransmission[17,165,230]. Angiotensin II may also interact with the atrial natriuretic peptide which antagonizes the effects of angiotensin II[128]. Activation of this system by coronary occlusion in vivo causes a constriction of arteriolar smooth muscle and increases systemic arterial pressure[68,118].

These properties could contribute to postischemic deterioration and even exhaust the myocardial energy status[11]. Inhibition of the angiotensin-converting enzyme (ACE) has a number of effects that could be beneficial to the (post)ischemic heart.

ACE inhibition

In vivo the actions of the inhibitors have only been partly clarified. In normal subjects angiotensin-converting-enzyme inhibitors lower blood pressure but do not affect cardiac output[119]. Reduction of angiotensin II release directly causes vasodilation but also decreases aldosterone production, interferes with the sympathetic nervous system, and affects the central nervous system. In addition angiotensin-converting enzyme prevents breakdown of the potent vasodilator bradykinin[209].

Captopril

Captopril, an angiotensin-converting-enzyme inhibitor, blocks the positive inotropic effect of angiotensin I in isolated cat heart[34]. On the other hand, perfusion with angiotensin I plus captopril reduces myocardial flow in isolated hamster hearts but increases left ventricle developed pressure, favouring a direct effect of angiotensin I[93]. In isolated rat heart, captopril reduces the ventricular fibrillation which was induced by regional ischemia followed by reperfusion[183,214] and consequently reduces the loss of purines[41,173,213] and that of noradrenaline[214]. Also in Langendorff rat hearts with segmental infarction, which was deteriorated by angiotensin I, captopril preserved the force of contraction and the release of creatine kinase[130].

In patients with ischemic heart disease and congestive heart failure, enhancement of myocardial performance after captopril[29,84] appears to be dependent on the reduction of angiotensin-mediated ventricular pre- and/or afterload[84,135]. The drug does not affect myocardial oxygen demand and consumption, or lowers them in patients with heart failure[84,170]. This reflects enhanced cardiac output without increased oxygen demand. In healthy dogs, captopril does not seem to have a direct cardiac effect[156], but after acute left anterior coronary artery occlusion, blockade of the renin-angiotensin system lowers systemic blood pressure and improves cardiac output[131]. Recently it has been reported that sulfhydryl-group containing substances can act as free radical scavenger. ACE inhibitors like captopril, containing such a group, proved to scavenge superoxide anions[223] and may therefore be superior in the treatment of reperfusion-induced myocardial dysfunction. Appendix 9 presents a study with captopril in the Langendorff rat heart. The drug blocked the conversion of angiotensin I to angiotensin II as shown by the increased coronary flow but did not affect the myocardial function during normoxia. The

postischemic myocardial function tended to recover faster in captopril-treated hearts than in untreated ones but this was not the result of reduced ATP breakdown during ischemia or a better high-energy phosphate recovery during reperfusion. We conclude that the rat heart contains an active angiotensin-converting enzyme which can be blocked by captopril. Regarding its cardioprotective effect, we speculate that the differences between various models are responsible for the variation in function recovery and energy preservation seen by various investigators. Many mechanisms that are affected by the renin-angiotensin system in the intact animal do not play a role in the isolated heart, perfused with Tyrode buffer and paced artificially. However, captopril prevents the direct effects of angiotensin I on the myocardial vasculature by blockade of the converting enzyme.

High-energy phosphate precursors

As described before, ischemia leads to a rapid fall in myocardial adenine nucleotides due to dephosphorylation of high-energy phosphates, followed by leakage of the purine nucleosides and oxypurines from the cell. Several compounds of the adenine nucleotide metabolism have been studied with respect to their possible protective effect during ischemic episodes. After an ischemic insult, it is imperative to improve the myocardial energy status. There are four pathways available to regenerate ATP in the heart[79]: 1) De novo synthesis; 2) Hypoxanthine or inosine salvage; 3) Adenine ribophosphorylation; 4) Adenosine phosphorylation. In this chapter we discuss the effects of some nucleotide cycle metabolites during ischemia and/or reperfusion.

Inosine

Inosine is a naturally occurring compound. In human blood the inosine concentration is about 1 μM[73,87]. Its concentration in coronary effluent of the salt perfused rat heart is about 0.2 μM (appendices 6,8). As many naturally existing compounds, inosine has a variety of effects both on the normoxic as well as on the ischemic heart. In the normoxic isolated rat heart infusion of concentrations of 30 μM and higher cause dose-dependent vasodilation[110,211]. Inosine increases contractility in rabbit heart[52,122] but not in rat heart[172,211].

In vivo the effects of inosine are much more complex due to interactions with, e.g., the adrenergic- and the renin-angiotensin system.

Inosine increased cardiac contractility in dogs[36,111,197,207], in open-thorax pig[210,225] and in man ([35], appendix 10). On the other hand, it did not alter or was negative inotropic in open-thorax guinea pig and rat heart[95]. Inosine can be used to regenerate ATP but must first be catabolized to hypoxanthine[55]. The incorporation rate is very low, compared with adenosine[5,55,88,231].

Added to a cardioplegic solution, inosine reduced the degradation of ATP and improved functional recovery in rat heart. When added to the reperfusion fluid as well as to the cardioplegic solution inosine further improved nucleotide levels and recovery of cardiac output[53].

Our experiments in the isolated rat heart (appendix 11) showed that inosine and adenine (20 μM, both, plus ribose), infused during reperfusion, did not affect the recovery of function and hardly affected nucleotide levels. The calculated incorporation

rate of inosine + ribose in glucose perfused heart was about 40 nmol·min^{-1}·g dwt^{-1} which is in line with the rate measured by others[5,88]. Only IMP levels had doubled when inosine was infused. Inosine administration during reperfusion increased the adenosine concentration in coronary effluent sixfold. In normoxic rat heart this has been described before[211]. Interference of inosine with adenosine metabolism could be explained by transport competition with the adenosine carrier[159]. Intravenous infusion of inosine to catheterized humans (appendix 10) increased arterial inosine concentrations and, more important, measurement of the arteriovenous difference revealed a substantial inosine uptake by the heart which was followed by improved hemodynamics.

Adenosine

Like inosine, adenosine is a catabolite of adenine nucleotide metabolism. In contrast to inosine, which has a relatively long half-life in blood, adenosine exists in blood plasma for less than one second[141]. In the circulation it may even be shorter because of the high activity of adenosine deaminase in endothelial cells (appendix 3). Consequently the adenosine concentration in blood is very low (ca. 0.01 μM)[39], and difficult to quantitate. In coronary effluent of normoxic isolated rat heart, the adenosine concentration is about 0.1 μM (see, e.g., appendix 8). Adenosine is rapidly broken down by adenosine deaminase and after cell entry mainly phosphorylated to AMP[54] in the erythrocytes and in the capillary endothelium[77]. It is doubtful whether the incorporation into myocytes is also rapid since adenosine transport over the membrane (which is a carrier-mediated process) is the rate-limiting step[20,55].

Adenosine and ATP recovery

Adenosine incorporation is the fastest pathway to increase the sum of adenine nucleotides[21,231]. The concentration used to attain a gain in ATP in cardiomyocytes must be rather high because adenosine in concentrations below 1 μM does not reach the interstitium[51]. In normoxic heart adenosine treatment elevates myocardial ATP content[18,19,97,103]. This higher level does not necessarily protect against function loss during subsequent ischemia and reperfusion[97]. In postischemic isolated rat heart treatment with adenosine for 30 min, did not increase the ATP content[179]. Treatment for hours restored ATP levels[178].

Other effects of adenosine

Adenosine has a number of effects on the myocardium. It is hypotensive, has negative chronotropic and dromotropic properties[12,176], reduces free-radical generation, inhibits platelet aggregation, provokes chest pain and interferes with the sympathetic nerve system.

The vasodilator effects are most pronounced and well-known for more than sixty years[58]. In isolated rat heart 0.1 μM adenosine causes vasodilation. Considering that such a low concentration does not reach the interstitium, the vasodilation must be mediated by the endothelium.

As described before adenosine is a catabolite of adenine nucleotide breakdown and thus released into the circulation. The vasodilator effect could reduce ischemia by regulation of the coronary blood flow[14], but could also aggravate ischemia by coronary steal[63]. Recent evidence suggests that adenosine provokes angina-like pain[31,123,124,205]. In this way it could function as a warning signal during ischemia. On the other end this pain

could also result of ischemia, caused by reduced perfusion pressure which is the consequence of vasodilation in an area in which the flow is limited by atheroma.

Adenosine inhibits impulse generation in the sinus node and the conduction in the AV-node. The effects on the sympathetic nerve system are not clear. Adenosine reduces noradrenaline release after sympathetic stimulation[222] in isolated rabbit hearts, has no effects in open-chest pigs[188] and even stimulates (nor)adrenaline release in humans[198]. The action of adenosine on heart rate reflects the effects on the electrical activity and on the sympathetic nerve system; a decrease in the isolated heart of several species, and an increase in conscious dogs and humans[16,30] which can be attenuated by ß-blockade and totally blocked by vagal section. In dog hearts subjected to ischemia and reperfusion in vivo, adenosine preserves endothelial integrity, prevents microvascular injury and thus the no-reflow phenomenon and may thereby protect parts of myocardium during reperfusion[6,158].

Adenosine inhibits neutrophil activation[32,33] which results in a reduced superoxide anion generation by neutrophils and may thereby limit infarct size[66,67]. Finally, extracellular accumulation of adenosine could suppress various lymphatic functions such as lymphocyte-mediated cytolysis[224,229].

Adenosine and cardioplegia

Arrest of the heart, often necessary during cardiac surgery, can be accomplished by cross-clamping of the aortic root. This leads to an ischemic cardiac arrest which results in metabolic and (ultra)structural damage which in turn gives rise to a reduced functional recovery after reperfusion. Nowadays most clinics use a cold cardioplegic solution containing high potassium to arrest and protect the heart during surgery. Nevertheless, some hearts need support from the extracorporeal circulation for some time early during reperfusion after cardiac surgery. The hearts need this support probably because of deprivation of the myocardial high-energy phosphate content during cardioplegia[113,200]. A large number of substances in various dosages have been tried as additive to improve the cardioplegic solution[28,48,90,139].

Adenosine could be valuable as additive to cardioplegic solutions. It inhibits the electrical activity of the SA- and AV-node[13] and the slow calcium channels[69,192]. These effects could accelerate cardiac arrest and thus reduce energy loss. In addition, adenosine is an ATP-regenerating metabolite. In isolated rat heart, addition of adenosine to the cardioplegic solution accelerated cardiac arrest and improved function during reperfusion[47]. Inhibition of the adenosine breakdown with the adenosine deaminase inhibitor EHNA could also be beneficial. It increases the intracellular adenosine concentration and facilitates re-incorporation in ATP during reperfusion.

Chapter 4

SUMMARY

Myocardial ischemia is a life-threatening situation. It leads to a cascade of events that can arbitrarily be divided into reversible and irreversible processes. The latter finally result in cell death and tissue necrosis. It is of decisive importance to abolish ischemia as quickly as possible. Breakdown products of myocardial energy catabolism proved to be suitable markers for the early detection of ischemia. In chapter 2 three models to study myocardial energy metabolism are described: rat cardiomyocyte cultures; isolated perfused heart of various species; and human heart in situ, i.e., during coronary angioplasty and stress testing. Isolated myocytes proved to be a suitable tools for studying energy metabolism, transport and enzyme kinetics. Appendix 1 describes the use of cultured heart cells for studying oxygen deprivation in a novel model for anoxia and ischemia. We conclude that ischemia, but not anoxia, exhausts intracellular ATP stores when glucose is present as substrate. In the absence of glucose, both anoxia and ischemia cause rapid adenine nucleotide breakdown. This study presents the kinetics of high-energy phosphate breakdown and concomitant nucleoside and oxypurine production.

In addition we used rat heart homogenates and isolated cells to study the age-related changes in the activity of the enzymes that regulate ATP metabolism (appendix 3). From this study we conclude that during development the catabolic pathways involved gain importance as the anabolic enzymes become relatively less important.

The activity of the catabolic enzyme xanthine oxidoreductase (XOD) was also measured in the isolated heart of a number of species (appendix 4). This enzyme plays an important role in the generation of oxygen free radicals. These oxygen species are implicated in the generation of cardiac damage. The activity of XOD varies from high (mouse, rat, guinea pig) to very low (rabbit, pig, man). Paradoxically, we measured significant urate release from the heart of catheterized patients (appendix 2). We think that, in man, the adhesion and penetration of leucocytes in diseased hearts may be responsible for XOD activity.

Chapter 3 highlights the application of several chemical compounds that could benefit the energy metabolism of the heart. Some improve coronary perfusion and/or reduce myocardial function, others are intermediates of high-energy phosphate metabolism. The studies in isolated heart indicate that some compounds with negative inotropic properties can protect the heart against ischemic damage. We observed a striking correlation between negative inotropy before ischemia and purine efflux after ischemia. On the other hand, the studies with nisoldipine (appendices 6 and 7) and with bepridil (appendix 8) show that concentrations which are too low to induce negative inotropy could reduce purine efflux to some extent. This indicates that factors other than negative inotropy protect the isolated heart against ischemic damage. Reduced efflux of biochemical breakdown products during ischemia was confirmed in patients during coronary angioplasty. Diltiazem diminished hypoxanthine and urate production but did not reduce lactate release (appendix 5).

We also studied captopril, an angiotensin-converting-enzyme inhibitor. This compound increased coronary flow and inhibited angiotensin I induced coronary vasoconstriction but did not affect myocardial function in isolated rat heart. It was also unable to reduce ischemic breakdown of myocardial high-energy phosphates or improve postischemic

cardiac function (appendix 9). Finally, the infusion of the ATP catabolite and ATP precursor inosine was studied in man (appendix 10) and in the isolated rat heart (appendix 11). In man inosine was apparently taken up by the heart; it improved cardiac function. In the rat heart neither the intracellular ATP content nor the cardiac function were improved. The differences between the models used could explain this discrepancy. In conclusion, the studies in this thesis show that ATP metabolites are suitable in the diagnosis of myocardial ischemia and in studying the effects of pharmacological treatment on ischemic heart. The metabolites themselves may, under certain conditions, be useful to improve myocardial ATP metabolism.

SAMENVATTING

Ischemie is een toestand waarbij een orgaan dusdanig slecht doorbloed wordt dat zijn functie daaronder lijdt. De te geringe doorbloeding is er de oorzaak van dat tijdens ischemie de aanvoer van zuurstof en substraat ontoereikend is en dat de afvoer van toxische afbraakprodukten van de stofwisseling eveneens onvoldoende is.

Ischemie van het hart is levensbedreigende toestand. Het leidt tot een reeks veranderingen die men kan onderscheiden in reversibele en irreversibele gevolgen. De laatste leiden uiteindelijk tot celdood en weefselnecrose. Het is voor het hart van doorslaggevend belang om een toestand van ischemie zo snel mogelijk op te heffen. De afbraakprodukten van de cardiale energiehuishouding bleken zeer geschikt om ischemie in een vroeg stadium te onderkennen.

In hoofdstuk 2 van dit proefschrift worden drie modellen besproken die gebruikt zijn om ischemie van het hart te bestuderen: gekweekte hartspiercellen van de rat; het geïsoleerde hart van verscheidene diersoorten; en het menselijk hart in situ, tijdens coronair angioplastie en tijdens een pacing stress test.

Gekweekte hartspiercellen bleken een geschikt hulpmiddel voor het bestuderen van de energiestofwisseling en transport en voor het bestuderen van enzymactiviteit.

Bijlage 1 beschrijft de ontwikkeling van een nieuw model van ischemie (onvoldoende doorstroming en onvoldoende zuurstof) en anoxie (voldoende doorstroming maar onvoldoende zuurstof) in gekweekte hartspiercellen. We komen tot de conclusie dat ischemie in tegenstelling tot anoxie de intracellulaire ATP voorraad in de gekweekte cellen uitput wanneer glucose aanwezig is als voedingsstof. Zonder glucose leiden anoxie en ischemie tot een snelle ATP afbraak.

Om meer inzicht te krijgen in veranderingen in de energiestofwisseling die aan leeftijd gerelateerd zijn, hebben we de activiteit gemeten van een aantal enzymen. Deze activiteiten zijn gemeten in gekweekte hartcellen en in homogenaten van harten van verschillende leeftijd (bijlage 3). De belangrijkste conclusie van dit onderzoek is dat tijdens het ouder worden de enzymen, die bij de afbraak betrokken zijn, belangrijker worden, terwijl de enzymen die voor de opbouw van het ATP zorgen minder actief worden.

De activiteit van het katabole enzym xanthine oxydoreductase (XOD) werd ook gemeten in het geïsoleerde hart van een aantal soorten (bijlage 4). Dit enzym speelt een belangrijke rol in de aanmaak van zuurstofradicalen. Deze worden ervan verdacht een rol te spelen bij het ontstaan van schade in het hart. Het bleek dat de activiteit van XOD per species sterk verschillend was. Zo werden hoge waarden gemeten in de muis, rat en cavia, terwijl de activiteit in konijn, varken en mens zeer laag bleek te zijn. Tot onze verbazing vonden we een hoge (schijnbare) activiteit in harten van gecatheteriseerde patienten (bijlage 2), althans we zagen een significante afgifte van urinezuur. We denken dat de hoge XOD activiteit die gemeten werd in gecatheteriseerde patienten veroorzaakt zou kunnen worden door neutrofielen, die zich gehecht hebben aan plaatsen waar het endotheel van bloedvaten beschadigd is.

Hoofdstuk 3 belicht het gebruik van een aantal stoffen die potentieel de energiestofwisseling van het hart kunnen verbeteren. Sommige verhogen de doorbloeding van de kransslagaders en/of laten het hart minder sterk kloppen, andere zijn intermediairen van de energiestofwisseling die in principe weer ingebouwd kunnen worden in ATP.

De studies in het geïsoleerde hart geven aan dat sommige stoffen die het hart minder

sterk laten kloppen bescherming bieden tegen ischemische schade. We vonden een goed verband tussen remming van contractiliteit vóór ischemie en de purine efflux tijdens ischemie. Toch bleken ook concentraties die te laag zijn om negatieve inotropie te veroorzaken een zekere bescherming tegen ATP afbraak te bieden. We hebben dat zowel voor nisoldipine (bijlagen 6 and 7) als voor bepridil (bijlage 8) kunnen aantonen. Dit houdt in dat er naast negatieve inotropie andere factoren zijn die het geïsoleerde hart tegen ischemie beschermen.

Metingen in patienten tijdens angioplastie bevestigden onze bevindingen in het geïsoleerde hart; de produktie van hypoxanthine en urinezuur door het hart kon verminderd worden door de patiënten te behandelen met de calcium antagonist diltiazem. Tijdens deze verlaging van het ATP catabolisme door diltiazem bleef de produktie van melkzuur onveranderd (bijlage 5).

In het geïsoleerde rattehartpreparaat hebben we ook de werking van captopril, een stof die de omzetting van het inactieve angiotensine I in het vaatvernauwende angiotensine II remt, bestudeerd. Captopril verhoogde de doorbloeding van de kransslagaderen en blokkeerde de door angiotensine I veroorzaakte vaatvernauwing, maar had geen effect op de hartfunctie en verbeterde het energiemetabolisme niet (bijlage 9).

Tenslotte bestudeerden we de werking van inosine, een intermediair van de energiestofwisseling, in de mens (bijlage 10) en in het geïsoleerde rattehart (bijlage 11). In de mens werd inosine door het hart opgenomen en verbeterde de functie van het hart. In de rat verbeterde noch het energiemetabolisme noch de hartfunctie. Mogelijk zijn de vele verschillen tussen de gebruikte modellen oorzaak van deze discrepantie.

Samenvattend kunnen we stellen dat de studies die in dit proefschrift gepresenteerd worden, laten zien dat ATP metabolieten bruikbaar zijn in de diagnose van myocardiale ischemie en daarnaast gebruikt kunnen worden om het effect van farmacologische behandeling van het ischemische hart te bestuderen. De metabolieten zelf kunnen, onder bepaalde omstandigheden, nuttig zijn om de myocardiale ATP huishouding te verbeteren.

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APPENDIX PAPERS

Appendix 1

STUDIES ON OXYGEN AND EXTRACELLULAR FLUID RESTRICTIONS IN CULTURED HEART CELLS: HIGH ENERGY PHOSPHATE METABOLISM

R. Vemuri, J.W. de Jong, J.A.J. Hegge, T. Huizer, M. Heller and A. Pinson,

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Studies on oxygen and extracellular fluid restrictions in cultured heart cells: high energy phosphate metabolism

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ABSTRACT Although cultured heart cells are increasingly used for the study of cardiac metabolism, relatively little is known about their energy turnover. We studied the effects of anoxia with simultaneous restrictions of the volume of the extracellular medium ("ischaemia") on high energy phosphate catabolism in cells from neonatal rat ventricles, cultured for 5 days. The cells were incubated for up to 4 h in Ham-F10 medium either in the presence or in the absence of glucose. High energy phosphates in cell extracts and AMP catabolites in the incubation medium were measured by high pressure liquid chromatography. ATP and creatine phosphate content in normoxic cells did not change significantly, either in the presence or absence of glucose, and the values were similar to those found in the heart in vivo. Energy rich phosphates decreased during anoxia, and were more rapidly depleted during simultaneous oxygen deprivation and volume restriction. Glucose delayed the decline in high energy phosphates. In the presence of glucose, hypoxanthine uptake was higher during normoxia than in anoxia, whereas in "ischaemic" conditions some hypoxanthine was produced. In the absence of glucose, only minor changes were observed in hypoxanthine levels during anoxia, but hypoxanthine production was marked when anoxia was coupled with extracellular volume restriction. Adenosine levels were below the limit of detection. Inosine release was relatively low under all conditions. Xanthine release did not show variation, and anoxia suppressed urate production. Oxygen and glucose deprivation thus led to various degrees of ATP and creatine phosphate breakdown in cultured neonatal heart cells both during anoxia and in simulated "ischaemia".

The heart is essentially an oxidative organ which uses fatty acids as a major source of energy. Thus cardiac contractility seems to depend predominantly on oxidative phosphorylation.[1 2] Under normal conditions, the heart continuously synthesises high energy phosphates to replace those utilised.[3] During oxygen restriction, increased carbohydrate catabolism via anaerobic glycolysis maintains high energy phosphate levels.[4 5] Subsequently both the production and levels of ATP rapidly decline, with a concomitant increase in the levels of degradation products, such as ADP, AMP and adenosine. The large amounts of adenosine formed during oxygen deprivation are rapidly converted by adenosine deaminase to inosine and then to hypoxanthine, xanthine and urate.[6–8] Indeed within 60 to 90 min of ischaemia, most of the ATP and other adenine nucleotides are converted to purine bases.[8] Figure 1 shows the breakdown of ATP to the level of urate. The release of these nucleosides and purine bases into the extracellular environment[9] may prevent the resynthesis of nucleotides after restoration of oxygenation and perfusion. Indeed, addition of hypoxanthine, inosine, or adenosine to the

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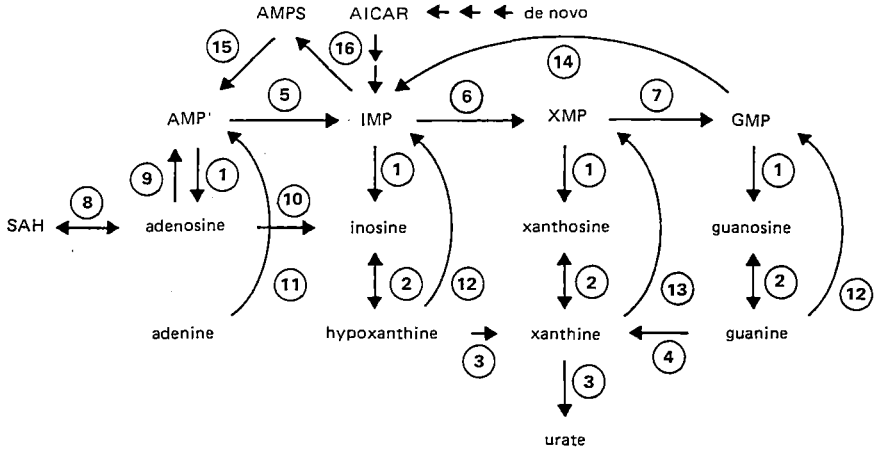

FIG 1 *Scheme showing myocardial metabolism of purine nucleotides. Enzymes catalysing the reactions labelled with a number are: (1) 5'-nucleotidase; (2) purine-nucleoside phosphorylase; (3) xanthine oxidoreductase; (4) guanine deaminase; (5) AMP deaminase; (6) IMP dehydrogenase; (7) GMP synthetase; (8) SAH hydrolase; (9) adenosine kinase; (10) adenosine deaminase; (11) adenine phosphoribosyltransferase; (12) hypoxanthine guanine phosphoribosyltransferase; (13) xanthine phosphoribosyltransferase; (14) GMP reductase; (15) AMPS synthetase; (16) AMPS lyase. Abbreviations: SAH, S-adenosyl homocysteine; AMPS, adenylsuccinate; AICAR, 5-amino-4-imidazolecarboxamide ribotide.*

reperfusion fluid decreases cellular damage and improves ATP regeneration.[3 10]

Cultured heart cells have been used to study oxygen deprivation at the cellular level.[11–13] However, in these studies enzyme release could only be detected after the cells had been kept under anoxic conditions for very long periods of up to 16 h. In addition, various agents such as dinitrophenol, cyanide, deoxyglucose and other metabolic inhibitors were employed in attempts to impair the respiratory chain or glycolysis.[14 15] Thus variables were introduced which are not present under "true" anoxic conditions.

Recently, we proposed a novel approach for the simulation of anoxic injury in cultured heart cells, which does not use artificial agents.[16] We have shown that if the volume of extracellular, anoxic, glucose depleted fluid is reduced, so that the height of the liquid above the cells (total volume 0.2-0.4 ml for a 35 mm diameter Petri dish) approximates to the diameter of a single cultured cell, the complete or partial reduction of the circulation at the cellular level is simulated. Anoxic conditions per se were achieved when the volume of the extracellular medium was 1.0-2.0 ml. We also described a suitable incubation unit and conditions of proper equilibration of the experimental medium with N_2/CO_2. Under these conditions, cellular damage, as reflected in enzyme release, was inversely proportional to the volume of the medium, and presumably attributable to the effects of catabolic products (ie, lactic acid) that remain in the vicinity of the cells.[16] Reduction of the extracellular volume per se did not have any deleterious effects. Furthermore, the onset of cellular damage, as reflected by enzyme release and high energy phosphate depletion, occurred at similar rates to that occurring in other experimental systems in vivo and in the perfused heart.[6 17] It certainly resembled these situations more closely than any other previously described studies in cultured cells.

Only a few investigations dealing with nucleotide and purine metabolism in anoxic heart cells in culture have been published. Mustafa *et al*[18] showed that in cultured cells from embryonic chick hearts, anoxia induces a twofold increase in the production of adenosine and its metabolic products. Surprisingly, the levels of ATP and ADP remained unchanged, while AMP levels increased. Van der Laarse *et al*,[19] using rat heart cells deprived of oxygen and metabolic substrates for up to 7 h, showed that hypoxanthine release, which reaches a maximum during the second hour of anoxia, precedes cellular enzyme depletion. Other researchers found an inverse relationship between the cellular ATP content and ability of phospholipase C to attack the cell membrane.[20 21] Our current studies were directed towards providing a more complete view of the energy state of cultured myocytes during anoxic injury by following the kinetics of high energy phosphate depletion and its subsequent degradation under anoxia or "ischaemia like" conditions.

Methods

CELL CULTURES

Heart cells cultured from one day old Wistar rats were prepared as previously described,[22] with seeding density of 1.8-2.0 × 10^6 cells per dish on 35 mm diameter Petri dishes (Falcon 3001). Under these conditions the initial proportion of 80 to 90% muscle cells is maintained for at least 6 d in culture.

OXYGEN DEPRIVATION AND VOLUME RESTRICTION

Cells cultured for 5 d were used for these experiments. The growth medium was discarded and the cells were immediately washed with Ham-F10 culture medium. Anoxia was achieved by maintaining the cells in a Petri dish with 2.0 ml medium, which had been pre-equilibrated with 95% N_2 and 5% CO_2 for at least 30 min. For anoxia combined with volume restriction, the same conditions were used except that the volume of the extracellular medium was 0.4 ml.[17] All the experiments, including those performed under normoxic conditions, were carried out in the special incubation device described previously.[17] In these studies, fatty acids were not included in the medium. In this report, the term anoxia refers to cell cultures incubated with 2 ml oxygen deprived medium per Petri dish, and "ischaemia" to plates with 0.4 ml medium.

REAGENTS

KH_2PO_4, H_3PO_4 and $HClO_4$ were obtained from Merck (Darmstadt, FGR). Adenosine triphosphate (ATP) and creatine phosphate (CrP) were from Boehringer (Mannheim, FGR). CH_3OH (HPLC grade) was purchased from Fisons PLC (Loughborough, UK). Uric acid and bovine serum albumin were supplied by Sigma (Saint Louis, MO, USA), the other standards by Kock-Light (Colnbrook, Bucks, UK). The protein dye was obtained from Bio-Rad (Munich, FGR). The water used was purified with the Milli-Ro4/Milli-Q system (Millipore-Waters, Bedford, MA, USA).

MEASUREMENT OF ADENOSINE TRIPHOSPHATE AND CREATINE PHOSPHATE

After normoxic, anoxic, or "ischaemic" periods, the plates were placed on ice. The media were transferred to Eppendorf tubes, and the cells were scraped off using 1.0 ml 4% $HClO_4$ (w/v), then placed in Eppendorf tubes and centrifuged at 4°C. The pellets were used for protein determination (vide infra). A 750 ml aliquot of supernatant fluid from each tube was brought to pH 5-7 with 2M KOH/1M K_2CO_3 and centrifuged again. A modification of the method of Harmsen *et al*[23] was used to determine the high energy phosphates with a high pressure liquid chromatography system (Millipore-Waters, Milford, MA, USA), consisting of two model 6000A pumps, two UV detectors in series (models 440 and 441, set at 254 and 214 nm, respectively). Samples were injected via a WISP 710B autosampler, kept at 4°C with a Ministat (Huber, Offenburg-Eigersweier, FGR). The HPLC equipment was controlled by an 840 data system (Millipore-Waters). Buffers were prepared fresh every 2 d and filtered through a 0.45 μm filter (Millipore). Buffer A consisted of 0.016M H_3PO_4 adjusted to pH 2.85 with freshly dissolved KOH; and buffer B of 0.75M KH_2PO_4, pH 4.5. The column (Partisil-10-SAX, 0.4 cm × 25 cm, particle size 10 μm; Whatman, Maidstone, UK) was eluted at a flow rate of 2 ml·min^{-1}. A guard column (0.4 cm × 3 cm; Valco, Houston, TX, USA) with Partisil-10-SAX (Whatman) was used. Five minutes after sample injection, a linear gradient was started reaching 100% at 20 min, and was followed by a 5 min wash period with 100% B. The column was brought back to buffer A via a linear gradient in 10 min and equilibrated for 15 min with buffer A before the next injection.

MEASUREMENT OF NUCLEOSIDES AND OXYPURINES

Purine release was determined in the extracellular medium collected in Eppendorf tubes at the end of the experimental periods. These tubes contained a final concentration of 0.02% NaN_3 to prevent purine breakdown by bacteria. Analysis was carried out by a modification of the HPLC method described by Harmsen *et al*,[24] using a Millipore-Waters system configuration: model M-45 pump, a cooled WISP 710B autosampler, a model 440 UV detector (254 nm) and an 840 data system. A C_{18} column (μBondapa k, 0.4 cm × 30 cm, particle size 10 μm; Millipore-Waters) was employed in combination with a guard column (0.4 cm × 3 cm; Valco), filled with Perisorb RP-18, particle size 30-40 μm (Merck). A 70mM KH_2PO_4, pH 4.6, plus 10% CH_3OH (v/v) buffer was used as eluent at a flow rate of 2 ml·min^{-1}. The buffer was filtered daily through a 0.45 μm filter (Millipore).

PROTEIN ASSAY

Pellets were dissolved in 1 ml 0.1N KOH and protein was determined according to Bradford[25] with a commercial dye (Bio-Rad, Munich, FGR). Bovine serum albumin was used as the standard.

STATISTICAL ANALYSIS

Three way analysis of variance was carried out on the results; the probability of 0.05 was taken as the limit of statistical significance. Significant interactions were tested with simple main effects. Differences between the means were detected by a reliable significant difference procedure.[26]

Results

When cells were maintained for 4 h in Ham-F10 medium under normoxic conditions in the presence of 6mM D-glucose, the variation in ATP and creatine phosphate levels was not statistically significant (fig 2). However, during anoxia, creatine phosphate decreased ($p<0.01$), whereas ATP levels remained virtually unchanged. Both ATP and creatine phosphate decreased quite rapidly during

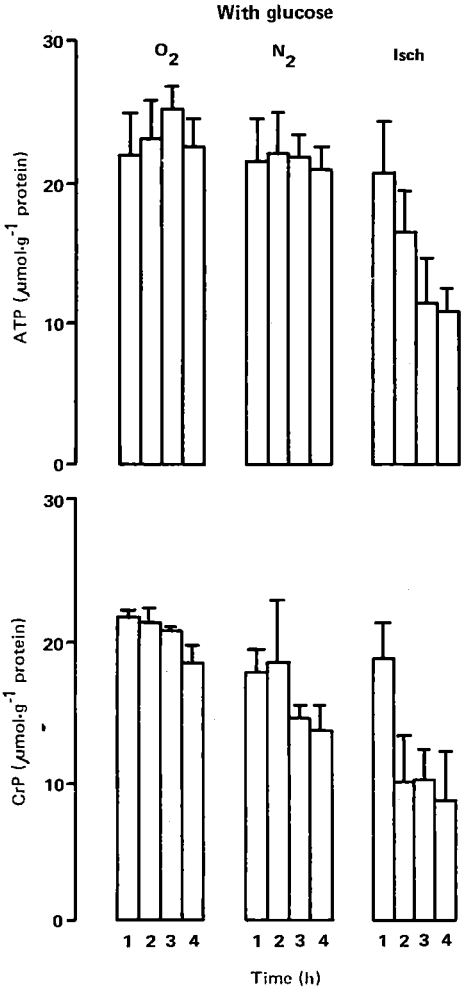

FIG 2 *Changes in cellular ATP levels and creatine phosphate (CrP) during 4 h of normoxia (O_2), anoxia (N_2) and "ischaemia" (Isch) in the presence of 6 mmol·litre^{-1} glucose in HAM-F10 incubation medium containing hypoxanthine. Results are means. Bars=SEM (n=3-4). The zero time controls for ATP and CrP were 18.4(1.5) μmol·g^{-1} (n=10) and 23(3) μmol·g^{-1} (n=7), respectively.*

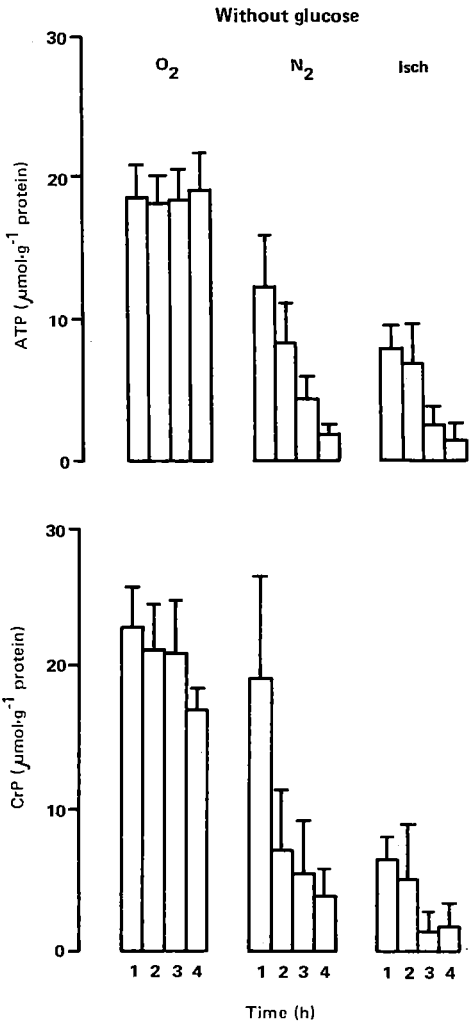

FIG 3 *Changes in cellular ATP and creatine phosphate (CrP) levels during 4 h of normoxia (O_2), anoxia (N_2) and "ischaemia" (Isch) in incubation medium without glucose. Results are means. Bars=SEM (n=3-4). For zero time controls, see legend to fig 2.*

"ischaemia". Although glucose was present in the medium, the decrease exceeded 50% after a 4 h incubation period ($p<0.01$, fig 2). In normoxic cells incubated without glucose, the high energy phosphate levels did not change significantly (fig 3), presumably because they used endogenous substrates and exogeneous hypoxanthine. During anoxia without glucose, an immediate and significant fall in high energy phosphates could already be observed after 1 h ($p<0.01$, compared to either anoxia with glucose, or normoxia without glucose). The cells were practically depleted of high energy phosphate reserves when anoxic injury was maintained for 2 h or longer. In the absence of glucose, high energy phosphate depletion was even more rapid in "ischaemic" cells than in anoxic cultures ($p<0.05$).

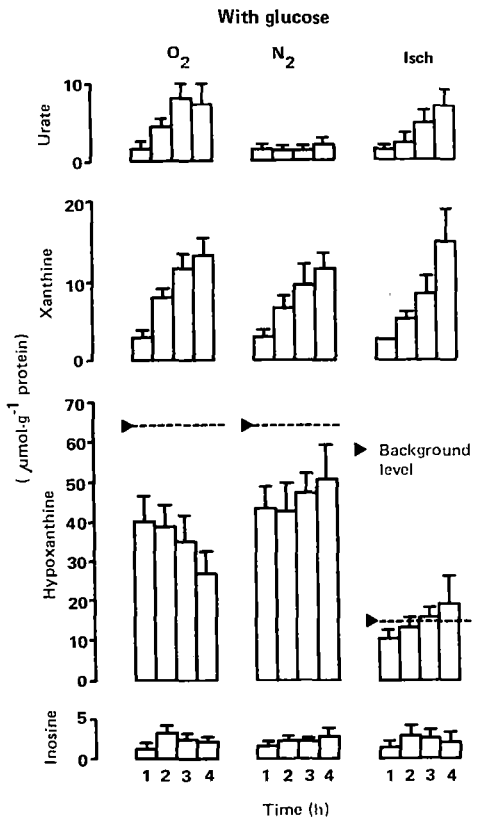

FIG 4 *Time courses of extracellular levels of purines (normalised with respect to total cell proteins) for cells maintained for up to 4 h under normoxic (O_2), anoxic (N_2) and "ischaemic" (Isch) conditions in HAM-F10 incubation medium, which initially contained 6 mmol·litre^{-1} glucose and 0.03 mmol·litre^{-1} hypoxanthine. Results are means. Bars=SEM (n=4-6).*

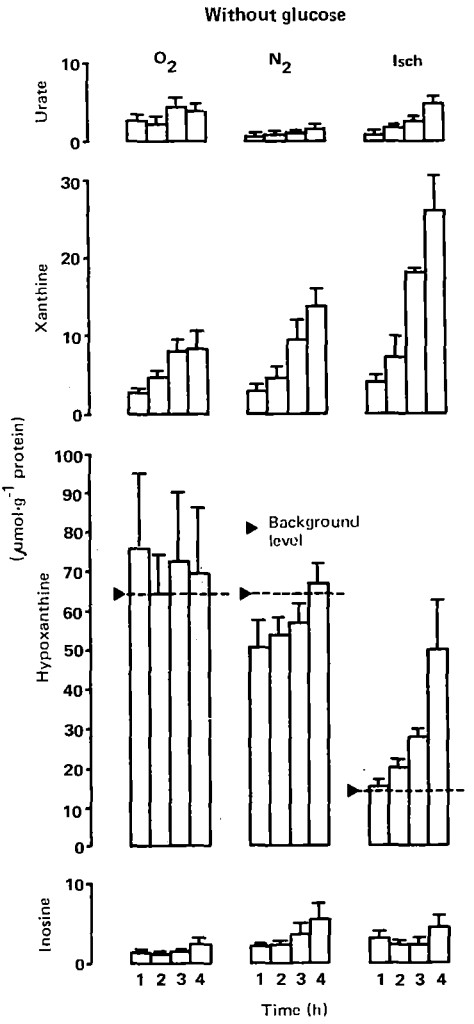

FIG 5 *Time course of extracellular levels of purines (normalised with respect to total cell proteins) for cells maintained for up to 4 h under normoxic (O_2), anoxic (N_2) and "ischaemic" (Isch) conditions in incubation medium without glucose, which initially contained 0.03 mmol·litre^{-1} hypoxanthine. Results are means. Bars=SEM (n=4-6).*

Concomitant with the fall in high energy phosphate levels, an increase in purine release from the cells could be observed. Extracellular urate remained low (about 2 μmol·g^{-1} protein) during anoxia, but approached 8 μmol·g^{-1} during normoxia and "ischaemia" ($p<0.01$ *v* anoxia, figs 4 and 5). We found a steady increase in xanthine levels in all experiments ($p<0.01$), with values ranging from 2 μmol·g^{-1} protein at 30 min (not shown) to 25 μmol·g^{-1} after 4 h of "ischaemia" (without glucose). The Ham-F10 medium initially contained 32 μmol·litre^{-1} hypoxanthine. In the presence of glucose, the cells removed more than 50% of the hypoxanthine after 4 h under normoxic conditions. This was decreased to about 20% during anoxia, whereas during "ischaemia" the cells produced hypoxanthine ($p<0.01$ *v* time zero, fig 4). Omission of glucose from the incubation medium resulted in increased hypoxanthine levels in normoxic, anoxic, and "ischaemic" cells ($p<0.01$, compare figs 4 and 5). In the absence of glucose, normoxic cells tended to produce hypoxanthine (fig 5). After 4 h we noted an insignificant hypoxanthine production in anoxic cells and the release of a large amount of hypoxanthine (30 μmol·g^{-1} protein) in "ischaemic" cells ($p<0.01$ *v* time zero, fig 5). Inosine production was relatively low under all conditions (figs 4 and 5). Adenosine was not detectable in the incubation fluids by our method (detection limit: 0.04 μmol·litre^{-1}).

Discussion

Anoxia disturbs the equilibrium between high energy phosphate utilisation and synthesis. In the absence of oxygen, cells depend on anaerobic glycolysis for continued high energy phosphate. In spite of complete cessation of contractility within a few minutes of anoxia,[27] the cells continue to use energy to maintain cellular functions, such as cationic pumps. Glycolytic flux, as expressed by glycogen depletion and lactate production,[27] although insufficient to satisfy all the energy needs of the cultured cells, helps to maintain high energy phosphates at an acceptable level during anoxia (fig 2). This is consistent with the findings of Neely and Morgan[28] who reported a similar phenomenon during high flow anoxia in the isolated heart perfused with glucose. In our experiments in the presence of 6 mmol·litre^{-1} glucose, both the cellular glycogen reserves[27] and a large volume of medium (2 ml) further contribute to the maintenance of high energy phosphate levels. Indeed, the amount of ATP did not fall significantly since creatine phosphate presumably served as a source for the replenishment of ATP. However, during "ischaemia", in spite of the presence of glucose, ATP levels declined by more than 50% after 4 h. The inability to maintain high rates of high energy phosphate production during "ischaemia" seems to be related to the low extracellular volume, which simulates low perfusion rates, and leads to an incomplete washout of metabolites such as lactate. Oxygen deprivation coupled with glucose deprivation led to depletion of high energy phosphate at a much faster rate, which is similar to the findings reported by Jennings *et al*[6] in the perfused heart.

In the presence of adenylate kinase, ADP formed from ATP is converted (2 ADP = ATP + AMP). AMP is either deaminated to inosine monophosphate (IMP) or dephosphorylated to adenosine (fig 1). Adenosine may be deaminated. However, under normal conditions, rephosphorylation is more likely to take place, since adenosine phosphorylation seems to predominate over deamination. This can be explained by the relatively low Km values of adenosine kinase, although its maximal activity is lower than that of adenosine deaminase.[29] When adenosine is not reutilised for high energy phosphate synthesis, it is rapidly degraded further to inosine.[6 17] Inosine is broken down to hypoxanthine, presumably by nucleoside phosphorylase.[30] The latter can either be converted to IMP and AMP (salvage), or catabolised to xanthine and urate. This probably explains why adenosine could not be detected in significant amounts in the present study, and inosine levels were relatively low. However some xanthine oxidase/dehydrogenase activity may be present in culture.[31] Thus the decreased levels of hypoxanthine in the medium can essentially be attributed to ATP synthesis with a low rate of conversion to xanthine, presumably carried out by endothelial cells[32] which constitute about 10% of the cultured cell population.[22] Since the incubation medium contained 0.03 mmol·litre^{-1} hypoxanthine, a fall in hypoxanthine levels, accompanied by relatively small rises in xanthine and urate levels, may be regarded as evidence of nucleotide synthesis. This is supported by the fact that, in the absence of glucose, purine release during "ischaemia" was much higher, since rephosphorylation did not continue as the energy loss was too great. It has been shown[33] that in normoxic cultured heart cells, the main flow of nucleotides is from IMP to AMP, whereas the rate of flow in the opposite direction is much slower. Thus AMP degradation proceeds via adenosine rather than via IMP. We speculate that the flow through this pathway is changed during oxygen deprivation. Since the conversion of AMP to adenosine is probably blocked during anoxia, this may lead to accelerated conversion of AMP to IMP. The increase of xanthine during anoxia could then be explained by the conversation of guanine or xanthosine, in addition to the xanthine derived from hypoxanthine (see fig 1). We do not as yet understand why urate production

during "ischaemia" is higher than during anoxia (figs 4 and 5).

CONCLUSION

In cultured heart cells, reducing the volume of extracellular medium, together with oxygen and glucose deprivation, undoubtedly best simulates the conditions at the cellular level during reduced flow. Heart cells in culture, undergoing anoxic injury, can conveniently be used for carrying out kinetic studies. Our approach of restricting the extracellular fluid resulted in a sequence of events which was similar, but somewhat more extended than that observed in other experimental systems such as the ischaemic perfused heart. This is presumably due to lower energy needs of the cultured cell, since external work is not carried out against flow resistance even though the ATP levels in cultured cells and in the tissues in vivo are similar. It should be noted that the duration of the events in the perfused heart is also extended as compared to in vivo ischaemia. As previously shown,[27] extracellular glucose and cellular glycogen may delay anoxic injury. In the present study, glucose prevented high energy phosphate depletion for a relatively long period. The presence of hypoxanthine in the culture medium seems to contribute to maintenance of high energy phosphate levels via the salvage pathway. However, in simulated "ischaemia like" conditions cellular metabolites are not washed away from the vicinity of the cells, and the fall in high energy phosphates is accelerated, with a concomitant increase in purine release. In this case, hypoxanthine does not contribute to the resynthesis of adenine nucleotides. On the contrary, it is either released by the myocytes or further metabolised. The contribution of non-muscle cardiac cells to inosine, hypoxanthine and xanthine catabolism remains to the elucidated. Comparison of the results of this study with previous findings,[16] suggests that high energy phosphate depletion corresponds with the onset of both lysosomal enzyme release and irreversible cellular damage.

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Appendix 2

URATE PRODUCTION BY HUMAN HEART

T. Huizer, J.W. de Jong, J.A. Nelson, W. Czarnecki, P.W. Serruys, J.J.R.M. Bonnier and R. Troquay,

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Urate Production by Human Heart

Tom Huizer,* Jan Willem de Jong,* J. Arly Nelson,† Wlodzimierz Czarnecki,‡ Patrick W. Serruys,* Johannes J. R. M. Bonnier,§ and Roel Troquay§

**Thoraxcenter, Erasmus University Rotterdam, The Netherlands; † Department of Experimental Pediatrics, University of Texas M.D. Anderson Hospital and Tumor Institute, Houston, Texas, USA; ‡Department of Medicine and Medical Physiology, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada; §Department of Cardiology, Catharina Hospital, Eindhoven, The Netherlands*

(Received 13 September 1988, accepted in revised form 3 March 1989)

T. Huizer, J. W. de Jong, J. A. Nelson, W. Czarnecki, P. W. Serruys, J. J. R. M. Bonnier and R. Troquay. Urate Production by Human Heart. *Journal of Molecular and Cellular Cardiology* (1989) **21**, 691–695. Xanthine oxidoreductase has been demonstrated in the heart of various species. However, its presence in human heart is still debated. In the literature, high to undetectable levels have been reported. We studied the arterial-venous urate difference across the heart of patients undergoing both routine cardiac catheterization and percutaneous transluminal coronary angioplasty. Urate is the end product of the reaction catalysed by xanthine oxidoreductase. In 10 patients, studied before angioplasty, the plasma urate level in the great cardiac vein exceeded the arterial one by 26 ± 10 nmol/ml ($P = 0.028$). In a further 13 patients, urate production was maximal immediately after the last of four consecutive occlusions (23 ± 8 nmol/ml, $P = 0.018$) and concomitant with increased coronary sinus hypoxanthine levels. We conclude that xanthine oxidoreductase is probably present in the heart of patients, suffering from ischemic heart disease, and responsible for the increase in urate production during transient myocardial ischemia.

Key Words: Xanthine oxidase; Uric acid; Myocardium; Ischemia; Human; Coronary angioplasty.

Introduction

Xanthine oxidoreductase activity has been demonstrated in the myocardium of a number of species (see Schoutsen and De Jong, 1987). Limited data are available on the enzyme in human heart. Autopsy material indicates high xanthine oxidase activity (Krenitsky *et al.*, 1974; Wajner and Harkness, 1988). Histochemical techniques have shown large amounts of the enzyme in human heart endothelium (Jarasch *et al.*, 1986). On the other hand, several authors have reported very low to undetectable xanthine oxidoreductase activity in human heart (Ramboer, 1969; Eddy *et al.*, 1987; Muxfeldt and Schaper, 1987). Preliminary observations assessing cardiac urate production in patients during pacing stress test at the University of Alabama (Nelson *et al.*, 1977) and in patients during coronary angiography in the National Institute of Cardiology, Warsaw (Czarnecki, 1988) have suggested that the human heart may be capable of urate production. We present evidence which shows that the human heart can produce significant amounts of urate. This observation suggests that a cardiac xanthine oxidoreductase is active in patients with ischemic heart disease.

Methods

Patients

Two studies were performed in patients, catheterized for percutaneous transluminal coronary angioplasty (PTCA). In 10 patients the urate concentrations of arterial and great cardiac vein plasma, obtained before PTCA,

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TABLE 1. Clinical characteristics of the study groups

Variable	Group 1 $n = 10$	Group 2 $n = 13$
Age (year), average	58	58
range	49 to 66	43 to 72
Gender (male/female)	7/3	10/3
CCS grade	III to IV	II to IV
Average severity of stenosis (%)		
before PTCA	78	79
after PTCA	44	37

CCS = Canadian Cardiovascular Society.

were assayed retrospectively (Group 1). Subsequently, in a prospective study (Group 2, 13 patients), urate and hypoxanthine concentrations were measured in arterial and coronary sinus plasma before, during and after angioplasty. In both studies, arterial blood was taken from the femoral artery. Great cardiac vein blood was sampled via the distal opening of a Webster flow catheter (Group 1) and coronary sinus blood via a diagnostic catheter (Group 2). All patients had a proximal stenosis < 1 cm from the origin of the left anterior descending artery and no collateral filling to the region supplied by the artery, seen at angiography. Amipaque or Isopaque contrast agents (Nyegaard, Oslo, Norway) were used for angiography. In all patients, vasoactive substances, except short-lasting nitrates, were discontinued at least 12 h before the study. The clinical characteristics are listed in Table 1.

Assays

To prepare plasma, blood was mixed in a heparinized tube with an equal volume of ice-cold 154 mM NaCl, containing 20 μM dipyridamole (Boehringer, Ingelheim, GFR) and 10 μM erythro-9-(2-hydroxy-3-nonyl)adenine (Wellcome, London, UK). These drugs were used to inhibit adenosine uptake and breakdown (Ontyd and Schrader, 1984; Edlund *et al.*, 1985). The plasma was kept at -80°C. Deproteinization was carried out with an equal volume of 8% $HClO_4$ (w/v) and the supernatant fraction neutralized with 2 M KOH/1 M K_2CO_3. HPLC-determination of urate and hypoxanthine concentrations in the plasma extract were performed on a μBondapak C_{18} column. A 100 μl sample was eluted with a mixture of CH_3OH (100 ml) and KH_2PO_4 (10 g/l, 1000 ml), pH 5-7, at a flow

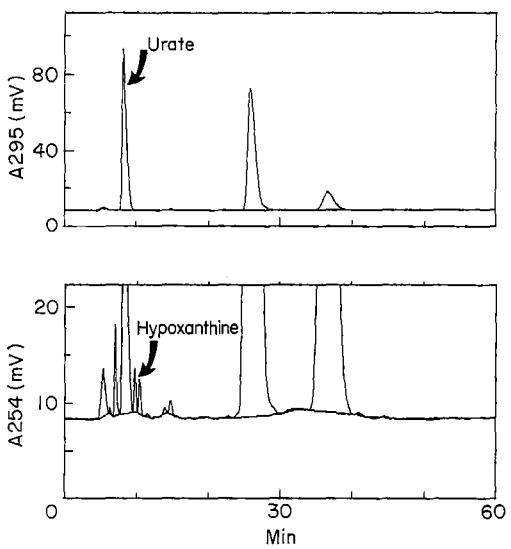

FIGURE 1. High-performance liquid chromatography of plasma extract. Urate was detected at 295 nm, hypoxanthine at 254 nm. The contrast agent used showed up in the chromatogram, but did not interfere with the peaks of interest.

TABLE 2. Arterial and venous urate levels before, immediately after four dilations, and during recovery

Patient no.	Before PTCA		After 1st dilation		After 2nd dilation		After 3rd dilation		After 4th dilation		Recovery	
	ART	CS	ART	CS	ART	CS	ART	CS	ART	CS	ART	CS
1	217	211	214	217	211	217	218	216	212	217	215	218
2	236	216	224	184	216	195	213	207	213	200	202	196
3	224	229	223	220	218	215	218	219	217	217	215	211
4	279	291	280	290	279	281	236	292	245	284	262	282
5	242	254	231	266	239	257	244	260	200	266	230	253
6	216	224	219	221	208	216	211	218	209	216	202	206
7	252	261	260	252	258	258	244	262	253	245	244	240
8	449	495	457	481	481	478	455	480	469	512	462	469
9	196	188	185	181	179	182	177	178	175	174	168	173
10	230	242	218	214	201	207	197	209	191	204	187	197
11	292	278	264	268	271	299	266	281	272	283	239	264
12	239	207	241	191	234	224	220	230	184	238	225	221
13	192	268	208	244	253	208	253	279	174	251	226	239
Mean	251	259	248	248	250	249	242	256	232	254	237	244
S.E.M.	18	21	19	22	21	21	19	21	21	23	20	21

ART = arterial; CS = coronary sinus. Data are in nmol/ml.

rate of 0.6 ml/min. The column was equipped with a LC-18 guard-column (Supelco, Bellefonte, PA). The Waters-HPLC equipment consisted of: WISP 710B cooled autosampler, Model 6000A pump, Model 490 multi wavelength detector, and Model 840 computer. Peaks were identified by retention times, internal standards and enzyme shifts. The optimal wavelengths for urate and hypoxanthine detection proved to be 295 and 254 nm as at these levels adsorption was maximal and disturbance by other materials minimal (Fig. 1). Sample preparation and assay were based on earlier work (Harmsen *et al.*, 1981). In 27 arterial and venous plasma samples of Group 2, urate was also assayed spectrophotometrically with uricase according to Scheibe *et al.* (1974). Enzyme was provided by Boehringer (Mannheim, GFR). Comparison of the data obtained with both methods showed that they correlated closely.

Data presented were analysed with Student's *t*-test for paired variates, or, where appropriate with two-way analysis of variance. A P value of <0.05 was considered as significant. The correlation test was done according to Bland and Altman (1986).

Results

In the preliminary studies, mentioned in the Introduction, hearts produced urate. In the American study, the arterial and venous blood urate levels were 59 ± 20 and 120 ± 23 nmol/ml, resp. ($n = 7$, $P = 0.003$). In the Polish study, these values were 96 ± 15 and 145 ± 25 nmol/ml resp. ($n = 6$, $P = 0.028$).

In Group 1, all patients had an isolated proximal left anterior descending artery stenosis and angina pectoris. In this group the arterial urate concentration was significantly lower than the coronary venous one (216 ± 17 and 242 ± 17 nmol/ml); a difference of 26 nmol/ml ($P = 0.028$). In seven out of 10 patients, the heart produced urate.

In a comparable patient population (Group 2, see Table 2), plasma urate concentrations were similar to those of Group 1, but the arterio-venous difference before coronary angioplasty was relatively small. Consequently we were unable to demonstrate significant urate production *before* coronary angioplasty. Analysis of data showed a significant increase in urate production during balloon inflations ($F = 2.85$; $P < 0.05$). *After* the third and fourth inflations, venous urate levels were significantly higher than arterial ones (Fig. 2). They differed 14 nmol/ml ($P = 0.009$) and 23 nmol/ml ($P = 0.018$), resp. Even after 15 min of recovery, urate production was still significant. The difference was 7 nmol/ml ($P = 0.033$).

In the latter study we also measured the arterial and coronary sinus hypoxanthine levels with HPLC. The arterial hypoxanthine

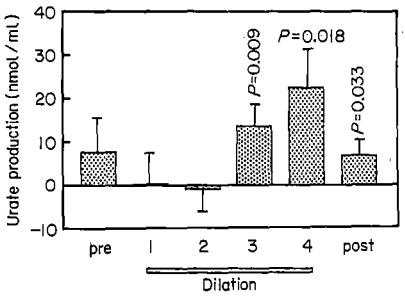

FIGURE 2. Urate production by the heart of 13 patients with single left anterior descending coronary artery stenosis, before coronary angioplasty (pre), after each balloon deflation (dilation one to four) and after 15 min of recovery (post). Mean coronary venous-arterial values are given with 1 S.E.M. Significant urate production was found immediately after the last two dilations, and during recovery.

plasma concentration slightly exceeded the venous one before angioplasty (0.58 ± 0.07 and 0.42 ± 0.07 nmol/ml, respectively, $P = 0.015$). Immediately following PTCA these values were 0.32 ± 0.06 and 1.28 ± 0.19 nmol/ml, respectively, $P < 0.001$ (average of four attempts). Thus cardiac uptake turned into production. Fifteen minutes after angioplasty arterial plasma hypoxanthine levels were not different from the venous ones.

Discussion

Xanthine oxidoreductase activity is detectable in the heart of a number of species (for reviews, see Schoutsen and De Jong, 1987; Downey *et al.*, 1988). In pig heart it seems to be absent (Podzuweit *et al.*, 1986; Muxfeldt and Schaper, 1987). In rabbit heart both Schoutsen *et al.* (1983) and Chambers *et al.* (1985) were unable to demonstrate the enzyme, but Wajner and Harness (1988) measured high activity. The literature on xanthine oxidoreductase in human heart is also conflicting. The reports vary from high (Krenitsky *et al.*, 1974; Jarasch *et al.*, 1986; Wajner and Harness, 1988) to (very) low levels (Watts *et al.*, 1965; Ramboer, 1969; Eddy *et al.*, 1987; Muxfeldt and Schaper, 1987). We want to emphasize that in these reports the number of samples assayed was often very small. Muxfeldt and Schaper (1987) found very low amounts of xanthine oxidoreductase in the two human heart biopsies studied. Krenitsky *et al.* (1974) reported data on one autopsy sample. These authors observed enzyme activity with ferricyanide as the electron acceptor but did not use NAD or oxygen as the cosubstrate. Allopurinol inhibited the activity. Eddy *et al.* (1987) could not demonstrate xanthine oxidoreductase in human ventricular tissue. Supposedly the four biopsies studied were not taken from ischemic hearts.

A possible explanation for the discrepancies in activity found could be a difference in quality of the hearts examined. Our data indicate that the enzyme could be active in the human heart *in vivo*. We cannot exclude that the urate production measured originated from xanthine oxidoreductase activity in polymorphonuclear neutrophils, adhering to areas of the coronary endothelium that are injured by the balloon during inflation.

In the American and Polish studies, mentioned before, *blood* was deproteinized with $HClO_4$ which causes a partial loss during sample clean-up. Never the less the arteriovenous differences in urate were significant. Moreover, they suggested that patients with a more severe ischemic heart disease produced the highest amounts of urate. In the American study, patients experiencing pain during a pacing stress test released lactate and showed the highest urate production. Czarnecki (1988) observed that patients with a history of subendocardial infarction produced high amounts of urate whereas patients with normal myocardium or extensive myocardial damage produced less. Our present results support this idea. Group 1, which comprised patients with CCS grades III and IV, showed significant urate production before PTCA. Group 2, in which four out of 13 patients were CCS grade II, only started to produce significant amounts of urate after several dilations. After each of the angioplasty attempts, venous plasma hypoxanthine [the relatively stable substrate for xanthine oxidoreductase (Harkness, 1988)] increased fourfold. The data suggest that ischemic myocardium at risk of infarction produces urate.

Patients of Group 1, all with a proximal stenosis of the left anterior descending coronary artery produced urate (Table 1). It is likely that this urate production was partly

due to endothelial damage, caused by insertion of the guide wire and the balloon catheter. In Group 2 urate production, which was not significant before PTCA, became obvious after repetitive angioplasty attempts (Fig. 2). Presumably, this is due to cardiac ATP breakdown, with a concomitant rise in hypoxanthine as a result of myocardial ischemia due to coronary occlusion by balloon inflation (see also Serruys *et al.*, 1989). Hypoxanthine serves as a substrate for xanthine oxidoreductase. We suggest that the human heart may contain active xanthine oxidoreductase.

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Appendix 3

ISCHEMIC NUCLEOTIDE BREAKDOWN INCREASES DURING CARDIAC DEVELOPMENT DUE TO DROP IN ADENOSINE ANABOLISM/CATABOLISM RATIO

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Ischemic Nucleotide Breakdown Increases During Cardiac Development Due to Drop in Adenosine Anabolism/Catabolism Ratio

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J. W. de Jong, E. Keijzer, T. Huizer and B. Schoutsen. Ischemic Nucleotide Breakdown Increases During Cardiac Development Due to Drop in Adenosine Anabolism/Catabolism Ratio. *Journal of Molecular and Cellular Cardiology* (1990) **22**, 000–000. Our earlier work on reperfusion showed that adult rat hearts released almost twice as much purine nucleosides and oxypurines as newborn hearts did [Am J Physiol **254** (1988) H1091]. A change in the ratio anabolism/catabolism of adenosine could be responsible for this effect. We therefore measured the activity of adenosine kinase, adenosine deaminase, nucleoside phosphorylase and xanthine oxidoreductase in homogenates of hearts and myocytes from neonatal and adult rats. In hearts the activity of adenosine deaminase and nucleoside phosphorylase (10–20 U/g protein) changed relatively little. However, adenosine kinase activity decreased from 1.3 to 0.6 U/g ($P<0.025$), and xanthine oxidoreductase activity increased from 0.02 to 0.85 U/g ($P<0.005$). Thus the ratio in activity of these rate-limiting enzymes for anabolism and catabolism dropped from 68 to 0.68 during cardiac development. In contrast, the ratio in myocytes remained unchanged (about 23). The large difference in adenosine anabolism/catabolism ratio, observed in heart homogenates, could explain why ATP breakdown due to hypoxia is lower in neonatal than in adult heart. Because this change is absent in myocytes, we speculate that mainly endothelial activities of adenosine kinase and xanthine oxidoreductase are responsible for this shift in purine metabolism during development.

Key Words: Adenosine deaminase; Adenosine kinase; Adult; Age; Development; Myocyte; Neonate; Nucleoside phosphorylase; Purine catabolism; Rat heart; Xanthine oxidoreductase.

Introduction

ATP metabolism plays an important role in myocardial function, e.g., contractility, ion transport, vasodilation. Most studies on this topic focus on adult heart. Consequently, relatively little is known about ATP metabolism in the newborn heart. We showed recently large age-related differences in cardiac purine release following ischemia [*1*]. We hypothesized that a change in anabolic/catabolic pathways of adenosine was responsible for this phenomenon. Therefore, we studied the activity of adenosine kinase, adenosine deaminase, nucleoside phosphorylase and xanthine oxidoreductase in (homogenates of) rat hearts and cardiomyocytes. Part of the results has been published in abstract form [*13*].

Materials and Methods

Chemicals

All chemicals used were of the highest grade available. Adenosine, inosine, hypoxanthine and uric acid were supplied by Janssen Chimica (Beerse, Belgium). Xanthine and [8-^{14}C]xanthine were from Boehringer (Mannheim, FRG) and Amersham (Little Chalfont, UK), respectively. Collagenase, hyaluronidase and calf serum were bought from Boehringer, M199 cell culture medium from Gibco (Paisley, UK). 5′-Iodotubercidine was obtained from Research Biochemicals (Natick, MA., USA), erythro-9-(2-hydroxy-3-nonyl)adenine·HCl from Burroughs Wellcome (Research Triangle Park, N.C.,

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USA), alpha, beta-methylene-adenosine-5'-diphosphate and bovine serum albumin from Sigma (St. Louis, Mo., USA). Allopurinol came from Wellcome (Beckenham, UK).

Neonatal preparations

Hearts were used of Sprague–Dawley rats (two days old; IFFA-Credo, Lyon, France). The sucklings were killed by decapitation. For the preparation of heart homogenates (5% w/v), a pool of ten ventricles was washed with 154 mM NaCl, O°C, then minced in a Virtis blender and a Potter-Elvehjem homogenizer at O°C. The homogenization buffer consisted of: 10 mM Tricine, 1 mM EDTA, 0.25 M sucrose, pH 7.4. Homogenates were stored below −80°C. Neonatal cells were prepared and cultured according to Link *et al.* [*20*]. Myocytes were separated from non-muscular cells with the method of Blondel *et al.* [*6*]. After a culture period of 2 days, the cells were washed three times with 154 mM NaCl, scraped from the Petri dishes into the homogenization buffer, and stored in liquid nitrogen. The purity of the cell culture was about 80% (May-Grünwald staining).

Adult preparations

Adult rats (about four months old; source: see above) were sedated intraperitoneally with 30–60 mg pentobarbitone. The hearts were isolated and the atria removed. To eliminate blood, the hearts were flushed retrogradely with 154 mM NaCl. The ventricles were homogenized essentially as described for the neonatal hearts. For the preparation of cardiomyocytes, the procedure of Farmer *et al.* [*16*] was partly followed. A modified Tyrode solution [*17*] was used with 0.1% collagenase, 0.1% hyaluronidase, 0.1% albumin and 50 μM $CaCl_2$ [*16*]. Hearts were perfused with this solution in a recirculating way at 37°C for 30–40 min [*21*]. Then the ventricles were cut from the perfusion apparatus and torn apart using two forceps. After 10 min incubation with the Tyrode solution (albumin raised to 1%), the remainder of the tissue was gently suspended further with a serological 10-ml pipette [*21*] and filtered through a 200-μm mesh sieve. The cells were washed in the Tyrode solution (no enzymes, 2% albumin, sterile [*16*]) once at 1 × g and twice at 12 × g for 1 min. Subsequently, the cells were suspended in sterile M199 medium, containing 4% fetal calf serum. They were purified using the method of Piper *et al.* [*21*] and kept in culture for 1 day. Then the cells were collected in homogenization buffer and stored in liquid nitrogen. With phase-contrast microscopy, only myocytes could be detected in the preparation.

Assays

Just before the assay of the (cytosolic) enzymes, carried out at 30°C, the samples (2–5 ml) were thawed, sonicated (M2/70, MSE, Crawley, UK) twice at O°C for 30 s, and spun in a Mikroliter centrifuge (Hettich, Tuttlingen, FRG) at 4°C for 5 min. Adenosine kinase was assayed with the radiometric method described by De Jong *et al.* [*11,12*]. Adenosine deaminase activity was determined according to Coddington *et al.* [*8*], using 45 μM adenosine. If the activity was too low for detection with the Hitachi U-2000 double-beam spectrophotometer, products were measured by high-performance liquid chromatography [*18*]. Nucleoside phosphorylase was determined at 293 nm, using 0.1 M $K–PO_4$ pH 7.4, 0.25 mM inosine and 0.2 U xanthine oxidoreductase (see Boehringer catalogue). Xanthine oxidoreductase was measured according to Schoutsen *et al.* [*23*]. Protein was assayed with Coomassie Brilliant Blue (Bio-Rad Laboratories, Munich, FRG) according to Bradford [*7*], using bovine serum albumin as the standard.

Statistics

Statistical analysis was done with Student's *t*-test for unpaired variates (two-tailed). Data are given as means ± S.E. Differences with $P < 0.05$ were considered significant.

Results

The adenosine kinase activity measured in ventricular homogenates decreased from 1.3 ± 0.2 U/g protein in neonatal hearts to 0.58 ± 0.12 U/g ($P < 0.025$) in adult hearts (Fig. 1). In isolated myocytes it also decreased about two-fold with increasing age (Fig. 1). In

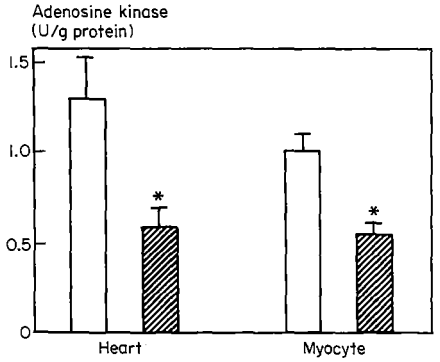

FIGURE 1. Adenosine kinase activities in ventricles and myocytes from neonatal (open bars) and adult (hatched bars) rat hearts. Mean values are given with S.E. ($n = 6$). *$P < 0.025$ vs. neonatal.

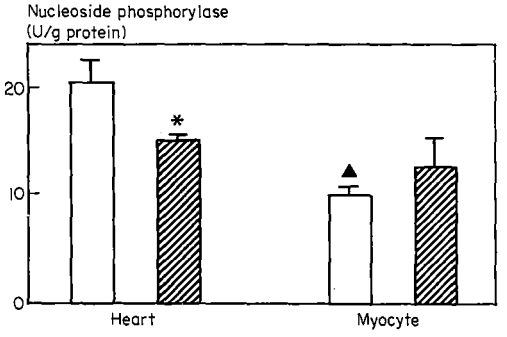

FIGURE 3. Nucleoside phosphorylase activities in ventricles and myocytes from neonatal (open bars) and adult (hatched bars) hearts. Mean values with S.E. ($n = 3$–6). *$P < 0.025$ vs. neonatal; ▲$P < 0.001$ vs. whole heart.

neonatal heart cells, we measured 1.02 ±0.10 U/g, in adult cells 0.56 ± 0.06 U/g ($P < 0.005$). The specific activities of the ventricular and myocyte preparations did not differ significantly.

The adenosine deaminase activity in ventricular homogenates was unaffected by age: 12.9 ± 1.1 U/g protein in neonatal hearts and 10.9 ± 0.2 U/g in adult hearts (Fig. 2). However, isolated myocytes differed in this respect: 6.8 ± 0.6 U/g (neonatal) versus 2.6 ± 0.2 U/g (adult, $P < 0.001$). The specific activity in both cell preparations was lower ($P < 0.001$) than that in the ventricular preparations.

The activity of nucleoside phosphorylase decreased from 20.6 ± 1.9 U/g protein in neonatal hearts to 15.1 ± 0.6 U/g ($P < 0.025$) in adult hearts (Fig. 3). We found 10.0 ± 0.7 U/g in neonatal myocytes, which was lower ($P < 0.001$) than that in whole hearts. Nucleoside phosphorylase activity in adult myocytes (12 ± 3 U/g) did not differ significantly from that in neonatal myocytes or adult hearts.

In heart homogenates xanthine oxidoreductase increased in activity during development from 0.018 ± 0.008 to 0.85 ± 0.15 U/g protein ($P < 0.005$, Fig. 4). In contrast, this change was absent in myocytes: 0.042 ± 0.015 U/g (neonatal cells), 0.026 ± 0.017 U/g (adult cells).

To check the specificity of the enzyme reactions studied, we tested several inhibitors in

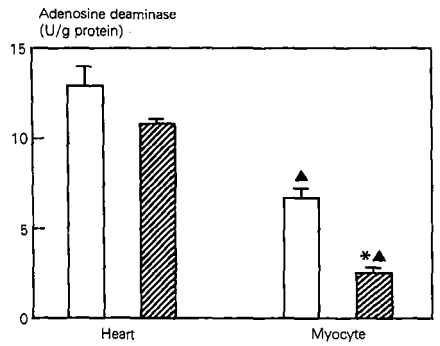

FIGURE 2. Adenosine deaminase activities in ventricles and myocytes from neonatal (open bars) and adult (hatched bars) hearts. Mean values with S.E. ($n = 5$–6). *$P < 0.001$ vs. neonatal; ▲$P < 0.001$ vs. whole heart.

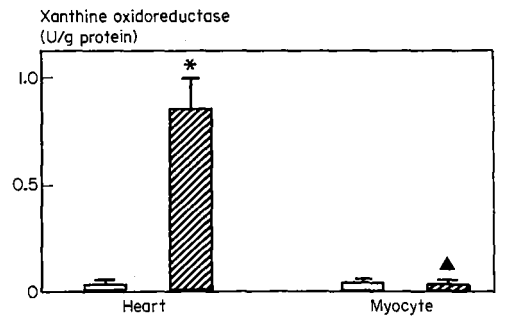

FIGURE 4. Xanthine oxidoreductase activities in ventricles and myocytes from neonatal (open bars) and adult (hatched bars) hearts. Mean values with S.E. ($n = 4$–6). *$P < 0.005$ vs. neonatal; ▲$P < 0.005$ vs. whole heart.

both neonatal and adult heart preparations. Erythro-9-(2-hydroxy-3-nonyl)adenine (10 μM) inhibited the adenosine deaminase activity by 98%, 5′-iodotubercidine (5 μM) that of adenosine kinase by 96% [*9*]. Addition of the adenosine deaminase inhibitor [*25*] or alpha,beta-methylene-adenosine-5′-diphosphate (50 μM), a 5′-nucleotidase inhibitor [*25*], did not stimulate the adenosine kinase reaction, showing that adenosine deaminase and 5′-nucleotidase did not interfere with the assay. Allopurinol (100 μM) inhibited the xanthine oxidoreductase activity >98% [*28*].

Discussion

A number of cardiac enzymes involved in ATP breakdown vary with age [*24,27*]. During hypoxia nucleotides catabolize to purines, which may cross cell membranes. Using neonatal and adult heart, we compared the activities of adenosine kinase and adenosine deaminase, enzymes sharing adenosine as a substrate. In addition we measured nucleoside phosphorylase and xanthine oxidoreductase, enzymes responsible for the ultimate breakdown of nucleosides. We do not know whether the different enzymic activities observed are due to different amounts of enzymes; xanthine oxidoreductase can occur in an inactive form [*26*]. The inhibitor studies proved that our enzymic assays were specific.

Adenosine kinase and adenosine deaminase

A new finding is the difference in adenosine kinase activity between neonatal and 4-month old ventricles (Fig. 1). The activity in the latter had decreased more than twice. We found this change also in isolated cardiomyocytes. Regardless of age, the specific activities in whole heart and myocytes were similar. This indicates that the bulk of adenosine kinase is present in the myocytes, which make up most of the cardiac mass.

The adenosine deaminase activity in hearts and myocytes was substantially higher than that of adenosine kinase (*cf.* Figs 1 and 2). It explains why deamination exceeds phosphorylation of adenosine in isolated, perfused hearts [*10*]. The enzymic measurements confirm the results of Arch and Newsholme [*3*] obtained in adult rat hearts. However, they contrast data from Dow *et al.* [*15*] who reported equal activities of the two enzymes in adult cardiomyoctes. The discrepancy is probably due to the difference in assay temperature used (see ref. 2).

The activities of adenosine deaminase in neonatal and adult hearts did not differ (Fig. 2), confirming literature data [*27*]. On the other hand, the specific activity in neonatal myocytes was about half of that in heart homogenates; it decreased 60% during development (Fig. 2). This indicates that adenosine deaminase activity is not evenly distributed among cardiac cells, especially in adult heart. Similarly, Dow *et al.* [*15*] stated that most of the myocardial adenosine deaminase is not located in (adult) myocytes. We postulate that this catabolic activity resides mainly in the endothelial cells.

Nucleoside phosphorylase and xanthine oxidoreductase

Nucleoside phosphorylase activity in adult heart was somewhat lower than in neonatal heart (Fig. 3). This finding is in agreement with published data [*27*]. Neonatal myocytes contained half the activity observed in heart homogenates. The adult ventricular and cellular preparations showed comparable activity. We conclude that the early preponderance of nucleoside phosphorylase activity in non-cardiac cells disappears later in life. We doubt that the enzyme in rat heart is predominantly located in endothelial cells, contrasting data for guinea-pig heart [*22*].

The data in Figure 4 confirm our earlier reports that xanthine oxidoreductase activity increases age-dependently [*24*]. Apparently this rise takes place almost exclusively outside the cardiomyocytes (Fig. 4), presumably in the microvascular endothelium [*4,19*]. We like to stress that xanthine oxidoreductase shows little activity in the (adult) heart of several species, including man [*14*].

Anabolism versus catabolism

The specific activities of adenosine kinase and xanthine oxidoreductase were more than ten times lower than those of the other enzymes. During development the ratio of the former,

rate-limiting enzymes for anabolism and catabolism, dropped from 68 to 0.68 in ventricles. In contrast, the ratio in myocytes remained unchanged, i.e., about 23. We conclude that the large difference, observed in heart homogenates, explains why ATP breakdown due to hypoxia is less in neonatal than in adult heart. Because this change is absent in myocytes, we speculate that endothelial activities of adenosine kinase and xanthine oxidoreductase are responsible for this shift in purine metabolism during development. Could the urate produced by the adult heart of some species act as a radical scavenger and antioxidant [2,5]?

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Appendix 4

XANTHINE OXIDOREDUCTASE ACTIVITY IN PERFUSED HEARTS OF VARIOUS SPECIES, INCLUDING HUMANS

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Circ. Res. 67 (1990) 770-773

Xanthine Oxidoreductase Activity in Perfused Hearts of Various Species, Including Humans

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Oxygen free radicals generated by xanthine oxidase have been implicated in cardiac damage. The activity of xanthine oxidase/reductase in adult rat heart is considerable. Its assay gives controversial results for other species, for example, rabbits and humans. Therefore, we perfused isolated hearts of various species, including explanted human hearts, to measure the conversion of exogenous hypoxanthine to xanthine and urate. We assayed these purines with high-performance liquid chromatography. The apparent xanthine oxidoreductase activities, calculated as release of xanthine plus 2× urate, were (milliunits per gram wet weight, mean±SEM) mice 33±3 (n=5), rats 28.5±1.4 (n=9), guinea pigs 14.4±1.0 (n=5), rabbits 0.59±0.09 (n=5), pigs <0.1 (n=6), humans 0.31±0.04 (n=7), and cows 3.7±0.8 (n=4). In rabbit heart the conversion of hypoxanthine to xanthine was slow, and that of xanthine to urate was even slower. On the other hand, guinea pig and human heart released little xanthine, indicating that xanthine breakdown exceeds its formation. We conclude that isolated perfused mouse, rat, guinea pig, and also bovine hearts show considerable xanthine oxidoreductase activity, contrasting rabbit, porcine, and diseased human hearts. (*Circulation Research* 1990;67:770–773)

In heart tissue, adenine nucleotides are broken down to adenosine, inosine, and hypoxanthine. These can be found in the cardiac effluent and can be used as markers for ischemia.[1] In rat heart, xanthine oxidoreductase catabolizes hypoxanthine to xanthine and urate.[2,3] Adult rat heart shows considerable activity of the enzyme,[4–8] in contrast to neonatal heart.[3,9] The oxidase form, which generates free oxygen radicals,[8] could potentially damage cardiac tissue. Whether the enzyme expresses itself in the hearts of humans[6,8,10–14] and several other species, including rabbits,[5–7,9,10] is controversial. The classical assay of the enzyme in homogenates may lead to erroneous results (e.g., because of endogenous inhibitors).[4,14] We decided to use the isolated, perfused hearts of various species, measuring the conversion of added hypoxanthine to xanthine and urate. This enabled us to estimate the cardiac xanthine oxidoreductase activity in a physiological environment.

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Materials and Methods

Perfusion of Rodent Hearts

Fed adult animals (BALB/c and hybrid C57 black/CBA mice, Wistar rats, Dunkin-Hartley guinea pigs, and New Zealand White rabbits) were anesthetized intraperitoneally with sodium pentobarbital in accordance with institutional guidelines. Hearts were removed, arrested, and perfused retrogradely with Tyrode's buffer at 9.6 kPa and 37° C as described previously.[1] Heart weight was 0.15–0.20 g (mouse), 0.9–1.6 g (rat), 1.6–2.2 g (guinea pig), and 8–12 g (rabbit). Cannulation of mouse aortas proved difficult and time consuming: about 25 minutes/aorta was needed. For the other species, 1 minute sufficed. In all hearts, except those from mice, apex displacement was used to monitor function and heart rate. Coronary flow was measured electromagnetically (Skalar, Delft, Netherlands) or by timed collection of perfusate. Unless otherwise indicated, a control period of 15 minutes was used; then, the perfusion medium was supplemented with hypoxanthine (Merck, Darmstadt, FRG), infused just above the aortic cannula, to give an optimal substrate concentration, usually 30–50 μM. In the coronary effluent, purines were assayed by high-performance liquid chromatography,[1] with detection at 295 nm (urate) and 254 nm (hypoxanthine, xanthine). For each species, perfusions with [8-^{14}C]hypoxanthine (5–40

Ci/mol, Amersham Int. plc, Little Chalfort, Buckinghamshire, U.K.) were also carried out. Radioactive chromatographic peaks were detected on-line with a 171 radioisotope detector (Beckman Instruments, Inc., Anaheim, Calif.).

Perfusion of Human, Porcine, and Bovine Hearts

Hearts of transplant patients in end-stage heart failure caused by ischemic heart disease or dilated cardiomyopathy were arrested in situ with St. Thomas' Hospital cardioplegic solution at 4° C.[15] Hearts from anesthetized, young adult pigs (hybrid Yorkshire/Danish Landrace, fasted overnight) underwent cardioplegic arrest just before or after excision at the conclusion of experiments performed for other purposes. Bovine hearts were flushed with the cardioplegic solution (with 5,000 IU/l heparin [Organon Teknika Nederland BV, Boxtel, Nederlands]) within 15–20 minutes after slaughter in a local abattoir.

Hearts were transported in ice-cold saline; cannulation time was 30–50 minutes. Then, retrograde perfusion of the aorta was started. The perfusion fluid (37° C) consisted of Tyrode's buffer[1] supplemented with 5 mM sodium pyruvate, 10 IU/l insulin (Novo, Industri AS, Bagsvaerd, Denmark), 5,000 IU/l heparin, and 25 g/l dextran (40,000 Da; Pharmacia, Uppsala, Sweden). After about 20 minutes of perfusion, 50 μM hypoxanthine was added. The perfusion fluid was oxygenated with 95% O_2-5% CO_2, using an S-070/S oxygenator (Shiley Laboratories Inc., Irvine, Calif.), with heat exchanger and defoaming membrane. The perfusion apparatus was also equipped with a roller pump, a manometer, an LPE-1440 filter (Pall BioSupport Corp., Glen Cove, N.Y.), a bubble trap, and a fluid reservoir. Blood was washed from the hearts for 10 minutes at a rate of 200 ml/min. Then, recirculation was started with 800–2,200 ml; perfusion pressure was 6.4–8.3 kPa. In human and porcine hearts, flow necessary to maintain perfusion pressure was 200–600 ml/min. Bovine hearts required 900–1,200 ml/min. Heart weight after the experiment was 300–800 g (humans), 200–300 g (pigs), and 1.9–3.2 kg (cows).

Results

Rodent Hearts

Because of their small size, we were unable to monitor function in mouse hearts. In the other hearts, changes in heart rate and apex displacement were minimal during the experiment. Control coronary flow (milliliters per minute per gram wet weight, mean±SEM) was 7.6±1.0 (mice), 7.3±0.5 (rats), 11.4±1.1 (guinea pigs), and 5.0±0.6 (rabbits). In the course of the experiment, flow decreased somewhat.

In contrast to its precursors,[16] infused hypoxanthine had no obvious inotropic or chronotropic effects; it was not vasoactive. We measured the catabolites xanthine and urate in the coronary effluent. Xanthine concentrations (micromolar) amounted to 2.9±0.4 (mice), 2.4±0.2 (rats), 0.23±0.08 (guinea pigs), and 0.106±0.014 (rabbits); urate concentrations (micromolar) were 1.3±0.3, 1.19±0.09, 0.74±0.09, and 0.018±0.004, respectively. The studies with radioactive hypoxanthine excluded a major contribution of xanthine from guanine. We found no evidence of urate breakdown to allantoin.

Table 1. Apparent Xanthine Oxidoreductase Activity in Isolated, Perfused Heart

Species	*n*	Xanthine (nmol/min/g)	Urate (nmol/min/g)	XOD (mU/g)
Mouse	5	18±2	8.3±1.4	33±3
Rat	9	14.1±1.1	7.8±0.4	28.5±1.4
Guinea pig	5	1.2±0.3	7.2±0.5	14.4±1.0
Rabbit	5	0.51±0.08	0.075±0.015	0.59±0.09
Pig	6	<0.05	<0.07	<0.1
Human	7	0.023±0.003	0.14±0.02	0.31±0.04
Cow	4	1.1±0.3	1.4±0.3	3.7±0.8

Data are expressed as mean±SEM and per gram wet weight. Hypoxanthine was infused into isolated hearts. From the xanthine and urate production rates, the xanthine oxidoreductase (XOD) activity was calculated as xanthine+2× urate. *n*, Number of experiments.

The apparent xanthine oxidoreductase activity, calculated from the concentrations mentioned above, was high in mouse hearts (33 mU/g). The activity was comparable in rat hearts, twice lower in guinea pig hearts, and very low in rabbit hearts (Table 1). In the latter, the conversion of hypoxanthine to xanthine was very slow, and that of xanthine to urate was even slower (Table 1). Exogenous hypoxanthine affected only the first reaction step in rabbit hearts. The guinea pig hearts released relatively little xanthine.

Porcine, Bovine, and Human Hearts

Human and porcine hearts beat regularly, whereas bovine hearts showed only atrial activity. Coronary flow (milliliters per minute per gram wet weight) was 0.82±0.12 (humans), 1.8±0.2 (pigs), and 0.43±0.07 (cows). Xanthine and urate concentration increased per minute by 0.012±0.001 and 0.08±0.02 μM, respectively, in human heart perfusate and by 0.72±0.10 and 1.2±0.4 μM, respectively, in bovine heart perfusate. We observed only marginal changes in the perfusate of porcine heart.

From Table 1 it is clear that relatively little activity was present in explanted human hearts, that is, 0.31 mU/g. Only porcine hearts showed a lower value: the activity was below the detection limit (0.1 mU/g). Bovine hearts had a 10-fold higher activity and fivefold lower urate/xanthine ratio than human hearts.

To preserve the hearts of the larger species, we administered a cardioplegic solution. To check whether this affected the xanthine oxidoreductase activity, we subjected rat hearts (which display a relatively high activity) to cold cardioplegia/ischemia. Figure 1 shows that this procedure hardly affected the enzymatic activity. We also tested in rat hearts whether the perfusion fluid used for the large hearts

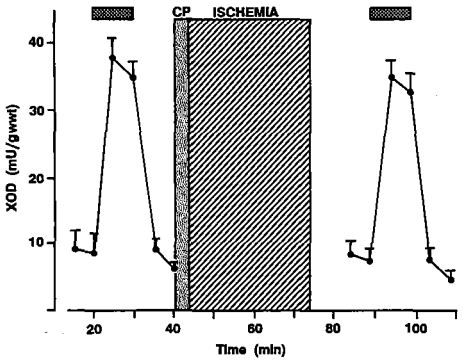

FIGURE 1. *Effect of cold potassium cardioplegia on apparent xanthine oxidoreductase (XOD) activity. After an equilibration period of 20 minutes, rat hearts were infused for 10 minutes with 50 μM hypoxanthine (horizontal bars). From the effluent xanthine and urate concentrations, the enzymatic activity was calculated. Ten minutes postinfusion, cold St. Thomas' Hospital cardioplegic solution (CP) was administered (3.0 ml/min), followed by an ischemic, hypothermic period of 30 minutes. After 15 minutes of reperfusion, hypoxanthine was again infused; the XOD activity measured was similar to that before cardioplegia/ischemia (Student's paired t test, p>0.05). Data are given as mean±SEM (n=4). wwt, Wet weight.*

influenced the enzyme. Again, a similar activity was found with both perfusion fluids. These control experiments make it unlikely that the low activity found in human, porcine, and bovine hearts is due to differences in experimental setup.

Discussion

Species Differences

Ronca-Testoni and Borghini[2] were the first to show urate production by the isolated, perfused rat heart. We confirmed their observation that urate composes the major purine released from rat hearts under basal conditions.[2-4] Gerlach et al[17] had similar observations in guinea pig hearts. We reported blockade of hypoxanthine breakdown during anoxia by allopurinol.[4] These observations strongly suggested that rat and guinea pig hearts contain xanthine oxidoreductase. Enzymatic measurements confirmed this hypothesis.[4,6,18] Reported discrepancies on the activity of cardiac xanthine oxidoreductase in various other species initiated the present study. The apparent xanthine oxidoreductase activity in mouse hearts (33 mU/g), measured with hypoxanthine, was of the same order of magnitude as that in rat and guinea pig hearts (Table 1). It exceeds the only reported value for mouse hearts by a factor of 10.[19] Our estimate for rat and guinea pig hearts is in reasonable agreement with values found in extracts.[4-8,18] The conversion of xanthine to urate seems to be faster in guinea pig than in mouse and rat hearts. This should be checked with xanthine as the substrate.

The activity found in isolated pig hearts was <0.1 mU/g. Several authors reported minimal activity of the enzyme in pig heart homogenates.[6,20] Our value for cow hearts agrees with data given in References 13 and 21 but is >30× higher than that reported in Reference 22. In guinea pig and human hearts, xanthine breakdown seems to be faster than xanthine formation. Rabbit hearts had little xanthine oxidoreductase activity, as calculated from the xanthine and urate production. Several authors reported minimal xanthine oxidoreductase activity in rabbit heart homogenate,[5-7,9] which is in line with our ex vivo observation. The high activity in homogenate found by Wajner and Harkness[10] is puzzling, as is the high activity reported for human heart autopsies.[10]

Xanthine Oxidoreductase in Heart Explants

Recently, we suggested that xanthine oxidoreductase is present in the hearts of patients suffering from ischemic heart disease.[23] This was based on the demonstration of cardiac urate production during transient myocardial ischemia. Autopsy material revealed high activity of the enzyme.[10,14] Limited histochemical work has shown substantial amounts of xanthine oxidoreductase in human heart endothelium.[11] On the other hand, several authors have reported very low to undetectable activity of the enzyme in human hearts.[6,8,12,24] Based on our isolated heart perfusions, we conclude that hearts from patients with cardiomyopathy or end-stage ischemic heart disease are almost devoid of xanthine oxidoreductase activity. We cannot exclude the possibility that the diseased human heart contains inactive xanthine oxidoreductase. Such enzyme has been described recently. Xanthine oxidase can destroy itself by self-generated O_2 metabolites.[25] At present, we assume that extracardiac factors, such as neutrophils or other blood components, are responsible for the apparent xanthine oxidoreductase activity observed in catheterized patients[23] or autopsies.[10,14]

Conclusion

Isolated, perfused mouse, rat, guinea pig, and also bovine hearts show considerable xanthine oxidoreductase activity, contrasting porcine, rabbit, and diseased human hearts.

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KEY WORDS • hypoxanthine • isolated heart • species differences • urate • xanthine oxidase

Appendix 5

MYOCARDIAL PROTECTION BY INTRAVENOUS DILTIAZEM DURING ANGIOPLASTY OF SINGLE-VESSEL CORONARY ARTERY DISEASE.

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Am. J. Cardiol. 66 (1990) 145-150

Myocardial Protection by Intravenous Diltiazem During Angioplasty of Single-Vessel Coronary Artery Disease

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The possible cardioprotective effect of diltiazem during ischemia caused by percutaneous transluminal coronary angioplasty was tested. Electrocardiograms and myocardial lactate, hypoxanthine and urate production were determined in 26 patients with a stenosis in the left anterior descending artery without angiographically demonstrable collaterals. Measurements took place before angioplasty, after each of 4 occlusions and 15 minutes after the last balloon inflation. Patients were randomly given placebo or DL-diltiazem (0.4 mg/kg as a bolus intravenously, followed by an infusion of 15 mg/hr). During angioplasty the ST-segment elevation for the anterior wall leads V_2, V_4 and V_6, and the intracoronary lead was similar for both groups, as was lactate release. Diltiazem significantly reduced cardiac hypoxanthine release immediately after angioplasty from 63 to 88% ($p < 0.05$). The drug diminished urate production after the last dilatation by 82% ($p < 0.05$). In conclusion, intravenous infusion of diltiazem reduced cardiac adenosine triphosphate breakdown during angioplasty as shown by diminished hypoxanthine and urate production. In contrast, diltiazem was unable to attenuate ST-segment elevation and lactate release.

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In the last decade, calcium antagonists have proven to be effective in the treatment of many cardiovascular disorders. A few of these drugs, including diltiazem, have been successful in the management of angina pectoris. One of the advantages of diltiazem is its ability to delay ischemic ventricular arrhythmias.[1] In addition, calcium antagonists may improve the protection afforded by potassium-cardioplegia for heart surgery.[2] They may also be useful for local cardioplegia during coronary angioplasty.[3–8] Diltiazem delayed the onset of pacing-induced ischemia.[9,10] In isolated hearts and open-chest animals, diltiazem[11–15] and other calcium antagonists[14,16–19] exert an energy-sparing effect during ischemia. In this study, we tested whether diltiazem could minimize adenosine triphosphate-breakdown during angioplasty and thus serve as a potential cardioprotective agent during the procedure.

METHODS

Patients: The study involved 26 patients with 1-vessel coronary artery disease undergoing percutaneous transluminal coronary angioplasty. The following characteristics were observed for each patient. Coronary artery disease was limited to an isolated proximal stenosis <1 cm in the left anterior descending artery (narrowing >70%) and no collateral filling was seen angiographically in the region supplied by this artery. There was no history of previous myocardial infarction, and nitrates were not required during the dilatation procedure (Table I). Antiplatelet drugs (including aspirin) and other cardioactive medications except short-acting nitrates were discontinued ≥48 hours before the procedure. With the exception of heparin, no drugs were given before completion of data acquisition. This research project was approved by the institutional committee on patient research. All patients gave informed consent before the study, and no complications related to the protocol were observed.

Coronary angioplasty: A normal El Gamal diagnostic 7Fr catheter (Cordis) was introduced through the right femoral vein in the great cardiac vein. Its position was confirmed with the injection of a small volume of nonionic contrast medium (Isopaque Coronar 370). An 8Fr guiding catheter was introduced percutaneously and advanced to the aortic root through the right fem-

TABLE I Clinical Characteristics of the Study Groups

	Placebo Group (n = 13)	Diltiazem Group (n = 13)
Age (yrs), average	58	58
Range	43–72	37–74
Sex: M/F	10/3	11/2
Patients in CCS		
Grade II	4	4
Grade III	7	6
Grade IV	2	3
Average severity of stenosis (%)		
Before angioplasty	79	82
After angioplasty	37	35
Average ejection fraction (%)	64	66
Mean aortic pressure during transluminal occlusion (mm Hg)	102	97
Mean heart rate during transluminal occlusion (beats/min)	74	68

None of the differences reached significance at $p < 0.05$.
CCS = Canadian Cardiovascular Society.

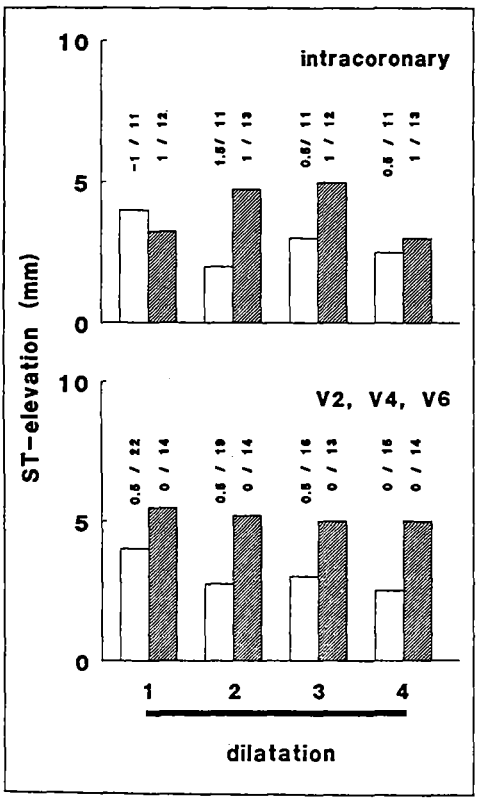

FIGURE 1. Effect of diltiazem on ST-segment elevation during angioplasty, measured with the intracoronary lead (*top*) and the precordial leads (*bottom*). *Bars* indicate the median values, ranges are given above the columns. *Open bars* = placebo; *hatched bars* = diltiazem. No significant effects

oral artery. The left anterior descending artery was visualized by injection of contrast medium after the first blood samples had been taken. Then, the angioplasty balloon (Advanced Cardiovascular System Inc.) was introduced. Coronary angioplasty was performed according to Simpson et al,[20] using a steerable guidewire (Advanced Cardiovascular System, high torque floppy, 0.014 inch). Balloon diameters were 3.0 or 3.5 mm. The maximal inflation pressures ranged from 6 to 12 atmospheres. For each patient, the dilatations were sustained for 90 seconds or until the onset of chest pain. In each patient, 4 consecutive dilatations were performed, with a 5 minute recovery between inflations. Control coronary angiography was then performed.

Sampling: Great cardiac venous and femoral arterial blood samples, taken before, immediately after each deflation and 15 minutes after the procedure, were treated as previously described.[21]

Lactate and purine analysis: In the deproteinized samples, lactate was determined enzymatically.[22] In addition, the adenine nucleotide catabolites hypoxanthine and urate were assayed by high-performance liquid chromatography,[23] as modified by Huizer et al.[21] Urate was detected at 290 nm.

Electrocardiograms: These were monitored from the precordial leads V_2, V_4 and V_6, and from the intracoronary lead, using the guidewire placed in the left anterior descending artery. Each lead was calibrated before the procedure (10 mm = 1 mV). Maximal ST elevation at the end of each inflation was measured 0.80 second after the J point, with the T-wave–P-wave interval as the isoelectric line.

Experimental regimen: The patients were randomly assigned to treatment with placebo or diltiazem (double-blind). Five minutes before the first dilatation, and immediately after data collection at rest, they received either DL-diltiazem (0.4 mg/kg intravenously [Lorex]), or solvent, as a bolus over 5 minutes, followed by a continuous infusion of either 15 mg/hr diltiazem or solvent. Plasma diltiazem was determined with the methodology described for verapamil.[24]

Statistical analysis: Results are given as median and range, unless indicated otherwise. Nonparametric statistical methods were used: for within-group analysis the Wilcoxon signed rank test, for between-group analysis the Mann-Whitney rank sum test. A p value <0.05 was considered statistically significant.

RESULTS

Clinical characteristics: The clinical characteristics of the study groups assigned to diltiazem or placebo are listed in Table I. Age, Canadian Cardiovascular Society grade, severity of the stenosis in the left anterior descending artery and left ventricular ejection fraction in the 2 groups were comparable. Inflation time (approximately 80 seconds), inflation pressure (approximately 9 atmospheres) and pain symptoms (in 10 or 11 of 13 patients) during the 4 consecutive inflations were comparable in the 2 groups. Cross-sectional area of the stenosis before and after coronary angioplasty was similar in both groups (approximately 80 and 35%, respectively). The baseline values of lactate, hypoxanthine and

TABLE II Effect of Diltiazem on Arterial and Great Cardiac Venous Plasma Lactate Concentrations During Angioplasty

Sampling Site	Treatment	No.	Lactate Concentration (mM)					
				Dilatation				
			Pre	1	2	3	4	Post
Arterial	Placebo	13	0.68 (0.41–1.54)	0.65 (0.41–1.48)	0.64 (0.44–1.57)	0.63 (0.38–1.61)	0.57 (0.38–1.54)	0.56 (0.38–1.85)
Arterial	Diltiazem	13	0.73 (0.43–0.99)	0.66 (0.40–0.91)	0.69 (0.36–0.93)	0.66 (0.39–0.84)	0.67 (0.39–0.86)	0.64 (0.34–0.91)
Venous	Placebo	13	0.53 (0.37–1.65)	1.54 (0.90–2.56)	1.45 (0.62–2.34)	1.48 (0.81–2.67)	1.50 (0.74–2.93)	0.59 (0.29–1.71)
Venous	Diltiazem	13	0.60 (0.34–0.86)	1.47 (0.54–2.39)	0.94 (0.45–2.32)	1.37 (0.41–2.51)	1.05 (0.43–3.24)	0.69 (0.35–0.98)

No. = number of observations. Median values are given with ranges in parentheses. No significant effects of treatment were observed.

TABLE III Effect of Diltiazem on Arterial and Great Cardiac Venous Plasma Hypoxanthine Concentrations During Angioplasty

Sampling Site	Treatment	No.	Hypoxanthine Concentration (μM)					
				Dilatation				
			Pre	1	2	3	4	Post
Arterial	Placebo	11	0.60 (0.23–0.96)	0.41 (0.07–0.63)	0.33 (0.07–0.79)	0.25 (0.05–0.55)	0.20 (0.05–0.79)	0.15 (0.02–0.48)
Arterial	Diltiazem	12–13	0.70 (0.25–1.60)	0.31 (0.11–0.87)	0.34 (0.08–0.84)	0.29 (0.09–0.52)	0.24 (0.06–0.54)	0.16 (0.08–0.49)
Venous	Placebo	11	0.41 (0.11–0.97)	0.94 (0.44–5.20)	1.30 (0.16–2.33)	1.31 (0.33–2.07)	1.04 (0.33–2.41)	0.42 (0.03–0.93)
p Value				0.017	0.017	0.023	0.017	
Venous	Diltiazem	13	0.31 (0.09–0.85)	0.42 (0.17–2.52)	0.33 (0.10–4.89)	0.43 (0.06–5.58)	0.37 (0.05–3.13)	0.18 (0.03–0.65)

No. = number of observations. Median values are given with ranges in parentheses. Where differences between placebo and diltiazem were statistically significant, p values are given.

TABLE IV Effect of Diltiazem on Arterial and Great Cardiac Venous Plasma Urate Concentrations During Angioplasty

Sampling Site	Treatment	No.	Urate Concentration (μM)					
				Dilatation				
			Pre	1	2	3	4	Post
Arterial	Placebo	13	236 (192–449)	224 (185–457)	234 (179–481)	220 (177–455)	212 (174–469)	225 (168–462)
Arterial	Diltiazem	12–13	246 (138–347)	244 (136–352)	240 (131–351)	226 (128–358)	243 (126–348)	238 (115–348)
Venous	Placebo	13	242 (188–495)	221 (181–481)	217 (182–478)	230 (178–480)	238 (174–512)	221 (173–469)
Venous	Diltiazem	13	244 (144–342)	250 (131–356)	251 (130–351)	251 (130–352)	247 (128–359)	238 (124–348)

No. = number of observations. Median values are given with ranges in parentheses. No significant effects of treatment were observed.

urate were similar for both groups (Tables II through IV).

Electrocardiographic measurements: No ST-segment or T-wave abnormalities were observed before angioplasty. Figure 1 shows the actual degree of ST-segment elevation during each occlusion. Electrocardiographic measurements taken from the intracoronary lead and the anterior wall leads V_2, V_4 and V_6 show that ST elevation tended to be smaller in the placebo group, but the differences were not significant. Some patients did not develop ST-segment elevation during transluminal occlusion. Although they did not show any collaterals on pretreatment coronary arteriography, it is possible that they developed collaterals during balloon occlusion. ST-segment depression during occlusion was observed on only 1 occasion.

Lactate release: The arterial lactate concentrations were not affected by diltiazem (Table II). After angioplasty great cardiac venous lactate increased 2- to 3-fold ($p < 0.01$) in both the placebo and diltiazem groups. Figure 2 shows the arteriovenous difference of lactate at control and after each balloon dilatation. Patients in both groups produced lactate immediately after the balloon deflations. Treatment with diltiazem tended to

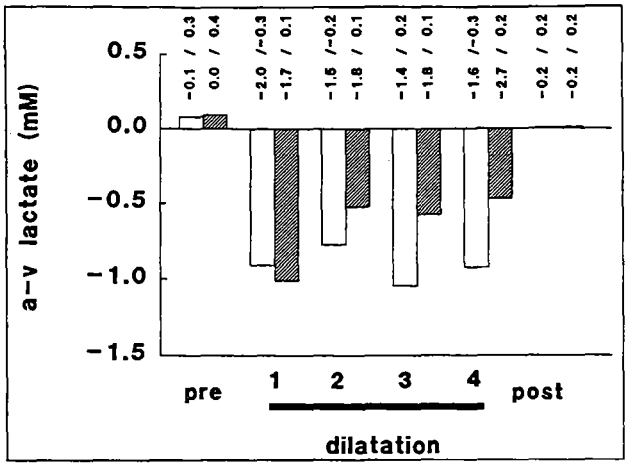

FIGURE 2. Effect of diltiazem on lactate uptake by the heart. Immediately after angioplasty, lactate production was noted, as the great cardiac venous concentration exceeded the arterial one. *Bars* indicate the median values, ranges are given above the columns. *Open bars* = placebo; *hatched bars* = diltiazem. No significant effects of treatment were found. a = arterial; v = venous.

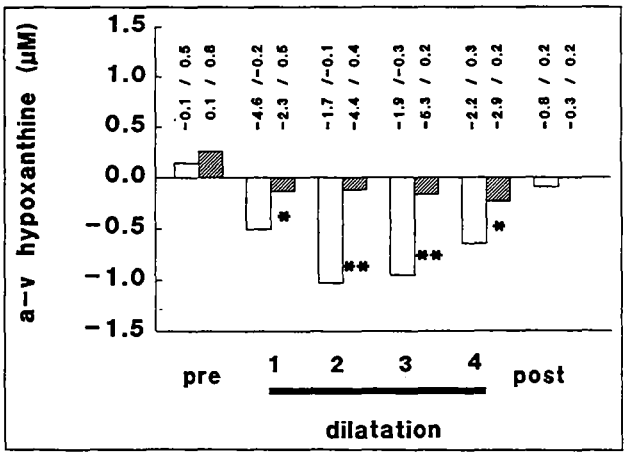

FIGURE 3. Effect of diltiazem on hypoxanthine uptake by the heart. Immediately after angioplasty, hypoxanthine production was noted, as the great cardiac venous concentration exceeded the arterial one. *Bars* indicate the median values, ranges are given above the columns. *Open bars* = placebo; *hatched bars* = diltiazem. Treatment with diltiazem lowered hypoxanthine production after each dilatation significantly. * $p < 0.05$; ** $p < 0.01$. Abbreviations as in Figure 2.

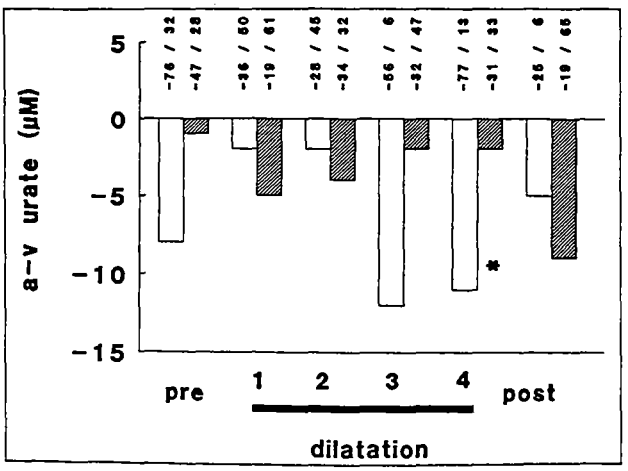

FIGURE 4. Effect of diltiazem on urate production by the heart. *Bars* indicate the median values, ranges are given above the columns. *Open bars* = placebo; *hatched bars* = diltiazem. Treatment with diltiazem significantly reduced the urate production found immediately after the last dilatation. * $p = 0.047$. Abbreviations as in Figure 2.

reduce lactate production after angioplasty was performed ($p > 0.05$).

Hypoxanthine metabolism: The hypoxanthine concentrations are listed in Table III. Diltiazem did not affect the arterial values. In the placebo group, the great cardiac venous hypoxanthine concentrations increased 3- to 5-fold ($p < 0.01$) after each dilatation. In contrast, only a minor increase was observed in diltiazem-treated patients (Table III). In these patients venous hypoxanthine was lower ($p \leq 0.02$) than that in the placebo group. Figure 3 shows the effect of placebo and diltiazem on hypoxanthine production by the heart. Samples taken directly after the 4 occlusions showed 63 to 88% lower hypoxanthine production in the diltiazem group than in the placebo group ($p < 0.05$).

Urate release: Diltiazem had no significant effect on the arterial and venous urate concentrations (Table IV). Figure 4 shows the effect of placebo and diltiazem on urate production by the heart. At rest and directly after the first 3 dilatations, there were no significant differences between the groups. Only immediately after the fourth dilatation was urate release smaller ($p = 0.047$) in the diltiazem group.

Diltiazem levels: The plasma concentrations of diltiazem, measured 5 minutes after the last deflation, varied widely (132 to 4,730 μg/liter, median 902).

DISCUSSION

Electrocardiographic data: We did not detect a statistically significant effect of diltiazem treatment on ST-segment changes during coronary angioplasty. The changes tended to be larger in the diltiazem group. In contrast, several investigators[5,7,8] observed that severity and time to onset of ischemic ST- and T-wave changes during coronary angioplasty were significantly reduced after treatment with this drug. In other studies, the administration of diltiazem has also been shown to reduce ST-segment elevations due to pacing-induced ischemia in dogs[9] as well as in man.[10] We cannot explain the discrepancies between our results and these reports.

Lactate production: In this study, diltiazem tended to reduce the amount of lactate produced by the heart subjected to coronary angioplasty. However, this effect was not statistically significant. Hanet,[4] Werner[6] and their co-workers reported a significant reduction in lactate production with other calcium antagonists used intracoronarily. Remme et al[10] observed that diltiazem could attenuate significantly lactate production induced by rapid atrial pacing.

Hypoxanthine and urate release: After ischemia the human heart releases hypoxanthine due to adenosine triphosphate catabolism. This has been demonstrated during pacing stress testing,[23,25] coronary angioplasty[26] and heart surgery.[2,27] In our study, hypoxanthine production was also obvious after each balloon deflation. Diltiazem could diminish this production. Based on animal studies,[11-15] it seems unlikely that the drug influenced purine uptake/release independent of adenosine triphosphate hydrolysis. An interesting aspect of the present investigation is the production of urate (Figure 4).[27] This could be partly suppressed by diltiazem. The appearance of urate indicates that the human heart contains xanthine oxidoreductase. This enzyme exists in the heart in a number of species,[28] but its presence in human heart is controversial.[29,30] We cannot exclude that the enzyme could be active in the intact human heart, generating free radicals in its oxidase form.

Diltiazem levels: We are puzzled by the large variation in plasma diltiazem concentrations. The same phenomenon has been noted in other clinical trials (W.J. Remme, personal communication). Whether distribution or metabolism of the drug varies greatly from patient to patient is not known. We did not see a correlation between diltiazem blood level and suppression of, for example, hypoxanthine release.

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Appendix 6

ENERGY CONSERVATION BY NISOLDIPINE IN ISCHAEMIC HEART

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Energy conservation by nisoldipine in ischaemic heart

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1 We studied the effect of the calcium entry blocker nisoldipine on ATP catabolism in the rat heart, perfused according to Langendorff. Even 1 nM nisoldipine induced vasodilatation; concentrations of 30 nM and higher caused significant negative inotropy.

2 The drug had a very strong affinity for silicon rubber tubing.

3 Myocardial ischaemia was induced by lowering the perfusion pressure, which reduced flow without nisoldipine by 85%. The efflux of purine nucleosides and oxypurines rose 14 fold. Nisoldipine reduced this efflux of ATP catabolites dose-dependently. The highest concentration, 300 nM, suppressed ischaemic purine production completely.

4 The action of the drug was antagonized by an increase in Ca^{2+}-concentration in the perfusion fluid.

5 We also showed the protective effect of nisoldipine on adenine nucleotides in freeze-clamped hearts. A concentration of 20 nM partially prevented the reduction of ATP and adenylate energy charge due to ischaemia.

6 We conclude that relatively low doses of nisoldipine effectively prevent ATP breakdown in ischaemic rat heart.

Introduction

Nisoldipine is one of the newer dihydropyridine derivatives, which act as powerful vasodilators by inhibiting transmembrane calcium influx. This Ca^{2+}-entry blocker reduces afterload and is an extremely potent coronary dilator (Kazda *et al.*, 1980). It seems to be a potential drug for the treatment of ischaemic heart disease (Vogt *et al.*, 1980).

Little is known about the effect of nisoldipine on myocardial metabolism. Takahashi & Kako (1983) found that nisoldipine suppresses the ischaemia-induced increase in phospholipid breakdown of cardiac sarcolemma. In the anaesthetized dog, Kazda *et al.* (1980) found no changes in myocardial lactate metabolism due to the drug. We tested whether it could prevent nucleotide catabolism in the ischaemic rat heart. Adenosine 5′-triphosphate (ATP) produces adenosine 5′-diphosphate (ADP) and adenosine 5′-phosphate (AMP) in the heart; an increased concentration of catabolites of the latter – adenosine, inosine, (hypo) xanthine and urate – occurs in the myocardial efflux during ischaemia (Schoutsen *et al.*, 1983). In an effort to elucidate the extent to which catabolism could be avoided and high-energy phosphates preserved, the efflux of these compounds was measured in the presence of various concentrations of the Ca^{2+}-entry blocker. Nisoldipine proved to be an effective inhibitor of ATP breakdown during myocardial ischaemia.

Methods

Heart perfusion

Male Wistar rats (230 to 380 g), with free access to food and water, were anaesthetized with 30 mg pentobarbitone i.p. Hearts were rapidly removed and cooled in ice-cold 0.9% w/v NaCl solution until beating ceased. Then retrograde perfusion of the aorta was started with a modified Tyrode buffer, gassed with 95% O_2 plus 5% CO_2 (pH 7.4, 37°C). Unless otherwise indicated the buffer contained (mM): D-glucose 10, NaCl, 128, KCl 4.7, $CaCl_2$ 1.4, $NaHCO_3$ 20, NaH_2PO_4 0.4, $MgCl_2$ 1.0. The perfusion temperature was measured in the aortic cannula. Pacing frequency was 300 beats min^{-1}. Other details of the perfusion are given elsewhere (De Jong *et al.*, 1984). Ischaemia was induced by lowering the perfusion pressure from 9.6 to about 2.2 kPa for 15 or 30 min. Where indicated, reperfusion took place for 15 min. Silicon rubber tubing, used in the perfusion apparatus, was supplied by Rubber-Technisch

Handels- en Adviesbureau, Hilversum, The Netherlands. Outer and inner dimensions were 6 and 4 mm, respectively.

Myocardial function

Apex displacement was measured as described previously (Stam & De Jong, 1977). This method gives relative values; the displacement found 5 min after the start of the perfusion was taken as 100%. Alternatively, developed tension was monitored with a P23Db transducer (Statham, Hato Rey, Puerto Rico) connected to the apex of the heart. Five minutes after the start of the perfusion, these hearts were adjusted to a resting tension of about 15 g.

Coronary flow

Perfusate was collected in a graduated cylinder for one minute periods at the end of normoxia, ischaemia and reperfusion.

Assay of purines

Perfusate samples were mixed with NaN_3 (0.02% final concentration) and kept on ice until analysis, usually within 12 h. Adenosine, inosine, hypoxanthine, xanthine, and urate were determined by high-performance liquid chromatography (h.p.l.c.), as described previously (Harmsen *et al.*, 1981; De Jong *et al.*, 1984).

Assay of adenine nucleotides

In freeze-clamped hearts, adenine nucleotides were determined by h.p.l.c. according to Harmsen *et al.*, (1982). From these nucleotides the adenylate energy charge, ([ATP]+0.5 [ADP])/([ATP] + [ADP] + [AMP]), was calculated.

Chemicals

All chemicals were of the highest grade available. Water was purified with the Millipore-Ro4/Milli-Q System (Millipore, Bedford, MA). Nisoldipine (isobutyl methyl 1,4-dihydro-2,6-dimethyl-4 (2-nitrophenyl)-3,5-pyridinedicarboxylate; Bayer, Wuppertal, GFR) was solved in absolute ethanol. The stock solution (2.5 mM) was diluted by adding perfusion buffer and vigorous stirring. Nisoldipine solutions were kept in the dark.

Statistical analysis

Two-way analysis of variance was employed. Further evaluations were made using Scheffé's method for multiple comparisons (Snedecor & Cochran, 1967).

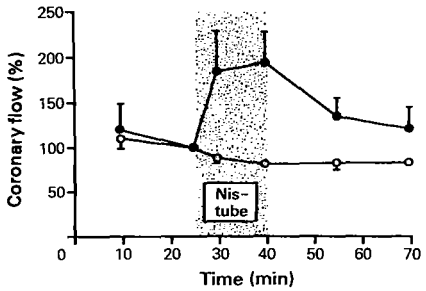

Figure 1 Increase in coronary flow due to residual nisoldipine (Nis) from perfusion tubing. For 15 min 100 nM of the drug, dissolved in perfusion medium, was pumped through 20 cm of silicon rubber tubing (internal diameter 4 mm) at a rate of 10 ml min^{-1}. Subsequently the tubing was washed for 1 min with four tube volumes of medium, and mounted in the perfusion apparatus. All hearts were perfused with standard medium for 25 min bypassing the tubing. Then the medium was forced to enter for 15 min the aorta either via the pretreated (●) tubing or via tubing which had been flushed similarly with standard medium (○). A significant ($P<0.001$) increase in flow, due to nisoldipine released from the tubing was observed. Flow data were calculated relative to the 25 min value; they are presented as means with vertical lines showing s.d. ($n=5$).

$P<0.05$ (two-tailed) was considered statistically significant.

Results

Affinity of nisoldipine for tubing

From initial experiments we got the impression that our standard wash-out procedures were insufficient to remove nisoldipine from the perfusion apparatus. The experiment depicted in Figure 1 showed that the drug had a very strong affinity for silicon rubber tubing. We pumped medium with nisoldipine through a piece of tubing, washed it, and tested to see whether this tubing affected coronary flow. Even after 15 min of perfusion, flow was doubled by the tubing treated with nisoldipine ($P<0.001$). We also noted some effect on apex displacement: it decreased by about 20% with this tubing ($P=0.009$; results not shown). In the studies, described below, we used only rubber tubing to connect glass pieces, with almost no contact between tubing and perfusate.

Coronary flow

From Figure 1 it is clear that very small amounts of nisoldipine induced vasodilatation. This is further

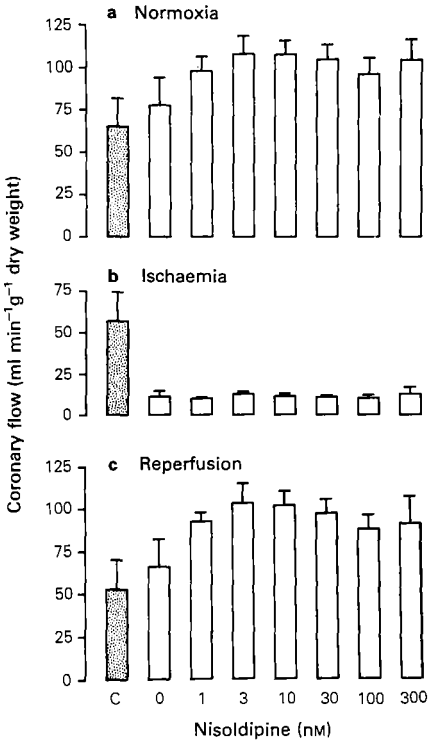

Figure 2 Vasodilatation by nisoldipine in (a) normoxic, (b) ischaemic and (c) reperfused hearts. At the end of the periods indicated, coronary flow data (in ml min^{-1} g^{-1} dry weight) were obtained from hearts perfused for 20 min under normoxic conditions (perfusion pressure 9.6 kPa), followed by 15 min of ischaemia (pressure lowered to 2.2 kPa), and 15 min of reperfusion at 9.6 kPa. Where indicated, treatment with nisoldipine started after 5 min of perfusion. Stippled columns (C) = control perfusion, with neither nisoldipine nor ischaemia. Columns show means and vertical lines s.d. ($n = 5-7$).

documented in Figure 2. Even 1 nM gave an increase in flow; all doses caused vasodilatation in normoxic hearts ($P < 0.001$). When the perfusion pressure was reduced from 9.6 ± 0.1 to 2.2 ± 0.2 kPa, coronary flow dropped to about 11 ml min^{-1} g^{-1} dry weight. This is about 85% less than flow in non-ischaemic control hearts. Nisoldipine did not cause significant differences in flow during ischaemia. However, in reperfused hearts the powerful vasodilator properties of nisoldipine were again apparent ($P < 0.001$). Already the lowest concentration (1 nM) caused an increase in flow.

Purine efflux

Nisoldipine, in a dose-range of 1 to 300 nM, did not significantly affect the efflux of adenosine, inosine, hypoxanthine or xanthine from normoxic hearts. For the four purines, mentioned above, the relative amounts were 2%, 39%, 35% and 24%, respectively. The total amount was 6 ± 3 nmol $min^{-1}g^{-1}$ dry weight after a 30 min perfusion period (Figure 3). The production of these purine nucleosides and oxypurines increased 14 fold as a result of ischaemia (Figure 3). The relative amounts found in the effluent of the ischaemic heart were 15% (adenosine), 51% (inosine), 20% (hypoxanthine) and 14% (xanthine). Nisoldipine suppressed purine efflux in a dose-dependent manner ($P < 0.001$). At the highest drug concentration, purine production was comparable to non-ischaemic control values. The reperfusion values were similar to those observed before the induction of ischaemia: no effect of nisoldipine.

We measured urate efflux in addition to the purine production mentioned above. It amounted to about 60 and 10% of total purines in the effluent of nor-

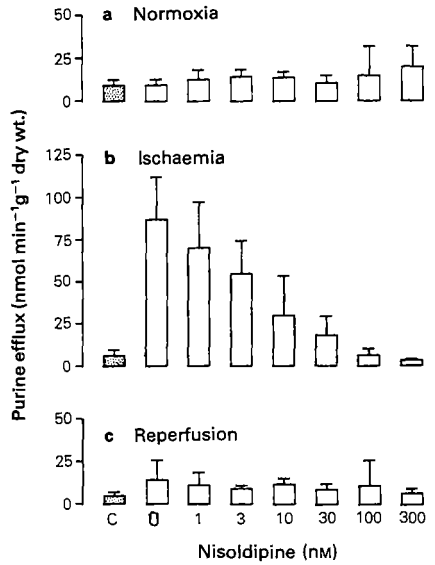

Figure 3 Reduction of purine efflux (in nmol $min^{-1}g^{-1}$ dry weight) from ischaemic heart by nisoldipine. The drug suppressed dose-dependently the production of adenosine, inosine, hypoxanthine and xanthine from ischaemic tissue. At the highest concentration of nisoldipine purine release was comparable to that from non-ischaemic controls (C, stippled columns). Other details are given in the legend to Figure 2.

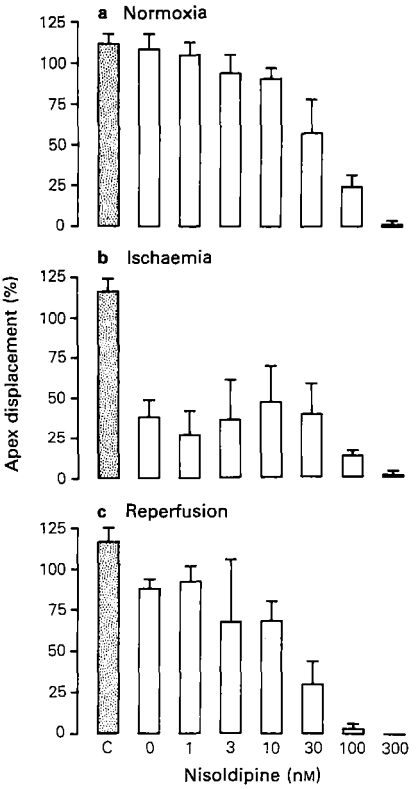

Figure 4 Effect of nisoldipine on apex displacement (%) of isolated perfused hearts. The value found 5 min after the start of the perfusion (before any drug was given) was taken as 100%. For other details, see legend to Figure 2. Please, note the dose-dependent negative inotropy before and after flow reduction. During ischaemia only the highest drug concentration reduced apex displacement significantly. Stippled columns (C) = non-ischaemic controls.

moxic and ischaemic heart, respectively. Nisoldipine (300 nM) also completely suppressed urate production from ischaemic heart. As the urate peaks in the high-performance liquid chromatograms were not always of a high quality, we decided to present only the data on the other purines in detail.

Myocardial function

We used apex displacement routinely as a measure of myocardial function. Figure 4 shows the dose-dependent ($P<0.001$) negative inotropic effect of nisoldipine in these isolated hearts. In the normoxic hearts, 30 nM nisoldipine decreased apex displacement by about 50%. A ten times higher concentration resulted in minimal contractile behaviour. Nisoldipine up to 30 nM did not affect apex displacement in ischaemic hearts. Apex displacement in these hearts was about 35% of non-ischaemic control values. Negative inotropy during ischaemia was seen with 100 and 300 nM nisoldipine. Apex displacement with 300 nM was significantly ($P<0.001$) smaller than that with the other doses. Apex displacement recovered to about 77% of control values without nisoldipine or with 1 nM of drug. Higher concentrations again gave dose-dependent negative inotropy ($P<0.001$).

In a number of hearts, we used developed tension to characterize myocardial function. Nisoldipine, in a concentration of 20 nM, decreased developed tension by 25% in normoxic hearts ($P<0.001$, data not shown). However, in both treated and untreated ischaemic hearts, developed tension was only a few % of that in non-ischaemic hearts ($P<0.001$, Figure 5). Nisoldipine treatment of the ischaemic heart did not significantly affect developed tension.

ATP breakdown

We studied the effect of nisoldipine on myocardial ATP content. In hearts with an initial preload of about 15 g, purine efflux was 13 ± 8 nmol min^{-1} g^{-1} weight. This increased to 128 ± 20 nmol min^{-1} g^{-1} due to ischaemia ($P=0.001$, Figure 5). Twenty nM nisoldipine reduced this efflux to 42 ± 12 nmol min^{-1} g^{-1}, which is still significantly different from the aerobic control ($P=0.05$, $P<0.001$ vs. non-treated). The hearts were freeze-clamped and the adenine nucleotides analysed. ATP content decreased by 49% due to ischaemia ($P=0.004$, Figure 6). With the relatively low dose of nisoldipine used, a non-significant decrease of 27% was found ($P=0.11$; $P=0.12$ vs. non-treated). Similar observations were made on adenylate energy charge; this ratio dropped by 18% ($P=0.001$) as a result of ischaemia (Figure 6), whereas this was only 6% with nisoldipine treatment, a non-significant decrease ($P=0.21$; $P=0.02$ vs. non-treated).

Perfusion with high calcium

When the Ca^{2+}-concentration in the perfusion medium was raised from 1.4 to 5.0 mM, apex displacement in the normoxic heart went up 1.6 fold ($P<0.001$, Figure 7). The negative inotropic effect of nisoldipine (100 nM) was less pronounced ($P=0.05$), both relatively and absolutely, than in hearts perfused with a low calcium concentration,

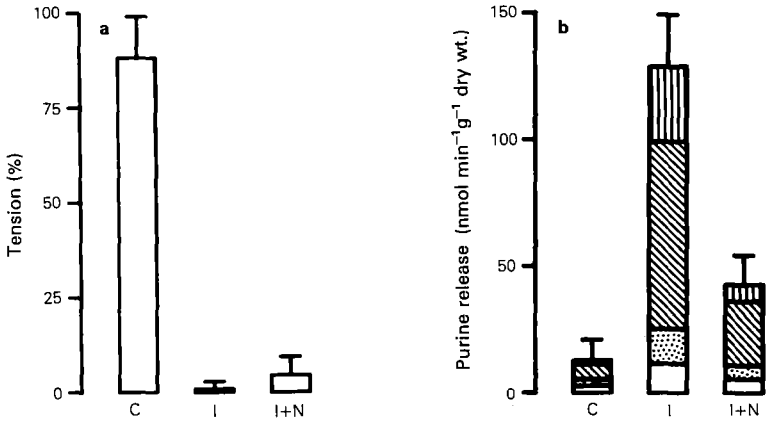

Figure 5 Effect of nisoldipine on developed tension (a) and purine efflux (b) from ischaemic myocardium. Rat hearts were perfused for 50 min under standard conditions, initially with a resting tension adjusted to about 15 g (control group: C). After 20 min of normoxic perfusion, 30 min of ischaemia (I) was induced in other hearts. Coronary flow was reduced by about 89%. Hearts in a third group were also made ischaemic, but they were perfused with medium containing 20 nM nisoldipine (I + N). This treatment began 15 min after the start of the perfusion, thus nisoldipine was already present before flow was reduced. Data, collected at the end of the perfusion, are presented. Developed tension at $t = 14$ min (22 ± 7 g) was taken as 100%. Mean values are given with vertical lines showing s.d. ($n = 4$). Striped section, adenosine; hatched section, inosine; stippled section, xanthine.

Despite the large differences in function of the normoxic heart, no significant differences in purine efflux were observed (Figure 8).

In contrast to the findings above, no significant differences in apex displacement were seen after 15 min of ischaemia, regardless of Ca^{2+}-concentration or the presence of nisoldipine (Figure 7). However, purine efflux from the ischaemic heart was increased by the higher Ca^{2+}-concentration ($P < 0.001$). Nisoldipine 100 nM suppressed purine production almost completely at low [Ca^{2+}], and reduced it by half at high [Ca^{2+}] (Figure 8).

Discussion

Nisoldipine showed a high affinity for silicon rubber tubing (Figure 1). We speculate that the isobutyl

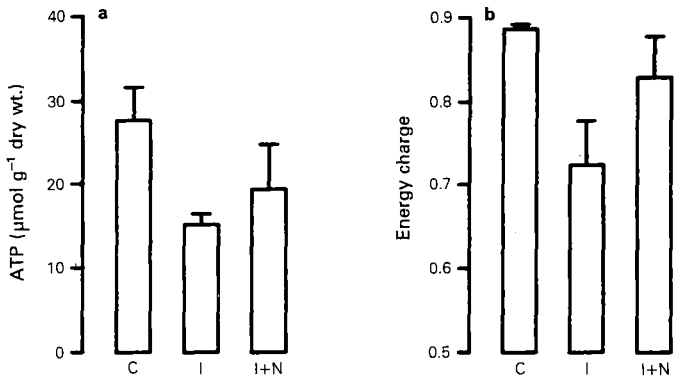

Figure 6 Protective effect of nisoldipine on ATP content (a) and adenylate energy charge (b) in ischaemic myocardium. Hearts were freeze-clamped after a control perfusion (C), or after a period of ischaemia (I). Where indicated by I + N, 20 nM nisoldipine was present. For other details, see legend to Figure 5.

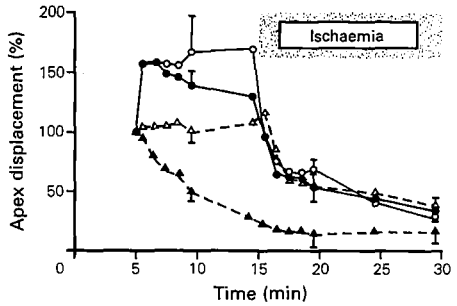

Figure 7 Effect of an increased Ca^{2+}-concentration in the perfusion medium on apex displacement (%). The value found 5 min after the start of the perfusion with standard medium was taken as 100%. Then perfusion took place with standard medium (1.4 mM $CaCl_2$) in the absence (△) or presence of 100 nM nisoldipine (▲), or medium containing 5.0 mM $CaCl_2$ in the absence (○) or presence of drug (●). Ischaemia was induced after 15 min normoxic perfusion by lowering the perfusion pressure from 9.6 to 2.3 kPa. Each point represents the mean and vertical lines s.d. ($n=4$).

group in nisoldipine is responsible for this property. Our finding could have implications for the use of nisoldipine in, for instance, heart surgery.

Kazda *et al.* (1980) described the powerful vasodilator properties of nisoldipine. They noted that negative inotropy in guinea-pig isolated hearts is only apparent when higher doses are used. From a comparison of Figures 2 and 4, it is also clear that in rat heart effects on the vasculature are seen with much lower doses of nisoldipine than those on the myocardium.

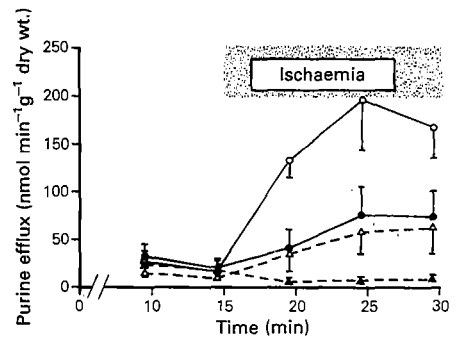

Figure 8 Effect of an increased Ca^{2+}-concentration in the perfusion medium on purine efflux (nmol min^{-1} g^{-1} dry weight). See legend to Figure 7. (△) Low Ca^{2+}, (▲) low Ca^{2+} + nisoldipine, (○) high Ca^{2+}, (●) high Ca^{2+} + nisoldipine.

In other studies, including those on calcium entry blocking agents, we used the efflux of purine nucleosides and oxypurines as a marker for myocardial ischaemia (see De Jong, 1979; De Jong *et al.*, 1982; 1984). Also in this study, ATP breakdown correlated well with the efflux of these purines (compare Figures 5 and 6). Nisoldipine proved to be an effective inhibitor of adenine nucleotide catabolism during ischaemia (Figures 3 and 5). About 50 nM reduced purine production by 50%. We estimate that the I_{50} is about 6 and 180 times higher for nifedipine and diltiazem, respectively.

It is likely that the protective effect of nisoldipine was caused by a reduction in contractile behaviour due to less Ca^{2+}-influx. Although at various doses of nisoldipine, apex displacement and developed tension in the ischaemic heart did not correlate with purine production (compare Figures 3 and 4, and see Figure 5), there is a strong correlation between normoxic function and ischaemic purine efflux (Figure 9). This occurs similarly with nifedipine and diltiazem (De Jong *et al.*, 1982; 1984). In addition, the sup-

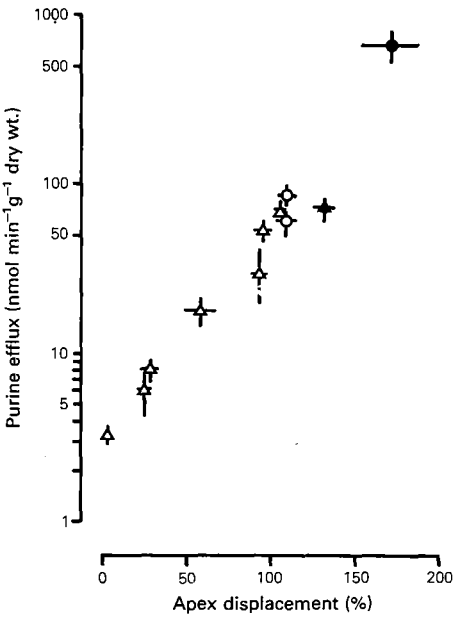

Figure 9 Correlation between normoxic function and ischaemic energy metabolism. Data were adapted from Figures 3, 4, 7 and 8. Open and closed symbols: 1.4 and 5.0 mM $CaCl_2$ in perfusion medium, respectively. Triangles: various concentrations of nisoldipine in perfusion medium. Each point shows mean and vertical and horizontal lines s.e. mean ($n=4$–7).

pression of ATP breakdown by nisoldipine is antagonized by increased Ca^{2+}-concentrations in the perfusion medium (Figure 8).

In a recent editorial, Drake-Holland & Noble (1983) rejected the idea that the protective effect of calcium antagonists is due to the negative inotropism and salvage of creatine phosphate and ATP. According to these authors, recovery from ischaemia is not correlated with preservation of high-energy phosphate scores. However, from our, and other, studies (e.g., Nayler, 1982), it seems clear that these drugs could prevent ATP breakdown under a number of conditions. Whether they increase recovery after an ischaemic insult is less obvious.

Pang & Sperelakis (1983) showed that nitrendipine, an analogue of nisoldipine, enters cardiac muscle cells and is accumulated, probably through binding to internal sites. Diltiazem not only inhibits the Ca^{2+}-influx, but also acts intracellularly (Saida & Van Breemen, 1983). It protects (partially) against ATP breakdown, when it is administered during ischaemia (De Jong *et al.*, 1984). Diltiazem, verapamil and nifedipine have no influence on beef heart phosphodiesterase (Daly *et al.*, 1983), but Norman *et al.* (1983) found that nisoldipine and four other dihydropyridine Ca^{2+}-entry blockers selectively inhibit a myocardial cyclic AMP phosphodiesterase. Therefore, the possibility cannot be excluded that Ca^{2+}-entry blockers, such as nisoldipine, could directly affect the enzymatic machinery, thereby conserving energy during myocardial ischaemia.

We greatly appreciate the encouragement by Prof. P.G. Hugenholtz and secretarial help of Mrs M.J. Kanters-Stam. We are indebted to Dr R. Gross (Bayer, Wuppertal, GFR) for his suggestions and gift of nisoldipine.

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Appendix 7

REDUCED GLYCOLYSIS BY NISOLDIPINE TREATMENT OF ISCHEMIC HEART

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Reduced Glycolysis by Nisoldipine Treatment of Ischemic Heart

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Summary: Calcium entry blockers seem useful for energy conservation in the ischemic heart. Their exact mechanism of action, however, remains uncertain. In this study we investigated the effect of 30 n*M* nisoldipine on carbohydrate metabolism in isolated rat heart perfused with glucose-containing medium. Nisoldipine increased flow 1.5-fold and reduced apex displacement 60%. We induced ischemia by lowering the perfusion pressure from 72 to 14 mm Hg, which resulted in a flow reduction in untreated hearts by 80%. Lactate production rose 16-fold, glucose utilization increased fourfold, and the heart glycogen content decreased by 32%. Nisoldipine treatment diminished ischemic lactate release by 77%. It decreased glucose utilization to normoxic levels and reduced glycogen breakdown to a value intermediate to the ischemic and normoxic ones. We conclude that nisoldipine reduces glycolysis in the ischemic heart. Consequently, it appears that the ATP-saving effect of nisoldipine during ischemia, reported elsewhere, is due to a lower energy demand rather than increased ATP production. **Key Words:** Calcium antagonist—Carbohydrate utilization—Glucose utilization—Myocardial ischemia—Nisoldipine.

Nisoldipine, a relatively new 1,4-dihydropyridine derivative, inhibits transmembrane calcium influx, which results in powerful vasodilatory effects. This calcium entry blocker therefore reduces afterload (1,2) and dilates the systemic vascular bed, even at extremely low doses (1–4). The positive effects on left ventricular systolic and diastolic performance (2) and suppression of life-threatening arrhythmias (5) indicate that nisoldipine could be a promising drug for the treatment of ischemic heart disease (3).

Hardly anything is known about the effects of nisoldipine on myocardial metabolism. Kazda et al. (1) concluded that it does not change myocardial lactate metabolism in the anesthetized dog. Takahashi and Kako (6) found that the ischemia-induced increase in phospholipid breakdown of cardiac sarcolemma is suppressed by the drug. We showed in earlier experiments (7) that nisoldipine preserves, dose dependently, adenine nucleotides in ischemic rat heart. In this study, we rule out the possibility that this is due to an increase of (anaerobic) glycolysis.

MATERIALS AND METHODS

Heart perfusion

Male Wistar rats (299 ± 33 g; mean ± SD; n = 26), with free access to food and water, were anesthetized intraperitoneally with pentobarbital. Retrograde perfusion of hearts was carried out at 37°C as described previously (7). A modified Tyrode buffer, containing 10 m*M* D-[2-^{3}H]glucose (0.02 Ci/mol), was used. After equilibration with carbogen, the pH was 7.4. Hearts were paced at 300 beats/min. Ischemia was induced for 15 min by lowering the perfusion pressure from 72 to 14 mm Hg.

Chemicals

D-[2-^{3}H]Glucose (17.0 Ci/mmol) was supplied by Amersham International plc (Amersham, U.K.). A 2.5-m*M* solution of nisoldipine (Bay k 5552; isobutyl methyl 1,4 - dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylate; Bayer, Wuppertal, G.F.R.) in absolute alcohol was diluted by addition of perfusion buffer under vigorous stirring. White light was excluded from the drug to prevent photolytic degradation. All chemicals were of analytical grade. Water was purified by ion-exchange and reverse osmosis (Millipore, Bedford, MA, U.S.A.).

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Myocardial function

Apex displacement was measured as described previously (8). The value, found 5 min after the start of the perfusion, was taken as 100%.

Coronary flow

Perfusate was collected in a graduated cylinder during 1-min periods at the times indicated.

Lactate assay

Perfusate samples were mixed with NaN_3 (0.03% final concentration) and stored at −20°C until analysis. L-Lactate was assayed enzymatically in these samples according to Apstein et al. (9) with the AutoAnalyzer II (Technicon, Tarrytown, NY, U.S.A.).

Measurement of glucose utilization

Utilization of exogenous glucose was estimated by measuring the rate of ^3H_2O production from [2-^{3}H]-glucose in the perfusate samples (10).

Assay of glycogen

At the end of the perfusions, hearts were quickly frozen with Wollenberger clamps cooled in liquid nitrogen. Tissue was pulverized in a deep-cooled mortar, transferred to a test tube, and denatured in a boiling-water bath. The content of the tubes was homogenized and stored at −20°C until analysis. Glycogen was converted to glucose according to Huijing (11). The glucose oxidase/peroxidase system (12), with diammonium 2,2′-azinobis(3-ethyl-6-benzothiazolinesulphonate) as the chromogen (13), was used to assay glucose.

Statistical analysis

One-way analysis of variance was employed. Further evaluations were made using Bonferroni's test for multiple comparisons (14). A p value of < 0.05 (two-tailed) was considered statistically significant. Results are expressed as means ± SD.

RESULTS

Coronary flow

Figure 1 shows that 30 n*M* nisoldipine increased flow in normoxic hearts 1.5-fold ($p < 0.001$). Coronary flow dropped when the perfusion pressure was reduced from 71.5 ± 1.1 to 14.4 ± 1.4 mm Hg. Flow in ischemic hearts was 80% lower than that in normoxic control hearts after 30 min of perfusion ($p < 0.001$). Treatment with nisoldipine was without influence on coronary flow during ischemia (Fig. 1).

Myocardial function

We measured apex displacement routinely as a (relative) indicator of myocardial function. Nisoldipine, at a dose of 30 n*M*, decreased apex displacement in the normoxic heart by 60% within 10 min ($p < 0.001$; Fig. 2). At the end of the ischemic period, apex displacement in treated and untreated hearts was ~30% of normoxic controls ($p < 0.001$).

Lactate

Nisoldipine did not affect myocardial lactate production in normoxic hearts. Myocardial ischemia increased lactate release 16 times ($p < 0.001$; Fig. 3). Nisoldipine reduced this release by 77% ($p < 0.001$, NS vs. normoxic control).

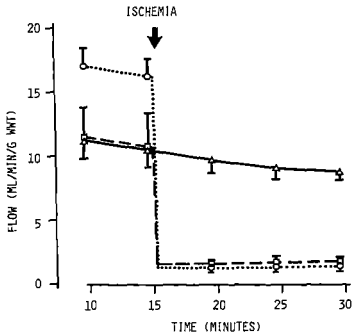

FIG. 1. Coronary flow in the isolated rat heart. Symbols: normoxic control hearts (triangle; solid line); hearts in which flow was reduced after 15 min of perfusion (square; broken line); hearts perfused with 30 n*M* nisoldipine from t = 5 min, underperfused from t = 15 min (circle; dotted line). Means ± SD (n = 8–9).

Glucose utilization

Treatment with nisoldipine did not significantly alter glucose utilization in normoxic hearts. As illustrated in Figure 4, ischemia increased this fourfold ($p < 0.001$). Nisoldipine treatment prevented this increase completely ($p < 0.001$, NS vs. normoxic control).

Glycogen

Glycogen content in hearts freeze-clamped after an ischemic period of 15 min was significantly lower than that in control hearts (2.1 ± 0.2 vs. 3.1 ± 0.5 mg/g wet weight, respectively; $p < 0.01$, n = 5). Hearts treated with nisoldipine showed an inter-

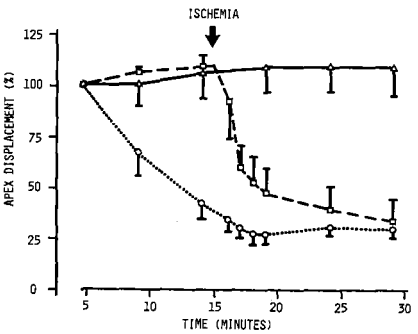

FIG. 2. Effects of nisoldipine and ischemia on myocardial apex displacement. Symbols as in Figure 1. The apex displacement observed at t = 5 min (before nisoldipine was given) was taken as 100%. Means ± SD (n = 6–8).

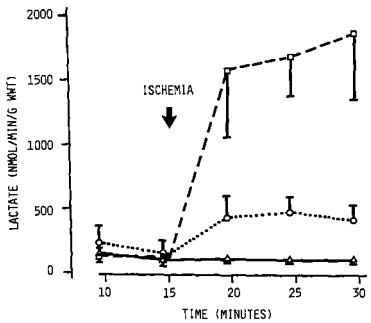

FIG. 3. Nisoldipine reduces ischemic lactate release. Symbols as in Figure 1. Means ± SD (n = 7–9).

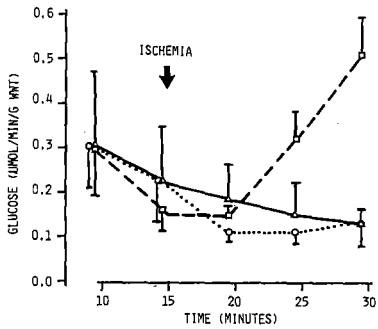

FIG. 4. Nisoldipine reduces glucose utilization in ischemic hearts. Symbols as in Figure 1. Glucose units, passing through the glycolytic pathway, were calculated from ^3H_2O produced. Means ± SD (n = 6–7).

mediate glycogen content (2.6 ± 0.2 mg/g wet weight, n = 5) that was not significantly different from either the normoxic or ischemic values.

DISCUSSION

In heart there is a delicate equilibrium between ATP production (mainly by oxidative phosphorylation) and ATP consumption (mainly by contractile proteins). During ischemia, ATP production is hampered owing to lack of oxygen. ATP catabolites can then be observed in the heart and its effluent. Under these conditions, calcium entry blockers have an ATP-sparing effect. This has been documented for verapamil (15–20), nifedipine (8,16,19), diltiazem (21,22), and, recently, also for bepridil (23) and nisoldipine (7). The reduced formation of ATP catabolites in the presence of calcium entry blocker could be due to either improved energy production or decreased energy utilization. We used the isolated heart, perfused with a glucose-containing medium, to test whether or not nisoldipine could increase (anaerobic) glycolysis. In this preparation the vasodilatory and negative inotropic properties of the drug, given during normoxia, were confirmed (Figs. 1 and 2). Nisoldipine did not influence lactate production or glucose utilization under these conditions. This is in agreement with the observations of Kazda et al. (1) in normoxic dog heart. After 15 min of ischemia, however, lactate production and glycolytic flux were drastically reduced by nisoldipine (Figs. 3 and 4), and myocardial glycogen breakdown was prevented. This indicates that myocardial glycolysis is not stimulated by the drug. It seems unlikely that myocardial lipid stores have provided substrates for energy utilization during the ischemic period; therefore, energy could only have been generated by carbohydrate breakdown, which is reduced by the drug. Clearly, the negative inotropy caused by nisoldipine in the well-oxygenated heart lowers its oxygen demands and the critical level of oxygen delivery. In other words, when nisoldipine is given, the critical level required to support the metabolic ATP demands of the tissue is lowered. Recently Brügmann et al. (24) demonstrated that nisoldipine has an efficacious anti-ischemic effect in patients; its mechanism may be similar in this setting.

In summary, nisoldipine reduces lactate production, glucose utilization, and glycogen breakdown in ischemic heart, ruling out the possibility that its ATP-saving effect is through increased glycolysis.

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Appendix 8

PROTECTION BY BEPRIDIL AGAINST MYOCARDIAL ATP-CATABOLISM IS PROBABLY DUE TO NEGATIVE INOTROPY

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Protection by Bepridil Against Myocardial ATP-Catabolism Is Probably Due to Negative Inotropy

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Summary: The protective effect of calcium antagonists on ischemic heart has been attributed to decreased energy expenditure. We administered one of the newer calcium antagonists, DL-bepridil (0.1–10 μ*M*), to Langendorff rat hearts 10 or 15 min before ischemia (flow reduction ~80%). Vasodilation during normoxia was already observed with 0.3 μ*M* DL-bepridil (flow increase 34%, $p < 0.005$). This concentration decreased normoxic contractility and ischemic purine release, a marker for ATP breakdown. In the absence of bepridil, purine release of hearts that were made ischemic was 8.5-fold higher than that of normoxic control hearts. With 1 μ*M* bepridil, the ischemic purine efflux was suppressed by 55% ($p < 0.05$), with negative inotropy ($p > 0.05$) during normoxia. At 3 and 10 μ*M*, bepridil decreased normoxic contractility by 40 and 75%, respectively ($p < 0.001$), concomitant with a decrease in ischemic purine release by 80 and 76%, respectively ($p < 0.01$). At the end of ischemia, myocardial ATP and creatine phosphate had decreased by 22 and 55%, respectively ($p < 0.05$), and ADP, AMP, and creatine had increased 1.5–3.5-fold ($p < 0.05$). Bepridil (3 μ*M*) normalized the adenine nucleotide values; creatine and creatine phosphate approached control levels. The dose-dependent protection of the ischemic heart by bepridil appears to arise from its negative inotropic action during normoxia. **Key Words:** Calcium antagonist—Myocardial ischemia—Bepridil—High-energy phosphates—Negative inotropy.

In ischemic heart, calcium antagonists exert an ATP-sparing effect which is of potential benefit for the functioning of the heart. This effect has been described for verapamil, nifedipine, nisoldipine, diltiazem, and bepridil (for review, see ref. 1). It seems likely that the protection originates from the negative inotropic properties of these drugs, which all inhibit the cytosolic Ca^{2+} accumulation following flow reduction. Calcium antagonists act by blocking slow calcium channels. Other (intracellular) actions of drugs like bepridil cannot be excluded. Bepridil accumulates in ventricular myocytes; it binds to, e.g., actin and calmodulin (2,3).

The antianginal and antiarrhythmic properties of bepridil are well documented (4–10), although the mechanism of action is not fully understood. De Leiris and co-workers (11) pretreated rats with bepridil intravenously (i.v.). They observed that bepridil did not significantly affect functional variables in isolated working hearts before ischemia was induced, but the postischemic function of the treated hearts improved. Nucleotide and carbohydrate metabolism benefited in a similar way. On the other hand, bepridil, applied i.v. to a blood donor cat, did not provide any protection of the isolated blood-perfused cat heart after normothermic global ischemia and reperfusion (12); treatment of the isolated preparation with bepridil did not prevent tissue accumulation of calcium or loss of tissue potassium and ATP upon ischemia and reperfusion (12).

We studied the effect of bepridil on coronary flow, functional variables, and myocardial ATP metabolism in the isolated perfused rat heart. The drug prevented ATP breakdown dose-dependently during ischemia followed by reperfusion, probably through its negative inotropic action before ischemia.

MATERIALS AND METHODS

Chemicals

All chemicals were of the highest grade available. Water was purified with the Millipore-Ro4/Milli-Q

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System (Millipore, Bedford, MA, U.S.A.). An aqueous stock solution (3 or 10 m*M*) was made of DL-bepridil (β-[(2-methylpropoxy)methyl]-*N*-phenyl-*N*-(phenylmethyl)-1-pyrrolidineethanamine monohydrochloride monohydrate; Organon, Oss, The Netherlands), and kept in the dark. Dilutions were made by adding perfusion buffer under vigorous stirring. The glass parts of the perfusion apparatus were brown to prevent photolysis of bepridil.

Perfusion of hearts

Male Wistar rats (253 ± 54 g, n = 68), with free access to food and water, were anesthetized intraperitoneally (i.p.) with 30 mg sodium pentobarbital (Abbott, Saint-Remy sur Avre, France). Hearts were rapidly removed and cooled in ice-cold NaCl, 154 m*M*. Retrograde perfusion of the aorta according to Langendorff was immediately started with a modified Tyrode's buffer, pH 7.4. The buffer consisted (in m*M*) of NaCl 128, KCl 4.7, $CaCl_2$ 1.4, $NaHCO_3$ 20, NaH_2PO_4 0.42, $MgCl_2$ 1.0, and D-glucose 10. The mixture was passed through a 0.45-μm filter and equilibrated with 95% O_2/5% CO_2. Atria were carefully removed, and a His-bundle block was made by a cut in the septum just below the atrioventricular-node or by coagulation of the His-bundle with a Cauterette I disposable coagulator (Concept, Clearwater, FL, U.S.A.). Pacing frequency was 300 beats/min (4 V, 2 ms; Grass Model 59C pacemaker, Quincy, MA, U.S.A.). A thin stainless-steel wire, inserted into the right ventricular wall, was used as one electrode; the stainless-steel perfusion canula was used as the other. The perfusion temperature was adjusted to 37°C with a thermocouple-regulated electric heater just prior to the perfusion canula. This was fitted with an A-F6 temperature probe, connected to a DU-3 monitor (Ellab, Copenhagen, Denmark). Ischemia was induced by lowering the perfusion pressure, measured with a Statham P23Db transducer (Hato Rey, PR, U.S.A.), from 9.6 to 2.3 kPa for 15 min, followed by 20 min of reperfusion at 9.6 kPa (see Fig. 1, protocol A). In another series of experiments, 25 min of ischemia was induced at 2.0 kPa (see Fig. 1, protocol B).

Myocardial function

In the dose–response experiments, developed tension was monitored with a Konigsberg F5-2 pseudoisometric force-transducer (Hugo Sachs, March/Freiburg, F.R.G.) connected to the apex of the heart. A resting tension of 10 g was applied. Five minutes after the start of the perfusion, resting tension was readjusted if necessary.

In the other experiments, left ventricular developed pressure was measured with a latex balloon (filled with 150 μl 154 m*M* NaCl), connected to an Ailtech ms 20 transducer (Eaton, City of Industry, CA, U.S.A.) by 20 cm of polyethylene tubing. A diastolic pressure of 1.3 kPa was applied and readjusted 5 min after the start of the perfusion if necessary.

Coronary flow

Perfusate was collected in a graduated cylinder over 1- or 5-min periods.

Assay of purines in myocardial effluent

After collection on ice, the perfusate samples were kept below 5°C in an autosampler (WISP 710B; Waters, Milford, MA, U.S.A.), connected to a cooling bath (Ministat; Huber, Offenburg-Elgersweier, F.R.G.). Adenosine, inosine, hypoxanthine, xanthine, and urate were

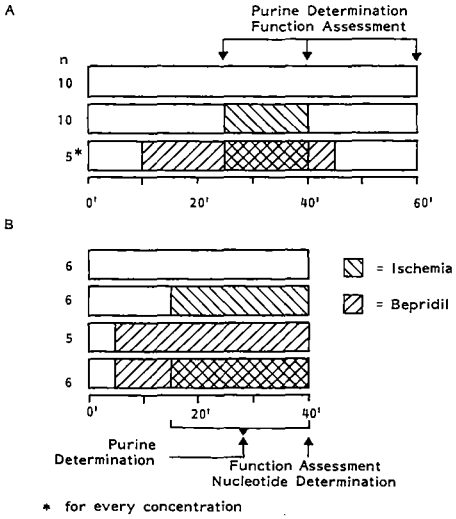

FIG. 1. Protocol A was used in the dose–response experiments. Developed tension was measured at the points indicated, and 1-min samples were then taken for purine analysis. In protocol B, perfusate was collected from 15 to 40 min for purine analysis. At the end of the experiment, developed pressure was measured and the heart was freeze-clamped for high-energy phosphate determination; n = number of experiments.

determined by a modification of our high-performance liquid chromatography (HPLC)-method (13). In brief, 50 μl of perfusate were injected onto a C_{18} μBondapak column (Waters) and eluted isocratically with a Model M-45 pump (Waters) at a rate of 1.0 ml/min with a mixture of methanol (100 ml) and KH_2PO_4 (10 g/L, 1,000 ml). The buffer was filtered (0.45 μm) and degassed. Peaks were detected at 254 nm by a Model 440 absorbance detector (Waters). Peaks were analyzed and compared with external standards using a Waters data system 840.

Analysis of myocardial adenine nucleotides

A series of ischemic hearts was immediately freeze-clamped after ischemia between alumina tongs, and the hearts were cooled in liquid nitrogen. The frozen hearts were then ground under liquid nitrogen. About one third was used for dry weight determination; the other part was thoroughly mixed with 3.0 ml 0.4 *M* $HClO_4$ at liquid nitrogen temperature, thawed, and centrifuged. The supernatant fluid was neutralized on ice with 2.0 *M* KOH/1.0 *M* K_2CO_3. The high-energy phosphates in the heart extracts were analyzed by HPLC, modified from Harmsen and co-workers (14). The HPLC system consisted of two pumps (Model 6000-A; Waters), a Partisil-10-SAX column (0.4 × 25 cm; Whatman, Maidstone, England) and two absorbance dectectors (Waters Model 440 and Model 441) set at 254 and 214 nm, respectively. Buffers consisted of 16 m*M* H_3PO_4, adjusted to pH 2.8 with KOH (buffer A) and 750 m*M* $NH_4H_2PO_4$, adjusted to pH 4.5 (buffer B). They were filtered through a 0.45-μm filter and degassed. Extracts were kept at 0–5°C in a Waters

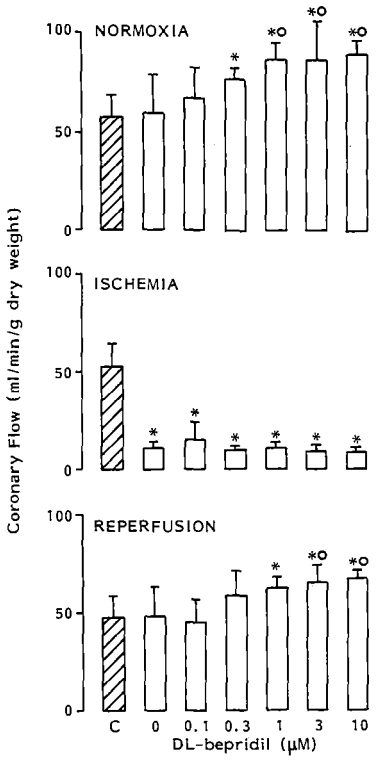

FIG. 2. Vasodilation by bepridil in normoxic and reperfused hearts. Coronary flow data were obtained after 25 min of normoxic perfusion (at 9.6 kPa), after 15 min of ischemia (perfusion pressure lowered to 2.3 kPa), and after 20 min of reperfusion at 9.6 kPa. Treatment with bepridil started 15 min before ischemia and was stopped after 5 min of reperfusion. Bars show means with SD; normoxic controls (C), and ischemic controls (0) n = 10; otherwise n = 5; * and ° indicate $p < 0.05$ as compared with C and 0 μM, resp.

autosampler. Five minutes after injection of a sample, which was eluted with buffer A at a flow rate of 2.0 ml/min, a gradient was started. Elution with buffer B was increased by 5%/min until 100%. Peaks were analyzed as described above. The adenylate energy charge, (ATP + 0.5 ADP)/(ATP + ADP + AMP), was calculated from the adenine nucleotides.

Statistical analysis

Statistical analysis was performed with Student's *t* test or with two-way analysis of variance; $p \geq 0.05$ was considered nonsignificant.

RESULTS

Coronary flow

Figure 2 shows that bepridil, in concentrations of ≥ 0.3 μM, induced significant vasodilation. Coronary flow increased up to 50% during normoxic perfusion. When the perfusion pressure was reduced from 9.6 kPa to 2.3 kPa, coronary flow dropped by 80% of control flow. Bepridil did not cause significant differences in flow during ischemia. When the perfusion pressure was restored, the vasodilatory properties of bepridil were again apparent ($p < 0.005$).

Myocardial function

In the dose–response experiments, myocardial function was monitored with a force-transducer. Figure 3 shows that the lower concentrations of bepridil did not affect the developed tension or the resting tension during normoxia. At concentrations of 3 and 10 μM, bepridil caused a negative inotropic effect ($p < 0.001$) and a concomitant increase in

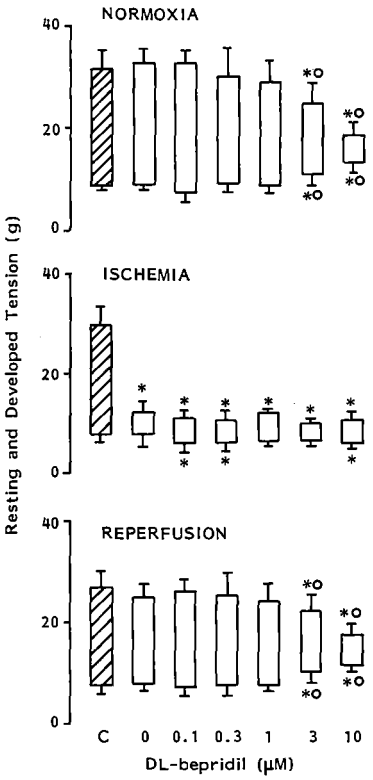

FIG. 3. Effect of bepridil on developed tension and resting (diastolic) tension of isolated perfused hearts. Developed tension decreased dose-dependently, and resting tension increased dose dependently during normoxia and reperfusion. Hatched columns depict nonischemic controls (C); * and ° indicate $p < 0.05$ versus C and 0 μM, respectively. Other details are given in legend to Fig. 2.

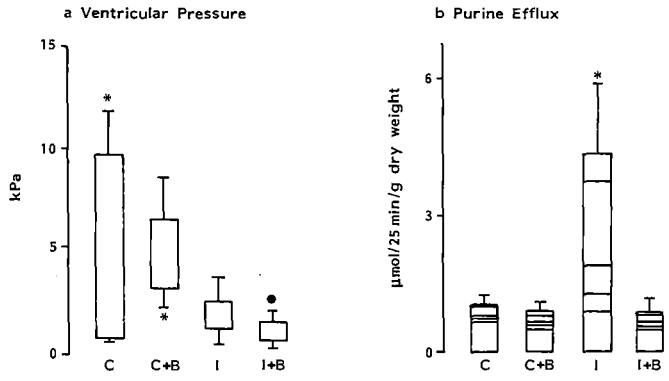

FIG. 4. Effect of bepridil on ventricular pressure and purine efflux. Hearts were perfused for 40 min under control conditions (C), or with 3 μM DL-bepridil (C + B), initially with a diastolic pressure of ~1.3 kPa. Bepridil infusion was begun 5 min after canulation. Ischemia (reduction in flow by 85% for 25 min) was induced after 15 min of normoxic preperfusion with or without bepridil (I + B and I, respectively). During ischemia, perfusate was collected and purine efflux was measured **(panel b).** From top to bottom, the division of the bars represents the quantities of adenosine, inosine, hypoxanthine, xanthine, and urate. Mean values are given with vertical lines showing SD for the sum of the purines (n = 5); * indicates $p < 0.05$ versus C + B; * indicates $p < 0.005$ versus all other conditions.

resting tension. Figure 4a shows that similar effects of bepridil were seen during normoxia when function was measured with an intraventricular balloon: developed pressure decreased and diastolic pressure increased ($p < 0.01$).

Ischemia decreased developed tension by 80% (Fig. 3) and developed pressure by 85% (Fig. 4a). No significant differences in ischemic function were observed with the various concentrations of bepridil used. In its absence, developed tension recovered to 55% of the preischemic value after 5 min of reperfusion; recovery with bepridil was 70–100% of the preischemic values (data not shown). After 20 min of reperfusion, of which the last 15 min were in the absence of bepridil, treated hearts did not show a better recovery than untreated hearts (Fig. 3). In control hearts, developed tension decreased by 15% over the entire experimental period. During reperfusion, the lower developed tension and higher resting tension were again apparent in hearts treated with the higher concentrations of bepridil (3 and 10 μM), when compared with normoxic controls ($p < 0.01$, Fig. 3). This effect remained even after 15 min of reperfusion in the absence of bepridil.

Purine efflux

In the experiments in which developed pressure was measured, the release of adenosine, inosine, hypoxanthine, xanthine, and urate was determined over the entire ischemic period. During ischemia, purine efflux increased significantly from 1 to 4 µmol/25 min/g dry weight. Bepridil (3 μM) reduced this ischemic purine release to control levels (Fig. 4b).

In the experiments in which developed tension was measured, bepridil showed a tendency to increase normoxic purine release (Fig. 5). The release of adenosine, inosine, hypoxanthine, and xanthine at the end of the normoxic period was about 10 nmol/min/g dry weight. Ischemia gave an increased release of these purines (to ~65 nmol/min/g dry weight). Bepridil suppressed the ischemic purine efflux in a dose-dependent way (Fig. 5). This reduction in purine release was significant at 1, 3, and 10 μM bepridil. During reperfusion, purine release declined to similar values in all experiments, with the exception of hearts perfused with 10 μM bepridil, which had an increased purine release. Bepridil concentrations of 30 and 100 μM were also tried, but these amounts of bepridil precipitated in the slightly alkaline perfusion buffer. Extensive gassing of the buffer with carbogen failed to dissolve the precipitate.

Adenine nucleotides

Figure 6a and c shows that ischemia caused a decrease in ATP-content (22%, $p < 0.005$) and adenylate energy charge (10%, $p < 0.05$), concomitant with 1.5- and 3.5-fold increase ($p < 0.05$) in ADP and AMP, respectively. Comparison of Figs. 4b and 6a makes clear that the loss in adenine nucleotides was reflected by the purine efflux due to ischemia. Bepridil (3 μM) abolished this loss completely.

Creatine phosphate and creatine

Figure 6b shows that ischemia decreased creatine phosphate content by 54% ($p < 0.001$) and increased creatine content by 47% ($p < 0.001$). With 3 μM bepridil, these contents approached control values.

DISCUSSION

Calcium antagonists have proven effective agents in the treatment of angina pectoris (15). Their use as an adjunct to cardioplegic solutions during heart

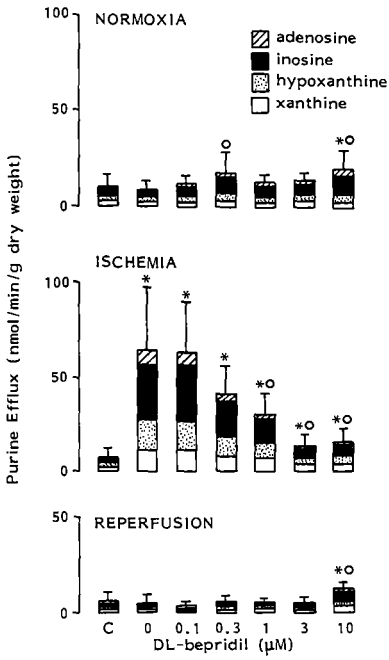

FIG. 5. Reduction of purine efflux from ischemic heart by bepridil. The drug suppressed the production of adenosine, inosine, hypoxanthine, and xanthine dose dependently; * and ° indicate $p < 0.05$ versus C and 0 μM, respectively. Other details are given in legend to Fig. 2.

surgery is promising (1). Our finding that concentrations of bepridil exceeding 10 μM could cause precipitation should be kept in mind if bepridil is to be used in cardioplegic solutions. Although calcium antagonists have several properties in common, it is more and more clear that their pharmacological profiles are different, as can be expected from such a structurally heterogenous group of drugs. Some of them, including bepridil, have beneficial effects on electrophysiological disturbances (8,9,16–19). All calcium entry blockers are more or less potent vasodilators (4,20–25). Moreover, they share negative inotropic properties; in isolated heart, however this cardiodepressive effect is always observed at higher doses than are necessary to induce vasodilation. Depending on the experimental approach and the drug studied, this difference in dosage varies between 3 and 100 times (cf. Figs. 2 and 3; refs. 20,26). Large differences in negative inotropic potency are observed for various Ca antagonists under different experimental conditions. In our study, bepridil depressed contractility dose-dependently during normoxia. This has also been described for other preparations (20,27,28). In contrast, in isolated working hearts from bepridil-pretreated rats, differences in hemodynamics, heart rate, and flow data during normoxia have not been observed (11). In in vivo and clinical studies, no or minor hemodynamic effects have been reported (7,10,25,29). In our study, bepridil, in concentrations which were clearly negative inotropic, increased resting tension or diastolic pressure before and after ischemia (Figs. 3 and 4a). Similarly, an i.v. infusion of a relatively high amount of bepridil in patients induces an elevation in left ventricular end-diastolic pressure (10,30). Severe ischemia in the isolated perfused heart is often accompanied by an increase in resting tension. Because only minor increases in ATP-catabolites (purines) and no significant changes in high-energy phosphate contents were noted (see Figs. 4–6), it seems unlikely that higher doses of bepridil cause ischemia in the oxygenated heart. Nevertheless, in isolated mitochondria under normoxic conditions bepridil diminishes ATP production (27).

In the isolated working heart, bepridil pretreatment does not affect normoxic coronary flow and function (11). After ischemia and subsequent reperfusion, however, adenine nucleotides and creatine phosphate are significantly higher in isolated hearts from bepridil-pretreated rats than in those from control rats (11). In contrast, Fuchs and colleagues (27) reported a dose-dependent decrease in aortic flow and increase in coronary flow in isolated working rat heart during normoxia, but aortic flow was not restored after 30-min reperfusion. With bepridil, Watts (31) did not observe an improvement in contractile function or an increase in high-energy phosphate stores during reperfusion. These apparent discrepancies are presumably due to differences in both the experimental models and drug concentrations used. We did not study ATP-restoration after reperfusion, but we did observe that bepridil decreased ATP catabolism during ischemia.

Administered as a single oral dose, the half-life of bepridil is 2 days, which is fivefold slower than that of diltiazem, nifedipine, and verapamil (32). Bepridil is very slowly metabolized in the liver, probably because it is strongly bound to plasma protein (32,33) resulting in a minute free fraction (32). Hearts treated with bepridil in concentrations that cause negative inotropy before ischemia recover better to preischemic contractile values than do untreated hearts (Fig. 3). Function remains less than that in hearts without bepridil treatment, however. Bepridil is highly lipophilic (32) and has a high affinity for plasma proteins (32,33). The persistent negative inotropy suggests therefore that bepridil remains bound to the heart in the absence of proteins in the perfusion buffer.

Whether bepridil can also effectively protect

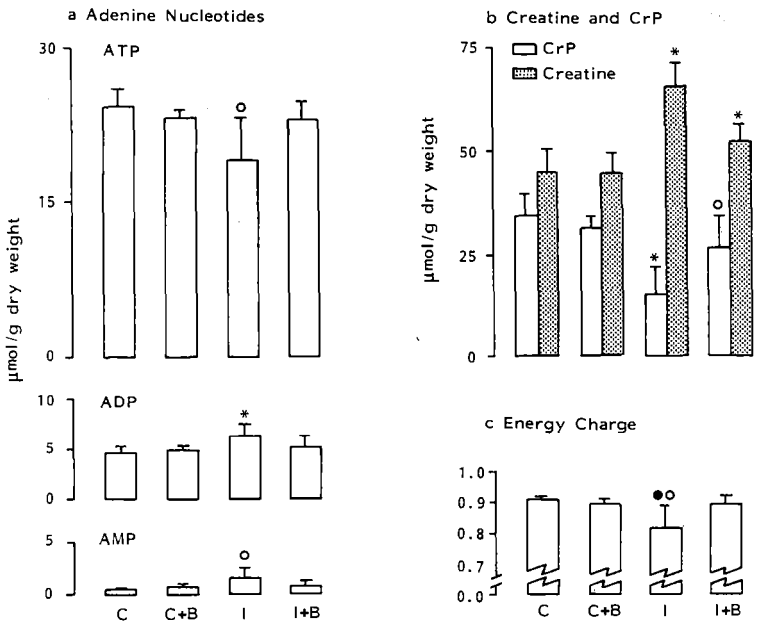

FIG. 6. Less breakdown of high-energy phosphates in ischemic hearts, treated with 3 µ*M* DL-bepridil. After 25 min of ischemia, hearts were freeze-clamped and analyzed for (a) adenine nucleotides, (b) creatine and creatine phosphate (CrP), and (c) adenylate energy charge. C, control; B, bepridil; I, ischemia, n = 5–6. Additional details are given in legend to Fig. 3; °, *, and * indicate $p < 0.05$ versus C, C + B, and all other conditions, respectively.

against ATP depletion, if applied after the onset of ischemia remains to be studied. Nisoldipine failed completely to protect against ATP depletion (34); diltiazem afforded some protection (22).

We conclude that bepridil proved an effective drug in preventing the ischemic breakdown of high-energy phosphates, when applied before ischemia, probably through its negative inotropic action before ischemia.

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Appendix 9

CAPTOPRIL RESTORES ANGIOTENSIN I INDUCED CORONARY FLOW REDUCTION IN ISOLATED RAT HEART BUT HAS NO EFFECT ON CONTRACTILITY OR ENERGY METABOLISM

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Captopril restores angiotensin I induced coronary flow reduction in isolated rat heart but has no effect on contractility or energy metabolism

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KEY WORDS: captopril, angiotensin I, angiotensin II, adenosine triphosphate, myocardial ischemia.

Abstract

Vasoconstriction, caused by activation of the renin-angiotensin system contributes to myocardial damage during ischemia; the converting enzyme inhibitor captopril suppresses angiotensin formation. We investigated the effects of angiotensin I, angiotensin II, and captopril on coronary flow, function and energy metabolism before, during and after ischemia in 59 Langendorff rat hearts.

Angiotensin I (100 nM) and II (10 nM) caused reduction of coronary flow at constant perfusion pressure by 31% ($p<0.005$) and 27% ($p<0.05$), respectively. During reperfusion these compounds decreased flow by 30% ($p<0.005$) and 12% ($p=0.40$), respectively. Captopril (0.4 mM) inhibited vasoconstriction caused by angiotensin I, but not by angiotensin II. The drug itself increased flow by 42% ($p<0.005$).

We did not detect significant effects of angiotensin I, angiotensin II, or captopril on cardiac function or high-energy phosphate content. Developed tension in captopril-treated hearts tended to recover faster from ischemia than controls, with concomitant lower ATP catabolism.

We conclude that the isolated rat heart contains an active angiotensin-converting enzyme. Captopril, used at a concentration of 0.4 mM, blocks its activity. The drug has no significant effects on myocardial function or energy metabolism but increases coronary flow during normoxic perfusion.

Introduction

Conversion of angiotensin I to angiotensin II takes place in heart tissue of several species[1,2,3,4]. Angiotensin II acts on its myocardial receptors to increase rate and force of contractions[5,6] and on its receptors located on sympathetic nerve terminals to facilitate neurotransmission[7]. The increased calcium current[8] in addition to noradrenergic stimulation mediates the positive inotropic effect of angiotensin[3,9]. In ischemic myocardium, positive inotropy may be deleterious.

Captopril, an angiotensin converting enzyme inhibitor, blocks the positive inotropic effect of angiotensin I in isolated cat heart[1]. In isolated rat heart, captopril increases ventricular fibrillation threshold and loss of purines from ATP[10,11]. In patients with ischemic heart disease and congestive heart failure, enhancement of myocardial

performance after captopril[12,13] appears to be dependent on reduction of angiotensin-mediated ventricular pre- and/or afterload[13,14]. The drug does not affect myocardial oxygen demand or consumption but may lower them in patients with heart failure[13,15]. This reflects enhanced cardiac output without increased oxygen demand. In healthy dogs, captopril does not seem to have a direct cardiac effect[16]. After acute canine left anterior coronary artery occlusion, blockade of the renin-angiotensin system lowers systemic blood pressure and improves cardiac output[17].

Relatively little is known about the local cardiac effects of the angiotensin system. We therefore investigated the effect of angiotensin I and II, as well as the converting enzyme inhibitor captopril, on coronary flow, function and energy metabolism in the isolated rat heart.

Materials and Methods

CHEMICALS

All chemicals were of the highest grade available. Water was purified with the Millipore-Ro4/Milli-Q System (Millipore, Bedford, MA, USA). Perfusion buffer with captopril (Squibb, Rijswijk, NL) was daily prepared by solving 100 mg inhibitor/liter of Tyrode, modified as described before[18]. For use as an infusion fluid, 133 mg captopril was prepared in 100 ml Tyrode buffer.

Aqueous stock solutions (100 μM) were made daily of angiotensin I and II (Sigma, St. Louis, MO, USA). Before the experiments the stock solutions were mixed with the perfusate while stirring, to final concentrations of 100 nM and 10 nM, respectively. In our model, these final concentrations showed equipotent levels of vasoconstriction.

PERFUSION OF THE HEARTS

Male Wistar rats (n = 59), were anesthetized i.p. with 30 mg sodium pentobarbital (Abbot, Saint-Remy sur Avre, F). Hearts were removed and cooled in ice-cold saline. Perfusion of the aorta according to Langendorff was started[18]. Hearts were paced at 300 beats $\cdot$ min^{-1} using a stainless steel wire inserted into the right ventricular wall, and the perfusion canula as electrodes. Ischemia was induced by lowering the perfusion pressure, measured with a Statham P23Db transducer (Hato Rey, PR, USA), from 9.6 to 1.9 kPa for 20 min. Ischemia was followed by 15 min of reperfusion at 9.6 kPa (for the perfusion protocol, see Fig. 1A). Alternatively, hearts were perfused according to the protocol shown in Fig. 1B. In these experiments captopril was infused into the perfusion line to exclude the formation of an inactive dimer of the drug[19]. Infusion rate was 600, 60 or 840 μl $\cdot$ min^{-1} during normoxic perfusion, (aggrevated) ischemia, and reperfusion, respectively, resulting in a captopril concentration of about 0.4 mM.

CORONARY FLOW AND MYOCARDIAL FUNCTION

Coronary flow was monitored with an electromagnetic flow meter (Transflow 601; Skalar, Delft, NL). A tension signal was measured with a Konigsberg F5-2 force-transducer (Hugo Sachs, March/Freiburg, FRG) connected to the apex of the heart. A resting tension of 10 or 5 g (protocol A and B, respectively) was applied and adjusted 5 min before the first measurement. The developed tension was calculated from the difference between peak systolic tension and resting tension.

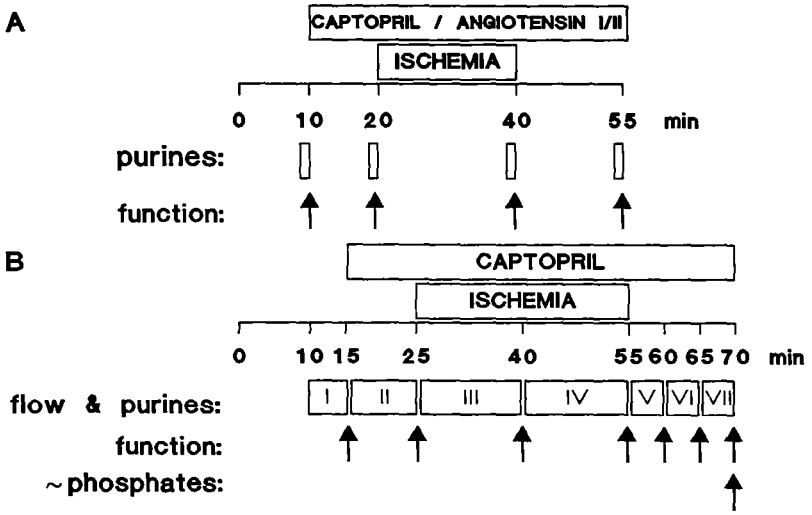

Figure 1. Perfusion protocols used in the experiments. In protocol A angiotensin I, angiotensin II, captopril, or a combination of drug with angiotensin I or II was added to the perfusion buffer. Developed tension was measured at the points indicated with arrows. Samples from the coronary effluent for analysis of purines were taken during the minute preceding these time points. In protocol B, captopril was infused into the perfusion line. In those experiments developed tension and high-energy phosphates were measured at the points indicated. Coronary effluent was collected over periods I to VII, and used for purine assay.

ASSAY OF EFFLUENT PURINES

After collection on ice, the perfusate samples were kept at 0 - 5 °C in an autosampler (WISP 710B; Waters, Milford, MA, USA), connected to a cooling bath (Ministat; Huber, Offenburg-Elgersweier, FRG). Adenosine, inosine, hypoxanthine, xanthine and urate were determined as previously described[18,20]. Perfusates were collected over the periods indicated in the protocols (Fig. 1). In the experiments according to protocol B, the total purine loss during the experiment was determined.

ASSAY OF CARDIAC HIGH-ENERGY PHOSPHATES

At the end of the infusion experiments (protocol Fig. 1B), hearts were freeze-clamped immediately with precooled alumina tongues, ground and mixed thoroughly with 3 ml 0.4 M $HClO_4$, all at -180 °C. After thawing and centrifugation, the supernatant extracts were neutralized using 6 M KOH/2 M K_2CO_3 and stored in liquid N_2 for later analysis. ATP, ADP, AMP, creatine phosphate, creatine, and NAD were determined using an HPLC-method modified from Harmsen et al.[21]. Briefly, the HPLC-equipment (Waters) consisted of an autosampler, two Model 6000-A pumps and a detector, set at 214 and 254 nm. A 50-μl sample was injected onto a Partisil-10-SAX column (0.4 · 25 cm,

Whatman, Maidstone, GB), guarded by a 3-cm precolumn of the same material, and eluted with buffer A, containing 0.01 M H_3PO_4, adjusted to pH 2.85 with KOH. After five minutes isocratic elution at a flow rate of 2.0 ml · min^{-1}, a gradient was started with an increase of 4% buffer B per minute up to 100%. Buffer B consisted of 0.75 M KH_2PO_4, pH 4.4. Absorption peaks were analyzed and compared with external standards using a data system 840.

STATISTICAL ANALYSIS

Values are expressed per gram dry weight (dwt) and reported as means ± SE. Statistical analysis was performed with Student's t-test (two-tailed). Differences at the $p < 0.05$ level were considered significant.

Results

CORONARY FLOW

Under normoxic conditions, coronary flow decreased slightly, but not significantly during the experiment. Captopril caused a 42% increase in coronary flow within two minutes ($P < 0.005$ vs. control, Fig. 2A). In contrast, angiotensin I (100 nM) and angiotensin II (10 nM) reduced flow by 31% ($P < 0.005$), and 27% ($P < 0.05$), respectively.

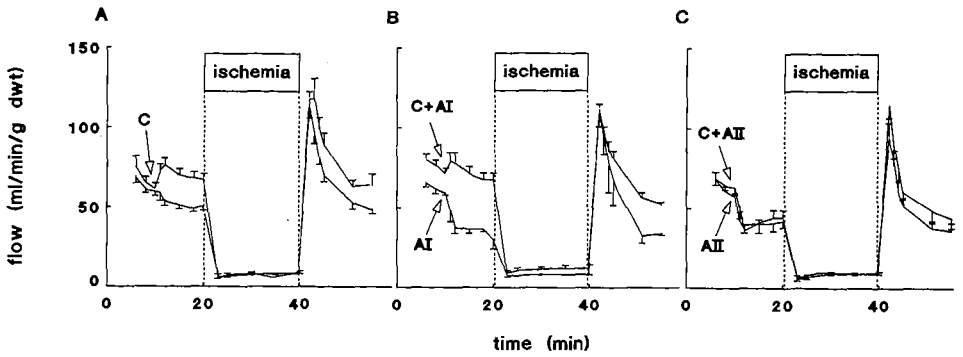

Figure 2. *Coronary flow patterns, observed during experiments following protocol A. Arrows indicate the point where treatment with captopril (C), angiotensin I (AI), angiotensin II (AII) or combinations of captopril with angiotensin I or angiotensin II started. The angiotensins were vasoconstrictive. Captopril inhibited vasoconstriction caused by angiotensin 1, but not of angiotensin 2; the drug itself increased flow. Means ± SE are presented; n = 4-6.*

Captopril completely blocked the flow reduction caused by angiotensin I (Fig. 2B). After two minutes perfusion with captopril + angiotensin I, coronary flow exceeded twice that in angiotensin I perfused hearts ($P < 0.001$). Vasoconstriction caused by angiotensin II could not be inhibited by captopril. When the perfusion pressure was lowered, coronary flow decreased by 83% in control hearts. Hearts treated with captopril + angiotensin I had a slightly higher flow during ischemia compared with the other hearts but differences

were not statistically significant. Reperfusion caused reactive hyperemia which was of the same magnitude and duration for all hearts. After 15 min reperfusion, flows in captopril and captopril + angiotensin I perfused hearts were significantly higher than those perfused without captopril ($P<0.05$ and $P<0.001$, respectively).

MYOCARDIAL FUNCTION

Developed tension decreased from 16.2 ± 1.4 g (control) to 3.7 ± 0.2 g 20 min after onset of ischemia (see Fig. 3A). Upon reperfusion, a partial recovery was observed: after 15 min function was 75% of the preischemic value. Compared to the normoxic controls, function had decreased by 22% ($P<0.05$) due to ischemia and reperfusion.
During normoxia, ischemia and reperfusion neither resting nor developed tension were affected by angiotensin I or II, captopril, or the combinations of captopril with angiotensin I or II. Recovery of developed tension varied from 51% to 75% when compared to pre-ischemic values, but no significant differences were present (Fig. 3A).

After 30 min of ischemia (protocol B), contractions had stopped in all experiments, except in one control heart and one captopril treated heart. During reperfusion, developed tension measured in hearts treated with captopril tended to recover better, but the differences did not reach statistical significance (Fig. 4A).

HIGH-ENERGY PHOSPHATE METABOLISM

Figure 3B shows the purine efflux during the experiments, using Protocol A. Efflux slowly decreased during the experiment from 73 ± 17 nmol · min^{-1} · g dwt^{-1}, 10 min after onset of normoxic perfusion, to 21 ± 4 nmol · min^{-1} · g dwt^{-1}, after 55 min. Ischemia, caused by an 83% reduction in coronary flow from control, increased purine efflux up to four-fold ($p<0.005$). After 5 min reperfusion, purine efflux was five times higher when compared to normoxic control. After 15 min reperfusion, purine efflux had approached the level of normoxic controls. Addition of either captopril, angiotensin I or II, or the combination of captopril with angiotensin I or II failed to alter the purine efflux during the experiment.

In Protocol B (Fig. 1B), a more severe degree of ischemia was induced by reduction of coronary flow to 12% of control for 30 min. In these experiments the efflux of high-energy phosphate catabolites was measured (Fig. 4B) as well as the myocardial high-energy phosphate content at the end of the experiments (Table 1). The normoxic purine efflux, which was 60 ± 4 nmol · min^{-1} · g dwt^{-1} after 10 min perfusion, decreased to 24 ± 2 nmol · min^{-1} · g dwt^{-1} after 70 min perfusion, resulting in a total purine loss of 2 μmol · g dwt^{-1}. Fifteen minutes of ischemic perfusion, increased the mean purine efflux to 190 nmol · min^{-1} · g dwt^{-1}, with a further increase to 250 nmol · min^{-1} · g dwt^{-1} after 30 min (Period IV, Fig. 4B). Infusion of captopril did not alter this purine loss. During the first 5 min of reperfusion, purine washout rose to 560 ± 60 nmol · min^{-1} · g dwt^{-1} in untreated hearts and to 446 ± 113 nmol · min^{-1} · g dwt^{-1} in captopril-treated hearts (n.s.). After 15 min reperfusion, purine efflux had decreased to preischemic levels, in both groups (Period VII, Fig. 4B). The total purine loss over the experiments amounted to 11.2 ± 0.7 μmol · g dwt^{-1} in untreated and 10.6 ± 1.1 μmol · g dwt^{-1} in captopril-treated hearts.

As a result of ischemia-reperfusion, myocardial ATP content had decreased by 44 ± 4% ($p<0.0001$, see Table 1). The ATP content after captopril treatment had decreased by 40 ± 7% ($p<0.001$) which was not significantly different from the untreated hearts.

Ischemia-reperfusion did not affect ADP, but AMP levels had doubled. Captopril had no effect on these adenine nucleotide levels. Adenylate energy charge, (ATP + ½ADP)/(ATP+ADP+AMP), decreased by 6 ± 1% during ischemia/reperfusion ($P < 0.0001$). Captopril could not prevent this decrease.

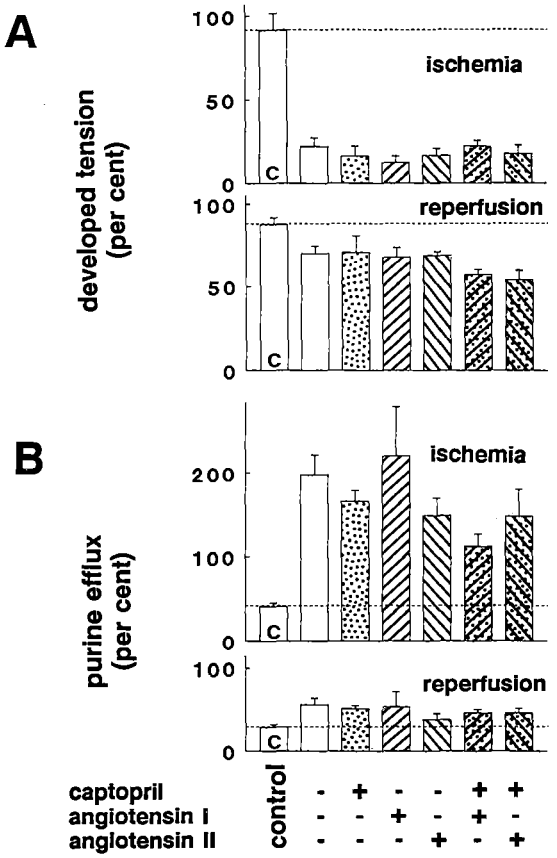

Figure 3 *Developed tension (panel A) in and purine efflux (panel B) from hearts expressed as percentage of the values, measured after 10 min preperfusion. From top to bottom in both panels, values after 20 min ischemia and after 15 min reperfusion (Protocol A) are displayed. No significant effects of treatment were observed. Data are expressed as means ± SE; n = 5-6. C = normoxic control.*

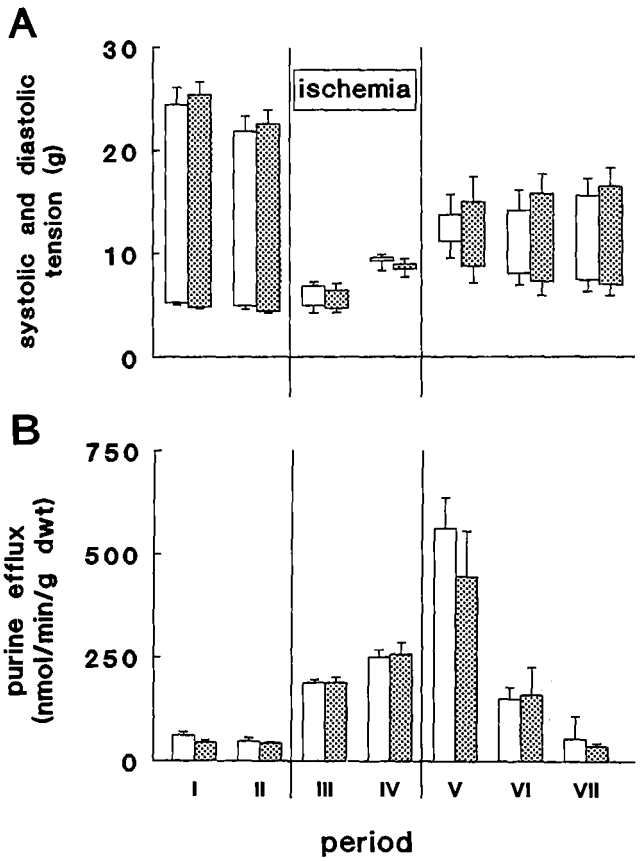

Figure 4. *Panel A shows the systolic and diastolic tension at the end of the periods indicated in Figure 1B. Open and dotted bars give data on untreated and captopril-treated hearts, respectively. Panel B depicts purine efflux from these hearts. Captopril did not affect either variable significantly. Data are expressed as means ± SE; n = 7-8.*

Variable	Control	Ischemia/ reperfusion	Ischemia/reperfusion + captopril
ATP	20.9 ± 0.6	11.6 ± 0.9	12.6 ± 1.5
ADP	2.7 ± 0.1	2.4 ± 0.1	2.3 ± 0.2
AMP	0.30 ± 0.03	0.69 ± 0.04	0.51 ± 0.09
CrP	40.0 ± 1.1	44.3 ± 1.6	41.0 ± 2.0
Cr	18.2 ± 1.4	22.1 ± 2.1	21.6 ± 2.5
EC	0.931 ± 0.003	0.872 ± 0.005	0.887 ± 0.008
n	6	7	8

Table 1. *High-energy phosphate status of the hearts freeze-clamped at the end of the experiments (Protocol B). Captopril treatment did not protect the heart against loss of ATP and creatine phosphate (CrP). Mean values ± SE are expressed in μmol per g dry weight except the EC (adenylate energy charge) which is a ratio. n = number of experiments, Cr = creatine.*

Discussion

The exact role of the renin-angiotensin system during myocardial ischemia hss yet to be clarified. Coronary occlusion causes a reduction in vascular tone which in turn activates the renin-angiotensin system. Activation of this system by coronary occlusion in vivo causes a constriction of arteriolar smooth muscle and increases systemic arterial pressure[6,22]. These properties could contribute to postischemic deterioration. The effects of the system on myocardial contractility are conflicting. Positive and negative inotropism have been described as well as no effect[17,23]. The latter is consistent with our findings. In vivo the actions of the inhibitors have only been partially elucidated. In normal subjects, angiotensin converting enzyme inhibitors lower blood pressure but do not affect cardiac output[24]. Reduction of angiotensin II release directly causes vasodilation but also decreases aldosterone production, interferes with the sympathetic nerve system and affects the central nerve system. In addition, angiotensin converting enzyme prevents bradykinin breakdown[25].

Our results obtained in isolated rat hearts confirm the existence of the converting enzyme in the rat myocardium. Perfusion with buffer containing 100 nM angiotensin I caused coronary vasoconstriction which could be totally prevented by 0.4 mM captopril. In contrast, vasoconstriction caused by angiotensin II could not be inhibited. We also observed an increase in coronary flow when captopril or captopril + angiotensin I was added to the perfusion buffer. During ischemia the coronary flow was not affected by angiotensin II. Undoubtedly other mechanisms which cause vasodilation (e.g., release of lactate, adenosine, prostaglandins, potassium) play a role during ischemia.

Li and Chen[26] found that 10^{-5} M captopril preserved force of contraction and release of creatine kinase in Langendorff rat hearts with segmental infarction, aggravated by angiotensin I. They ascribed the protection to reduced production of angiotensin II and increased prostacyclin release. In our experiments we did not detect a significant effect of captopril on myocardial function. Neither preload nor developed tension were

affected. We only observed a nonsignificant improvement in developed tension after reperfusion when captopril was infused (Fig. 4A, periods V - VII). In isolated hamster hearts, captopril reduced myocardial flow rate and increased left ventricular developed pressure[27]. Both were more pronounced in myopathic hearts than in normal hearts.

Neither angiotensin I, angiotensin II nor captopril affected adenylate energy metabolism. This conflicts with the results reported by Van Gilst and collaborators[11,28]: In their experiments captopril reduced purine loss during reperfusion and induced a better postischemic recovery of ATP-stores. However, they did not observe an ATP-sparing effect by captopril during normoxia and hypothermic global ischemia. Our data also contrast those of Becker et al.[29], who reported angiotensin induced exhaustion of the energy status.

Van Gilst et al.[30] reported fewer arrhythmic events, concomitant with reduced purine and noradrenaline release during reperfusion of rat heart after regional ischemia. Similarly, Rochette et al.[31] found that captopril attenuated reperfusion arrhythmias in both isolated rat hearts and anesthetized rats after left anterior descending coronary artery ligation, despite unaltered noradrenaline release. In the isolated rat heart, induction of regional ischemia is known to provoke ventricular fibrillation during subsequent reperfusion[32]. The protective effects of captopril[11,31,30] probably originate from a reduction of arrhythmias, but this is no direct effect of captopril[33]. Angiotensins were not arrhythmogenic in our preparation, although they have been in the hands of others[29]. The failure of captopril to prevent deterioration of function and metabolism is probably due to this lack of arrhythmogenesis. We conclude that the rat heart contains an active angiotensin-converting enzyme which can be blocked by captopril. Regarding its cardioprotective effect, we speculate that the differences between various models are responsible for the variation in function recovery and energy preservation seen by various investigators. Many mechanisms that are affected by the renin-angiotensin system in the intact animal do not play a role in the isolated heart, perfused with Tyrode buffer and paced artificially. However, captopril prevents the direct effects of angiotensin I on the myocardial vasculature by blockade of the converting enzyme.

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Appendix 10

APPARENT INOSINE UPTAKE BY THE HUMAN HEART

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Cardiovasc. Res. 23 (1989) 484-488

Apparent inosine uptake by the human heart

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ABSTRACT Although inosine has been used clinically to support the myocardium, no data are available on the fate of exogenous inosine in the human heart. We therefore infused six patients, catheterised for coronary angiography, with inosine (5 $mg \cdot kg^{-1} \cdot min^{-1}$ intravenously) for 6 minutes. Before infusion, the arterio-venous difference of inosine, hypoxanthine and xanthine across the heart was nil. During infusion, arterial inosine increased substantially, exceeding the coronary sinus concentration by a maximum of 200 (SEM 53) $\mu mol \cdot litre^{-1}$, $p = 0.02$, at the fourth minute. Arterial hypoxanthine and xanthine also increased, while the arterio-venous difference became 16(11) and 10(3) ($p = 0.04$) $\mu mol \cdot litre^{-1}$, respectively. Left ventricular dP/dt_{max} increased by 22(7)% ($p = 0.04$) at the end of infusion. Thus, there seemed to be substantial uptake of inosine by the human heart, followed by improvement in haemodynamics.

In recent years, interest in inosine, a degradation product of tissue adenine necleotides, has produced vast amounts of data concerning the cardiovascular effects and the mechanism of action of this substance. The release of inosine from the heart exposed by hypoxia or ischaemia,[1–3] and its ability to increase myocardial blood flow[4 5] and contractility[4–6] stimulated experimental studies on its role in myocardial ischaemia[7] and in acute left ventricular failure.[8] Beneficial effects of inosine in limiting infarct size[7 9] and counteracting left ventricular failure[8] have been ascribed to direct vasodilatation of the coronary arteries and to a positive inotropic effect at low energy cost.[5 8] Inosine promotes enhanced nucleotide resynthesis in (post)ischaemic hearts[10 11] via breakdown to hypoxanthine, subsequent ribophosphorylation, and conversion to AMP (see fig 1).

These positive effects of inosine on myocardial ischaemia and in left ventricular failure encouraged the therapeutic application of this agent in the management of the low output state and cardiogenic

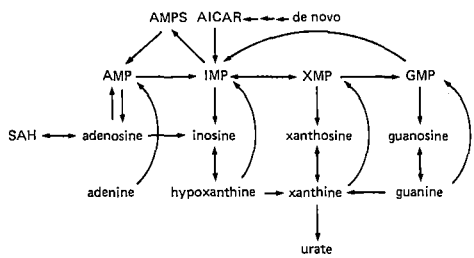

FIG 1 *Scheme showing cardiac metabolism of purine nucleotides (AMPS=adenylsuccinate, AICAR=5-amino-4-imidazolecarboxamide ribotide, SAH=S-adenosylhomocysteine). Note breakdown of inosine to hypoxanthine, xanthine and urate, and incorporation of inosine in AMP via "salvage pathway" (hypoxanthine, IMP, AMPS, AMP).*

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Key words: nucleotide regeneration; inosine infusion; hypoxanthine; xanthine; inotropy; human heart.

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shock.[12] Although inosine has been used as a clinically applicable myocardium supporting agent, no data are available so far on the fate of exogenous inosine in man. Undertaking this study, we wished to answer the following questions: (1) Is exogenous inosine taken up by the human heart? (2) Is the inotropic response to inosine related to uptake?

Methods

POPULATION

The study group consisted of six patients with ischaemic heart disease, catheterised for coronary angiography. The study protocol was approved by the Committee on Human Investigations. The patients gave their consent to participate in the study, Patient characteristics are listed in table 1. Their mean age was 46 (SEM 4) years.

PROTOCOL

The inosine study was performed after the coronary sinus catheter was positioned, visualised with a bolus injection of contrast agent (Uropolinum 75%, Polfa, Starogard, Poland), and before angiography. The aim of this procedure was to avoid as far as possible the use of angiographic contrast which disturbs high performance liquid chromatography (HPLC) of purines. Inosine (Trophicardyl iv, Laboratoire Innothéra, Chantereau, France) was infused at a rate of 5 $mg \cdot kg^{-1} \cdot min^{-1}$ (4% solution) into a peripheral vein. Blood was sampled from the aorta and the coronary sinus immediately before the infusion and after 2, 4 and 6 min of inosine administration.

HAEMODYNAMICS

Left ventricular pressure was obtained from a saline filled catheter-manometer system, using a Statham P23Db pressure transducer (Hato Rey, PR, USA). Pressures were measured at the level of the left ventricular mid plane. The first derivative of left ventricular pressure (dP/dt_{max}) was obtained from the resistance-capacitance differentiating circuit (using a contractility calculator model 868; Siemens-Elema, Solna, Sweden) and was calibrated by known slope. The upper frequency limit depends on the measurement range for the derivative channel; in our apparatus the 3 dB limit was 20, 40, 80, 200 Hz for the four channels.

PURINE ASSAY

Two ml blood were immediately mixed with 2.0 ml cold 8% $HClO_4$, present in preweighed tubes. The exact amount of each blood sample was determined by weighing. Denatured proteins were removed by centrifugation in the cold. The supernatant fluid was collected and stored below –15°C. An aliquot of this fluid was neutralised with 6 $mol \cdot litre^{-1}$ KOH/2 $mol \cdot litre^{-1}$ K_2CO_3. Potassium chlorate was removed by centrifugation in the cold. Nucleotides in the deproteinised, neutralised blood (1.5 ml) were adsorbed on prewashed Al_2O_3 (0.6 g) in a plastic column (internal diameter 8 mm), and purines eluted with 5 ml 10 $mmol \cdot litre^{-1}$ Tris-HCl, pH 7.4. The eluate was stored at –15°C. HPLC of purines (100 μl) took place on a μBondapak C_{18} column (Millipore-Waters, Milford, MA, USA) with 1% K_2HPO_4 – 1% methanol, pH 4.7, at a flow rate of 1.0 $ml \cdot min^{-1}$. The column was protected by a precolumn of Perisorb RP-18 (Merck, Darmstadt, GFR). Waters HPLC equipment used included: WISP 710B cooled autosampler, Model 6000A pump, Model 490 detector, Model 840 computer. The nucleoside and oxypurine peaks were identified by retention times, enzyme shifts, absorption at various wavelengths, and internal standards. Figure 2 shows the separation of urate, hypoxanthine, xanthine and inosine by HPLC. Peaks were routinely detected at 254 nm. Sample clean up and purine assay are based on our earlier work.[13]

TABLE 1 *Patient characteristics*

Patient No	*Sex*	*Age (yr)*	*History of the disease*			*Coronary catheterisation findings*		
			MI	*Angina*	*Other*	*LV volume*	*Atheroscl*	*Other*
1	F	30	Inf	+	ventricular ectopic beats	normal	+	akinesis of Inf wall
2	M	56	Ant	+	–	normal	+	hypokinesis of Ant wall
3	M	44	–	+	hyperlipidaemia Type IIA	normal	+*	–
4	M	48	Ant and Inf-Lat	+	–	abnormal	++	akinesis of Ant-Lat wall
5	M	49	sub-endocardial	+	–	normal	+**	—
6	M	47	Ant-Lat	+	–	abnormal	++	akinesis of Ant-Lat wall, aneurysm

Coronary bypass surgery performed six weeks* and three weeks** later. Ant=anterior; Atheroscl=coronary atherosclerosis; Inf=inferior; Lat=lateral; LV=left ventricular; MI=myocardial infarction in the past.

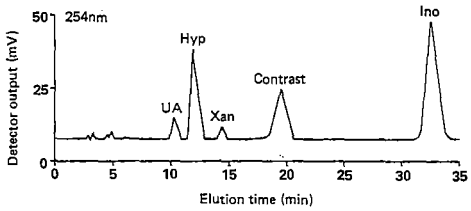

FIG 2 *High performance liquid chromatogram of blood extract. The blood sample was taken from the coronary sinus 6 min after the inosine infusion started. See Methods for extraction procedure. Urate (UA), hypoxanthine (Hyp), xanthine (Xan) and inosine (Ino) were routinely detected at 254 nm.*

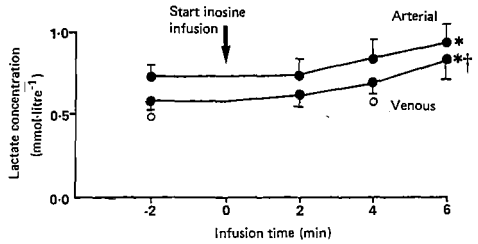

FIG 3 *Increase in both aortic and coronary sinus blood lactate concentration during inosine infusion in man. Results are means. Bars=SEM, n=5-6.*
**p<0.05 v baseline (t=–2 min), †p<0.02 v arterial.*

LACTATE ASSAY

The acid supernatant fluid (see Purine assay) was used to determine lactate with the Boehringer UV-Test (Boehringer, Mannheim, GFR).

STATISTICS

Analysis of variance or Student's *t* test for paired observations was used. Differences with $p<0.05$ (two-tailed) were considered statistically significant. Values are presented as means (SEM).

Results

HAEMODYNAMIC DATA

As shown in table 2, infusion of inosine had no significant effect upon either heart rate, or systolic, diastolic and end diastolic blood pressure. Left ventricular dP/dt_{max} increased in all patients. The increase varied from 6 to 44% in individual patients; the average rise was 22(7)% ($p = 0.04$) at the sixth minute of inosine infusion (table 2).

BIOCHEMICAL DATA

During the study, the lactate levels rose gradually (fig 3). At the end of the infusion period, the arterial and venous lactate concentrations had increased by 20(16)% ($p = 0.02$) and 42(13)% ($p = 0.04$), respectively. However, the arterio-venous difference of this carbohydrate was not significantly affected by the infusion of inosine.

Figure 4 shows the arterial concentrations of the purines. During infusion, there was a substantial increase of inosine and its catabolites hypoxanthine and xanthine. From initial values ≤ 1 mol·litre^{-1}, increases were observed in inosine, to 459(87) μmol·litre^{-1} ($p = 0.003$), hypoxanthine, to 142(9) μmol·litre^{-1} ($p<0.001$), and xanthine, to 38(6) μmol·litre^{-1} ($p = 0.001$).

Figure 5 shows the arterio-venous differences in inosine and its catabolites. Before the infusion of inosine, the differences were virtually nil. During infusion of inosine, a part of this nucleoside was converted to hypoxanthine and xanthine. At the fourth

TABLE 2 *Haemodynamic effects of inosine in man*

Patient No	*Ejection fraction (%)*	*Heart rate (beats·min^{-1})*		*Left ventricular pressure (mm Hg)*						*Left ventricular dP/dt_{max} (mm Hg·s^{-1})*		
				Systolic		*Diastolic*		*End diastolic*				
		Control	*Ino*	*Control*	*Ino*	*Control*	*Ino*	*Control*	*Ino*	*Control*	*Ino*	*%*
1	61	90	121	130	125	8	5	18	9	1760	2540	44
2	82	70	77	181	171	5	1	17	15	1720	2100	22
3	86	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4	ND	102	107	136	140	14	20	41	44	1280	1360	6
5	72	90	97	147	180	7	10	18	18	2550	3200	25
6	ND	67	67	152	136	10	8	35	32	1280	1440	12
Mean	75	84	94	149	150	8.8	9	26	24	1718	2128*	22
SEM	6	7	10	9	11	1.5	3	5	6	232	345	7

The ejection fraction was measured after the inosine protocol was completed and the infusion discontinued. Ino=measurements at 6th minute of inosine infusion; ND=not determined.
*p=0.04 *v* control.

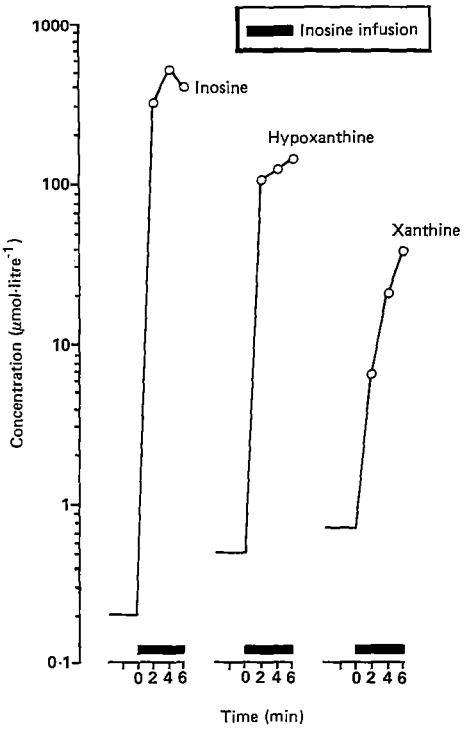

FIG 4 *Increase in the arterial levels of inosine, hypoxanthine and xanthine during inosine infusion in man.*

minute of infusion, the arterio-venous differences were 16(11) and 10(3) ($p = 0.04$) μmol·litre^{-1}, respectively. The production of these catabolites, however, accounted for only a small percentage of the inosine that had disappeared. Thus a short lasting, substantial uptake of inosine seemed to take place, the arterio-venous difference being 200(53) μmol·litre^{-1} ($p = 0.02$) at that time.

We were unable to detect adenosine in the blood samples with the HPLC condition employed by Harmsen *et al*[13] (detection limit 0.1 μmol·litre^{-1}). We do not report data on urate, because the recovery of that purine from the Al_2O_3 column was poor.

SIGNS OF ISCHAEMIA

There were no signs of myocardial ischaemia, such as pain or ECG changes.

Discussion

Inosine is a therapeutic agent used in several countries outside the United States.[14] Nevertheless clinical data

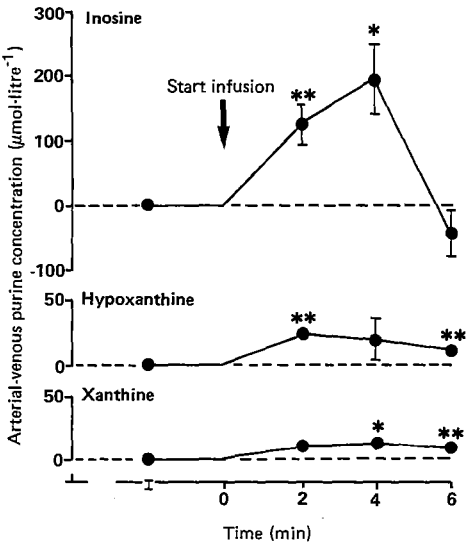

FIG 5 *Apparent uptake of inosine and its catabolites by the human heart. Results are means. Bars=SEM, n=5-6. *p<0.05, **p<0.01 v baseline value (t=–2 min).*

on this subject are scarce. We show here that inosine induced an increase in contractility measured by left ventricular dP/dt_{max} in man, without a statistically significant change in heart rate (table 2). The observed small changes in heart rate cannot account for the effect of inosine on dP/dt_{max}.[15] As the increase in contractility did not accompany a significant increase in heart rate, we expect the metabolic cost of the action of inosine to be relatively low. Our assumption is based on the concensus that heart rate is an important determinant of myocardial oxygen consumption. For example, increase in heart rate has been shown to induce an increase in oxygen consumption.[16] Jones and Mayer[5] found that myocardial oxygen consumption did not increase during inosine infusion in dogs (see also [17]).

The positive inotropic effect of inosine in dog heart is well established.[6 8 14 18] The nucleoside also increases contractility in the frog,[19] and pig[7 20] and rabbit.[21] In contrast, inosine seems to have no effect on myocardial contractility in the guinea pig[22] and a negative effect in the rat.[22 23] Thus, the effect of inosine on contractility is probably species dependent.

An interesting observation in this study is the very large arterio-venous difference in inosine 2 and 4 minutes after the start of the infusion (fig 5). We did not note this apparent cardiac inosine uptake at the sixth minute of inosine infusion. Whether

redistribution of inosine among various intra- and extracellular compartments took place during the second part of the infusion remains to be studied. On the basis of animal experiments,[10 11] inosine incorporation into cardiac adenine nucleotides is not unlikely. It is tempting to speculate that such inosine incorporation is responsible for the positive inotropic effect observed (but see [24]).

Using the pure β-blocker sotalol in the dog, Czarnecki and Noble[18] found that the β-adrenergic system partly governs the nucleoside induced increase in contractility. The magnitude of the adrenergic dependent inotropic effect may rely on the actual sympathetic drive to the heart. Explanations of this supersensitivity of the myocardium to catecholamines include an increased number of, or affinity for, β receptors. Alternatively, inosine could increase the cardiac cAMP level through decreased phosphodiesterase activity, as shown in other organs.[25] Finally, inosine might act intracellularly, preventing the catecholamine induced loss of Ca^{2+} sensitivity of the contractile apparatus.[26]

Inosine increased myocardial carbohydrate metabolism in the isolated rabbit heart[21] and the closed chest dog preparation.[8] In our patients the arterio-venous lactate difference across the heart did not change significantly. Supposedly inosine causes coronary vasodilatation in man. We therefore postulate that in our study lactate uptake also increased, due to the higher arterial lactate concentration following inosine infusion (fig 3). The latter could be caused by a shift away from gluconeogenesis due to the purine load.

We conclude that inosine deserves further attention as a cardiotonic agent. Its mechanism of action has still to be elucidated.

We are grateful to Ms M J Kanters-Stam for her outstanding secretarial help.

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Appendix 11

EFFECT OF INOSINE AND ADENINE ON NUCLEOTIDE LEVELS IN POST-ISCHEMIC RAT HEART PERFUSED WITH AND WITHOUT PYRUVATE

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Cardioscience, 1 (1990) 241-246

EFFECTS OF INOSINE AND ADENINE ON NUCLEOTIDE LEVELS IN THE POST-ISCHEMIC RAT HEART, PERFUSED WITH AND WITHOUT PYRUVATE

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Reports on enhanced nucleotide regeneration by purines during reperfusion are conflicting. We have, therefore, evaluated the effects of inosine or adenine, administered after ischemia, on adenine nucleotide levels and function in isolated rat hearts. The hearts were perfused with a Tyrode solution, containing 10 mM D-glucose, with or without 5 mM pyruvate. After 15 minutes without flow, the hearts were reperfused for 45 minutes with 20 μM purine and 0.5 mM D-ribose. Adenine nucleotide levels tended to recover better in the purine-treated groups. The purines decreased the ATP/ADP ratio by 10-15% ($p<0.05$) if pyruvate was absent. The IMP level in the inosine/glucose group exceeded that in all other groups by a factor of two ($p<0.001$). Inosine increased the adenosine concentration in the effluent sixfold ($p<0.005$). The hypoxanthine concentration rose up to four times following adenine treatment ($p<0.05$). The administration of purine, with or without pyruvate, did not affect mechanical recovery, heart rate or coronary flow. We conclude that inosine and adenine failed to improve cardiac function and hardly affected nucleotide levels in the reperfused heart.

Key words: adenine, inosine, pyruvate, nucleotide regeneration

Cardioscience 1990; 1: 241-246

INTRODUCTION

Ischemia reduces the myocardial content of adenine nucleotides; and the regeneration of nucleotides is a relatively slow process. Purines, such as inosine and adenine, can enhance the synthesis of nucleotides through the salvage pathways (for a review, see reference 1).

Inosine may alter the metabolic and functional state of the heart, possibly with secondary effects on the nucleotides. It dilates coronary arteries and increases myocardial contractility in the dog[2] in contrast to its negative inotropic effect in the rat heart[3]. We administered inosine in the reperfusion period, combined with ribose. The latter accelerates repletion of the ATP pool during recovery from reversible ischemia[4].

Isolated cardiomyocytes rapidly incorporate adenine into adenine nucleotides[5] but few reports describe such incorporation in the isolated heart or the heart in vivo[6]. Some authors observed enhanced ATP levels if they provided inosine during reperfusion, even though the incorporation rate of ^{14}C-labeled inosine was too low to explain the observed elevation[7,8]. The maximal activity of adenylosuccinate synthetase, one of the enzymes in this salvage pathway, does not permit a rapid regeneration[9]. In addition, the ^{14}C-incorporation rate over-estimates the net gain of nucleotide because even the normoxic heart catabolizes its AMP to adenosine.

Exogenous pyruvate increases glycolysis and improves mechanical recovery in the (post)ischemic heart[10,11,12,13]. The reduction in free AMP content, caused by pyruvate in hearts perfused with glucose, lowers the efflux of adenosine and inosine[14]. Pyruvate also decreases the NADH/NAD$^+$-ratio and may, thereby, unblock an inhibition in the salvage of hypoxanthine and thus also of inosine[15]. We assessed whether adenine and inosine, administered with or without pyruvate, could accelerate nucleotide regeneration and mechanical recovery after ischemia.

MATERIALS AND METHODS

Chemicals

All chemicals were of the highest grade avail-

able. Water was purified with the Millipore-RO4/Milli-Q System. Inosine and D-ribose were purchased from Merck; adenine was from Koch-Light, sodium pyruvate from Boehringer.

Cardiac perfusions

We studied 40 rats weighing 252±4 g. Details of the animals, anesthesia and perfusion have been described elsewhere[16]. The atria were not removed; the His-bundle was left intact.

Mechanical activity was monitored with a force transducer[16] using a resting tension of 5 g. Developed tension was calculated as peak systolic tension minus resting tension. The heart rate was registered using the tension signal. Flow was measured with an electromagnetic flow probe (Transflow 601, Skalar) or by timed collection of effluent.

Extraction and high-performance chromatography of tissue adenylates and high-energy phosphates took place as described earlier[16]. Purines in the effluent were measured in 200-μl samples as given in detail elsewhere[16]. The retention times, in minutes, were: urate 7.5, hypoxanthine 8.6, xanthine 9.4, inosine 11.9, adenine 13.6, adenosine 27.7. Peaks were identified by comparing retention times and wave-length ratios of 254 and 290 nm (purines) and 254 and 214 nm (high-energy phosphates) with those of standards. Compounds were quantified from the areas of their peaks.

Protocol

After a 15-minute equilibration period, followed by a 15-minute period before ischemia, the hearts were made globally ischemic for 15 minutes. When the heart rate dropped below 300 beats per minute, the hearts were paced at that rate. They were reperfused for 45 minutes with standard medium with one of the six following additions: none, inosine + ribose, adenine + ribose, pyruvate, pyruvate + inosine + ribose, pyruvate + adenine + ribose. The concentrations of inosine and adenine were 20 μM; those of ribose and pyruvate were 0.5 mM and 5 mM, respectively. Finally, a standard perfusion buffer was given during one minute before the hearts were frozen with a clamp cooled in liquid nitrogen.

Statistics

All values are presented as average ± standard error. Hearts treated with purine were compared with their respective controls. Analysis of variance was used, followed by Student's t-test (two-sided). Hearts perfused with glucose were not compared with those perfused with glucose/pyruvate, as the effects of pyruvate have been described in the literature, and because the two series were not randomized.

RESULTS

Contractile force, flow and heart rate

Before ischemia, pyruvate did not affect contraction, flow or heart rate. Developed tension was 15±2 and 14±3 g in the hearts perfused with glucose and pyruvate/glucose, respectively. Coronary flow was 8.0±0.3 and 9.0±0.3 ml/min and the heart rate 300±6 and 310±7 beats/min, respectively.

Addition of adenine or inosine to the reperfusion buffer did not significantly alter the recovery of developed tension. (The values in brackets are percentages of pre-ischemic values.) Developed tension recovered to 9±1 g (glucose, 65±14%) and to 14±0 g (pyruvate/glucose, 89±14%). Coronary flow at the end of reperfusion was 6.2±0.3 ml/min (glucose, 78±3%) and 9.2±0.3 mi/min (pyruvate/glucose, 102±4%). The product of developed tension and heart rate was 2600±800 g/min (60±15%) and 3900±500 g/min (85±14%), respectively. The heart rate was similar in all groups.

Adenylates and high-energy phosphates

Hearts treated with inosine plus glucose tended to show a higher content of total adenine nucleotides ($p = 0.09$, see Fig 1). The addition of inosine increased the ADP levels from 2.88±0.09 to

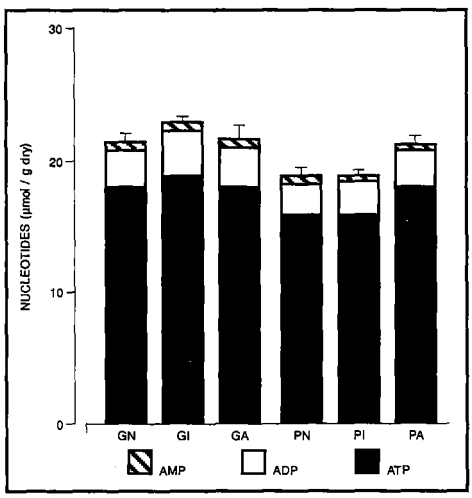

Figure 1. *Effect of purine administration during reperfusion on adenine nucleotide content. Hearts treated with inosine plus glucose tended to show a higher total nucleotide content. The addition of inosine increased the ADP levels 21% ($p < 0.005$) in this group. Means ± SEM, $n = 6$-9. G = glucose; P = pyruvate/glucose; N = no purine; I = inosine/ribose; A = adenine/ribose.*

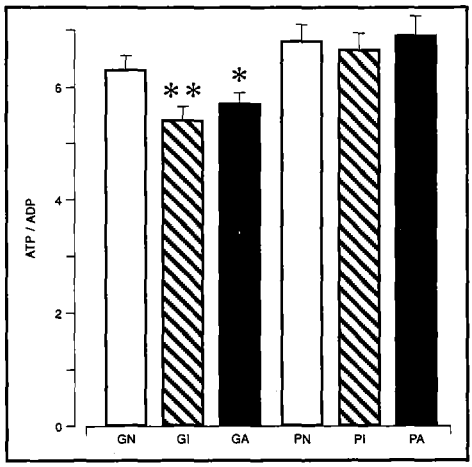

Figure 2. *Effect of purine treatment during reperfusion on the ATP/ADP ratio. Inosine and adenine, both combined with ribose, decreased the ratio in the glucose group. Means ± SEM, n = 6-9. G = glucose; P = pyruvate/glucose; N = no purine; I = inosine/ribose; A = adenine/ribose. * p<0.05; ** p<0.01.*

3.51±0.15 μmol/g dry weight ($p<0.005$). A decreased ($p<0.01$) ATP/ADP ratio reflected this increase in ADP (Fig 2). The IMP levels doubled when inosine was present ($p<0.001$, Fig 3). Addition of adenine to the buffer containing glucose caused smaller alterations in the nucleotide levels, but in the same direction; the ADP level increased 13% ($p = 0.06$) and the ATP/ADP ratio decreased 8% ($p<0.05$). Purine treatment did not improve the nucleotide levels in the hearts treated with pyruvate plus glucose, although they prevented the decrease in ATP/ADP ratio, mentioned above. Addition of purines did not significantly alter the creatine phosphate and creatine levels (data not shown).

Nucleosides and oxypurines

Before ischemia, the average purine concentration (adenosine + inosine + hypoxanthine + xanthine + urate) in the effluent was 0.57 ± 0.03 μM. Per minute, the heart lost 29 nmol/g dry weight of purine compounds, amounting to about 0.1% of the myocardial adenine nucleotide content. In samples, drawn from the effluent collected 0-5 min after ischemia, the sum of AMP catabolites had increased to 12±1 μM in the untreated glucose group, i.e., 1300 nmol/min per g dry weight. The untreated pyruvate/glucose group, and the groups treated with adenine showed similar results. The same was true for the groups in which inosine had been added, provided the purine concentrations measured were corrected for the nucleoside administered. The breakdown of AMP, as reflected by effluent catabolites, decreased gradually to the levels observed before ischemia. We calculated the wash-out of nucleotide catabolites during reperfusion. These values show that nucleotide breakdown in the groups was comparable.

Addition of inosine increased the effluent concentration of adenosine 3-4 times in the samples collected 45 minutes after ischemia (see Fig 4). The addition of inosine also increased the concentration of inosine, hypoxanthine, xanthine and urate (not shown).

Addition of adenine increased the hypoxanthine concentration 2-4 times 45 minutes after ischemia (Fig 5). There was also a non-significant rise in urate and xanthine, but inosine and adenosine levels were unaltered (data not shown). The uptake/breakdown ratio of adenine was highest shortly after ischemia and decreased in the following samples (Fig 6).

DISCUSSION

When the requirement for oxygen exceeds the oxygen consumption, ATP breaks down and its dephosphorylated catabolites leak out of the cell. Without treatment it takes days to reach pre-

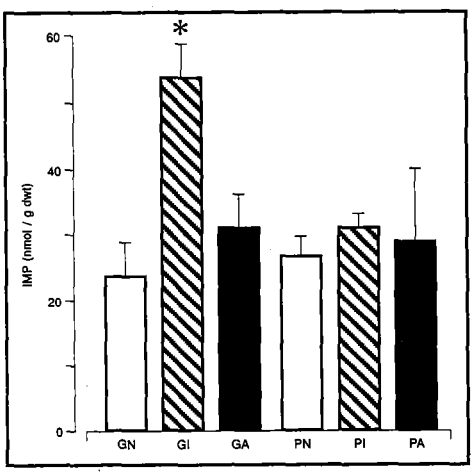

Figure 3. *The hearts showed a higher IMP content in the glucose group after treatment with inosine/ribose. Means ± SEM n = 6-9. G = glucose; P = pyruvate/glucose; N = no purine; I = inosine/ribose; A = adenine/ribose. * p<0.001.*

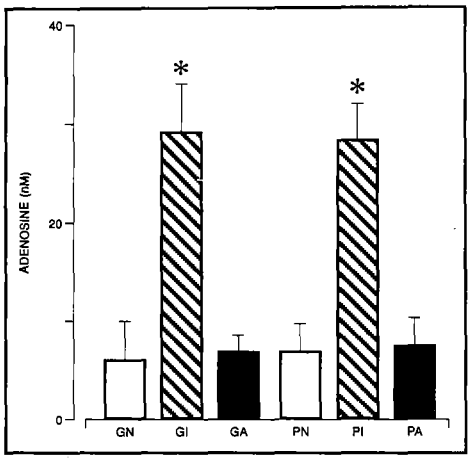

Figure 4. *Adenosine concentration in the effluent at the end of reperfusion, The concentration was higher in the inosine-treated hearts (GI, PI) than in the control hearts (GN, PN). Means ± SEM, n = 6-9. G = glucose; P = pyruvate/glucose; N = no purine; I = inosine/ribose; A = adenine/ribose.*
** p < 0.005.*

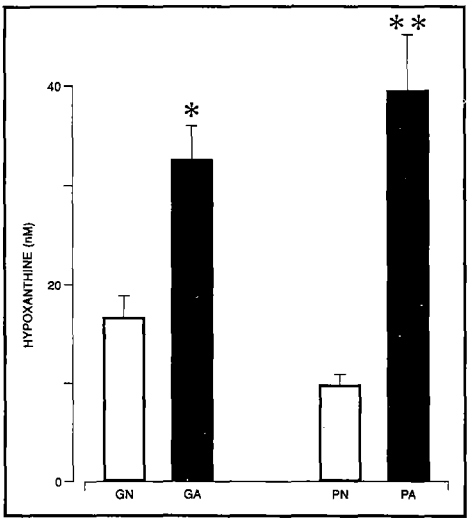

Figure 5. *Concentration of hypoxanthine in the effluent at the end of reperfusion. The concentration was higher in the hearts treated with adenine (GA, PA) than in the control hearts (GN, PN). Means ± SEM, n = 6-9. G = glucose; P = pyruvate/glucose; N = no purine; I = inosine/ribose; A = adenine/ribose.*
** p < 0.05; ** p < 0.005.*

ischemic ATP values[17]. There are four pathways for adenine nucleotide regeneration: adenosine incorporation, salvage of hypoxanthine (inosine), salvage of adenine, and de novo synthesis. Adenosine incorporation is the fastest regeneration pathway, de novo synthesis the slowest (for a review, see reference 1). The hemodynamic effects of adenosine and its very short half-life in blood limit its use as substrate for enhanced nucleotide synthesis. Therefore other precursors are more promising as potential treatments.

Salvage pathways

Hypoxanthine-guanine phosphoribosyl transferase catalyzes the incorporation of hypoxanthine into IMP; adenylosuccinate synthetase and lyase, in turn, convert IMP to AMP. Cardiomyocytes use ribose at a rate of 50 nmol/min per g dry weight to synthesize 5-phosphoribosyl-1-pyrophosphate[9], the rate-limiting co-substrate of the transferase. The administration of ribose for 24 hours increased the available phosphoribosylpyrophosphate pool in the rat heart fourfold[6]. The adenylosuccinate synthetase activity is rate-limiting for IMP synthesis: 15 nmol/min per g. This is theoretically the maximal incorporation rate of hypoxanthine and inosine[9].

Nucleoside phosphorylase catalyzes the conversion of inosine to hypoxanthine and

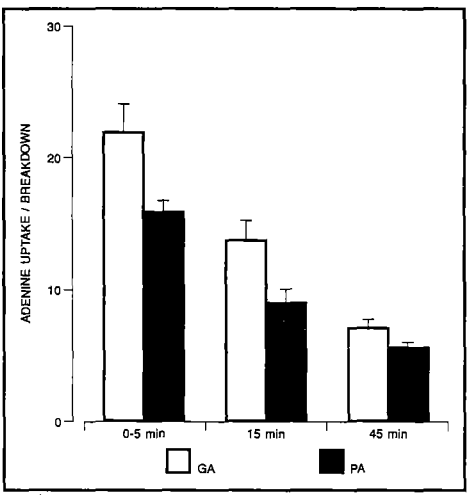

Figure 6. *Uptake/breakdown of adenine decreased during reperfusion in both adenine-treated groups. Values were calculated as the difference between the infused concentration and the effluent concentration, multiplied by coronary flow. Means ± SEM, n = 6. GA = glucose + adenine/ribose; PA = pyruvate/glucose + adenine/ribose.*

ribose-1-phosphate before incorporation[18]. The phosphate, an intermediate in the synthesis of phosphoribosylpyrophosphate, will probably remain within the endothelial cell[19,20], unused for enhancing the concentration of the latter in the myocytes.

The incorporation rates of inosine measured by various authors vary between 0.4 and 40 nmol/min per g dry weight[7,21,22]. Inosine/ribose, added to the hearts perfused with glucose, increased the adenine nucleotide content in our study from 21.6 to 23.0 μmol/g (Fig 1). This indicates an average increase of 35-40 nmol/min per g. However, the change was not statistically significant. The regimen decreased the ATP/ADP ratio (Fig 2), as noted before[7,21,23]. The twofold increase in IMP, due to inosine/ribose (Fig 3), may have resulted from incorporation. Compared to the adenine nucleotide levels (Fig 1), the concentrations of IMP are low. We cannot exclude that AMP-deaminase generated the IMP.

With adenine/ribose we observed only trends towards higher nucleotide levels (Fig 1), although this treatment caused a significant decrease of the ATP/ADP ratio in the glucose group (Fig 2). In dog hearts, repletion of adenine nucleotides took ten days without treatment, but adenine/ribose treatment shortened this to one day[17]. The ATP regeneration rate was about 20 nmol/min per g during the first four hours after ischemia, but it subsequently decreased rapidly[17].

Pyruvate and nucleotides

Addition of pyruvate to an isolated heart, perfused with glucose, decreases the concentrations of inorganic phosphate, AMP and ADP[11,14]. In line with these observations is our finding that pyruvate prevented the decrease in ATP/ADP induced by purines (Fig 2). Pyruvate also inhibited the increase in IMP content, found after inosine administration (Fig 3). Bünger showed that pyruvate lowers the production of adenosine and inosine in the guinea-pig heart, and speculated that reduced AMP levels would decrease purine production[24]. Our values did not show this result. This point needs further evaluation.

Concentrations of purine in the effluent

In the inosine groups, the adenosine concentration increased (Fig 4). This phenomenon, which we described earlier in the normoxic rat heart[25] therefore also occurs after ischemia.

The adenine uptake by the heart is highest soon after the start of the adenine infusion (Fig 6). Because of the reactive hyperemia, the effluent concentration of adenine is minimal after 15 minutes of reperfusion. In intact dogs, Ward et al.[17] measured an adenine uptake of 38% in one passage through the heart (arterial concentration 18 μM). Our value in the isolated rat heart was less than 10%. Adenine increased the production of hypoxanthine (Fig 5), confirming data in the literature.[5]
The absence of increased concentrations of inosine suggests that the deamination of adenine is not routed through AMP and adenosine, or through IMP.

Purines and mechanical function

Purine derivatives affect hemodynamics. Inosine causes vasodilation; it increases contractility in some species[3,26]. Our earlier work showed that inosine, at concentrations more than 30 μM, exceedingly increases coronary flow in the isolated rat heart[25]. Several authors have described protection of the heart by inosine, during and after ischemia[13,22,27,28]. In our animals, mechanical activity improved during the first half hour after reperfusion and then stabilized. We found no differences between hearts treated with or without purines.

In our experiments, the mechanical activity of the hearts perfused with pyruvate recovered well and coronary flow was high. On the other hand, we failed to see an improved recovery of adenine nucleotides. These observations are in line with data in the literature[11].

Conclusion.

Using isolated rat hearts, we tried to accelerate the incorporation of purine by perfusing with adenine or inosine supplemented with ribose and pyruvate. We raised adenine nucleotide levels only marginally, but failed to improve mechanical function.

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