METABOLIC AND FUNCTIONAL EFFECTS OF PURINES IN THE HEART

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Metabole en functionele effecten van purines in het hart

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Ter nagedachtenis aan mijn vader

Aan mijn moeder
Aan Evelien

CONTENTS

Chapter 1

General introduction

This thesis discusses metabolism and functional effects of purine nucleosides and oxypurines in the heart. The study is mainly limited to the nucleosides adenosine and inosine and the oxypurines: hypoxanthine, xanthine and urate, all adenine-nucleotide catabolites. It is confined to effects in mammals, especially rats, pigs, and humans.

Within certain disciplines, various aspects of the purines have been explored extensively. Neuropharmacologists have researched the adenosine receptors. Purine analogues have been synthesized for the treatment of neoplasms and virus infections. The erythrocyte membrane has been used to evaluate the transport across membranes. The study of some metabolic diseases and renal calculus formation provided knowledge of normal purine metabolism.

There are several reasons why an important part of this thesis is devoted to adenosine. The knowledge of adenosine-induced effects is the most detailed. Adenosine has stronger effects than, for example, inosine. There are indications for the involvement of adenosine in the effects of other nucleosides.

Purines are important in cardiac research. Ischemia rapidly elevates purine concentrations. This elevation may serve as a diagnostic tool for the quantification of ischemia. The difference in enzyme activities between various species causes concentration differences. Both endogenous and exogenous nucleosides affect hemodynamics and metabolism. Several drugs modify purine metabolism or purine induced effects. Purinergic receptors mediate effects of the nucleosides. The effects in vivo and in isolated organs are different, as nucleosides have both direct effects on cardiac cells, and indirect through the autonomic system.

The objective of this thesis is the evaluation of purine function and metabolism in the heart, in the hope that this knowledge is useful for extended basic research and for the treatment of the cardiological patient. Transport and metabolism are discussed to demonstrate what concentrations may be expected and which conditions alter purine levels. The functional effects of the nucleosides in the heart are shown and their modification by the autonomic system.

Finally, effects in pathological hearts, interaction with drugs, and some clinical applications are presented.

Organization of this thesis

Chapter 2 discusses the concentrations of purines in several species, their localization, with emphasis on the role of vascular endothelium. Furthermore, it shows the effect of some metabolic diseases on the purine concentrations. Chapters 3 and 4 give a description of the metabolism of adenine nucleotides and purines: chapter 3 describes the catabolic pathways and the rate of nucleotide depletion during ischemia; chapter 4 shows the anabolic pathways and the (im)possibilities for rapid adenine-nucleotide level repletion after ischemia. Chapter 5 discusses nucleoside transport across the cell membrane. Chapter 6 and 7 evaluate the functional effects: chapter 6 focusses on the effects in isolated heart, the direct hemodynamic effects, and the adenosine receptors; chapter 7 concentrates on the effects in vivo and discusses the modifying effects of the other systems, like the autonomic system. Chapter 8 assesses the potential clinical applications of purine administration, effects of purines on diseased tissue and the modification by several drugs of purine metabolism and functional effects. Chapter 9 summarizes the thesis.

Chapter 2

Purine concentrations

Intravascular concentrations

Purine concentration depends on species and localization. Humans have a high plasma urate concentration, and 100 times lower precursor concentrations. The variation in adenosine concentrations is primarily due to the differences between individuals; the concentration for a given individual is relatively constant[48]. The half-life of adenosine in blood is only a few seconds due to a rapid uptake in erythrocytes[71]. Nevertheless, there is a more or less stable concentration of about 0.1 μM adenosine in blood. In an isolated heart, there is no arterial supply of adenosine. The administration of exogenous adenosine in this concentration markedly influences the effects of inosine infusion [appendix 2]. It may also alter nucleoside incorporation in isolated heart (see chapter 4).

Pigs have high plasma inosine and hypoxanthine concentrations, but urate levels are low [appendix 3].

Adenosine, inosine, hypoxanthine and xanthine are usually assessed in deproteinized blood plasma by high pressure liquid chromatography (HPLC). Urate is usually measured enzymatically in serum, but HPLC also provides very accurate measurements[63].

The role of endothelium

Vascular endothelium forms an active metabolic barrier for a number of purines. It incorporates or catabolizes adenosine and inosine; in the heart it is the most important localization of the enzymes which catabolize inosine and hypoxanthine[9,46]. There are often large differences between the interstitial and the plasma concentrations. Several authors measured differences in adenosine concentration between the venous effluent and the interstitium of the heart. The interstitial adenosine concentration is 0.8 μM in rat heart[57] and 0.18 μM in isolated rat or guinea-pig heart[27]. In the dog heart the interstitial adenosine concentration amounts to about 0.1 μM[49]. All authors find a higher concentration in interstitium than in the effluent or in the veins. Infusion of adenosine increases the

concentration in the veins, but only marginally or not at all in the interstitium. Exogenous adenosine in concentrations below 1 μM does not reach the interstitium. Therefore its effects must be mediated by the endothelium[31,88]. The interstitial inosine concentration is about 0.6 μM[27].

Detection of nucleotide breakdown

Purine levels increase by ATP-breakdown. The adenosine levels rise, but as a consequence of the short half-life never reach high levels. Inosine and even better hypoxanthine are useful indices of ATP breakdown. We measure differences between aorta and coronary sinus/great cardiac vein concentration of hypoxanthine to evaluate possible protection of drugs during procedures like coronary angioplasty[115] or atrial pacing stress test[53]. Hypoxanthine has also been used to assess the severity of birth asphyxia in neonates[64,111] or to follow the condition of critically ill patients[109].

Metabolic disease

Metabolic diseases may influence purine levels[69]. Gout is a disorder of urate metabolism, restricted to humans. It is characterized by hyperuricemia and recurrent attacks of acute arthritis. Its usual cause is a reduced excretion of urate by the kidney. It may also be secondary to conditions with increased urate production. The basis of the Lesch-Nyhan syndrome is a deficiency of the enzyme hypoxanthine-guanine phosphoribosyltransferase. The blockade of hypoxanthine salvage induces an increased rate of de novo purine synthesis[52]. The levels of urate in blood, urine and cerebro-spinal fluid are increased. Urate deposits are found in kidneys and joints, leading to nephropathy and gout. The neurological symptoms include severe mental retardation, spasticity and choreoathetosis. Patients with adenine phosphoribosyltransferase deficiency often present with 2,8-dioxyadenine stones[69]. Adenosine deaminase and purine nucleoside phosphorylase deficiency cause a severe combined immunodeficiency by an accumulation of deoxyadenosine triphosphate. Xanthinuria, urinary xanthine stones and myopathy are features of xanthine oxidase deficiency.

Patients with Duchenne muscular dystrophy have lower adenine, but higher adenosine and xanthine levels than normal controls[14].

Drugs may alter the concentration of purines. Patients treated with cytostatics have often very high urate levels due to cell necrosis. Diuretics, like the thiazide derivatives, increase urate levels by decreased kidney excretion[125]. Some anti-epileptics decrease urate levels by an unknown mechanism[72]. Dipyridamole increases adenosine concentration by an inhibition of cellular uptake. Mioflazine, which acts by a similar mechanism, is being tested as hypnotic. Allopurinol decreases urate production by inhibiting the enzyme xanthine oxidoreductase. Some of these drugs will be discussed in chapter 8.

Chapter 3

Adenine nucleotide catabolism

The energy for cardiac contraction is derived from breakdown of ATP to ADP. The available ATP within a myocyte is only sufficient for a few beats[65]. Under normal (normoxic) conditions, ADP is rapidly rephosphorylated by oxidative phosphorylation. Measurements with nuclear magnetic resonance spectroscopy indicate that the creatine kinase reaction:

$$ATP + Cr + H^+ \rightleftharpoons ADP + CrP$$

is in equilibrium (Cr = creatine, CrP = creatine phosphate). The ADP concentration measured in homogenized tissue is higher than the free concentration by binding of ADP to contractile proteins.

An imbalance between oxygen demand and supply reduces oxidative phosphorylation. ATP levels fall and ADP levels rise. AMP levels rise by the adenylate kinase reaction:

$$ATP + AMP \rightleftharpoons 2\ ADP$$

AMP is catabolized by 5'-nucleotidase with formation of adenosine, or to IMP by AMP-deaminase. IMP is rapidly converted to AMP in skeletal muscle. In heart, however, this conversion is very slow[124]. 5'-Nucleotidase also catalyzes the breakdown of IMP to inosine. During normoxia hydrolysis of S-adenosylhomocysteine contributes significantly to adenosine production, but during ischemia most adenosine is produced by AMP[1].

Even when oxygen supply to a myocardial cell has completely stopped, depletion of ATP will take more than a few seconds. Creatine phosphate may act as an energy buffer for seconds. Its function is probably the transport of high-energy phosphate bonds from mitochondria to contractile proteins.

Nucleoside breakdown

Adenosine and inosine pass the cell membrane through the nucleoside transporter (see chapter 5). Adenosine is rapidly taken up into the cell and phosphorylated by adenosine kinase or deaminated to inosine by adenosine deaminase. Nucleoside phosphorylase breaks down inosine to hypoxanthine and

ribose-1-phosphate. The activity of this enzyme is almost absent in cardiac myocytes, but high in the endothelium[108]. Breakdown of hypoxanthine to xanthine and of xanthine to urate is catalyzed by an endothelial enzyme, xanthine oxidoreductase. In some species, but not in primates, uricase catabolizes urate to allantoin.

Rate of catabolism

In the isolated rat heart, net ATP breakdown occurs even if it is perfused with a well-oxygenated buffer. Shortly after preparation of the heart, the efflux of purines is high due to the inevitable period of ischemia. In the experiments described in appendix 6, we found a purine concentration of 0.57 μM in the effluent after equilibration. Consequently the decrease in adenine nucleotide levels is about 25 nmol/min per g dry weight (dwt). We found similar values in other experiments. There are several explanations for the purine loss. It could be the normal turnover rate of adenine nucleotides. The rate of nucleotide formation in vivo is equal to the rate of breakdown. In the isolated preparation, no substrates are provided for generation, and consequently, there is net breakdown. Another explanation is an increased rate of catabolism in isolated heart. The absence of erythrocytes in the perfusion buffer may limit the oxygenation in this model.

After 15 min stop-flow ischemia, the purine release is about 6 μmol/g dwt in the first five minutes reperfusion [appendix 6]. Van Bilsen et al. found that, ATP levels decreased from 18.9 to 9.3 μmol/g dwt by 30 minutes stop-flow ischemia, and to 5.7 μmol/g dwt by 90 minutes ischemia[128]. The rate of nucleotide breakdown depends on the model and the method of simulating ischemia. Pacing the heart during a period of hypoperfusion increases damage, as it elevates the oxygen demand. The purine efflux is higher, if the oxygen is removed from the buffer (anoxia), than if flow to the heart is stopped. A high flow causes washout of purines. Heart function just before ischemia may affect the severity of the ischemic injury. In isolated rat heart, time to onset of contracture is inversely related to pre-ischemic function [appendix 5]. Reduction of pre-ischemic function may be the mechanism by which some drugs provide protection against ischemia in various models and the clinical situation[29].

In several models purine production is a good quantitative measure of ischemia. The production of nucleosides depends on the AMP level. Addition of pyruvate reduces the AMP level in a normoxic glucose-perfused isolated heart preparation[45]. Pyruvate increases the CrP concentration and reduces the level of inorganic phosphate. Bünger et al. found a lowering of adenosine and inosine production by pyruvate in isolated guinea-pig heart[13]. Our results do not suggest such an effect in isolated rat heart [appendix 6].

To protect a heart against ischemic damage during heart surgery, it is arrested by high potassium and cooled. We evaluated adenosine as a possible adjunct to potassium cardioplegia. Before a 20-minute stop-flow period, isolated rat hearts were arrested with a three-minute high-potassium infusion [appendix 4]. Adenosine addition to this high-potassium cardioplegic solution accelerated cardiac arrest and improved post-ischemic function recovery. Addition of the adenosine deaminase inhibitor erythro-6-amino-9-(hydroxy-3-nonyl)purine (EHNA) buffer did not reduce the post-ischemic purine production. EHNA did increase the adenosine/inosine ratio after reperfusion. Therefore, the adenosine deaminase activity is presumably not important for the rate of purine production after ischemia. Zoref-Shani et al. concluded the same from experiments in cardiomyocytes[149].

Xanthine oxidoreductase

Xanthine oxidoreductase (XOD) activity is localized in the endothelium. Reports on the activity of this enzyme in homogenates of hearts of various species (especially man) are conflicting. We compared the apparent XOD-activity in isolated hearts from four rodent species [appendix 7]. In rat and mouse heart XOD activity was highest, in rabbit heart it was low, while guinea-pig heart showed intermediate activity. In the rabbit heart, increasing hypoxanthine concentrations caused increased production of xanthine, without altering the production of urate. The urate production in guinea-pig heart is comparable to that in rat and mouse, but xanthine production is low. We conclude that there are not only differences in XOD-activity between species but also in the ratio of the products. This difference in ratio contradicts the argumentation of Smolenski et al., who maintain that the absence of xanthine production in human heart provides evidence that it has no XOD activity[119].

Inosine infusion in isolated rat heart increases the hypoxanthine, xanthine and urate concentration. Elevation of the inosine infusion rate, increases in the xanthine and hypoxanthine concentrations, but reduces the urate concentration [appendix 2]. An explanation for this phenomenon is a competition between hypoxanthine and xanthine for XOD.

During coronary angioplasty, there is a difference between the urate levels of aorta and coronary sinus/great cardiac vein[63]. This finding suggests urate production and therefore XOD activity in the human heart. The activities in homogenates of human heart vary from less than 0.1 mU/g wet weight (wwt)[38,119] to more than 50 mU/g wwt73,130. In perfused human hearts, we found a low but detectable urate production (XOD = 0.31 ± 0.12 mU/g wwt) [appendix 7].

Chapter 4

Synthesis of adenine nucleotides

Pathways for adenine nucleotide synthesis

After reduction of the adenine nucleotide levels, repletion to normal concentrations is slow. Appendix 1 reviews the pathways for generation of these nucleotides. Briefly, there are four pathways:

1. phosphorylation of adenosine to AMP.
2. ribophosphorylation of adenine to AMP.
3. ribophosphorylation of hypoxanthine to IMP, followed by conversion to AMP.
4. de novo synthesis of IMP followed by conversion to AMP.

Many authors have investigated enhanced nucleotide regeneration by providing substrates for these pathways.

Adenosine phosphorylation

The incorporation of adenosine is faster than the other pathways[11,148]. The transport through the cell membrane limits the incorporation rate, not the adenosine kinase activity[9,36]. Reibel and Rovetto showed that hours of adenosine treatment restored post-ischemic ATP levels in isolated rat heart[105]. In an earlier study, they found no increase after 30 minutes treatment[104]. The elevation of adenosine concentration to augment nucleotide regeneration provides practical problems. The adenosine half-life in blood is only seconds[71]. The nucleoside affects hemodynamics at low concentrations (see chapter 6). Furthermore, the endothelium has an active adenosine metabolism and therefore acts as a barrier between the capillary lumen and the interstitium.

Adenine ribophosphorylation

The maximum rate of adenine incorporation into cardiac myocytes is about 50% of that of adenosine[11,148]. There are few reports on enhanced nucleotide regeneration by adenine in the in vivo/ex vivo heart. Ward et al. described

increased myocardial ATP levels by 24 hours adenine/ribose administration in the post-ischemic open-thorax dog[132]. In an earlier (preliminary) report, they found no effect on nucleotide levels after 90 minutes treatment[131]. We found no significant improvement after 45 minutes treatment in isolated rat heart [appendix 6]. The difference in species and model may explain why we did not find elevations in adenine nucleotide levels. Possibly the severity of ischemia and the length of treatment were not enough to find a marked improvement. Blood banks use adenine for the preservation of erythrocytes. Toxic effects on kidney after chronic adenine feeding have been described, but adenine/ribose preservation is in use for transplantation kidneys.

Hypoxanthine/inosine salvage

The incorporation of hypoxanthine is 10 times slower than adenosine incorporation in cardiomyocytes[11,148]. Inosine is only useful for regeneration after breakdown to hypoxanthine[36,87]. There is a considerable difference in nucleotide levels between isolated hearts treated with inosine during reperfusion and untreated hearts[54,144]. This difference is larger than can be explained from measurements of ^{14}C inosine incorporation[54]. The incorporation rates of inosine vary from 0.4 to 40 nmol/min per g dwt[2,36,54,148]. We were unable to find increases of nucleotides by inosine treatment in post-ischemic rat heart [appendix 6]. A 6-minute infusion of inosine in the pig did not increase cardiac nucleotide levels, but altered the ATP/ADP ratio [appendix 3]. The maximum conversion rate of IMP to AMP measured in cardiomyocytes is about 15 nmol/min per g dwt[11,36].

Infused inosine may affect adenine nucleotide levels in the post-ischemic myocyte in several ways:

1. Exogenous inosine enters an endothelial cell through the nucleoside transporter. Subsequently the nucleoside is broken down to hypoxanthine, which leaves the cell through a nucleobase transporter (see chapter 5). The hypoxanthine diffuses to a similar transporter of a myocyte and enters into the cell. Then hypoxanthine is incorporated into IMP and converted to AMP.
2. Infused inosine passes unaltered either through the endothelium or through clefts between the cells. It enters the myocyte through the nucleoside transporter. Then inosine competes with adenosine for transport both into

and out of the cell (see chapter 5). Adenosine kinase rapidly rephosphorylates the increased amount of intracellular adenosine.

3. Inosine affects both hemodynamics and metabolism, and may thereby alter nucleotide levels.

De novo synthesis

The production of IMP from precursors is very slow. Swain et al. reported that aminoimidazole carboxamide riboside (AICAriboside), which is converted in two steps to IMP by enzymes of the de novo pathway, may enhance nucleotide regeneration[120]. Other authors were unable to reproduce the improvement in ATP levels[60,83]. The agent increases left ventricular compliance in the post-ischemic isolated cat heart[84]. It also causes a progressive deterioration of function in the post-ischemic region in the open-chest dog[60].

Ribose

The incorporation rate of hypoxanthine into IMP, and adenine into AMP, and the de novo pathway are dependent on the availability of phosphoribosyl pyrophosphate (PRPP). The synthesis rate of PRPP of rat-heart myocytes with ribose as substrate is 50 nmol/min per g dwt[36]. Harmsen showed that hypoxanthine incorporation was faster when ribose was added[54]. We added ribose in our inosine and adenine incorporation study for a maximal incorporation rate [appendix 6].

Chapter 5

Transport of nucleosides and nucleobases across the cell membrane

There are four ways for purines to cross the cell membrane[99]:

1. facilitated transport of nucleosides
2. active nucleoside transport
3. nucleobase transport
4. non-mediated transport

Facilitated transport

Almost all cells possess a low affinity, high capacity, non-concentrative (passive) nucleoside transporter. There are, however, large differences in properties and numbers of transporters between species and cell types. Transporters of freshly isolated human, pig and guinea-pig erythrocytes exhibit directional symmetry (no difference between influx and efflux kinetics), but differential mobility of empty and loaded carriers. For instance the uridine-loaded carrier moves on the average 6 times faster than the empty carrier. The transporter of human erythrocytes stored for 1 or 2 weeks under blood bank conditions becomes asymmetrical. In isolated rat heart, adenosine transport (< 50 μM) follows Michaelis-Menten kinetics with a Km of 5 μM and a Vmax of 10 nmol/min per g tissue[62]. At concentrations above 50 μM non-saturable transport becomes important[43]. Rat-heart cells have a common transporter for adenosine, inosine and guanosine[47]. Geisbuhler et al. measured a Km value for adenosine of 6 μM, guanosine 18 μM, and an intermediate value for inosine[47]. Other authors find similar values for the affinity of adenosine[9]. Heaton and Clanachan argue that a Km of 10 μM represents an underestimation, which is caused by long incubation and interference of intracellular adenosine metabolism[56]. These authors measured a value of 146 μM in guinea-pig cardiomyocytes. The Vmax for adenosine transport in these cells is about 3 nmol/s per g protein[43,47].

Transporters can be blocked by inhibitors. Cell-types can be characterized by the percentage nucleoside transporters blocked by a nanomolar concentration of nitrobenzylthioinosine (NBTI) or by the inhibition of dipyridamole. In contrast to its effect in guinea-pig heart, dipyridamole does not inhibit transport in rat heart[62].

Inhibitors of the nucleoside transporter include many, mainly hydrophobic, substances with various structures like nimodipine, diltiazem, verapamil, lidoflazine and diazepam. Nucleoside transport inhibition of calcium channel antagonists occurs at concentrations far above those necessary for their effects on calcium channels. In cultured endothelial cells from calf aorta, 10 μM dipyridamole causes a 96% transport inhibition, while the inhibition by papaverine, prazosine and nifedipine is only 30-60%[82].

Inhibition of nucleoside uptake may decrease the intracellular adenosine concentration, and therefore the incorporation of adenosine. As the affinity for adenosine of adenosine kinase is higher than that of adenosine deaminase, inhibitors affect incorporation less than deamination. Dipyridamole shifts the balance between adenosine phosphorylation and deamination in human blood[26]. In case of high adenosine concentrations, transport inhibition may reduce substrate inhibition of adenosine kinase, and thus increase adenosine incorporation. Dipyridamole also inhibits the activity of purified adenosine kinase at low adenosine concentrations, and stimulates the activity under optimal conditions[28].

In dogs dipyridamole and mioflazine inhibit both adenosine uptake and release[12,74]. Therefore dipyridamole may limit nucleotide depletion during ischemia[59]. Uptake inhibition occurs, however, at lower inhibitor concentrations than release. This is an argument for an asymmetric transporter.

The nucleoside transporter bears similarities to the glucose transporter. Isolation of the nucleoside transporter is complex due to copurification with the glucose transporter, which is in about 20-fold excess[142].

Inosine infusion in isolated rat heart increases the concentration of adenosine in the effluent [appendices 2,6]. Combined infusion of inosine and adenosine results in a much higher effluent adenosine concentration than infusion of only adenosine. As inosine does not affect adenosine metabolism[36], inosine probably competes with adenosine for the nucleoside transporter. Geisbuhler et al. described competitive inhibition of adenosine transport by inosine in cardiomyocytes with an apparent Ki of 112 μM[47]. Our findings suggest that inosine in a nearly physiological concentration modifies adenosine transport, and therefore both intracellular and extracellular adenosine concentrations.

Active nucleoside transport

Active sodium-dependent nucleoside transport has only been described in a few specialized cell types, such as intestinal epithelium. Inhibitors of facilitated nucleoside transport may increase the nucleoside concentration in these cells, as they inhibit the efflux of the nucleosides.

Nucleobase transport

Cell types differ in the way they transport hypoxanthine. In some, hypoxanthine is transported by the same carrier as the nucleosides. In these cells dipyridamole inhibits hypoxanthine transport. In others there is a special nucleobase transporter for hypoxanthine. Human erythrocytes are equipped with a specific transporter with a Km of 200 μM[98]. This transporter exhibits directional symmetry. Its mobility is not influenced by hypoxanthine loading. NBTI, dipyridamole or nucleosides don't inhibit hypoxanthine transport in rat-heart cells[11]. Therefore, there is a separate hypoxanthine transporter.

Little is known about adenine transport. In human erythrocytes, there is a specialized transporter for adenine, distinct from the hypoxanthine transporter. This transporter is symmetrical and adenine loading does not alter its mobility. However, its affinity for adenine is so low (Km > 5 mM) that its measurement is hindered by the solubility of adenine[98]. Brown et al. report a high affinity (Km 1.6 μM) transporter for adenine in rat cardiomyocytes[11].

Non-mediated transport

Transport not mediated by a carrier has been described both for nucleosides and nucleobases. The rate is at least 20 times slower than carrier-mediated transport. It only becomes important with extremely high purine concentrations or when inhibitors are used.

Chapter 6

Hemodynamic effects of purines in isolated heart

Adenosine receptors

The action of adenosine in several cell systems involves external cell membrane receptors. The receptors are classified by an activation or inhibition of adenylate cyclase and the rank order of potency of adenosine analogues[40]. Adenosine analogues that are often used for receptor discrimination are N-phenylisopropyl-adenosine (PIA) and 5'-N-(ethyl-carboxamide)adenosine (NECA). The presence of A1 and A2 receptors in heart has been confirmed, an A3 receptor is under debate. The A1 receptor is characterized by:

* an inhibition of adenylate cyclase
* a rank order of potency of PIA > adenosine > NECA

The A2 receptor is characterized by:

* a stimulation of adenylate cyclase
* a rank order of potency of NECA > adenosine > PIA

An A3 receptor, without effect on c-AMP, but acting directly on calcium channels has been postulated in rat brain and pig ventricle[107].

In broken cell preparations, apart from extracellular adenosine receptors, there is a further class of sites which are especially sensitive for the purine domain of the adenosine molecule (P-sites). Presumably, the P-site is intimately associated with the adenylate cyclase catalytic subunit. Activation of this site by adenosine invariably inhibits enzyme activity. Cardiomyocytes possess A1 receptors[58], A2 receptors are only found in coronary arteries[20] (on the smooth muscle cells), and on the endothelium[113].

In isolated hearts infusion of adenosine causes vasodilation, in addition to negative chronotropy, dromotropy and inotropy. Adenosine is a potent vasodilator. Berne hypothesized that adenosine was the mediator of coronary autoregulation, reactive hyperemia and metabolic vasodilation[5,6]. There is a good correlation between coronary flow and the interstitial adenosine concentration during intra-coronary infusion of various amounts catecholamines[49]. Infusion of adenosine deaminase

(ADA) attenuates reactive hyperemia, but has no influence on autoregulation[35]. Decking et al. found no direct relationship between coronary conductance and interstitial adenosine concentration, while the adenosine levels were modified by hypoxia and infusion of ADA or coformycin[27]. The role of adenosine in coronary vasoregulation is not completely unravelled[41]. The increase of the coronary artery concentration of adenosine to a physiological level (0.1 μM) in isolated rat heart causes a small vasodilation [appendix 2]. The very slow rate of this increase in flow is surprising.

Endothelial receptors probably mediate the vasodilation induced by exogenous adenosine, because exogenous adenosine (< 1 μM) does not reach the interstitium (see chapter 2). Adenosine-analogues increase the level of c-AMP in isolated endothelial cells, with a potency corresponding to their affinity for the A2 receptor. However, the concentrations to exert this effect are 10 times higher than the levels which affect coronary flow[31]. A concentration of 1 mM has less effect than 0.1 mM, but this may be a P-site effect. Although adenosine-induced vasodilation parallels the increase in endothelial c-AMP, inhibition of adenylate cyclase does not alter vasoregulation[89]. Adenosine relaxes coronary artery rings better after contraction by prostaglandin F2α than by high potassium[103]. This observation may be important to unravel adenosine's mechanism of vasodilation.

Adenosine has a negative chronotropic and dromotropic effect on isolated preparations. Some authors suggest that adenosine mediates the bradycardia early during ischemia[139]. We showed that a high adenosine concentration added to potassium cardioplegia decreased arrest time [appendix 4]. Adenosine causes a hyperpolarization of the sinoatrial node cells by an activation of potassium channels[4] [appendix 4]. Acetylcholine has a similar effect, but in contrast to adenosine it is inhibited by atropine[141]. Adenosine potentiates the negative effects of the vagus on heart rate[95]. Besides an effect on potassium channels, there is also a reduction of calcium currents, and slow-time and voltage-dependent inward currents, but only after stimulation by isoproterenol[4].

Adenosine reduces the contractility of isolated atria, but only in very high concentrations. This effect probably depends on the level of cyclic-AMP (see chapter 7). Cerbai et al. state that the potassium agonist but not calcium antagonist effect of adenosine cause the hyperpolarization, shortening of action potentials and

negative inotropy[15].

Inosine and other purines

Inosine causes coronary vasodilation [appendix 2]. It is, however, 200-300 times less potent than adenosine[68]. Infusion of 30 to 400 μM inosine increased coronary flow dose dependently in an isolated rat-heart preparation. However, inosine transiently decreased flow half a minute after starting the infusion [appendix 2]. Inosine and adenosine were also infused together. Inosine (10 μM) alone did not cause vasodilation, and adenosine (0.1 μM) induced only a partial and slowly developing vasodilation. The combination, however, decreased flow within 30 seconds. This was followed by a rapid and (almost) complete vasodilation. Infusion of inosine together with ADA or the adenosine-receptor antagonist 8-phenyltheophylline induced a lower coronary flow than without these substances [appendix 2].

While adenosine lowers contractility, inosine may increase cardiac function. In isolated rabbit heart inosine increases dP/dt[32,75]. The influence of inosine on function and heart rate is negligible in isolated rat heart [appendix 2].

Some of the effects induced by inosine are similar to the effects of adenosine, while others are the opposite. We speculate that in the case of the former, they are mediated by increased adenosine. Inosine infusion increases the concentration of adenosine (see chapter 5). The effects of inosine, which differ from those induced by adenosine, may be caused by a number of speculative mechanisms. There may be a special receptor for inosine. Inosine may compete with adenosine for binding with a receptor, and thereby antagonize the effects of adenosine. However, inosine also reduced coronary flow when the adenosine receptors were blocked by 8-phenyltheophylline [appendix 2]. Which effect of inosine prevails depends on the local adenosine concentration, adenosine transport and number and type of receptors. In contrast to adenosine, inosine may reach considerable intracellular concentrations. Therefore it may exert its effect also inside the cell.

Adenine, hypoxanthine, xanthine and urate have no effect on heart function.

Chapter 7

In vivo effects

The purine effects in vivo are more complex than those described in the previous chapter. In vivo the purines interact with other systems such as the adrenergic system.

Effects of adenosine on autonomic nerves

Autonomic nerves have purinergic receptors. In the isolated rabbit heart, 1-100 μM adenosine reduces noradrenaline release after sympathetic stimulation via an effect on presynaptic receptors[137]. However, adenosine infusion does not alter the effects of sympathetic nerve stimulation in an open-thorax dog model[112]. In awake human volunteers, adenosine increases the levels of both adrenaline and noradrenaline[118]. The dissimilarity in model and species may cause the difference between these observations.

Depression of contractility by adenosine

A1-receptor activation inhibits the adenylate cyclase activity in the heart. Most authors find an inhibition of the catecholamine-induced rise in c-AMP by adenosine[33,34]. Adenosine itself, in contrast to its A1 selective analogues, consistently inhibits c-AMP generation in heart cells[114]. A P-site rather than a receptor-mediated effect of the nucleoside may be assumed. However, adenosine inhibits the effects of forskolin, a receptor-independent activator of adenylate cyclase[140]. Dipyridamole increases and theophylline decreases this effect, indicating that it is adenosine-receptor mediated. Removal of adenosine by ADA, or receptor inhibition by 8-phenyltheophylline, augments the effects of catecholamines in isolated rat heart[55]. However, the removal reduces the efficiency of the heart, possibly secondary to a metabolic effect. Adenosine does not inhibit the effects of the stabile c-AMP analogue dibutyryl-c-AMP[140]. It does reduce the positive inotropic effect of the phosphodiesterase inhibitor 3-iso-butyl-1-methylxanthine, without an effect on c-AMP or c-GMP levels[8].

In summary:

1. Adenosine reduces adenylate-cyclase activity via the extracellular A1-receptor or the P-site, and thus lowers the level of c-AMP.

2. The nucleoside decreases the effects of phosphodiesterase inhibitors without changing the concentration of cyclic AMP or cyclic GMP, and therefore alters the effects of these cyclic nucleotides.

3. However, adenosine does not inhibit the effects of a stabile c-AMP analogue.

We conclude that the mechanism is not fully clarified.

Effects of adenosine on heart rate

Although adenosine has negative chronotropic properties in isolated rat heart, it increases heart rate in conscious man[17,18,136]. Propranolol does not reduce this effect, but a combination of propranolol plus atropine does[18].

Effects of adenosine on blood pressure

In patients under anesthesia adenosine is being tested to accomplish controlled hypotension (see chapter 8). Recently Biaggioni et al. compared the effects in healthy volunteers and patients with autonomic failure[7]. In healthy volunteers a bolus injection of adenosine caused an early (30 s) increase in both systolic and diastolic blood pressure. A drop in systolic and diastolic blood pressure and bradycardia followed. These hemodynamic changes coincided with a deepening of thorax excursions. A continuous infusion increased systolic and decreased diastolic pressure and heart rate. There was an elevation of noradrenaline and adrenaline levels. Patients with autonomic failure were much more sensitive to adenosine. They reacted with a decrease in systolic and diastolic blood pressure, and a decrease in heart rate. The deepening of thorax excursions was less clear than in the healthy volunteers. The authors explain these effects by an activation of carotid chemoreceptors. This activation increases heart rate, systolic blood pressure and respiration. The respiratory effect stimulates the pulmonary stretch receptors, causing tachycardia.

Effects of inosine on heart function

Inosine increases contractility under several experimental conditions[24,30,67,117,127,143]. In the open-thorax rat preparation, inosine reduces dP/dt_{max}, heart rate and mean arterial pressure[61]. Inosine increases the potency of noradrenaline, while decreasing its maximal effect in rat atria[110]. The nucleoside has no effects in the open-thorax guinea pig[61].

We hypothesized that the positive inotropy induced by inosine would be mediated by c-AMP. Therefore, we infused inosine intravenously into an open-chest pig model [appendix 3]. We found an increase in dP/dt_{max} and a reduction of mean arterial pressure. There was no effect on left ventricular end diastolic pressure or heart rate. We found no increase in the cyclic AMP levels, and could, therefore, not confirm our theory.

Effects of adenosine in the kidney

One of the (side) effects of a low adenosine concentration is renal vasoconstriction. High adenosine concentrations induce vasodilation. An afferent-arteriolar A1-receptor mediates vasoconstriction and A2-receptors on both afferent and efferent arterioles mediate vasodilation[86]. Occupation of A1- and A2-receptors inhibits and stimulates renin secretion in the kidney[16].

Purine effects on glucose metabolism

Adenosine alters the insulin responsiveness in adipose tissue, skeletal muscle and heart. In adipocytes adenosine enhances glucose transport and oxidation, and inhibits lipolysis. In skeletal muscle adenosine reduces the effects of insulin. Adenosine increases glucose uptake in isolated rat heart[79]. In dogs the effect is similar, with a concomitant reduction of lactate uptake[66]. Adenosine potentiates the increase of glucose uptake by insulin[78]. Addition of ADA to adenosine annihilates this potentiation. The potentiation is independent of changes in blood flow, as nitroprusside is ineffective. Inhibition of the adenosine receptors annihilates the effects of insulin on glucose uptake.

As adult-pig erythrocytes lack a functional glucose transporter, the high inosine concentration in pigs may serve as energy source[142,145,146]. In dogs inosine shifts myocardial metabolism towards increased uptake of carbohydrates and away

from free fatty acid utilization[116]. The nucleoside increases the insulin levels[117]. In isolated mouse pancreas ß-cells, inosine stimulates insulin production. Nucleoside phosphorylase inhibitors attenuate the inosine-induced hormone production, but a combination of hypoxanthine and ribose is ineffective[10].

Adenosine and the immune system

Adenosine modulates the function of several inflammatory cells. Human B- and T-lymphocytes, polymorphonuclear leucocytes, monocytes, basophils and platelets possess an A2-receptor. Lymphocytes and neutrophils also have an A1-receptor[80]. Adenosine reduces the superoxide production of human neutrophils, stimulated with N-formylmethionylleucylphenylalanine (FMLP), but not all other stimulants[133]. Occupation of the A2-receptor of neutrophils both prevents adhesion and injury to endothelial cells[21].

Chapter 8

Influence of drugs on purine-induced effects and potential clinical applications

Modification of adenosine-induced effects

It is possible to administer purines to exert certain effects directly. Alternatively, transport inhibitors can increase the effects of endogenous purines. Adenosine analogues can selectively stimulate or inhibit purine receptors.

Dipyridamole enhances the adenosine concentrations by inhibiting nucleoside transport across the cell membrane. Through this mechanism dipyridamole potentiates the effects of adenosine[19]. It inhibits platelet aggregation and induces vasodilation (e.g., for thallium-201 scintigraphy).

The methylxanthines theophylline and caffeine block purinergic receptors. Only concentrations that are far above the normal (therapeutical) concentrations inhibit phosphodiesterase[44,102]. Caffeine antagonizes the effects of a continuous adenosine infusion[118]. Heart rate increases less and diastolic blood pressure decreases less. The effects of caffeine on blood pressure and, hypothetically, vasoconstriction may be the cause of the association between coffee consumption and cardiovascular morbidity and mortality.

Adenosine-provoked pain

Healthy human volunteers tolerate only limited doses of adenosine. High concentrations cause discomfort or pain in thorax, neck and jaws, sometimes mimicking angina pectoris, palpitations, colicky pains in the stomach, dry mouth, stuffed nose and restlessness[17,18,81,118]. Patients report that the pain provoked by adenosine is similar to the pain from angina pectoris or myocardial infarction[122]. Adenosine-provoked pain is reduced by theophylline and aggravated by dipyridamole[121]. Therefore Sylvén et al. suspect adenosine to play a role in the pain due to ischemic heart disease[121]. The mechanism of angina-like pain and vasodilation are different. There are differences in the dose-response relation and the time of onset of the effects[123]. The lowest pain-producing dose induces maximal vasodilation. Patients with duodenal ulcer state that the epigastric pain induced by

adenosine is equivalent to their ulcer pain[135]. In asthmatic patients inhalation but not intravenous infusion of adenosine provokes bronchospasm[76].

Adenosine and arrhythmias

In patients with spontaneous supraventricular arrhythmias, adenosine is able to slow heart rate or terminate the arrhythmias[134]. In a small number of patients, the nucleoside terminated the tachycardia except in the patients with an atrium flutter[134]. Addition of dipyridamole reduced the effective dose tenfold[134]. The ability of adenosine to block atrioventricular conduction allows diagnosis and treatment for most supraventricular tachycardias. The short half-life and the absence of negative inotropic effects make it moderately safe if administered during ventricular tachycardia[51].

In a dog model with a total occlusion of the left anterior descending coronary artery (LAD), intraventricular administration of adenosine or dipyridamole reduces the number of arrhythmias. There is also a reduced number of arrhythmias, including ventricular fibrillations, after release of the occlusion. Adenosine may serve as a natural anti-arrhythmic agent in vivo[129].

Adenosine-induced controlled hypotension

Adenosine is a potent vasodilator. This property has brought some investigators to test adenosine for the induction of controlled hypotension during surgery. Controlled hypotension reduces blood loss and facilitates surgery. Öwall et al. reported on successful adenosine-induced hypotension in five relatively young patients without reported lung or heart disease during neurolept anesthesia[93]. A 30 % reduction in systolic tension was associated with a 42% reduction of myocardial oxygen consumption. In another series, adenosine induced hypotension in six patients without alterations of heart rate, myocardial oxygen consumption, catecholamine levels or lactate extraction. Two patients had marginal ST-segment changes. However, in a third, there was a severe ST-segment depression possibly caused by coronary steal[92]. The myocardial effects of adenosine-induced hypotension were therefore tested in dogs with an 80% reduction of the left anterior descending coronary artery[91]. The aggravation of ischemia by coronary steal and reduced perfusion pressure balanced the protection by a decrease in heart rate and left-

ventricular pressure, which led to a reduction of oxygen consumption. Subendocardial ECG and lactate extraction, indices of ischemia, were not affected. To assess the influence of adenosine on coronary steal, patients with stabile angina pectoris received theophylline during exercise testing. Theophylline reduced both pain and ST-segment depression during submaximal workload, and increased the maximal work load[39]. This indicates that adenosine possibly induces coronary steal in cardiac patients. In the anesthetized pig adenosine was compared to sodium nitroprusside and nitroglycerine for the induction of controlled hypotension[90]. In contrast to the other substances, adenosine caused a stable reduction of systolic pressure, without effect on heart rate. Perfusion, measured with microspheres, of cerebrum, cerebellum, the spinal area, muscles, heart and the splanchnic area was unaltered or increased. Adenosine did not reduce the renal perfusion, but there was a dramatic reduction in urine production.

Cardiac protection

In several models purine derivatives have been shown to protect against the effects of myocardial oxygen shortage. We showed the benefits of the addition of adenosine to potassium cardioplegia in the isolated rat heart [appendix 4]. Inosine prevents the slow post-ischemic deterioration of function in isolated guinea pig heart[144]. Takeo et al. showed protection by adenosine, inosine and hypoxanthine in isolated rabbit heart[126]. The mechanism of protection by inosine remains unclear.

Inosine protects against myocardial damage by adrenaline administration[22,23]. A potential mechanism is the increase in adenosine levels, causing a reduced increase in c-AMP levels by the catecholamines.

Administration of large quantities of purines may provoke attacks of gout or induce stone formation. Urate levels should therefore be monitored carefully.

Xanthine oxidase (XO) is one of the possible mediators of reperfusion damage by free radicals. Ischemia may partially convert xanthine dehydrogenase (XD) to XO. During reperfusion, there is the combination of a high hypoxanthine concentration with the reintroduction of molecular oxygen. This situation can cause a high XO activity and thereby production of superoxide radicals.

Allopurinol, a drug employed in the treatment of gout, is both substrate and inhibitor of XOD. The inhibition is competitive with low allopurinol concentrations,

and non-competitive with high concentrations[42]. Oxypurinol, the product of allopurinol breakdown by XOD, is an even stronger non-competitive inhibitor of XOD.

A number of studies evaluated allopurinol or oxypurinol for protection against ischemia or reperfusion induced damage. Damage can be measured as infarct size, number of arrhythmias, loss of cardiac function. Several studies in various models showed protection[3,25,50,77,96,101,138]. However, in comparable studies these drugs were ineffective[37,70,94,101,106]. There are several explanations for the differences. Allopurinol may delay cell death, but does not limit infarct size after a longer reperfusion period[85]. There may be a critical length of ischemia, during which allopurinol protects, while it is ineffective against longer or shorter ischemic periods. Allopurinol works better, if given hours before the ischemic period. This time may be necessary to allow breakdown of allopurinol to oxypurinol. However, oxypurinol has little effect, just as allopurinol, if given shortly before reperfusion[70]. An alternative explanation is that an unknown product of allopurinol metabolism is able to protect the heart. This would explain, why allopurinol has the best effects when given about 18 hours before ischemia. By facilitating electron transport, allopurinol could also enhance the reduction of triphenyl tetrazolium chloride, one of the dyes most commonly used for the detection of vital tissue[97].

Protection may be independent of the XOD activity. In rat heart, protection by allopurinol is similar to that by hypoxanthine, which is a substrate for XO[77]. Even in species in which XOD is hardly detectable, like man, pig or rabbit[25,37,100] [appendix 7], allopurinol may exert a protective effect. There are, however, mechanisms of protection by allopurinol different from XO inhibition. Allopurinol has weak scavenger activity. It scavenges ClO_2 and HOCl, but not superoxide or hydroxyl radicals[25]. Allopurinol may also act as an electron transfer agent from ferrous iron to ferric cytochrome c[97]. Allopurinol does not modify antioxidant properties of extracellular fluid, because the scavenging potential of normal biological fluids is tremendous[147].

Chapter 9

Summary

There are several reasons why purines are important in the heart. Under normal, physiological, conditions these substances may exert several effects. During oxygen shortage, purines rapidly leak out of the cells. Under these conditions the nucleosides probably cause vasodilation and bradycardia. Purines are potentially useful both for diagnostics and for therapy in patients.

In many species the normal plasma concentration of the nucleosides adenosine and inosine is below 1 μM. Concentration differences may exist between the compartments of the heart. The endothelium is a metabolically active barrier between the capillary lumen and the interstitium. Enzymes in the endothelium break down adenosine and inosine, which are produced by adenine nucleotide degradation. Xanthine oxidoreductase catabolizes hypoxanthine further to xanthine and urate in some species, but not in man.

The ischemic purine production of a heart gives a good indication of the amount of adenine nucleotide breakdown, and therefore of the severity of oxygen shortage. Both activity and the properties of xanthine oxidoreductase are species-dependent. During ischemia xanthine dehydrogenase may be converted to xanthine oxidase. The oxidase form may induce free radical formation.

The ischemic purine release depletes the adenine nucleotide content. Without treatment nucleotide levels remain depressed for days. Administration of purines may accelerate the nucleotide regeneration. Adenosine has the highest incorporation rate in isolated cells. In vivo treatment with adenosine is useless. The half-life of adenosine in blood is only a few seconds. Adenosine in a low concentration does not reach the interstitium, high concentrations of the nucleoside affect hemodynamics. Incorporation of inosine (via hypoxanthine) is much slower. Inosine competitively inhibits adenosine transport through the cell membrane, as both nucleoside use the same transporter. In theory inosine may reduce adenosine transport both into the cell as well as out of the cell.

A1 and A2 receptors mediate the hemodynamic effects of adenosine. Activation of the A2 receptor induces vasodilation. The A1 receptor mediates

negative inotropy, chronotropy and dromotropy. Adenosine derived from cardiac cells acts directly on smooth muscle cells, while exogenous adenosine probably activates receptors on the endothelium. Inosine also causes vasodilation, but only at a concentration, which is 200-300 times higher than the active adenosine concentration. Part of the effects of inosine may be attributed to an increase of the adenosine concentration. Other effects of inosine include a transient vasoconstriction and an increase in heart function.

The effects of adenosine in vivo are markedly different from the effects in an isolated organ. Adenosine modulates sympathetic effects. The nucleoside reduces noradrenaline release after sympathetic nerve stimulation. Activation of the A1 receptor inhibits adenylate cyclase. Furthermore, adenosine may also antagonize the intracellular effects of cyclic-AMP. Adenosine also acts on the renin system and on glucose metabolism.

Healthy volunteers react with an increase in heart rate and blood pressure to adenosine administration. High doses of adenosine may provoke an angina pectoris like pain. Another potential application of adenosine is the induction of controlled hypotension during anesthesia. However, maximal coronary vasodilation can cause a steal phenomenon. Adenosine may improve the protection by potassium cardioplegia, by accelerating the time to arrest. Its ability to block atrioventricular conduction may be of help in the diagnosis of arrhythmias.

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Samenvatting

Er zijn verschillende redenen waarom purines belangrijk zijn in het hart. Onder normale, fysiologische, omstandigheden oefenen deze stoffen diverse effecten uit. Tijdens zuurstoftekort lekken purines snel uit de cel. Nucleosiden kunnen dan o.a. vaatverwijding en bradycardie veroorzaken. Purines zijn mogelijk bruikbaar zowel voor diagnostiek als bij behandeling van patiënten.

Onder normale omstandigheden zijn de intra- en extracellulaire concentraties van de nucleosiden adenosine en inosine laag. Tussen de compartimenten van het hart kunnen er concentratie verschillen bestaan. Het endotheel vormt een metabool actieve barrière tussen het capillaire lumen en het interstitium. Adenosine en inosine, gevormd bij adenine nucleotide afbraak, worden in het endotheel verder gecatabolizeerd tot hypoxanthine. In sommige species breekt xanthine dehydrogenase hypoxanthine verder af tot xanthine en vervolgens uraat. De activiteit van dit enzym bij de mens is echter laag.

De ischemische purine productie geeft een goede indicatie van de hoeveelheid afgebroken nucleotiden, en vormt daarom een maat voor de ernst van het zuurstof tekort. De activiteit en de eigenschappen van xanthine dehydrogenase verschillen per species. Tijdens ischemie kan dit enzym omgezet worden tot xanthine oxydase, wat tijdens reperfusie vrije radicalen kan produceren.

Door het weglekken van purines tijdens en na ischemie ontstaat er een verlaagd adenine nucleotide gehalte in de hartspiercel. Zonder behandeling duurt het dagen voordat dit tekort is opgeheven. Versneld herstel van de nucleotide gehalten is in principe mogelijk door het aanbieden van purines die ingebouwd kunnen worden. Adenosine heeft de hoogste inbouwsnelheid in geïsoleerde cellen, doch in het bloed wordt het binnen seconden afgebroken. Bovendien passeren lage concentraties de endotheel barrière niet en hebben hoge concentraties hemodynamische effecten. Inbouw van inosine (via hypoxanthine) verloopt vele malen trager. Inosine remt competitief het adenosine transport over de celmembraan, daar beide nucleosiden van een gemeenschappelijk transporteiwit gebruik maken. Theoretisch kan inosine daarom zowel de uitwas, als ook de (her)opname van adenosine verminderen.

De hemodynamische effecten van adenosine verlopen via A1- en A2-

receptoren. Activatie van A2-receptoren veroorzaakt vaatverwijding, A1-receptor activatie heeft negatieve inotropie, chronotropie en dromotropie tot gevolg. De vaatverwijding bij intracoronaire toediening van adenosine komt mogelijk tot stand via endotheliale A2-receptoren. Inosine veroorzaakt eveneens vaatverwijding, doch bij een 200-300 maal hogere concentratie dan adenosine. Een belangrijk deel van de effecten van inosine is te verklaren uit een door inosine veroorzaakte verhoging van de adenosine concentratie. Bij sommige effecten, zoals contractiliteitsverhoging is de werking van inosine echter tegengesteld aan die van adenosine.

In vivo zijn de effecten van adenosine duidelijk anders dan in het geïsoleerde hart. Adenosine beïnvloedt zowel het sympathische als het parasympathische systeem. Er is een werking op sympathische zenuwcellen, op adenylaat cyclase, en mogelijk ook op intracellulaire effecten van cyclisch AMP. Verder beïnvloedt adenosine het renine systeem en het glucose metabolisme.

Ondanks het vaatverwijdend effect van adenosine veroorzaakt toediening ervan bij gezonde vrijwilligers een verhoging van de hartfrequentie en de bloeddruk. Verder kunnen zij een pijnlijk drukkend gevoel op de borst krijgen, lijkend op angina pectoris. Bij patiënten onder narcose kan adenosine gebruikt worden om de bloeddruk tijdelijk laag te houden, wat bij bepaalde ingrepen belangrijk kan zijn. De coronaire vaatverwijding, die hierbij optreedt, kan echter een 'steal fenomeen' veroorzaken. Het toevoegen van adenosine aan een oplossing met hoog kalium gehalte versnelt het stilleggen van een hart door deze oplossing. Een diagnostische toepassing is toediening van adenosine tijdens ritmestoornissen om op veilige wijze achter de aard van deze stoornissen te kunnen komen.

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UITLEG VOOR DE LEEK

ATP is een stof die zorgt voor de overdracht van de energie. Deze energie komt vrij bij de verbranding van suikers en vetten en wordt overgedragen aan energie-verbruikende processen, zoals het samentrekken van een spier. Afbraakproducten van ATP zijn onder andere adenosine, inosine en hypoxanthine. Deze afbraakproducten, purines, komen in het bloed voor in concentraties die verschillen per soort en die afhankelijk zijn van omstandigheden.

In het hart treedt bij zuurstoftekort afbraak van ATP op. De concentraties van purines in het uit het hart afkomstige bloed worden dan hoger. De productie van purines kan als maat worden gebruikt voor de ernst van het zuurstoftekort. In een aantal zoogdiersoorten kan hypoxanthine in het hart verder afgebroken worden tot xanthine en vervolgens tot uraat. Het enzym dat voor deze omzetting zorg draagt, xanthine dehydrogenase, kan tijdens zuurstoftekort omgezet worden tot xanthine oxydase. Dit laatste enzym is in staat zuurstofradicalen te produceren, die de oorzaak kunnen zijn van celbeschadiging. Bij doorstroming van geïsoleerde harten van enkele knaagdiersoorten kon hoge activiteit van xanthine dehydrogenase worden aangetoond; dit in tegenstelling tot het bij hart-transplantatie vrijgekomen mensehart. Xanthine oxydase is daarom hoogst waarschijnlijk niet verantwoordelijk voor beschadiging in het menselijk hart.

Na een periode van zuurstoftekort is het ATP gehalte van een cel gedaald. Zonder behandeling duurt het dagen voordat een tekort, ontstaan door een kwartier zonder doorstroming van het hart, is hersteld. In principe kan het ATP gehalte versneld worden genormaliseerd door het aanbieden van een aantal purines. In een geïsoleerd rattehartmodel bleek het echter niet mogelijk het ATP gehalte te verhogen door gedurende 45 minuten inosine of adenine aan te bieden.

Het transport van adenosine en inosine door de celmembraan vindt plaats door middel van het nucleoside-transport eiwit. Adenosine en inosine remmen elkaars transport over de celmembraan, omdat zij een competitie aangaan voor het gemeenschappelijke transporteiwit. Inosine toediening aan een geïsoleerd rattehart veroorzaakt een stijging van de hoeveelheid adenosine die uit het hart weglekt. Dit is waarschijnlijk het gevolg van een competitie tussen adenosine, wat normaal na vrijkomen opnieuw in de cel wordt opgenomen, en inosine. De cellen die de wand

van de (haar)vaten vormen, de endotheel cellen, vormen een metabool actieve barrière tussen de hartspiercellen en het bloed.

In een geïsoleerd hart geeft adenosine in een lage concentratie vaatverwijding. Hogere concentraties veroorzaken een vermindering van de hartfrequentie en de contractiekracht. Adenosine samen met kalium legt het hart sneller stil dan kalium alleen. Inosine veroorzaakt een kortdurende vaatvernauwing, gevolgd door een blijvende vaatverwijding. De oorzaak van de vaatverwijding is een verhoogde adenosine concentratie.

Toediening van adenosine aan gezonde vrijwilligers geeft, in tegenstelling tot het effect in een geïsoleerd hart, een verhoging van de hartfrequentie. Bovendien wordt de bloeddruk door adenosine verhoogd en treedt een op angina pectoris lijkende pijn op de borst op. Een belangrijke oorzaak voor de verschillen tussen de effecten van adenosine in een geïsoleerd orgaan en een intact organisme is het effect van adenosine op het autonome (onwillekeurige) zenuwstelsel. Mogelijke toepassingen van adenosine zijn bloeddruk-verlaging tijdens operaties, toediening voor diagnostiek van hartritmestoornissen en verbeterde bescherming van het voor een operatie stilgelegde hart.

APPENDIX PAPERS

1: P. van der Meer and J.W. de Jong. Regeneration of adenine nucleotides in the heart. In: J.W. de Jong (ed) Myocardial Energy Metabolism, Martinus Nijhoff Publishers, Dordrecht/Boston/Lancester 1988;283-289

2: P. van der Meer and J.W. de Jong. Inosine transiently decreases coronary flow, but potentiates vasodilation by adenosine. Am J Physiol 1990 (in press)

3: P. van der Meer, W. Czarnecki, and J.W. de Jong. Cardiac effects of inosine in the pig are not mediated by c-AMP. (Submitted)

4: J.W. de Jong, P. van der Meer, H. van Loon, P. Owen, and L. Opie. Adenosine as adjunct to potassium cardioplegia: effect on function, energy metabolism and electrophysiology. J Thorac Cardiovasc Surg 1990 (in press)

5: R. Stroeve, J.W. de Jong, and P. van der Meer. Relationship between pre-ischemic heart function and onset of contracture. (Submitted)

6: P. van der Meer, T. Huizer, and J.W. de Jong. Effect of inosine and adenine on nucleotide levels in post-ischemic rat heart with and without pyruvate. (Submitted)

7: J.W. de Jong, P. van der Meer, A.S. Nieukoop, T. Huizer, R.J. Stroeve, and E. Bos. Xanthine oxidoreductase activity in perfused heart of various species, including man. (Submitted)

Appendix 1

Regeneration of Adenine Nucleotides in the Heart

P. van der Meer and J.W. de Jong

ABSTRACT

The fast adenine nucleotide degradation, which takes place in the ischemic heart, contrasts with the slow regeneration after ischemia. There are 4 pathways for adenine nucleotide regeneration:
1. De novo synthesis. This is a slow process. IMP is built from small molecules in a number of steps. The process can be accelerated to some extent by administering ribose. Ribose is incorporated in 5-phosphoribosyl-1-pyrophosphate (PRPP) and the amount of PRPP is rate-limiting. Administration of 5-aminoimidazol-4-carboxamide ribose (AICAribose), which can be converted to an intermediate of this pathway, has also been tried for a faster regeneration, but this compound produced a deterioration of heart function. The conversion of IMP, end product of both de novo synthesis and salvage pathway, to AMP is very slow.
2. Salvage of hypoxanthine or inosine. Inosine has to be catabolyzed to hypoxanthine before it can be incorporated. IMP is formed from hypoxanthine in one step, with subsequent conversion to AMP. PRPP is rate-limiting, so adding ribose enhances the incorporation rate. Inosine has some hemodynamic effects.
3. Adenine ribophosphorylation. Adenine is incorporated in AMP in one step. PRPP is again rate-limiting. No nucleotide conversion is necessary. Toxic effects of long-term adenine administration have been described.
4. Adenosine phosphorylation. This is the fastest pathway for adenine nucleotide regeneration. Adenosine causes a renal vasoconstriction together with a systemic vasodilation (hypotension). These adverse reactions limit the practical value of adenosine.
Even though there is limited clinical experience with some of these compounds that enhance postischemic ATP pool repletion, none have been examined for this purpose in humans.

INTRODUCTION

Ischemia leads to a rapid fall in the adenine nucleotide content of the heart. The rate of ATP decrease depends on factors like tissue perfusion (collaterals), heart function, temperature, species. The dephosphorylated breakdown products of ATP, the purine nucleosides and oxypurines, leak out of the myocardial cell. A simple scheme of ATP breakdown is shown in Fig. 1. The amount of adenine nucleotides will be roughly halved within the first hour of ischemia. A return of flow will cause a restoration of the adenine nucleotide levels (if the cells are not irreversibly damaged), but this takes days to accomplish.

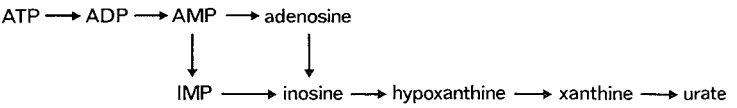

Fig. 1. Adenine nucleotide catabolism: ATP loses its phosphate groups. AMP can be converted to either adenosine or IMP. They are both broken down to inosine. This is catabolyzed to hypoxanthine, xanthine and urate.

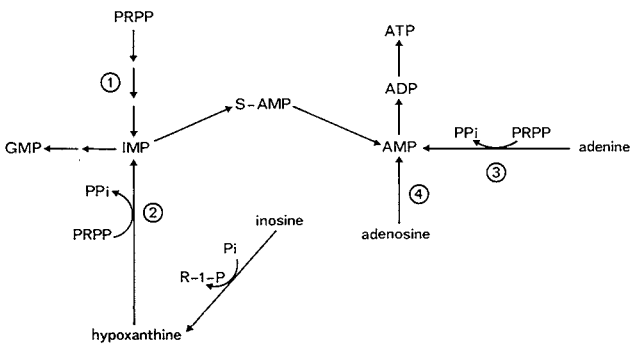

Fig. 2. Pathways for adenine nucleotide regeneration: 1. De novo synthesis; 2. Salvage of hypoxanthine and inosine; 3. Adenine ribophosphorylation; 4. Adenosine phosphorylation. The pathways 1 to 3 utilize 5-phosphoribosyl-1-pyrophosphate (PRPP). Pi = inorganic phosphate, PPi = inorganic pyrophosphate, R-1-P = ribose-1-phosphate, S-AMP = adenylosuccinate.

The effects of ischemia on a cardiac myocyte are complex and their mechanisms have not fully been unravelled. There is a good correlation between the effect of myocardial protection during ischemia and the postischemic content of adenine nucleotides. Thus it is plausible that a fast restoration of ATP levels will promote the recovery of the postischemic heart. To regenerate ATP in the heart, four pathways are available, as was pointed out by Goldthwait[14] 30 years ago:

1. De novo synthesis;
2. Salvage of hypoxanthine or inosine;
3. Adenine ribophosphorylation;
4. Adenosine phosphorylation.

A scheme of these pathways is shown in Fig. 2.

1. De novo Synthesis

This pathway involves the production of the purine skeleton from small molecules in 10 steps (see Fig. 3). The first step is the formation of 5-phosphoribosylamine (PRA) from 5-phosphoribosyl-1-pyrophosphate (PRPP) and an amino group donor like glutamine. The last step is the formation of IMP. There are no branching points between PRA and IMP. The conversion of IMP, endproduct of both de novo synthesis and salvage pathway, to AMP or GMP is slow.

Little is known about the value of enhanced guanine nucleotide regeneration. Adenylosuccinate synthetase catalyzes the reaction: IMP + aspartate + GTP -> adenylosuccinate + GDP + P_i. The activity of this enzyme is very low[5]. Adenylosuccinate lyase catalyzes the reaction: Adenylosuccinate -> AMP + fumarate, but the same enzyme is also involved in one of the last steps of the de novo synthesis.

Zimmer et al.[39] estimated that 0.04% of the nucleotide pool can be regenerated per hour this way. The rate of ATP synthesis is enhanced after ischemia[26], and limited by the concentration of PRPP[11].
Ribose induces a considerable enhancement of the PRPP-concentration and of the rate of adenine nucleotide synthesis in heart[38], although the ribokinase activity in most tissues is low[2]. There is some clinical experience with ribose. Zöllner et al.[40] described the successful symptomatic treatment of a patient suffering from an AMP-deaminase deficiency with high oral doses of ribose (maximum 60 g per day). Segal et al.[29] described a hypoglycemic effect of ribose. Human lymphocytes, exposed to extremely high ribose concentrations (25-50 mM), showed inhibition of their DNA repair synthesis[41].

Another possibility is to give 5-amino-4-imidazolcarboxamide riboside (AICAriboside), which can be phosphorylated to its ribotide (AICAR). This intermediate in the pathway becomes IMP in two steps. Swain et al.[31] showed that a 24-hour infusion of AICAriboside caused an accelerated repletion of ATP and GTP pools in postischemic canine myocardium. In an isolated cat heart Mitsos et al.[27] described an increase in postischemic left ventricular developed pressure, but this was accompanied by a considerable increase in left ventricular compliance.

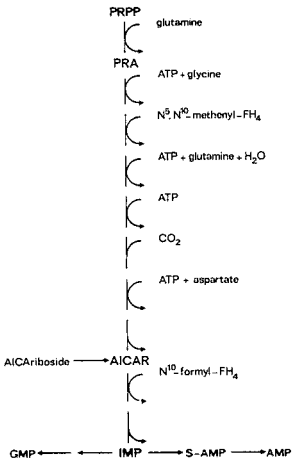

Fig. 3. De novo synthesis: Formation of IMP from small molecules in 10 steps. IMP is converted to AMP. AICAR = 5-aminoimidazol-4-carboxamide riboside, FH4 = tetrafolate, PRA = phospheribosylamine, PRPP = 5-phosphoribosyl-1-pyrophosphate, S-AMP= adenylosuccinate

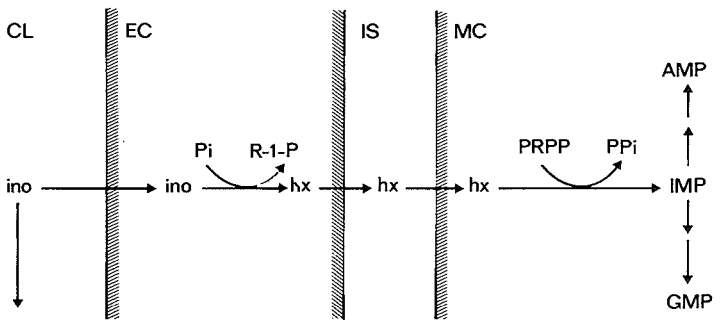

Fig. 4. Inosine has to be broken down to hypoxanthine within the endothelium before incorporation in adenine nucleotides of the myocytes. (cl = capillary lumen, ec = endothelial cell, hx = hypoxanthine,ino= inosine, is = interstitial space, mc = myocyte, Pi = inorganic phosphate, PRPP = 5-phosporibosyl-1-pyrophosphate.)

In an open-chest dog model, Hoffmeister et al.[18] observed a marked impairment of regional function by postischemic AICAriboside. This deterioration progressed in time. We conclude that the value of accelerating de novo synthesis is limited.

2. Salvage of Hypoxanthine or Inosine

Hypoxanthine can be utilized to restore the ATP content of the heart. The enzyme hypoxanthine guanine phosphoribosyl transferase catalyzes the reaction hypoxanthine + PRPP -> IMP + PP_i. PRPP is rate-limiting[38]. Harmsen et al.[16] found that adding ribose stimulates the hypoxanthine incorporation rate. (For a discussion about the conversion of IMP to the other nucleotides, we refer to the part on de novo synthesis.) Although the rate of adenine nucleotide production from this pathway is faster than the de novo synthesis, it is limited by the availability of PRPP and the activity of adenylosuccinate synthetase. Hypoxanthine enters the cell by simple diffusion. As a substrate for xanthine oxidase (XO), hypoxanthine may cause generation of free oxygen radicals in species showing XO activity in the heart. Its presence in the human heart has to be clearly demonstrated. Addition of a xanthine oxidase inhibitor could be important.

Inosine has been described as a cardioprotective agent[1,13,32]. Mammalian cells lack inosine kinase[35]. Therefore inosine has to be broken down by nucleoside phosphorylase: Inosine + P_i -> hypoxanthine + R-1-P before it can be incorporated in the adenine nucleotides. As nucleoside phosphorylase is not located in the cardiomyocyte[28], the ribose-1-phosphate can be utilized for PRPP production, but presumably not in the myocyte. It is possible that inosine incorporation in the myocyte can be enhanced by ribose addition, but as far as we know this has never been tried.

In contrast to hypoxanthine, inosine does effect contractility, heart rate and blood flow. Juhász-Nagy and Aviado[22] found an increase in myocardial contractility in dog heart, while Hoffmeister et al.[17] observed a decrease in rat heart. There is conflicting evidence about the effect on heart rate[21,22]. Coronary vasodilation has been reported by many authors[21,32]. Inosine inhibits the uptake of adenosine in the heart[25], both nucleosides use the same carrier to enter the cell[36]. Although inosine is used in Mediterranean countries as a cardiotonic, the effects of this nucleoside on humans have not extensively been investigated. Recently de Jong et al.[7] described the apparent inosine uptake by the human heart together with an increase in dP/dt. We conclude that inosine and hypoxanthine are possibly valuable to the postischemic heart, but more research on this subject is necessary.

3. Adenine Ribophosphorylation

Little research has been devoted to this pathway. The enzyme adenine phosphoribosyl transferse catalyzes the reaction: Adenine + PRPP -> AMP + PP_i. The adenine concentration in blood is very low[24]. If adenine is administered, the amount of PRPP becomes rate-limiting. Addition of ribose can enhance the incorporation rate. Ward et al.[33] gave adenine + ribose to a dog heart, in which the ATP levels had been halved. They repleted ATP in 24 hours, while it took 5 days without treatment. In isolated ventricular cells Dow et al.[8] compared the incorporation rate of adenosine, of adenine + ribose, and of hypoxanthine + ribose. They showed that

adenosine incorporation is twice as fast as adenine + ribose, and twenty times as fast as hypoxanthine + ribose. Adenine lacks hemodynamic effects; it has no effect on adenosine transport[25]. Clifford and Story[6] described growth disturbances in young animals fed a high adenine diet. Recently Yokozawa et al. [37]showed that metabolic abnormalities resembling chronic renal failure could be produced by long-term feeding of adenine to rats. Human experience with adenine is available from its use as an erythrocyte preservative in blood banks. We conclude that adenine could be a valuable compound, provided its safety for postischemic ATP regeneration can be proved.

4. Adenosine Phosphorylation

This pathway provides the fastest possibility to regenerate adenine nucleotides. The enzyme adenosine kinase catalyzes the reaction: Adenosine + ATP -> AMP + ADP. Adenosine has been used for cardioprotection with varying success[9,15,18,19,20,23,30]. Gerlach et al. showed that adenosine incorporation is very fast in endothelial cells[12]. Breakdown of adenosine in blood is very fast: a half life of 10 seconds has been estimated. It is possible that most of the infused adenosine will be either catabolyzed or incorporated in the endothelial cells, with little adenosine incorporated in the myocardial cells. Infused adenosine is also taken up very rapidly by other cells (especially in the lungs). Adenosine transport is mediated by a carrier. Dow et al.[8] reported that in isolated cardiac myocytes adenosine transport is rate-limiting for adenosine incorporation.

Adenosine exerts other effects mediated by purinergic receptors. It is a potent vasodilator and plays presumably an important role in coronary vasoregulation[4]. Some groups described the slowing of atrioventricular impulse conductance and a decrease in heart rate[3,34]. Foker et al.[10] reported that adenosine caused a vasoconstriction of the renal vessels. In humans, adenosine is used against arrhythmias (superventricular tachycardias). We conclude that adenosine is potentially useful although it has many side effects.

CONCLUSION

There is no clinical experience with enhanced ATP regeneration in the post ischemic heart. For this purpose inosine or adenine in combination with ribose seem the most promising compounds.

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Appendix 2

Inosine transiently decreases coronary flow, but potentiates vasodilation by adenosine

Peter van der Meer, and Jan Willem de Jong

ABSTRACT

To test whether inosine interferes with the action of adenosine, we investigated the effects of inosine, adenosine, 8-phenyltheophylline and adenosine deaminase on isolated rat heart. Inosine did not change heart rate or function, but increased the effluent adenosine concentration. It also decreased exogenous adenosine uptake and breakdown. After the start of the inosine infusion (20 μM), a transient 30% decrease of coronary flow took place within a half minute. It caused sustained vasodilation, dependent on the concentration (30 to 400 μM). After the infusion we observed a transient vasodilation. A non-vasoactive inosine concentration (10 μM) combined with an adenosine concentration that increased flow by 60% (0.1 μM) raised vasodilation by another 60%. Infusion of adenosine, adenosine deaminase or 8-phenyltheophylline did not influence the inosine-induced transient decrease in flow, indicating that this decrease is independent of adenosine and its receptor. We conclude that inosine 1) potentiates the vasodilation induced by adenosine, 2) is a coronary vasodilator, but probably also a vasoconstrictor.

INTRODUCTION

The blood concentration of inosine, a purine nucleoside, is below 1 μM in humans, rats and dogs, and about 10 μM in pigs[4,7,12,13]. It increases when adenine nucleotide breakdown occurs. In heart the nucleoside is incorporated in both adenine and guanine nucleotides, or catabolized to hypoxanthine, xanthine and urate[14]. Inosine inhibits myocardial adenosine uptake[20,23,24].

Inosine has a number of hemodynamic effects, including coronary vasodilation. However, it is far less potent than adenosine, its precursor[18]. In humans and dogs it has a positive inotropic effect[6,8,19], but it decreases contractility in rat heart[15]. Inosine also increases heart rate in dogs[19]. Adenosine has negative inotropic, dromotropic and chronotropic properties[2,9,29].

Little attention has been paid to the interference of inosine with the action of adenosine. We hypothesized that inosine potentiates exogenous and endogenous adenosine vasodilation through its inhibition of adenosine uptake. Because we noted transient flow reduction by inosine, we designed experiments to document this effect. Thus we investigated the biochemical and functional effects of adenosine/inosine on isolated rat heart, using adenosine deaminase to destroy endogenous adenosine and 8-phenyltheophylline to block adenosine receptors.

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). Inosine and adenosine were purchased from Merck, Darmstadt, FRG. The purity of the inosine and adenosine was 98%. The inosine contained some adenosine (about 0.001%). Adenosine deaminase from calf intestine was purchased from Boehringer, Mannheim, FRG; it was desalted with a PD-10 column (Pharmacia, Uppsala, Sweden). 8-Phenyltheophylline was supplied by Sigma, St. Louis, MO, USA.

Heart perfusion

Male Wistar rats (249 ± 26 g, n=29), with free access to food and water, were anesthetized intraperitoneally with 0.5 ml (30 mg) sodium pentobarbital. Hearts were rapidly removed and cooled in ice-cold 0.9% w/v NaCl solution until they stopped beating. Retrograde perfusion of the aorta according to Langendorff was performed with a modified Tyrode's buffer, pH 7.4. The buffer consisted (in mM) 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. Depending on the protocol inosine, adenosine, 8-phenyltheophylline or adenosine deaminase was added (see Protocols). Mixtures were passed through a 0.45-μm filter and equilibrated with 95% O_2/5% CO_2. The perfusion temperature was adjusted to 37°C with a thermocouple-regulated electric heater just prior to the perfusion canula. The canula was fitted with an A-F6 temperature probe, connected to a DU-3 monitor (Ellab, Copenhagen, Denmark). The perfusion pressure, measured with a Statham P23Db transducer (Hato Rey, PR, USA), was held constant at 70 mmHg. The perfusion apparatus had a valve for rapid switching between two perfusion buffers. All hearts were stabilized for 15-20 min before the protocols were carried out.

Purine assay

Adenosine, inosine, hypoxanthine, xanthine and urate in perfusion buffer and myocardial effluent were determined in 200-μl samples by our high performance liquid chromatographic method[17]. The samples from the first protocol were frozen and kept below -20°C until determination. The samples from the second protocol were kept at 4°C and determined as soon as possible, but always within 48 hours.

Myocardial function

Function was monitored with a Konigsberg F5-2 pseudoisometric force transducer (Hugo Sachs, March/Freiburg, FRG) connected to the apex of the heart. Developed tension was calculated as the difference between peak systolic tension and resting tension. The applied resting tension was five gram at the start of the experiment.

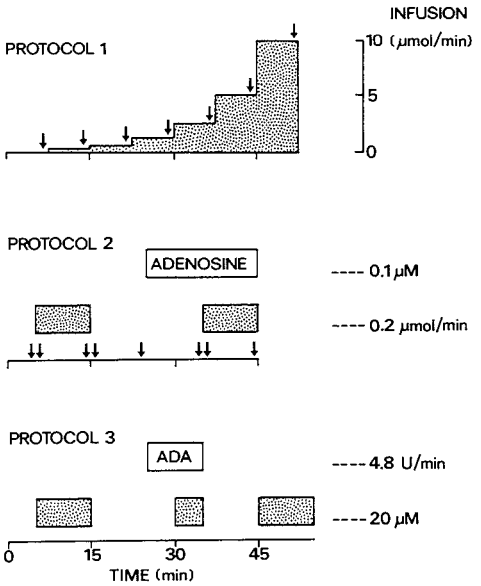

FIG. 1. Protocols 1-3: Inosine infusion is represented by the shaded areas. Samples for purine assay were collected at times indicated by arrows. Essentially, protocol 3 was also used to test the effect of 5 μM 8-phenyltheophylline; it then replaced adenosine deaminase (ADA).

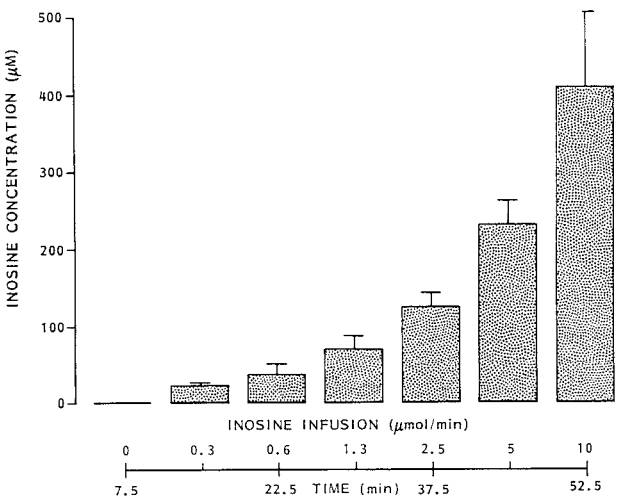

FIG. 2. Protocol 1: The inosine concentration in the effluent as a function time and inosine infusion rate (means ± SD, n=7).

Coronary flow

An electromagnetic flow transducer (Skalar Transflow 601, Delft, The Netherlands) was used to measure the flow in the perfusion line.

Statistical analysis

Results are expressed as means ± SD. Statistical analysis was performed with two-way analysis of variance or Student's t-test; $p > 0.05$ (two-tailed) was considered nonsignificant.

Protocols:

Protocol 1. A fixed volume of inosine solution was infused into a mixing chamber in the perfusion apparatus with a rate-adjustable roller pump (Ismatec MV-MS3, Zürich, Switzerland). In this chamber the inosine solution was diluted with standard perfusion buffer. The amount of test-compound, infused per minute, was held constant, while its concentration depended on the flow through the heart. The mixture was warmed and gassed with 95% O_2/5% CO_2. After a 7.5-min control period, every 7.5 min the inosine infusion rate was doubled (see Figure 1). The initial infusion rate was 0.31 μmol/min, the final one 10 μmol/min. At the end of every 7.5-min period, function, heart rate and coronary flow were assessed and both effluent and perfusion buffer samples were collected. A 7.5-min period was chosen because this was the time necessary to reach a steady state.

For comparison a group of control perfusions was performed in which hemodynamics were assessed and samples were collected at 7.5 and 52.5 min. These equal the time before inosine infusion and that of the highest inosine infusion rate, respectively.

Protocol 2. A concentrated inosine solution was added with the pump to the perfusion buffer just prior to the canula. After a 5-min control period, inosine was infused for 10 min at a rate of 0.2 μmol/min. After a 10-min washout period, the perfusion buffer was switched to one containing 0.1 μM adenosine. When a test-compound was given by switching the valve, the buffer concentration was fixed, while the amount per minute varied. This adenosine infusion lasted 20 min. During the second half, inosine was again infused at a rate of 0.2 μmol/min (see Figure 1). Samples for purine assay were collected at the times indicated in Figure 1. Function, heart rate and coronary flow were assessed every minute.

Protocol 3. After a 5-min control period, the heart was perfused for 10 min with buffer containing inosine (20 μM). Following a 10-min washout period, adenosine deaminase (ADA, 4.8 U/min) was added with a roller pump to the buffer for 10 min. During the last 5 min, ADA was infused into inosine-containing buffer. After a second 10-min washout period, the heart was again perfused for 10 min with inosine-containing buffer alone to exclude time effects on the preparation. Hemodynamics were monitored continuously.

Essentially the same protocol was used for the experiments with 5 μM 8-phenyltheophylline (8-PT). In those studies the receptor blocker replaced ADA.

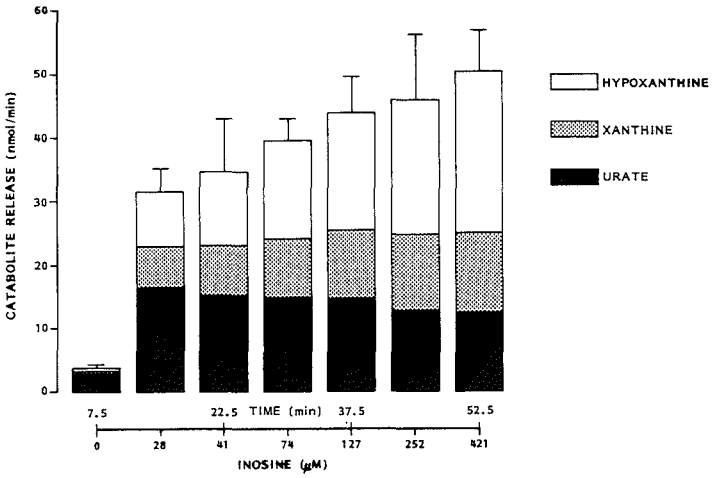

FIG. 3. Protocol 1: Catabolite production. Hypoxanthine and xanthine production increased with increasing inosine infusion rates, while urate production decreased. Total inosine breakdown decreased from 10% at the lowest to <1% at the highest infusion rate. On the x-axis time and inosine concentration in perfusion buffer are shown. Means ± SD, n=5-7.

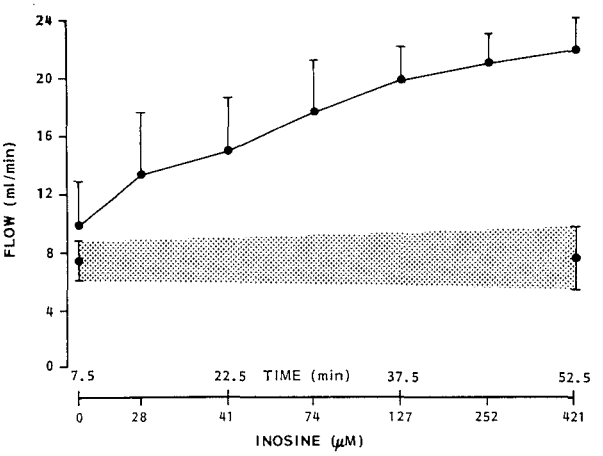

FIG. 4. Protocol 1: Flow increased with increasing inosine concentrations. The shaded area represents the values in the control group. Means ± SD, n=6-7.

RESULTS

Protocol 1

Figure 2 shows the inosine concentration in the effluent represented as a function of the amount infused. The inosine concentration in the buffer, measured in samples drawn from the perfusion apparatus just prior to the heart, compared well with the values calculated from the pump speed and the flow through the heart. At the highest infusion rate, the effluent inosine concentration was 409 ± 97 μM. The differences between the inosine concentration in effluent and perfusion buffer were very small, except for the lowest inosine infusion rates. We calculated the production of catabolites by multiplying the concentration in the effluent by the flow. At all inosine infusion rates, the production of hypoxanthine, xanthine and urate exceeded that of the pre-infusion values (Figure 3). Concomitant with the increasing inosine concentrations, we found a rise in the production of hypoxanthine ($p<0.001$) and xanthine ($p<0.001$), but a decrease of the urate production ($p<0.05$). Figure 4 depicts the dose-dependent increase of the coronary flow by inosine. The flow at the beginning of the experiment is within the ranges reported by other authors. At all inosine infusion rates, the flow increased compared to the pre-infusion values. When the inosine infusion started, a transient vasoconstriction took place, but we did not quantify this phenomenon using protocol 1. The coronary flow did not change in the control group. In the course of the experiment, developed tension went down to 76 ± 8% (inosine group) and 88 ± 12% (control group). Resting tension and heart rate showed no differences between the two groups.

Protocol 2

Figure 5 shows the effluent concentration of adenosine and inosine. Within one minute of inosine infusion (about 18 μM), the adenosine concentration increased from 14 ± 7 to 30 ± 15 nM ($p<0.005$), to remain 26 ± 13 nM ($p<0.005$ vs. the value before inosine infusion) till the end of the first inosine infusion. The switch to 0.1 μM adenosine increased the effluent adenosine concentration from 7 ± 5 to 17 ± 5 nM ($p<0.001$). The combined infusion of inosine and adenosine increased the perfusate adenosine concentration to 44 ± 13 nM ($p<0.001$) within the first minute and to 52 ± 16 nM ($p<0.001$ vs. the value before inosine infusion) after 10 min. Figure 6 shows the effects on the coronary flow. Within the first minute of inosine infusion, flow decreased transiently from 9.1 ± 1.8 to 6.3 ± 1.5 ml/min ($p<0.001$), followed by a return to the pre-infusion value. After the inosine infusion, a transient vasodilation was seen from 9.6 ± 1.3 to 10.7 ± 1.4 ml/min ($p<0.001$). While adenosine was infused, the coronary flow slowly increased from 8.6 ± 1.3 to 14 ± 2 ml/min ($p<0.001$). Within a minute after inosine infusion started, coronary flow decreased to 11 ± 3 ml/min ($p<0.001$), followed by a rapid increase to 18.8 ± 1.3 ml/min ($p<0.001$ vs. the value before inosine infusion). During the experiment there was some decrease in developed tension, but resting tension (and heart rate) did not change.

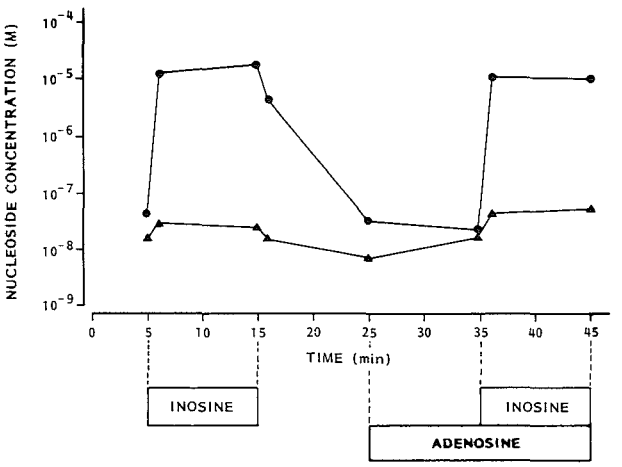

FIG. 5. Protocol 2: Concentration of adenosine (triangles) and inosine (dots). Increase in the inosine concentration caused a rapid increase in the concentration of inosine breakdown products (not shown) and adenosine. Inosine (0.2 μmol/min) infused together with adenosine (0.1 μM) increased the effluent concentration of adenosine more than separate infusion of inosine and adenosine. Mean values, n=8. For SD, see text.

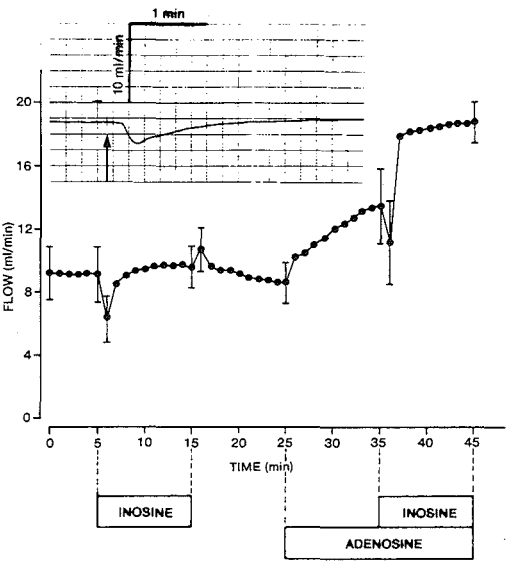

FIG. 6. Protocol 2: Potentiation of adenosine's vasodilator effect by inosine. Start of inosine infusion (0.2 μmol/min) without adenosine caused a transient vasoconstriction, followed by a return to the pre-infusion values (inset: actual flow record; arrow indicates start inosine infusion). The flow increased slowly during infusion of 0.1 μM adenosine. When inosine and adenosine were infused together, the vasoconstriction was followed by a rapid vasodilation. Means ± SD, n=8.

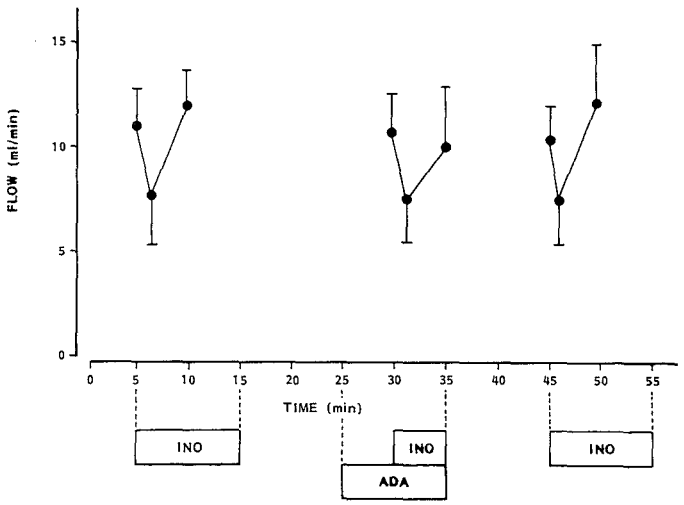

FIG. 7. Protocol 3: Inosine (INO, 20 μM) was given with and without adenosine deaminase (ADA, 4.8 U/min). The switch to inosine-containing buffer caused transient vasoconstriction in all cases. Five minutes after the start of inosine, ADA plus inosine gave a lower coronary flow than inosine alone. Replacement of ADA by 8-phenyltheophylline, an adenosine receptor antagonist, gave similar results. Means ± SD, n=4.

Protocol 3

We used this protocol to compare three conditions: inosine perfusion without adenosine deaminase or 8-phenyltheophylline, inosine perfusion during the second half of an ADA or 8-PT infusion, and inosine perfusion starting 10 min after the washout of enzyme or blocker. Within the first minute of inosine infusion, flow decreased about 30% in all three conditions (Figure 7). ADA had no effect on flow during perfusion without inosine or on the transient decrease of flow due to inosine infusion. After 5 min of inosine infusion, coronary flow changed from 10 ml/min to 12 ± 2, 10 ± 3 and 12 ± 3 ml/min before, during and after ADA infusion, respectively. The flow during the combined ADA and inosine infusion was significantly lower then before ($p<0.01$) and after ($p<0.05$).

With 8-PT in stead of ADA, essentially the same results were obtained: in four experiments coronary flow was 8.3 ± 1.9, 6.9 ± 1.6 and 7.6 ± 1.2 ml/min before, during and after drug infusion, respectively. The values during the combined 8-PT and inosine infusion were significantly lower than before ($p<0.02$) and after ($p<0.001$) combined infusion.

DISCUSSION

In vivo nucleoside concentrations

In human blood the inosine concentration is 0.2 μM[13]. Pacing-induced angina increases the plasma concentration in the coronary sinus from 0.6 to 1.5 μM (22).

In infarcted heart tissue, inosine amounts to 2.9 μmol/g dry weight, which suggests extremely high interstitial concentrations[11]. In pigs the coronary venous inosine concentration increases from 15 to more than 100 μM due to local ischemia (7). In rat blood the concentration varies between 0.2 and 1.6 μM[4]. Thus, the inosine concentrations used in our experiments are supra normal, but likely to occur under pathological conditions.

In humans the normal values for adenosine are in the range from 0.06 to 0.14 μM[3]. It follows that the adenosine concentration used is within the normal range.

Xanthine oxidase/dehydrogenase activity

We were able to recover most of the inosine infused in the effluent. Uptake and incorporation or catabolism were relatively small. Hypoxanthine production increased with increasing inosine concentrations. Augmentation of the inosine concentration infused increased xanthine production and decreased that of urate, but the sum of xanthine and urate production remained constant (Figure 3). Our values for xanthine and urate production during inosine infusion compare well with the activity of xanthine oxidase/dehydrogenase found in rat-heart homogenate after removal of low molecular weight inhibitors[26]. This finding suggests that in the intact heart these inhibitors do not affect enzyme activity.

We considered the possibility that vasoconstriction may result from conversion of xanthine dehydrogenase to xanthine oxidase: Diversion of urate production through the oxidase pathway would generate superoxide and other noxious humors that can destroy endothelial derived relaxing factor. However, the ischemic conditions needed for this conversion[26] were presumably not met during isolation and perfusion of the rat hearts.

Increased adenosine concentration by inosine

In protocol 2 inosine doubled the adenosine concentration within a minute (Figure 5). The inosine-containing buffer was contaminated with some adenosine, but this amounted to less than 10% of the adenosine found in the effluent. The major fate of adenosine infused was uptake, followed by catabolism or incorporation. Infusion of both adenosine (0.1 μM) and inosine (18 μM) increased the adenosine concentration in the effluent to about 50% of the infused concentration. Using dog heart Olsson et al.[23] described the inhibition of adenosine uptake by inosine. Aussedat et al.[1] demonstrated a decrease in [^{14}C]inosine incorporation in isolated rat heart when adenosine was added. The findings of inosine interference with adenosine metabolism could be explained by a competition with the adenosine carrier. Dipyridamole fails to inhibit the carrier in rat heart, in contrast to its effect in other species[16]. Alternative explanations for the increased adenosine concentration are inhibition of adenosine deaminase or increased adenosine production. Dow et al.[10] state that inosine (1-200 μM) does not alter the activity of adenosine deaminase, adenosine kinase, 5'-nucleotidase or AMP deaminase. We conclude that increased adenosine production is unlikely because in that case the increase in adenosine concentration would have been smaller with exogenous adenosine.

Effects of inosine on contractility and heart rate

Inosine did not change heart rate or function. Thus we failed to find the negative inotropy described in an open-chest rat-heart model[15]. Its effect on heart rate is controversial[6,8,19,21]. In dog and human heart[6,8,19], inosine has a positive inotropic effect. We ascribe these discrepancies to differences in model and function assessment.

As discussed above, inosine induced a rise in effluent adenosine. The concentration of the latter, however, was too low to affect function[2].

Inosine as vasodilator

In the isolated rat-heart preparation, vasodilation by inosine occurs at high concentrations, i.e., between 30 and 400 μM (cf. Figure 4). In the second protocol, we showed that 0.2 μmol/min inosine (18 μM) did not cause significant vasodilation (Figure 6). Adenosine, 0.1 μM, increased flow by 60%. This vasodilation developed slowly in about 10 min. The combination of adenosine and inosine had a rapid effect (total increase of 120%, Figure 6). Decreased adenosine uptake may have caused this effect. ADA or 8-PT infusion together with inosine gave lower coronary flows than inosine infusion alone (cf. Figure 7). The infusion of ADA or 8-PT without inosine had no effect. These findings suggest that part of inosine's vasodilation is mediated through endogenous adenosine. However, the effect of ADA or 8-PT was rather small (<20%). Possibly higher concentrations of ADA would have had more effect. On the other hand, a similar infusion in isolated guinea-pig heart, markedly attenuated coronary vasodilator responses to global hypoxia[28]. In addition, a number of adenosine analogues[5] or pharmacological doses of inosine[21] potentiate the coronary dilator action of adenosine, given intravenously to the dog. This could be due to decreased uptake and breakdown outside the heart. Olsson et al.[23] showed, however, the correlation between apparent adenosine transport inhibition and vasodilation in canine heart to be weak. We conclude from the above that inosine-induced vasodilation is mediated by adenosine.

Inosine, a vasoconstrictor?

To the best of our knowledge, a decrease in coronary flow by inosine (Figures 6, 7) has not been described before. Trachtenberg and Sullivan[27] found that inosine increased blood pressure in the rat. Cessation of the inosine infusion caused a transient vasodilation (Figure 6). This suggests that inosine causes sustained vasoconstriction that is over-shadowed by adenosine vasodilation. Sakai et al.[25] reported vasoconstriction in hindlimb by inosine and adenosine, presumably mediated by a tryptaminergic mechanism. As the adenosine concentration has already increased at the time of vasoconstriction (Figures 5, 6), a decrease of adenosine concentration cannot be responsible. A possible mechanism for coronary vasoconstriction would be a competition between adenosine and inosine for a receptor, if inosine had a lower intrinsic activity. However, our experiments with 8-phenyltheophylline strongly suggest that the inosine-induced vasoconstriction is not mediated by an adenosine receptor.

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Appendix 3

Cardiac effects of inosine in the pig are not mediated by c-AMP.

Peter van der Meer, Wlodzimierz Czarnecki, Jan Willem de Jong

ABSTRACT

In order to evaluate the mechanism of inosine's positive inotropy, we examined the haemodynamic and biochemical effects of intravenous infusion of inosine (5 mg/min per kg) in nine anaesthetized open-chest pigs. After 6 minutes of infusion, we saw a 12 (SEM 4) % decrease in left-ventricular peak-systolic pressure ($p<0.05$), a 15 (5) % decrease in mean aortic pressure ($p<0.05$) and a 24 (6) % increase in dP/dt(max) ($p<0.005$). Heart rate and left-ventricular end-diastolic pressure were not affected. During the infusion the concentration of inosine increased from 15 (4) to 554 (40) μmol·litre^{-1} in the aorta, and from 11 (1) to 380 (17) μmol·litre^{-1} in the coronary sinus. The difference between the aorta and coronary sinus concentration was statistically significant after 4 and 6 minutes of infusion ($p<0.001$). The hypoxanthine and xanthine concentration increased too, but no significant arterial-venous differences were present. Inosine infusion did not change the cardiac levels of c-AMP or high-energy phosphates, except for a higher ADP level.
We conclude that inosine administration led to an increase in heart function, concomitant with a decrease in the mean aortic pressure and left-ventricular peak-systolic pressure. In the heart no breakdown in the heart of inosine and hypoxanthine could be detected. During the infusion there was uptake of inosine. Cyclic-AMP did not change, indicating that the inosine-induced increase in heart performance is not mediated by this compound.

INTRODUCTION

In an earlier study, we evaluated the effect of inosine on the human heart[1]. The results prompted a further investigation in the mechanism of the inotropic action of inosine using the open-chest pig model. We chose the pig because its cardiac microcirculation resembles that of man. Inosine increases myocardial performance, both during normoxia and ischemia, in many species[2-6].

Inosine is a breakdown product of adenosine, which in turn is a catabolite of adenine nucleotides. Adenosine and inosine are also precursors for adenine nucleotide generation (for a review, see ref. 7). Both compounds use the nucleoside carrier to pass the cell membrane. In contrast to inosine, adenosine is rapidly catabolised, never reaching high intracellular concentrations[8]. Inosine potentiates the effects of adenosine, by increasing the adenosine concentration, as it inhibits the (re-)uptake and breakdown of adenosine[9]. Both adenosine and inosine cause coronary vasodilation, but adenosine is 300 times more potent. It is possible that the inosine-induced vasodilation is partially mediated by adenosine. Aminophylline, an adenosine receptor inhibitor, decreases vasodilation by inosine[10].

Heart function depends on the interaction of calcium with the contractile proteins. The calcium concentration is regulated by Ca^{2+}-channels and Ca^{2+}-pumps.

Furthermore the calcium concentration is modified by Na^+, K^+ and H^+ through effects on the action potential and Na^+/Ca^{2+} exchange. The sensitivity of the contractile proteins to Ca^{2+} may be modified through effects on troponin. c-AMP affects heart function by an increased influx through the slow Ca^{2+}-channels, modulation of the Ca^{2+}-handling of the sarcoplasmatic reticulum and a reduced sensitivity of the contractile proteins for calcium by phosphorylation of troponin-I.

c-AMP levels may be increased by direct or indirect activation of adenylate cyclase or an inhibition of phosphodiesterase. Blockade of the ß-adrenoceptor reduces the effect of inosine on heart function[5,6]. Adenosine inhibits adenylate cyclase, mediated both through a membrane receptor (A1-site) and directly (P-site)[11]. Methylxanthines, with a structure similar to inosine, inhibit phosphodiesterase. We hypothesized, that inosine might increase the cardiac levels of c-AMP and thereby increase heart function. Thus the purpose of this study was to evaluate c-AMP as a possible mediator of inosine's positive inotropy.

METHODS

Chemicals

All chemicals were analytical grade. Water (used for liquid chromatography buffers) was purified with the Milli-Ro4/Milli-Q system (Millipore, Bedford, MA). Inosine was purchased from Boehringer (Mannheim, FRG).

Pig experiments

Nine domestic pigs of either sex, weighing 22-26 kg, were fasted overnight. They were anaesthetized initially with ketamine hydrochloride (20 mg/kg i.m.) followed by chloralose (100 mg/kg i.v.). Artificial ventilation with room air was maintained through an endotracheal tube with end-expiratory pressure of 0.5 kPa of water. Adequacy of ventilation was monitored by arterial pH, pCO_2 and pO_2 measurements. The temperature of the animals was kept within the range of 35 to 37°C with a heating pad.

A midline sternotomy was performed and the heart was suspended in a pericardial cradle. The coronary sinus catheter was placed under manual control via the right atrial appendage and secured with a purse string silk suture. A 6F catheter was positioned in the aortic arch via the carotid artery for collection of blood samples. Aortic blood pressure was measured from the catheter (filled with 0.9% NaCl) inserted into the femoral artery, advanced to the aorta and attached to a Statham P23Db pressure transducer (Hato Rey, PR, USA). A Gaeltec pressure transducer (type 16 CT) which had been mounted on the tip of a double lumen No 8 USCI catheter was introduced retrogradely via the right carotid artery into the left ventricle to obtain left-ventricular pressure (LVP) recordings.
The maximum rate of rise of LVP (LVdP/dtmax) was obtained by differentiation of pressure using a low-pass filter set at a frequency of 50 Hz. The haemodynamic parameters were recorded on a Watanabe WA 294 recorder (Watanabe Instruments, Tokyo, Japan) at a paper speed of 50 mm/s.

Inosine dissolved in 0.9% NaCl was infused in a dose of 5 mg/kg per min into the jugular vein. The compound was administered for 6 minutes to analyse the steady-state response. Pressure variables were recorded before and at the 6th minute of infusion to assess the function of the heart. Blood was collected from the

aorta and from the coronary sinus for purine analysis at the 4th and 6th minute of infusion. At the same time myocardial biopsies were taken for adenine nucleotide determinations.

Purine assay

Two ml of blood were immediately mixed with 2.0 ml of cold 8% $HClO_4$, present in pre-weighed 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 neutralized with 6 mol·litre^{-1} KOH plus 2 mol·litre^{-1} K_2CO_3. Potassium chlorate was removed by centrifugation in the cold. Nucleotides in the deproteinized, neutralized blood (1.5 ml) were absorbed on pre-washed Al_2O_3 (0.6 g) in a plastic column (internal diameter 8 mm), and purines eluted with 5 ml 10 mmol·litre^{-1} Tris-HCl pH 7.4. The column did not absorb considerable amounts of the purine compounds except for urate. The eluate was stored at -15°C. Purines were determined by high pressure liquid chromatography (HPLC), as described by Huizer et al[12].

High-energy phosphates

Transluminal myocardial biopsies were taken with a Tru-cut biopsy needle (Travenol, Deerfield, IL, USA), quickly washed in ice-cold 0.9% NaCl, frozen in liquid nitrogen and kept in liquid nitrogen until determination. The biopsies were homogenized by a micro-dismembrator (Braun, Melsungen, FRG) at liquid nitrogen temperature together with 0.5 ml of 4% perchloric acid. After centrifugation in the cold, 350 μl of the supernatant fluid was used for the nucleotide determination; the pellet was used for the protein determination. The high-energy phosphates were measured by a method described by Huizer et al[12]. The protein content was estimated according to Bradford[13] using a commercial protein assay (Bio-Rad, Munich, FRG) and bovine serum albumin as the standard.

c-AMP

The measurement of c-AMP was carried out using a commercial assay kit (Amersham, Amersham, UK).

Statistical methods

Statistical significance was evaluated by Student's t-test for paired observations. For multiple comparisons we applied a Bonferroni correction. $P<0.05$ was considered significant. All results are expressed as means ± SEM.

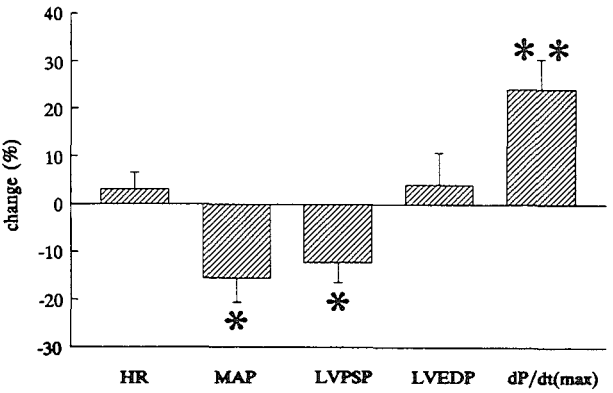

Fig. 1. Haemodynamic effects after six minutes of inosine infusion. There was an increase in left-ventricular dP/dt(max), while the mean aortic pressure and the left-ventricular peak-systolic pressures were decreased. The heart rate and left-ventricular end-diastolic pressure were unchanged. Means + SEM, n=7. HR = heart rate, MAP = mean aortic pressure, LVPSP = left-ventricular peak-systolic pressure, LVEDP = left-ventricular end-diastolic pressure, dP/dt(max) = maximum rate of rise of left-ventricular pressure, * $p<0.05$ and ** $p<0.005$ vs. before infusion.

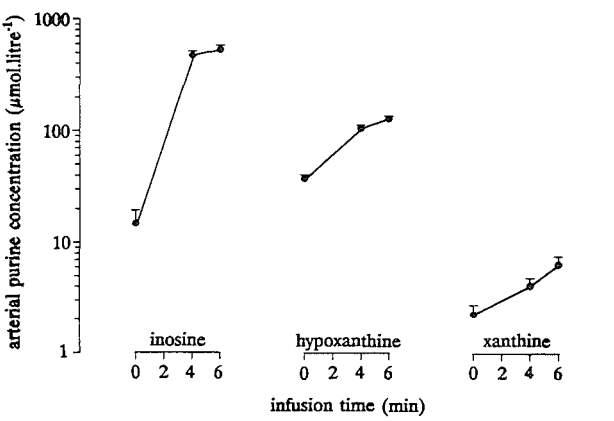

Fig. 2. Inosine infusion caused an increase in the arterial concentration of inosine, hypoxanthine and xanthine. Means + SEM, n=9.

RESULTS

Haemodynamic data

After six minutes of inosine infusion the mean aortic pressure (MAP) and left-ventricular peak-systolic pressure (LVPSP) decreased from 87 ± 6 to 73 ± 5 mmHg ($p<0.05$, $n=7$) and 102 ± 10 to 89 ± 8 mmHg ($p<0.05$, $n=7$), respectively (see figure 1). There were no significant changes in heart rate (HR) and left-ventricular end-diastolic pressure (LVEDP). The dP/dt(max) showed a 24% increase from 1400 ± 200 to 1700 ± 200 mmHg/s ($p<0.005$, $n=7$).

Biochemical values

The arterial and coronary sinus concentration of inosine increased from 15 ± 4 to 554 ± 41 μmol·litre^{-1} ($p<0.001$, $n=9$, figure 2) and 10.4 ± 1.2 to 375 ± 47 μmol·litre^{-1} ($p<0.001$, $n=9$), respectively. After four and six minutes of infusion, the difference between the arterial and coronary sinus concentration was also statistically significant ($p<0.001$, figure 3A). Both the arterial (figure 2) and coronary sinus hypoxanthine levels increased, but no significant differences appeared between the arterial and coronary sinus concentration (figure 3B). The same was true for the xanthine concentrations (figures 2 and 3C). The ATP, AMP, creatine phosphate and c-AMP levels in the ventricular muscle biopsies taken during inosine infusion were not statistically different from the levels before infusion (see table 1 and figure 4). After four min inosine infusion, the ADP level was increased ($p<0.01$). However after six minutes this increase was no longer significant.

DISCUSSION

Purine metabolism

Inosine infusion into the jugular vein increased the inosine concentration in both the aorta and the coronary sinus. During the infusion there is a large, statistically significant, concentration difference between the arterial and the venous side of the heart: the aortic value exceeded that in the coronary sinus by 150 μmol·litre^{-1}. We also found increases in the concentration of hypoxanthine and xanthine, the breakdown products of inosine. However, hypoxanthine and xanthine levels in the aorta and coronary sinus were not significantly different. The activity of xanthine oxidase/reductase, the enzyme which catabolizes the breakdown of hypoxanthine and xanthine, is very low in the pig[14]. Therefore, there is hardly any urate production. The difference between aortic and coronary sinus inosine concentration represents uptake in the heart.

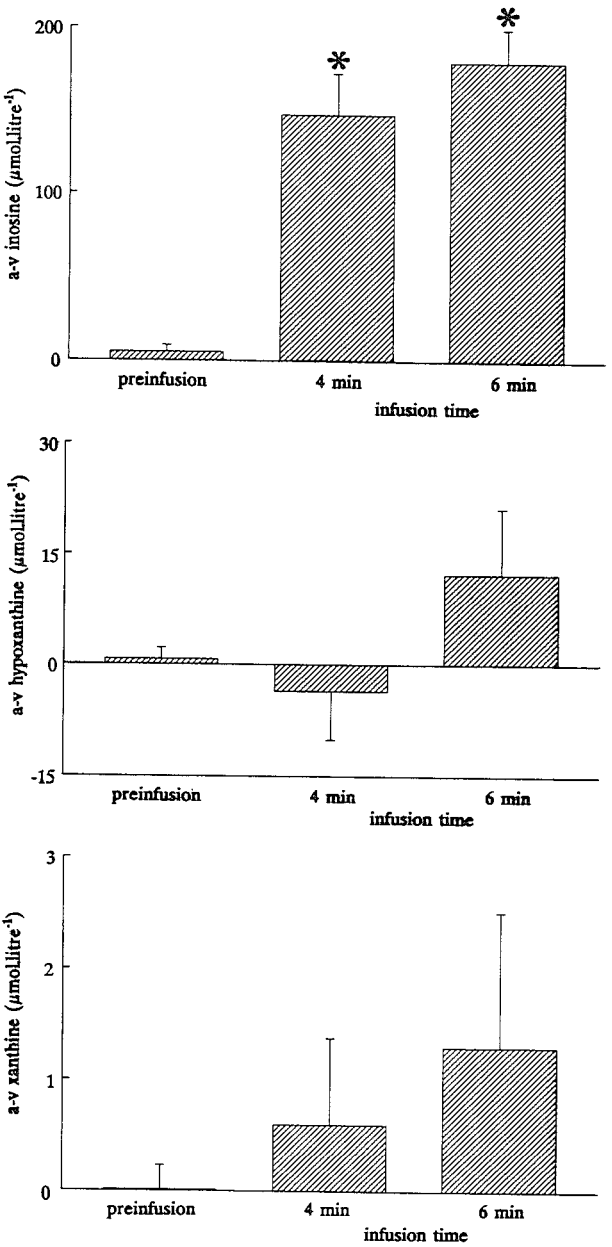

Fig. 3a-c. After 4 and 6 minutes of inosine infusion, there was cardiac uptake of inosine (a). The uptake or production of hypoxanthine (b) and xanthine (c) was not statistically significant. Means + SEM, n=9. * $p<0.001$ vs. before infusion.

Nucleotide metabolism

Inosine is a precursor in the adenine nucleotide salvage pathway. It is broken down to hypoxanthine, followed by incorporation into IMP. Subsequently IMP is converted to AMP. In our experiments some of the inosine may have been incorporated into IMP and subsequently into the adenine nucleotides. We did not find a significant rise of ATP. The incorporation of inosine is slow. In isolated rat heart incorporation rates between 0.4 to 20 nmol·min^{-1} per g dry weight have been described[15,16]. If the highest rate would apply to the pig heart, the increase would have been about 0.1 μmol per g protein, i.e., less then 0.5% of the normal value. We therefore assume that during the infusion inosine is present within the cell in a high concentration and most of it is not metabolized.

We did find a significant increase in the level of ADP after four min. After six min there was no significant increase. Kypson and Hait found reduced ATP and increased ADP levels, along with increased function in isolated rabbit heart[5]. Other authors report that inosine does not affect the nucleotide levels in the in situ dog heart[4]. More research is necessary to evaluate these effects.

Glucose metabolism

In the dog inosine increases the release of insulin, and shifts myocardial metabolism towards increased uptake of carbohydrates relative to free fatty acids[17]. Compared to other species, pigs have a high blood inosine concentration. (The concentration is even so high that inosine may serve as energetic substrate for erythrocytes in pigs[18,19]).

Haemodynamics

The mean arterial pressure decreased during inosine infusion (figure 1). Although we were unable to measure adenosine levels (detection limit 0.2 μmol·litre^{-1}), the effect of inosine on arterial pressure may have been secondary to adenosine. The latter is a very potent vasodilator. Adenosine causes hypotension, when administered during anaesthesia, but in awake healthy human volunteers, adenosine even increased blood pressure[20]. This difference is ascribed to the influence of anaesthesia on the autonomic nerve system. The breakdown products of inosine have no haemodynamic effects.

Inosine showed no effects on heart rate (figure 1). Maximum rate of rise of ventricular pressure was used to estimate directional changes in contractility induced by inosine, since it was shown to be useful to assess acute changes in inotropic state of the heart[21,22]. Studies in humans as well as in animals have demonstrated that peak dP/dt is largely independent of changes in afterload[23-25]. The enhanced dP/dt(max) during inosine administration despite of lowered aortic pressure, shown in this study, strongly suggests a positive inotropic action of this nucleoside. If inosine would improve heart function via a vasodilating action, a decrease in dP/dt(max) would be more likely to occur[24]. Since inosine did not affect heart rate and left-ventricular end-diastolic pressure we conclude that increased dP/dt(max) reflects increase in myocardial contractility. In a similar open-chest pig model, Woollard et al. examined the effects of 0.3-1.2 mmol·litre^{-1} inosine[2].

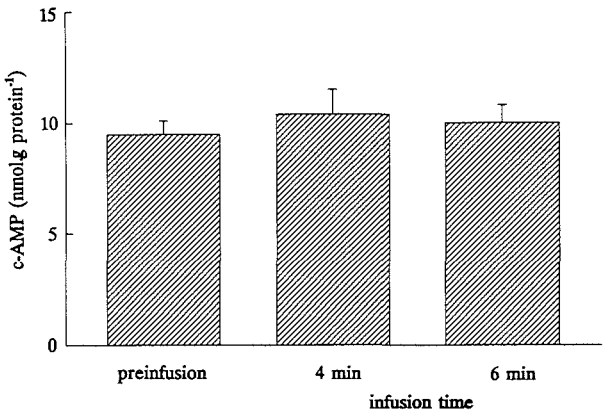

Fig. 4. There were no significant differences between the levels of c-AMP. Means + SEM, n=5.

Table 1. Inosine infusion did not significantly change the ATP and AMP levels, the energy charge (EC) nor the level of creatine phosphate (CrP). After 4 minutes the ADP level was, however, increased. Means ± SEM, n=5.

Variable	before infusion	during infusion	
		4 min	6 min
ATP (μmol·g protein^{-1})	26.3 ± 1.2	30.2 ± 1.7	28.3 ± 2.2
ADP (μmol·g protein^{-1})	5.4 ± 0.4	6.0 ± 0.4 *	5.8 ± 0.4
AMP (μmol·g protein^{-1})	0.47 ± 0.09	0.50 ± 0.13	0.56 ± 0.13
EC	0.900 ± 0.008	0.908 ± 0.011	0.897 ± 0.008
CrP (μmol·g protein^{-1})	42 ± 7	50 ± 9	58 ± 7

* $p<0.01$ vs. before infusion

They showed that the increase in dP/dt(max) by inosine was not secondary to effects on the mean aortic pressure. At the end of the infusion, the dP/dt(max) was still increasing, while blood pressure returned to normal[2].

Wetzel and Hauel described seven mechanisms for cardiotonic agents: ß-adrenergic stimulation, phosphodiesterase inhibition, α-adrenergic stimulation, Na^+/K^+ ATPase inhibition, enhanced sensitivity of contractile elements for Ca^{2+}, Ca^{2+}-channel agonism, and slowed closing of Na^+-channels[26]. The effects of adenosine on function are complex. In isolated rabbit heart adenosine inhibits noradrenaline release by an effect on presynaptic purine receptors[27]. However, in dog heart this mechanism is not found[28]. Adenosine attenuates the effects of isoproterenol on c-AMP levels, but reduces vagal tone[29,30]. The mechanism for inosine's effects are unknown. In rabbit heart the inotropic effects of inosine are abolished by propranolol[5]. In the in situ dog heart, Jones et al. found no alteration of the effects of inosine on the contractile force by propranolol[4]. Czarnecki and Noble reported that in the same species another ß-blocker, sotalol, partially reduced the increase in dP/dt(max) by inosine, but that the nucleoside caused a supersensitivity to the positive inotropic effect of adrenaline[6]. In the same series verapamil had no reproducible effect. c-AMP levels were not affected in our experiments (figure 4). This makes the ß-adrenergic mechanism, the phosphodiesterase inhibition or a direct effect on adenylate cyclase unlikely, as they are mediated through increased c-AMP levels. An alternative mode of action may involve increased intracellular calcium, either by a direct effect on the Ca^{2+}-channel, or secondary through effects on K^+, Na^+, H^+, or by α-adrenergic stimulation. A completely different explanation is the sensitization of contractile proteins. A c-AMP dependent protein kinase phosphorylates troponin-I, resulting in a reduced myofibrillar ATP-ase activity. The dephosphorylation of troponin-I involves a c-GMP dependent phosphoprotein phosphatase. This dephosphorylation therefore enhances the sensitivity of the contractile proteins for Ca^{2+}. As we found no increase in c-AMP, we speculate that the inosine-induced increase in performance involves either of these two mechanisms.

Comparison to other species

The effects of inosine in pigs are quite similar to our previous study in humans[1]. Both pig and human hearts take up inosine. In both species mean aortic pressure decreases, heart rate remains unchanged and dP/dt(max) increases. Similar effects have also been described for rabbits and dogs[3-6]. In rats inosine decreases myocardial performance[31].

We conclude that inosine, a naturally occurring substance, enhances heart performance in the pig. The mechanism of its effects remains unclear. We demonstrated that inosine does not increase function by increasing c-AMP levels.

ACKNOWLEDGEMENTS

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Appendix 4

Adenosine as adjunct to potassium cardioplegia: effect on function, energy metabolism and electrophysiology

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ABSTRACT

Adenosine is known to induce rapid cardioplegic arrest and to improve post-ischemic arrest in the isolated rat heart. However, long exposures to high doses of adenosine impair post-ischemic recovery. In this paper we test the combination of low-dose adenosine (1 mM) with potassium 26 mM, with the aim of achieving rapid arrest (as with high-dose adenosine), but eliminating the need for post-arrest washout of adenosine. Cardioplegic solutions tested were: (1) Krebs-Henseleit potassium 26 mM (K); (2) K plus adenosine 1 mM (KA); (3) K plus an adenosine deaminase inhibitor [erythro-9-(2-hydroxy-3-nonyl)adenine EHNA)] 0.1 mM (KE); and as control (4) Krebs-Henseleit potassium 6 mM (C). We induced cardiac arrest in Langendorff-perfused rat hearts by infusing the cardioplegic solution for 3 min at 3 ml/min. Total ischemia lasted 20 min at 37°C, followed by reperfusion for 30 min. High-potassium decreased the arrest time from 260 ± 16 (group C) to 22 ± 4 s (group K, mean values ± SEM). A further decrease to 10 ± 2 s was observed with KA (p = 0.016 vs. K). KE, which increased endogenous adenosine, gave intermediate effects. All hearts recovered during reperfusion: the product of developed tension and heart rate (g/min) was superior in KA hearts (6,250 ± 740 vs. K hearts 4,380 ± 390, p = 0.021). KE gave an intermediate result (5,290 ± 900), while C showed the worst recovery (3,180 ± 830). Our electrophysiological studies with sinus node and atrial tissue suggest that adenosine-induced hyperpolarization and an increase in potassium permeability, thereby arresting the sinus node prior to depolarization of the membrane by potassium 26 mM. We conclude that low-dose adenosine as an adjunct to potassium shortens the arrest time in this model and improves post-ischemic recovery.

INTRODUCTION

Adenosine has several theoretical qualities which make it attractive as a component of a cardioplegic solution. Adenosine, by its activation of the potassium channel, inhibits the sinus and atrioventricular nodes.[1,2] Adenosine also indirectly inhibits myocardial contractility,[3] enhances the tolerance of the myocardium to ischemic arrest,[4] and may improve recovery of cardiac contractile force after hypoxia.[5]

Despite the rapid cardiac arrest induced by high concentrations of adenosine (10 mM) and improved post-ischemic recovery,[6] a distinct disadvantage is that the adenosine had to be washed out after induction of cardiac arrest.

We theorized that a lower dose of adenosine (1 mM) could still rapidly cause mechanical arrest of the heart in combination with potassium (26 mM) without

requiring washout.[6] Secondly, we wished to assess whether low-dose adenosine when combined with potassium could improve recovery from cardiac arrest, in contrast to high-dose adenosine plus potassium which gave results no better than with potassium alone.[6] Thirdly, we wished to distinguish between the electrophysiological mechanisms of action of potassium, adenosine and their combination. Fourthly, we examined whether an inbibitor of the breakdown of endogenous adenosine could achieve the same benefits as added adenosine. Part of this study has been reported in abstract form.[7]

MATERIALS AND METHODS

Chemicals

All chemicals were of the highest grade available. Adenosine was supplied by Merck or Boehringer, and erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA) by Burroughs Wellcome.

Animals

Fed male rats weighing 200 - 250 g were used. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" (National Society for Medical Research) and the "Guide for the Care and Use of Laboratory Animals" (National Academy of Sciences). For perfusion studies they were anesthetized intraperitoneally with 30 mg sodium pentobarbital. Hearts were rapidly removed and cooled in ice-cold NaCl 154 mM. For electrophysiological studies rats were anesthetized with diethyl ether and heparinized. Hearts were rapidly removed and cooled in ice-cold Krebs-Henseleit buffer (see below).

Perfusion of hearts

Retrograde perfusion of the aorta according to the method of Langendorff was immediately started with a modified Krebs-Henseleit buffer, pH 7.4, at an aortic pressure of 9.2 kPa. The buffer consisted of (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, D-glucose 11. The mixture was passed through a 0.45-um filter, pre-warmed and equilibrated with 95% O_2/5% CO_2. The heart was kept in a double-walled chamber. The perfusion temperature was adjusted to 37° C with a thermocouple-regulated electric heater just prior to the perfusion canula. The latter was fitted with an A-F6 temperature probe, connected to an Ellab DU-3 monitor. After 15 min equilibration at 9.2 kPa, cardioplegic solution was given for 3 min with a roller pump, which delivered 3 ml/min. The following cardioplegic solutions were used at random: Krebs-Henseleit buffer K^+ 26 mM (K), K^+ 26 mM + adenosine 1 mM (KA), or K^+ 26 mM + EHNA 0.1 mM (KE); or control group Krebs-Henseleit buffer K^+ 5,9 mM (C). The ischemic control group C served to show (partial) protection by potassium cardioplegia. The study also included a normoxic control group N, to estimate the maximally obtainable benefit. Stop-flow ischemia was induced for 20 min, followed by 30 min of reperfusion. The perfusion pressure was measured with a Hewlett Packard model 1280 transducer. This variable, and the hemodynamic parameters mentioned below, were monitored on a Graphtec Linearcorder FWR3701.

Myocardial function

Resting and peak systolic tension were measured continuously with a Konigsberg F5-2 pseudoisometric force transducer (Hugo Sachs), connected to the apex of the heart with a ligature[8,9]. A resting tension ("preload") of 5 g was applied. During the first 15 min of perfusion, resting tension was readjusted if necessary. Developed tension was calculated as the difference of peak systolic and resting tension.

Heart rate was measured from the tension tracing. The product of developed tension and heart rate was subsequently calculated. The heart was considered in arrest if the tension-rate product was smaller than 5% of the 15-min equilibration value.

Coronary flow

Flow was monitored with a Skalar electromagnetic flow probe, mounted in the perfusion line. During reperfusion, effluent from the heart was collected (for purine assay) in a graduated cylinder over periods indicated in Fig. 1. The two methods to measure flow correlated well.

Assay of purines

Samples were kept on ice until analysis, within one day. Purine concentrations were determined by automated high-performance liquid chromatography (HPLC), essentially as described before.[10,11] The uBondapak C_{18} column (Millipore-Waters) was equipped with a 5-um Supelguard LC-18 guard column (Supelco). Flow rate of the buffer, 1% KH_2PO_4 - 10% CH_3OH (pH 4.7), was 0.6 ml/min. Urate was detected at 295 nm, hypoxanthine, xanthine, inosine and adenosine at 254 nm (Waters Model 490 absorbance detector). The retention times were 9, 10, 12, 16 and 40 min, respectively.

Analysis of cardiac high-energy phosphates

At the end of the reperfusion period, hearts were freeze-clamped at liquid nitrogen temperature. The extraction and automated HPLC-analysis of high-energy phosphates was essentially done as described before.[12,13] The Partisil-10-SAX column (Whatman) was equipped with a 5-μm Supelguard LC-SAX guard column (Supelco). The gradient of H_3PO_4 16 mM (pH 2.85) and $NH_4H_2PO_4$ 750 mM (pH 4.5) started 10 min after sample injection; the second buffer increased 4% per min until 100%. Flow rate was 2.0 ml/min. The nucleotides were detected at 254 nm, the creatine compounds at 214 nm (Model 490 absorbance detector). The retention times of creatine, AMP, creatine phosphate, ADP and ATP were 2, 3, 18, 27 and 37 min, respectively. The adenylate energy charge, (ATP + 0.5 ADP)/(ATP + ADP + AMP), was calculated from the adenine nucleotides.

Table I. *Tension-rate product during the first 30 seconds of cardioplegia*

Function	Percentage of hearts															
	0"				10"				20"				30"			
	C	K	KA	KE	C	K	KA	KE	C	K	KA	KE	C	K	KA	KE
>90%	100	100	100	100		14	9	14								
>20-90%					100	86	18	57	100	29	9		100	14		
arrhythmic							9	29						14		
5-20%							9			14	9					
<5%							54			57	82	100		71	100	100

After 15 min of equilibration, hearts were perfused at 3 ml/min with Krebs-Henseleit buffer (C, n = 6); buffer containing K^+ 26 mM (K, n= 7); K^+ 26 mM + adenosine 1 mM (KA, n = 11); or K^+ 26 mM + EHNA 0.1 mM (KE, n= 6). The percentage of hearts belonging to each function class is listed. Function, calculated as developed tension times heart rate, at t = 15 min was taken as 100%.

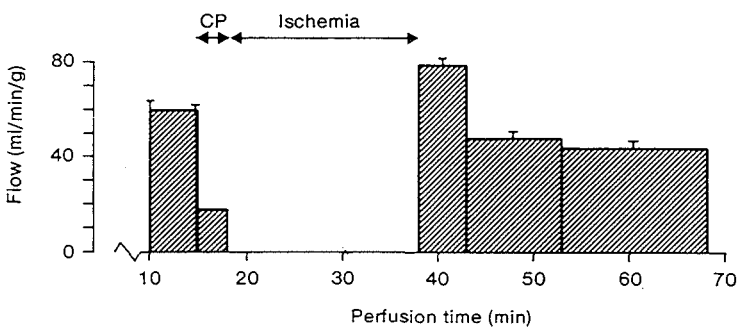

Fig. 1. Average coronary flow data in ml/min per gram dry weight of all groups (n = 23). After an equilibration period of 15 min (perfusion pressure 9.2 kPa), the cardioplegic solution (CP) containing high K^+, high K^+/adenosine, high K^+/EHNA, or standard K^+, was infused with a roller pump at a rate of 3 ml/min (18 ml/min per g dry weight) for 3 min. Total ischemia was subsequently induced for 20 min, followed by 30 min of reperfusion at 9.2 kPa with standard buffer. Means ± SEM are given.

Electrophysiological studies

The electrophysiological techniques used are described by Coetzee and Opie.[14] Briefly, sinus-node (SA) and atrial tissue was isolated at 37°C and mounted in an incubation bath (0.5 x 1.0 cm). The preparation (<0.5 x 1.0 mm) was superfused at 3 ml/min at 35-36°C with the oxygenated Krebs-Henseleit buffer described above. The preparation was impaled with a microelectrode, containing KCl 3 M. The reference electrode was positioned in the fluid close to the preparation; action potentials were monitored on an oscilloscope (Tektronix 5103 N) and recorded on video-tape after digitization (Sony PCM-501 ES, Unitrade). After a stabilization period of 15 min, the cardioplegic solutions described above were randomly superfused for 3 min in one preparation, followed by Krebs-Henseleit buffer until control values were reached again.

Statistics

Data are reported as means ± SEM. Anova analysis was not performed, as the large differences between the control and other groups could mask differences between the high-potassium and the high-potassium/adenosine groups. For contrast of the high-potassium/adenosine group versus the high-potassium group, data were analyzed with Student's t-test (two-tailed). Differences with p-values < 0.05 were considered statistically significant.

RESULTS

A) Function in normoxic control hearts

In the normoxic control hearts (N perfused for one hour), resting and developed tension were 5.0 ± 0.0 and 30 ± 2 g (10-min value) and 3.1 ± 0.5 and 28 ± 3 g (60-min value), respectively. The values for heart rate in that period were 321 ± 16 and 261 ± 6 beats/min, respectively. Consequently, cardiac function, expressed as the tension-rate product, was 9,790 ± 880 g/min after ten minutes, and 7,340 ± 630 g/min after one hour. Coronary flow was 58 ± 5 ml/min per gram dry weight, ten minutes after the start of the perfusion, and 37.4 ± 1.7 ml/min per g after one hour.

B) Function in cardioplegic hearts

1. Cardiac arrest

Table I shows that administration of either adenosine 1 mM or EHNA 0.1 mM, in addition to K^+ 26 mM, accelerated the arrest of the hearts. After 30 seconds of reduced flow, all hearts in groups KA or KE were arrested, while only five out of seven hearts in group K were arrested. Arrest time for group K was 22 ± 4 s, KE decreased the time to 14 ± 1 s, while KA gave the shortest arrest time, 10 ± 2 s ($p=0.016$ vs. K). In group C all hearts were still beating, with arrest only occurring after 260 ± 16 s in addition during ischemia, they resumed some contractile activity.

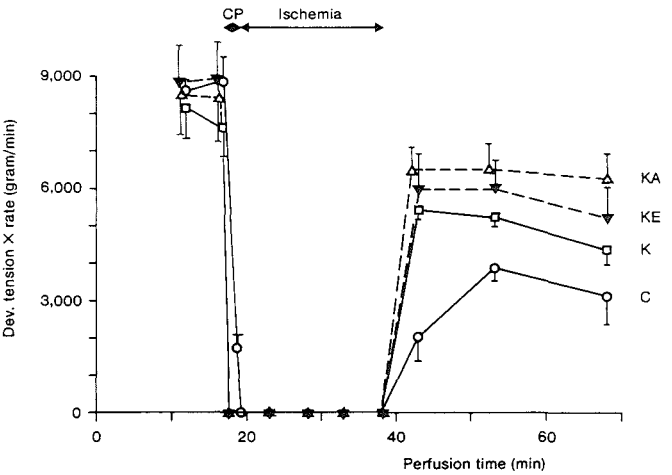

Fig. 2. Effect of potassium cardioplegia, with and without adenosine or EHNA on resting tension and peak systolic tension during ischemia and reperfusion. With the normal K^+-concentration (group C) in the buffer, infused from 15 to 18 min, the hearts arrested slowly and resting tension rose steeply during ischemia and reperfusion. High-potassium (K), with adenosine (KA) or EHNA (KE), induced quick arrest and minimized the rise in resting tension. The arrow indicates the start of reperfusion. Means ± SEM are given (n=5-6).

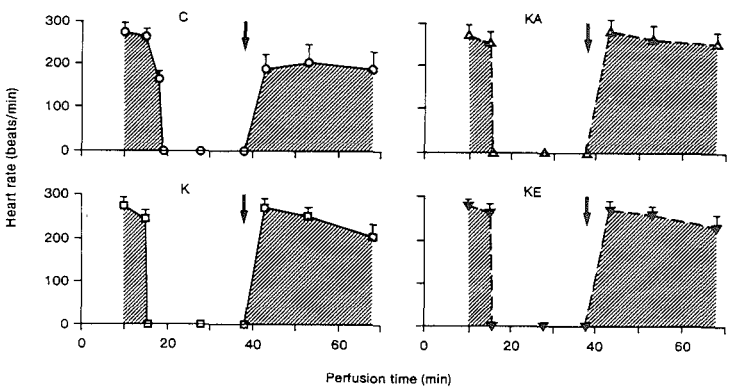

Fig. 3. Effect of potassium cardioplegia, with and without adenosine or EHNA, on heart rate. After an equilibration period of 15 min, flow was reduced to 3 ml/min for 3 min. This decreased heart rate partly (panel C). If K^+-cardioplegia was employed, the hearts stopped completely (panel K). This also occurred with K^+-cardioplegia plus adenosine (panel KA) or plus EHNA (panel KE). After this infusion, ischemia was induced. Reperfusion started at the point indicated with an arrow. At the end of reperfusion, heart rate was highest in the group treated with potassium and adenosine (KA). Mean values with SEM (n=5-6).

2. Tension-rate product

We used the product of developed tension times heart rate as an index of cardiac function. During equilibration the differences between the groups were small (Fig. 2). Just
before cardioplegia, the average tension-rate product was 8,500 g/min. At the end of the cardioplegic infusion, function in the K, KA and KE groups was zero, while control hearts (C) had reduced function (1,670 ± 320 g/min). After five minutes of reperfusion, all hearts had regained function. The highest values were found in the KA group (6,550 ± 629 g/min), intermediate values with the groups KE (6,050 ± 950 g/min) and K (5,490 ± 290 g/min, $p < 0.001$ vs. KA), while the control group gave the lowest function (2,060 ± 659 g/min). At the end of reperfusion, function in the KA group remained constant and significantly higher than the K group which had deteriorated slightly (6,250 ± 740 and 4,380 ± 390 g/min, respectively, $p = 0.05$).

3. Heart rate

Figure 3 shows the effect of cardioplegia, ischemia and reperfusion on heart rate. At the end of the 3-min cardioplegic buffer infusion, all high-potassium hearts (K, KA and KE) had arrested, while the heart rate in group C only fell from 268 ± 16 to 168 ± 18 beats/min, with final arrest occurring three minutes later (Fig. 3).

At the end of reperfusion, the heart rate was lowest in control hearts (191 ± 43 beats/min), and group K (209 ± 27 beats/min). The addition of adenosine to the cardioplegic solution did not inhibit the recovery of the post-ischemic heart rate, and the group KA gave the highest recovery (259 ± 30 beats/min ($p=0.2$). Addition of EHNA gave an intermediate value (Fig. 3).

4. Resting tension

Pre-ischemic resting tension in all hearts was about 5 g. It remained unchanged during ischemia in all groups except the control group C, where it increased to 9.1 ± 0.7 g in the end of ischemia (Fig. 4). After two minutes of reperfusion, the resting tension in all hearts increased; group C gave the largest increase (17 ± 3 g), whereas the values in the remaining groups were 7.2 ± 0.8 g (K), 6.6 ± 0.7 g (KA), and 6.4 ± 0.5 g (KE). At the end of the 30-min reperfusion period, the resting tension in all groups had returned to the pre-ischemic value (around 5 g), while it was still elevated to 8 ± 2 g in group C (Fig. 4).

5. Coronary flow

All groups of hearts undergoing cardioplegia, ischemia and reperfusion, showed comparable values throughout the experiment. Flow in the first five minutes of reperfusion was 76 ± 6 ml/min per gram dry weight (group C), 72 ± 2 ml/min per g (group K), and 82 ± 4 ml/min per g (group KA), and 80 ± 5 ml/min per g (group KE). For illustrative purposes, the data were combined (Fig. 1).

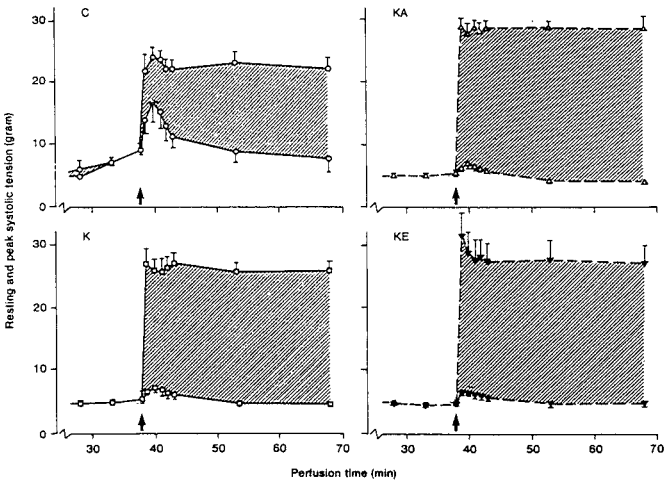

Fig. 4. Improved recovery of function after ischemia with adenosine as adjunct to potassium cardioplegia. The product of developed tension and heart rate is depicted during the last 5 min of the equilibration period, cardioplegia (CP), ischemia and reperfusion. Cardioplegia was carried out with high K^+ (curve K), high K^+ + adenosine (curve KA), or high K^+ + EHNA (curve KE). As a control, standard medium was infused (curve C). Mean values with SEM are presented (n=5-6).

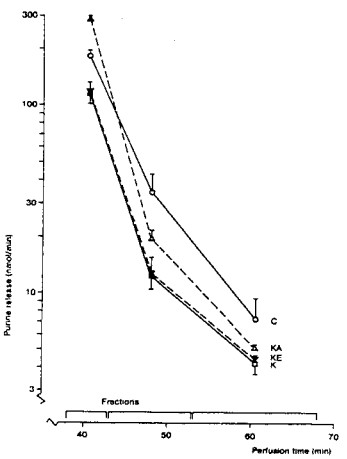

Fig. 5. Semilogarithmic plot of ATP-catabolite release versus time. Effluent adenosine, inosine, hypoxanthine, xanthine and urate were measured with HPLC during 30 min of reperfusion. Purine production in the three fractions collected is depicted. EHNA did not reduce the release of ATP-catabolites. The initial high purine release in the K^+/adenosine group (KA) is presumably caused by washout of adenosine(catabolite), employed during cardioplegia. Mean values with SEM (n = 5-6).

C) Metabolism

1. Purine release

Figure 5 depicts the production of purine nucleosides and oxypurines after ischemia. The control hearts (C) released 183 ± 13 nmol/min in the first five minutes of reperfusion. In K and KE-treated hearts, purine release was lower: 116 ± 15 nmol/min and 119 ± 12 nmol/min, respectively, while release was in KA-treated hearts was 290 ± 10 nmol/min. At the end of reperfusion the rate of purine release was 10 nmol/min in all groups. The adenosine deaminase inhibitor EHNA changed the adenosine/inosine ratio in the effluent drastically. During early reperfusion the ratio increased from 0.24 ± 0.02 (K group) to 3.3 ± 0.3 (KE group) (Fig. 6). This was due to a 5.8-fold rise in adenosine concentration and a 2.5-fold fall in inosine concentration. At the end of reperfusion, these differences had almost disappeared.

2. High-energy phosphates

At the end of reperfusion the ATP values were depressed when compared to normoxic hearts (N); ATP values fell from 25 ± 3 μmol/g dry weight to 16.3 ± 1.2 μmol/g (group C) and about 20 μmol/g for groups K, KA and KE. We found a comparable pattern for ADP (Table II).

The amount of creatine phosphate in normoxic control hearts was 22 ± 2 umol/g (Table II). After reperfusion, both treated and untreated hearts contained about 30 umol/g.

3. Water content

The water fraction was about 0.87 in all groups, indicating that ischemia followed by reperfusion did not cause swelling (Table II).

C) Electrophysiological studies

Potassium 26 mM (K) initiated depolarization of the atrial SA-node membrane, 20 seconds after being added (Fig. 7). It took a further 20 s for depolarization to be complete and for the action potential to be abolished. When adenosine 1 mM (KA) was added, there was initial hyperpolarization during the first few seconds prior to depolarization to similar values by the high-potassium in the cardioplegic solution. Cessation of the action potential occurred prior to depolarization of the membrane by the high-potassium concentration. In four preparations KA shortened the arrest time by 18 ± 4 s when compared to K alone ($p = 0.004$, $n = 7$ impalements). KE gave similar results to K alone, showing no hyperpolarization and arrest alone when depolarization was near completion.

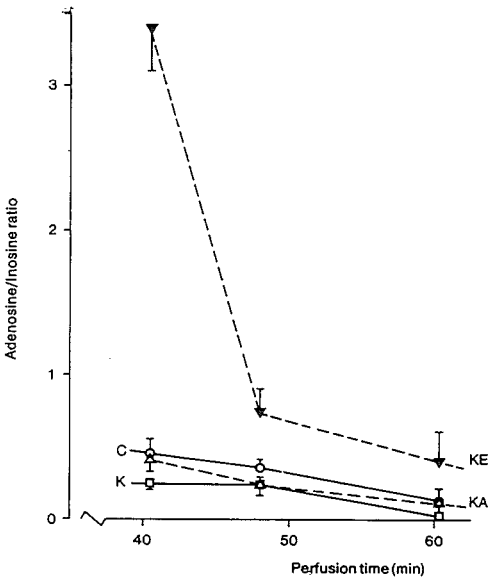

Fig. 6. EHNA, present during cardioplegia and stopped-flow ischemia, increased the adenosine/inosine ratio in the cardiac effluent during reperfusion. Effluent was collected from 38 to 43 min, from 43 to 53 min, and from 53 to 68 min. The nucleoside concentrations were determined by HPLC and their ratios calculated. The following additions were made to the perfusion buffer during cardioplegia: None (C), 20 mM K^+ (K), 20 mM K^+ + 1 mM adenosine (KA), or 20 mM K^+ + 0.1 mM EHNA (KE). Mean values are given with SEM (n = 5-6).

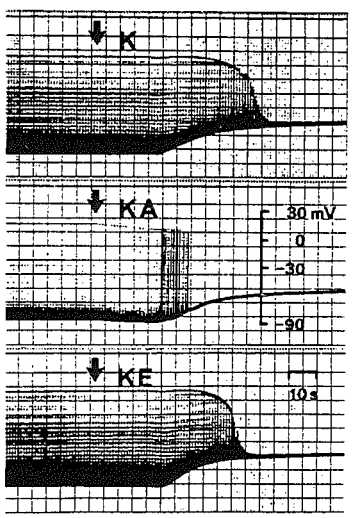

Fig. 7. Accelerated arrest of the atrial-sinus-node preparation with adenosine as adjunct to potassium cardioplegia. A typical example is given of action potential measurements in one single cell, superfused with Krebs-Henseleit buffer (3 ml/min). Arrest was induced by addition of 20 mM K^+ (K), 20 mM K^+ + 1 mM adenosine (KA), or 20 mM K^+ + 0.1 mM EHNA (KE).

Table II. The effect of potassium cardioplegia, with and without adenosine or EHNA, on myocardial ATP and related metabolites in reperfused hearts

Variable	N	C	K	KA	KE
ATP	25 ± 3	16.3 ± 1.2	20.3 ± 0.6	20.1 ± 0.6	21.1 ± 1.1
ADP	4.9 ± 0.4	3.05 ± 0.17	3.47 ± 0.09	3.60 ± 0.07	3.57 ± 0.20
AMP	0.98 ± 0.18	0.65 ± 0.11	0.75 ± 0.04	0.61 ± 0.05	0.66 ± 0.05
ATP/ADP	5.1 ± 0.5	5.3 ± 0.4	5.8 ± 0.3	5.5 ± 0.2	5.9 ± 0.3
Sum AdNu	31 ± 3	20.0 ± 1.3	24.5 ± 0.5	24.3 ± 0.6	25.6 ± 0.9
AEC	0.888 ± 0.005	0.887 ± 0.011	0.898 ± 0.005	0.900 ± 0.003	0.903 ± 0.003
CrP	22 ± 2	30 ± 2	30.3 ± 0.8	27.8 ± 1.0	30.0 ± 1.1
Cr	48 ± 3	31.5 ± 1.3	36.3 ± 1.6	38.0 ± 1.4	36 ± 4
CrP/Cr	0.45 ± 0.04	0.98 ± 0.13	0.81 ± 0.06	0.75 ± 0.05	0.90 ± 0.13
CrP+Cr	70 ± 5	60.5 ± 1.5	66.0 ± 1.4	65.8 ± 1.2	67 ± 3
H_2O fr.	0.861 ± 0.005	0.876 ± 0.004	0.872 ± 0.006	0.867 ± 0.004	0.870 ± 0.002
n	4	6	6	6	5

After equilibration hearts underwent cardioplegia, ischemia and reperfusion as described under Materials and methods. The hearts were freeze-clamped, extracted and analyzed by HPLC for metabolites. N = normoxic non-ischemic, C = control (normal K^+ in "cardioplegic solution"), K = high K^+ in cardioplegic solution, KA = high K^+ + adenosine, KE = high K^+ + EHNA, AdNu = adenine nucleotides, AEC = adenylate energy charge, CrP = creatine phosphate, Cr = creatine. H_2O fr= water fraction. Data are in umol/gram dry weight, except the ratios which are dimensionless. Means ± SEM.

DISCUSSION

Overall effects of adenosine

In this study the addition of low-dose adenosine to potassium decreased the time to cardiac arrest, in confirmation of our earlier study.[6] The new data here presented concern (i) improved speed of cardiac arrest achieved by low-dose (1 mM) adenosine, when added to potassium (26 mM) as judged by the rate tension product; (ii) the electrophysiological mechanism whereby cardiac arrest is achieved by adenosine; and (iii) the effects of endogenous adenosine raised by EHNA (vide infra); and (iii). However, high-potassium alone was also effective in preventing reperfusion damage, as judged by prevention of the further rise in diastolic tension upon reperfusion (Fig. 4). The addition of low-dose adenosine to potassium accelerated arrest without improving recovery of high-energy phosphates or patterns of release of purine when compared with potassium alone. The mechanism of the benefit of added low-dose adenosine on function in the reperfusion period is unclear; we cannot exclude an improved energy status at the end of the ischemic period as a consequence of the accelerated cardiac arrest, although the patterns of purine release during the reperfusion period were unaltered by the addition of adenosine.

Metabolic and myocardial function effects of adenosine and EHNA

Effects of adenosine

Previous authors suggest metabolic benefits due to adenosine and EHNA which we did not find. Takeo et al.[5] reported increases in ATP and creatine phosphate due to adenosine(catabolite) administration to hypoxic rabbit heart. Kao and Magovern[15] showed that adenosine 1 mM in a hypothermic cardioplegic solution prevented ATP breakdown during and after ischemia of the isolated rat heart. Large aberrations in myocardial glucose-6-phosphate, creatine phosphate and lactate content, however, were still present. In the anesthetized dog,[16] a 3-h infusion of adenosine after myocardial ischemia, repleted nearly 50 percent of the ATP lost, but regional and global myocardial function was not improved. Recently Wyatt et al.[17] found in the same preparation that adenosine (plus hypoxanthine and ribose) maintains myocardial ATP content during ischemia and reperfusion and enhances functional recovery during the post-ischemic period. Silverman et al.[18] claimed that adenosine (with or without EHNA) during reperfusion of the dog heart, after a period of hypothermic K^+-cardioplegic arrest, improved the myocardial ATP content. However, they failed to compare the groups (which seem to be from an inhomogeneous population) and they did not supply data on myocardial function with the regimens studied.

Effects of EHNA

In our study we put emphasis on the effect of exogenous adenosine and less on endogenous adenosine. Increasing endogenous adenosine by means of the inhibitor of adenosine deaminase EHNA (Fig. 6), gave functional results similar in trend to those achieved by addition of exogenous adenosine to potassium. Thus the time to cardiac arrest was diminished and post-ischemic cardiac function was improved by EHNA. We speculate that adenosine formed in the ventricle accelerates the arrest of the sinus-node of EHNA-perfused hearts.

Dhasmana et al.[19] showed that a very high dose of EHNA, given before and after ischemia, improved functional recovery of the rat heart. However, this benefit was also not correlated with ATP recovery during reperfusion nor did it prevent ventricular fibrillation. In dogs on cardiopulmonary bypass, treatment with EHNA before ischemia, and with adenosine during and after ischemia, resulted in some improvement in myocardial ATP.[20] It is not clear whether this improvement was statistically significant. However, mean aortic pressure remained low, and renal vasoconstriction took place. Hull-Ryde et al.[21] reported that EHNA (plus dipyridamole) in a cardioplegic solution, administered to dogs on cardiopulmonary bypass, increased the myocardial ATP content during reperfusion from 4.0 to 5.0 umol/g wet weight. In their paper, the emphasis is on nucleotide analysis, and they supplied only few details about their animal preparation and infusates. We failed to see an improvement of ATP by EHNA in our model (Table II). Humphrey et al.[22] treated rat hearts with EHNA before ischemia. They found some increase in adenine nucleotides after reperfusion, but ventricular function failed to recover. We failed to see an improvement of ATP by EHNA added to potassium arrest in our model (Table II).

Electrophysiological actions of adenosine

Adenosine produces a dose-dependent increase in potassium permeability which leads to hyperpolarization of the membrane thus active in a similar way to acetylcholine; relatively high doses of adenosine alone caused complete arrest associated with hyperpolarization of 12-15 mV,[1,2] as also confirmed by our unpublished data. In whole-cell voltage clamp experiments, adenosine activated a time-dependent potassium current which at -50 mV was approximately 30 pA in a normal Tyrode's solution and 50 pA in high-potassium Tyrode's solution.[2] The rapid cardiac arrest produced by adenosine (1 mM) in the presence of high-potassium (26 mM) was probably due to increased potassium permeability causing early arrest of the sinus-node. EHNA may have been ineffective in this model due to the high superfusion rate of 3 ml/min which could have prevented any accumulation of adenosine.

In our study we only added adenosine and EHNA to the cardioplegic solution which was administered for 3 min prior to 20 min of total ischemia. The reperfusion solution contained no adenosine or EHNA and the potassium concentration was 6 mM. Although we did not find an improvement in high-energy phosphates at the end of the reperfusion period, we cannot exclude an improvement during ischemia or early in reperfusion. The benefits of adenosine and EHNA as shown by improved myocardial function were not translated into higher levels of ATP at the end of reperfusion.

Reservations

We studied the rat heart, thought to be 'hypercontractile', and, therefore, the results may not be applicable to other species, let alone man. Also, we did not assess whether adenosine, when added to the standard cardioplegic combination of hypothermia plus hyperkalemia, would still give any added benefit. Therefore, further work is required before adenosine can be recommended as a standard adjunct to potassium cardioplegia.

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Appendix 5

Relationship between pre-ischemic heart function and onset of contracture

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ABSTRACT

We expected an inverse relationship between pre-ischemic function and onset of myocardial contracture. To test this, we manipulated the pre-ischemic inotropic state of 45 perfused rat hearts by adapting the buffer concentration to: potassium (20 mM), magnesium (15 mM), calcium (4 mM), nifedipine (0.3 μM), DL-verapamil (0.9 μM), or dopamine (6 μM). High-calcium had a positive inotropic effect, dopamine affected function transiently, the other substances were negative inotropic. Then, we stopped the flow until contracture started. Time until contracture was delayed or shortened with negative or positive inotropic treatment, respectively. Despite these differences in total ischemic time, we found no significant differences between the groups in developed or resting tension, coronary flow or purine release during reperfusion, apart from a lower resting tension in the calcium-treated group. Thus, in this model: 1) an inverse relationship exists between pre-ischemic heart function and onset of contracture; 2) Ca-antagonists fail to show benefits above other negative inotropic treatments.

INTRODUCTION

Prolonged myocardial ischemia or hypoxia leads to a depletion of ATP, a loss of contractile function and a rise in resting tension (onset of contracture). Full-blown contracture or "stone heart"[5, 21] is now a rare complication in heart surgery. However, mild degrees of contracture are common occurrence after heart surgery and after acute coronary occlusion. Two factors seem to be of major importance in the onset and development of contracture[11, 12, 16, 19]: 1) A critical ATP-depletion, which leads to rigor bond formation between actin and myosin; the contracture itself further accelerates the ATP-hydrolysis[2]; 2) Ca^{2+}-accumulation near the active sites of actin.

Several authors[9, 10, 16] using different protocols could delay the ischemic rise in resting tension by pretreatment of the hearts with agents like high-potassium cardioplegia or Ca-antagonists. These substances influence heart function. Our earlier work[6,7] already showed the relation between pre-ischemic heart function and ATP-breakdown during ischemia. Now, our objective was to investigate in one model the relationship between pre-ischemic heart function and onset of contracture. We pretreated hearts with a positive or negative inotropic agent; then we made them ischemic until they had reached a fixed rise in resting tension. We analyzed the relation between the inotropic response and the time to onset of contracture.

MATERIALS AND METHODS

Heart perfusion. Male Wistar rats (255 ± 34 g) with free access to food and water were anesthetized with 50 mg pentobarbitone intraperitoneally. The heart was

rapidly excised and cooled in ice-cold 0.9% w/v NaCl until beating ceased. Then retrograde perfusion of the aorta according to Langendorff was started with a modified Tyrode's buffer containing (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. Water was purified with the Millipore-Ro4/Milli-Q System (Millipore, Bedford, MA, USA). The buffer was gassed with 95% O_2 plus 5% CO_2. The perfusion temperature was 37°C. The buffer was delivered to the aorta at a pressure of 9.3 kPa. Pressure and temperature were measured above and in the aortic canula, respectively. During the entire experiment, the heart was paced at a frequency of 300 beats/min (4 V, 2 ms). Myocardial resting tension (preload) and developed tension (peak systole minus resting tension) were measured with a force transducer (Hugo Sachs F5-2, March/Freiburg, FRG) connected to the apex of the heart. Perfusate from the heart was collected in a graduated cylinder for 5-min periods. The cylinder was placed in ice to diminish purine catabolism. An electromagnetic flow probe (Transflow 601, Skalar Medical, Delft, The Netherlands) was used to monitor flow. During the period of no-flow ischemia, a water-jacketed chamber, sealed with Parafilm, prevented the heart from cooling. A thermistor was inserted into the left ventricular cavity to measure the intra-cardial temperature. Upon reperfusion this probe was removed from the ventricle.

Protocol. Five hearts were perfused for one hour to check the stability of the preparation. Another 40 hearts were randomized into seven groups receiving the treatments described below. After being mounted on the canula (t=0 min), the hearts were equilibrated for fifteen minutes with standard buffer. During this period, preload was adjusted to 5 g by manipulating the force-transducer; then it was corrected no more. The developed tension at t=15 min was taken as 100% function. After equilibration the hearts were perfused with a modified solution for ten minutes. (During this period the untreated control group was perfused with standard medium.) Function at t=25 min was used to determine the effect of the modified solutions during normoxia. At t=25 min the hearts were made globally ischemic by stopping flow to the hearts completely. Reperfusion with standard medium was started when the resting tension had increased by 10% of the developed tension at t=15 min above the nadir. The reperfusion period lasted twenty minutes (Fig. 1).

Treatments. The modified solutions consisted of standard medium adjusted to or fortified with one of the following: KCl (20 mM), $MgCl_2$ (15 mM), $CaCl_2$ (4 mM), dopamine (6 μM), nifedipine (300 nM), or DL-verapamil (880 nM). To correct for osmotic differences, the NaCl-content of the buffer was adapted. Nifedipine was obtained from Bayer (Leverkusen, FRG), verapamil from Knoll (Almere, The Netherlands), and dopamine from Brocacef (Rotterdam, The Netherlands). Nifedipine solutions were kept in dark brown reservoirs to avoid breakdown by light.

Purine analyses. Perfusate samples were stored on ice and analyzed for purine nucleosides and oxypurines by high-performance liquid chromatography[15,17], usually within hours. In some experiments the samples were kept at -20°C up to two days before they were assayed.

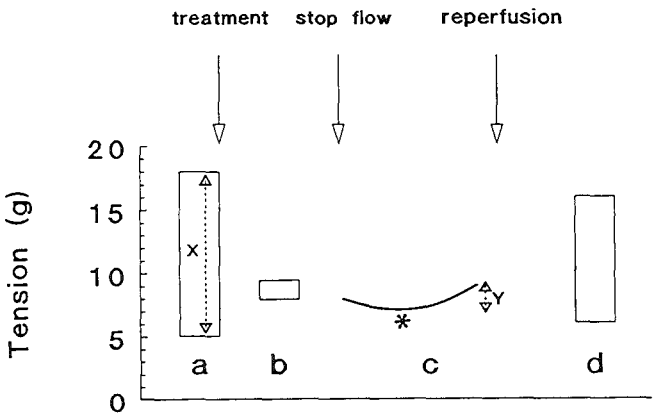

FIG. 1. Schematic representation of an experiment (taken from the high-magnesium group. a: equilibrium period, b: treatment period, c: ischemia, d: reperfusion. x: developed tension before treatment, y: required rise in resting tension (y=10% of x), *: nadir.

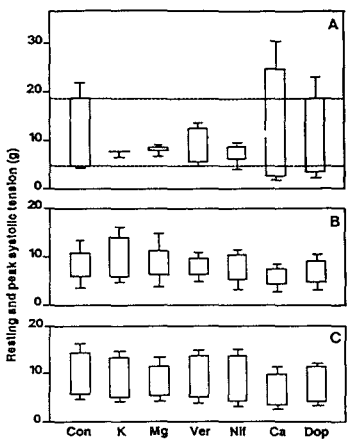

FIG. 2. Effect of various agents on contractility during normoxia (A), after reperfusion for 5 min (B) and 20 min (C). During ischemia developed tension became virtually zero (not shown). The dotted lines depict the average pooled values just prior to drug or ion administration; resting tension was (adjusted to) 5 g, developed tension was 14 ± 4 g (n=45). Con = ischemic control group; K = potassium group; Mg = magnesium group; Ver = verapamil group; Nif = nifedipine group; Ca = calcium group; Dop = dopamine group.

Statistical analyses. For statistical comparisons the one-way ANOVA-test was used with six degrees of freedom. Differences were further identified within each group using Student's unpaired t-test with Bonferroni correction. Chi-square analysis revealed statistical differences in time needed to regain function. Differences with a probability >0.05 were considered not statistically significant (NS). Unless otherwise stated, P values refer to comparisons with the untreated hearts (ischemic control group). Data are presented as means ± SD.

RESULTS

Normoxic controls. In the normoxic control group tension, flow and purine release did not change. Developed tension in the control group decreased from 14 ± 3 g at t=15 min to 13 ± 2 g after 60 min. Resting tension increased from 5.0 ± 0.0 to 5.7 ± 0.7 g. The release of AMP-catabolites (purines) decreased from 6 ± 2 to 3.9 ± 1.3 nmol/min per g wet weight. Flow remained about 13 ml/min per g.

The first 15 min of all experiments was an equilibration period. We measured at the end of this period, developed and resting tension, flow and purine release. There were no statistically significant differences between the various groups during the equilibration period.

Myocardial function. We used developed and resting tension (preload) as a measure of cardiac function. Fig. 2 shows resting and peak systolic tension; we obtained the developed tension by subtracting resting tension from the peak systolic tension. At t=25 min, developed tension in the ischemic control group (14 ± 4 g) had not changed from t=15 min. Due to high-potassium, developed tension dropped to zero ($P<0.001$ vs. untreated control, Fig. 2A). This figure also shows the negative inotropic effect of high magnesium, verapamil and nifedipine. Developed tension fell to 0.7 ± 0.4 g ($P<0.001$), 6.9 ± 1.0 g ($P<0.01$), and 2.5 ± 1.0 g ($P<0.001$), respectively. High calcium gave a marked positive inotropic response: Developed tension increased to 22 ± 6 g ($P<0.001$). Developed tension in the dopamine-treated group increased transiently, but did not significantly differ from the untreated control group at t=25 min.

Preload increased from 5.0 ± 0.0 to 7.8 ± 1.3 g in the high-potassium group ($P<0.001$, Fig. 2A) and to 8.0 ± 1.1 g in the high-magnesium group ($P<0.001$). Due to increased calcium, the preload decreased to 2.8 ± 0.6 g ($P<0.002$). The preload in the other groups did not differ significantly from the untreated control group.

During ischemia developed tension in the high-potassium group remained zero. In all other groups developed tension decreased precipitously. In all ischemic hearts, resting tension decreased first and rose later. The nadir in the ischemic control group was 4.1 ± 1.0 g. There were no significant differences between the groups (F-value: 0.79 with 6 degrees of freedom (group) and 32 degrees of freedom (event).

After reperfusion most hearts took some time to resume their rhythmic heart rate. After 30 s all hearts in the high-potassium group had a rhythmic function, while in the other groups only a few hearts resumed a normal contraction pattern ($P<0.01$, potassium vs. other groups).

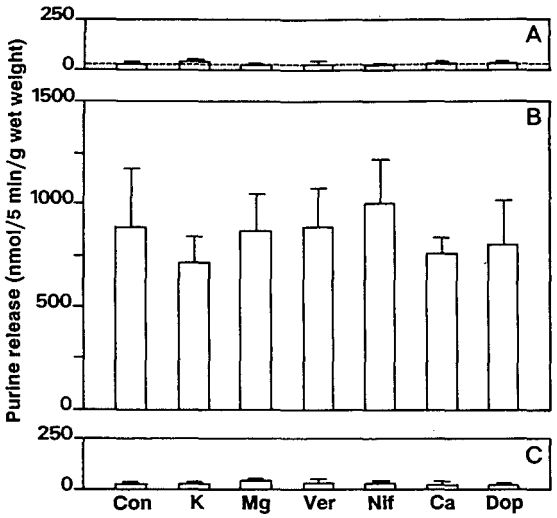

FIG. 3. Effect of various agents on the release of AMP-breakdown products. Purine release before treatment, depicted by the dotted line, was 33 ± 10 nmol/5 min per g wet weight (n=45). For other details, see legend to Fig. 1.

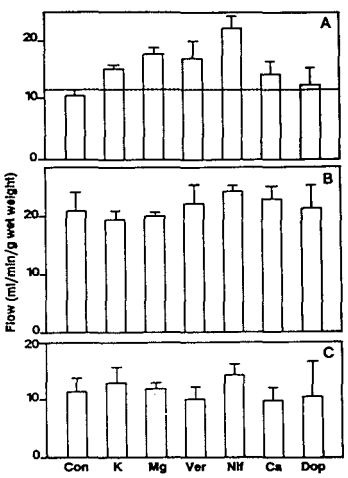

FIG. 4. Effect of various agents on coronary flow. Flow before treatment, depicted by the dotted line was 11.4 ± 1.7 ml/min per g wet weight (n=45). For other details, see legend to Fig. 1.

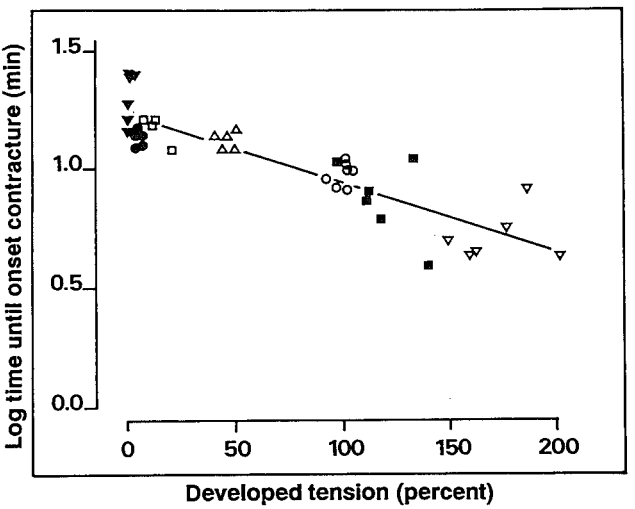

FIG. 5. Relation between inotropic response (tension at t=25 min expressed as percentage of t=15 min) and time until the onset of contracture. For details, see Materials and Methods. o = ischemic control group; = potassium group; = magnesium group; = verapamil group; = nifedipine group; = calcium group; = dopamine group.

Upon reperfusion the preload rose steeply and decreased subsequently. After 5 min of reperfusion, none of the groups had a preload significantly different from the ischemic control group (6 ± 3 g). Developed tension was not different from the ischemic control group (5 ± 3 g) either (Fig. 2B). Likewise, after 20 min of reperfusion, there were no statistically significant differences in developed tension between the groups (Fig. 2C). However, the preload in the high-calcium group was lower than that in the ischemic control group (3.6 ± 0.7 vs. 5.8 ± 1.0 g, $P<0.05$).

Purine release. Fig. 3A shows the effect of the various agents on myocardial purine release. In the high-potassium group, purine release at 25 min, i.e., before ischemia, was 42 ± 10 nmol/5 min per gram wet weight. This is significantly higher than that in the untreated group (28 ± 6, $P<0.05$). The other groups did not differ significantly from the latter. After ischemia there were no statistically significant differences in purine release between the groups (Figs. 3B and 3C).

Flow. The effect of the various agents on the flow at t=25 min, i.e., before ischemia, can be seen in Fig. 4A. Flow (in ml/min per g wet weight) in the untreated control group was 10.6 ± 1.0. It increased by high potassium (to 15.1 ± 0.6, $P<0.001$), high magnesium (to 18 ± 1, $P<0.001$), verapamil (to 17 ± 3, $P<0.001$), nifedipine (to 22 ± 2, $P<0.001$) and high calcium (14 ± 3, $P<0.01$). Only minor increases in flow were seen with dopamine.

Reperfusion resulted in a marked reactive hyperemia. During reperfusion there were no significant differences in flow between the groups (Figs. 4B and 4C).

Time until onset of contracture. The hearts were made ischemic until the

resting tension had increased above the nadir by 10% of the developed tension at t=15 min. There were marked differences between the groups in the time elapsed before the preload had reached such an increase. The time until onset of contracture was prolonged by negative inotropic treatment and shortened by positive inotropic treatment.

In the untreated hearts, contracture started to develop at 9.7 ± 1.1 min. Treatment with high concentrations of potassium or magnesium delayed this to 21 ± 5 min ($P<0.001$) and 13.9 ± 1.4 min ($P<0.05$), respectively. For the Ca-antagonists these values were: nifedipine (14.5 ± 1.9 min, $P<0.05$); verapamil (13.4 ± 1.2 min, NS). In the dopamine group contracture started at 8 ± 3 min (NS), while in the calcium group it began at 5.5 ± 1.6 min ($P<0.05$). If we expressed the function after the inotropic intervention as a percentage of the developed tension at t=15 min and plotted this against the logarithm of the time to onset of contracture, an inverse linear relationship was apparent ($r=-0.876$, $P<0.001$; see Fig. 5).

DISCUSSION

Methodological aspects. We used developed tension as an indicator for heart function[13, 24]. We avoided an intraventricular balloon, because of the resulting subendocardial ischemia[18].

We used purine nucleoside and oxypurine release as an indicator of ATP-breakdown. Although part of the ATP metabolized remains in the cell as ADP or AMP, purine release rises in accordance with ATP-breakdown[1, 20].

Myocardial function. The effect of the six different inotropic agents on heart function were in accordance with their known modes of action[3, 9, 28]. No statistically significant differences in heart function were found during reperfusion, except for the lower resting tension in the high-calcium group at the end of reperfusion. Although wash-out of Ca-antagonists is notoriously slow, the hearts pretreated with Ca-antagonists did not exhibit a lower function. We cannot exclude that drug residues, causing negative inotropy, masked improved function due to protection against ischemic injury. The high-potassium group resumed contractile activity significantly earlier than all other groups. Probably in the former, ionic homeostasis was restored faster.

Purine release. The effects of the various agents on purine release were mostly non-significant. Only during perfusion with high potassium did the purine release rise significantly. This ion probably induces increased calcium-influx through the depolarized membrane[14], causing increased energy turnover[25].

During reperfusion there were no significant differences in purine release between the ischemic control group and the treated groups. Our earlier work showed the inverse relationship between pre-ischemic myocardial function and ischemic purine release[6].

The similarity of purine release after ischemia in the present experiments indicates that ATP breakdown was comparable in the treated groups, and remained unaltered by the variation in duration of ischemia. We assume that the ATP-sparing effect of the negative inotropic treatments was masked by the longer duration of the ischemic period.

Coronary flow. All agents but dopamine increased coronary flow significantly. This effect is due to either a direct vasodilation (potassium, magnesium, nifedipine and verapamil) or to increased demand (calcium). These results are in line with the

known effects of the agents or drugs.

After reperfusion there was no difference in flow between the groups. The interplay between coronary flow regulation and myocardial metabolism remains complex and may involve various factors[26]. When compared for flow, all hearts exhibited a similar recovery.

Time until the onset of contracture. The time until the onset of contracture in our ischemic control group (about 10 min) is in accordance with the reports by other authors[16, 27]. We were particularly interested in the relationship between pre-ischemic heart function and the time until onset of contracture. The fairly strong inverse relation found stresses the influence of pre-ischemic heart function on the ensuing ischemic period. This relation was also found in an in vivo dog model[23]. In that study infarct size was used instead of onset of contracture. We think the same mechanisms apply in both studies. We reported earlier that ischemic function and ischemic purine release did not correlate[8]. On the other hand, ischemic purine release seemed dependent on pre-ischemic heart function. A lower cardiac function at the onset of ischemia probably results in reduced ischemic ATP-catabolism[6]. This agrees with the observation that different, equi-active negative inotropic treatments result in equivalent ischemic ATP-content (and recovery of heart function)[28]. Slower ischemic ATP-breakdown probably underlies the delay in onset of ischemic contracture due to hypothyroidism[4].

Calcium-antagonists are thought to be beneficial through a variety of mechanisms: increasing collateral flow, decreasing preload and afterload, reducing calcium-influx and preserving mitochondrial function[22]. We expected an additional protective effect of the calcium-antagonists compared to potassium cardioplegia. We did not find such an effect. Collateral flow or reduced afterload have no role in an isolated, non-working heart model. The preload in an intact organism is different from the in vitro preload. Remains the reduced calcium-influx which probably causes the negative inotropic effect of calcium-antagonists. So calcium-antagonists probably have a broader (and better) effect in an in vivo situation than in an isolated heart preparation. We think that in our model, their main mode of action is through negative inotropy.

The exponential relation between the inotropic response and the time until contracture suggests that, at low levels of heart function, a further small reduction of heart function results in a markedly longer time until the onset of contracture.

We cannot exclude that the higher pre-ischemic resting tension in the negative inotropic groups resulted in a longer time until contracture. Further research is necessary to evaluate this possibility.

Clinical implications. This paper further stresses the importance of pretreatment in therapeutic interventions that are accompanied by transient ischemia (percutaneous transluminal coronary angioplasty, bypass surgery, heart transplantation).

CONCLUSIONS

In an isolated rat heart preparation: 1) an inverse relationship exists between pre-ischemic function and onset of contracture; 2) Ca-antagonists fail to show benefits above other negative inotropic treatments.

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Appendix 6

Effect of inosine and adenine on nucleotide levels in post-ischemic rat heart perfused with and without pyruvate

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ABSTRACT

Reports on enhanced nucleotide regeneration by purines during reperfusion are conflicting. Therefore we evaluated the effects of post-ischemic inosine or adenine administration on adenine nucleotide levels and function recovery in the isolated rat heart. Experiments were performed with glucose-containing modified Tyrode solution both with and without addition of 5 mM pyruvate. After 15-min stop-flow ischemia, hearts were reperfused for 45 min with 20 μM purine and 0.5 mM ribose.

Adenine nucleotide levels tended to recover better in the purine-treated groups. The purine-treated hearts with pyruvate exhibited a higher energy status, as expressed by an elevation of the ATP/ADP ratio. The level of IMP was twice higher in the inosine/glucose group than in all other groups.

Purine treatment, with or without pyruvate, did not affect function recovery, heart rate or coronary flow. Inosine treatment increased the adenosine concentration in the effluent, the hypoxanthine concentration rose with adenine treatment.

We conclude that after 45 min reperfusion, inosine or adenine do not improve heart function and hardly affect nucleotide levels.

INTRODUCTION

Ischemia reduces the myocardial adenine nucleotide content. De novo synthesis of nucleotides is very slow. Purines, like inosine and adenine, can enhance nucleotide regeneration via the salvage pathways (for a review, see ref. 30).

Isolated heart cells rapidly incorporate adenine into adenine nucleotides[3], but there are few reports on incorporation in isolated heart or in the heart in vivo. Some authors report enhanced ATP levels, if inosine is provided during reperfusion, even though the incorporation rate of ^{14}C-labeled inosine is too low to explain the observed elevation[11,26]. The maximal activity of adenylosuccinate synthetase, one of the enzymes in this salvage pathway, does not permit a rapid regeneration[6]. In addition, the ^{14}C-incorporation rate overestimates net nucleotide gain, because even the normoxic heart catabolizes its AMP to adenosine.

Inosine may alter the metabolic and functional state of the heart, possibly with secondary effects on the nucleotides. Inosine causes coronary vasodilation and increases myocardial contractility in the dog[14], but it is negative inotropic in rat heart[12]. It shifts the use of free fatty acids to glucose[25]. Inosine also inhibits adenosine uptake[15].

Exogenous pyruvate increases glycolysis and improves function recovery in the (post-)ischemic heart[17,18,21,28]. The reduction in free AMP content, caused by pyruvate in glucose-perfused hearts, lowers the efflux of adenosine and inosine[4]. Pyruvate also decreases the NADH/NAD$^+$-ratio, and may thereby deblock an inhibition in the salvage of hypoxanthine, and thus also of inosine[20].

We wanted to assess the contribution of adenine and inosine in the post-

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). Inosine and D-ribose were purchased from Merck, Darmstadt, FRG; adenine from Koch-Light, Haverhill, Suffolk, UK; and sodium pyruvate from Boehringer, Mannheim, FRG.

Perfusion of the hearts

Male Wistar rats (252 ± 27 g, n=40) with free access to food and water were anesthetized by an intraperitoneal dose of 30 mg pentobarbital. Hearts were rapidly removed and immediately cooled in ice-cold saline until they stopped beating. Retrograde perfusion according to Langendorff was performed with a modified Tyrode buffer. The buffer consisted (in mM) 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. Mixtures were passed through a 0.45 μm filter and equilibrated with 95% O_2/5% CO_2. The perfusion temperature was adjusted to 37°C with a thermocouple-regulated electric heater just prior to the perfusion canula. The canula was fitted with an A-F6 temperature probe, connected to a DU-3 monitor (Ellab, Copenhagen, Denmark). Cooling of the heart was prevented by keeping it in a double-walled chamber. The perfusion pressure, measured with a Statham P23Db-transducer (Hato Rey, PR, USA) was stabilized at 72 mmHg.

Protocol After a 15-min equilibration period, followed by a 15-min pre-ischemic period, hearts were made globally ischemic for 15 min. When heart rate dropped below 300 beats per minute, the hearts were paced at that rate. They were reperfused for 45 min with standard medium with one of the six following additions: none (GN), inosine + ribose (GI), adenine + ribose (GA), pyruvate (PN), pyruvate + inosine + ribose (PI), pyruvate + adenine + ribose (PA). The concentrations of inosine and adenine were 20 μM, those of ribose and pyruvate 0.5 mM and 5 mM, respectively. Finally, standard perfusion buffer was given during one min before the hearts were freeze-clamped with a Wollenberger clamp cooled in liquid nitrogen.

Function was monitored with a force transducer (Hugo Sachs F5-2, March/Freiburg, FRG). During the equilibration period, resting tension was adjusted to 5 g. Developed tension was calculated as peak systolic tension minus resting tension. Flow was measured with an electromagnetic flow probe (Transflow 601, Skalar Medical, Delft, The Netherlands) or by timed collection of effluent. Heart rate was assessed and the rate tension product (RTP) was calculated as the product of heart rate and developed tension.

	GN	GI	GA	PN	PI	PA
AMP	0.69 ± 0.15	0.59 ± 0.21	0.71 ± 0.16	0.46 ± 0.10	0.46 ± 0.12	0.48 ± 0.20
ADP	2.88 ± 0.28	3.51 ± 0.39***	3.25 ± 0.36	2.44 ± 0.28	2.51 ± 0.17	2.66 ± 0.39
ATP	18.0 ± 1.2	18.9 ± 1.6	18.5 ± 2.1	16.6 ± 1.4	16.6 ± 1.2	18.2 ± 1.6
TAN	21.6 ± 1.4	23.0 ± 1.7	22.5 ± 2.4	19.5 ± 1.5	19.6 ± 1.2	21.3 ± 1.8
IMP	0.022 ± 0.015	0.053 ± 0.013****	0.030 ± 0.013	0.026 ± 0.008	0.030 ± 0.006	0.028 ± 0.026
CrP	34.5 ± 3.6	32.4 ± 9.0	32.9 ± 3.3	45.3 ± 3.1	43.6 ± 3.7	33.9 ± 16.4
ATP/ADP	6.3 ± 0.5	5.4 ± 0.5**	5.7 ± 0.4*	6.8 ± 0.7	6.7 ± 0.7	6.9 ± 1.0
dwt	0.15 ± 0.02	0.14 ± 0.04	0.18 ± 0.03	0.20 ± 0.01	0.18 ± 0.02	0.18 ± 0.02

Table 1. The concentration of high-energy phosphates (in μmol/g dwt), ATP/ADP ratio and dry weight (in gram) measured in freeze-clamped heart. Inosine increases the ADP and IMP concentration and decreases the ATP/ADP ratio in the glucose groups. Adenine also decreases ATP/ADP ratio. In the pyruvate-treated groups, addition of purine compounds did not cause significant differences. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001, for p values: GI and GA were compared with GN, PI and PA were compared with PN; G=glucose, P=pyruvate, N=no purine, I=inosine, A=adenine, TAN=total adenine nucleotides, CrP=creatine phosphate, dwt=dry weight, means ± SD, n=6-9.

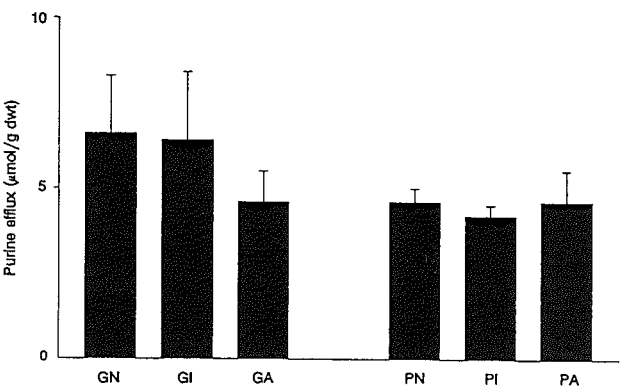

Fig. 1. Purine efflux in the first five-minute period of reperfusion after subtraction of infused inosine was comparable in all groups (means ± SD, n=6-9; G: glucose, P: pyruvate + glucose, N: no purine, I: inosine, A: adenine).

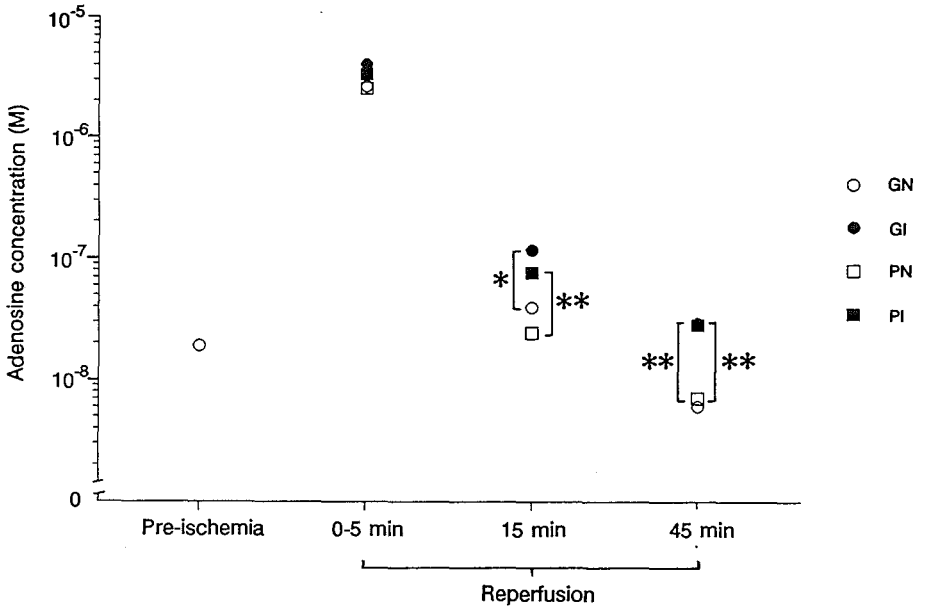

Fig. 2. Adenosine concentration in the effluent before ischemia and during 45 min of reperfusion. The adenosine concentration was higher in the inosine- treated hearts (GI, PI) than in the control hearts (GN, PN) (For abbreviations, see legend to Fig. 1, *p<0.05, **p<0.005, means, n=6-9).

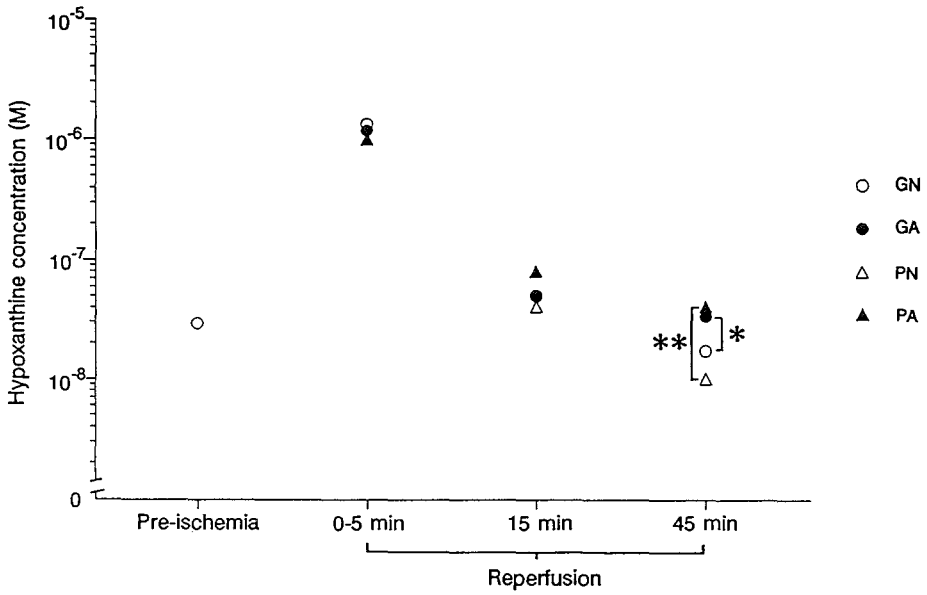

Fig. 3. Effluent hypoxanthine concentration before ischemia and during reperfusion. The hypoxanthine efflux was higher in the adenine-treated hearts (GA, PA) than in the control hearts (GN, PN) after 45 min of reperfusion (For abbreviations, see legend to Fig. 1, *p<0.05, **p<0.005, means, n=6-9).

Cardiac high-energy phosphates, AMP and IMP were measured by our HPLC-method[13]. Samples were taken from the effluent for the purine assay just before ischemia, 15 and 45 min after ischemia, and also from the effluent collected during the first five min reperfusion. Adenosine, inosine, adenine, hypoxanthine, xanthine and urate were determined in 200-μl samples as described before[13]. The retention times (min) were: urate 7.5, hypoxanthine 8.6, xanthine 9.4, inosine 11.9, adenine 13.6, adenosine 27.7. Identification of HPLC peaks was done by comparing retention times and wavelength ratios of 254 and 290 nm (purines in effluent) and 254 and 214 nm (high-energy phosphates in tissue) with standards. Compounds were quantified from peak areas.

Statistics

All values are presented as average ± standard deviation. GI and GA were compared with GN, PI and PA were compared with PN. Student's t-test was used (two-sided) for statistical analysis. G groups were not compared to the P groups, as the effects of pyruvate have been described in the literature, and because the G and P groups were not randomized.

RESULTS

Function, flow and heart rate

Before ischemia the values of the groups, which were to be treated with the purines and those that were not, were similar. Developed tension was 14 ± 3 g in the glucose-perfused groups (G) and 15 ± 2 g in the groups with pyruvate addition (P). Coronary flow was 8 ± 1 ml/min (G) and 9 ± 1 ml/min (P). Heart rate was 310 ± 35 beats/min (G) and 300 ± 24 beats/min (P).

Addition of adenine or inosine to the reperfusion buffer did not significantly alter the recovery of developed tension. (The values between brackets are percentages of pre-ischemic values.) The developed tension recovered to 9 ± 3 g (G, 65 ± 14%) and to 14 ± 2 g (P, 89 ± 14%). Coronary flow at the end of reperfusion was 6 ± 2 ml/min (G, 76 ± 20%) and 9 ± 2 ml/min (P, 103 ± 30%) The product of developed tension and heart rate was 2600 ± 800 g/min (60 ± 15%) and 3900 ± 500 g/min (85 ± 14%) in the glucose- and pyruvate-perfused groups, respectively. Heart rate was equal in all groups.

Adenylates and high-energy phosphates

Hearts treated with inosine plus glucose (GI), had only a tendency towards higher total adenine nucleotides (TAN) ($p=0.09$ vs GN, see Table 1). Inosine addition increased ADP levels from 2.88 ± 0.28 (GN) to 3.51 ± 0.39 (GI) μmol/g dwt ($p=0.004$). This increased ADP was reflected in a decrease of the ATP/ADP ratio ($p=0.008$). The IMP levels more than doubled when inosine was added ($p=0.0006$ GI). Addition of adenine to the glucose buffer caused smaller alterations in the nucleotide levels, but in the same direction. The ATP/ADP ratio decreased ($p=0.03$) and the ADP level slightly increased ($p=0.06$). Purine treatment did not improve nucleotide levels in the pyruvate-treated hearts; they exhibited a higher energy status, as expressed by an elevation of the ATP/ADP ratio. Addition of the purines did not alter the creatine phosphate (CrP) levels.

	GN	GI	GA	PN	PI	PA
preperfusion	0.63 ± 0.18	0.54 ± 0.09	0.52 ± 0.12	0.59 ± 0.17	0.62 ± 0.19	0.52 ± 0.23
0-5 min	12.2 ± 2.9	30.9 ± 2.7	11.7 ± 3.8	11.4 ± 1.7	29.8 ± 1.0	10.2 ± 1.4
15 min	0.95 ± 0.31	20.0 ± 0.3	0.84 ± 0.24	0.74 ± 0.14	20.4 ± 0.2	1.23 ± 0.30
45 min	0.45 ± 0.19	19.8 ± 0.3	0.54 ± 0.14	0.38 ± 0.07	19.6 ± 0.2	0.58 ± 0.16

Table 2. Sum of the concentration (μM) of urate, xanthine, hypoxanthine, inosine and adenosine in the effluent, before and after ischemia (means ± SD, n=6-9).

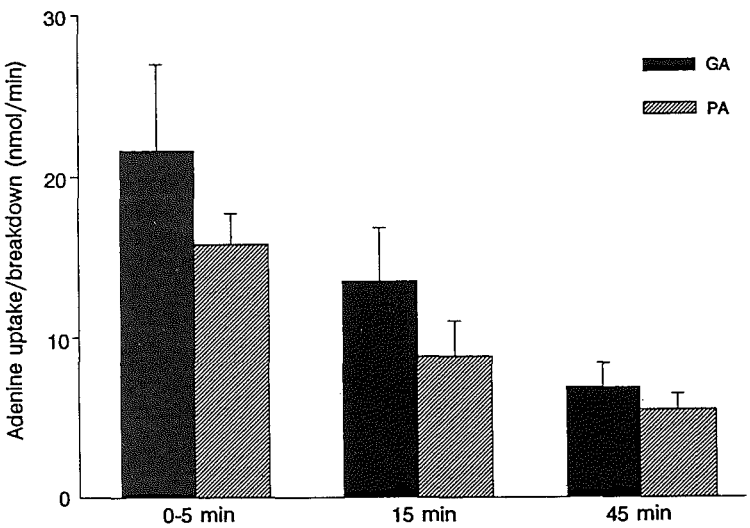

Fig. 4. The uptake/breakdown of adenine decreased during reperfusion in both adenine-treated groups (GA = glucose-adenine, PA = pyruvate adenine; means ± SD, n=6).

Nucleosides and oxypurines

In Table 2 the sum of the concentrations of AMP catabolites (adenosine + inosine + hypoxanthine + xanthine + urate) are shown before, 0-5, 15 and 45 min after ischemia.

Before ischemia, the average purine concentration in the effluent was 0.57 ± 0.16 μM. Per minute, the heart lost 25 nmol/g dwt of purine compounds, which was about 0.1 % of the myocardial adenine nucleotide content. In samples, drawn from the effluent collected from 0-5 min after ischemia, the sum of AMP catabolites had increased to 12 ± 3 μM in the GN group. The GA, PN and PA groups showed similar results. When inosine was added, the sum was much higher (Table 2). We corrected the sum of catabolite concentrations for the added inosine, and calculated the washout of nucleotide catabolites during the first 5 min of reperfusion. These values, presented in Figure 1, show that the nucleotide breakdown in the groups is comparable.

Figure 2 shows that addition of inosine increased the effluent concentration of adenosine 3-4 times in the samples collected 15 and 45 min after ischemia. Inosine addition also increased the concentration of inosine, hypoxanthine, xanthine and urate (not shown).

Addition of adenine increased the hypoxanthine concentration 2-3 times 45 min after ischemia (see Fig. 3). There was also a nonsignificant rise in urate and xanthine, but inosine and adenine levels were unaltered (data not shown). The uptake/breakdown of adenine is highest shortly after ischemia and decreases in the following samples (Fig. 4). These values were calculated as difference of infused concentration and effluent concentration multiplied by the coronary flow.

DISCUSSION

Nucleotide loss

When oxygen requirement exceeds oxygen consumption, ATP is broken down and dephosphorylated catabolites leak out of the cell. In isolated rat heart, 30 min stop-flow ischemia decreases the ATP level by 50%[22]. Reperfusion causes a rapid phosphorylation of ADP and AMP, but ATP recovers only to 60% within 1 h. Without treatment it takes days until pre-ischemic values are reached[33]. In our untreated glucose group, the ATP concentration after 15 min stop-flow ischemia and 45 min reperfusion was 18 μmol/g dwt. In a previous study, we found a pre-ischemic ATP level of 23 μmol/g[13]. The decrease of total adenine nucleotides would therefore be about 6 μmol/g, which fits very well with the purine loss during the first 5 min of reperfusion.

To find a net increase in adenine nucleotide concentrations, the formation has to be higher than the loss. In our pre-ischemic hearts, the average loss was 25 nmol/min per g, while at the end of the reperfusion period this was 15 nmol/min per g. These losses compare well with values earlier described by us[11]. There are four pathways for adenine nucleotide regeneration: adenosine incorporation, salvage of hypoxanthine (inosine) or adenine and de novo synthesis.

Adenosine incorporation

Adenosine incorporation is the fastest regeneration pathway. Dow et al.[6] measure an incorporation rate of about 400 nmol/min per g dwt. in adult rat-heart myocytes. Transport across the cell membrane is rate-limiting[6]. Other authors find maximal adenosine transport and incorporation rates which are 7 times lower[7,23,35]. Adenosine metabolism in the endothelium is very high. Therefore, conclusions drawn from studies in isolated myocytes can not be extrapolated to whole heart.

Reibel and Rovetto[22,23] found that 30 min of adenosine infusion did not increase post-ischemic nucleotide levels, but 5-h infusion restored ATP levels which had been halved by ischemia.

The hemodynamic effects of adenosine and its very short half-life in blood may limit its use as substrate for enhanced nucleotide synthesis. Therefore other precursors are more promising as potential treatments.

Hypoxanthine incorporation

Hypoxanthine is incorporated into IMP by hypoxanthine-guanine phosphoribosyl transferase (HGPRT). The conversion of IMP to AMP is catalyzed by adenylosuccinate synthetase and lyase. Hypoxanthine incorporation is a factor 10 slower than adenosine incorporation in neonatal cardiomyocytes[35]. The activity of HGPRT is limited by the concentration of phosphoribosyl-pyrophosphate (PRPP). Ribose-phosphate formed through the hexose-monophosphate shunt is used for PRPP production. The rate of synthesis of phosphoribosylpyrophosphate of whole heart cells with ribose as substrate is 50 nmol/min per g dwt[6]. The adenylosuccinate synthetase activity is extremely low, i.e. 15 nmol/min per g, therefore this should theoretically be the highest incorporation rate of hypoxanthine and inosine possible[6].

Inosine incorporation

Inosine is catabolized to hypoxanthine and ribose-1-phosphate before incorporation[19]. This reaction is catalyzed by nucleoside phosphorylase, mainly localized in the endothelium[2,24]. The ribose-1-phosphate will probably remain within the endothelial cell, unused for enhancing the PRPP concentration in the myocytes. Hypoxanthine and adenosine inhibit inosine incorporation, possibly by competition for HGPRT and the nucleoside carrier, respectively[1,19]. Inosine does not inhibit the activities of adenosine deaminase, 5'-nucleotidase, adenosine kinase and AMP-deaminase[6].

The incorporation rates of 20 μM inosine measured by various authors differ widely. Aussedat et al.[1] found a ^{14}C-inosine incorporation rate of 10 nmol/min per g dwt in isolated rat heart, while Harmsen et al.[11] reported 0.4 nmol/min per g. The former found no increase in ATP in hearts after 30-min inosine treatment during reperfusion. The increase in adenine nucleotides of 3.7 μmol/g dwt by 20 min reperfusion with inosine, described by the latter, is much higher than could be explained by the rate of inosine incorporation. The adenine nucleotides content in our series increased from 21.6 (GN) to 23.0 (GI) μmol/g (Table 1). (This would indicate an average increase of 35-40 nmol/min per g dwt, but the increase was not statistically significant.)

Reperfusion with 200 μM inosine in guinea-pig heart resulted in an increase

of adenine nucleotides of 35-40 nmol/min per g[34]. The increases in adenine nucleotides are higher than might be expected from the ^{14}C-incorporation rates or the in vitro activity of adenylosuccinate synthetase minus the amount of purine bases leaking out of the heart. A potential explanation for this discrepancy is an inhibition by inosine of adenosine transport out of the cell.

The increase in IMP from 22 to 53 nmol/g may have resulted from incorporation (Table 1). However, IMP may also have been generated through AMP-deaminase. In cardiomyocytes Geisbuhler et al.[9] measured ten times higher amounts than we do.

Adenine incorporation

In isolated heart cells the maximum adenine incorporation rate is 50% of adenosine incorporation[6]. Adenine uptake does not interfere with adenosine metabolism[6]. In theory adenine incorporation should not be faster than the rate of PRPP synthesis (maximum 50 nmol/min per g dwt in rat cardiomyocytes). In our series ther were only trends towards higer ATP levels. In a dog-heart model, a decrease of adenine nucleotides took 10 days to recover without treatment, but was replenished with adenine/ribose treatment in one day[33]. With ca. 18 μM adenine, the ATP regeneration was ca. 20 nmol/min per g during the first 4 h after ischemia, but this rate decreased rapidly in the following hours. In an earlier, preliminary paper, however, the same authors found no effect on ATP levels after 90 min adenine/ribose treatment[32].

Pyruvate and high-energy phosphate

Addition of pyruvate to a glucose-perfused isolated heart decreases the concentrations of inorganic phosphate, AMP and ADP, but increases the level of CrP[4,8,18]. Bünger showed that pyruvate lowered adenosine and inosine production in guinea-pig heart, and speculated that reduced AMP levels would decrease purine production[5]. Our values do not show this result. This point needs further evaluation.

Pyruvate could decrease the inhibition of hypoxanthine incorporation by NADH in an isolated cardiomyocyte preparation[20]. In our model, however, there was merely a tendency towards an increased nucleotide level in the glucose/inosine group (GI) compared to the glucose group (GN), and in the pyruvate/adenine group (PA) compared to the pyruvate group (PN). These differences were not significant. We were unable to find evidence that this mechanism[20] functions in our model.

Purines and energy status

In the glucose-perfused group, addition of purines decreased the energy status of the heart (Table 1). We found an increase in ADP and a decrease in the ATP/ADP ratio. A similar tendency in the inosine group has been reported earlier[11]. Inosine reduced ATP and increased ADP in isolated rabbit heart and reduced CrP in rat heart[1,16].

Purine concentrations in the effluent

Inosine infusion caused the expected increase in effluent inosine and its breakdown products during reperfusion. In the inosine group -both with and without pyruvate- the adenosine concentration increased (Fig. 2). This phenomenon, which we earlier described in normoxic rat heart[31], therefore also occurs after ischemia.

The adenine uptake by the heart is highest soon after the start of the adenine infusion (Fig. 4). Because of the reactive hyperemia, the effluent concentration of adenine is lowest after 15 min reperfusion.

In intact dogs Ward et al.[33] measured an adenine uptake of 38% in one passage through the heart (arterial concentration 18 μM). Our value in isolated rat heart was less than 10%. Adenine perfusion caused an increase in the levels of hypoxanthine, xanthine and urate. Adenine increased hypoxanthine production, confirming literature data[3]. The absence of increased inosine concentrations suggests that adenine deamination is not routed via AMP and adenosine, or via IMP.

Purines and function

Purine derivatives affect hemodynamics. Inosine causes vasodilation, it increases contractility in some species[12]. Our earlier work showed that inosine (>30 μM) increases coronary flow in isolated rat heart[29].

Many authors describe protection of the heart during and after ischemia by inosine[9,27,28,34]. Yoshiyama et al.[34] showed that in an isolated guinea-pig model, after 2 h of post-ischemic inosine treatment, function was better than in a control group. Shortly after ischemia they found the highest function, but this deteriorated later. Inosine decelerated this rate of decline. In our model function improved the first half hour after reperfusion and than stabilized. We found no differences between hearts treated with or without purines.

The hearts perfused with pyruvate had a very good function recovery. Coronary flow was high. In post-ischemic working rat heart, it caused an earlier start of function, enhanced function and increased flow, but no increase in ATP[18].

CONCLUSION

In an isolated rat-heart model, we tried to create optimal conditions for purine incorporation by perfusing the heart with ribose and pyruvate supplemented with adenine or inosine. We raised adenine nucleotide levels only marginally, but failed to improve function.

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Appendix 7

Xanthine Oxidoreductase Activity in Perfused Heart of Various Species, Including Man

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ABSTRACT

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 in other species, e.g., rabbit and man. 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 2x urate, were (mU/g wet weight, mean ± SE): 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=5); 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 conclude that isolated perfused mouse, rat, guinea-pig, and also bovine hearts show considerable xanthine oxidoreductase activity, contrasting rabbit, porcine and human hearts.

INTRODUCTION

In heart adenine nucleotides are broken down to adenosine, inosine and hypoxanthine. These can be found in the cardiac effluent, and 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 heart of man[6,8,10,11-14] and several other species, including rabbit,[5-7,9,10] is controversial. The classical assay of the enzyme in homogenates may lead to erroneous results, e.g., due to endogenous inhibitors.[4,14] We decided to use the isolated, perfused heart 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.

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 pentobarbital in accordance with institutional guidelines. Hearts were removed, arrested, and perfused retrogradely with Tyrode buffer at

9.6 kPa and 37°C as described before.[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). Canulation of mouse aorta proved difficult and time-consuming: about 25 min were needed. For the other species one 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) or by timed collection of perfusate. Unless otherwise indicated, a control period of 15 min was used; then the perfusion medium was supplemented with hypoxanthine (Merck), infused just above the aortic canula, 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, also perfusions with [8-^{14}C]-hypoxanthine (5-40 Ci/mol, Amersham) were carried out. Radioactive chromatographic peaks were detected on line with a 171 Radioisotope detector (Beckman).

Perfusion of human, porcine and bovine heart

Hearts of transplant patients in end-stage heart failure due to ischemic heart disease or dilated cardiomyopathy were arrested in situ with St. Thomas' Hospital cardioplegic solution 4°C (ref. 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 5000 IU/liter heparin (Organon Teknika)] within 15-20 min after slaughter in a local abattoir.

Hearts were transported in ice-cold saline; canulation time was 30-50 min. Then retrograde perfusion of the aorta started. The perfusion fluid (37°C) consisted of Tyrode buffer[1], supplemented with 5 mM Na-pyruvate, 10 IU/liter insulin (Novo), 5000 IU/liter heparin and 25 g/liter dextran (40,000 dalton; Pharmacia). After ca. 20 min 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), with heat exchanger and defoaming membrane. The perfusion apparatus was also equipped with a roller-pump, a manometer, an LPE-1440 filter (Pall), a bubble trap and a fluid reservoir. Blood was washed from the hearts for 10 min at a rate of 200 ml/min. Then recirculation started with 800-2200 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 900-1200 ml/min. Heart weight after the experiment was 300-800 g (man), 200-300 g (pig) and 1.9-3.2 kg (cow).

RESULTS

Rodent hearts

Due to their small size, we were unable to monitor function in mouse heart. In the other hearts, changes in heart rate and apex displacement were minimal during the experiment. Control coronary flow (ml/min per g wet weight) was 7.6 ± 1.0 (mouse, mean ± SE), 7.3 ± 0.5 (rat), 11.4 ± 1.1 (guinea-pig) and 5.0 ± 0.6 (rabbit). In the course of the experiment, flow decreased somewhat.

Table 1. Apparent xanthine oxidoreductase activity in isolated, perfused heart

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

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
Man	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

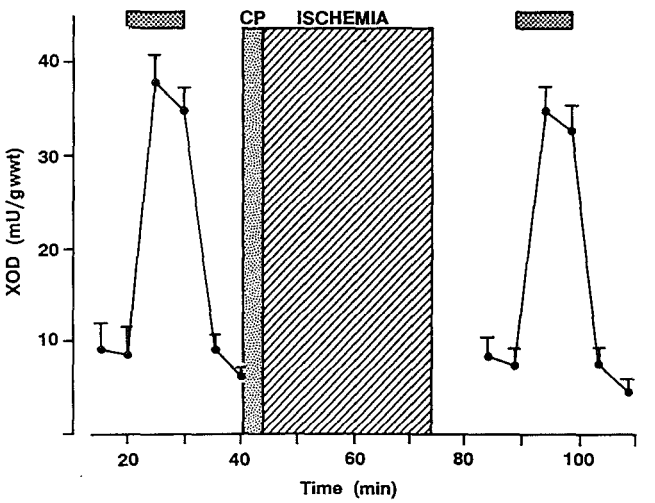

FIG. 1. Effect of cold potassium cardioplegia on apparent xanthine oxidoreductase activity. After an equilibration period of 20 min, rat hearts were infused for 10 min with 50 μM hypoxanthine (horizontal bar). From the effluent xanthine and urate concentration, the enzymatic activity was calculated. Ten minutes post-infusion, cold St. Thomas' Hospital cardioplegic solution was administered (3.0 ml/min), followed by an ischemic, hypothermic period of 30 min. After 15 min reperfusion, hypoxanthine was again infused; the xanthine oxidoreductase activity measured was similar to that before cardioplegia/ischemia (Student's paired t-test, $P > 0.05$). Data are given as means + SE (n=4). XOD = xanthine oxidoreductase. wwt = wet weight.

Contrasting 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 (μM) amounted to 2.9 ± 0.4 (mouse), 2.4 ± 0.2 (rat), 0.23 ± 0.08 (guinea-pig) and 0.106 ± 0.014 (rabbit); urate concentrations (μM) 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.

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

Pig, cow and human hearts

Human and porcine hearts beat regularly, whereas bovine hearts showed only atrial activity. Coronary flow (ml/min per gram wet weight) was: 0.82 ± 0.12 (man), 1.8 ± 0.2 (pig) and 0.43 ± 0.07 (cow). 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 heart, i.e., 0.31 mU/g. Only porcine heart showed a lower value: the activity was below the detection limit (0.1 mU/g). Bovine heart had a tenfold higher activity and fivefold lower urate/xanthine ratio than human heart.

Upon collection of 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 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 set-up.

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 comprises the major purine released from rat heart under basal conditions.[2-4] Gerlach et al.[17] did similar observations in guinea-pig heart. We reported blockade of hypoxanthine breakdown during anoxia by allopurinol.[4] These observations strongly suggested that rat and guinea-pig heart 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 heart (33 mU/g), measured with hypoxanthine, was of the same order of magnitude as that in rat and guinea-pig heart (Table 1). It exceeds the only reported value for mouse heart by a factor ten.[19] Our estimate for rat and guinea-pig heart 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 heart. This should be checked with xanthine as 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 heart agrees with data given in refs. 13 and 21, but is >30x higher than that reported in ref. 22. In guinea-pig and human hearts, xanthine breakdown seems to be faster than xanthine formation. Rabbit heart 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

We suggested recently that xanthine oxidoreductase is present in the heart 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 heart.[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. 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 human hearts.

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