PURINES, FREE RADICALS AND ANTIOXIDANT SYSTEMS

A study in hearts of various species, including humans

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PURINES, VRIJE RADICALEN EN BESCHERMENDE SYSTEMEN
Een studie in harten van verschillende dieren en de mens

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The stars have not dealt me the worst they could do:
My pleasures are plenty, my troubles are two.
But oh, my two troubles they reave me of rest,
The brains in my head and the heart in my breast.

(A.E. Housman 1859-1936, Collected Poems (1939), Additional Poems, 17)

Unfortunately we have known each other for a short time
From this particular period I have no clear memories
Maybe unconsciously you have told me
To understand and find a cure for the disease
Which has ended your live, without knowing mine.

In memory of my father

CONTENTS

Chapter 1

INTRODUCTION

The heart provides the mechanical pump to circulate blood with necessary nutrients and oxygen for bodily functions. Under normal conditions blood flow regulates the supply to fulfill the demand. If supply is lower than the demand, because of for instance coronary artery disease, ischemia develops. This may be life threatening due to development of arrhythmias and pump failure. The length and the severity of the ischemic period determines the extent of the damage. If a severe period of ischemia is longer than 20-30 minutes the heart becomes irreversibly damaged. Stores of high-energy phosphates such as ATP and phosphocreatine decrease during ischemia. Measurement of high-energy phosphates and their breakdown products can be used as indicators of the metabolic status of the heart (appendix 1).

If oxygen is not available, ATP cannot be regenerated from ADP and the latter is further broken down to the purine catabolites. Although depletion of ATP itself can already be lethal for the cell, accumulation of metabolites might even worsen the situation. Within the catabolic pathway of ATP the last enzyme, xanthine oxidoreductase, might play an important pathological role during ischemia-reperfusion of the heart[110]. During reperfusion, this enzyme can produce superoxide radicals, which can be converted to even more cytotoxic compounds (Figure 1).

Free radicals are atoms or molecules which have gained or lost a single electron. These compounds are highly reactive due to the presence of this unpaired electron[153,178,289]. Oxygen-derived free radicals may play a critical role in human disease processes[33], such as inflammation, aging and reperfusion injury of various organs, including the heart[110,188,189]. Damage may occur to DNA, proteins, lipids, and other organic compounds, and thus impair the function of vital systems[247] (see reference 154 for review on cardiovascular injury). Measurement of free radicals is difficult due to their short half-life. Indirect measurement is possible with malondialdehyde (MDA) as indicator of lipid-peroxidation. However, reported levels are controversial and might be caused by a false detection method[37]. Chapter 2 highlights the controversies concerning MDA.

Levels of free radicals are not only modulated by their sources such as leukocytes[203,296], oxidation of catecholamines[128], prostaglandin pathways[147], mitochondrial respiratory chain[246] or xanthine oxidase, but also by endogenous scavenging systems, like catalase, superoxide

dismutase and glutathione peroxidase. Little knowledge exists about these enzymes in human myocardium. Large species differences in activities could exist in analogy with xanthine oxidoreductase or uridine phosphorylase. Additionally the glutathione system may be used as an indicator of oxidative stress after ischemia. The second part of chapter 2 describes these endogenous antioxidant systems.

As already mentioned xanthine oxidoreductase could play an important role in ischemia/reperfusion damage. The relationship between xanthine oxidoreductase, superoxide radicals and detoxifying systems is depicted in Figure 1.

Chapter 3 is devoted to xanthine oxidoreductase and the controversial data presented in the literature about this enzyme. We measured the activity of the enzyme in an isolated perfused heart model and heart homogenates. In analogy with purine catabolism pyrimidine breakdown seems to happen in the ischemic myocardium[257]. Little knowledge exists about pyrimidine degradation in human myocardium. More information could be useful, because pyrimidine metabolizing enzymes regulate levels of anti-cancer drugs. Secondly, the detection of pyrimidine catabolites may provide an additional diagnostic tool for assessing the severity of myocardial ischemia.

In the early 80's investigators thought that repeated ischemia induced cumulative damage to the myocardium[55]. However, short ischemic periods of intermittent anoxia gradually decrease ATP breakdown in rat hearts[55]. Multiple transient coronary occlusions during coronary angioplasty (PTCA) seem to reduce angina symptoms and ECG changes and improve hemodynamic function[49,58]. One or more brief ischemic periods reduce infarct size in dogs if they precede a prolonged severe ischemic period[205]. Until this moment it is unclear what mechanism causes this protection. The effects of repeated short ischemic periods on myocardial energy metabolism will be described in chapter 4. Furthermore we speculate about the role of adenosine as protecting substance in this chapter.

It is obvious that long lasting ischemia results in irreversible damage. However, reperfusion, although necessary for myocardial survival, may cause irreversible damage. Since myocardial reperfusion damage may occur during life-saving cardiac surgery, it is vital to know what source(s) produce(s) these radicals, how they are counteracted and how to intervene in their formation. The understanding of the protective mechanism of repeated short ischemic periods provides potentially a useful tool to treat high risk cardiac patients.

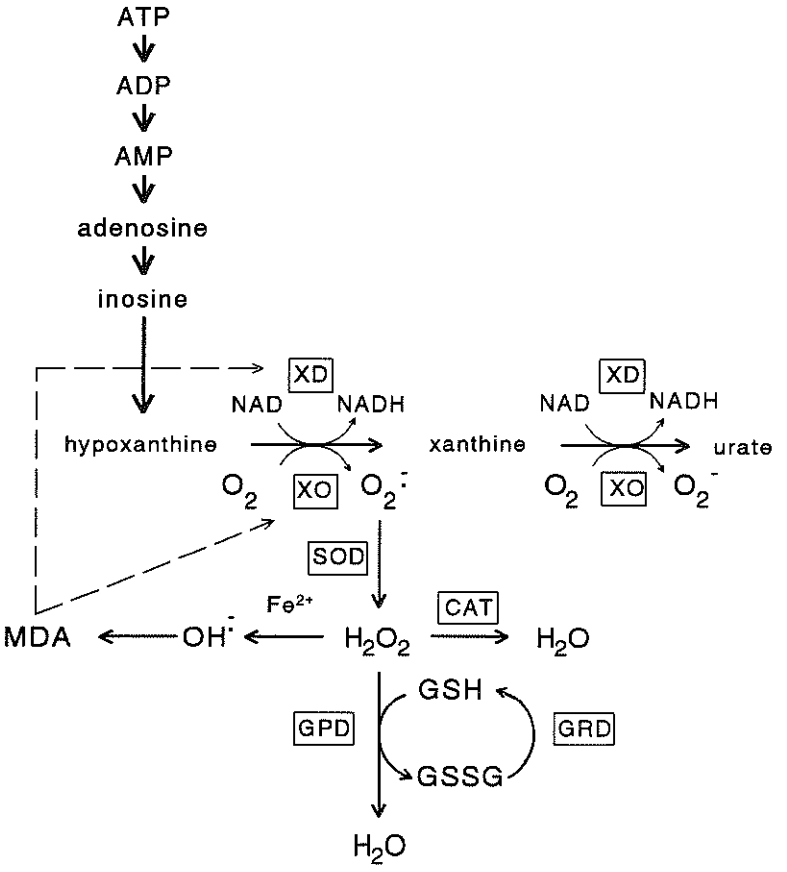

Figure 1. *Schematic relationship between xanthine oxidoreductase, oxygen-derived free radicals and protective enzymes. Ischemia causes the breakdown of ATP and the conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO), which produces superoxide radicals upon reperfusion. The free radicals are detoxified by the various antioxidant enzymes shown. GPD = glutathione peroxidase, GRD = glutathione reductase, SOD = superoxide dismutase, CAT = catalase, GSH = reduced glutathione, GSSG = oxidized glutathione, MDA = malondialdehyde, stippled line see reference 102, for the role of iron (Fe^{2+}) see reference 289.*

Chapter 2

FREE RADICALS

In the last two decades numerous studies have provided evidence for the relationship between free-radical damage and various pathological states[33], including cardiovascular injury[105,153]. In every living cell which uses molecular oxygen, oxygen derived free radicals can be produced. The role played by these radicals in myocardial ischemic injury is indirectly proven by the beneficial effects of "free radical scavengers". As can been seen from table 1 various sources and endogenous or exogenous defences are known. Direct detection of free radicals is almost impossible, so indirect methods are often applied to prove the existence of these radicals. This chapter highlights the use of malondialdehyde, the end-product of lipid peroxidation, as an indirect marker for oxygen-derived radicals and focuses on the natural scavenging systems.

Sources

Various free radical sources are known, including leukocytes[203,296], oxidation of catecholamines[128], prostaglandin pathways[147], and mitochondrial respiratory chain[246]. Within the catabolic pathway of ATP the last enzyme, xanthine oxidoreductase (XOD), might play an important pathological role during ischemia-reperfusion of the heart[110].

In the 70's Hearse described for the first time the occurrence of reoxygenation injury in rat myocardium[109]. The hypothesis that XOD could play a role in myocardial reperfusion injury was based on two observations, 1) the possible role of xanthine oxidoreductase in gut ischemia[90] and 2) the beneficial effects of the XOD blockers allopurinol (see chapter 3) and tungsten[32], an inactive analog of molybdenum which inactivates both dehydrogenase and oxidase form, on myocardial performance during reperfusion. The native dehydrogenase form of the enzyme can be converted to the oxidase form during ischemia by proteolysis[16,57,294] (irreversible) or oxidation of thiol groups[22,57,133,294] (reversible). Data on the time span of conversion are controversial[42,61,67]. The oxidase form uses molecular oxygen as electron acceptor, producing superoxide radicals during the breakdown of hypoxanthine or xanthine. ATP breakdown during ischemia provides high concentrations of the substrate and reperfusion the necessary oxygen to induce the production of superoxide radicals by

xanthine oxidoreductase. This enzyme is described in Chapter 3.

Sources	Action	Reference/chapter
Activated leukocytes	produce superoxide radicals	203,296
Xanthine oxidase	produces superoxide radicals	chapter 3
Mitochondria	produce superoxide radicals	246
Arachidonic acid metabolism	produce superoxide radicals	147
Catecholamines	radicals through autooxidation	128
Natural defences		
Superoxide dismutase	scavenges superoxide radical	188,191, chapter 2
Catalase	scavenges hydrogen peroxide	chapter 2
Glutathione peroxidase (GPD)	scavenges hydrogen peroxide	chapter 2
GSH	co-substrate of GPD	chapter 2
Urate	scavenges superoxide	
	scavenges hydroxyl radical	6,17,18
Therapeutical possibilities		
Allopurinol/oxypurinol	blocks xanthine oxidase	chapter 3
	scavenges hydroxyl radical	112,200
Mannitol	scavenges hydroxyl radical	73
Dimethylthiourea	scavenges hydroxyl radical	27, but see 304
Deferoxamine	chelates iron, blocks Haber-Weiss reaction	11,155,236,291
GSH precursors	increase cellular GSH	41,145,228
Vit E (alpha-tocopherol)	terminates radical chains by donation of H^+	14,184

Table 1. *Free radical sources, defences and therapeutical interventions. The items shown in italics will be discussed in this thesis. GSH = reduced glutathione.*

Detection methods	Action	Reference/chapter
EPR spectroscopy	direct visualisation of radicals	92,309
Spin trapping	EPR spectroscopy, formation of stable radical	31,234,309
Aromatic hydroxylation	hydroxyl radical, detection of products	93,135,215,231
Fluorescent probes	hydrolysis+fluorescence	136
Scavengers SOD+CAT	effects on ischemia/reperfusion	82,84,303
GSSG	$GSH+H_2O_2$->*GPD*->$GSSG+H_2O$	chapter 2
Malondialdehyde	end-product of lipidperoxidation	chapter 2

Table 2. *Direct and indirect free radical detection methods frequently used. The items shown in italics will be discussed in this chapter. EPR = electron paramagnetic resonance, CAT = catalase, GSH = reduced glutathion, GSSG = oxidized glutathione, GPD = glutathione peroxidase, SOD = superoxide dismutase.*

Detection

Methods

Direct detection of oxygen-derived free radicals is almost impossible, because of their instability and reactivity and consequent short half-life. Electron-spin resonance or spin trapping might provide a direct method to measure free radical formation[234,308]. Indirect probing can be done by various techniques (Table 2). Firstly, the influence of endogenous or exogenous free-radical scavengers, like superoxide dismutase or catalase or other compounds might give a clue about the causative role of free radicals in reoxygenation injury of the myocardium. Secondly, hydroxyl radicals can be trapped by aromatic compounds[93,135,231], with subsequent measurement of the hydroxylation products by high performance liquid chromatography (HPLC). Onodera and Ashraf detected hydroxyl radicals in rat heart with salicylic acid as trap[215]. Recently 2,3-dihydroxybenzoic acid, one of the hydroxylation products of salicylic acid which is absent in healthy subjects, was

detected in patients with myocardial infarction[275]. Explanted human hearts subjected to 20 minutes of ischemia and 30 minutes of reperfusion release one of the hydroxylation products of phenylalanine if the latter was given 10 minutes prior to ischemia and during the reperfusion phase (unpublished data). Thirdly, compounds associated with the detoxification of free radicals like oxidized glutathione can be measured (see section **Defences**). Finally, products such as malondialdehyde, generated during the free radical attack can be used as indirect markers.

It has been shown that lipid peroxidation occurs during a variety of pathological conditions[222], including aging and myocardial damage. Lipid peroxidation is an autocatalytic free-radical-mediated destructive process. Polyunsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides during this process (Figure 2). The latter decompose to form low molecular mass hydrocarbons, hydroxy aldehydes and fatty acids, ketones, alkenals and alkanals and the end-product malondialdehyde. MDA can react with important biological compounds like amino acids, proteins and nucleic acids.

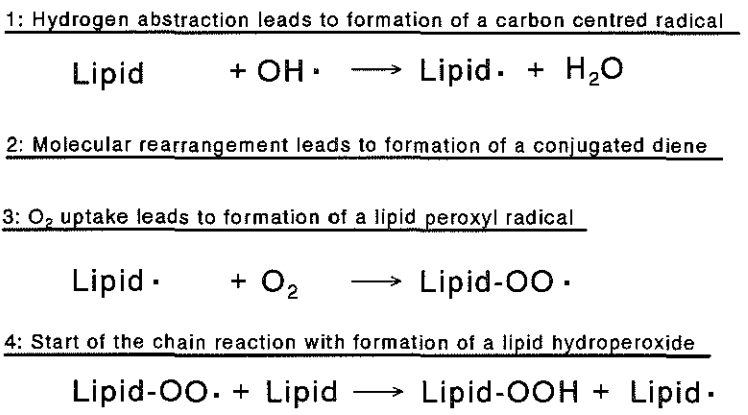

Figure 2. *Initiation and propagation of lipid peroxidation. Lipid hydroperoxides can react with Fe^{2+} or Fe^{3+} to form lipid alkoxyl radicals or lipid peroxyl radicals, respectively*[289].

MDA determination

Several methods have been developed to quantify MDA formation in vitro and in vivo. In the past most of the detection methods were based on the reaction of MDA with thiobarbituric acid (TBA), in which one molecule of MDA reacts with two molecules of TBA to form a stable red chromogen[212]. The MDA-TBA adduct was measured by spectrophotometry or fluorometry. However, the TBA-test is often criticized due to its lack of specificity in complex biological systems because: 1) TBA can react with various other compounds[123,142], and 2) the strong acidic or heating conditions might induce artificial MDA formation during determination[103].

To circumvent the first problem, HPLC separation of the MDA-TBA adduct was developed[298]. This method increased the specificity of the assay; however still included the acid and heating step. For a couple of years a sensitive direct HPLC detection method, without the use of TBA, is available[34,159].

Clinical studies

In a variety of pathological heart conditions MDA formation was investigated. Belch and co-workers showed that there was a direct relationship between cardiac function and plasma MDA in patients with congestive heart failure[20]. Patients with myocardial infarction showed increased levels of MDA in the blood[177]. Reduction of the bloodflow like in coronary angioplasty[56,232], or coronary surgery[269], as well as a disturbance in supply/demand of the heart (pacing stress test[213]) seems to induce lipid peroxidation. Recently Plachta and co-workers suggested that the blood MDA concentration may provide important supplementary information in patients with symptoms of atherosclerosis[220]. Furthermore they proposed that peroxidation of lipids, especially low-density lipoprotein (LDL), might be the cause of atherosclerosis (see also reference 105). One study shows that patients with atherosclerosis have higher levels of oxidized LDL[295]. However the presence of oxidized LDL in the circulation seems peculiar because it is possibly not produced in the blood due to high levels of antioxidants or taken up by the Kupffer cells in the liver[280]. It is important to note that all these studies applied the TBA-assay and the results should therefore be viewed with caution.

Ex-vivo studies

Literature data on MDA formation are controversial and depend on the detection method used. Studies performed with the TBA test clearly show the formation of TBA reactive material in myocardial tissue as a consequence of ischemia-reperfusion[5,13,40,59,84,99]. The increase in TBA-reactive material seems to depend on the duration of the ischemic period[99] and the time of reoxygenation[48]. However, experiments with detection of MDA by HPLC,

failed to observe increased MDA formation in rabbit[40,68] and rat[13] (but see references 60,261) myocardial tissue during ischemia/reperfusion. Others could only detect MDA in the coronary effluent during artificial induction of lipid peroxidation by hydroperoxide[132,150].

It is important to notice the discrepancy of the time scale between ex-vivo studies with rodent hearts and the clinical studies, especially coronary angioplastic procedures. In ex-vivo studies MDA formation, measured with TBA, is only observed after relatively long ischemic periods (> 10 minutes), whereas during PTCA procedures balloon inflation for maximal 1-2 minutes leads to MDA production. Furthermore, ex-vivo MDA formation seems to depend on the detection method used. These contradictory results could be due to different factors: 1) the use of the unspecific TBA-test; 2) errors by interfering blood constituents during in-vivo measurement of MDA; and 3) species differences in free radical producing potential, like xanthine oxidase, or scavenging abilities.

To investigate some of the aspects mentioned above, we studied the effect of ischemia/reperfusion on MDA formation in isolated perfused rat and human hearts (appendix 2). To exclude the influence of blood constituents we perfused the hearts with a salt-buffer. Since in this kind of setup MDA production has only been reported after ischemic periods of more then 10 minutes, we subjected the hearts to 20 minutes of global ischemia. MDA was measured in effluent and myocardial tissue with HPLC. We observed no MDA formation after this ischemic period in both species. Cumene hydroperoxide induced however the release of MDA in rat hearts. Furthermore human myocardial tissue contained negligible amounts of free MDA (<4 nmol/gram protein), contrasting rat hearts displaying levels of approximally 50 nmol/g protein. Ischemia and reperfusion did not change the tissue content. These differences in MDA content between the two species may be caused by species differences in susceptibility to lipid-peroxidation, as described recently[248]. Peroxide stress increased the amount of MDA in tissue in both species. These results suggest that in both rat and human myocardium MDA is only formed under extreme conditions.

We made one peculiar observation: 40 minutes cardioplegia prior to the perfusion decreased the levels of MDA in rat hearts by 50% (appendix 2), an observation for which we have no explanation.

MDA as free radical indicator

A few studies compared the TBA-test with the HPLC method[13,40,68]. Levels of TBA-reactive materials were 100x higher than HPLC-measured concentrations of MDA in tissue. Interestingly, detection by HPLC showed that MDA levels decreased during ischemia, whereas TBA-adduct levels had a tendency to increase. Ceconi and co-workers speculated that the oxygen tension in the tissue regulates the MDA content[40]. That MDA returned to

pre-ischemic values during reperfusion further strengthened this observation.

Our ex-vivo data show that MDA formation, measured by HPLC, is absent in human heart after 20 minutes ischemia (appendix 2). This suggests that MDA formation found in clinical studies with relative short ischemic periods (<2 minutes) might have an extracardiac source. MDA could be formed by platelets or released from the atherosclerotic plaque during inflation of the balloon during coronary angioplasty. Apart from other possible extracardiac sources, the positive results could be caused by the non-specific TBA-assay. Therefore, reliable conclusions can only be drawn if blood MDA is measured by HPLC. Furthermore, seems the susceptibility for lipid peroxidation species dependent[248]. This has to be investigated further, especially in the human heart.

Defences

The extent of free-radical injury depends on the balance between the amount of free radicals produced and the activity of scavenging systems, especially the glutathione redox system. As xanthine oxidoreductase varies in different species this might also be true for antioxidant enzymes. Numerous data of different species are available in literature, but comparison is complicated because of differences in detection methods and tissue sources. Furthermore, activities of antioxidant enzymes in human myocardium are only reported in atrial tissue or post-mortem material. We therefore studied the activities of the free radical scavenging enzymes in various species (see also Figure 1).

Superoxide dismutase

Superoxide dismutase (SOD), an enzyme discovered in 1969, detoxifies the superoxide radical to hydrogen peroxide[191] in the following reaction:

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Hydrogen peroxide can react with iron (Fe^{2+}) or copper (Cu^{2+}) to form the highly cytotoxic hydroxyl radical (Haber-Weiss reaction)[32,223,291]. For a historical overview of this enzyme one is referred to reference 190. Cytosol contains CuZn-SOD, whereas mitochondria contain Mn-SOD[77]. Additionally, an extracellular and interstitial superoxide dismutase have been described[181]. The extracellular enzyme has a subtype, called C, which can bind to endothelial cells[250]. Organs with high respiration, like myocardium, have higher Mn-SOD activities[181]. Myocardial activities in various mammals differ little[87], however aging seems

to increase total enzyme activity in rat hearts[101,129]. Ischemia diminishes SOD activity in rabbit hearts, making them more susceptible to superoxide damage during reperfusion[59,71].

Little is known about this enzyme in human myocardial tissue. Marklund and co-workers reported the activities of SOD in human tissues; however they studied only a limited number of autopsies[181,183]. Appendix 3 describes our study on the SOD (CuZn-SOD + Mn-SOD) activity in pig, rat, guinea-pig and human myocardial tissue. Human heart contains about 7400 U/g protein, which is significantly lower than in the other species. This could suggest that human myocardium is less protected against superoxide radicals.

Although endogenous SOD is important for protection against superoxide radicals, this enzyme might even be more important in therapy. Recombinant extracellular superoxide dismutase type C given prior to ischemia seems beneficial[106,250]. Likewise superoxide dismutase coupled to polyethylene glycol to increase plasma half-life (PEG-SOD), might protect against changes in antioxidant defences during ischemia[81], or improve post-ischemic function when added to cardioplegia[82]. Despite these positive reports negative findings are also reported (for review see references 178,182) and could be the result of the experimental model used[290].

Catalase

Catalase, as well as glutathione peroxidase (see below), converts hydrogen peroxide to water:

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

The kinetics of catalase do not obey the normal pattern. It can not be saturated within the feasible concentration range for the spectrophotometer and is inactivated by hydrogen peroxide concentrations above 0.1 M. The enzymic decomposition of hydrogen peroxide is a first-order reaction. Therefore the enzyme activity can be expressed as the first order rate constant. In the species examined we observed similar low constants (appendix 3). This confirms literature data[70]. Catalase plays probably a minor role in the detoxification of hydrogen peroxide. However Thayer reported that although catalase is present in low concentrations it serves as a major route of hydrogen peroxide breakdown[264]. As with SOD, literature data on the beneficial effects of catalase are conflicting[178,182].

The glutathione system

The glutathione system consists of glutathione peroxidase (GPD), glutathione reductase

(GRD) and reduced glutathione (GSH). GSH converts electrophilic centers to thioether bonds, through enzymatic and chemical mechanisms, and serves as a substrate for selenium containing glutathione peroxidase (GPD). GSH regulates protein synthesis, degradation, structure and function. The availability of GSH has been related to some disease states. This system plays an important role in the detoxification of hydrogen peroxide and lipid peroxides, i.e. protection against oxidative damage. Since catalase activity is low (see above + references 70,129), this system plays a major role in the destruction of hydrogen peroxide, preventing the formation of the highly cytotoxic hydroxyl radical. The principal reaction schemes are:

$$2GSH + H_2O_2 \xrightarrow{GPD} GSSG + 2H_2O$$

$$2GSH + ROOH \xrightarrow{GPD} GSSG + ROH + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GRD} 2GSH + 2NADP^+$$

Tissue enzyme activities

Lawrence and Burk reported different glutathione peroxidase activities in various organs of the rat and species differences in liver tissue[163]. Human and pig hearts showed 10 times less activity than rat heart. In myocardial tissue this enzyme has been extensively studied under various conditions, including aging and pathological conditions. Aging decreases myocardial activity[36,107,129,252] whereas hypertrophied rat hearts contain higher levels[101]. Reported activities in rat vary between 16 and 200 mU/mg protein depending on the method used. On the other hand, few data on glutathione reductase are available in different species[71,87].

Little is also known about glutathione peroxidase and reductase in human myocardial tissue. Marklund, followed by Carmagnol and co-workers, found activities in human hearts between 16 and 69 mU/mg protein. However, they analyzed postmortem material[38,183]. Recently glutathione peroxidase and glutathione reductase were determined in atrial tissue from patients undergoing coronary bypass-surgery[2,193]. This might not reflect reality because: 1) enzyme activities could be different in other parts of the hearts, as described for other enzymes[45,53]; 2) ischemia-reperfusion injury largely concerns the ventricles and

diseased hearts might have activities different from normal hearts.

Since comparison of the activities reported in the literature in different species is difficult because of the use of different measurement methods we studied glutathione peroxidase and reductase in ventricles of different species, including humans. Appendix 3 shows that the activity in rat hearts is one order of magnitude higher than that in guinea-pig, pig and explanted diseased human hearts. Analysis of healthy human myocardium revealed values similar to those found in diseased myocardium. The latter suggests that the diseased state might not have influenced the activities of both enzymes. Based on these results rat hearts seem relatively better protected against hydrogen peroxide.

One would expect that glutathione peroxidase, glutathione reductase and GSH would act in concert. However GSH content varied little in the species examined, despite the large variations in glutathione peroxidase activities. Linear regression analysis of our data on enzyme activities and GSH content revealed no correlations. This supports the hypothesis by Godin and Marnet that the levels of these myocardial antioxidants are regulated individually[87].

Changes during myocardial ischemia

The glutathione system is a valuable means of assessing oxidative damage in myocardium and other organs (liver[122], kidney[171]) during ischemia and reperfusion[70,118]. It is unclear whether ischemia influences the activity of glutathione peroxidase. No effect of ischemia is observed in rabbits[71,144]. On the other hand, 40 to 90 minutes of cardioplegic arrest in humans is already sufficient to increase enzyme activity[2]. Glutathione reductase activity is unaffected by ischemia[2,71,144], but correlates weakly with basal GSH levels[144].

The change of tissue GSH and oxidized glutathione (GSSG) and the release of GSSG in the coronary effluent can easily be determined. GSSG efflux is crucial for the cell to avoid highly oxidative states. An energy-driven transport mechanism has been described in various tissues[216,227], including rat myocardial tissue[119,120]. The amount of myocardial GSH decreases during both ischemia and reperfusion, concomitantly with an increase of GSSG and GSH in the effluent. The ratio of GSH and GSSG in normoxic tissue decreases in various species during ischemia from >50 to 30[50,72] and even lower with hydroperoxide challenge (appendix 2). Since GSH is in equilibrium with cellular sulfydryl groups[72], its decline during ischemia decreases the protective role of glutathione peroxidase and possibly changes the activities of enzymes such as Na^+-K^+ ATPase or the Ca^{2+}-pumps in the sarcoplasmatic reticulum[227].

To investigate the importance of GSH during ischemia/reperfusion different techniques can be used. GSH concentrations in myocardium (and other organs as well) can be depleted by buthionine sulfoximine (BSO) which inhibits gamma-glutamylcysteine synthetase, an

enzyme involved in the GSH synthesis[91]. Regeneration of GSH from GSSG by glutathione reductase can be blocked with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)[25]. On the other hand, cellular cysteine or N-acetylcysteine[41], methionine[228] and gamma-glutamylcysteine ethyl ester[145], i.e., sulfur-containing precursors, increase GSH biosynthesis. Enhancement of GSH levels is also possible by exogenous GSSG[100] or glutathione esters[277]. The absence of selenium in the diet impairs the function of the selenoenzyme glutathione peroxidase[148].

Blaustein and co-workers observed an improved recovery in GSH-depleted rat hearts when the reperfusion buffer was supplemented with GSH[24]. Infarct size in GSH-depleted pigs was larger than in controls and smaller if GSH was infused during reperfusion[249]. Ceconi and co-workers described the protective effects of N-acetylcysteine on contractile recovery and post-ischemic GSSG levels in rabbits[41]. Supplementation of cardioplegic solution with N-acetylcysteine improves postarrest recovery of function in rat[192].

We found that rat hearts released substantial amounts of GSH, but no GSSG, after a cardioplegic period. This washout may be partly prevented by adding GSH precursors to the cardioplegic solution[192]. Rat hearts receiving 40 minutes cardioplegia prior to the perfusion protocol, released 10 times less GSH and GSSG than rat hearts without a cardioplegic period. This can be explained in two ways. Firstly, the washout of GSH after the cardioplegic period decreases the levels to a point where the systems become compromised and less GSSG can be formed. If this is true then the addition of GSH-precursors to the cardioplegic solution might be beneficial[192]. Secondly, the cardioplegic period protects against a subsequent period of ischemia, thereby protecting membranes (less washout) and decreasing free radical formation.

The fact that ischemic heart disease correlates with plasma selenium levels[256] further underlines the importance of this system. Although the effects of this system on post-ischemic function have been reported, it remains unclear through which mechanism this occurs. Especially the effects on enzyme activities need further investigation.

An indicator of oxidative stress

Based on the active transport of GSSG out of the cell[119], and the observations in different animals, this compound may be used as index of oxidative stress in man. Ferrari and co-workers tested this in patients undergoing coronary bypass grafting[69]. They clearly showed that oxidative stress occurred and that the magnitude depended on the duration of ischemia. However GSSG release could also be the result of active blood components.

To establish if human hearts release GSSG in a similar way as other species we studied the glutathione system in salt-perfused explanted human and rat hearts (appendix 2). After 20 minutes of global ischemia these hearts release GSSG in the first minutes of reperfusion. GSH/GSSG ratios decreased from 50 to 30 after 30 minutes reperfusion. This suggests that

oxidative stress has occurred in these explanted human hearts confirming clinical observations[69]. Therefore, the release of GSSG in the clinic might be a useful tool to investigate the occurrence of oxidative stress.

Sources versus Defences

We studied xanthine oxidoreductase as one of the possible sources of oxygen-derived free radicals and the important antioxidant enzymes (chapter 3). It is logical to assume that sources and defences are delicately balanced. We hypothesized that levels of xanthine oxidoreductase, and thus exposure to free radicals, might modulate the activities of superoxide dismutase, catalase, and glutathione peroxidase. Linear regression analysis of our XOD data and corresponding antioxidant activities in the various species revealed no correlations.

It is obvious that a simple relationship between XOD and the antioxidant enzymes does not exist. Possibly other factors such as non-enzymic antioxidants could be as effective as enzymic and could vary between species, as suggested by Godin and Marnet[88], counterbalancing the observed differences in myocardial activities of superoxide dismutase and glutathione reductase (human) or glutathione peroxidase (rat). Additionally, other free radical sources may differ between the species examined.

Based on our findings of different antioxidant profiles we want to caution against extrapolation of data from one species to another in respect with free radical generation, protection and damage.

Chapter 3

ASPECTS OF PURINE AND PYRIMIDINE METABOLISM

The catabolic breakdown of ATP involves many enzymes. We focused our investigations on the last enzyme in the purine cascade, xanthine oxidoreductase (XOD), since it might be involved in free-radical mediated myocardial injury. Literature data on the presence of this enzyme in human myocardium are however controversial. In this chapter the activity of this enzyme in various species, the effects of allopurinol, and its role in ischemia reperfusion injury will be discussed. Furthermore, in ischemic myocardium pyrimidine breakdown seems to parallel purine catabolism. Little is known about pyrimidine metabolism in man, and we therefore studied the activity of uridine phosphorylase in human and rat myocardium.

Xanthine oxidoreductase

XOD is a metal-flavoprotein containing molybdenum, flavin adenine dinucleotide (FAD) and iron/sulphur redox centers. The enzyme has broad substrate specificities[30,151]. However, its most important function is the breakdown of hypoxanthine to xanthine and xanthine to urate. Histochemical studies of XOD in human, bovine and rat myocardial tissue revealed its localization in the endothelial cells[125,238] (but see reference 149).

Assay methods and models

The activities of both XOD forms (see chapter 2 section **Sources**) can be determined by measuring the conversion of (radioactive) hypoxanthine or xanthine in heart homogenates by high-performance liquid chromatography[209,240], spectrophotometry[63,292], or thin-layer chromatography[202]. Xanthine has been favored as substrate, because it forms only one product, making the enzyme assay relatively easy. With hypoxanthine as substrate, the two-step reaction complicates the calculation of XOD activity. Other detection methods, including fluorometry[19], chemiluminescence[12], and colorimetry[79], make use of different substrates (e.g., pterin) or electron-accepting dyes (methylene blue, tetrazolium salts). It is important to mention the inactivation of xanthine oxidoreductase by proteolysis or thiol

group oxidation during processing of the tissue samples and the presence of endogenous inhibitors. Furthermore it is known that xanthine oxidoreductase can destroy itself by self generated O_2 metabolites[262].

To circumvent all these problems De Jong and co-workers perfused hearts with hypoxanthine and calculated xanthine oxidoreductase activity from the formation of xanthine and urate. They found that XOD activities in 7 species varied considerably[54]. Furthermore the ratio of xanthine and urate release seemed different, especially in the guinea-pig which released little xanthine. It was hypothesized that xanthine breakdown exceeded its formation in this species. To study this phenomenon we perfused rat and guinea pig hearts with hypoxanthine or xanthine and measured xanthine and/or urate production (appendix 4). This differential breakdown hypothesis could not be confirmed since the Michaelis constants were similar for both substrates in both species. At this moment we have no explanation for the low xanthine release in guinea-pig hearts. However, we made another unexpected observation. The estimated xanthine oxidoreductase activity was lower with xanthine than that with hypoxanthine. The measured distribution of xanthine over the intra- and extracellular space, using ^{14}C-sorbitol, suggests that a transport barrier exists for xanthine into the cell. A possible explanation for this barrier is that hypoxanthine can be used in the salvage pathways[306], whereas xanthine is not salvaged by the myocardium and will be degraded to urate. Thus hypoxanthine is preferable as substrate in this experimental model to investigate free-radical damage caused by XOD.

Species differences

Considerable differences exist between various mammals with regard to xanthine oxidoreductase activities. Pig hearts, as well as rabbit hearts, contain little XOD[54,62,98,209,263,292]. In contrast rat, guinea-pig and dog hearts display relatively high activities[42,61,96,209,265]. Reported activities in human hearts vary from high[125,151,292] to very low[54,63,96,149,209,221,225,251,293]. We can not explain these controversial results easily. They could be the result of different or limited tissue sources (biopsies of normal and diseased tissue or post-mortem material), various assay methods, or tissue processing (see above).

However, data from cardiac patients, suggest that the human myocardium can produce urate and thus contains active xanthine oxidoreductase [56,116,210]. Based on the ex-vivo results of De Jong and co-workers[54] and the experiments described in appendix 3 we conclude that human myocardium contains only negligible amounts of active enzyme. These results must however been looked at with some caution, because these hearts came from patients with cardiomyopathy or end-stage ischemic heart disease. We can not rule out the possibility that diseased human heart contains inactive xanthine oxidoreductase as described by Edmondson and co-workers[64]. Urate production found in clinical studies might come from extracardiac

factors like neutrophils or other blood components. This could explain the difference found between in-vivo and ex-vivo studies (with blood-free solutions).

Influence of the pathological state

The possibility that the pathological state of the myocardial tissue might influence the xanthine oxidoreductase activity led us to investigate XOD activity in myocardial homogenates of spontaneous hypertensive (SHR) and hypercholesterolemic rats (Yoshida) and its relationship with age. We found a linear increase of XOD activity with age in the SHR rats (appendix 5), which was absent in their non-hypertrophic controls, suggesting that hypertrophied myocardium is more vulnerable during an ischemic period, especially in view of the age dependent decrease of detoxifying systems (see chapter 2). The hypercholesterolemic state did not change the XOD activities.

Influence on the pathological state

It is absolutely essential that the conversion of xanthine dehydrogenase to xanthine oxidase takes place, otherwise no free-radical injury can be induced during reperfusion of the heart. Until now data on the conversion rate are controversial. Under normoxic conditions the oxidase form comprises approximally 10% of the total activity (appendix 4). Engerson and co-workers observed that after 7 hours of ischemia 50% was present in the oxidase form in rat hearts[67], whereas the group of Downey observed that during global ischemia the conversion was maximal after 5 minutes, i.e. 30% of the total activity[61]. We did not see an increased xanthine oxidase activity after 30 minutes ischemia in rat and guinea-pig hearts (unpublished data).

Although the majority of the literature data suggests that human myocardium is almost devoid of XOD, we can not exclude the possibility that this enzyme still plays a role in ischemia-reperfusion injury. This is based on the fact that an inactive desulfo form exists under physiological conditions in rat liver[117]. Recently Abedah and co-workers demonstrated this form in human milk[1]. The desulfo form is able to produce superoxide radicals, without the conversion of the dehydrogenase to the oxidase form, through the FAD group.

Allopurinol

In principle allopurinol competes with hypoxanthine and xanthine for binding and oxidation by XOD. The product formed is oxypurinol, which forms a stable complex. This suggests that, although hypoxanthine or xanthine breakdown is blocked, free radicals could be formed during the conversion of allopurinol.

Nevertheless various authors showed that infarct size decreases in open-chest dogs pretreated with allopurinol[3,42,61]. It is interesting to notice that all studies using allopurinol

as a pretreatment for days observed beneficial effects, whereas the single study with a brief pretreatment (10 minutes prior to occlusion), lacked positive effects[139]. This might be explained by the fact that effective XOD blockade occurs when allopurinol is converted to oxypurinol. This could take place in the pretreatment period. In terms of pharmacokinetics, the halflife of oxypurinol is 20 times longer than the halflife of allopurinol[204]. This would give high levels of oxypurinol at the time of intervention as suggested by Tabayashi and co-workers[259].

We tested the influence of allopurinol, since it is commonly used, on purine release in rat, guinea pig, pig and human hearts (appendix 3). The blockade was 95% in rat and guinea-pig hearts and 40% in human hearts. In pig hearts XOD activity was too low to measure.

Allopurinol improves contractile function, coronary flow and phosphocreatine levels in tissue and decreases the incidence of arrhythmic activity, ultrastructural changes during ischemia/reperfusion, creatine kinase release and free radical formation in rat[11,43,265], rabbit[86,263], guinea-pig[254], and pig hearts[51]. Others could not find beneficial effects in rabbits[61,97]. The positive effects are surprising because rabbit, pig (and human) seem devoid of xanthine oxidase, in contrast with rat hearts[54]. The beneficial effect of allopurinol in species with low XOD activity can be explained in two ways: 1) only small amounts of XOD are necessary to induce injury as proposed by Terada and co-workers[263], 2) the blockade of hypoxanthine breakdown in rat heart increased its salvage and therefore ATP levels[161]. Chambers an co-workers rejected this hypothesis because they did not observe any differences in ATP levels in allopurinol pretreated or control rat hearts during either ischemia or reperfusion[43], 3) the protective effect of allopurinol is through another mechanism.

In species with low levels of xanthine oxidoreductase activity the protective mechanism probably does not involve xanthine oxidase inhibition or purine salvage. Purine salvage in these species might already be maximal because hypoxanthine is not degraded. Increased flow by increased adenosine levels might be beneficial. However the effect of allopurinol on flow is also seen in rabbits and could be the result of an unknown effect[263].

Despite the fact that human myocardial XOD activity is almost absent, allopurinol is used during thoraxsurgery. The drug is also present in the University of Wisconsin cardioplegic solution. In patients undergoing coronary artery bypass grafting, allopurinol as adjunct to cardioplegia reduces the number of chromosomal aberrations found after crossclamping by 60%[66]. In some studies pretreatment (2-4 days before surgery) with the drug decreases enzyme release, the incidence of cardiac complications[26,130,226,259] and improves post-operative cardiac performance[26,130]. Thus allopurinol is effective through another mechanism than XOD blockade. Allopurinol, and in particular oxypurinol, are free

radical scavengers by itself[112,200]. This would explain the effect in species with low or absent xanthine oxidase. Although the overall picture clearly shows a beneficial effect of allopurinol in different species, including man, the exact mechanism is still not completely understood.

Uridine phosphorylase

Importance

Relatively little is known about pyrimidine nucleotide catabolism. The degradation of these nucleotides seems to follow the same pattern as breakdown of purines in ischemic myocardium. The pyrimidine uridine might have physiological functions in analogy with adenosine. Its inotropic action in rabbit myocardium has been described, as well as its positive effects on glucose uptake and glycogen content[156]. Furthermore, key enzymes within the breakdown pathway play an important role in activation or breakdown of anticancer drugs[170,218,241,299].

Species differences

Based on the above and the observed species differences of enzyme activities in the purine breakdown pathways, we studied formation and breakdown of uridine in human and rat hearts during ischemia and reperfusion (appendix 6). Human myocardium hardly catabolizes uridine, as can be concluded from clinical observations and experiments with explanted perfused hearts and tissue homogenates. The activity of uridine phosphorylase, catalyzing the conversion of uridine to uracil, was 60 times higher in rat heart homogenates. Consequently, uracil was mainly found after an ischemic period in rat hearts.

Implications

Because uridine accumulates in the myocardium and is released after an ischemic period, this compound could be used as an indicator of myocardial ischemia. Based on the large differences between rat and human myocardium in enzyme activity, experiments with rat hearts are invalid to test the cardiotoxicity of anticancer drugs. Further discussion about this subject lies beyond the scope of this thesis.

Chapter 4

REPEATED ISCHEMIA

Repeated short periods of ischemia occur naturally in man, but clinical interventions, such as percutaneous transluminal coronary angioplasty (PTCA), also provoke them. It was thought that the deleterious effect of recurrent ischemia would be cumulative in terms of function decrease or metabolic derangement[85]. In 1979 Verdouw and co-workers already showed in pigs that a second similar reduction of flow reduced lactate and inosine formation in comparison with the first[287]. Subsequently various studies showed that no cumulative loss in function[158], tissue damage[15,211] or ATP levels[113,157] occurred during repeated periods of ischemia. In 1986 Murry and co-workers observed infarct-size reduction in dogs induced by multiple brief ischemic periods followed by a prolonged period[205]. They called this finding "ischemic preconditioning" and this observation opened up a new field of investigations (Figure 3). This chapter describes the effect of multiple ischemic periods in rodent hearts on ATP catabolism and speculates about its role in preconditioning.

Characteristics

Since the observation of infarct-size reduction in dogs by repeated short ischemic periods through 4 x 5 minutes ischemia followed by 40 minutes sustained ischemia various investigators also observed a similar protection in other species, i.e. pig, rabbit and rat. Depending on the species the preconditioning stimuli may vary in number[168] or duration. Additionally, brief ischemic periods in one segment of dog hearts seem to protect against damage caused by a sustained ischemic period in a non-preconditioned part of the heart[224]. This was however not found in a limited amount of pig hearts in our department. Hypoxia[160,244,245], ventricular pacing[286] (but see reference 180), potassium channel openers[94] and specific activation of adenosine A_1-receptors[95,266] also can induce the effect. It seems that the reperfusion period between the brief and prolonged ischemic period should be at least 1 minute[4].

Duration of the protective effect disappears rapidly. In dogs after 2 hours the infarct-size limiting effect was attenuated[206]. In rabbits the duration of full protection has been reported to be 30 minutes[195,282], gradually decreasing until it is completely lost after 2 hours[282].

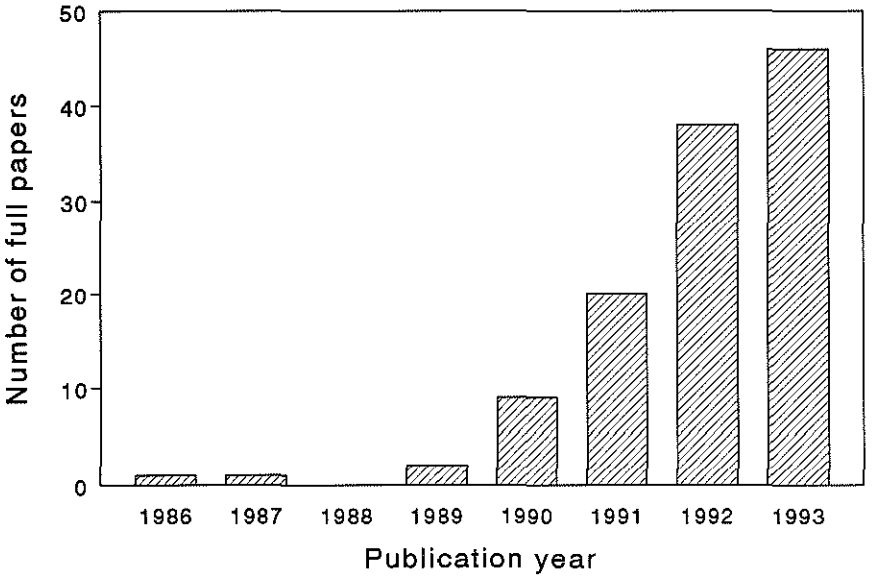

Figure 3. *Number of articles concerning preconditioning in the international literature.*

In rat[169] and pig[237] the infarct-size limiting effect is lost after 1 hour. Experiments with pigs performed in our department show that after 2 hours the protective effect still exists in some animals but is completely lost in others, suggesting an all or nothing phenomenon[146]. It must be kept in mind however that various studies used different protocols and that this may affect the duration of the protection. Recently in rabbits the initial protection could be reinstated if a second preconditioning period was given two hours after the initial stimulus[300]. In a limited number of pigs, this was only possible after 4 days and not after 1 hour (at which point the protective effect of the first stimulus was abolished)[237]. The authors hypothesized that the effect was caused by a mediator from an exhaustible pool. Possibly, if such a mediator exists, it is not derived from the blood since preconditioned blood-perfused hearts have similar protective capabilities as buffer-perfused hearts[239].

In addition to infarct-size reduction, preconditioning also improves recovery of contractile function[138,168,297] and reduces the incidence of post-ischemic ventricular arrhythmias[104,243]. In guinea-pig hearts four sequences of 5 minutes of ischemia and

reperfusion had no effect on contractile function after a 20-minute ischemic period[255]. In rats the beneficial effects on arrhythmias last for more then one day[243] (but see reference 169). In patients multiple transient coronary occlusions during PTCA reduce anginal symptoms and ECG changes and improve hemodynamic function[49,58], but others have failed to find beneficial effects[242]. Table 3 summarizes some of the important changes seen during ischemic preconditioning, while table 4 describes the changes seen during subsequent sustained ischemia/reperfusion.

Parameter	Reference
Expression of proto-oncogens	29,52
Formation of heat-shock proteins	7,23,52,143,302
5'-nucleotidase activity	141
Adenosine release	141
Glycogen breakdown	288,297
Antioxidant enzyme activity	52,115

Table 3. *Factors that increase due to brief periods of ischemia and reperfusion.*

Metabolic changes during and after the preconditioning period

High energy phosphates and purine metabolism

Murry and co-workers observed a reduction of ATP breakdown in dogs in the first 15 minutes of sustained ischemia after 4 x 5 minutes preconditioning[205,208]. This beneficial effect was transiently as at 60 minutes myocardial ATP levels were similar in control and preconditioned groups. Others confirmed these findings in pig[137,138,198]. Conflicting reports exist on rat hearts[104,126,255,288,297].

We focused our attention on the high-energy phosphate metabolism during short recurrent ischemia/reperfusion preconditioning periods for two reasons. First, short repetitive ischemic periods mimic coronary angioplastic procedures. Second, these short periods play a key role in the preconditioning phenomenon. We studied the changes in high-energy

phosphates and their catabolites during repeated ischemia in rat and rabbits hearts (appendices 7 and 8, respectively). We used a protocol of 6 sequences of 2 minutes ischemia and 3 minutes reperfusion. This ischemia-reperfusion period seems to be sufficient to elicit preconditioning in rat hearts[288].

In both species the first ischemic period induced a decrease of function and phosphocreatine, which stabilized throughout the subsequent occlusions. Purine release progressively decreased after each cycle. The pattern of recovery during each reperfusion period of P_i, phosphocreatine (PCr) and ATP is consistent with the data of Kida and co-workers[137]. If the 6 sequences were followed by 6 sequences of 4 minutes ischemia and 3 minutes reperfusion followed, purine release increased after the first 4 minute ischemic period and decreased in the subsequent ischemia/reperfusion periods. Thus the apparent blockade of ATP breakdown can be overcome by a more aggressive ischemic period. ATP levels remained the same during the protocol. However, other investigators found a decline of ATP levels during the preconditioning period[126,255].

Reduction in/of	**Reference**
Infarct size	169,174,208,237,239,301
Ultrastructural damage	137,208
Severity arrhythmias	104,169,174,206,243
Stunning	138,168,239,255,288,297,301
Time to contracture	9
High energy phosphate breakdown (transient)	9,126,137,198,208,255,283
Anaerobic glycolysis/lactate production	9,126,208,283, but see 124
Acidosis	9,137,198,255,297
Free Mg^{2+}	28
Ca^{2+}	255
Fatty acids	131

Table 4. *Effects of preconditioning on structural, physiological, and biochemical parameters during subsequent sustained ischemia/reperfusion.*

This decrease in purine release might be the result of 1) adenine nucleotide depletion; 2) reduced ATP synthesis; 3) impaired contractile function. The first explanation seems

invalid since ATP levels remained constant. Decreased ATP synthesis would lead to a reduction of the phosphorylation potential ([ATP]/[ADP]*[P_i]), increasing AMP and therefore purine release. Purine release is directly related to the rate of energy consumption and thus contractile function[307] or energy demand. Pre-ischemic function correlated well with post-ischemic purine release in the protocol with 2 minutes occlusion (appendix 8) as observed before[55]. This correlation decreased in the protocol with 4 minutes occlusion and might be the beginning of the dissociation between contractile function and energy metabolism which is observed after ischemic periods[21]. Thus purine release is related to the duration of the ischemic period and the contractile state. From this we may conclude that purine release as a parameter for ischemia has to be looked at with some caution.

We speculate that the phosphorylation potential regulates adenosine production -and consequent receptor effects- to achieve a new metabolic state of the myocardium. Adenosine would reduce cardiac work and consequently energy demand. This idea is further supported by the fact that A_1-receptor blockade diminishes the preconditioning effect (see below). Through this mechanism energy demand after the first ischemic period is reduced, protecting the heart in subsequent ischemic periods. Interestingly, activity of 5'-nucleotidase increases due to preconditioning, further increasing the levels of adenosine[141].

Role of adenosine

Adenosine changes the energy demand/supply ratio by decreasing function/heart rate or increasing flow (for review see references 65,114). The energy demand seems to be regulated through A_1-receptors, which are primarily located on the myocytes[111], while changes in oxygen supply are mediated by A_2-receptors on the endothelial cells[235]. Adenosine is supposed to be an important mediator of recovery of function during the post-ischemic period or preconditioning. This hypothesis seems logical, since adenosine builds up in the cardiomyocytes during ischemia (but see reference 279). It has been proposed that nucleoside transport inhibitors potentiate the infarct-size limiting effect of preconditioning possibly by enhancement of interstitial adenosine levels[44,197]. The effects of the adenosine receptors on ischemia can be investigated with the use of specific receptor agonists or antagonists, such as R(-)N6-2-phenylisopropyl adenosine (R-PIA) and 8-phenyltheophylline, respectively. Adenosine improves post-ischemic function through A_1 receptor activation in rat hearts[162]. A_1-receptor agonists attenuate H_2O_2-induced dysfunction[134]. It may be possible that via the A_1-receptor adenosine modulates the glutathione system through the pentose-phosphate cycle, increasing the resistance against hydrogen peroxide[305]. Furthermore, adenosine reduces glycolysis and glycogenolysis thereby preserving intracellular pH and preventing calcium overload[74,283].

Does adenosine mediate the preconditioning effect? Increased levels of adenosine are

only present for a short period because of rapid metabolism or washout upon reperfusion. The initial high release of adenosine decreases during subsequent preconditioning periods. Thus the protective effect possibly occurs after the first ischemic period. Stabilizing the myocardium in a new metabolic state would also lead to smaller variations in the phosphorylation potential and consequently adenosine release. Until now the role of adenosine is only partly supported[160,172] and needs further investigation.

Glycogenolysis and glycolysis

Deutsch and co-workers observed less lactate formation during coronary angioplasty after the second balloon inflation in patients[58]. This was however not found by others[242]. Lactate release in rabbits during repeated ischemia showed only minimal variations (appendix 8); peak variations depended on the duration of the ischemic period. We also observed a decrease in glycogen content during repeated ischemia, confirming the data of Volovsek and co-workers[288]. Furthermore, we found glycogen in both acid soluble as insoluble fractions. The acid-insoluble fraction is probably proglycogen, a low molecular weight form of glycogen with a high protein content[179]. During repeated ischemia both forms decrease whereas continuous ischemia blocks proglycogen breakdown.

Sustained ischemia after preconditioning reduces the breakdown of glycogen as well as glycolysis, as can be measured by the total glycogen content or glycolytic intermediates[127,205,208,283,288]. Recently Wolfe and co-workers showed a correlation between glycogen content and infarct size and they proposed that limited substrate availability reduces anaerobic glycolysis thereby preserving intracellular pH[297]. It remains questionable if this explains also the beneficial effect of only one 5 minute preconditioning period. Others also observed the beneficial effect of preconditioning on intracellular pH[9,137,255]. Steenbergen and co-workers proposed that preconditioning decreases the generation of H^+ which in turn leads to less Na^+-H^+ exchange and consequently reduced levels of intracellular Ca^{2+} by Na^+-Ca^{2+} exchange[255].

Stunning and preconditioning

One of the observations in our studies (appendix 7) was that after the first preconditioning period function decreased to a larger extent than in the following periods. However, from these experiments we can not conclude that the decrease of function during the preconditioning period might influence the outcome of a sustained ischemic period. Others have tried to investigate this hypothesis (see below). This decrease in myocardial

function, which is reversible, is termed "stunning", which refers to the reduced contractile function (hours to days) after a brief ischemic period, despite restoration of the coronary flow, without apparent cellular damage. Although a causal relationship between stunning and preconditioning seems unlikely because function during stunning can be depressed for days whereas the preconditioning effect lasts only a few hours, investigators have tried to establish this link. A decrease of the protective effect of preconditioning by extending the time between preconditioning period and the sustained ischemic period was observed even though a severely depressed function was seen[206]. Jennings and co-workers diminished the effect of futile contractile activity at the beginning of the sustained ischemic period, and thus ATP expenditure, by infusion of high-potassium cardioplegia before the sustained ischemic period. They observed less energy consumption in the preconditioned group, despite quick cardiac arrest[126]. Others restored contractility before the sustained ischemic period with dobutamine. However, the beneficial effects of preconditioning on infarct size remained[89,185,196]. This still does not prove that the decreased function is not related to preconditioning since 1) multiple systems might be affected; 2) dobutamine only changes the calcium transient, thereby increasing contractility; 3) dobutamine might act via catecholamines, which may be a mechanism for preconditioning.

Repeated ischemia: Epilogue

The investigations concerning this topic have increased considerable since the observation of the infarct-size limiting effect of repeated short ischemic periods in 1986 (Figure 3). However, although the beneficial effect of preconditioning has been demonstrated under various conditions major controversies still exist. Firstly, species differences in threshold and duration of the effect make it difficult to extrapolate results from one species to another. Secondly, data on the possible mechanism within one species are controversial (Table 5). This could be partly due to different preconditioning protocols and length of period of sustained ischemia, different anesthesia or even temperature. Furthermore, various compounds are used to study the adenosine receptor or the K_{ATP}-channel. Different binding properties for various compounds might exist. Whereas the number of receptors could explain the controversies between species. The nature of the adenosine receptor should be investigated further.

At this moment it is still unclear if one mediator or multiple compensatory mechanism cause the preconditioning effect. The protection of virgin myocardium against a sustained ischemic period by preconditioning another segment of the heart suggests that such a mediator exists[224], however this phenomenon is still not confirmed by others. This would

further explain why a certain reperfusion period is needed between the brief and sustained ischemic period. Interestingly, McClanahan and co-workers observed that transient renal ischemia was as effective as myocardial preconditioning in reducing myocardial infarct size in rabbits[187].

Based on the observations of Thornton and co-workers, using a protein synthesis blocker, it is unlikely that synthesis of a protective protein is the mechanism of protection[267]. However, they used cycloheximide and actinomycin D, which do not completely block protein synthesis. Others speculated that the formation of heat-shock proteins induce myocardial protection[7,23,52,143,302]. The synthesis of these proteins possibly occurs within one or two days, and therefore can not explain the shortterm protective effects of preconditioning.

Induction of multiple compensatory mechanism leading to a higher metabolic capacity could have positive effects in or after a second ischemic period. The induction of these mechanism could also explain the disappearance of the effect, possibly coinciding with the reinstatement of "physiological" intracellular conditions after a certain time. However, the duration of the preconditioning effect and stunning have different time scales. This suggests that normal conditions are not present after preconditioning has disappeared or that the reduction of contractile function is not related to regaining normal cell viability.

The fact that preconditioning is found in animal studies leads to the speculation that patients who have periods of angina might be better protected in a subsequent ischemic period that occurs within a certain time frame. Furthermore, understanding of the mechanism may provide a useful tool to decrease infarct size in high-risk patients.

Hypothesis	Reference				
	Dog	**Pig**	**Rabbit**	**Rat**	**Man**
Decreased ATP utilization	126,208	75,137,198,297		104	
Increased PCr levels		137,198		255,297(-)	
Decreased lactate production	126,208			9,288	58
Adenosine + A_1-receptor activation	95,141,283	281	35,176,197,272, 273,274,278	39(-),160(±),167(-), 172(±),186,224,266	
ATP sensitive K^+-channels	10,94,95,284		260,268(-),270,271	76(-),172(-)	
Formation of free radicals			121(-),214(-)		
Increased collateral flow	46(-)				49
Decreased contractility ("stunning")	127,185(-),206,217(-)	198(-),233(-)	196(-)	194(-)	
Increased antioxidants	115	80(-)	276(-)	52	
Neutrophils		201(-)	175(-)		
Prostanoids	285		174,258	8,166(-)	
G-Proteins		80		164(-),165,173(-),219	
Heat-shock proteins		7	108,143,152(-)	52	

Table 5. *Hypotheses on the protective effect of preconditioning. This table clearly shows the controversies which still exist in the literature between various species. Possibly free radicals or neutrophils play no role in the protective effect. Data on collateral flow are limited, but this mechanism may be of no importance since the protective effect of preconditioning also exists in species without collaterals such as rats, rabbits or pigs. It is obvious that the exact mechanism still has to be elucidated. (-) = hypothesis could not be proved. (±) = hypothesis partly supported.*

Chapter 5

SUMMARY

ATP is vital for contractility and basal metabolism, i.e. maintenance of ions through membrane pumps. Derangement in high-energy substrates levels due to ischemia could lead to cell death or prolonged post-ischemic dysfunction. Furthermore, other lethal substances, like free radicals, can be formed during reperfusion and cause injury. ATP catabolites might have beneficial or detrimental effects on myocardial function.

Hypoxanthine builds up during myocardial ischemia and is broken down to xanthine and urate. The enzyme xanthine oxidoreductase (XOD), which catalyzes this reaction, could be converted during ischemia to the oxidase form which produces superoxide radicals during reperfusion. The first part of chapter 2 deals with free radicals with emphasis on malondialdehyde formation as indirect free radical indicator. Despite the reports of malondialdehyde (MDA) formation in clinical studies, we were unable to measure this compound in explanted human and rat hearts subjected to ischemia (appendix 2). Probably MDA is only formed under extreme oxidative stress like cumene peroxide. Positive reports in clinical studies could be caused by the non-specific thiobarbituric acid test or an extracardiac source of malondialdehyde.

Since the extent of free-radical damage is not only modulated by the sources but also by the defences we investigated the activities of catalase, superoxide dismutase and glutathione-related enzymes. This is described in the second part of chapter 2. Human myocardium is less protected against superoxide radicals in comparison with the other species. Rat hearts have higher glutathione peroxidase activities (appendix 3). Although the various species have different enzyme profiles, there was no relationship between antioxidant levels and xanthine oxidoreductase activity. This suggests that they are not regulated by XOD. In view of these differences in protective capabilities one should be cautious with extrapolation of results from one species towards another.

Appendix 2 describes, in addition to malondialdehyde formation, the release of oxidized and reduced glutathione from hearts after a cardioplegic and ischemic period. We conclude that glutathione release after a cardioplegic period could make the hearts more susceptible to oxidative stress upon reperfusion. Furthermore these compounds could be used as indicators of oxidative stress in a clinical setting.

Chapter 3 is devoted to the enzyme xanthine oxidoreductase as a possible source of free radicals during ischemia/reperfusion. In this thesis two models (heart perfusions and

homogenates) are described to measure XOD activity in hearts (appendix 4). We propose to use hypoxanthine in a Langendorff model as substrate, since xanthine transport into the cell is limited. Since enzyme activities might change during pathological states or aging we investigated the cardiac XOD activity in hypertensive and hypercholesterolemic rats (appendix 5). XOD in hypercholesterolemic rats did not differ from their controls, whereas hypertensive rats have increased levels of XOD at old age. This might disturb the balance between XOD and antioxidant systems, which activities decrease during aging. Additionally we tested the effect of allopurinol on hypoxanthine breakdown in various species. From these studies and literature data we must conclude that xanthine oxidoreductase activity is virtually absent in human heart, and that this enzyme probably plays a minor role in free radical damage. In analogy with the purine metabolism myocardial uridine metabolism in man is low in comparison with rats (appendix 6). The release of uridine might prove to be a valuable detection method for cardiac ischemia in man.

Since the observation of the phenomenon called "preconditioning", i.e. infarct-size reduction after a severe ischemic period if this period was preceded by one or more brief ischemic periods, investigators have tried to understand the mechanism involved. The effects of repeated brief ischemic periods on high-energy phosphates and purine release are described in chapter 4. Brief periods of repeated ischemia did not induce cumulative ATP decrease or cumulative purine release or functional deterioration (chapter 4) contrasting the decrease after one long period. We propose that adenosine might play a regulatory role in the equilibrium between contractile function and high-energy phosphate metabolites and modulates the protective effect of preconditioning

In conclusion the studies in this thesis show that 1) the activity of xanthine oxidoreductase as a free radical source varies considerably between species, with very low activity in man; 2) the antioxidant profiles differed in the species studied; 3) glutathione, but not malondialdehyde release, can be used as indicator of oxidative stress; 4) uridine may be used as a new ischemic marker in man; 5) adenosine may regulate the phenomenon observed during repeated ischemia.

SAMENVATTING

De stof adenosine tri-fosfaat (ATP) is noodzakelijk voor de contractiliteit en de basale stofwisseling van het hart. Verandering van ATP concentraties door ischemie (aanbod van zuurstof is kleiner dan de vraag) van de hartspier kan leiden tot celdood of tot langdurige funktie verstoring. Tevens kunnen bij het herstel van de bloedtoevoer vrije radicalen worden gevormd, die beschadiging van de hartspier kunnen veroorzaken.

Tijdens ischemie wordt ATP afgebroken tot hypoxanthine wat zich ophoopt in de cel. Het enzym xanthine oxidoreductase welke de afbraak van hypoxanthine naar xanthine en urinezuur cataliseert, kan tijdens ischemie veranderen in een oxidase vorm die in staat is om superoxide radicalen te vormen. Hoofdstuk 2 beschrijft de diverse aspecten van vrije radicaal vorming, met name de productie van malondialdehyde, en de enzymen welke het hart beschermen tegen deze radicalen. Hoewel de vorming van malondialdehyde is aangetoond bij patienten, konden wij deze stof niet aantonen in geexplanteerde menseharten of ratteharten na ischemie (bijlage 2). Waarschijnlijk wordt deze stof alleen tijdens extreme omstandigheden gevormd, zoals de blootstelling aan hydroperoxiden. De aanwezigheid van malondialdehyde bij patienten zou veroorzaakt kunnen worden door de niet specifieke thiobarbituurzuur test of door vorming buiten het hart.

De schade veroorzaakt door vrije radicalen hangt niet alleen af van de bronnen maar ook van de beschermende systemen. We hebben de activiteit onderzocht van catalase, superoxide dismutase en enzymen van het glutathion systeem. Dit wordt in het tweede deel van hoofdstuk 2 beschreven. In vergelijking met de andere diersoorten is de mens minder beschermd tegen superoxide radicalen. Terwijl ratteharten een hogere activiteit van glutathion peroxidase hebben. Hoewel de verschillende diersoorten verschillende enzym profielen hadden was er geen relatie tussen de activiteit van deze enzymen en die van xanthine oxidoreductase. Dit suggereert dat de beschermende systemen niet worden gereguleerd door dit enzym. Door de verschillen in enzym activiteit moet worden opgepast bij extrapolatie van resultaten van de ene naar de andere diersoort.

Bijlage 2 beschrijft de vorming van glutathion na een cardioplegische of ischemische periode van het hart. Het verlies van glutathion na een cardioplegische periode vermindert de bescherming tijdens reperfusie. Glutathion zou in de kliniek gebruikt kunnen worden als een indicator van oxidatieve stress.

Hoofdstuk 3 is gewijd aan het enzym xanthine oxidoreductase, dat een mogelijke oorzaak is van vrije radicalen tijdens ischemie en reperfusie. Bijlage 4 beschrijft twee modellen (perfusies en homogenaten) om de activiteit van dit enzym te meten in het hart.

Op basis van de resultaten concluderen we dat hypoxanthine tijdens een hartperfusie gebruikt moet worden wanneer men de activiteit van xanthine oxidoreductase wil weten. De opname van xanthine in de cel is zonder verklaarbare rede verminderd. Omdat enzym activiteiten tijdens ziekten of veroudering kunnen veranderen hebben we de activiteit van xanthine oxidoreductase gemeten in ratten met verhoogd cholesterol of hoge bloeddruk van verschillende leeftijden (bijlage 5). De activiteit in de ratten met verhoogd cholesterol was vergelijkbaar met de controle ratten. Ratten met hoge bloeddruk vertoonden echter een toename van de activiteit tijdens toenemende leeftijd. Dit zou de balans tussen xanthine oxidoreductase en de beschermende system nadelig kunnen beinvloeden omdat de activiteit van de beschermende systemen afneemt tijdens veroudering. Voorts hebben we gekeken naar de invloed van allopurinol op de afbraak van hypoxanthine in verschillende diersoorten. Uit onze experimenten en literatuur gegevens moeten we concluderen dat de activiteit van dit enzym bij de mens verwaarloosbaar laag is en dus geen rol speelt bij vrije radicaal vorming. In analogie met het purine metabolisme is het uridine metabolisme in de mens erg laag in vergelijking met de rat (bijlage 6). De vorming van uridine zou een extra parameter kunnen zijn voor ischemie van het hart bij de mens.

Wanneer een lange ischemische periode wordt vooraf gegaan door één of meer kortdurende ischemische perioden neemt de grootte van het beschadigde gebied af. Dit fenomeen wordt "preconditioning" genoemd. Het effect van herhaalde kortdurende ischemie op ATP en purine uitscheiding wordt beschreven in hoofdstuk 4. Kortdurende ischemische perioden veroorzaken geen cumulatieve verandering in ATP concentraties, purine vorming of funktie vermindering in vergelijking met een lange periode. We suggereren dat adenosine een regulerende rol speelt tussen het ATP verbruik en de hartfunktie en mogelijkerwijs ook bij het beschermende effect van herhaalde ischemie.

Concluderend tonen de studies aan dat 1) de activiteit van xanthine oxidoreductase als een vrije radicaal producent behoorlijk verschilt tussen verscheidene diersoorten en praktisch afwezig is bij de mens; 2) de beschermende enzymen vertonen verschillende activiteiten in verschillende diersoorten; 3) glutathion, maar niet malondialdhyde, kan gebruikt worden als indicator van oxidatieve stress; 4) uridine gebruikt zou kunnen worden als nieuwe marker voor ischemie bij de mens; 5) adenosine mogelijkerwijs een regulerende funktie heeft tijdens herhaalde ischemie.

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APPENDICES

Appendix 1

High-energy phosphates and their catabolites

J.W. de Jong, T. Huizer, M. Janssen, R. Krams, M. Tavenier and P.D. Verdouw

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High-energy phosphates and their catabolites

J.W. de Jong, T. Huizer, M. Janssen, R. Krams, M. Tavenier, and P.D. Verdouw

"Energy is Eternal Delight"
William Blake (1757-1827)
The Voice of the Devil

1. Introduction

In this chapter we put emphasis on the use of high-energy phosphates and their breakdown products to characterize the metabolic status of the heart before, during and after cardiac surgery. We address the relationship between these compounds and cardiac function, e.g., during stunning. In addition we describe their role for cardioprotection, e.g., during cardioplegia.

2. Energy metabolism

High-energy phosphates are energy-rich molecules such as phosphoenolpyruvate, phosphoglyceroyl phosphate, phosphocreatine, ATP and ADP. The free energy, ΔG, liberated from these molecules by hydrolysis of an anhydride bond, supports energetically unfavorable reactions. Dependent on the standard free-energy change $\Delta G°$, these compounds release more or less energy. As discussed below, ATP, phosphocreatine and phosphoenolpyruvate are useful for therapeutic and/or diagnostic purposes. Figure 1 shows the formation and breakdown of purine and pyrimidine 5'-triphosphates. Adenine nucleotides play an important role storing and transferring metabolically available energy. ATP is the most important carrier of free energy ($\Delta G° = -7.3$ kcal mol^{-1}). Phosphocreatine, with a higher standard free-energy change (-10.3 kcal mol^{-1}), can transfer its phosphoryl group to ADP, generating ATP. In this way ATP shuttles between the mitochondria, where it is synthesized from ADP by oxidative phosphorylation, and the myofibrils, where it delivers energy for contraction.

Anaerobic metabolism can also generate ATP, but it produces only 3 moles of ATP for every mole of glucose metabolized, compared with 38 moles generated by aerobic metabolism. The heart uses its ATP at a rapid rate. Mechanically quiescent hearts consume approximately 10 μmol ATP min^{-1} g wet $weight^{-1}$ to maintain ionic homeostasis. A heart rate of 75 beats min^{-1} requires another 23 μmol min^{-1} g^{-1}. Thus, per beat the heart uses about 5% of its energy stores. (This organ can store up to 5-6 μmol g^{-1}.) Some energy reserve is available from phosphocreatine but this suffices only for a few minutes [67].

Other high-energy compounds, such as GTP, UTP and CTP, drive several biosynthetic reactions; their concentration is ≤5% of that of ATP. UTP is the immediate phosphate donor for many reactions leading to polysaccharide synthesis. CTP is the energy donor in lipid biosynthesis. We were unable to locate any literature data on cardiac XTP and ITP.

3. Catabolism

During hypoxia, abundant myocardial ATPase activity ensures the rapid breakdown of ATP to ADP, with subsequent catabolism to AMP by the myokinase reaction. Adenosine 5'-monophosphate is not a high-energy phosphate because its dephosphorylation by 5'-nucleotidase to the regulatory metabolite adenosine produces relatively little energy ($\Delta G° = -3.4$ kcal mol^{-1}). The alternative pathway, deamination to IMP (see Figure 1), is relatively inactive in heart muscle, but its postulated role is preservation of the purine ring inside myocytes (as nondiffusable IMP) [114]. Breakdown of adenosine depends on the ubiquitous adenosine deaminase. Little is known about 5'-nucleotidase and adenosine deaminase in human myocardium; their cardiac activities vary considerably among species [86]. The activity of 5'-nucleotidase is lower in ventricles than in atria accompanied with a transmural distribution across the left ventricle wall, with the highest values in the subepicardial and the subendocardial regions [31]. During ischemia 5'-nucleotidase activity decreases, whereas adenosine deaminase activity increases [22]. Purine nucleoside phosphorylase provides hypoxanthine, the substrate for the xanthine oxidoreductase reaction, which catalyzes the breakdown of hypoxanthine to xanthine and urate. Histochemical studies of xanthine oxidoreductase in human, bovine and rat myocardial tissue have revealed its localization in the endothelial cells [64,108]. During ischemia and reperfusion, proteases convert the native form of the enzyme, xanthine dehydrogenase, to the oxyradical-generating oxidase form. Reported activities in human myocardium vary from high [64,140] to very low [34,45,97,118].

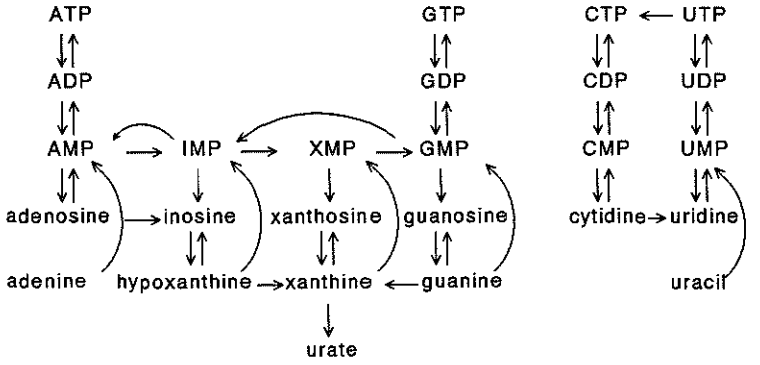

Figure 1.
Formation and breakdown of high-energy phosphates. Cardiac AMP formation from IMP takes places via adenylosuccinate. Direct GMP formation from guanosine by adenosine kinase or (low activity) guanosine kinase is not unlikely [41].

Various tissue sources and assay techniques, as well as self-inactivation [127], could explain these large differences. In patients undergoing coronary angioplasty, we observed urate release [59], an indication of active xanthine oxidoreductase. However, our group found also that explanted (diseased) human hearts, perfused with hypoxanthine, released little urate [27]. Urate production during angioplasty and heart surgery might come from extracardiac factors like neutrophils or other blood components. This could explain the difference found between clinical studies [59,118] and ex vivo studies with blood-free solutions [27]. Although the sum of the literature data suggests that human myocardium is almost devoid of xanthine oxidoreductase, we cannot exclude the possibility that a desulpho form -which is inactive with hypoxanthine and xanthine- generates oxyradicals (cf. [1]).

3.1. High-energy phosphates as markers of ischemia

In animal studies, but also in clinical work, high-energy phosphates have been used to characterize the metabolic status of the heart. Analysis of ATP and phosphocreatine takes place in biopsies frozen immediately in liquid nitrogen. The presence of blood makes it difficult to distinguish between myocardial and erythrocyte adenine nucleotides. In deproteinized samples the compounds of interest can be determined with HPLC, phospholuminescence, and other sensitive techniques. Usually the data are expressed on a non-collagen protein basis. Magnetic resonance spectroscopy (see next subsection) offers an alternative to the chemical analysis of high-energy phosphates.

In anesthetized dogs, circumflex occlusion gives rise to myocardial adenine nucleotide breakdown and accumulation of nucleosides and bases [102]. ATP depletion is more rapid in the endocardial layers. Quite a few authors measured myocardial high-energy phosphates to study the efficacy of calcium antagonists as adjuncts to cardioplegia (for review, see [29]). In coronary-bypass patients, the adenylate and phosphocreatine contents decrease substantially in the infarcting heart; in patients without infarction, severe stenosis of the left anterior descending coronary artery results in myocardial dysfunction associated with adenylate pool depletion, but mitochondrial function remains intact [37].

3.1.1. Magnetic resonance spectroscopy

Slowly nuclear magnetic resonance (NMR) spectroscopy finds its way into the heart clinic. The technique has the advantage of *non-invasive* assessment of high-energy phosphates, inorganic phosphate and pH, but it is insensitive and expensive. In addition, blood contribution to cardiac NMR spectra, e.g., from the ventricular cavities, is a potential problem. Using surface-coils on rat and dog hearts, various investigators [18,39,49] showed that changes in high-energy phosphate metabolism could predict rejection of heterotopic cardiac allografts. Bottomley et al. [14] found that patients with heart transplants had a 20% lower ratio of anterior myocardial phosphocreatine to ATP compared with that of healthy control subjects. Ratios of phosphocreatine to inorganic phosphate also appeared lower whenever detectable. The authors concluded, however, that ^{31}P-

NMR spectroscopy did not permit reliable identification of patients who required augmented therapy for rejection. Nevertheless regular monitoring post-transplantation, using patients as their own control, might be useful. The same group demonstrated that resting humans with ischemic and idiopathic dilated cardiomyopathy have also reduced myocardial phosphocreatine/ATP ratios [47]. The investigators corrected their ^{31}P-spectra for contaminating blood components.

3.2. Purines as markers of ischemia

One can measure the catabolites of high-energy phosphates in biopsies or in myocardial effluent. In patients undergoing bypass grafting, Flameng et al. [37] observed that nucleosides, in particular inosine, accumulated in the infarcting heart. During heart surgery, Smoleński et al. [115,118] measured substantial increases in adenosine, inosine and hypoxanthine in coronary effluent collected during subsequent infusions of cardioplegic fluid into the coronary root. They did not detect xanthine, and attributed the release of urate to washout of this oxypurine accumulated in the myocardium [118]. The much higher release of purines (and lactate as well as phosphate) in children as opposed to adults provides evidence for more severe metabolic injury during cardioplegic arrest to the juvenile heart [115].

3.3. Inhibitors of purine catabolism/uptake

3.3.1. Adenosine deaminase inhibitors

Prevention of adenosine catabolism is insufficient for adequate (rat) ventricular recovery unless tissue [ATP] remains above about 1.0 μmol g^{-1}. Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) + adenosine (low Ca^{2+}) conserve ATP, improve functional recovery of hearts markedly, and thus may play a role to play in myocardial preservation during elective cardiac arrest [62] (cf. table 1).

3.3.2. Xanthine oxidase inhibitors

Allopurinol and its catabolite oxypurinol are xanthine oxidase inhibitors, useful to prevent damage by free radicals. Table 1 shows that these inhibitors, administered during potassium cardioplegia, improve function and metabolism. Addition of allopurinol to blood cardioplegia increases function in severely ischemic dog ventricles [138]. Allopurinol and oxypurinol also improve function when given during reperfusion of rabbit hearts [79], with varying results in rat hearts [21,134]. This is surprising, because rabbit (and human) hearts seem devoid of xanthine oxidoreductase, in contrast to rat hearts [27]; allopurinol may be effective, because it is a weak radical scavenger. Allopurinol pretreatment of donor dogs diminishes malondialdehyde production, which is an indicator of free radical generation; concomitantly function of the transplanted hearts

Table 1.
"Purine blockers" used adjunctive to potassium cardioplegia

Compound	Mode of action	Effect	Reference
EHNA Deoxycoformycin	ADA inhibition	>Function recovery; (>ATP)	[13,26,145]
Allopurinol Oxypurinol	XO inhibition; radical scavenging?	<CK release; >function recovery;	[9,21,92,95,119]
R75231 Dipyridamole NBMPR Mioflazine Lidoflazine Dilazep	Nucleoside transport inhibition (anti-platelet aggregation; Ca^{2+}-entry blockade; vasodilation?)	>Heart transplantation; <ventricular fibrillation; >function recovery	[38,46,50,81,136]

Abbreviations: ADA, adenosine deaminase; CK, creatine kinase; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; NBMPR, 6-[(4-nitrobenzyl)-mercapto]purine ribonucleoside; XO, xanthine oxidase.

improves [5] (cf. [43]). The drug is present in the University of Wisconsin cardioplegic solution (see below). In patients undergoing coronary artery bypass grafting, allopurinol as adjunct to cardioplegia reduces the number of chromosomal aberrations found after crossclamping by 60% [35]. In some studies pretreatment with the drug decreases drastically enzyme release and the incidence of cardiac complications in heart surgery [68,100,124]. However, the effect of allopurinol and oxypurinol on infarct size is variable (for review, see [141]), and may depend on the efficiency of myocardial protection with cardioplegia and cooling [10]. Allopurinol increases hyperemic flow after embolization [57]. The literature data leave the overall impression that allopurinol is useful for cardioprotection during heart surgery.

3.3.3. Nucleoside transport blockers

Dipyridamole blocks the (re)uptake of adenosine. By raising local plasma concentrations of this nucleoside, it reduces arrhythmias due to ischemia and reperfusion in dogs [139]. It also reduces platelet activation and depletion during bypass surgery [126]. Another blocker, 6-[(4-nitrobenzyl)-mercapto]purine ribonucleoside (NBMPR), in combination with EHNA, protects against ATP depletion and heart function loss due to ischemia in a canine model [2]. Yet other blockers, including R75231, appear useful as adjunct to potassium cardioplegia (table 1). Using dogs, Flameng et al. [38] assessed the effect of nucleoside transport inhibition with R75321 in the cardioplegic fluid on 24 hour's preservation of donor hearts for transplantation. Serial transmural left-ventricular biopsies revealed moderate ATP catabolism during cold storage in the control group. On reperfusion ATP content declined further, accompanied with washout of the accumulated nucleosides. In the treated group, ATP breakdown was similar during cold storage and continued up to 1 hour after reperfusion. The nucleosides adenosine and inosine, however, did not wash out. ATP content recovered completely after 2 hours of reperfusion. Nucleoside transport blockers as cardioprotectants deserve more attention.

3.4. Pyrimidines as markers of ischemia

Much more is known about purine metabolism than about pyrimidine metabolism. Pyrimidine nucleotide breakdown in ischemic myocardium seems to parallel degradation of adenylates [80,122]. We reported recently that during heart transplantation procedures the uridine content in human donor myocardium increases during cold storage; during reperfusion, after aorta declamping, the implanted heart also releases uridine. During corrections of congenital or acquired heart defects, we observed a continuous degradation of uridine nucleotides in the ischemic myocardium [117]. Isolated, perfused human hearts release uridine, rat hearts uracil, as the end product of ischemic pyrimidine nucleotide breakdown. It follows that uridine phosphorylase activity in human heart is very low compared to rat heart. This agrees with the activity of the enzyme measured in homogenates of rat and human heart, 153 and 2.7 mU g wet weight^{-1}, respectively [117]. The opposite is true for another catabolic enzyme, cytidine deaminase, which is responsible for the production of uridine. Its activity is 14 mU g^{-1} in human heart and <1.0 mU g^{-1} in rat heart [116]. In rat and human heart, release of uracil and uridine is a potential marker for ischemia, respectively.

4. Cardiac energetics and function

4.1. Correlation between ATP content and function

During prolonged ischemia, myocardial high-energy phosphate content will decrease. The extent of this reduction depends on the duration of ischemia. Several studies show a good correlation between myocardial ATP content and cardiac function [32,66,101]. However, in some experimental models, an acceptable myocardial performance takes place at seriously deprived [ATP] [63,93,106]. Until the early eighties, biochemical recovery from myocardial ischemia was assumed to be complete within a short period. However, Reimer et al. [101] demonstrated that it takes at least a week to regenerate myocardial nucleotides lost during ischemia. The phenomenon "myocardial stunning", the temporary incomplete recovery of function in reperfused heart, seems to confirm the importance of metabolic recovery (see next subsection).

Figure 2 shows that myocardial function *before* ischemia determines ATP breakdown *during* ischemia. We calculated whether decreased purine efflux from the heart reflected better preservation of myocardial ATP or resulted from reduced membrane permeability. The close correlation between ATP loss and purine efflux confirmed a lower ATP breakdown. We speculate that during heart surgery hypothermia and chemical arrest contribute to the efficacy of cardioplegic solutions through the phenomenon described in Figure 2.

4.2. Cardiac function and ATP during stunning

After brief periods of ischemia cardiac function remains depressed for hours to days, while tissue necrosis is undetectable [51]. This is a clinical entity often observed after coronary angioplasty, silent ischemia and cardioplegia. To explain the relatively long depression of cardiac function, obtained after one occlusion in animal experiments, Heyndrickx et al [52] postulated that the decreased ATP synthesis rate became the rate-limiting step in ATP delivery to the contractile machinery. They based this hypothesis on the relationship between depression in intracellular [ATP] and duration of occlusion; the former changes in concert with severity of cardiac dysfunction [66]. Furthermore, regeneration of nucleotides from purines and de novo synthesis is slow (for review, see [28]), which could account for the slow return of cardiac function. Some fundamental, theoretical arguments do not support this hypothesis: 1) The [ATP] not necessarily reflects the ATP-turnover rate (balance between ATP-synthesis and hydrolysis), but merely the cellular outward diffusion of catabolites from ATP like adenosine, inosine and hypoxanthine (see section 3). 2) The myofibrils have a low K_m-value for ATP [69], which implies that the [ATP] has to decrease to very low values before it becomes the rate-limiting step in cross-bridge cycling and therefore in cardiac function. Literature estimates indicate that developed left-ventricular pressure is independent of the [ATP] unless it falls to values <50%

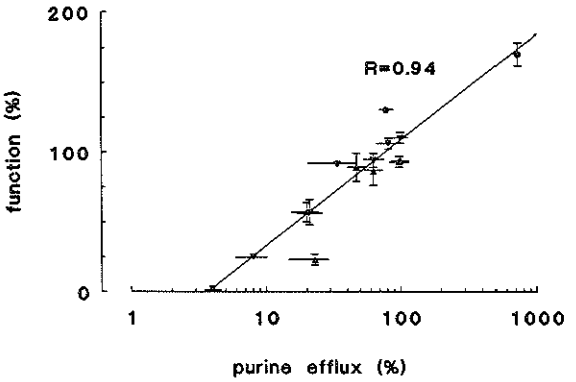

Figure 2. Correlation between normoxic function and ischemic energy metabolism. Data were obtained in rat hearts perfused according to Langendorff [30,60]. The figure shows that cardio-protection before ischemia is imperative. Closed dots: 5.0 and 1.4 mM $CaCl_2$ in perfusion medium. ▲ and ▼: various concentrations of bepridil and nisoldipine in the perfusion medium, respectively. Each point shows the mean of 4-6 observations; vertical and horizontal lines indicate s.e.m. Function 100% = function of untreated hearts; purine efflux 100% = efflux of untreated hearts. From [61].

of normal [93,125]; such low values are rare during brief periods of ischemia and subsequent reperfusions. In addition a considerable body of evidence also argues against a role of a decreased rate of ATP synthesis as a cause of myocardial stunning. Phosphocreatine increases immediately and transiently upon reperfusion, indicating the quickly restored capability of the mitochondria to rephosphorylate creatine after reinstitution of blood supply [109]. Several authors have challenged the stunned myocardium by inotropic stimulation [3,8,17,84,85]. They showed invariably that inotropic stimulation can recruit cardiac function, thereby indirectly confirming that the ATP-synthesis rate is not the rate-limiting step. Cardiac function did not detoriate after withdrawal of the inotropic agent and the [ATP] did not decrease during stimulation, suggesting that the increase of ATP-synthesis rate during inotropic stimulation matches the myofibrillar ATP-hydrolysis rate [3].

Since these arguments are rather qualitative, there was a need for further support. A modified ^{31}P-NMR technique, which enabled the simultaneous measurement of the net ATP production rate [107] and myocardial O_2-consumption (MVO_2), provided non-invasively data on phosphorylation efficacy in the post-ischemic myocardium. These were similar to those in control hearts, indicating unchanged efficiency of the Krebs-cycle, despite a 50% reduction in [ATP] [107]. However, in that study MVO_2 was normal, notwithstanding depressed cardiac function. The relatively high MVO_2 indicates a high ATP turnover, whereby conversion to mechanical energy fails to take place (see below). Unfortunately during most of the in vivo experiments, MVO_2 has not been measured during inotropic stimulation. Furthermore, in vivo, cardiac function is often assessed by systolic segment-length shortening or systolic wall thickening. These indexes of cardiac function

are highly load-dependent. Coupling of inotropic recruitment and ATP-turnover are therefore debatable.

These combined theoretical and experimental arguments strongly suggest a relationship between decreased function of post-ischemic myocardium and defective ATP-utilization [109], rather than impaired ATP synthesis.

To avoid load-dependent indexes of myocardial contractile function, we have applied the time-varying elastance concept, introduced by Suga and Sagawa for the whole heart [121], to regionally stunned myocardium of open-chest pigs. This approach allows, besides the determination of a load-independent index of contractility (E_{max}), also the calculation of external work (EW), potential energy (PE, see figure 3) together with the pressure-length area (PLA = EW + PE) and the efficiency of energy transfer (EET = EW/PLA*100%). E_{max}, PLA and basal metabolism are the major determinants of MVO_2 [120,121]. Myocardial stunning does not affect the basal metabolic rate, which implies that changes in E_{max} and PLA are responsible for alterations in MVO_2. By relating changes in these compounds to those in MVO_2 before

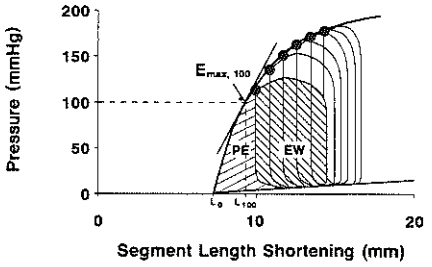

Figure 3. *Theoretical graph illustrating the calculations possible by applying the time-varying elastance concept [121]: PLA = PE + EW and EET = EW/PLA (see section 3.2). $E_{max,100}$ (mmHg min^{-1}) is the index of contractility at 100 mmHg. Together with segment shortening at 0 and 100 mmHg (L_0 and L_{100}, respectively), it describes the end-systolic pressure-length relation ESPLR. PE is the area of the triangle below the ESPLR. Abbreviations: PLA, pressure-length area; PE, potential energy; EW, external work; EET, efficiency of energy transfer.*

and after inotropic stimulation with dobutamine, we obtained a better insight into the mechanism underlying the disturbance of ATP-utilization. The stunned myocardium, induced by 2 periods of 10-min occlusion and 30 min of reperfusion, showed decreased contractility (50% decrease of E_{max}), in accordance with the postulated decrease of myofibrillar Ca^{2+}-sensitivity [74], and decreased MVO_2 (a 33% drop). due to the decrement in contractility, external work decreased [120,121]. Because of the regional nature of the stunning protocol, end-systolic pressure changed little, however. Consequently end-systolic segment length increased. As a result PE rose, thereby counterbalancing the decrease in EW (figure 4). Since PE is not used for pump

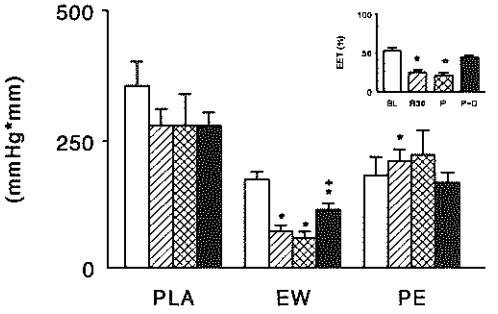

Figure 4. *Results of the mechanical determinants, pressure-length area (PLA), external work (EW), potential energy (PE); and the resulting efficiency of energy transfer (EET, inset) in stunned swine myocardium. Open bars: baseline (BL); hatched bars: after 30 min of reperfusion (R30); crosshatched bars: after atrial pacing (P); filled bars: after pacing plus inotropic stimulation with dobutamine (P+D). *$p<0.05$ vs baseline; $^{+}p<0.05$ vs pacing. The difference between pacing alone and pacing plus dobutamine is due to pure inotropic effects of the drug. Mean ± s.e.m., $n = 10$.*

function and PE eventually degrades to heat, the efficiency of energy transfer fell from 55% to 25% (figure 4, inset). Thus, not only MVO_2 and therefore ATP-utilization decreased, but also ATP proved to be converted less efficiently. During dobutamine infusion contractility (E_{max}) of the stunned myocardium recovered, in addition to a return to baseline of PLA and its distribution in EW and PE (and thus EET). In concert with these mechanical parameters, MVO_2 normalized, indicating that ATP-utilization and mechanical function were still matched during inotropic stimulation of the stunned myocardium. One must keep in mind, however, that dobutamine induced a 20% drop in peripheral vascular resistance, possibly contributing to the beneficial effects.

Nevertheless, our data indicate that in the stunned myocardium contractility and EW are reduced to lower MVO_2, thereby redistributing its available cellular energy to processes that keep cellular integrity intact [109]. As a consequence, however, PE increases, offsetting the benefit of decreased contractility plus EW and reduced efficiency of energy transfer. The balance between the decrease of contractility plus EW and the increase of PE determines whether MVO_2 changes; it potentially explains the variability of MVO_2 in stunned myocardium reported [6,25,76]. Reversibility of the process was confirmed by the short-lasting inotropic stimulation with dobutamine. Apparently inotropic stimulation with dobutamine overrides the energetic downregulation of the contractile machinery, without an extra need for energy requirements. Inotropic stimulation up to 1 hour is without unwanted side effects [109]; it is questionable whether longer periods of stimulation do not induce tissue necrosis.

5. Anabolism

5.1. High-energy phosphates

The alleged protective properties of extracellular high-energy phosphates are controversial [50]. Table 2 lists the effects seen after the use of ATP, phosphocreatine and phosphoenolpyruvate for cardioprotection. ATP and phosphocreatine seem to be useful for postischemic recovery [15,71,146], the latter possibly also in man [19,24,88,111] (but see [20,135]). Their mechanism of action remains to be elucidated, but it is unlikely that direct uptake of these compounds by the cardiomyocyte takes place. The effect of phosphoenolpyruvate is unclear [128,130,131,132]. Exogenous ATP suppresses supraventricular tachycardias in patients [99].

5.1.1. High-energy precursors

5.1.1.1. Adenosine

Figure 5 depicts the role of (endogenous) adenosine in the energy demand/supply balance. Adenosine given during normoxic perfusion in Langendorff rat hearts increases tissue ATP and phosphocreatine but does not affect postischemic functional recovery or ATP [54]. Adenosine alone or adjunctive to high potassium cardioplegia arrests rat hearts quicker [26,110], which seems advantageous. It is, however, ineffective in arresting fibrillating baboon hearts [11]. Recently, we observed that adenosine, added to St. Thomas' Hospital cardioplegic solution at concentrations of 0.05, 0.5 and 5 mM did not improve high-energy phosphate metabolism in working rat hearts. However, the lower two concentrations improved recovery of function

Table 2.
High-energy phosphates used adjunctive to potassium cardioplegia

Compound	Mode of action	Effect	Reference
ATP Phosphocreatine (Phosphoenol- pyruvate)	Membrane stabilization; energy donor??	>Function recovery; >ATP; >phosphocreatine; <CK release; >birefringerence	[15,19,20,24,50,71, 72,73,88,98,103,104,105, 111,112,113,128,129,133]

Abbreviation: CK, creatine kinase

(Huizer et al., unpublished observations). Table 3 shows that the nucleoside, given adjunctive to potassium cardioplegia, improves functional recovery under various experimental conditions. Concomitant inhibition of adenosine deamination could be beneficial [13].

Adenosine is one of the many ingredients of the University of Wisconsin cardioplegic solution. It has provided excellent preservation for the pancreas, kidney, and liver after extended cold ischemic storage times [123]. The solution may be useful for heart transplantation as well [65,90,123] (but see [89]); studies are underway to determine whether adenosine (and other components) are essential for its cardioprotective action [142]. Adenosine administration during reperfusion increases rabbit- and canine-heart ATP content, without concomitant improvement in contractility [4,53]. The opposite is true in rat heart [77]. Through receptor activation, the nucleoside may play a role in "preconditioning" [70,78,91,137]. It reduces infarct size and

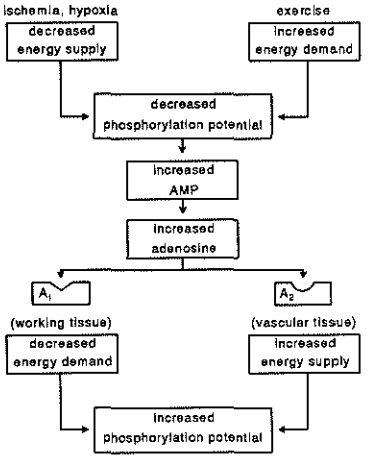

Figure 5. *Role of (endogenous) adenosine in the energy demand/supply balance. A_1 and A_2 depict adenosine receptors. Adapted from [16].*

Table 3.
ATP-precursors used adjunctive to potassium cardioplegia

Compound	Mode of action	Effect	Reference
Adenosine	Hyperpolarization; nucleotide synthesis; vasodilation; adrenergic modulation	Quicker arrest; >function recovery; >ATP	[11,12,13,26,50]
Inosine	Salvage pathway; vasodilation??	>Function recovery; >ATP	[33]
AICAr	De novo nucleotide synthesis? adenosine production	>Function recovery; >ATP	[40]
Ribose (+ purines)	Through PRPP?	>ATP	[143]

Abbreviations: AICAr, 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamide riboside; PRPP, α-5-phosphoribosyl-1-pyrophosphate.

improves regional ventricular function in the ischemic zone in dogs [55,94,96]. A raised local plasma adenosine concentration reduces the incidence and severity of the life-threatening ischemia/reperfusion arrhythmias [99]. However, the intravenous infusion of adenosine in man provokes angina-like chest pain [23,75].

5.1.1.2. Adenosine catabolites

Inosine enhances reperfusion ATP [48,144]. Addition of inosine to the reperfusion fluid as well as the cardioplegic solution further improves nucleotide levels and recovery of cardiac output [33] (table 3). Urate, produced by the heart of many species [27], is a radical scavenger [7]; it protects against reperfusion damage. Of course its plasma concentration in man is already very high, in comparison to that of other species.

5.1.1.3. De novo synthesis of purines

Postischemic administration of acadesine (AICAr = 5-amino-1-ß-D-ribofuranosylimidazole-4-carboxamide riboside, see Figure 6), which can be metabolized to ATP and GTP via IMP, does not increase rabbit-heart ATP or contractility [4]. AICAr pretreatment augments adenosine release from ischemic canine heart nearly 10-fold, accompanied by increased collateral blood flow and decreased arrhythmias [36,44]. In a similar set-up, AICAr does not change cardiac adenine nucleotide, but it increases IMP content 13- to 25-fold, concomitant with early recovery of regional function [42]. In earlier work, AICAr administration during reperfusion proved to be detrimental for heart function [53], although nucleotide synthesis rate increased [82]. In cat heart AICAr improved postischemic function, but not ATP [87]. It may decrease infarct size [83] and myocardial ischemia in coronary microembolization [56]. We conclude that AICAr deserves further attention as an agent for cardioprotection. Ribose stimulates the rate of myocardial ATP synthesis during reperfusion after coronary artery occlusion in the dog, although to a (much) smaller extent than adenosine or AICAr [82]. Using NADH fluorometry to monitor rat myocardial metabolism, Horvath et al. [58] observed that a ribose-enriched perfusate improved glycogen content and myocardial function, but not high-energy phosphates levels.

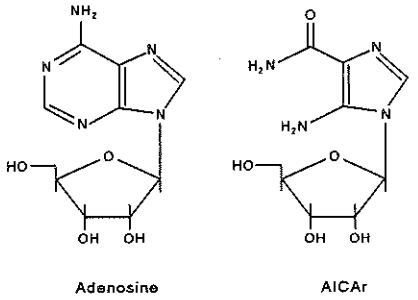

Figure 6. Structural relationship between adenosine and acadesine (AICAr = 5-amino-1-ß-D-ribofuranosylimidazole-4-carboxamide riboside). Although initially used for de novo synthesis of purines/ nucleotides, the latter may be useful for cardioprotection through a rise in endogenous adenosine.

6. Conclusions

The measurement of myocardial high-energy phosphates (especially ATP and phosphocreatine) with biopsy or NMR spectroscopic techniques provides information about the metabolic status of the heart. They could predict rejection of heart transplants. Breakdown products of high-energy phosphates, such as purines and pyrimidines, seem useful as markers of cardiac ischemia. They can be measured in the cardiac efflux. The correlation between energy content and function is often poor. Decreased function of post-ischemic myocardium seems related to defective ATP utilization rather than decreased ATP synthesis. Adenosine and related compounds could play a role in cardioprotection. Such agents often protect heart function and ATP content against the ill effects of ischemia/reperfusion, if used, e.g. adjunct to potassium cardioplegia.

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Appendix 2

Malondialdehyde and glutathione production in isolated perfused human and rat hearts

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Circ. Res., in press

Malondialdehyde and glutathione production in isolated perfused human and rat hearts

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A number of studies show the relationship between oxygen free radicals and cardiac ischemia/reperfusion injury. However, little is known about oxidative stress in the human heart, which can be measured by oxidized glutathione (GSSG) and malondialdehyde (MDA) formation. Furthermore, data on MDA production by rat hearts are controversial, possibly because of the use of the aspecific thiobarbituric acid assay. Therefore GSSG and MDA were measured, with colorimetric and high performance liquid chromatography assays, respectively, in buffer-perfused explanted human hearts and normal rat hearts made temporarily ischemic. Human hearts received cardioplegia; rat hearts were studied in a control and an ischemic group with or without cardioplegia. Baseline GSSG release was <0.01 nmol·min^{-1}·g wet wt^{-1} in both species. During reperfusion GSSG release from human hearts, ischemic and cardioplegic/ischemic rat hearts peaked at 0.24±0.12, 1.1±0.4 and 0.19±0.04 nmol·min^{-1}·g^{-1} respectively. MDA was undetectable (<0.02 nmol·min^{-1}·g^{-1}) in the effluent of both species and in human heart (<4 nmol/g protein). Rat heart reduced glutathione levels decreased 32% as a consequence of cardioplegia and ischemia. Cardioplegia induced a 41% (P=.08) decrease in rat heart MDA content, whereas cumene hydroperoxide increased it 3.6 times (P<.01). Thus, after ischemia human and rat hearts release GSSG, indicating that oxidative stress has occurred. Apparently, lipid peroxidation takes place in normal rat hearts, decreases after cardioplegia, but does not increase after ischemia/reperfusion. Human hearts lack MDA under normoxic and ischemic conditions. This novel finding seems to reflect a low MDA-forming potential in both situations. Substantial amounts of MDA are only formed under extreme conditions, like perfusion with cumene hydroperoxide, when scavenging systems cannot cope with the amounts of free radicals formed. MDA found in clinical studies could come from extracardiac source or could be due to the use of the aspecific thiobarbituric acid assay. (*Circulation Research* 1993;73:●●●-●●●)

Key Words ● explanted human hearts ● glutathione ● ischemia ● malondialdehyde ● rat

A considerable number of studies show that oxygen-derived free radicals can cause injury, related to cardiac ischemia/reperfusion.[1] Various free radical sources, like the mitochondrial respiratory chain, arachidonic acid, leukocytes and endothelial xanthine oxidoreductase, could play a role. McCord[2] postulated that, during myocardial ischemia, xanthine dehydrogenase is converted to xanthine oxidase, which forms superoxide radicals upon reperfusion.

Superoxide dismutase converts superoxide to hydrogenperoxide,[3] which can react with iron to form the highly cytotoxic hydroxyl radical (Haber-Weiss reaction).[4-6] The latter can initiate the peroxidation of polyunsaturated fatty acids in cell membranes and thus impair or destroy cell function. Since catalase activity in myocardial tissue is very low,[7] glutathione peroxidase seems to be crucial in detoxifying hydrogen peroxide via reduced glutathione (GSH). The excess of oxidized glutathione (GSSG) inside the cell is converted back to GSH by glutathione reductase or actively transported out of the cell.[8]

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Direct detection of oxygen-derived free radicals is almost impossible, because of their instability and consequent short half-life. Electron spin resonance or spin trapping provides a new direct method to measure free radical formation.[9,10] Indirect probing can be done by measuring compounds associated with the detoxification, by assaying products generated, or by applying free-radical scavengers.

GSSG levels in tissue and coronary effluent rise during lack of oxygen.[7,8] Likewise malondialdehyde (MDA), one of the many products of lipidperoxidation, can be demonstrated.[11] GSSG and MDA are therefore useful as indices of myocardial oxidative stress.

Various authors have reported MDA formation during coronary angioplasty procedures[12,13] and pacing stress testing,[14] with ischemic periods of a few minutes, contrasting the negative reports for rabbit and rat hearts with ischemic periods up to 60 minutes.[15,16] These contradictory results could be due to three factors: (1) errors by interfering blood constituents during in-vivo measurement of MDA, (2) the use of the aspecific thiobarbituric acid assay for MDA, and (3) species differences. The second hypothesis is based on the observation that studies reporting MDA production use the thiobarbituric acid assay,[12-14,17-19] whereas the negative findings are obtained with a more specific method.[15,16] To circumvent the possible influence of blood components, the isolated, buffer-perfused heart is

a useful model to investigate the effects of ischemia/reperfusion. To shed light on the MDA controversy described above, we subjected human and rat hearts to temporary ischemia, and measured MDA, GSH and GSSG in coronary effluent and tissue samples. To validate the MDA detection method, we perfused rat hearts with cumene hydroperoxide, which is known to induce MDA formation.[20] Our data show that oxidative stress caused by a 20-minute period of ischemia followed by reperfusion does not result in MDA release in isolated human and rat hearts. A novel finding is the absence of MDA in normoxic human myocardial tissue, whereas rat hearts contain sizable amounts, which do not increase after ischemia/reperfusion.

Materials and Methods

Human Heart Perfusions

Hearts (n=8) were removed from patients undergoing cardiac transplantation for end-stage heart failure due to ischemic heart disease or dilated cardiomyopathy. They were arrested in situ with ice-cold St. Thomas' Hospital cardioplegic solution,[21] removed, and transported within approximally 15 minutes in gassed (95% O_2-5% CO_2) ice-cold cardioplegic solution to the laboratory. For details about the perfusion system, see Smoleński et al.[22] Briefly, the hearts were perfused in the Langendorff mode with a roller pump. The perfusion fluid consisted of a modified Tyrode's buffer,[23] containing glucose and dextran (40000 D, Isodex, Pharmacia, Uppsala, Sweden). It was oxygenated with 95% O_2-5% CO_2, using a baby oxygenator. Retrograde aortic perfusion started 30 to 50 minutes after cardiac arrest. Blood was washed from the hearts for 10 minutes, then recirculation started with 1.0 to 1.5 L perfusion fluid. After a 30-minute control period, the hearts were subjected to 20 minutes of no-flow ischemia, followed by a 30-minute reperfusion period. Arterial and venous samples were collected at regular intervals. Biopsies (0.1 g) were taken from multiple locations of the left ventricle with a Tru-cut needle (Travenol Laboratories, Deerfield, IL) before the onset of the preparation period and after 30 minutes of reperfusion; they were immediately frozen with liquid nitrogen.

In a pilot experiment, perfusion took place 30 minutes with buffer supplemented with 0.5 mmol/L cumene hydroperoxide (Merck, Amsterdam, The Netherlands), to induce lipid peroxidation.

Rat Heart Perfusions

Adult male Wistar rats (400 to 450 g) were anesthetized intraperitoneally with 60 mg/kg pentobarbital (Nembutal, Sanofi, Paris, France) in accordance with guidelines of the American Physiological Society. Hearts were removed, arrested in ice-cold saline, and retrogradely perfused with a modified Tyrode's buffer, oxygenated with 95% O_2-5% CO_2 at 72 mm Hg and 37°C. Cannulation of the aorta was done <1 minute after arrest. Coronary flow was measured electromagnetically (Skalar, Delft, The Netherlands). Hearts were perfused in a non recirculating mode for 30 minutes, then recirculation was started with a total volume 100 mL. At this point the hearts were divided into three groups. Control hearts (n=9) were recirculated for 60 minutes. In the ischemic group (n=6), hearts were subjected to 20 minutes ischemia, followed by 30 minutes reperfusion. The third group (n=6) was perfused for 30 minutes with buffer supplemented with 0.5 mmol/L cumene hydroperoxide, to induce lipid peroxidation.

To check the influence of a prolonged transportation and preparation period (compare with human hearts), two additional groups (n=7) of rat hearts were investigated. Hearts were removed from the thorax as described above and immediately flushed for 3 minutes with gassed (95% O_2-5% CO_2) ice-cold St. Thomas' Hospital cardioplegic solution.[21] The organs, submerged in the cardioplegic solution, were put on melting ice for 40 minutes. Then they were connected to the perfusion system, and subjected to the protocols (cardioplegic control and cardioplegic/ischemic groups) described above.

In the groups mentioned above, samples were collected every 5 minutes. Because of the minimal arterio-venous differences, release was calculated from the increase in catabolite concentration divided by the time interval. Because of the lag time in the recirculating system, sampling was omitted during the first few minutes of reperfusion. At the end of the experiments, hearts were freeze clamped at liquid-nitrogen temperature.

To investigate if GSSG release reflected production or increased washout, we subjected hearts (n=6) to 20 minutes ischemia after a 30-minute stabilization period and perfused them for 1 minute before they were freeze clamped.

Glutathione Determination

Effluent samples were deproteinized with 4% $HClO_4$ (final concentration), centrifuged at 10,000*g* for 2 minutes, and neutralized with 6 mol/L KOH/2 mol/L K_2CO_3. Tissue was crushed in liquid nitrogen and deproteinized with 5% sulfosalicylic acid.[24] After centrifugation at 10,000*g* for 2 minutes, the supernatant fluid was neutralized with 6 mol/L KOH/2 mol/L K_2CO_3. Total glutathione (GSH+GSSG) and GSSG were analyzed according to Ceconi et al[25] on a double-beam spectrophotometer (model U-2000, Hitachi, Tokyo, Japan). Contents were expressed per gram protein to circumvent weight problems due to (1) cumene hydroperoxide-induced loss of tissue water and (2) edema formation in isolated human hearts. The detection limit of GSSG in perfusion fluid was 0.01 nmol·min^{-1}·g wet wt^{-1}.

MDA Determination

Tissue samples, crushed in liquid nitrogen, and effluent samples were deproteinized with 4% $HClO_4$ (final concentration), centrifuged at 10,000*g* for 2 minutes, and neutralized with 6 mol/L KOH/2 mol/L K_2CO_3. MDA was assayed by high-perfomance liquid chromatography (HPLC) according to Bull and Marnet,[26] with some modifications. A μBondapak C_{18} (10-μm particle diameter; Waters-Millipore, Milford, Mass), preceded

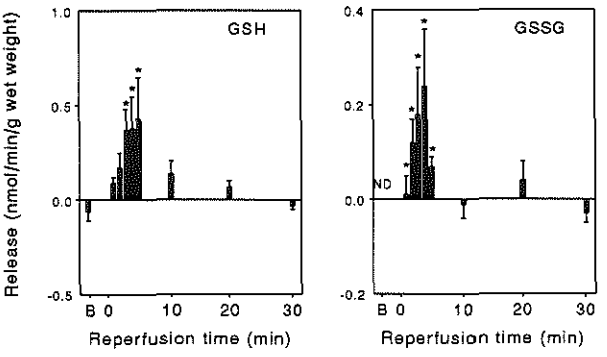

Fig 1. *Bar graphs show the release of reduced glutathione (GSH) and oxidized glutathione (GSSG) after 20 minutes of no-flow normo-thermic ischemia from isolated hu-man hearts. Arterial and venous samples were collected and ana-lyzed for GSH and GSSG. The data strongly suggest that oxidative stress had occurred. Values are mean±SEM (n=7). The value be-fore the onset of ischemia repre-sented the baseline (B); ND indi-cates not detectable (<0.01 nmol min^{-1} g wet wt^{-1}).* *P<.05 *vs baseline.*

by a Supelguard LC18 guard column (5-μm particle diameter, Supelco, Bellafonte, Pa), was eluted with 20% acetonitrile (vol/vol) in 1.5 mmol/L myristyltrimethylammonium bromide, 20 mmol/L sodium phosphate buffer (pH 7.2). Detection was done at 267 nm with a forward optical scanning detector (Spectra-Physics Analytical, San Jose, Calif). Peaks were identified by comparison of retention times and spectra (multiwavelength scan) of the standard and the samples. MDA standard was prepared by hydrolysis of 1,1,3,3-tetraethoxypropane (Merck, Darmstadt, Germany), according to Largilliere and Melancon.[27] The detection limit was 4 nmol/g protein for tissue and 0.02 nmol·min^{-1}·g wet wt^{-1} for effluent.

Protein Assay

Tissue protein was assayed with Coomassie Brilliant Blue (Bio-Rad Laboratories, Munich, Germany) according to Bradford,[28] using bovine serum albumin as the standard.

Statistics

All data represent mean±SEM. Rat and human effluent data were analyzed with two-tailed analysis of variance (least significant difference and Tukey's test, respectively). Hemodynamic data and tissue measurements were analyzed with Student's paired *t* test. A value of $P<.05$ was considered a significant difference.

Results

General

The weight of the human hearts was 480±62 g (n=8). Because of the perfusion, the weight increased 25% ($P<.05$). The hearts beat spontaneously within 5 minutes after connection to the perfusion system. The preischemic and postischemic heart rate was 52±6 beats per minute; average flow was 1.0 mL·min^{-1}·g wet wt^{-1}. This relatively low coronary flow seems to be adequate, since baseline lactate production was virtually absent (data not shown). Early during reperfusion flow did not increase. The heart perfused with cumene hydroperoxide stopped beating after 25 minutes of drug infusion.

Rat heart weight (1.48±0.07 g, n=41), was similar before and after the experiments. During the stabilization period, hearts had a rate of 254±9 beats per minute; the postischemic rate was 12 % less (P=NS). Hearts stopped beating 20 minutes after the start of cumene hydroperoxide infusion.

In rat hearts coronary flow was initially 10.5±0.5 mL·min^{-1}·g^{-1}. In the control groups, flow decreased little in the course of the experiment. In the groups subjected to transient ischemia, flow increased 32% and 47% ($P<.005$) early during reperfusion in the ischemic and treated ischemic groups, respectively, and decreased subsequently towards baseline. In hearts perfused with cumene hydroperoxide, flow increased 49% ($P<.005$ versus baseline), declining quickly after 20 minutes to 30% of the initial flow ($P<.05$ vs baseline).

Table 1. Decrease in Human Heart Glutathione Content due to Ischemia/Reperfusion

Variable	Control	Ischemia/reperfusion
GSH,μmol/g protein	25.3±4.5	12.8±1.5*
GSSG,μmol/g protein	0.61±0.12	0.84±0.24
Ratio	42.0±2.5	20.5±3.4*

GSH indicates reduced glutathione; GSSG, oxidized glutathione; and ratio, GSH/GSSG. Values are mean± SEM (n=7).

Hearts were removed from patients undergoing cardiac transplantation. They were arrested with cold cardioplegia and subsequently perfused according to the Langendorff procedure. After baseline perfusion for 30 minutes, hearts were subjected to normoxic perfusion for another 30 minutes (control) or to 20 minutes global ischemia and 30 minutes reperfusion (ischemia/reperfusion). Tissue biopsies were taken before the ischemic period and after reperfusion. The data indicate that ischemia/reperfusion lowers the cardiac GSH, but does not increase GSSG content.

*$p<0.05$ *vs control.*

Glutathione in Human Heart Effluent and Tissue

Baseline arterial and venous GSH concentrations in the ischemic/reperfused hearts did not differ significantly. Early during reperfusion, GSH release increased rapidly to 0.43±0.22 nmol·min^{-1}·g^{-1} (P<.05 versus baseline, Fig 1). Efflux decreased towards baseline after 10 minutes reperfusion. GSSG was undetectable in the control period. After 4 minutes of reperfusion, the release amounted to 0.24±0.12 nmol·min^{-1}·g^{-1} (P<.05 versus baseline). GSSG efflux ceased after 5 minutes (Fig 1).

Postischemic myocardial GSH content was 50% lower than control (P<.05, Table 1). GSSG increased 28% in ischemic tissue when compared with control (P=NS). The ratio between GSH and GSSG in control hearts (42±2) decreased twofold in ischemic hearts (P<.05). There seems to be an imbalance between the decrease in tissue GSH and the amount found during 30 minutes reperfusion in the effluent: Tissue GSH plus GSSG content decreased 700 nmol/g wet wt, whereas the total amount released in 30 minutes was only 10 nmol/g.

After the start of cumene hydroperoxide infusion, effluent GSH and GSSG release increased rapidly to 0.8 and 0.3 nmol·min^{-1}·g wet wt^{-1}, respectively. These values —— somewhat higher than those after ischemia (compare with Fig 1) —— decreased little during the remaining perfusion period. At the end of the experiment, tissue GSH and GSSG levels were 7.95 and 0.70 nmol/g protein, respectively. These levels were slightly lower than those observed after ischemia/reperfusion (compare with Table 1).

Glutathione in Rat Heart Effluent and Tissue

Control and cardioplegic control rat hearts released no GSH and GSSG. GSH efflux increased rapidly to

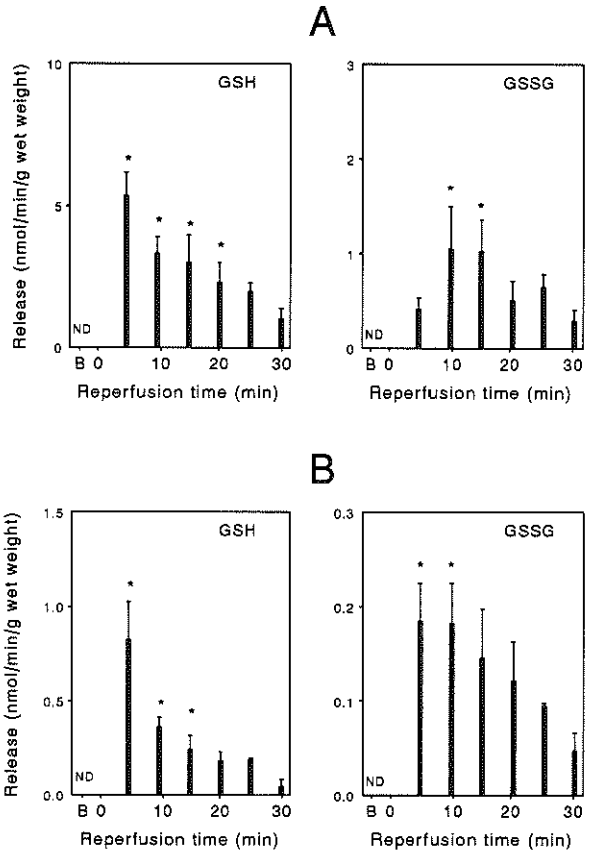

Fig 2. *Bar graphs show the release of reduced glutathione (GSH) and oxidized glutathione (GSSG) in isolated rat hearts. Data were obtained after 20 minutes of no-flow ischemia (A) or after 40 minutes of cardioplegia plus 20 minutes of no-flow ischemia (B). The data indicate that signs of oxidative stress are reduced in hearts that underwent long-term hypothermic cardioplegia. Values are mean±SEM (n=6 to 7). Baseline values (B) before the onset of ischemia were not detectable (ND, <0.01 nmol·min^{-1}·g wet wt^{-1}). Note the difference in scale of the y axes.* *P<.05 *vs baseline.*

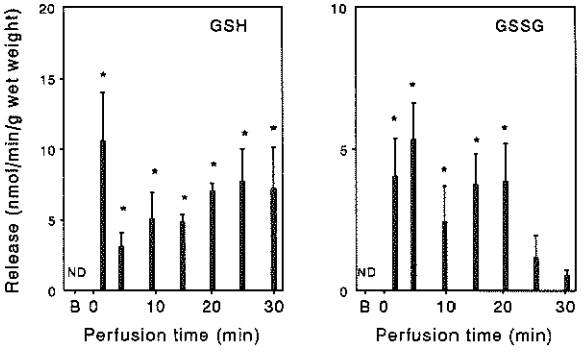

Fig 3. *Bar graph show the release of reduced glutathione (GSH) and oxidized glutathione (GSSG) from rat hearts during peroxide infusion. After a stabilization period of 30 minutes, hearts were perfused for 30 minutes with 0.5 mmol/L cumene hydroperoxide in a recirculating manner. Peroxide induces a bi-phasic massive release of GSH and GSSG. Data are expressed as mean±SEM (n=6). *P<.05 versus baseline (B). ND indicates not detectable.*

5.4±0.8 and 0.8±0.2 nmol·min^{-1}·g wet wt^{-1} in the ischemic (Fig 2A) and cardioplegic/ischemic (Fig 2B) group, respectively (P<.001). In the ischemic group, GSSG efflux amounted to 1.0±0.3 nmol·min^{-1}·g^{-1} at 10 minutes reperfusion (P<.05 versus baseline, Fig 2A). In the cardioplegic/ischemic group, the pattern of GSSG release was similar to that in the ischemic group; however, the values were 5 times lower (P<.05; compare Fig 2A and 2B).

Infusion of cumene hydroperoxide in normoxic hearts caused a biphasic release of both GSH and GSSG (Fig 3). GSH release was of the same order of magnitude as in rat hearts made ischemic (compare with Fig 2A). GSSG efflux was 5 times higher than in human and is ischemic rat hearts, about 10 nmol·min^{-1}·g^{-1} (see Figs 1 and 2A). After 15 minutes infusion, there was a second burst of GSH and GSSG release, coinciding with arrest of the hearts and a decrease in flow.

Table 2. Decrease in Rat Heart Glutathione due to Cardioplegia and/or Ischemia/Reperfusion

Variable	Without cardioplegia		After cardioplegia	
	Control	Isch/Rep	Control	Isch/Rep
GSH, µmol/g protein	14.5±1.3	9.4±0.7*	9.9±0.7*	9.4±0.8
GSSG µmol/g protein	0.22±0.03	0.23±0.03	0.16±0.02	0.15±0.01
Ratio	70.9±7.3	47.1±8.2*	67.0±8.8	62.3±4.8

Isch/Rep indicates ischemia/reperfusion; GSH, reduced glutathione; GSSG, oxidized glutathione; and ratio GSH/GSSG. Values are mean±SEM (n=6 to 9).

Two groups of hearts received 40 minutes cardioplegia before the start of the perfusion, and 2 groups did not. After baseline perfusion for 30 minutes, hearts were subjected to normoxic perfusion for another 30 minutes (control), or to 20 minutes global ischemia and 30 minutes reperfusion (Isch/Rep). Hearts were freeze-clamped at the end of the perfusion. The data, indicate that Isch/Rep lowers the cardiac GSH but does not increase GSSG content. In addition, they show that cardioplegia induces loss of GSH.

**P<.05 versus control without cardioplegia.*

Tissue GSH content was 35% lower in the ischemic group than in controls (P<.05, Table 2). GSSG content was similar in these two groups. The ratio between GSH and GSSG in the control group (71±7) decreased 34% in the ischemic group (P<.005 versus the control group). The hearts subjected to 40 minutes of cardioplegia showed a different pattern (Table 2): GSH content of the control group showed a 32% (P<.05) decrease due to cardioplegia. Cardioplegia with and without ischemia/reperfusion did not affect the GSH/GSSG ratio (Table 2).

Tissue GSH and GSSG content in the ischemic group at 1 minutes reperfusion was 11.5±1.1 and 0.22±0.08 µmol/g protein, respectively. These are similar to those at 30 minutes (Table 2).

Hearts subjected to cumene hydroperoxide contained 3.9±0.8 µmol GSH/g protein, ie, three times lower than GSH in control hearts (P<.05). GSSG content was higher than control hearts (0.45±0.11 µmol/g protein, P=NS). The ratio of GSH to GSSG had decreased by a factor of 6 (P<.005).

In rat hearts, an imbalance existed also between loss of tissue glutathione and total efflux, although it was less pronounced than in human preparations. Rat heart content decreased by 400 nmol/g wet wt, whereas GSH+GSSG release in 30 minutes was 200 nmol/g.

MDA in Effluent and Tissue of Both Species

No MDA was detected in the human arterial or venous samples during the whole period of perfusion and reperfusion (detection limit, 0.02 nmol·$^{-1}$min·g wet wt^{-1}). In addition, we could not detect any MDA in control or ischemic/reperfused human tissue samples (detection limit, 4 nmol/g protein). MDA efflux could not be measured due to an interfering peak that coincided with MDA. This peak was not present in the effluents of the ischemic hearts and must be related to the peroxide stress. Tissue MDA amounted to 14 nmol/g protein at the end of drug infusion (n=1).

Control and ischemic/reperfused rat hearts, with or without cardioplegia, released no detectable MDA.

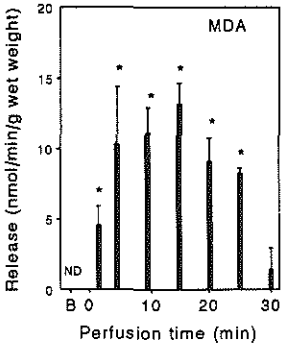

Fig 4. *Bar graph shows the release of malondialdehyde (MDA) from rat hearts during peroxide infusion. After a stabilization period of 30 minutes, hearts were perfused for 30 minutes with 0.5 mmol/L cumene hydroperoxide in a recirculating manner. MDA was determined by high performance liquid chromatography. The data suggest that substantial lipid peroxidation due to hydroperoxide infusion occured. Data are expressed as mean±SEM (n=6).* *$P<.05$ *vs base line.*

Cumene hydroperoxide infusion caused a rapid increase in MDA release, reaching a maximum of 13.2±1.5 nmol·min^{-1}·g wet wt^{-1} after 20 minutes perfusion (Fig 4).

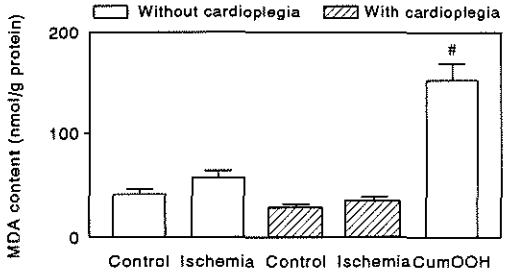

Fig 5. *Bar graph shows malondialdehyde (MDA) content of rat myocardial tissue subjected to cardioplegia, ischemia/reperfusion or peroxide stress. After baseline perfusion for 30 minutes, control hearts were subjected to normoxic perfusion for another 30 minutes, and ischemic hearts to 20 minutes global ischemia and 30 minutes reperfusion. A series of normoxic hearts was infused for 30 minutes with cumene hydroperoxide (CumOOH). Two additional groups received 40 minutes cardioplegia prior to the protocol for the control or ischemic hearts. Hearts were freeze-clamped at the end of the perfusion protocol. The data indicate that only a drastic stress such as CumOOH induced a significant rise in MDA. In contrast cardioplegia lowered the MDA content. Values ae mean±SEM (n=6 to 9).* #$P<.05$ *versus control.*

Tissue MDA content determined in rat hearts is shown in Fig 5. Tissue MDA content in ischemic/reperfused rat myocardium at the end of 30 minutes of reperfusion was similar to control values, as was MDA in the cardioplegic groups. Cardioplegia induced an insignificant decrease in MDA content, both in control and ischemic/reperfused hearts. MDA content in rat hearts perfused with cumene hydroperoxide increased 3.6 times ($P<.01$ versus control hearts).

Discussion

Glutathione as Indicator of Oxidative Stress

Tissue GSH content after ischemia/reperfusion decreases due to a combination of washout and conversion to GSSG, thereby decreasing the ratio of tissue GSH to GGSG.[29] GSSG release after ischemia is an important indicator for oxidative stress.[29,30] We observed GSH and GSSG release from human and rat hearts after ischemia (Figs 1 and 2), with changes in tissue contents in accordance with literature data. This release of GSSG reflects a production, because tissue GSSG levels are comparable after 1 and 30 minutes of reperfusion. Tissue content does not increase because GSSG is actively transported from the cell.[8] Our isolated human heart data are in agreement with the clinical results of Ferrari et al.[31] Because of cardioplegia, rat hearts lost large amounts of glutathione (Table 2). This could explain the relatively low release from the human hearts, which were also subjected to cardioplegia.

We cannot rule out the possibility that the diseased human hearts used differ in scavenging ability and behavior from normal hearts. In this respect is is noteworthy that hypertrophied rat hearts have higher glutathione-peroxidase activities.[32]

In our experiments there was an apparent loss of GSH as evidenced by the imbalance between GSH + GSSG release and decrease of tissue content. Others attributed a similar finding to the formation of glutathione-S-conjugates,[8] mixed disulfides or dipeptides,[33] during reperfusion.

MDA Detection

MDA, generated during lipid peroxidation, can react with thiobarbituric acid, forming a colored complex, but so do various other compounds. Several reports confirm that the colorimetric assay, based on this principle, is not a reliable index for lipid peroxidation.[15,16,34] With these studies in mind, we have opted for the direct quantitation of MDA by HPLC.[26] A novel finding is the absence of MDA in human myocardial tissue and the lack of MDA release after a considerable ischemic period.

MDA as Free Radical Indicator

MDA formation, assessed by the thiobarbituric-acid assay, was significant in humans during pacing stress testing[14] and coronary angioplasty.[12,13] Both interventions disturb the cardiac oxygen supply/demand balance for short periods, ie, minutes. In view of the discrepant literature data described in the introduction, we subjected isolated hearts from humans and rats to ischemia/

reperfusion. Under these conditions MDA was undetectable (<0.02 nmol·min^{-1}·g wet wt^{-1}) in the cardiac effluent of both species during reperfusion, regardless of the use of cardioplegia; this finding confirms the (rat heart) data of Julicher et al.[35] Under physiological conditions, MDA was present in rat myocardial tissue (41 nmol/g protein), in accordance with literature data.[15,16]

The lack of MDA in human ischemic myocardial tissue could be explained in several ways. First, cardioplegia could have affected MDA content (we took our control biopsies just before ischemia). Second, the state of these diseased hearts may have interfered. We used hearts from patients undergoing transplantation for end-stage cardiac disease. Turkeys with cardiomyopathy are known to have lower lipid peroxidation.[36] Nevertheless, our preliminary data indicate that cumene hydroperoxide can induce MDA formation in human heart, but the tissue value remained 10 times lower than that in rat hearts. Third, species-dependent membrane susceptibility to lipid peroxidation could explain differences between human and rat MDA tissue content.[37]

Rat heart MDA content after ischemia/reperfusion in the non cardioplegic group showed a tendency to increase (Fig 5), in agreement with data of Ceconi et al.[15] Subjected to more drastic stress (cumene hydroperoxide), rat hearts released substantial amounts MDA (Fig 4), as reported in earlier work.[20,30] The myocardial MDA content increased significantly in comparison with control hearts (Fig 5). This suggests that MDA release from heart or an increase in its myocardial content occurs only under extreme conditions like exposure to toxic hydroperoxide concentrations; then the endogenous scavenging systems apparently cannot cope with the large amounts of free radicals formed.

The absence of MDA formation in the isolated human heart preparations sheds doubt on the reports on MDA found in clinical studies. Until now, the source of free radicals is unclear. False-positive reports could be ascribed to the classical, aspecific, colorimetric thiobarbituric acid assay for MDA, which also measures other aldehydes.

Effect of Cardioplegia on Glutathione and MDA Content

Although cardioplegia followed by ischemia might be beneficial for postischemic functional recovery, restoration of blood flow paradoxically poses a threat to the myocardium. Concomitant with reoxygenation, superoxide radical formation, as well as washout of important cellular compounds, takes place.

We found that GSH levels decreased by a factor of 1.5 as a result of cardioplegia in control rat hearts (Table 2). This can be explained by two factors: (1) massive oxidative stress upon reperfusion with conversion of GSH to GSSG or (2) washout of large amounts of GSH. At the start of the perfusion GSH release is extensive without detectable amounts of GSSG (unreported data). Therefore, we think that the second explanation is valid. The imbalance in human and rat hearts increased as a result of cardioplegia; it is probably caused by this loss at the start of the perfusion. Thus supplementation of a cardioplegic solution with GSH may have beneficial effects. In line with this hypothesis, Menasché et al[38] recently showed that the addition of *N*-acetylcysteine to cardioplegia improved hemodynamic recovery.

Ischemia did not induce a decrease in GSH content in cardioplegic rat hearts (Table 2). In contrast, GSH levels in human hearts decreased (Table 1). We are unable to offer an explanation for this difference.

Hypothermic cardioplegia lowers the MDA content nonsignificantly in rat hearts (Fig 5). Ceconi et al[15] observed a decrease of MDA tissue content during ischemia; MDA returned to aerobic values after readmission of flow. These workers hypothesized that MDA levels might be closely related to oxygen tension. Since flow was absent following the cardioplegic period, oxygen levels could have decreased and therefore MDA content.

Source of Free Radicals or of MDA

In vivo chemotaxis of leukocytes to injured myocardial tissue, eg, after myocardial infarction, is known to induce free radical formation.[39,40] The severity of the myocardial injury is correlated with the serum MDA concentration.[41] Such a mechanism, however, does not explain the immediate release of MDA after short ischemic periods. Also, platelets adhere to arterial lesions and arteriosclerotic plaques, and MDA formation is closely related to prostaglandin synthesis by platelets.[42] MDA formation observed during coronary angioplasty may originate from the arteriosclerotic lesion, as balloon inflation crushes the plaque and adhering platelets. On the basis of literature data, we assume that xanthine oxidoreductase plays only a minor role in free radical generation in human hearts (for review, see references 23 and 43). However, Abadeh et al[44] found an inactive form of human-milk xanthine oxidase which produces superoxide radicals through NADH oxidation by the flavin adenine dinucleotide group. Human myocardium could contain a similar XOD, responsible for radical-induced damage.

On the basis of the tissue GSH content and release of GSSG, human and rat hearts seem to have a similar vulnerability for oxidative stress. However, other systems like the pentose-phosphate cycle or GSH-transferases can also change GSH and GSSG levels inside the cell under ischemic conditions.

Limitations of the Study

GSH release may have been underestimated, since we were unable to collect samples every minute early during reperfusion in rat hearts. GSH could have been converted to GSSG during recirculation, resulting in an overestimation of the GSSG efflux data. GSH and GSSG can be taken up by the heart.[45] Consequently this would change tissue content and underestimate glutathione efflux. We are unable to assess from the experiments conducted to what extent these processes affected our results.

The increase in human heart weights evidences edema, despite the presence of the polymer dextran in

the perfusion buffer. Dextran used in rat-heart perfusions did not influence glutathione or MDA levels (data not shown). We are not sure whether edema has influenced GSH and GSSG release.

As already mentioned, the explanted diseased human hearts may differ from normal hearts in their MDA-producing potential or scavenging ability. However, hearts of cardiac patients undergoing coronary angioplasty or other procedures could have changed to a similar, but smaller, extent.

Conclusion

After ischemia isolated human and rat hearts released GSSG, which indicates that oxidative stress has occurred. Lipid peroxidation (as measured by MDA formation) took place in control rat hearts, decreased non significantly after cardioplegia, but did not increase after ischemia/reperfusion. Substantial amounts of MDA were formed under extreme conditions, ie, perfusion with cumene hydroperoxide. Then scavenging systems seem unable to cope with the amounts of free radicals formed. The novel finding that human hearts lack MDA under normoxic conditions and after ischemia possibly reflects a lower MDA-producing potential. MDA found in clinical studies could come from another source or could be due to the use of the aspecific thiobarbituric-acid assay.

Acknowledgements

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Appendix 3

Antioxidant defences in rat, pig, guinea pig and human hearts: comparison with xanthine oxidoreductase activity

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Cardiovasc. Res., in press

Antioxidant defences in rat, pig, guinea-pig and human hearts: comparison with xanthine oxidoreductase activity

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Key words: Catalase, Glutathione system, Isolated heart perfusion, Species differences, Superoxide dismutase, Xanthine oxidoreductase

Abstract

Objective: Cardiac injury, related to ischaemia and reperfusion, may be caused by the action of oxygen free radicals. Xanthine oxidoreductase activity (XOD) may be an important free-radical source. During cardiac ischaemia, the native dehydrogenase form may be converted to the oxidase form, which uses molecular oxygen to form superoxide radicals. Superoxide dismutase (SOD) converts the latter to H_2O_2, which is detoxified by catalase (CAT) and glutathione peroxidase (GPD). In view of the large differences in XOD in various species, we investigated whether the activity of these antioxidant enzymes also varied to a large extent. **Methods:** Normal rodent and porcine as well as explanted human hearts were perfused according to Langendorff. After a 30-min stabilization period, hypoxanthine was added to the perfusion buffer to estimate XOD. Hearts or biopsies were freeze-clamped after 90 min. Effluent xanthine (X) and urate (UA) were assayed with HPLC, tissue GSH content and the activity of SOD, CAT, GPD, glutathione reductase (GRD) were determined spectrophotometrically. Apparent XOD was calculated as X + 2x UA production. **Results:** XOD was (mU/g protein, mean ± SEM, n = 5-7): Rat, 470±40; guinea pig, 270±41; pig, <1.5; and man, 5.4±1.0. SOD activities were (U/g protein): Rat, 13,370±1,030; guinea pig, 10,100±1,110; pig, 12,800±450; and man, 7,400±450. CAT activity (k≤10/g protein) was low in all species studied. GPD activity was 93±7 U/g protein in rat heart, and 10x lower in the other species. GRD activity was (U/g protein): Rat, 15.0±1.6; guinea pig, 10.4±1.3; pig, 16.0±1.5; and man, 26.6±2.0. Tissue GSH concentrations were (μmol/g protein): 13.5±0.8, 18.5±0.9, 11.1±2.9, 17.2±1.7, respectively. **Conclusions:** Considerable species differences in XOD activity exist, contrasting the smaller variations in antioxidant enzyme activities. In the species examined catalase activities were very low. Rat hearts are far better protected against hydrogen-peroxide than the other three species. XOD-induced free-radical damage probably plays a minor role in pig and human hearts. Human myocardium seems less protected against superoxide radicals. (*Cardiovascular Research* 0000;**00**:000-000)

Introduction

A number of studies provide evidence that cardiac injury, related to ischaemia and reperfusion, may be caused by the action of oxygen-free radicals. Various sources of the latter are known, including leucocytes,[1] oxidation of catecholamines,[2] prostaglandin pathways,[3] mitochondrial respiration,[4] and xanthine oxidoreductase (XOD).[5] XOD catalyzes the breakdown of hypoxanthine to xanthine and xanthine to urate. In normal cardiac tissue, XOD is mainly present in the dehydrogenase form (XD). In contrast to the dehydrogenase form, the oxidase form (XO) uses molecular oxygen as electron acceptor. It is claimed that during myocardial ischaemia, XD is converted to XO, which contributes to the reperfusion damage.[5,6] In the ischaemic period, ATP breakdown gives rise to large amounts of hypoxanthine, a substrate for XO. A simplified scheme of events which might occur from this point is depicted in Fig. 1. Superoxide dismutase (SOD) converts superoxide to hydrogen peroxide,[7] which can react with iron to form the highly cytotoxic hydroxyl radical (Haber-Weiss reaction).[8,9] The latter can initiate the peroxidation of polyunsaturated fatty acids in cell membranes, and thus impair or destroy cell function. Although myocardial SOD activities seem to differ little in various mammals, data on human tissue are limited.[10]

Hydrogen-peroxide is converted to water by catalase and by glutathione peroxidase (Fig. 1). The latter oxidizes the co-substrate reduced glutathione (GSH). Glutathione reductase recycles the oxidized glutathione (GSSG) to GSH. Liver glutathione peroxidase activities vary between species.[11] Activities in human atrial biopsies have been measured,[12 13] whereas Carmagnol et *al.* reported activities present in post-mortem material.[14]

Various investigators found that GSH depletion makes hearts more susceptible to ischaemia-reperfusion injury;[15 16] in contrast, GSH enhancement improves cardiac function,[16] implicating that GSH is an important factor in maintaining the glutathione redox system.

The extent of free-radical injury depends on the amount of free radicals produced and the activity of scavenging enzymes. Species differences in antioxidant capacity might influence the effect of xanthine oxidoreductase on ischaemia-reperfusion damage between species.

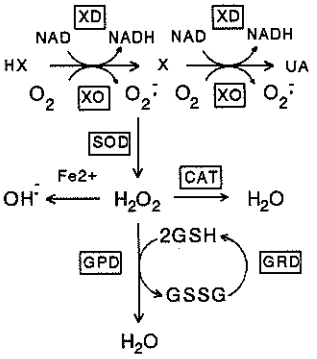

Figure 1. *Myocardial defences against hydrogen peroxide and superoxide radical damage. CAT: catalase, GPD: glutathione peroxidase, GRD: glutathione reductase, GSH: reduced glutathione, GSSG: oxidized glutathione, HX: hypoxanthine, O_2^-: superoxide radical, OH^-: hydroxyl radical, SOD: superoxide dismutase, UA: urate, X: xanthine, XD: xanthine dehydrogenase, XO: xanthine oxidase.*

In view of the large differences in XOD activity in various species,[17] we investigated whether the endogenous antioxidant enzymes also varied to a large extent. Therefore, we infused hypoxanthine into isolated rat and guinea-pig hearts (displaying relatively high XOD activity[17]) as well as pig and human hearts (relatively low activity[17 18]). We estimated the ex-vivo XOD activity, and determined the activities of the antioxidant enzymes as well as GSH and GSSG content. Furthermore we tested the ability of allopurinol to inhibit xanthine oxidoreductase activity.

The large species differences in XOD activity contrast with the smaller variations in antioxidant enzyme activities. Human myocardium has lower activity of superoxide dismutase and higher levels of glutathione reductase in comparison with several other species.

Methods

Rat and guinea-pig heart perfusions

Adult Wistar rats and Dunkin-Hartley guinea pigs were anaesthetized intraperitoneally with 60 mg/kg pentobarbital (Nembutal; Sanofi, Paris, France) in accordance with the institutional guidelines. Hearts were removed, arrested in ice-cold saline, and perfused retrogradely according to Langendorff with a modified Tyrode's buffer, oxygenated with 95% O_2/5% CO_2, at 9.6 kPa and 37°C, as described previously.[19] Cannulation of the aorta was done <1 min after arrest. Coronary flow was measured electromagnetically (Skalar, Delft, The Netherlands). The perfusion system consisted of two reservoirs. One contained medium, the other medium with 50 μM hypoxanthine (Merck, Darmstadt, FRG). After a 30-min stabilization period, medium plus hypoxanthine was infused for 60 min. Then, hearts were freeze-clamped. An additional group

of hearts underwent the same protocol, with perfusion buffer containing hypoxanthine and 100 μM allopurinol (Wellcome, Beckenham, UK).

Human and pig-heart perfusions
Explanted hearts of patients in end-stage heart failure due to ischaemic heart disease or dilated cardiomyopathy were used. Hearts were also obtained from young adult pigs (hybrid Yorkshire/Danish Landrace), used as control in other experiments. Pigs were sedated with azaperone 4 mg/kg i.m. (Stresnil; Janssen Pharmaceutica, Beerse, Belgium) and anaesthetized with 5 mg/kg metomidate iv (Hypnodil; Janssen). Both human and porcine hearts were arrested in situ with ice-cold St. Thomas' Hospital cardioplegic solution,[20] and transported in ice-cold saline to the laboratory. Approximate transportation time was 5 and 15 min for pig and human hearts, respectively. Retrograde aortic perfusion started 30-50 min after cardiac arrest. The perfusion apparatus was equipped with a roller pump (Verder, Vleuten, The Netherlands), externally controlled by the perfusion pressure (10.6 kPa). To minimize pump pulsations, a Wind Kessel that also functioned as fluid reservoir and heat exchanger (37°C) was used. The perfusion fluid consisted of Tyrode's buffer,[19] supplemented with 10 IU/litre insulin (Novo Industri AS, Bagsvaerd, Denmark), 5,000 IU/litre heparin, and 25 g/litre dextran (40 kDa; Pharmacia, Uppsala, Sweden). The perfusion fluid was oxygenated with 95% O_2/5% CO_2, using a baby oxygenator (Polystan, Vaerløse, Denmark). Blood was washed from the hearts for 10 min, then recirculation started with 1.5-2.0 litre perfusion fluid. After a 30-min control period, 50 μM hypoxanthine final concentration was infused, during 60 min, just above the aortic cannula. Then a left ventricular biopsy (about 2 g) was taken and immediately freeze-clamped. Another group of hearts underwent the same protocol, where the infusate contained hypoxanthine and 100 μM allopurinol.

Oxypurine assay
Human and porcine heart arterial and venous samples, as well as rodent-heart coronary effluent, were collected at regular intervals. To prevent adenosine catabolism, the former were mixed with dipyridamole (Boehringer, Mannheim, FRG) and erythro-9-(2-hydroxy-3-nonyl)adenine (Burroughs Wellcome, Research Triangle Park, NC, USA); final concentration 10 and 20 μM, respectively. Purines were assayed by HPLC as described previously,[19 21] with detection at 295 nm (urate) and 254 nm (hypoxanthine, xanthine). The amount of enzyme required to produce 1 μmol of xanthine + 2 μmol of urate per minute is defined as 1 XOD unit.[17]

Glutathione determination
Tissue (0.4 g) was crushed in liquid nitrogen and deproteinized with 5% sulpho-salicylic acid.[22] After centrifugation at 10,000 g for 2 min (4 °C), the supernatant fluid was neutralized with 6 M KOH/2 M K_2CO_3. Total glutathione and GSSG were analyzed as described by Ceconi *et al.*,[23] on a double-beam spectrophotometer (Hitachi, model U-2000, Tokyo, Japan), measuring the rate of reduction of 5-5'-dithiobis-(2 nitrobenzoic acid) (Sigma-Aldrich, Brussels, Belgium) at 412 nm, in the presence of glutathione reductase (Boehringer). GSSG was determined after the removal of GSH with N-ethyl maleimide.

Superoxide dismutase assay
Total superoxide dismutase (Cu/Zn-SOD and Mn-SOD) was determined according to Flohé and Ötting.[24] Briefly, tissue was crushed in liquid nitrogen in 10 volumes of 50 mM K^+-phosphate buffer, pH 7.2, thawed and centrifuged at 18,000 g for 15 min (4 °C). Activity was determined at 25 °C in 50 mM K^+-phosphate buffer, pH 7.8, containing (final concentration); 0.1 mM EDTA, 20 μM cytochrome-c (Sigma-Aldrich), 2 μM natrium azide and 50 μM xanthine. Xanthine oxidase (Boehringer) was added to this reaction mixture to reduce ferricytochrome-c at a rate of approximally 0.025 absorbance unit/min at 550 nm. The amount of enzyme required to inhibit this reduction by 50% is defined as 1 SOD unit.[24]

Catalase assay
Catalase was assayed according to Aebi after incubating 100 volumes supernatant fluid (see SOD assay) with 1 volume of 95% ethanol to decompose complex II.[25] The rate of H_2O_2 decomposition was measured at 240 nm for 30 sec. The calculated rate constant (K) correlates with the catalase activity in the samples.[25]

Glutathione peroxidase and glutathione reductase determination
Enzyme activities were analyzed as described by Ferrari *et al.*[26] Briefly, tissue was crushed in liquid nitrogen

in 10 volumes of 50 mM K^+-phosphate buffer, pH 7.2, centrifuged at 3,000 g for 10 min. The supernatant fluid was used for enzymatic determinations. The selenium containing enzyme glutathione peroxidase was assayed; the reaction volume (2.0 ml) contained 50 mM Tris-HCl, pH 7.3, 0.1 mM EDTA, 0.12 mM NADPH, 0.25 mM GSH, 1 U/ml glutathione reductase and sample (0.1 ml). The reaction was started by addition of 50 µl H_2O_2 (final concentration 0.25 mM). The disappearance of NADPH was monitored at 340 nm. The reaction mixture to assay for glutathione reductase contained 0.1 M Tris-HCl, pH 8.0, 0.94 mM EDTA, 4.6 mM GSSG, 0.16 mM NADPH (final volume 2.0 ml). The reaction was initiated by adding supernatant fluid (0.1 ml). The amount of enzyme required to oxidize 1 µmol of NADPH per minute is defined as 1 GPD or GRD unit.

Protein assay

Protein was assayed with Coomassie Brilliant Blue (Bio-Rad Laboratories, Munich, FRG) according to Bradford[27] using bovine serum albumin as the standard.

Statistics

Data are presented as mean ± SEM. Purine release and enzyme activities were analyzed by one-way analysis of variance, followed by a Bonferroni test to assess the significance of intergroup differences. $p<0.05$ was considered a significant difference.

Results

General

At the start of the perfusion, weights were 1.5±0.1, 1.8±0.2, 167±37 and 531±52 gram for rat (n=10), guinea-pig (n=8), pig (n=5) and human hearts (n=12), respectively. The weight of rodent hearts at the end of the experiment was similar to the initial value; in pig and human hearts it increased 30-40% ($p<0.005$ vs initial weight). The hearts were not paced; they had a frequency of about 260 (rat), 230 (guinea pig), 100 (pig) and 50 (human) beats/min throughout the experiment. Coronary flow in rodent hearts remained about 11 ml/min/g wet weight. Pig and human hearts had a coronary flow of 2.6 and 1.0 ml/min/g wet weight, respectively, decreasing some 6% during the course of the experiment.

Table 1. *Release of xanthine and urate from isolated hearts*

Species	Base line				Hypoxanthine infusion			
	Xanthine		Urate		Xanthine		Urate	
Rat	0.36 ± 0.08		3.42 ± 0.37		17.9 ± 1.5*		9.78 ± 0.78*	
Guinea pig	0.48 ± 0.08		3.29 ± 0.77		2.18 ± 0.48*		7.91 ± 0.95*	
Pig	0.014 ± 0.004		0.003 ± 0.001		0.014 ± 0.003		0.003 ± 0.001	
Human	0.039 ± 0.013		0.13 ± 0.06		0.073 ± 0.019#		0.10 ± 0.04	

After a 30-min stabilization period, the hearts were infused with 50 µM hypoxanthine. At fixed time intervals, effluent samples were collected and analyzed by HPLC. Xanthine and urate production was calculated after steady-state conditions were reached. Data are expressed in nmol/min/g wet weight, means ± SEM (n = 5-7). # *$p<0.05$ vs base line,* * *$p<0.001$ vs base line.*

Apparent xanthine oxidoreductase activity

We observed high base-line hypoxanthine release in pig and human hearts, about 1.4 nmol/min/g wet weight. Rodent hearts released 5x less hypoxanthine. Base-line urate formation in rodent hearts was 25 and 1000x higher than in human and pig hearts, respectively (Table 1). Xanthine production followed the same pattern as urate formation, high in rodent and low in human and pig hearts (Table 1).

The release of xanthine and urate during hypoxanthine challenge, shown in Table 1, reflects the situation under steady-state conditions. During hypoxanthine infusion, xanthine and urate formation in pig heart was

identical to base-line conditions. Xanthine, but not urate, release increased significantly in human hearts (Table 1). In rodent hearts xanthine and urate production both rose substantially vs base line (Table 1). Compared to rat hearts, guinea-pig hearts released relatively little xanthine during the hypoxanthine infusion: the xanthine/urate ratios were 1.8 and 0.3, respectively. Table 2 shows the xanthine oxidoreductase activities in the species examined, estimated from the xanthine and urate production during hypoxanthine infusion. If 100 μM allopurinol was included in the perfusion buffer, xanthine oxidoreductase activity in rat and guinea-pig hearts decreased 93 and 97%, respectively. In pig hearts allopurinol was not tested since activity with hypoxanthine alone was already below the detection limit. In human hearts the low XOD activity decreased 43% due to allopurinol.

Table 2. *Effect of allopurinol on xanthine oxidoreductase activities in perfused hearts*

Species	XOD (HX)	XOD (HX+ALLO)
Rat	470±40	32±2*
Guinea pig	270±41	8.8±0.1*
Pig	<1.5	ND
Human	5.4±1.0	3.1±0.2*

*During perfusion with hypoxanthine (HX) or HX + allopurinol (ALLO), effluent samples were collected and subsequently analyzed by HPLC. Xanthine and urate release was measured and xanthine oxidoreductase (XOD) activity calculated as xanthine + 2x urate production. Data are expressed in mU/g protein, means ± SEM (n=5-7), * $p<0.05$ vs XOD (HX), ND = not determined.*

Antioxidant defences

Activities of superoxide dismutase are depicted in Fig. 2. Enzyme activity was lowest in human myocardial tissue, i.e., 7,400 U/g protein ($p<0.05$ vs rat and pig) and highest in rat: 13,400 U/g. Catalase activity (Fig. 3) did not differ significantly among the four species examined. Activities of glutathione peroxidase and glutathione reductase are depicted in Fig. 4. The activity of glutathione peroxidase in rat hearts (93 U/g) was 10x higher in comparison with the other species ($p<0.001$). Glutathione reductase in human heart, i.e. 27 U/g, was 2-3x higher than that in the other species ($p<0.05$). Non-perfused healthy human myocardial tissue displayed similar enzyme activities in comparison with explanted human hearts (data of pilot study not shown).

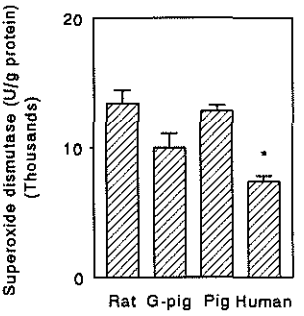

Figure 2. *Superoxide dismutase activity in myocardial tissue. After 90 min of normoxic perfusion, rat and guinea-pig hearts were freeze-clamped. At that time, a biopsy was taken from pig and human left ventricles and immediately frozen in liquid nitrogen. The activity was calculated from the decrease of cytochrome-c reduction at 550 nm. G-pig = guinea pig, means ± SEM (n = 5-7).* * *$p<0.05$ vs rat and pig.*

Myocardial GSH content (Fig. 5) varied between 10 and 20 μmol/g protein. Significant differences existed between pig and guinea-pig hearts ($p<0.05$). Tissue GSSG content (Fig. 5) was of the same order of magnitude in all species, i.e., between 0.4 μmol/g protein (rat and guinea-pig heart) and 1.0 μmol/g (human heart). No linear correlations were found between the levels of the various antioxidant enzymes. GSH levels did not correlate with GPD and GRD activities, nor did antioxidant activity correlate with XOD activity.

Discussion

Xanthine oxidoreductase

Xanthine oxidoreductase activities differ substantially among the species studied. XOD activity in pig hearts is undetectable. The value for human hearts (5.4 mU/g protein) is two orders of magnitude lower than that in rat and guinea-pig hearts. This agrees with values published before.[17] It implicates that free-radical formation by xanthine oxidase in pig and human hearts is probably very low. Thus this enzyme plays probably a minor role in free-radical-induced damage during myocardial ischaemia/reperfusion in these species.

Allopurinol can reduce the apparent XOD activity in rat, guinea-pig and human hearts significantly (Table 2). Despite its low activity in human hearts, allopurinol seems beneficial against ischaemia/reperfusion injury: Two to 4 days of drug treatment before surgery decreases drastically enzyme release and the incidence of cardiac complications,[28-30] and improves post-operative cardiac performance.[29] Allopurinol, and to a larger

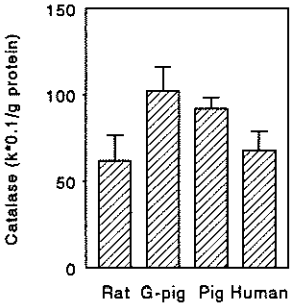

Figure 3. *Catalase activity in perfused hearts. Samples were taken as described in the legend of Fig. 2. The activity, expressed as the rate constant k, was calculated from the disappearance of hydrogen peroxide at 240 nm. G-pig = guinea pig, means ± SEM (n = 5-7).*

extent its catabolite oxypurinol, is a free-radical scavenger.[31-33] The beneficial effects of allopurinol observed in species with low myocardial XOD activity may thus be due to free-radical scavenging instead of blockade of XOD.

Antioxidant enzymes

Marklund studied superoxide dismutase activities in 9 species, which showed comparable levels of the enzyme.[10] However, human myocardial tissue was obtained post-mortem, which may have undergone enzymatic changes. Activities in our study are comparable with those reported by Marklund.[10] SOD activity in human myocardium was significantly lower compared with that in pig and rat hearts. This suggests that protection against superoxide radicals is relatively meagre in human hearts.

Since catalase kinetics show abnormal behaviour, activity was expressed in terms of the rate constant of the first order reaction.[25] Activities were similar in all species and very low in comparison with other organs.[34] Therefore this enzyme plays probably a minor role in detoxifying hydrogen peroxide in the myocardium,

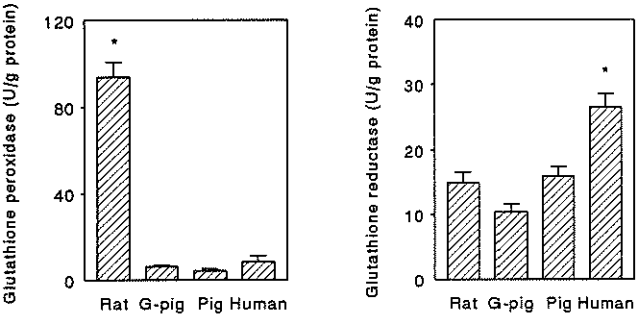

Figure 4. *Glutathione peroxidase and glutathione reductase activity in isolated perfused hearts. Samples were taken as described in the legend of Fig. 2. Activities were measured by analyzing the decrease in NADPH at 340 nm. G-pig = guinea pig, means ± SEM (n = 5-7). * $p<0.05$ vs the other species.*

contrasting the notion of Thayer that catalase serves as a major route of cardiac hydrogen peroxide breakdown,[34] despite its low activity.

Unlike xanthine oxidoreductase activity, glutathione peroxidase activity is of the same order of magnitude in man, guinea pig and pig. However, rat hearts seem to have better protection against hydrogen peroxide. Our data on glutathione peroxidase activity in rat hearts agree with those reported in the literature;[35-38] in human ventricle they are 3x lower than those reported for human atria.[12 13]

We observed little variation in GRD activity among the species examined. Glutathione reductase activities in human ventricles are comparable to those found in atria.[12] The hearts of the species studied seem to have equal potential to cycle GSSG back to GSH. With the exception of the rat, GRD activity is higher than GPD.

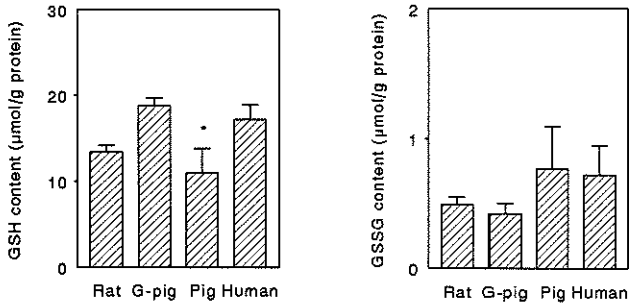

Figure 5. *Reduced glutathione (GSH) and oxidized glutathione (GSSG) content in isolated perfused hearts. Samples were taken as described in the legend of Fig. 2. The amount of GSH was calculated from total glutathione and GSSG, assayed spectrophotometrically. G-pig = guinea pig, means ± SEM (n = 5-7). * $p<0.05$ vs guinea pig.*

This would mean that during oxidative stress rat hearts are less capable of regenerating GSH and consequently antioxidant capacities might be more affected in comparison with the other species.

Intracellular GSSG determines the amount of GSSG transported out of the cell.[34 39] If GSSG is used as indicator of oxidative stress, this would lead to an overestimation in the rat heart, since GPD activity exceeds that of GRD. It is therefore preferable to measure the ratio of tissue GSH/GSGG to evaluate this stress.

GSH is a co-substrate of glutathione peroxidase in its action upon hydrogen peroxide. The integrity of the glutathione redox system depends on the GSH available inside the cell. The close relationship between GSH and enzyme function, by stabilizing sulphhydryl groups, reflects its importance for cell function.[40] Myocardial GSH levels in the species examined are of the same order of magnitude (Fig. 5). On a wet weight basis, our

GSH value for rat heart agrees with data given in ref. 39. GSSG levels were one order of magnitude lower than GSH levels, in line with literature values.[16 41] The relatively high GSSG content in pig and human hearts could be due to the preparation time before the perfusion was started. Intracellular GSH levels are modulated by GPD and GRD, thus a close relationship between GSH levels, GPD and GRD activities may be present. However, our values did not correlate. These enzymes are probably regulated independently, as proposed by Godin and Garnett.[38]

Although in this study significant differences exist in antioxidant profiles between the various species, it is impossible to hypothesize to what extent this influences oxidative damage. Recently species differences were reported in susceptibility to oxidative challenge that cannot be explained by the variations in antioxidant enzymes.[42] Other non-enzymic antioxidants, like vitamine E, could vary between species, modulating the response to free radicals.

Speculation

Firstly, the nature of the low xanthine release in guinea-pig hearts is unclear. It seems not due to a different affinity of XOD for xanthine and hypoxanthine.[43]

Secondly, we reported an age-dependent increase in XOD activity.[44] In contrast antioxidant capacity seems to decrease during aging,[36 45] suggesting a decreasing ability to detoxify H_2O_2. Consequently the balance between production and elimination of H_2O_2 might change during life. We are not sure whether species differences exist regarding these changes in free-radical producers and protective capabilities.

Limitations of the study

The stability of the pig and human heart preparations may have influenced the enzyme activities. Edema poses problems in Tyrode-perfused hearts, even if a high-molecular weight compound, such as dextran, is included to augment oncotic pressure. However, throughout the perfusion the hearts released no lactate, indicating adequate perfusion.

Gupta and Singal reported higher glutathione peroxidase activities in hypertrophic hearts, in comparison with control hearts.[37] We used diseased human hearts for our experiments, which could have had different enzyme activities. Our preliminary data in non-perfused healthy human myocardial tissue would argue against such changes.

The hearts may have been "preconditioned" by their diseased state. Preconditioning does not involve antioxidant defences in rabbit hearts.[46] On the other hand, Hoshida et al. reported recently small but significant increases in antioxidant activity in preconditioned canine hearts.[47]

Conclusions

Considerable species differences in XOD activity exist, contrasting the smaller variations in antioxidant enzyme activities. Xanthine-oxidase-induced free-radical damage probably plays a minor role in pig and human hearts. Human myocardium seems less protected against superoxide radicals than hearts of other species. Catalase activities are similar, but very low in the species examined. Rat hearts are far better protected against hydrogen peroxide than the other three species.

Acknowledgements

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Appendix 4

In vitro and ex vivo xanthine oxidoreductase activity in rat and guinea-pig hearts using hypoxanthine or xanthine as substrate

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In vitro and ex vivo xanthine oxidoreductase activity in rat and guinea-pig hearts using hypoxanthine or xanthine as substrate

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Through oxyradical formation xanthine oxidoreductase (XOD) could play a role in the etiology of cardiac damage. Its measurement poses problems, due to little substrate specificity, self-inactivation and endogenous inhibitors. Perfusion of guinea-pig hearts with hypoxanthine gave rise to only little xanthine release; in contrast rat hearts showed vivid xanthine production. Therefore, xanthine breakdown was hypothesized to exceed its formation in guinea-pig hearts. The kinetics of both substrates for XOD in cardiac homogenates were therefore compared with those obtained in perfused hearts. Oxypurine contents and effluent catabolites were determined by HPLC. Regardless of substrate, V_{max} values in homogenates were about 38 and 13 mU/g for rat and guinea-pig heart, respectively. K_m values were in the 3–5 μM range; therefore the hypothesis concerning the low xanthine release in guinea-pig hearts must be rejected. Activities in hearts perfused with hypoxanthine (50 μM) were 40 and 18 mU/g for rat and guinea pig, respectively; perfusion with xanthine produced $< 50\%$ of the activities observed with hypoxanthine ($p < 0.002$). Intracellular xanthine concentration, estimated from sorbitol distribution space and myocardial xanthine content was negative in both species, contrasting intracellular hypoxanthine levels, which approached extracellular concentrations. This disparate distribution indicates that hypoxanthine transport across the cell membrane far exceeds that of xanthine. Consequently, hypoxanthine is preferable to xanthine as substrate in perfused hearts to estimate XOD activity in situ.

Introduction

Xanthine oxidoreductase (XOD) catalyzes the breakdown of hypoxanthine to xanthine, and of xanthine to urate. It contains molybdenum, Fe/S and FAD redox centres, and has broad specificities for substrates [1]. In rat myocardium the enzyme is localized in the endothelial cells [2]. In normal cardiac tissue, XOD is mainly present in the dehydrogenase form (EC 1.1.1.204). Proteolysis [3] or oxidation of thiol groups [4,5] induces conversion to the oxidase form (EC 1.1.3.22). Several authors postulated that during myocardial ischaemia, xanthine dehydrogenase is converted to xanthine oxidase, which forms superoxide radicals upon reperfusion [6,7].

The activities of both XOD forms can be determined by measuring the conversion of (radioactive) hypoxanthine or xanthine in heart homogenates by high-performance liquid chromatography [8,9], spectrophotometry [10,11] or thin-layer chromatography [12]. Xanthine has been favoured as substrate, because it forms only one product, making the enzyme assay relatively easy. With hypoxanthine as substrate, the two-step reaction complicates the calculation of XOD activity. Other detection methods, including fluorometry [13], chemiluminescence [14] and colorimetry [15], make use of artificial substrates (e.g., pterin) or electron-accepting dyes (methylene blue, tetrazolium salts). The controversial literature data concerning differences in XOD activity, especially in human-heart homogenates (see review in Ref. 16), could be due to the use of different assay techniques, varying tissue sources, and uncontrolled self-inactivation of the enzyme.

An alternative approach to estimate XOD activity in situ is the infusion of substrate into isolated beating hearts, with measurement of the breakdown products in the effluent [16]. It allows the investigation of XOD's role during ischaemia-reperfusion injury. Application

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of this method assumes that substrate transport across the cell membrane is not rate-limiting. Using isolated perfused hearts, we observed earlier little xanthine release with hypoxanthine as substrate in guinea-pig hearts, compared with a substantial release in rat hearts. We hypothesized that xanthine breakdown exceeds its formation in guinea-pig hearts [16]. To test this hypothesis, we studied the kinetics of hypoxanthine and xanthine as substrate for XOD in heart homogenates and compared these in vitro data with results obtained in isolated perfused hearts, i.e., ex vivo. We also determined the myocardial oxypurine distribution during hypoxanthine or xanthine infusion, using [^{14}C]sorbitol as the extracellular tracer. The data suggest that rat and guinea-pig hearts contain a transport barrier for xanthine, but not for hypoxanthine.

Materials and Methods

Heart perfusions

Adult Wistar rats ($n = 14$) and Dunkin-Hartley guinea pigs ($n = 11$) were anaesthetized intraperitoneally with pentobarbital in accordance with institutional guidelines. Hearts were removed, arrested, and perfused according to Langendorff with modified Tyrode's solution at 9.6 kPa and 37°C [17]. Cannulation of the aorta was done within 1 min after cold arrest. Apex displacement was used to monitor function and heart rate [18]. Coronary flow was measured with a flow probe (Skalar, Delft, Netherlands).

After a stabilization period of 30 min, the hearts were infused for 15 min with 50 μM hypoxanthine or xanthine (Merck, Amsterdam, Netherlands). This purine infusion was followed by a washout period of 15 min, then the alternative substrate was given for 15 min.

At fixed time points, coronary effluent samples were collected. They were assayed by high-performance liquid chromatography (HPLC) as described by Smoleński et al. [19], with detection at 295 nm (urate) and 254 nm (hypoxanthine, xanthine). When hypoxanthine was used as substrate, XOD activity was calculated as xanthine + 2 × urate production. During xanthine infusion xanthine oxidoreductase activity equalled urate production. Activity is expressed in mU/g wet weight, 1 U being defined as the amount of enzyme that converts 1 μmol substrate per min.

Determination of intracellular hypoxanthine and xanthine content

After a stabilization period of 30 min, the rodent hearts were perfused for 8 min with buffer containing 50 μM hypoxanthine or xanthine, in addition to 5 μM [^{14}C]sorbitol (Amersham 's-Hertogenbosch, Netherlands; 6000 dpm/ml). Effluent samples were collected every minute. At the end of the perfusion, the hearts were freeze-clamped. Adhering frozen medium was carefully removed and heart weight determined. Then tissue was ground in liquid nitrogen. An aliquot was freeze-dried to determine water content. The remainder was thoroughly mixed with 4% $HClO_4$ (final concentration), thawed, centrifuged at $3000 \times g$ for 15 min at 4°C, and neutralized with 6 M KOH/2 M K_2CO_3. To determine the extracellular volume (ECV), the amount of [^{14}C]sorbitol in the supernatant fluids and in the effluent samples was counted with a Tri-Carb Liquid Scintillation Analyzer (Model 2500 TR, Packard Instrument Company, Meriden, CT, USA), using 10 ml Instagel (Packard) and 0.5 ml sample. Oxypurine content of the samples was determined by HPLC [19].

Intracellular volume (ICV) was calculated by subtracting ECV from total water content. Extracellular content was calculated from ECV and infused hypoxanthine or xanthine concentration. Intracellular content was calculated from the total hypoxanthine or xanthine content minus the extracellular content, assuming for both substrates homogeneous distribution over the total extracellular space, i.e., the sum of the intravascular and interstitial space.

Myocardial homogenate

Five rats and 5 guinea-pig hearts were anaesthetized as described above. Hearts were cooled in and washed with ice-cold saline. Then 5% (w/v) homogenates were made in 50 mM K_2HPO_4/1 mM EDTA.Na_2 (pH 8.3) with a Potter-Elvehjem homogenizer. The buffer contained 1 mM dithiothreitol to prevent oxidative conversion of xanthine dehydrogenase to xanthine oxidase [4], and 1 mM phenylmethylsulphonyl fluoride to prevent proteolysis [20]. The homogenate was centrifuged at $25000 \times g$ (r_{av} 8 cm) for 10 min at 4°C, and the supernatant fluid used immediately.

Xanthine oxidoreductase assay in homogenates

Just before the assay, the supernatant fluid was passed through a Sephadex G-25 column (PD-10, Pharmacia, Uppsala, Sweden), prewashed with the homogenization/incubation buffer, to remove low-molecular-weight compounds including endogenous inhibitors [21]. The method described by Terada et al. was used [20]. Briefly, each sample of column effluent was incubated (30°C, pH 8.3) with (hypo)xanthine, in the presence of 100 μM NAD^+ (xanthine dehydrogenase + xanthine oxidase activity) or ambient oxygen (xanthine oxidase activity). To prevent enzyme inhibition by NADH, LDH (70 U/ml) and pyruvate (1.75 mM) were present. In addition, blanks (without substrate) and negative controls (+100 μM allopurinol final concentration) were determined. The relationship between amount of sample and urate production was linear between 0.1 and 0.6 ml sample (end volume 1.0 ml). Routinely, a sample volume of 0.2 ml (0.14 mg protein)

was used. Incubations were stopped with 30 μl 3 M $HClO_4$, and neutralized with 6 M KOH/2 M K_2CO_3. Xanthine and urate were determined by HPLC [19].

Calculation of Michaelis constants in homogenates

With xanthine as substrate, column effluent was incubated with 1–100 μM final concentration. For each concentration the incubations were stopped at 20, 30 and 40 min. Urate formation was measured with HPLC in deproteinized samples. K_m and V_{max} were calculated from Lineweaver-Burk plots [22].

In addition, incubations took place with 100 μM hypoxanthine and 100 μM NAD^+. Complete hypoxanthine degradation; formation and degradation of xanthine; and urate formation were followed by stopping the reaction at fixed time points (every 15 min for rat and every 30 min for guinea-pig), and measuring oxypurines by HPLC in deproteinized samples. Computations of the kinetic parameters K_m and V_{max} with hypoxanthine as substrate were performed by a program (Tutsim™, Palo Alto, CA, USA), in which Eqns. 1–3, described by Escribano et al. [23] were substituted. The purine data were fitted by non-linear regression coupled to numerical integration of the three equations. The K_m and V_{max} with xanthine were used as initial estimates, in addition to a K_i of 1.6 mM for urate [24]. V_{max} values are expressed in mU/g wet weight.

Statistics

Values are presented as mean ± SEM. Data were evaluated with Student's *t*-tests. $P < 0.05$ was considered a significant difference.

Results

Perfusion experiments

Changes in heart rate and apex displacement were minimal during the experiment. Coronary flows (ml/min per g wet weight) were 10.7 ± 1.0 (rat) and 10.9 ± 1.2 (guinea pig). In the course of the experiment, flow decreased 10% in both species. Rat-heart's basal release of hypoxanthine, xanthine and urate was 0.20 ± 0.02, 0.36 ± 0.04 and 3.42 ± 0.20 nmol/min per g, respectively. Guinea-pig hearts released 0.40 ± 0.01, 0.48 ± 0.04 and 3.29 ± 0.41 nmol/min per g, respectively.

Fig. 1 shows the xanthine and urate production during the perfusion with hypoxanthine or xanthine. The washout periods between the infusions were sufficient for the xanthine and urate release to return to baseline values. With hypoxanthine as substrate, urate production was of the same order of magnitude in both species: about 10 nmol/min per g. Under these conditions guinea-pig hearts released 10x less xanthine than rat hearts did. Urate production during xanthine infusion rose twofold in rat hearts, when compared to hypoxanthine infusion. Urate production from xanthine by guinea-pig hearts equalled that from hypoxanthine. The apparent xanthine oxidoreductase activity in perfused rat hearts was > 2 × higher than that in guinea-pig hearts, both with hypoxanthine ($P < 0.001$) and xanthine ($P < 0.05$) as substrate (Fig. 2).

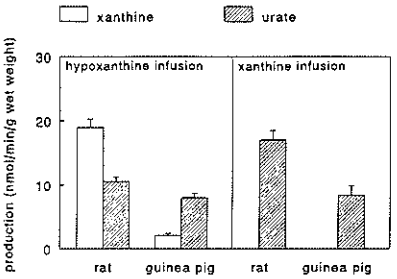

Fig. 1. Guinea-pig hearts, infused with hypoxanthine, released much less xanthine than rat hearts did. Depicted is the release of xanthine and urate in coronary effluent from isolated hearts, perfused with 50 μM hypoxanthine or xanthine. Oxypurines were assayed by HPLC and expressed as mean ± SEM ($n = 4–7$).

Intracellular hypoxanthine and xanthine content

Extracellular volumes in rat and guinea-pig hearts were 0.51 ± 0.01 and 0.60 ± 0.02 ml/g, respectively. Table I shows the extra- and intracellular oxypurine content of these hearts. From recovery studies with known amounts of hypoxanthine and xanthine added to a rat-heart homogenate, recoveries were found of about 99% and 87%, respectively. In both species calculation of intracellular xanthine content and concentration produced negative values; consequently the

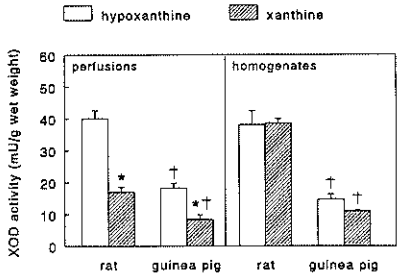

Fig. 2. In perfused hearts, xanthine proved to be inferior to hypoxanthine as a substrate. During ex vivo hypoxanthine perfusion, the apparent XOD activity was calculated as xanthine + 2 × urate production; with xanthine as substrate, XOD activity equalled urate production. V_{max} values during in vitro incubations were derived from a computer model (hypoxanthine) or Lineweaver-Burk plots (xanthine). Data expressed as mean ± SEM ($n = 4–7$). * $p < 0.005$ vs. hypoxanthine, † $p < 0.05$ vs. rat.

TABLE I

Extra- and intracellular content of hearts perfused with hypoxanthine or xanthine suggests transport barrier for xanthine

Hearts were perfused with 50 μM hypoxanthine ($n = 4$) or xanthine ($n = 3$). The perfusion buffer was supplemented with 5 μM [^{14}C]sorbitol to calculate the extracellular space. Oxypurines were determined by HPLC. We assumed that both substrates were homogeneously distributed over the extracellular space. This was true for hypoxanthine (see text). However, the negative values calculated for intracellular content (ICC) of xanthine demonstrated that this was not the case for this substrate; the resulting overestimation of the extracellular xanthine pool is consistent with a transport barrier. Data are expressed in nmol/g wet weight. Values are means ± SEM, ECC = extracellular content.

Species	Hypoxanthine		Xanthine	
	ECC	ICC	ECC	ICC
Rat	24.5 ± 1.4	10.4 ± 2.6	26.8 ± 1.5	−17.6 ± 1.8
Guinea pig	24.8 ± 3.5	13.2 ± 1.5	36.3 ± 0.3	−8.9 ± 2.6

initial assumption (see Materials and Methods) that xanthine was homogeneously distributed throughout the extracellular space overestimated its extracellular pool size. In contrast, the extracellular hypoxanthine concentration in rat hearts, 47.8 ± 0.5 μM, was of the same order of magnitude as its intracellular concentration, 28 ± 10 μM. Guinea-pig hearts displayed similar results, 50 ± 8 μM intracellular vs 60.1 ± 0.4 μM extracellular hypoxanthine.

Xanthine oxidoreductase assay with xanthine

The relationship between urate formed and incubation time was linear up to 40 min for each xanthine concentration used. Urate formation in the blank was negligible (< 0.02 nmol/ml per min). Degradation of xanthine did not take place in the presence of 100 μM allopurinol. Xanthine oxidoreductase activity in homogenates is presented in Fig. 2. Rat hearts had about 3-fold higher activity than guinea-pig hearts ($p < 0.001$). The oxidase form of XOD comprised < 20% in the hearts of both species studied (Table II). The K_m for xanthine, obtained from the Lineweaver-Burk plots, was about 5 μM in both species studied (Table III). These plots revealed the same K_m values for xanthine oxidase and XOD.

Xanthine oxidoreductase assay with hypoxanthine

The complete breakdown of 100 μM hypoxanthine in guinea-pig heart homogenates was similar to that in rat-heart homogenates, 4 and 11 h for rat and guinea-pig hearts, respectively. Because the same K_m values with xanthine were found for xanthine oxidase and XOD, and the incubation time for xanthine oxidase would become excessive, the measurements were restricted to XOD. No degradation took place if 100 μM allopurinol was present in the mixture. The initial

TABLE II

Xanthine oxidoreductase in rat and guinea-pig heart homogenates contain similar fractions of the oxidase form.

The activity of both forms of XOD was assayed with 100 μM xanthine as described in the Materials and Methods section. The data show that < 20% of XOD existed in the oxidase form in both species. Activities are expressed in mU/g wet weight. Values are means ± SEM, N = the number of experiments.

Species	N	Oxidase	Dehydrogenase
Rat	5	6.7 ± 0.3	31.9 ± 1.1
Guinea pig	5	1.1 ± 0.2	9.9 ± 0.2

formation rate of xanthine with hypoxanthine was identical to that of urate during experiments with xanthine. Xanthine accumulated during the hypoxanthine oxidation until it reached a concentration of approx. 50 μM; it disappeared subsequently (Fig. 3). Urate production increased exponentially.

The K_m and V_{max}, calculated from the curve of either hypoxanthine or xanthine or urate, varied only slightly. If the K_m and V_{max}, obtained with xanthine as substrate, were used as additional variables in the model, the calculated values were similar to the values found in the xanthine assay. The hypothetical curves for hypoxanthine breakdown, xanthine formation and breakdown, and urate formation (stippled lines, Fig. 3) showed a high concordance with the experimental data (Fig. 3). The K_m values were similar for both substrates and both species (Table III). The V_{max} values with hypoxanthine were about 3-fold higher in rat-heart homogenates than in guinea-pig homogenates ($p <$

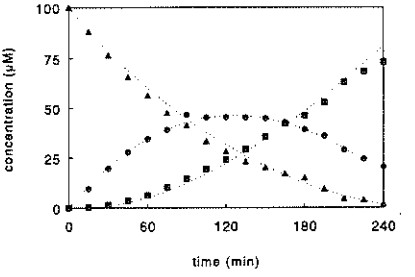

Fig. 3. High concordance between hypothetical curves and hypoxanthine breakdown in rat and guinea-pig heart homogenates. The hypoxanthine (▲), xanthine (●) and urate (■) concentrations measured during a typical incubation with rat heart homogenate are indicated. The stippled lines represent the fitted curves of the purine data by non-linear regression coupled to numerical integration of the equations, describing the hypoxanthine breakdown (see Materials and Methods). Estimated kinetic parameters were: K_m with xanthine = 4.54 μM, V_{max} with xanthine = 0.856 nmol/min, K_m with hypoxanthine = 2.81 μM, V_{max} with hypoxanthine = 0.760 nmol/min, and K_i (urate) = 1.60 mM.

0.002, Fig. 2). The V_{max} values with xanthine for both species were similar (Fig. 2).

Discussion

Incubation vs perfusion: differences between hypoxanthine and xanthine as substrate

In heart homogenates of both species, XOD activity was similar for hypoxanthine and xanthine (Fig. 2). The degradation curves for hypoxanthine were similar in both species. These data confirm the findings of Kaminski and Jezewska [25]. The increased urate production during perfusion with hypoxanthine and xanthine demonstrates that both substrates enter the endothelial compartment. However, there were distinct differences in estimated in situ XOD activities. The apparent activity of XOD in hearts perfused with hypoxanthine was similar to that in the homogenates in both species. In contrast, when xanthine was used as perfusion substrate, the estimated activities in situ were 2–3 × lower than those in the homogenates.

One possible explanation for these discrepancies could be the assumption that xanthine transport across the endothelial cell membrane is limiting xanthine degradation by XOD. Transport was clearly not a limiting factor for hypoxanthine, since the activities in perfusions and incubations were not different. Indeed, the removal of membrane barriers by homogenization lead to similar activities with hypoxanthine and xanthine.

Hypoxanthine transport across the cell membrane comprises facilitated diffusion into the cell [26] and probably also simple diffusion. Although the chemical structures of hypoxanthine and xanthine are very similar, it is not known whether xanthine can enter the cell via the hypoxanthine carrier [26] or whether cellular xanthine transport occurs only by simple diffusion.

Our observations support the notion that xanthine transport is impaired in intact tissue compared to that of hypoxanthine. Not only are the XOD activities in situ considerably lower with xanthine as substrate than those with hypoxanthine (Fig. 2), but there is also a marked disparity between the distribution of hypoxanthine and xanthine in perfused hearts. As expected, 50 μM hypoxanthine near-equilibrated across the cell membrane (Table I). Xanthine (50 μM), on the other hand, seemed to be essentially excluded from most of the intracellular space. We conclude this from the negative values found for intracellular xanthine concentration, assuming a homogeneous distribution of xanthine throughout the calculated extracellular space. Obviously, this assumption lead to an overestimation of the extracellular xanthine pool size.

From a physiological point of view, transport of hypoxanthine into the endothelial cell and across the endothelial cell membranes is beneficial for the myocardium, because hypoxanthine is a known substrate for salvage pathways. Xanthine, on the other the hand, is not a substrate for these pathways. Based on the lower in situ XOD activity found during xanthine perfusions, combined with the peculiar tissue distribution of xanthine, we hypothesize that in both species the mechanism of xanthine transport into the endothelial cell is smaller than that of hypoxanthine. Possibly, transport of xanthine occurs mainly by simple diffusion, whereas that of hypoxanthine is facilitated by a special carrier [26].

Tarantola et al. [27] reported a better ventricular function with xanthine than with hypoxanthine during reperfusion after an ischaemic period. Since cellular transport of xanthine appears small compared to that of hypoxanthine, native XOD would only operate at submaximal capacity with less free-radical production if xanthine is present in the perfusion medium. We conclude that hypoxanthine is preferable as substrate when the role XOD-derived free radicals or the effect of free-radical scavenging compounds is investigated.

As for cardiac patients, the potential for damage caused by xanthine oxidase-derived free radicals during ischaemia-reperfusion remains questionable because human myocardium is presumably almost devoid of XOD activity [10,16,28, but see Ref. 29]. Nevertheless, Abedah et al. [30] recently found an inactive form of human-milk xanthine oxidase which can produce superoxide through the FAD group. It is not known whether human myocardium contains a similar XOD isoform which could be responsible for radical damage.

Low xanthine release from perfused guinea-pig hearts

Our earlier suggestion [16] that guinea-pig hearts catabolize xanthine faster to urate than rat hearts requires revision. This can be concluded from the Michaelis constants, which were identical for hypoxanthine and xanthine (Table III). An impairment of xanthine release from the guinea-pig hearts is excluded because (1) the release of xanthine and urate during the washout period was similar in both species, and (2) the intracellular levels of xanthine during hypoxanthine

TABLE III

Comparable Michaelis-Menten constants of xanthine oxidoreductase in rat and guinea-pig heart homogenates, incubated with hypoxanthine (hx) or xanthine (x)

K_m values during incubations were derived from a computer model (hx) or Lineweaver-Burk plots (x). Values are means ± SEM, N = number of experiments.

Species	N	K_m hx (μM)	K_m x (μM)
Rat	5	3.0 ± 0.3	4.5 ± 0.4
Guinea pig	5	2.5 ± 0.7	4.8 ± 1.3

infusion were similar in both species. Thus, we are currently at loss to explain the observed difference in xanthine release between rat and guinea-pig hearts.

Limitations of the study

We are aware of the fact that a multitude of factors can influence the XOD activity, both in intact cells and homogenates. We believe that interference by endogenous purines during perfusions is negligible, as the infused concentration was 1000-fold higher than the intracellular hypoxanthine and xanthine concentrations measured before oxypurine perfusion (data not shown). In the perfused hearts, endogenous inhibitors, such as NADH, are of course still present; in homogenates they were removed. This would only account for different activities between perfused hearts and myocardial homogenates, but not between hypoxanthine and xanthine.

Concluding Remarks

Substrate-dependent differences exist in xanthine oxidoreductase activities between homogenates and perfused hearts, combined with the disparate distributions of hypoxanthine and xanthine, the latter being clearly excluded from most of the intracellular compartment and possibly subcompartmentalized in the extracellular space. We therefore propose that endothelial transport for hypoxanthine occurs readily compared to that of xanthine, in both rat and guinea-pig hearts. We conclude that (1) the relatively low release of xanthine in guinea-pig hearts is not the result of different kinetics of XOD towards both substrates, 2) hypoxanthine is preferable as substrate to estimate XOD activities in perfused hearts. The latter is important in studies where relationships between xanthine oxidoreductase and free radicals are investigated.

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Appendix 5

Myocardial xanthine oxidoreductase in hypertensive and hypercholesterolemic rats

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MYOCARDIAL XANTHINE OXIDOREDUCTASE ACTIVITY IN HYPERTENSIVE AND HYPERCHOLESTEROLEMIC RATS

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In several species, xanthine oxidoreductase activity seems to be a major source of free radicals in myocardial tissue. Its activity changes during development and aging, at least in the rat heart. Hardly any data are available about its activity in two important diseases, hypertension and hypercholesterolemia, in which the production of free radicals induced by xanthine oxidoreductase activity could play a role. Therefore we measured the activity of xanthine oxidase and dehydrogenase in myocardial tissue of spontaneously hypertensive, Wistar (control hypertensive), Yoshida (hypercholesterolemic) and Brown Norway (control hypercholesterolemic) rats of various ages. Cytosolic fractions were incubated at 30°C, pH 8.3, with 60 μM xanthine, and the formation of urate was measured with high performance liquid chromatography. In the Wistar group, xanthine oxidoreductase activity was relatively constant during aging (about 1.8 U/g protein). In the hypertensive group, the activity increased gradually from 1.7 to 2.3 U/g at 18 months ($p<0.05$ compared with Wistar at 18 months). Xanthine oxidase was about twice as high in both groups at 18 months ($p<0.001$ compared with 2 and 6 months). The ratio of xanthine dehydrogenase to xanthine oxidase had decreased 42% at this age ($p<0.001$). In the Yoshida and Brown Norway groups, xanthine oxidoreductase activity was similar, with a peak at 6 months. These data suggest that the hypercholesterolemic state does not influence xanthine oxidoreductase activity. In contrast, in hypertrophied myocardium, xanthine oxidoreductase activity was higher than in the control, suggesting a different potential for free-radical generation. The combined effect of aging and hypertrophy could make these hearts more susceptible to damage from ischemia and reperfusion, especially in the light of the decrease in detoxifying systems with age.

Key Words: hypercholesterolemia, hypertension, xanthine oxidoreductase

Cardioscience 1993; 4: 25-29

INTRODUCTION

Xanthine oxidoreductase catalyzes the breakdown of hypoxanthine to xanthine and xanthine to urate. In cardiac tissue it is mainly present in the NAD^+-dependent dehydrogenase form. Proteolysis[1] or oxidation of thiol groups[2] can induce conversion to the oxidase form, which uses molecular oxygen as electron acceptor and produces superoxide radicals. This conversion takes place during myocardial ischemia, followed by the generation of superoxide radicals during reperfusion[3]. The superoxide radical can be converted to hydrogen peroxide and the highly cytotoxic hydroxyl radical. Free radicals could play a role in the etiology of two important diseases, hypertension and hypercholesterolemia[4]. Hardly any data are available on cardiac xanthine oxidoreductase activity under these pathological conditions. Therefore, we measured the activity in spontaneously hypertensive and hypercholesterolemic rats at various stages of development.

MATERIALS AND METHODS

Inbred spontaneously hypertensive rats (SHR) were used, with genetically comparable Wistar Kyoto (WKY) rats as control. The latter develop no hypertension during life. In addition, the inbred Yoshida (hypercholesterolemic) strain and Brown Norway control rats (BN) were investigated. Three age groups (2, 6 and 18 months) in each strain were investigated, with 6 animals for each age. The

Table 1. *Age-dependent activity of xanthine oxidase and xanthine dehydrogenase in heart homogenates of hypertensive (SHR) and hypercholesterolemic (Yoshida) rats, and in their respective control strains (Wistar and Brown Norway). Activities in U/g protein, mean ± SEM (n = 6).*

A $p<0.001$ compared with 6 months; B $p<0.05$ compared with hypertensive rats 18 months; C $p<0.001$ compared with 2 and 6 months; D $p<0.003$ compared with Yoshida 2 months; E $p<0.001$ compared with 2 and 18 months; F $p<0.003$ compared with Yoshida 6 months; G $p<0.01$ compared with 6 and 18 months; H $p<0.05$ compared with 6 months.

WKY = Wistar Kyoto; SHR = spontaneously hypertensive; BN = Brown Norway.

Strain	Xanthine oxidase			Xanthine dehydrogenase		
	2 mo	6 mo	18 mo	2 mo	6 mo	18 mo
WKY	0.36±0.06	0.31±0.01	0.49±0.03^{AB}	1.51±0.08	1.36±0.10	1.33±0.10^{B}
SHR	0.36±0.04	0.33±0.01	0.64±0.05^{C}	1.36±0.11	1.47±0.04	1.66±0.10
BN	0.42±0.03^{D}	0.73±0.05^{EF}	0.37±0.02	1.84±0.04^{D}	1.96±0.13	1.91±0.06
Yoshida	0.31±0.01^{G}	0.46±0.03	0.41±0.03	1.43±0.09^{G}	2.19±0.16	1.85±0.041^{H}

animals were anesthetized intraperitoneally with pentobarbital, according to institutional guidelines. Hearts were removed, cooled in and washed with ice-cold saline. They were stored in liquid nitrogen.

After the hearts had been thawed, 5% homogenates were made in 50 mM K_2HPO_4, 1 mM EDTA.Na_2 (pH 8.3) with a Potter-Elvejhem homogenizer. The buffer contained 1 mM dithiothreitol[2] to prevent oxidative conversion of xanthine dehydrogenase to the oxidase form, and 1 mM phenylmethylsulfonyl fluoride[5] to prevent proteolysis. The homogenate was centrifuged at 25000 g for 10 minutes at 4°C, and the supernatant fluid immediately used. The latter was passed through a Sephadex G-25 column (PD-10, Pharmacia, Uppsala, Sweden), prewashed with the homogenization/incubation buffer, to remove low molecular weight compounds including endogenous inhibitors[6].

Xanthine oxidoreductase activity was measured according to Terada *et al*[5]. The column effluent was incubated at 30°C, pH 8.3, with 60 μM xanthine. In addition, a blank (without xanthine) and a negative control (100 μM allopurinol + xanthine) were measured. There was a direct correlation between the amount of urate produced at a fixed time point and different amounts of sample. This relation was linear between 0.1 and 0.6 ml of sample (end volume 1.0 ml). The routine sample volume was 0.2 ml, containing about 0.14 mg protein. To measure the amount of dehydrogenase and oxidase forms, the column effluent was incubated with or without NAD^+ (using ambient oxygen). For each homogenate, the incubation was stopped at 20, 30 and 40 minutes with 3 M $HClO_4$. Samples were neutralized with 6 M KOH, 2 M K_2CO_3. The concentration of urate was measured by high-performance liquid chromatography as described by Smolenski *et al*[7]. Protein was assayed with Coomassie Brilliant Blue (Bio-Rad Laboratories, Munich, Germany) according to Bradford[8], using bovine serum albumin as the standard. Values are presented as mean ± SEM. Data were evaluated with analysis of variance, $p<0.05$ being considered a significant difference.

RESULTS

General

In the assay, the relation between urate production and time was linear up to 40 minutes. Baseline formation of urate was negligible (<0.02 nmol/min). Degradation of xanthine did not take place in the presence of 100 μM allopurinol.

Hypertensive group

Figure 1A presents the xanthine oxidoreductase activity (XOD) measured in the hearts of hypertensive animals and those from a control strain. The activity in the hypertensive and Wistar groups did not differ significantly at 2 months. In the control Wistar rats, xanthine oxidoreductase activity remained relatively constant during aging; in the hypertensive group it gradually increased during life. In the hypertensive group, xanthine oxidoreductase activity correlated linearly with age ($r = 0.993$). At 18 months, the activity of both xanthine oxidase and dehydrogenase was 24% higher in the hypertensive group than in the control group (Table 1). The contribution of xanthine oxidase activity to total xanthine oxidoreductase activity changed from 18% to 27% in both groups at the age of 18 months ($p<0.001$ compared with 2 and 6 months). Cardiac protein content increased about 18% in both the hypertensive and the control group at 18 months ($p<0.005$ compared with 2 and 6 months).

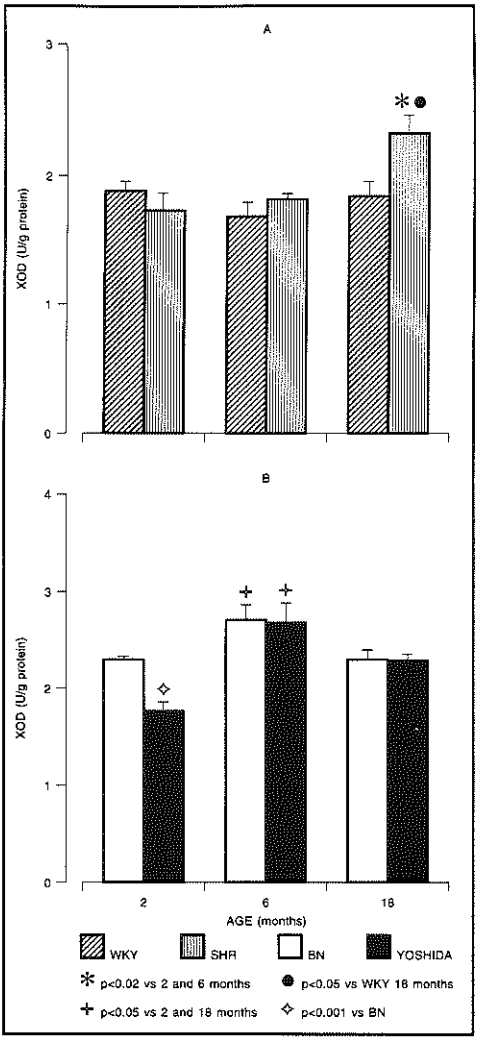

Figure 1. *Panel A. Myocardial xanthine oxidoreductase activity (XOD) in hypertensive (SHR) and control (WKY) rats at different ages.*

Panel B: Myocardial xanthine oxidoreductase activity in hypercholesterolemic (Yoshida) and control (BN) rats at different ages.

Data expressed as mean ± SEM, n = 6 for each group.

Hypercholesterolemic group

The xanthine oxidoreductase activity measured is depicted in Figure 1B. Apart from the values at 2 months, xanthine oxidoreductase activity was similar in the Yoshida and Brown Norway groups. In both strains xanthine oxidoreductase activity was significantly higher at 6 months. Xanthine dehydrogenase and oxidase activities in 2 months old Yoshida rats were lower than those at 6 and 18 months (Table 1). In the hearts of those animals, the ratio of xanthine dehydrogenase to xanthine oxidase remained constant. At 6 months the Brown Norway group displayed about twice as much xanthine oxidase activity (Table 1). The ratio of xanthine dehydrogenase to xanthine oxidase at 2 and 18 months in the Brown Norway rats was identical to the ratio found in the Yoshida group of the same age. Cardiac protein content was similar in both groups and at various ages.

DISCUSSION

General

In several species, cardiac xanthine oxidoreductase seems to be an important source of free radicals during ischemia and reperfusion[9]. The activity changes during development and aging[10]. The formation of free radicals is counteracted by several enzymes. Superoxide dismutase converts the superoxide radical to hydrogen peroxide. The latter can be detoxified by catalase or glutathione peroxidase. The activity of these enzymes decreases during life[4,11,12]. Although hypertrophied myocardium seems to have a higher antioxidant status[4,13] the balance between producers and scavengers of free radicals may be disturbed at old age.

Hypertensive group

We found relatively constant levels of cardiac xanthine oxidoreductase activity in the control Wistar group throughout life. Our data suggest that the hypertrophied myocardium differs from control in its potential to generate free radicals, with an increase during life. This could explain the higher production of xanthine and uric-acid in hypertensive rats at the age of 18 months, in addition to the higher vulnerability of hypertrophied myocardium towards injury from ischemia and reperfusion[14,15]. There was an increase not only in the total activity but also in the percentage of xanthine oxidase present under aerobic conditions. We observed this also in the control group. We speculate that a lower activity of the glutathione system at a higher age plays a role. Oxidation of thiol groups can induce conversion of xanthine dehydrogenase to the oxidase form[2]; the glutathione

system stabilizes these thiol compounds[16]. The question remains if the higher xanthine oxidoreductase activity is a cause or a consequence of this pathological condition. Recently Terada *et al* reported that hypoxia increases endogenous xanthine oxidoreductase activity in pulmonary endothelial cells[17]. Nakazono *et al* described the relation between superoxide radicals and the pathogenesis of the hypertension of spontaneously hypertensive rats; however, they failed to observe higher xanthine oxidoreductase activities in aortic endothelium, despite the increased release of uric acid[15]. Whether this mechanism also applies to myocardial tissue has to be elucidated further.

Hypercholesterolemic group

Hypercholesterolemic rats did not show the gradual increase in activity observed in the hypertensive animals. We found the same pattern of activity in the Yoshida and the Brown Norway groups, with a peak at 6 months. Perhaps old age, combined with hypercholesterolemia, induces enzymic self-inactivation[18]. The hypercholesterolemic state does not seem to be associated with an increased potential for generating free radicals.

Speculation

Various studies on human myocardial tissue have failed to detect xanthine oxidoreductase activity[19,20,21,22] but other investigations showed low[23,24] or high[25,26] activity. Abadeh *et al*[27] suggested that xanthine oxidoreductase in human myocardium is present in the inactive desulfo-form, which can still produce free radicals. This raises the question whether patients with myocardial hypertrophy could have higher levels of the desulfo-form of xanthine oxidoreductase, making their hearts more susceptible to ischemic injury. This requires further investigation.

Limitations of the study

We expressed our data on a protein basis. Xanthine oxidoreductase activities normalized in that way did not differ appreciably from activities expressed per gram wet tissue. Because we observed the increase in protein content during aging in both the Wistar and hypertensive groups, we are confident that other ways of normalization (based on DNA-content, cell number, or marker enzymes) would not change the overall picture. We believe that the increase in activity in the hypertensive group is not caused by a change in K_m; in pilot experiments we found the K_m to be independent of age, i.e. about 4 μM. Therefore we measured the xanthine oxidoreductase activity at one, saturating, concentration.

Conclusion

Our data from rat hearts indicate that the hypercholesterolemic state has no influence on xanthine oxidoreductase activity. In contrast, the activity in hypertrophied myocardium was higher than control, suggesting a different potential for the generation of free radicals. The combined effect of aging and hypertrophy could make these hearts more susceptible to ischemia-reperfusion damage by xanthine oxidase, especially in the light of the age-dependent changes in detoxifying systems.

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Appendix 6

Formation and breakdown of uridine in ischemic hearts of rats and humans

R.T. Smoleński, J.W. de Jong, M. Janssen, D.R. Lachno, M.M. Żydowo, M. Tavenier, T. Huizer and M.H. Yacoub

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Formation and Breakdown of Uridine in Ischemic Hearts of Rats and Humans

Ryszard T. Smoleński[1], Jan W. de Jong[2], Maarten Janssen[2], David R. Lachno[3], Mariusz M. Żydowo[1], Monique Tavenier[2], Tom Huizer[2] and Magdi H. Yacoub[3]

[1]*Department of Biochemistry, Academic Medical School, Gdańsk, Poland;* [2]*Cardiochemical Laboratory, Thoraxcenter, Erasmus University Rotterdam, Rotterdam, The Netherlands;* [3]*Thoracic and Cardiac Surgical Unit, Harefield Hospital, Harefield, UK*

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R. T. Smoleński, J. W. de Jong, M. Janssen, D. R. Lachno, M. M. Żydowo, M. Tavenier, T. Huizer, and M. H. Yacoub. Formation and Breakdown of Uridine in Ischemic Hearts of Rats and Humans. *Journal of Molecular and Cellular Cardiology* (1993) **25**, 67–74. In contrast to cardiac purine metabolism, little is known about pyrimidine catabolism in heart. We therefore investigated uridine and uracil formation in ischemic rat and human hearts. Human donor hearts accumulated uridine 3× ($P<0.05$) before implantation. Hearts released this pyrimidine during implantation or correction of cardiac defects. During the former systemic blood uridine rose 38% ($P<0.05$). In explanted human hearts, uridine was the only pyrimidine released during reperfusion; isolated, perfused rat hearts produced initially 3× more uracil than uridine. Uridine phosphorylase activity in human heart homogenate was 3.4 mU/g wet weight, i.e. 60× lower than that in rat myocardium (198 mU/g, $P<0.02$); its purine counterpart, nucleoside phosphorylase, differed much less in activity (0.32 and 1.12 U/g, respectively; $P<0.001$). Thus human heart is virtually devoid of uridine phosphorylase, contrasting rat heart. Consequently uridine accumulates in ischemic human heart while uracil production predominates in rat heart.

Key Words: Cardiac surgery; Cardioplegia; Myocardial ischemia; Perfused human heart; Purine nucleoside phosphorylase; Pyrimidines; Uracil; Uridine phosphorylase.

Introduction

End-products of nucleotide metabolism include purines and pyrimidines. Our knowledge of cardiac pyrimidine breakdown is limited but catabolism of pyrimidine nucleotides in ischemic myocardium seems to parallel degradation of adenylates (Swain *et al.*, 1982). More information about pyrimidine nucleotide breakdown is useful because of (i) the specific role of pyrimidine derivatives in lipid and carbohydrate metabolism (Lortet *et al.*, 1987); (ii) the interconversions of anticancer drugs by pyrimidine metabolizing enzymes (Lin and Williams, 1988; Peters *et al.*, 1986, Schwartz *et al.*, 1985; Woodman *et al.*, 1980); (iii) the potential diagnostic value of pyrimidine catabolites.

Our report focusses on the cardiac formation and release of uridine, which turned out to be the major pyrimidine nucleotide catabolite in ischemic human but not rat heart. Uridine phosphorylase proved to be virtually absent in the human heart, in contrast to its purine counterpart, purine nucleoside phosphorylase.

Materials and Methods

Uridine concentration in human heart, blood and cardioplegic effluent

Human myocardial tissue was collected during heart surgery with the approval of Ethical Committees at the Academic Medical School in Gdańsk and at Harefield Hospital. For nucleotide catabolite determination, blood-free coronary effluent was collected during corrections of congenital or acquired heart

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defects at the time of subsequent infusions of cardioplegic solution. For details of sample collection and processing, see Smoleński *et al.* (1989). Also for nucleotide catabolite assay, biopsy specimens, coronary sinus and arterial blood were collected during heart or heart-lung transplantation (see protocol in Fig. 1). Biopsies were processed as described previously (Smoleński *et al.*, 1990). Blood sample preparation before analysis was similar to that described for coronary effluent with some modifications (Smoleński *et al.*, 1989). Briefly, immediately after collection 0.8 ml of coronary sinus or arterial blood was put into Eppendorf tubes containing 0.8 ml of cold 1.3 M $HClO_4$. After vigorous mixing, the tubes were frozen in liquid nitrogen. At the time of analysis, they were allowed to thaw; after centrifugation the supernatant fluids were neutralized with 2 M KOH and analyzed for uridine by HPLC (vide infra). Reproducibility of this procedure, evaluated by analysis of five samples from a blood pool, gave coefficients of variation of 11.9% for coronary effluent collected 1 min after reperfusion (mean = 4.5 μM) and 5.9% for arterial blood (mean = 1.6 μM). The uridine concentration in coronary sinus blood remained stable if samples had been mixed with $HClO_4$ within 2.5 min. Simultaneously significant changes of purine metabolities were observed (data not shown).

Uridine release from isolated, perfused human heart

Explanted hearts of patients with end-stage heart failure due to ischemic heart disease or dilated cardiomyopathy were used. Hearts were arrested *in situ* with St. Thomas' Hospital cardioplegic solution and transported in ice-cold saline to the labortory. They were perfused in the Langendorff mode with a roller pump (Verder, Vleuten, The Netherlands), externally controlled by the perfusion pressure (100 mmHg). To minimize pump pulsations, a Wind Kessel that also functioned as fluid reservoir and heat exchanger (37°C) was used. The perfusion fluid consisted of a modified Tyrode buffer (De Jong *et al.*, 1990b). It was oxygenated with 95% O_2/5% CO_2, using a baby oxygenator (Polystan, Vaerløse, Denmark). Blood was washed from the hearts for 10 min, then recirculation started with 1 l perfusion fluid. After a stabilization period of 30 min, hearts were subjected to 20 min of global normothermic ischemia, and arterial and venous effluent samples were collected throughout reperfusion. Uridine was measured using HPLC as described previously (Smoleński *et al.*, 1990).

Uridine and uracil production in isolated, perfused rat heart

Wistar albino rats weighing 250–300 g were anesthetized intraperitoneally with 60 mg/kg pentobarbital (Sanofi, Paris, France) in accordance with the "Guiding Principles for Research Involving Animals and Human Beings" (American Physiological Society). Hearts were removed and arrested in ice-cold saline. Perfusion fluid consisted of a modified Tyrode buffer (Huizer *et al.*, 1987), oxygenated with 95% O_2/5% CO_2. Hearts were initially perfused according to Langendorff at 72 mmHg and 37°C. After a 20-min equilibration period, the system was switched to the working mode. Then St. Thomas' Hospital cardioplegic solution was administered (3 min at 63 mmHg) and hearts were subjected to 30 min of global ischemia. Coronary effluent for pyrimidine catabolite determination

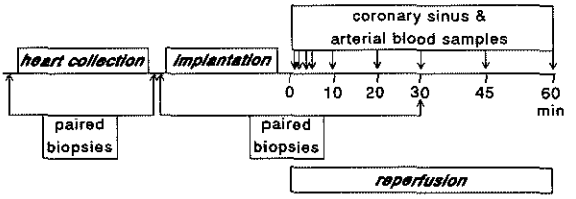

FIGURE 1. Study protocol during heart transplantation. Arterial blood was collected from the arterial line during extracorporeal circulation.

(Smoleński *et al.*, 1990) was collected during three subsequent 5-min periods during reperfusion in the Langendorff mode.

Analysis of metabolite concentration using HPLC

Uridine concentration was evaluated with reverse phase HPLC in perchloric acid extracts of tissue, coronary sinus and arterial blood (Smoleński *et al.*, 1990). Peak identity was confirmed with a diode array detector. The uracil formed had a retention time of about 3 min, identical to that of standard uracil. Chromatographic procedures applied for the analysis of coronary efflux of cardioplegic solution, collected during corrections of heart defects, have been described previously (Smoleński *et al.*, 1989).

Determination of uridine phosphorylase and purine nucleoside phosphorylase activity

For the determination of uridine phosphorylase (EC 2.4.2.3), a sample of human left ventricular free wall from explanted diseased or normal hearts, human papillary muscle, or rat ventricular myocardium was homogenized in 9 volumes/g tissue of buffer consisting of 50 mM Na-P_i (pH 6.9), 1 mM EDTA and 0.1 mM dithiothreitol, using a Potter-Elvehjem type homogenizer. Before incubation, homogenates were diluted three times with P_i-buffer. Incubation was started by adding 0.1 ml of diluted homogenate to 0.1 ml of P_i-buffer containing 2 mM uridine. Incubations carried out at 37°C were routinely terminated after 15 min by adding 0.2 ml of 8% $HClO_4$. After centrifugation the supernatant fluid was neutralized with 2 M KOH/1M K_2CO_3 and the conversion of uridine to uracil measured by HPLC (Smoleński *et al.*, 1990).

The same homogenization and incubation procedure was followed to determine purine nucleoside phosphorylase (EC 2.4.2.1), with the exception of a 10-fold dilution of homogenates and the replacement of uridine by inosine.

For both enzymes the reaction was linear with time for at least 30 min and with amounts of enzyme up to three times those routinely used.

Statistics

Values are presented as the mean ± S.E. Data were evaluated (Glantz, 1981) with the Wilcoxon signed rank test (Figs 2 and 3, Fig. 4 – third *vs.* second cardioplegic infusion), the Mann-Whitney *U*-test (Fig. 4 – second and third *vs.* first cardioplegic infusion), and Student's *t* test (Figs 5–7). $P<0.05$ was considered a significant difference.

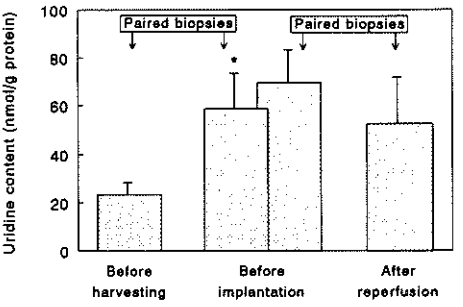

FIGURE 2. Uridine content in human heart increased during transplantation. Biopsy specimens were collected from the apex of the hearts and analyzed by HPLC after extraction. Values represent the mean ± S.E. of paired samples collected before harvesting the hearts and before implantation ($n=7$), or before implantation and after 30 min of reperfusion ($n=6$). *$P<0.05$ *vs.* before harvesting.

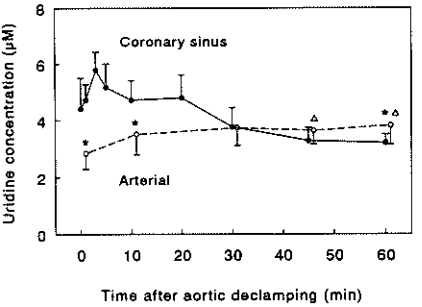

FIGURE 3. Implanted human hearts released uridine during reperfusion. Deproteinized whole blood samples, collected at the times indicated, were analyzed by HPLC. Values represent the mean ± S.E. ($n=6$). *$P<0.05$ *vs.* coronary sinus concentration, △$P<0.05$ *vs.* first arterial sample after declamping.

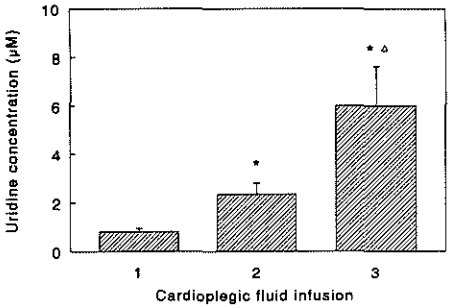

FIGURE 4. Uridine concentration increased in blood-free coronary sinus effluent, collected during corrections of congenital or acquired heart defects at the time of cardioplegia. The first amount of cardioplegic fluid was administered just after clamping of the aorta, the second after 30–50 min of ischemia, and the third after 60–80 min of ischemia. Samples were analyzed by HPLC after acid extraction. Values represent the mean ± S.E. Sample size for the first infusion: $n=5$, for the second and third infusions: $n=7$. $^{*}P<0.05$ *vs.* first infusion. $^{\Delta}P<0.05$ *vs.* second infusion.

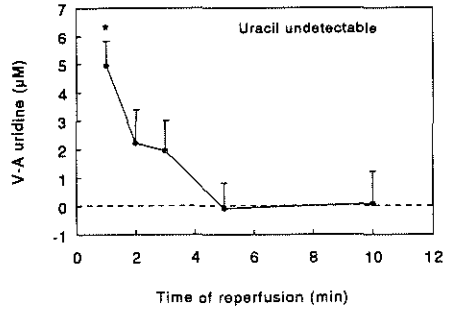

FIGURE 5. Perfused, explanted human hearts released uridine, not uracil. The arteriovenous difference in uridine concentration was determined during reperfusion of explanted hearts subjected to 20 min of global normothermic ischemia. HPLC showed that the uracil concentration was <0.1 μM, both in venous and arterial samples. Values represent the mean ± S.E. ($n=6$). $^{*}P<0.05$: significant uridine release. V = venous, A = arterial.

Results

Uridine concentration in heart, coronary effluent and arterial blood during heart surgery

Figure 2 gives the uridine content in human donor myocardium during transplantation. We found a 3-fold increase ($P<0.05$) in uridine content during cold storage of the heart (2–4 h); the partial reversal was not statistically significant. In a few hearts, we noted that the breakdown product of uridine, uracil, was undetectable.

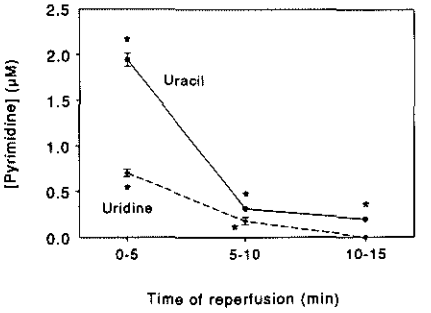

FIGURE 6. Uracil and uridine concentration in coronary effluent of rat hearts after 30 min of global normothermic ischemia. Samples of coronary effluent were collected over three subsequent 5-min periods of reperfusion, and analyzed by HPLC. Values represent the mean ± S.E. ($n=6$). $^{*}P<0.05$: significant pyrimidine release.

Figure 3 shows the uridine concentration in the coronary sinus and arterial blood during reperfusion of the human heart after implantation. Reperfused human myocardium released significant amounts of uridine for at least 10 min. Uridine release became uptake from blood after 60 min of reperfusion. Arterial blood uridine rose gradually by 38% ($P<0.05$) after aortic declamping. Interestingly, the majority of this increase took place in the initial phase of reperfusion, when release of uridine from the heart was greatest.

Washout of uridine formed in the human heart during corrections of congenital or acquired heart defects was also observed (Fig. 4). The concentration in the coronary effluent increased from 0.8 μM after the first infusion of cardioplegic solution to 6 μM after the third ($P<0.05$), indicating a continuous degradation of uridine nucleotides in the ischemic myocardium.

Release of uridine and uracil from perfused human and rat hearts

Figure 5 shows the release of uridine during reperfusion of explanted human hearts after 20 min of global ischemia. A peak value of 5 μM was demonstrated in the early reperfu-

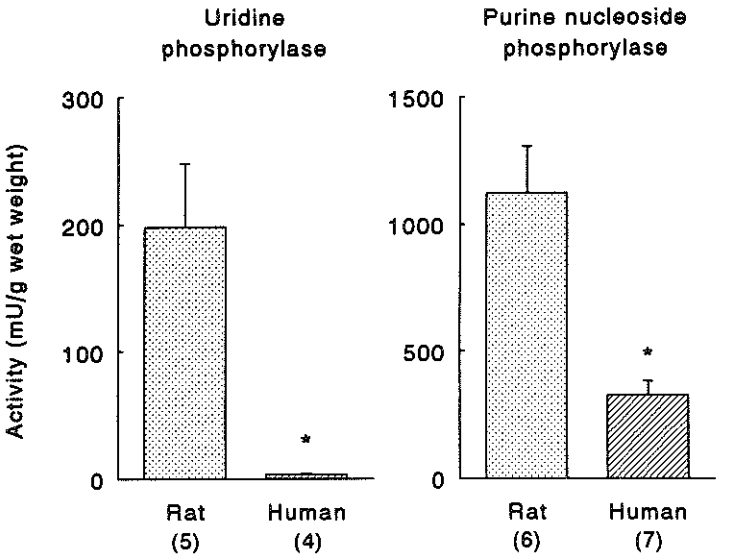

FIGURE 7. Activity of uridine and purine nucleoside phosphorylases in human and rat myocardial tissue. Incubations were carried out at 37°C for 15 min in phosphate buffer (pH 6.9) containing 1 mM substrate, and stopped with acid. Conversion of uridine to uracil, or inosine to hypoxanthine, was followed by HPLC. Values represent the mean ± S.E. In brackets: number of experiments. $*P<0.02$ *vs.* rat heart.

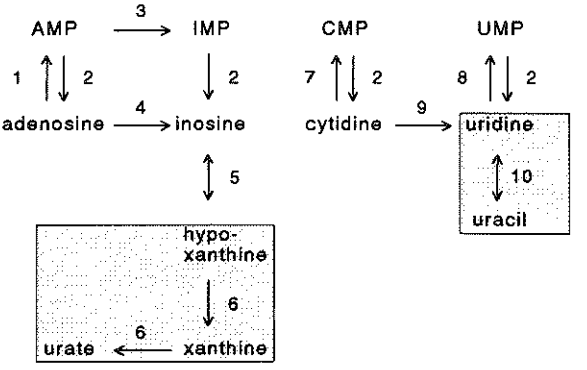

FIGURE 8. Scheme depicting mammalian metabolism of inosine and uridine. (1) Adenosine kinase; (2) 5'-Nucleotidase; (3) AMP deaminase; (4) Adenosine deaminase; (5) Purine nucleoside phosphorylase; (6) Xanthine oxidoreductase; (7) Cytidine kinase; (8) Uridine kinase; (9) Cytidine deaminase; (10) Uridine phosphorylase. Shaded areas indicate pathways virtually absent in human heart, but present in rat heart.

sion period. In contrast uracil production was nil (detection limit 0.1 μM). Figure 6 presents the concentration of uracil and uridine in the effluent of reperfused rat heart. Uracil was the predominant pyrimidine catabolite (peak value 2 μM). In the first 10 min of reperfusion, the uracil concentration was 2–3 fold higher than that of uridine.

Activities of uridine phosphorylase and purine nucleoside phosphorylase in human and rat hearts

Figure 7 depicts the activities of uridine phosphorylase and purine nucleoside phosphorylase. The former was about 60 times less active in human myocardium than in rat heart ($P<0.02$). For comparative reasons purine

nucleoside phosphorylase was also assayed. The enzyme proved to be substantially more active in both species than uridine phosphorylase; its activity in human heart was only threefold less than that in rat heart ($P<0.001$).

Discussion

Low uridine phosphorylase in human heart

The major finding of this study is the demonstration that ischemic human heart hardly catabolizes uridine. We conclude this from observations in the clinical setting, experiments with explanted, perfused hearts subjected to ischemia and reperfusion, and direct enzymatic evaluation of uridine catabolism in myocardial homogenates. Uridine accumulated in donor hearts during cold storage for implantation, as well as in hearts undergoing correction of congenital or acquired defects. The explanted, perfused heart released uridine, but not uracil. Human heart fails to catabolize uridine, because it virtually lacks phosphorylase activity. Since the activity was also minimal in samples from donor human heart and from hearts with valvular defects, it is unlikely that the pathological state of the explanted human hearts affected the uridine phosphorylase activity. The myocardial uridine content was high, even after 30 min of reperfusion, possibly due to the elevated uridine blood concentration and/or due to further degradation of myocardial pyrimidine nucleotides during reperfusion. Literature data on uridine concentration in human plasma (Harkness, 1988: 3.0–4.5 μM) are comparable to our results, suggesting that the concentration in plasma and whole blood is similar.

In contrast to human heart, rat heart does catabolize uridine to uracil (cf. Deuticke and Gerlach, 1966). In effluent of ischemic rat heart, the uracil concentration exceeded several-fold the uridine concentration. We subsequently could demonstrate substantial uridine phosphorylase activity in rat heart homogenate.

In a comparative study, we showed that both human and rat heart possess relatively high purine nucleoside phosphorylase activity. This finding – novel for human heart – explains the significant production of hypoxanthine by the heart of both species (Harmsen *et al.*, 1981; Huizer *et al.*, 1987). No literature data are available on the activity of purine nucleoside phosphorylase in human heart; a limited number of samples from donor hearts, analyzed in our laboratory, revealed an activity similar to that in diseased hearts. The next step, i.e., conversion to xanthine and urate, is virtually absent in human heart (De Jong *et al.*, 1990b; see Fig. 8).

Physiological implications

Uridine production may be of physiological significance bearing in mind the cardiovascular roles of another nucleoside – adenosine (Newby *et al.*, 1984). Uridine has a positive inotropic action in rabbit myocardium; it increases glucose uptake and glycogen content in the myocardial cell (Kypson and Hait, 1977). Claims that uridine corrects contractility disturbances in the non-ischemic portion of infarcted rat heart (Meerson and Dosmagambetova, 1985) await confirmation.

In rat heart cytidine, but not uridine, plays a prominent role in the maintenance of the pyrimidine nucleotide pool (Lortet *et al.*, 1986, 1987; Olivares and Rossi, 1988). However, the low activity of uridine phosphorylase in human myocardium may favor uridine as the substrate for pyrimidine nucleotide synthesis. The high activity of uridine phosphorylase in rat glomerulus could be a major factor preventing uridine incorporation into uracil nucleotides, as application of a uridine phosphorylase inhibitor markedly increases uridine incorporation (Cortes *et al.*, 1988). This shows that principles of cardiac pyrimidine metabolism derived from animal experiments could be irrelevant for man.

Clinical significance

Drug therapy

Uridine phosphorylase is the key enzyme either generating or catabolizing active metabolites of some pyrimidine analogs used in anticancer therapy (Lin and Williams, 1988; Peters *et al.*, 1986; Schwartz *et al.*, 1985; Woodman *et al.*, 1980). The prominent difference between the activity in human and rat heart makes it difficult to predict cardiotox-

icity of these drugs. In the heart of several species, uridine phosphorylase activity is present in the endothelial cells (Rubio and Berne, 1980). This strategic localization can lead to a complete change in pattern of pyrimidine metabolites reaching myocardial cells from the blood.

Diagnosis

Uridine determination may be important where one evaluates the cardioprotective action of purine compounds, and where determination of purine catabolites has limited value (De Jong *et al.*, 1990a; Harkness, 1988). It is also important that uridine metabolism in human blood is very slow (Tseng *et al.*, 1971), which contrasts purine catabolism (Harmsen *et al.*, 1981; Moser *et al.*, 1989). Coronary sinus uridine has potential value as indicator of myocardial ischemia.

Conclusions

We conclude that human myocardium shows very little activity of uridine phosphorylase. Consequently, during ischemia and reperfusion, the heart accumulates and releases uridine. The active uridine phosphorylase in rat heart explains why uracil, and not uridine, is the major pyrimidine released in that species.

Acknowledgements

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Appendix 7

Changes in phosphorylation potential and purine release during recurrent myocardial ischaemia

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Submitted for publication

Changes in phosphorylation potential and purine release during recurrent myocardial ischaemia

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Key words: Adenosine, Adenylate charge, AMP, Energetic index, HPLC, NMR, Phosphate, Phosphocreatine, Preconditioning, Reperfusion

Abstract

Objective: We investigated why purine release declines in rodent hearts after repetitive short ischaemic periods, such as those used during preconditioning and coronary angioplasty. **Methods:** We monitored cardiac high-energy phosphates by ^{31}P-NMR and effluent ATP catabolites by HPLC. More specifically, we investigated the possible relationships between phosphorylation potential ([ATP]/([ADP]•[P_i]) and purine release. The recurrent ischaemia protocol was 6 x (2 min ischaemia + 3 min reperfusion) in 22 Langendorff rat hearts. **Results:** Phosphocreatine, baseline 7.6±0.7 mM (mean±SEM), fell to 40-50% during every ischaemic period. During reperfusion, recovery was 70-80%. P_i, 3.2±0.3 mM, rose by 59% during the first ischaemic period, with a return to baseline levels during reperfusion; this increase was only 30% during the 6th occlusion ($p<0.05$ *vs* 1st ischaemia). ATP, 4.0±0.4 mM, and pH, 7.04±0.02, remained unaffected. The phosphorylation potential after 6 short cycles of ischaemia and reperfusion exceeded that observed during normoxia by 31% ($p<0.05$), demonstrating that the proportion of high-energy phosphate in the nucleotide pool had improved relative to non-ischaemic controls. The phosphorylation potential correlated (r=0.97) positively with adenylate charge, and negatively with free [AMP]. Purine release correlated ($r\geq0.90$) negatively with phosphorylation potential, and positively with [P_i]. Baseline release of adenosine and total purine were 0.39±0.15 and 9.2±1.0 nmol min^{-1} g^{-1} wet weight, respectively. Early during the first reperfusion, this efflux rose to 2.9±0.3 and 61±7 nmol min^{-1} g^{-1}, respectively. The release declined gradually. For adenosine it reached baseline levels early during the 6th reperfusion: 0.61±0.11 nmol min^{-1} g^{-1} ($p<0.001$ *vs* 1st reperfusion). Total purine release dropped to 40(6) nmol min^{-1} g^{-1} ($p<0.05$). Phosphorylation potential correlated in a negative exponential way with total purine release (r=0.90), and with adenosine + inosine release (r=0.81). **Conclusion:** Our data strongly suggest that adenosine plays a role in the energy supply-demand balance during repeated ischaemia (*cf.* preconditioning). They support the notion that the phosphorylation potential regulates AMP catabolism to adenosine. (*Cardiovascular Research* 0000;**00**:000-000)

Introduction

One or more brief episodes of myocardial ischaemia activate adaptive changes which quickly lead to increased myocardial tolerance to a later ischaemic insult.[1] This "preconditioning" markedly limits the size of the myocardial infarct resulting from ischaemia up to 1 h followed by reperfusion. The phenomenon was first observed in dogs,[2,3] and has been confirmed in pigs, rabbits and rats.[4,5,6] Recent studies by our group show that preconditioning effects are elicited in the rat by four periods of 2 min of ischaemia separated by 3 min of reperfusion.[7] The mechanism of the protective effect is still a matter of discussion.[8,9] Several authors have proposed that intermittent reperfusion allows an efficient washout of many potentially harmful metabolites,[2,10] which would otherwise accumulate in the tissue.

The protective effect has been associated, either causal or consequential, with a marked decrease in energy

utilization. Murry *et al.*[3] have suggested that preconditioning reduces myocardial energy demand during ischaemia, and leads to a reduction in the use of high-energy phosphates. The preservation of ATP and/or the limitation of ATP-catabolite accumulation may be responsible for the slower rate of ischaemic cell death.

Recent work indicates that the adenosine released due to a preconditioning occlusion might mediate the beneficial response through stimulation of cardiac A_1 receptors.[11] Adenosine limits myocardial ischaemia-reperfusion injury through vasodilation, adaptation of cardiac adrenergic responses, inhibition of neutrophil function, and modulation of cardiac energy supply-demand.[12 13 14] Whether adenosine can precondition rat hearts is controversial.[6 15 16 17 18]

Also the results in humans are conflicting. Studying patients undergoing percutaneous transluminal coronary angioplasty, Deutsch *et al.*[19] interpreted changes detected, *a.o.*, in lactate metabolism (after two sequential 1.5-min periods of ischaemia, separated by 5 min of reperfusion) as evidence of adaptation to myocardial ischaemia. Using a similar protocol, we found no significant variation in lactate and purine release after four sequential periods of ischaemia.[20] Other groups also failed to find biochemical evidence for preconditioning during coronary angioplasty.[21 22]

We showed recently that recurrent *anoxia* in rat heart leads to a decrease in adenosine production.[23] In the present study, we investigated the effect of repetitive *ischaemia* on cardiac high-energy phosphate metabolism in relation to purine release from the heart, thus extending the early observations in canine hearts.[24 25 26] Our ^{31}P-NMR spectroscopic approach allowed the non-invasive determination of changes in cytosolic metabolism with a time resolution of 120 s. The results, which have been partly published in abstract form,[27 28] indicate that the phosphorylation potential regulates adenosine release during recurrent ischaemia.

Materials and methods

Heart perfusion

This investigation followed the principles expressed in "Position of the American Heart Association on Research Animal Use" [Circulation (1985) 71; 849]. Male Sprague-Dawley rats of 150-175 g body weight were stunned and bled. The heart was rapidly removed and cooled in saline. Retrograde, not recirculating perfusion of the aorta according to Langendorff at constant flow (7 ml/min) was immediately started using a modified Tyrode buffer (NaCl 137.0 mM; KCl 5.4 mM; $MgCl_2$ 0.52 mM; $NaHCO_3$ 12.0 mM; $CaCl_2$ 1.8 mM; NaH_2PO_4 0.46 mM; glucose 11.0 mM; pH 7.4) saturated with O_2-CO_2 (95:5) at 37°C.

Experimental protocol

Two groups of animals were used for NMR measurements: Group A, treated (n=10), and Group B, control (n=6). In a parallel third group, purine release was determined: Group C, treated (n=6). For Groups A and C the protocol was as follows: after an equilibration period of 30 min (t=0 min), the flow was stopped for 2 min and resumed for 3 min; this cycle was repeated six times, i.e., 6 x (2 min ischaemia + 3 min reperfusion). In Group B, after an equilibration period of 30 min, constant flow was maintained for another 30 min.

Assessment of myocardial function

Myocardial function was monitored as either developed pressure or developed tension. Pressure was measured using an intraventricular balloon connected to a pressure transducer; the filling pressure was individually adjusted to 21 ± 0.1 mmHg in order to achieve maximal contractile performance. Tension was assessed by means of a pseudoisometric force transducer connected to the apex of the heart, which was tuned in order to read the values of developed tension in grams. A resting tension of 5 g was applied, and adjusted during the equilibration period if necessary. Heart rate values were obtained from the function tracing.

Biochemical assays

Samples for purine analysis were taken at the end of the equilibration period, and every minute during the reperfusion periods. Adenosine, inosine, hypoxanthine, xanthine and urate were determined using HPLC.[29 30] Briefly, the perfusate was injected onto a C_{18} μBondapak™ column and isocratically eluted with a mixture of methanol and KH_2PO_4. Peaks were detected at 254 and 295 nm by means of a multiwavelength absorbance detector.

NMR analysis

^{31}P NMR spectra were obtained using a Varian XL-300 spectrometer (7.05 Tesla) operating at a resonance frequency of 121.1 MHz.[31] The experimental parameters were: 30 μs 90° pulse; 1 s recycle time; 64-96-160 transients, respectively corresponding to 2, 3, and 5 min of accumulation time, and 10 Hz line broadening.

Cytosolic metabolite concentrations

Relative concentrations of phosphocreatine (PCr), ATP and P_i were determined by integrating the relevant peaks.[31] Peak areas of PCr, ß-ATP and P_i were corrected for saturation by comparison with spectra obtained in fully relaxed conditions. For quantification, a capillary inserted close to the organ in the NMR tube and containing Na-methylenediphosphonate (100 mM) was used as reference standard.

In each preparation, the [PCr]+[creatine] was checked to be constant. The PCr phosphorylation state, $[PCr]/([Cr]\bullet[P_i])$, was evaluated by means of the relationship:[32]

$$[PCr]/([Cr]\bullet[P_i]) = 0.13\bullet([PCr]/[P_i])^{1.27} \qquad \text{Eqn. 1}$$

in which Cr=creatine.

The phosphorylation potential, $PP=[ATP]/([ADP]\bullet[P_i])$, was calculated from the values obtained for PCr, P_i and pH_i. In particular it was estimated from the creatine kinase equation in the following form:

$$[ATP]/([ADP]\bullet[P_i]) = \{[PCr]/([Cr]\bullet[P_i])\}\bullet([H^+]/K_{ck}) \qquad \text{Eqn. 2}$$

where $[H^+]$ is the cytosolic H^+ concentration, and K_{ck} the pH- and Mg^{2+}- dependent creatine-kinase equilibrium constant.[33] The cytosolic free $[Mg^{2+}]$ was determined as described in refs. 34 and 35:

$$[Mg^{2+}] = K_D\bullet\{[(\delta_{\alpha\beta} - \delta'_{\alpha\beta})/(\delta''_{\alpha\beta} - \delta'_{\alpha\beta})] - 1\} \qquad \text{Eqn. 3}$$

in which K_D is the dissociation constant of ATP, and $\delta_{\alpha\beta}$, $\delta'_{\alpha\beta}$, and $\delta''_{\alpha\beta}$ respectively represent the chemical shift differences between α-P and β-P resonances of ATP in the heart, in solutions with and without saturating Mg^{2+}. Intracellular pH (pH_i) was measured from the chemical shift difference of P_i and PCr; the titration curve determined for our buffer was found not different from the one generally used.[36]. In the absence of significant changes in intracellular pH (pH_i) and free Mg^{2+}, as detected by NMR, the following relationship was used to calculate $[H^+]/K_{ck}$:[32]

$$\log[H^+]/K_{ck} = -0.87\bullet pH_i + 8.31 \qquad \text{Eqn. 4}$$

No corrections were made for the effects of $[Mg^{2+}]$ on K_{ck} and K_{mk} (see below).

Cytosolic free [AMP] was calculated from the myokinase equilibrium:

$$[AMP] = K_{mk}\bullet([ADP]^2/[ATP]) \qquad \text{Eqn. 5}$$

assuming near-equilibrium conditions and an equilibrium constant (K_{mk}) of 1.12.[33]

The value of cytosolic free [ADP] used was calculated from the creatine kinase equilibrium, a rearrangement of Eqn. 2:

$$[ADP] = ([ATP]\bullet([Cr]/[PCr])\bullet(K_{ck}/[H^+]) \qquad \text{Eqn. 6}$$

Statistical analysis

All data are presented as mean ± SEM. Student's paired and unpaired t-tests were used for comparisons. Analysis of variance was used when necessary. Differences with $p\leq0.05$ were considered significant. Correlation coefficients were computed for linear or power fits; the residuals of the fits were analyzed by means of the Durbin-Watson and Wilk-Shapiro tests, using Statistix® version 3.1 (NH Analytical Software,

Roseville, Minn). The lowest values usually accepted for these statistics are near to 1.5 and 0.85, respectively.[37]

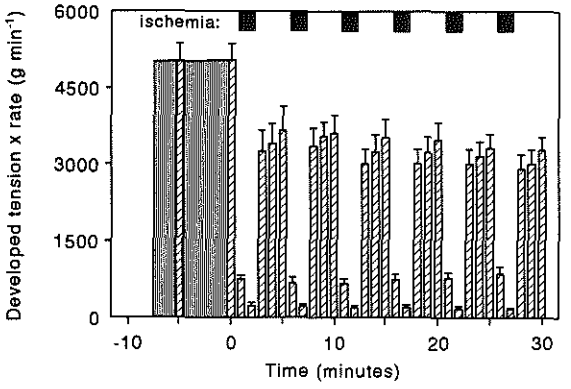

Figure 1. Decline in function of rat hearts during repeated ischemia. The product of developed tension and heart rate is shown. Flow was 7.2 ml min^{-1}. After 30 min equilibration 2-min periods of stop-flow ischemia (indicated by black boxes at the top of the graph) alternated with 3-min reperfusion periods. Mean ± SEM (n=6).

Results

Function

In Group C, the product of heart rate and developed tension was 5,040±320 g min^{-1} at the end of the equilibration period (t=0 min). Due to a 2-min occlusion, contractile function fell to 5% of the initial value ($p<0.001$ *vs* baseline: Fig. 1), but recovered upon reperfusion to 73% ($p<0.02$ *vs* baseline). This pattern of falling and recovering function took place throughout the subsequent ischaemia-reperfusion cycles, although these did not significantly affect function any further. At t=30 min, function was 65% of the initial value (Fig. 1). In Group A, the product of heart rate and developed pressure was 27,900±3,100 mmHg min^{-1} at baseline; its pattern was identical to that of Group C (data not reported). In the non-ischaemic control Group B, the product of heart rate and developed pressure remained unchanged throughout the experiment.

Purine efflux

Baseline efflux of purine nucleoside and oxypurines was 9.2±1.0 nmol min^{-1} g^{-1} wet weight; it peaked early during the 2nd reperfusion, reaching values of 72±9 nmol min^{-1} g^{-1} ($p<0.001$ *vs* baseline: Fig. 2, upper panel). Purine release gradually declined during the subsequent cycles, reaching a final peak value of 40±6 nmol min^{-1} g^{-1} ($p<0.05$ *vs* 2nd reperfusion; $p<0.01$ *vs* baseline).

Effluent adenosine was always <5% of the total purine concentration. Adenosine efflux followed a pattern which was similar to that of total purine, although the former had already declined after the first cycle (Fig. 2, lower panel). Baseline adenosine efflux was 0.39±0.15 nmol min^{-1} g^{-1} wet weight. Early after the 1st ischaemia, adenosine release peaked at 2.9±0.3 nmol min^{-1} g^{-1} ($p<0.01$ *vs* baseline). This release gradually declined during subsequent ischaemia-reperfusion cycles, returning to baseline efflux levels during the 6th reperfusion: 1st min, 0.61±0.11 nmol min^{-1} g^{-1} ($p<0.001$ *vs* 1st reperfusion; $p=0.4$ *vs* baseline); 2nd and 3rd min, 0.09±0.06 nmol min^{-1} g^{-1}. As early as the 2nd min of reperfusion after the 3rd ischaemic-reperfusion period, adenosine release was already significantly lower than baseline (Fig. 2).

Throughout the experiment, both hypoxanthine and xanthine efflux accounted for 10% of total purine release. The fraction of the major purine produced (urate, 62% of total purine release at baseline) temporarily dropped by 1/3 as the result of the first few periods of ischaemia. Inosine release made up the difference, doubling its baseline value (data not shown).

Metabolic responses in the heart

The NMR approach was chosen because it allows sequential determinations to be performed in the same organ, and thus provides a better statistical accuracy. In particular, it has been extensively used to measure cytosolic high-energy phosphates in the evaluation of a number of metabolic indices.[38 39 40 41]

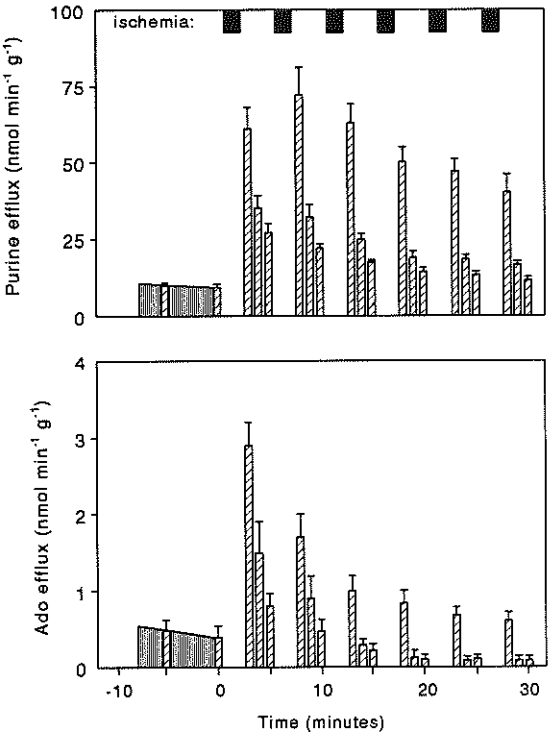

Figure 2. *Decline in purine efflux, measured by HPLC in cardiac effluent after repeated periods of ischemia. The top panel depicts the total release of adenosine, inosine, hypoxanthine, xanthine, and urate; the bottom panel shows the efflux of adenosine (Ado). For further details, see legend to Fig. 1.*

After 30 min equilibration, NMR analysis indicated that PCr was 7.6±0.7 mM, ATP 4.0±0.4 mM, P_i 3.2±0.3 mM, and pH_i 7.04±0.02 in Groups A and B. In Group A, PCr decreased by 41% ($p<0.005$ *vs* baseline) after 2 min of ischaemia and recovered to 83% ($p=0.15$ *vs* baseline) upon reperfusion (Fig. 3). This pattern was almost constant for every intervention. At the end of the experiment, PCr was 75% of the initial value, no different from that of controls when compared at the same time (Group B, Fig. 3).

P_i increased by 59% in Group A ($p<0.02$ *vs* baseline) during the 1st ischaemia, and approached the initial value upon reperfusion (Fig. 3). A similar pattern occurred during the subsequent interventions, although P_i production gradually declined. The P_i level during the 6th ischaemia was only 30% higher than baseline ($p<0.02$ *vs* 1st ischaemia). However, during the 6th reperfusion, it was 19% lower than that of the control Group B ($p<0.05$), which had undergone a spontaneous linear increase of 25% over the same period of time (Fig. 3). While PCr and P_i were very sensitive to ischaemic intervention, ATP remained constant in Group A, and did not differ from the values observed in control Group B (Fig. 3).

Intracellular pH did not change significantly. In Group A, it varied between 7.04±0.03 (t=0 min) and 6.99±0.03 (t=30 min). In Group B, these values were 7.05±0.01 and 7.00±0.01, respectively.

The cytosolic [Mg^{2+}] determined by ^{31}P-NMR spectra remained stable at about 0.30-0.35 mM throughout

the recurrent ischaemia protocol (*cf.* ref. 41).

Metabolic indices

The values of the PCr phosphorylation state, [PCr]/([Cr]•[P_i]), are reported in Table 1. The baseline value in Group A, 0.40±0.03 mM^{-1}, decreased during the 1st ischaemia by 32% ($p<0.001$ *vs* baseline), recovering to 68% ($p<0.005$) at reperfusion. Subsequent cycles produced an almost identical oscillation in values, indicating that the additional repetitive ischaemia-reperfusion treatments had a marginal influence on the PCr phosphorylation state. In the non-ischaemic control Group B, the PCr phosphorylation state deteriorated as predicted by the decreased PCr and increased P_i levels: at t=30 min, it was 49% lower than baseline ($p<0.001$ *vs* t=0 min). Comparison between Groups A and B at the end of the protocols indicated that the PCr phosphorylation state was enhanced by 12% ($p<0.05$) in treated hearts at almost constant pH_i.

The phosphorylation potential, PP=[ATP]/([ADP]•[P_i]), followed the pattern described for the PCr phosphorylation state. At t=0 min, PP was 62±5 mM^{-1} in Group A. It decreased by 66% ($p<0.001$) after the 1st ischaemia, and returned to 82% of baseline ($p<0.002$) upon reperfusion (Table 1). During subsequent cycles, PP reached almost constant values after each ischaemic period (22 mM^{-1}). It showed a gradual decline after the subsequent reperfusions, reaching a value which was 24% lower than the initial one at the end of the experiment (t=30 min, $p<0.05$). In the control Group B, PP decreased by 44% ($p<0.001$). The value in Group A exceeded that in Group B by 31% ($p<0.05$, Table 1).

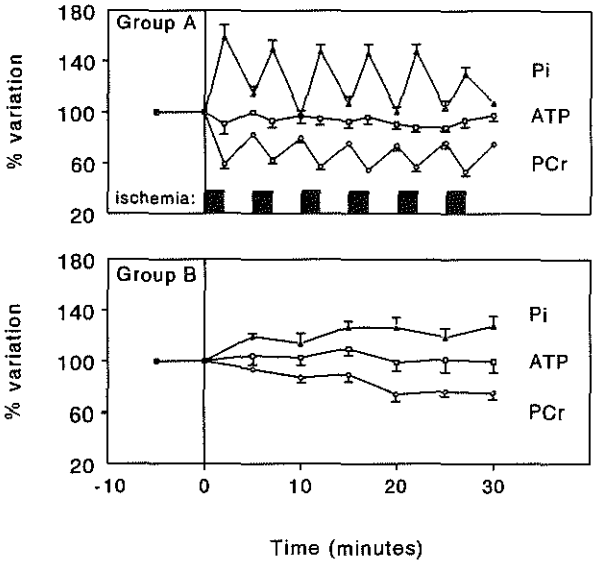

Figure 3. *^{31}P-NMR measurements of cardiac phosphates during recurrent ischemia (Group A, n=10) and during control perfusion (Group B, n=6). Data expressed as % of baseline values (in mM): P_i, 3.2 ± 0.3; ATP, 4.0 ± 0.4; phosphocreatine (PCr), 7.6 ± 0.7. For further details, see legend to Fig. 1.*

The AMP values reported in Table 1 were used to calculate the cytosolic adenylate energy charge, an alternative metabolic index, which links the formation of adenosine to energy metabolism:[42]

([ATP]+0.5•[ADP])/([ATP]+[ADP]+[AMP]) Eqn. 7

in which [ADP] is taken from Eqn. 6. The linear correlation found between adenylate energy charge and PP was very good (r=0.97, Fig. 4).

The energetic index provides yet another way of describing the metabolic status of the heart:[43]

([ATP] + [PCr])/([ATP] + [PCr] + [P_i]) Eqn. 8

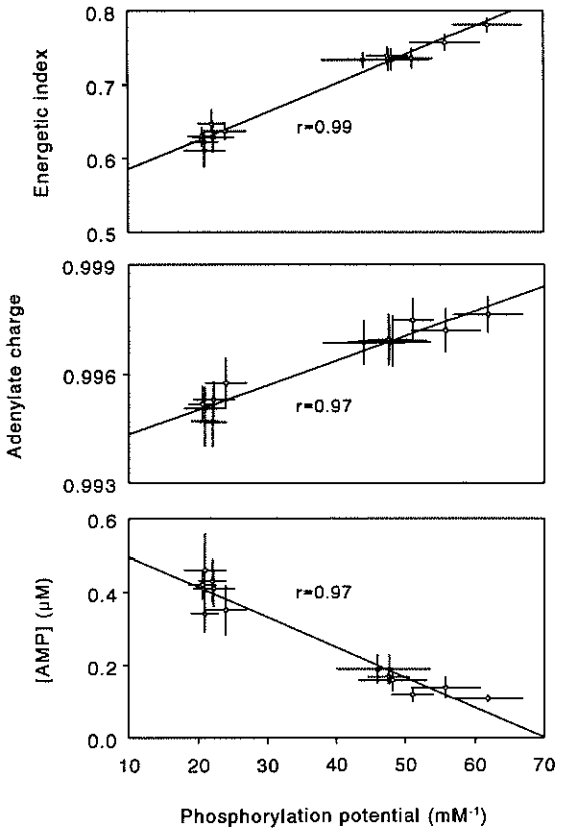

Figure 4. *Correlations of phosphorylation potential, vs energetic index, vs adenylate charge, and vs the cytosolic [AMP]. For all r-values, $p<0.001$. Mean ± SEM ($n=6$-10).*

Table 1. *Calculated free AMP, PCr phosphorylation state, and phosphorylation potential during recurrent ischemia*

Time (min)	Intervention	[AMP] (μM)		[PCr]/([Cr]•[P$_i$]) (mM^{-1})		[ATP]/([ADP]•[P$_i$]) (mM^{-1})	
		Group A	Group B	Group A	Group B	Group A	Group B
0		0.11±0.01	0.21±0.02	0.40±0.03	0.41±0.03	62±5	62±5
2	1st ischemia	0.46±0.10		0.13±0.02		21±3	
5	reperfusion	0.12±0.02	0.23±0.05	0.27±0.02	0.31±0.02	51±3	45±3
7	2nd ischemia	0.35±0.07		0.13±0.01		24±3	
10	reperfusion	0.14±0.03	0.37±0.09	0.31±0.02	0.29±0.03	56±5	43±5
12	3rd ischemia	0.42±0.04		0.12±0.01		21±2	
15	reperfusion	0.19±0.04	0.33±0.06	0.27±0.02	0.27±0.03	48±6	42±3
17	4th ischemia	0.41±0.05		0.12±0.02		22±3	
20	reperfusion	0.17±0.02	0.54±0.09	0.27±0.02	0.21±0.02	48±3	32±3
22	5th ischemia	0.34±0.05		0.12±0.01		21±2	
25	reperfusion	0.16±0.03	0.44±0.09	0.28±0.03	0.23±0.02	48±5	37±3
27	6th ischemia	0.43±0.06		0.13±0.01		22±2	
30	reperfusion	0.19±0.02	0.42±0.07	0.25±0.02	0.21±0.02	46±4	35±2

Hearts in group A were made repeatedly ischemic for 2-min periods. Group B consisted of non-ischemic controls. Data are expressed as mean ± SEM. Group A, n=10, Group B, n=6.

To calculate this index, quantities directly derived from the NMR analysis were used; once again, an excellent correlation with PP was found (r=0.99, Fig. 4).

Durbin-Watson and Wilk-Shapiro test results were in the acceptable range, strengthening confidence in the r-values reported for these linear correlations.

Correlations between purine formation and energy metabolism

The metabolic indices defined above correlated with free [AMP], the release of adenosine (+ its metabolite inosine), and the release of total purine. Given the very good linear correlations found between phosphorylation potential and the other metabolic indices, only the correlations of the metabolites with PP are reported. Phosphorylation potential displayed an excellent negative linear correlation with free [AMP] (r=0.97, Fig. 4); residual analysis of the linear fit showed no evidence of model mis-specification. PP also correlated well (in a negative exponential way) with total purine release (r=0.90) and, to a lesser extent, with adenosine + inosine release (r=0.81) and adenosine release (r=0.67, Fig. 5). The Wilk-Shapiro test showed that the randomness of the standardized residuals was within acceptable limits, although the rigorous Durbin-Watson test detected correlated errors distorting SEM and hence the r-values.

The anomalous behaviour of the decrease in P_i production along the recurrent ischaemia protocol correlated in a power fit with purine release: $[P_i]$ *vs* total purine efflux, r=0.93; *vs* adenosine + inosine release, r=0.90; *vs* adenosine release, r=0.80 (Fig. 6). Analysis of the residuals showed that only the $[P_i]$ *vs* total purine efflux fit could be considered acceptable. A good exponential correlation was found between the [AMP] and the $[P_i]$ (r=0.87, data not shown).

Discussion

Hypothesis

The injury produced by a short period of cardiac ischaemia followed by reperfusion does not worsen when repeated similar cycles occur within a short time span. On the contrary, this preconditioning adapts the tissue, protecting it against major insults such as prolonged ischaemia.[2 4 6 11] Although the phenomenon is well-documented, the underlying mechanism is far from clear.[8 9] Adenosine could play an important role in preconditioning, as revealed by a plethora of studies with adenosine receptor (ant)agonists or exogenous adenosine. On the other hand, endogenous adenosine production during repeated ischaemia has received relatively little attention.[3 24 25] Hoffmeister *et al.*[26] observed increased tissue adenosine after 5, but not 20, occlusions of dog-heart coronary artery; in contrast, Henrichs *et al.*[44] failed to find adenosine and inosine during intermittent occlusion.

We hypothesized that the phosphorylation potential regulates variations in purine production during recurrent ischaemia and reperfusion. Our protocol of recurrent ischaemia elicits the preconditioning phenomenon[7 45] when followed by 30 min no-flow ischaemia and 30 min reperfusion; our results on cardiac function, pH_i, and high-energy phosphates parallel recently published data.[46]

Function

The first cycle of ischaemia-reperfusion abruptly impaired contractile function (Fig. 1). However, we did not observe any further deterioration after subsequent ischaemic insults, suggesting that the first cycle effectively preconditions the heart by bringing about an adaptation that protects the heart from subsequent injuries. In our scheme of repetitive ischaemia, pH_i did not decrease. Literature data show acidosis to be constant and prominent in sustained ischaemia. The lack of H^+-accumulation is probably due to the repeated periods of reperfusion, which allow efficient washout of acidic equivalents produced during preceding ischaemic periods. This, in turn, prevents the Na^+-inflow and Ca^{2+}-overload triggered by Na^+/Ca^{2+}-exchange, thus preserving ionic homeostasis and precluding contracture. To explain the absence of a cumulative impairment of contractile performance during recurrent ischaemia, we concentrated on the role of adenosine. We found that the first ischaemia induced a marked production of total purines and adenosine (Fig. 2), showing that an ischaemic period as short as 2 min is a pathogenic trigger for this parameter. Given that a pump was used to dictate flow, vasodilation could not have affected energy supply. Adenosine may have been beneficial by reducing cardiac work and, consequently, energy demand.[47 48] This hypothesis is in line with a report that the inhibition of adenosine A1 receptors cancels the beneficial effects of preconditioning evaluated as infarct size.[11] Furthermore, Headrick *et al.*[40] showed that adenosine production, estimated from epicardial or interstitial concentrations, closely parallels the extent of the metabolic response to either hypoxia or $ß_1$-receptor

stimulation.

Myocardial energy state

The present study well documents the modification of the myocardial energy state produced by ischaemia and the ensuing reflow. Although we noticed increased purine release due to ischaemia, significant changes in ATP content did not take place. Bailey *et al.*[49] also found no decline within the first few minutes of ischaemia. These authors estimated the $t_{1/2}$ for ATP loss during ischaemia at 9 min, far longer than the ischaemic period used in our study. PCr decreased 41% after the first occlusion (Fig. 3), but subsequent occlusions did not produce any additional decrease. Recovery remained almost constant (75% of control) during the following cycles of ischaemia and reperfusion. These results are in line with earlier studies: Repetitive episodes of regional ischaemia in open-chest dogs do not produce a cumulative decrease in cardiac high-energy phosphates.[24 25 26]

We did not expect P_i production to decrease due to recurrent episodes of ischaemia-reperfusion. To explain the low P_i levels, we suggest that this effect is due to the high concentration of endogenous adenosine produced during the first ischaemia. In fact, this adenosine might induce metabolic alterations not linked to cellular acidosis.[50 51] Mentzer *et al.*[48] postulated this process when they studied the effects of adenosine on

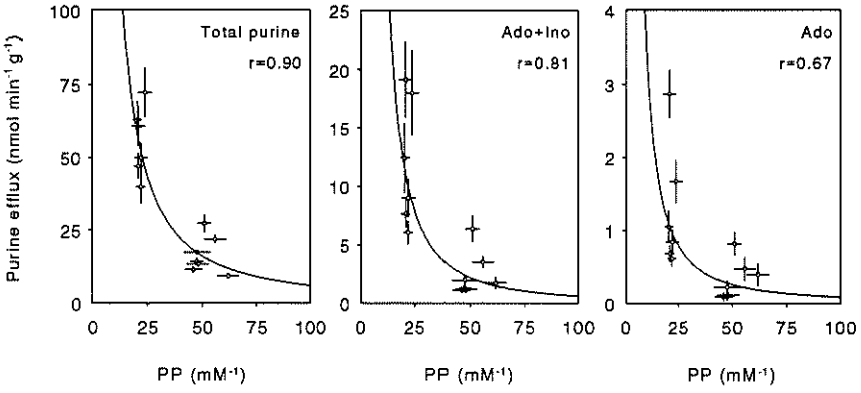

Figure 5. *Correlations between phosphorylation potential (PP) and purine release. The NMR data collected during the periods of ischemia and reperfusion are plotted against the HPLC data obtained early and late, respectively, during the reperfusion periods. Ado = adenosine, Ino = inosine. Mean ± SEM (n=6-10).*

glycolytic intermediates in ischaemic hearts. Our data are in line with this possibility, since the accumulation of ischaemic P_i progressively decreased with the number of ischaemic episodes. In a broader sense, this can be seen in the increase in the PCr phosphorylation state.

Metabolic indices

A different approach confirms the validity of the hypothesis, regulation of purine production by the phosphorylation potential during recurrent ischaemia. We calculated the cardiac energy state according to three different metabolic indices: phosphorylation potential, cytosolic adenylate charge and energetic index. They correlate with purine release and cardiac free [AMP], confirming data obtained during low-flow ischaemia or metabolic manipulation.[38 52] Our observations support the hypothesis that the energy status of 5'-nucleotidase regulates its activity (see also ref. 53). Interestingly, Kitakaze *et al.*[54] recently reported a twofold increase in activity after preconditioning of canine heart.

The data on contractile work, which does not return to baseline values during reperfusion, show the effect

of adenosine on energy demand. In our opinion, the values of the energy-state indices are major points for regulating cardiac performance during repeated ischaemia-reperfusion following the first cycle. The rationale is as follows. By the second cycle, the heart has "adapted" to the insult, *i.e.*, it has gained a stable metabolic

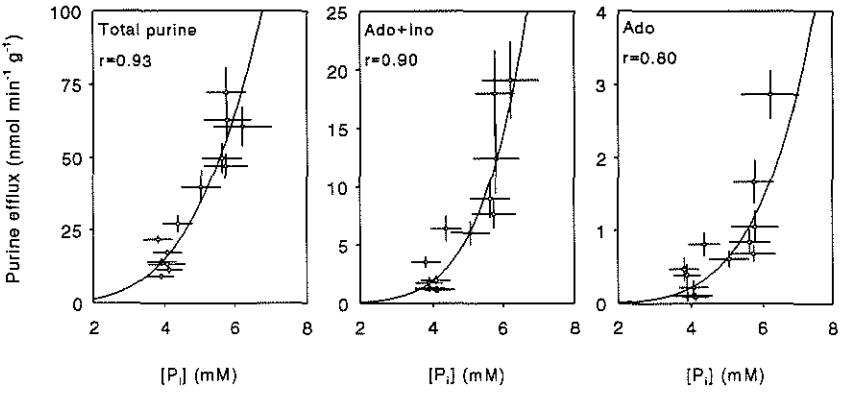

Figure 6. *Correlations between cytosolic [P_i] and purine release. For other details, see legend to Fig. 5.*

state (as measured by the three indices) with decreased function which induces a proportional decrease in adenosine production and release. The balance between the release (and pharmacological activity) of adenosine and the new steady-state of tissue metabolism allows the heart to tolerate subsequent cycles of ischaemia-reperfusion -up to 6 in our investigation. In this view, the observed decrease in purine and adenosine release associated with the last 3-4 cycles indicates a progressive improvement in the general condition of the heart. The organ regulates its adenosine production -and consequent receptor effects- according to the necessity of maintaining the new metabolic state which, as the indices show, becomes stable.

Thus, a metabolic index might be the key factor regulating the response of the heart to recurrent ischaemic insults, and this might occur through the ensuing regulation of AMP supply, and therefore of adenosine production and release. This is in line with the results of Mentzer *et al.*[48], who found that high doses of adenosine can induce a parallel modification of PP, similar to that shown in this study for endogenous adenosine. We would like to stress a further point. To assess the metabolic state of the heart, we examined phosphorylation potential, adenylate charge and the energetic index. As expected from the parameters involved in the respective equations, these correlated with each other. However, several reasons support the view that PP should receive major attention: 1) We based the only assumption we had to make on the creatine-kinase equilibrium, which in turn depends on [Mg^{2+}] and pH_i; but [Mg^{2+}] was relatively stable during short periods of ischaemia, while pH_i remained constant throughout the experiment. 2) Unlike the other indices, PP responses varied widely (observed range of individual data during ischaemia-reperfusion: 9-90) while, *e.g.*, adenylate charge showed minor changes (range 0.992-0.999), although we calculated the indices from the same parameters. 3) Only PP is stoichiometrically related to, for example, Na^+,K^+-ATPase or Ca^{2+}-ATPase, two key systems known to regulate the ionic homeostasis of the cell.

The good correlations found between the metabolic indices and [AMP], as well as total purine release, strongly suggest that a link exists between the metabolic state and purine turnover, in agreement with results obtained after 10 min of hypoxia.[39]

Limitations of the study

NMR measurements provide an overall assessment of the concentration of high-energy phosphates in the entire heart. Although the alteration in metabolite levels due to ischaemia is expected to be transmurally non-uniform, our experimental approach does not allow any estimate to be made of this possible heterogeneity.[55] Adenosine breakdown is also likely to vary in different cardiac cell types. We believe, however, that

adenosine catabolism in the species used (rat) takes place to a major extent in the cardiomyocyte.[56]

We averaged the NMR values for the 2 min of ischaemia and for the 3 min of reperfusion. In an attempt to obtain faster results, we collected NMR data every 15 s during the first ischaemia-reperfusion cycle (data not reported) and found a behaviour analogous to that presented by Clarke *et al.*[51] regarding ATP, P_i, and PCr levels. Nevertheless, the use of such a short time for sampling high-energy phosphate data was not suitable for the protocol adopted in this study.

In the non-ischaemic control experiments, several sensitive biochemical parameters changed (Table 1). However, contractility remained stable.

We collected data on cardiac metabolites and indices in NMR experiments, and purine data in parallel experiments. The correlation coefficients between total purine release and PP, as well as P_i, were quite high. However, fit analysis of residuals warns us to regard these coefficients with some caution; this is even truer for the correlations reported for the rapidly catabolized adenosine (± inosine). Reason for further concern is the non-even distribution of data points in some correlation studies.

Conclusion

It is a well-established principle that the myocardial energy state, evaluated using adenylate charge or phosphorylation potential, is related to purine formation and release. Still, quantitative relationships between these entities are not well-defined.[37] The original outcome of this study is the finding that myocardial purine formation and energy state are also related during recurrent ischaemia. Our ^{31}P-NMR data support the assumption that adenosine formation is linked to myocardial metabolism through the phosphorylation potential.[32 57] Adenosine sparing may prime the metabolic and functional performance at a lower level of equilibrium, thus preventing any further impairment of the heart. We consider the release pattern of purine (and in particular that of adenosine) as being indicative of cardiac adaptation to subsequent ischaemic insult.

Clarke *et al.*[51] indicated that the cytosolic phosphorylation potential might be the metabolic factor that determines cardiac contractile function. In our study, neither phosphorylation potential (which decreased after the first ischaemia-reperfusion cycle) nor contractility were altered by the subsequent cycles. Our data strongly suggest that adenosine plays a role in the energy supply-demand balance during repeated ischaemia: we propose the regulation of AMP breakdown by the phosphorylation potential.

Acknowledgements

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Appendix 8

Repeated versus continuous ischemia: effects on energy and carbohydrate metabolism as well as catecholamine release

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Repeated versus continuous ischemia: effects on energy and carbohydrate metabolism as well as catecholamine release

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Key words: Endogenous adenosine, Norepinephrine, Preconditioning, (Pro)glycogen

Abstract

Background. Hearts tolerate intermittent ischemia better than continuous ischemia of the same duration. Assessing metabolic aspects, we sought to get insight in the mechanisms(s) involved.
Methods and Results. We perfused rabbit hearts (n=30) retrogradely at 22 mL/min, with flow interrupted either intermittently (for 12x2 or 6x2 + 6x4 minutes) or continuously (for 24 or 36 minutes). After 2 minutes occlusion, adenosine efflux rose temporarily 9x ($P<0.001$). Subsequent occlusions induced substantially less release. Postischemic purine efflux correlated well ($r=0.95$) with preischemic function. More severe ischemia overcame the decrease in ATP breakdown. After permanent ischemia, purine efflux was ≤6x higher than with repeated ischemia ($P<0.001$). In contrast, lactate release after short ischemic periods changed little; it was lower after continuous ischemia. Then the cardiac glycogen/proglycogen ratio dropped 8x ($P<0.001$). Norepinephrine release took only place after continuous ischemia. Investigations on intermittently perfused rat hearts (n=28) confirmed the purine findings. ^{31}P-NMR spectroscopy showed that recurrent ischemia attenuated the fall in phosphorylation potential.
Conclusions. 1) Intermittent ischemia induces a decline in breakdown of ATP, but not carbohydrate; continuous ischemia exacerbates purine release, attenuates lactate production, and induces norepinephrine efflux. 2) The correlation between postischemic purine release and preischemic function suggests that the inotropic state determines the fate of energy metabolism during ischemia. 3) Recurrent ischemia does not prevent total glycogen breakdown. However, it seems to attenuate glycogen catabolism, at the expense of proglycogen. We speculate that the glycogen/proglycogen ratio, regulated by adenosine, plays a role in preconditioning. (*Circulation* 0000;00:000-000)

Introduction

The concept of ischemic preconditioning, i.e., adaptation following brief ischemic periods, has attracted widespread attention.[1] Preconditioning probably involves adenosine, acting via the A1 receptor, although other mechanisms are possible.[1] The nucleoside, derived from ATP via AMP, forms during brief periods of ischemia or anoxia, in animal models and in man.[2,3] Its role in preconditioning is likely in many species, but unclear in the rat. In the case of this rodent, controversial results are present in the literature.[4,5,6,7,8,9] The occurrence of preconditioning in man lacks convincing evidence: Deutsch et al.[10] and Cribier et al.[11] claimed that the phenomenon occurs during percutaneous transluminal coronary angioplasty, but others failed to find the effect.[12,13,14]

We noted before that adenosine production in isolated rat hearts during short periods of intermittent *anoxia* gradually decreases, notwithstanding the presence of substantial amounts of ATP.[2] This block in ATP breakdown could be overcome with a more aggressive anoxic protocol. Using isolated rabbit and rat hearts,

we investigated now whether the above phenomena could be observed after short periods of *ischemia*, thus imitating repeated ischemia during coronary angioplasty attempts and anginal attacks. In addition, we sought to clarify why hearts tolerate intermittent ischemia better than continuous ischemia of the same duration. We studied specifically the role of norepinephrine, a candidate for the genesis of ischemia-induced arrhythmias.[8] Parts of this study appeared in abstract form.[15,16]

Materials and methods

Heart perfusions

Rodents were treated conform the guiding principles of the American Physiological Society. Male New Zealand white rabbits (2.0 - 2.3 kg, n=30), maintained on a standard diet, were stunned by a blow on the head; the hearts were quickly removed and cooled in Krebs-Henseleit buffer (0°C). After being cleaned from connective tissue, the hearts were perfused by the Langendorff technique using a modified Krebs-Henseleit buffer containing (in mmol/L): NaCl 115; $NaHCO_3$ 25; KCl 4.0; KH_2PO_4 0.9; $MgSO_4$ 0.65; $CaCl_2$ 1.7 and D-glucose 11. The perfusion solution was heated at 37°C, bubbled with 95% O_2 and 5% CO_2, and transported at a rate of 22 mL/min to the aortic cannula with a Gilson Minipuls 2 rotary pump. The hearts were jacketed (40°C) to provide a constant myocardial temperature of 37°C checked by a model CTD 85 thermometric probe (Ellab, Copenhagen) in the pulmonary artery. They were paced throughout the experiment at a rate of 180 beats/min as described before.[17]

The following protocols were used after 30 minutes equilibration: A) twelve 2-minute periods of stopped-flow, alternated with 3-minute reperfusion periods; B) six 2-minute and six 4-minute periods of total ischemia, alternated with 3-minute reperfusion periods; C) and D) 24 and 36 minutes of ischemia followed by 30 minutes reperfusion, respectively; E) normoxic control perfusion at 22 mL/min.

Protocols A, B and E were also carried out with Sprague-Dawley rats (n=28). Their isolated hearts were perfused without pacing at 7 mL/min with the buffer described above.

Left-ventricular function measurements

To obtain an isovolumetrically beating preparation, a fluid-filled balloon was inserted into the left-ventricular cavity via the atrium. The balloon was connected by a fluid-filled polyethylene catheter to a Hewlett Packard transducer (model 1290A OPT 002). Alternatively, function was measured as developed tension.[18]

Biochemical Assays

Coronary-effluent fractions were collected in glass vials (0°C). An aliquot of perfusate (2.0 mL) was added to 30 µL of 10 mmol/L $Na_2S_2O_5$ for HPLC catecholamine determination;[19] another aliquot (0.5 mL) was added to 1.0 mL of $HClO_4$ (6%) for enzymic lactate determination.[20] All samples were stored below -80°C; these included 2-mL aliquots of perfusate for HPLC purine determination with a Model 990 Photodiode Array Detector (Waters).[21,22] At the end of the experiments, the hearts were clamped by Wollenberger tongs, cooled in liquid N_2, and stored below -80°C. Acid extraction, and neutralization of the supernatant fraction, took place as described before[23] (NaOH replaced KOH if glycogen was to be determined). The neutral supernatant fractions were analyzed for nucleotides (HPLC),[23] (phospho)creatine (HPLC),[24] glycogen (enzymic),[24,25] inorganic phosphate (colorimetric),[26] and catecholamine (HPLC).[19] Glycogen[24,25] and protein[27] were determined in the acid precipitate, with rabbit liver glycogen and bovine serum albumin, respectively, as the standard.

NMR studies

The majority of the rat hearts were perfused in a ^{31}P-NMR spectrometer (Varian XL-300; 7.05 Tesla, 121.1 MHz), as described before.[28] The experimental parameters were: 30-µs 90° pulse; 1-s recycle time; 64-96-160 transients, corresponding to 2, 3, and 5 minutes accumulation time, respectively; 10-Hz line broadening.

Statistics

Where appropiate, the groups were compared with analysis of variance (ANOVA), followed by Bonferroni's *t* test. Student's paired and unpaired *t* tests were used for comparisons within and between groups, respectively. Differences with $P \leq 0.05$ (2-tailed) were considered significant. Data are reported as mean±SEM.

Table 1. *Effect of staccato ischemia on energy metabolism in isolated rat hearts*

Intervention	Function	Tissue			Effluent	
		ATP	PCr	P_i	Nucleosides	Oxypurines
Occlusion 1	4.7 ± 1.0	93 ± 8	61 ± 4	165 ± 13	-	-
Reperfusion 1	72.8 ± 7.7	101 ± 2	82 ± 2	113 ± 5	1,580 ± 390	560 ± 50
Occlusion 6	3.5 ± 0.4	90 ± 4	52 ± 3	139 ± 8*	-	-
Reperfusion 6	65.9 ± 5.0	95 ± 3	74 ± 3	107 ± 3°	570 ± 170°	480 ± 87°

*Per cent change from baseline during the first parts of Protocols A and B: 6 bouts of 2-min stop-flow ischemia, with 3 min reperfusion (n=6-10). Tissue-phosphate data were obtained by NMR-spectroscopy, peak purine release data by HPLC. Baseline values: Function (heart rate x developed tension), 5,040 ± 320 g/min; ATP, 4.0 ± 0.4 mM; phosphocreatine (PCr), 7.6 ± 0.7 mM; P_i, 3.2 ± 0.3 mM; purine nucleoside and oxypurine release, 1.7 ± 0.5 and 7.5 ± 0.6 nmol/min/g protein, respectively. Mean± SEM. *p<0.05 vs Occlusion 1, °p<0.05 vs Reperfusion 1 (paired t-test).*

Results

Function

At the start of the experiment, developed pressure in rabbit hearts was similar in the 5 protocols (pooleddata: 58±2 mmHg). Due to a 2-min occlusion, developed pressure fell to 6.4±0.4 mmHg (P<0.001), but recovered upon reperfusion to 57±2 mmHg (Fig. 1). Thirty minutes after the start of the interventions, following 6 occlusions/reperfusions, developed pressure had decreased to 56±2 mmHg (P=0.004), i.e., by 11%. Upon continuation of protocol A, in the next 2-minute occlusion, it diminished to 7.4±0.2 mmHg, and recovered to 54±3 mmHg (P=0.052, reperfusion 7 vs 6; Fig. 1A). A sharper drop was seen at the end of 4 minutes ischemia, i.e., to 3.4±0.5 mmHg (P<0.001, protocol B vs A), and recovery was lower (46±3 mmHg, P<0.001 vs 6th reperfusion value, P=0.092 vs A). At the end of the experiments, developed pressure was 52±4 mmHg in protocol A, 42±3 mmHg in protocol B, and 57±2 mmHg in control hearts E (P<0.01 vs B). In hearts submitted to 24 minutes (protocol C) and 36 minutes (protocol D) of ischemia, followed by 36 minutes of reperfusion, it was 37±5 mmHg (P=0.050 vs A) and 21±5 mmHg (P=0.007 vs B), respectively.

In rat hearts, submitted to protocols A and B, cardiac function showed essentially the same picture (see Table 1).

Purine efflux

After the first 2-minute occlusion, effluent ATP-catabolites (adenosine, inosine, hypoxanthine) rose from basal levels of 1.07±0.12 to 9.8±0.4 μmol/L (P<0.001, Fig. 2A-B). After subsequent occlusions, the maximal purine concentration observed declined about 65% (P<0.001) after 6 occlusions. This apparent inhibition of ATP breakdown could be overcome by doubling the ischemic periods: In protocol B, the peak purine concentration found after the first 4-minute occlusion was 15.8±1.1 μmol/L, whereas the comparable value in protocol A was 3.0±0.3 μmol/L (P<0.001). Purine release also declined in B after subsequent occlusions (by 53%, P<0.001). At the end of the experiments, purine release in A and B was similar to that in control hearts E (0.70±0.09 μmol/L).

Adenosine, the ATP-catabolite with pronounced cardiovascular activity, followed a release pattern very similar to that of total purine efflux (Fig. 3). During the first reperfusion, effluent adenosine exceeded basal levels up to 8.6× (P<0.001). Peak concentrations decreased during subsequent interventions: After the 6th occlusion, it had declined by 67% (P<0.001, Fig. 3A-B). Doubling the ischemic period led to a new peak level; the adenosine concentration in B exceeded that in A 3.7× (P<0.001). Adenosine peaked 51% lower due the last 4-minutes occlusion (P=0.002 vs first 4-minute period). At the end of the experiments, control hearts (E) released 0.19±0.02 μmol/L adenosine, i.e., >2-3× than in hearts subjected to protocols A and B (P<0.005).

Fig. 4 shows the striking correlation between developed pressure *before* the ischemic periods and purine release *after* those periods.

Similar data were obtained in rat hearts in which the purine fraction also comprises xanthine and urate. A

2-minute occlusion induced a rise in adenosine effluent concentration from 0.05±0.03 to 0.27±0.03 μmol/L (P<0.001 vs baseline); a marginal increase (to 0.06±0.01 μmol/L) was seen after six 2-minute occlusions (P<0.001 vs reperfusion 1).

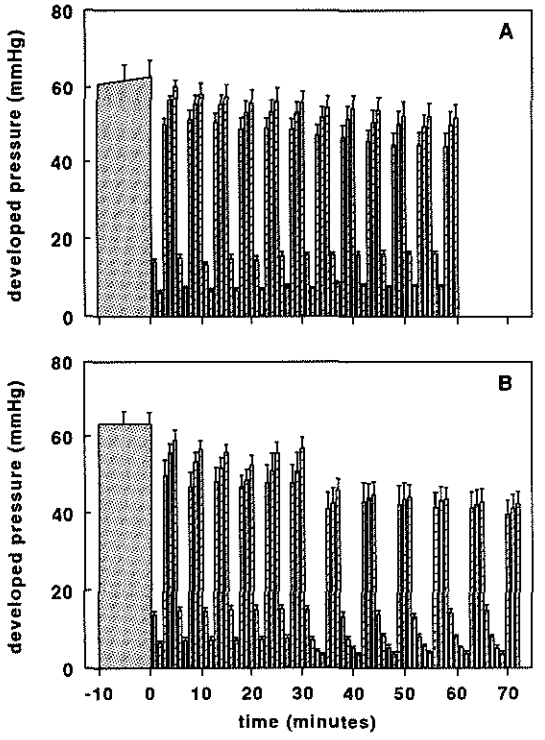

Figure 1. *Function in paced rabbit hearts due to twelve 2-minute periods of ischemia (protocol/panel A, n=5), or six 2-minute plus six 4-minute periods of ischemia (protocol/panel B, n=5). At the start of the experiments, the amount of fluid in the balloon was adjusted to obtain a diastolic pressure of 0 mmHg. Flow alternated between 22 and 0 mL/min. Mean values+SEM.*

Table 1 shows that qualitatively similar changes were observed in total release of the nucleosides adenosine + inosine, and of the oxypurines (hypo)xanthine + urate.

Rabbit-heart ATP catabolism, as indicated by purine release, was up to 6x higher if coronary flow was interrupted continuously (Table 2).

Lactate efflux

After the first 2-minute occlusion, lactate concentrations in the effluent rose from basal levels of 0.27±0.05 mmol/L to peak levels of 1.31±0.05 mmol/L (P<0.001, Fig. 5). After subsequent occlusions, peak lactate concentration decreased only 15% (P=0.016). Following more severe ischemia (protocol B), peak lactate concentration was 1.76±0.12 mmol/L, which differed from that in protocol A (1.02±0.10 mmol/L, P=0.002). In contrast to the peak purine levels, the maximal lactate concentrations did not decline after subsequent occlusions.

Lactate production was up to 45% less if flow was stopped continuously for 36 minutes (P<0.05 vs intermittent ischemia, Table 2).

Table 2. *Repeated versus continuous ischemia in rabbit hearts: effect on energy and carbohydrate metabolism, as well as norepinephrine release*

Ischemia Protocol (min)		Cardiac Content after Reperfusion(s), per g protein					Release during Reperfusion(s), per g protein/36 min			
		AdNucl (μmol)	PCreat (μmol)	Creatine (μmol)	Glycogen (mg)	Proglycogen (mg)	Total glycogen (mg)	Purines (μmol)	Lactate (mmol)	Norepinephrine (nmol)
12x2	A	39 ± 2	66 ± 3	66 ± 2	15.6 ± 2.1*	4.9 ± 0.8	20.5 ± 2.2*	3.8 ± 0.3*	1.00 ± 0.08*	0.27 ± 0.02
24	C	18 ± 2*°	46 ± 7	46 ± 4*	5.5 ± 1.2*°	12.3 ± 1.9*°	17.8 ± 1.9*	23.1 ± 2.5*°	0.74 ± 0.14	5.6 ± 1.5*°
6x2+6x4	B	35 ± 3	66 ± 5	51 ± 3*	11.7 ± 2.0*	3.3 ± 1.0	15.0 ± 2.6*	7.1 ± 0.5*	1.45 ± 0.17*	0.22 ± 0.02
36	D	9 ± 1*°	42 ± 8	72 ± 7°	3.0 ± 1.1*	7.7 ± 1.3	10.7 ± 1.9*	29.6 ± 3.2*°	0.81 ± 0.05°	13.1 ± 3.0*°
0	E	42 ± 2	56 ± 3	72 ± 4	28.6 ± 2.8	7.3 ± 0.4	36.0 ± 3.0	1.3 ± 0.1	0.66 ± 0.07	0.22 ± 0.04

*Means±SEM (n=5-7). AdNucl, adenine nucleotides; PCreat, phosphocreatine; NorE, norepinephrine. ANOVA: *p<0.05 vs 0' ischemia (normoxic control E); °p<0.05 vs recurrent ischemia (A vs C; B vs D)*

Table 3. *Recurrent short periods of ischemia attenuate the fall in rat-heart cytosolic atp phosphorylation potential, seen in control hearts*

Reperfusion	Protocol		
	A	B	E
Baseline	59.6 ± 5.1	65.2 ± 9.0	61.9 ± 4.7
One	46.3 ± 1.8	58.2 ± 4.2[a]	44.7 ± 3.1
Six	43.9 ± 6.0	44.5 ± 4.2	35.2 ± 2.2
Twelve	49.8 ± 8.5[b]	35.4 ± 3.8	21.6 ± 1.0

Data were calculated from ^{31}P-NMR measurements carried out on hearts submitted to protocol A [12x(2 minutes ischemia + 3 minutes reperfusion), n=6]; protocol B [6x(2 minutes ischemia + 3 minutes reperfusion) + 6x(4 minutes ischemia + 3 minutes reperfusion)], n=4; or protocol E (normoxic control), n=6. Values are in L/mmol, mean±SEM. ANOVA: [a]P<0.05, [b]P<0.005 vs E.

Catecholamine release

Small and decreasing amounts of norepinephrine were released during baseline perfusion. Neither protocol A nor protocol B affected the catecholamine concentrations found in the effluent (<.5 nmol/L). The total release was similar to that in control hearts E (Table 2). In contrast, in protocols C and D, after 24 and 36 minutes of ontinuous ischemia, total norepinephrine release increased 25x (P=0.007 vs A) and 68x (P=0.006 vs B), respectively (Table 2).

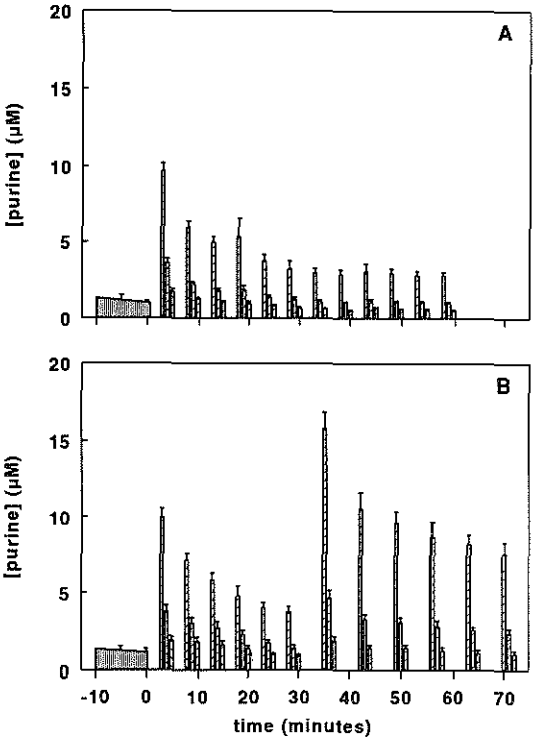

***Figure** 2. Rabbit-heart effluent concentration of adenosine + inosine + hypoxanthine in protocols A and B. For details, see legend to Fig. 1.*

Cardiac adenine nucleotides

Table 2 shows the rabbit-heart total adenine nucleotide levels measured at the end of the experiments. After intermittent ischemia, only the ADP content differed significantly. The control value (protocol E, 5.9±0.3 μmol/g protein) declined in protocols A and B, by 19 and 12%, respectively (P<0.05 vs E). As expected, total adenine nucleotide content had declined more from the control levels E (42.1±1.6 μmol/g) in B (by 17%) than in A (by 6%), but the changes were not statistically significant. Much larger decreases in adenine nucleotides were observed after continuous ischemia (Table 2). The purines found in the effluent accounted for the drop in nucleotides (Table 2).

With ^{31}P-NMR spectroscopy, small, insignificant changes in ATP content could be detected in rat hearts undergoing the repeated ischemia protocols (Table 1). Cardiac phosphorylation potential was computed from the measured ATP and calculated ADP data. Table 3 shows that recurrent short periods of ischemia attenuate the fall in this potential in comparison with nonischemic controls.

Cardiac creatine derivatives

The NMR-studies in rat hearts showed that phosphocreatine decreased reversibly during each cycle of ischemia/reperfusion, whereas inorganic phosphate increased reversibly (Table 1).

At the end of protocol D (36 minutes of continuous ischemia/reperfusion), rabbit hearts had a significantly higher creatine level compared to those submitted to the intermittent ischemia protocol B; however, this pattern was absent in hearts undergoing the less severe protocols C and A (Table 2). The phosphocreatine values after continuous ischemia were (non-significantly) lower than those after intermittent ischemia (Table 2). The inorganic phosphate levels varied only little between the groups studied (from 118±3 to 129±5 μmol/g protein).

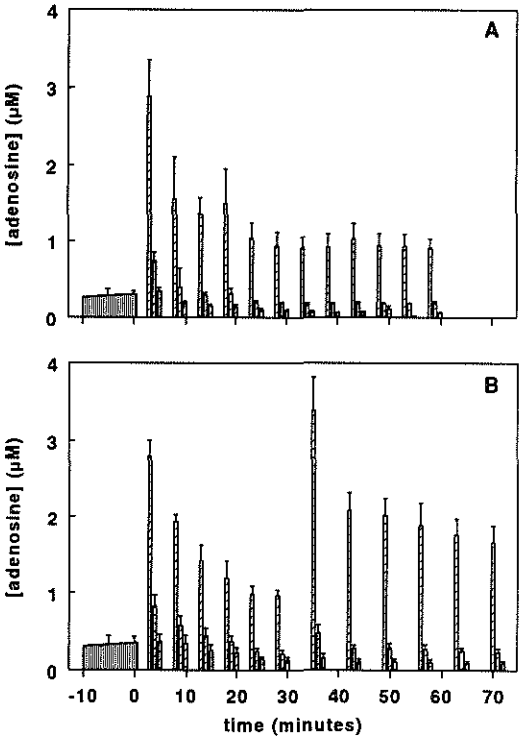

***Figure** 3. Adenosine concentration in rabbit cardiac effluent in protocols A and B. For details, see legend to Fig. 1.*

Tissue levels of glycogen and NAD

Glycogen was measured in freeze-clamped heart homogenates, separated into an acid-soluble and an acid-insoluble fraction. Table 2 shows that the control values in the supernatant fraction (glycogen) declined after protocols A and B (by 44 and 59%, respectively; $P<0.01$). Analysis of the acid-soluble fraction, which consists presumably of the protein-containing low-molecular form of glycogen (proglycogen), revealed a similar pattern. The proglycogen content had declined both in A and B (by 33 and 65%, respectively; $P<0.01$).

Continuous ischemia caused a further decrease in glycogen, but proglycogen rose (Table 2). The ratio glycogen/proglycogen was found to be about 4 in control hearts and those made intermittently ischemic; it

dropped to about 0.5 in hearts which had undergone the continuous ischemia protocols ($P<0.001$).

Intermittent ischemia did not affect the NAD levels significantly, but continuous ischemia induced a drop of some 30% ($P<0.02$ vs control and vs A and B; data not shown).

Discussion

High-Energy phosphate catabolism

Adenosine + inosine + hypoxanthine produced during the reperfusions by the rabbit hearts accounted completely for the decreases seen in total adenine nucleotide content (Table 2). This indicates that hypoxanthine breakdown due to xanthine oxidoreductase does not occur in rabbit heart (see ref. 18 and that salvage of hypoxanthine or inosine to IMP and AMP is relatively unimportant (cf. ref. 29

Using dogs, Lange et al.[30,31] observed that one 15-min coronary occlusion causes severe depression of myocardial high-energy phosphates; however, two additional occlusions do not cause a further decrease when intermittent reperfusion is allowed for 30 minutes. In a similar preparation, Hoffmeister et al.[32] showed that ATP breakdown per 3-minute occlusion period diminished with increasing number of ischemic periods. In our normoxic rat hearts, the cytosolic free ATP phosphorylation potential declined 65% after 1 hour perfusion (Table 3). This is due to a relatively small decrease in free ATP and relatively small increases in inorganic phosphate and free ADP. In contrast, hearts which underwent protocol A showed only a 16% decrease in phosphorylation potential (Table 3). We speculate that the decreased production of adenosine

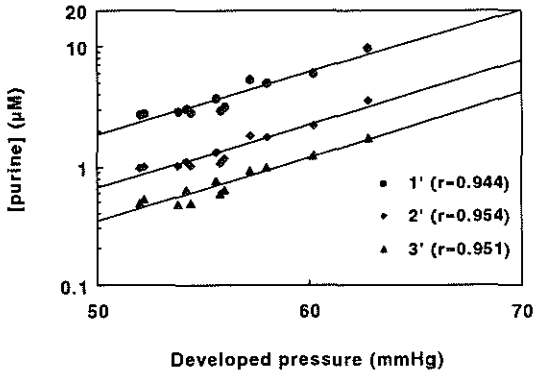

Figure 4. *Correlation between developed pressure before the ischemic periods and effluent purine concentration after those periods (protocol A). 1', 2' and 3' refer to the first, second and third reperfusion minute of rabbit hearts. For further details, see legend to Fig. 1. Mean values are given (SEM can be found in Figs 1 and 2).*

after the first periods of ischemia (Fig. 3) is due to the smaller changes in phosphorylation potential. In fact, Mentzer et al.[33] suggested recently that adenosine exerts its cardioprotective effect via enhanced cellular phosphorylation potential.

The declining production of adenosine after repetitive ischemia, clearly demonstrated in rabbit and rat hearts, apparently contradicts the data of Kitakaza et al.,[34] indicating that preconditioned dog hearts have increased 5'-nucleotidase activity, possibly by activation of protein kinase C, as demonstrated in hypoxic rat cardiomyocytes.[35]

Glycogen breakdown

In freeze-clamped hearts, we found glycogen both in the acid-soluble and acid-insoluble fraction. The former is likely to be high-molecular weight glycogen, whereas the latter presumably consists of proglycogen, a low-molecular-weight form of glycogen with a high protein content.[36] Skeletal muscle proglycogen contains

glycogenin, the priming protein for glycogen biogenesis.[37] Repeated ischemia did not prevent the breakdown of total glycogen; the ratio glycogen/proglycogen remained comparable to that of control hearts (Table 2). In the continuous ischemia protocols, it dropped 8x. These data suggest that glycogen breakdown stops at proglycogen during continuous ischemia. We speculate that adenosine regulates this process.

Different pattern of purine, lactate and norepinephrine release

We induced ischemia in isolated rodent hearts in a manner comparable to that in patients during coronary angioplasty: Repeated short periods in which flow completely ceased. In both situations hearts release ATP-catabolites and lactate during reperfusion (see ref. 38 and Fig. 2). An important observation in our study was the decline in purine production during reperfusion, similar to findings of Reimer et al.[39] They showed that substantial quantities of purines were produced in a first ischemic episode, but much less in later episodes. In our study, a lack of substrate was not responsible for the lower ATP breakdown: Doubling the ischemic period (second part of protocol B) gave rise to even higher peak purine levels during reperfusion. Furthermore, NMR analysis showed that ATP content in rat hearts, subjected to the same protocols, varied little due to 2- or 4-minute periods of ischemia (Table 1). Also in rabbit hearts, quickfrozen at the end of the experiments, contained substantial amounts of nucleotides.

Lactate production showed only minimal variation due to repeated ischemia. More severe ischemia resulted in higher, but constant, peak levels of lactate in the perfusate (Fig. 5).

Rabbit hearts did not release norepinephrine during reperfusion due to short-periods of ischemia, contrasting purine and lactate release. Carlsson et al.[40] reported that rat hearts produced norepinephrine after 7.5 minutes of coronary artery occlusion, whereas Schömig et al.[41] claimed that within the first 10 minutes

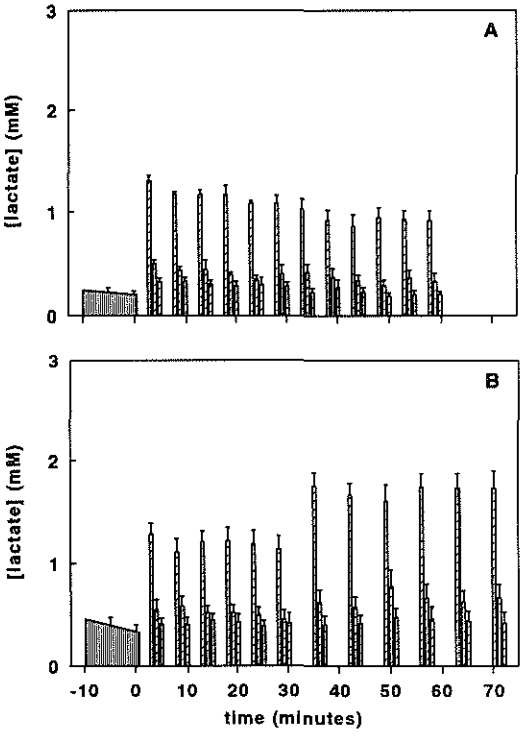

Figure 5. *Lactate concentration in rabbit cardiac effluent (protocols A and B). For details, see legend to Fig. 1.*

of ischemia the myocardium is protected from excessive catecholamine release. We found substantial release of norepinephrine after 24 and 36 minutes of continuous ischemia (Table 2). In our experiments, energy production via anaerobic glycolysis is supposedly sufficient to support the nerve terminal in retaining the transmitter during intermittent, but not continuous ischemia.[42] The differences in metabolite efflux, observed in this study, indicate that the apparent inhibition of ATP breakdown during intermittent ischemia is a rather specific phenomenon.

Correlation between function and metabolism

In the isolated heart, purine release is directly related to the rate of energy consumption, and inversely related to the rate of energy production.[43] In our study, function temporarily decreased drastically during each occlusion, reaching very low values after the more severe part of protocol B. During reperfusion, recovery of developed pressure was also less in B, concomitant with higher ATP-catabolite and lactate release. The correlation between preischemic developed pressure and postischemic catabolite appearance was excellent, at least in protocol A (Fig. 4). If similar mechanisms act in human heart, purine release as a marker for ischemia[38,44] should be used with caution. Apparently not only the degree of ischemia determines the efflux of purines, also the contractile state plays an important role. Kaukinen et al.[45] in fact reported that myocardial catecholamine release seemed more sensitive than purine or lactate production as indicator of myocardial ischemia during bypass surgery.

Intermittent versus continuous ischemia

Geft et al.[46] occluded canine coronary arteries up to 18x for periods of 5, 10 or 15 minutes. They observed creatine kinase release concomittant with morphologic proof of necrosis in 11, 24 and 34% of the dog hearts, respectively. Using morphologic and biochemical criteria, the groups of Jennings[39,47] and Schaper[48,49] concluded on the other hand that dogs tolerate intermittent myocardial ischemia much better than permanent ischemia of the same duration. In our study, with occlusion periods of 2 to 4 minutes up to 12x, intermittent ischemia was also clearly beneficial: The severe deterioration of function, excessive ATP breakdown and profound norepinephrine release observed after continuous ischemia were absent. We believe that adenosine release during short bouts of ischemia is responsible for this difference.

Relevance to preconditioning phenomenon

Did we use preconditioning conditions? In separate experiments rat hearts underwent a sequence of 4x2 minutes of ischemia with 3-minute reperfusions, followed by 30 minutes of total ischemia and 30 minutes reperfusion. A comparison with hearts, only submitted to the second part of the protocol, showed that preconditioning had indeed occurred.[50] Also other workers showed that this protocol elicits the phenomenon.[51]

Did endogenous adenosine lower energy demand? We believe that adenosine released during the first bout of ischemia could have played a crucial role in the anticumulative effect of repeated ischemia on ATP catabolism and function deterioration. The drop in phosphorylation potential during the first short period of ischemia (Table 3) is probably the signal for increased 5'-nucleotidase activity.[33] This gives rise to increased adenosine production,[34] which induces a negative inotropic effect through the A_1-receptor (cf. Fig. 4). The lowered energy demand at the initiation of subsequent short periods of ischemia is responsible for smaller changes in phosphorylation potential, with minimal activation of 5'-nucleotidase. It is conceivable that variations in species, experimental set-up and protocol are responsible for differences in decline of adenosine during repeated ischemia. This could explain the large differences in efficacy of preconditioning studies reported.[33]

What roles do (pro)glycogen and glycolysis play? Whether glycogen depletion, observed during preconditioning[52] and in repetitive ischemia experiments (Table 2), is a protective factor against the deleterious effects of long-term ischemia remains to be firmly established. The correlation between duration of protection by preconditioning and time-course of glycogen resynthesis[52] is striking. Future research will tell us whether the degradation of proglycogen during repeated, but not during continous ischemia (Table 3) is important in this context. How adenosine affects glycolysis is controversial.[33,53,54,55] We speculate that the glycogen/proglycogen ratio, regulated by adenosine, plays a role in preconditioning.[16]

Conclusions

This study demonstrates that intermittent ischemia induces a decline in breakdown of ATP, but not carbohydrate; continuous ischemia exacerbates purine release, attenuates lactate production, and induces

norepinephrine efflux. The correlation between postischemic purine release and preischemic function suggests that the inotropic state determines the fate of energy metabolism during ischemia. Recurrent ischemia does not prevent total glycogen breakdown. However, it seems to attenuate glycogen catabolism, at the expense of proglycogen. We speculate that the glycogen/proglycogen ratio, regulated by adenosine, plays a role in preconditioning.

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