ADENOSINE, CARBOHYDRATES, AND ISCHEMIC PRECONDITIONING

ADENOSINE, KOOLHYDRATEN EN ISCHEMISCHE PRECONDITIONERING

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Aan mijn ouders
en grootmoeder

Contents

1. Ischemic preconditioning

In order to sustain normal function, the heart is dependent on an adequate delivery of oxygen and substrates to the heart to meet the energy demands of the contracting muscle. Myocardial ischemia can be defined as "an imbalance between the amount of oxygen and substrates supplied to the heart and the amount needed to perform normal function" [1]. Ischemic heart disease is one of the major causes of death in the world [2,3] including developing countries [4]. Depending on factors like the amount of collateral flow, myocardial necrosis develops after 10-20 min of ischemia [5]. 'Ischemic preconditioning', one of the 'New Ischemic Syndromes' [6,7], refers to the mechanism that short periods of (nonlethal) ischemia and reperfusion protect the heart from injury during a subsequent prolonged period of ischemia. It was first described in 1986 by Murry et al. [8] (Figure 1). In dog hearts, these investigators showed that infarct size resulting from 40-min coronary artery occlusion was reduced by 75% when ischemia was preceded by four cycles of 5 min coronary artery occlusion and reperfusion (Figure 1).

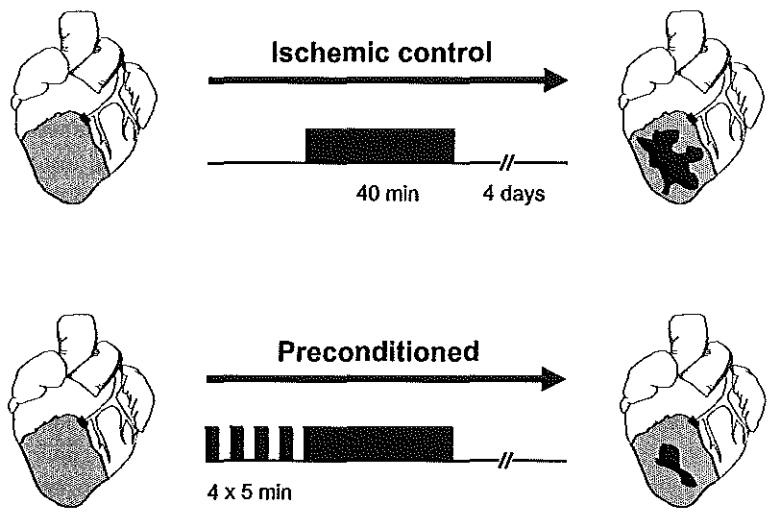

Figure 1. Schematic depiction of the infarct-size limiting effect of ischemic preconditioning as originally proposed by Murry et al. (1986). In this pioneering study, infarct size (dark area in the heart) expressed as a percentage of the area at risk (grey area in the heart) amounted to 29% after 40 min coronary artery occlusion (black bar) and 4 days reperfusion in an in vivo dog model (top panel). However, when 40 min coronary artery occlusion was preceded by four cycles of 5-min occlusion /5-min reperfusion (4 x 5 min), infarct-size was reduced to 7% of the area at risk (75% infarct-size reduction; lower panel). They termed this phenomenon 'preconditioning with ischemia'. (Adapted from Gho [9] with permission.)

After early reperfusion, preconditioning probably is the strongest form of endogenous protection against ischemic injury. It should be kept in mind that preconditioning only delays the development of infarction. Hence, preconditioning is only effective during temporary occlusions and not during permanent or prolonged occlusion (> 60-90 min). The window of protection from preconditioning is bimodal. Classic preconditioning (also called the first window of protection, FWOP) lasts only for 1-3 hours after which protection weans off; a second window of protection (SWOP) reappears 24 hours after

preconditioning lasting 12-72 hours [10]. To date, ischemic preconditioning has been shown to exist in all laboratory animals studied [1] and in other tissues than the heart [11]. Moreover, short ischemia preconditions not only the tissue within but also outside its perfusion territory (remote preconditioning). Remote intracardiac [12] and organ [9,13] ischemic preconditioning protect the heart against sustained ischemia. Apart from reduced infarct size, preconditioning also reduces contractile dysfunctioning [14], arrhythmias [15,16], and apoptosis (programmed cell death) [17,18], depending on the animal model studied. Reduced contractile dysfunctioning in preconditioned hearts most likely reflects reduced infarct size [19-22] since ischemic preconditioning does not reduce stunning [22-24].

2. Mechanism of ischemic preconditioning

Many studies have attempted to elucidate the mechanism of ischemic preconditioning for pharmacological exploitation in humans. The mechanism of ischemic preconditioning includes: 1) triggers, 2) mediators, and 3) end-effectors (Figure 2). The mechanisms of protection of the FWOP and the SWOP are most likely not the same. The FWOP does not involve stunning [25,26], recruitment of collateral vessels [8], and protein synthesis [27] whereas protein synthesis (e.g., heat shock proteins) is important in the SWOP. This thesis will exclusively deal with classic preconditioning (FWOP). Common to all proposed end-effectors of ischemic preconditioning is that they intervene in the sequence of events which lead to the irreversible loss of transmembrane ion gradients resulting in membrane rupture. The events leading to cell death include: 1) inhibition of Na^+/K^+ ATPase activity by ATP depletion and extrusion of protons produced in anaerobic glycolysis by Na^+/H^+ exchange; these two events lead to Na^+-overload, and 2) exchange of Na^+ for Ca^{2+} by the Na^+/Ca^{2+} exchanger leading to calcium overload and membrane rupture (cf. [28]). Ischemic preconditioning is accompanied by attenuation of the rise in intracellular Na^+ and Ca^{2+} [29,30].

2.1. Triggers of ischemic preconditioning

Protection by preconditioning is receptor mediated. Humoral factors released during preconditioning ischemia trigger protection by binding to their respective receptors. These paracrine/autocrine factors include adenosine [31-33], catecholamines [34,35], bradykinin [36], acetylcholine [37], opioids [38-40], endothelin [41,42], and angiotensin II [43] (Figure 2). However, probably only adenosine, bradykinin, and opioids are released in sufficient quantities during transient ischemia to activate their receptors. The importance of each receptor may vary between species. Furthermore, bradykinin is more important during short preconditioning ischemia periods whereas adenosine is more important during preconditioning ischemia of longer durations [44]. Goto et al. [36] proposed the threshold hypothesis based on the great redundancy in receptor systems triggering preconditioning. All receptor systems involved in preconditioning couple to G-proteins and phospholipases to activate protein kinase C (PKC), which may phosphorylate an unknown end-effector. A threshold of PKC activation must be reached to achieve protection (see Chapter 3, Figure 1). Thus, signals from different receptors converge at PKC to reach the threshold activation of the kinase necessary to induce protection. Above the threshold, preconditioning is a graded phenomenon [44].

2.2. Mediators of ischemic preconditioning

As indicated in the previous section, PKC has been proposed to be the mediator of ischemic preconditioning. The PKC hypothesis [45,46] states that receptor signals converge at PKC, which is activated by translocation (of specific isoenzymes) from the cytosol to the sarcolemma. This explains the 'memory' of ischemic preconditioning (ca. 2 hours) because PKC is already activated at the beginning of long ischemia providing protection by phosphorylation of end-effector protein(s) [47]. Preconditioning can be mimicked by PKC activators and abolished by PKC inhibitors [45,46,48-50]. However, PKC activation does not seem to be involved in larger species like the dog [51] and the pig [52,53]. The major problem in these studies is the poor selectivity of the PKC activators/inhibitors. Furthermore, PKC consists of many isoenzymes [54], which may 1) be differently affected by ischemic preconditioning, and 2) may have a different time-frame of activation, which makes the assay for PKC rather difficult. Furthermore, the lack of isoform- and cell-specific PKC assays hampers research in this area [55]. Immunohistochemical [50] and quantitative Western blot analysis [56] revealed that translocation of either PKC-α, -δ, or -ϵ isoforms mediate ischemic preconditioning of rat hearts.

Recent evidence suggest that also tyrosine kinases are involved in the signalling cascade of ischemic preconditioning [16,57-62], either downstream [57,60], upstream [57], or parallel to PKC [61]. In line with the involvement of kinases in preconditioning, inhibition of phosphatases protects rabbit hearts from infarction [63,64].

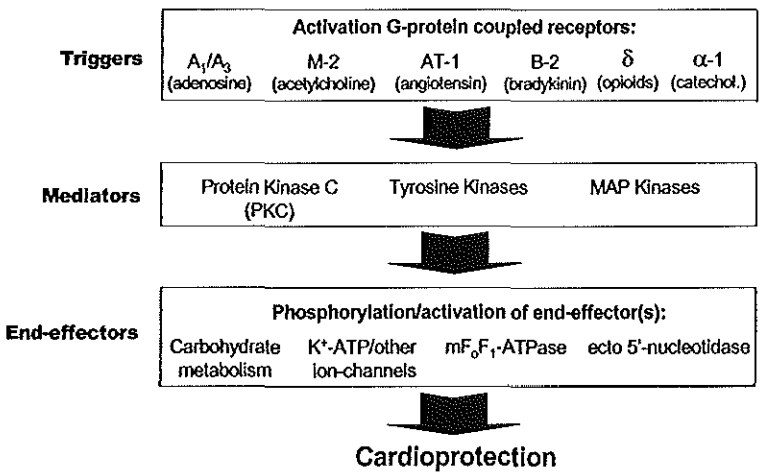

Figure 2. Triggers, mediators, and end-effectors proposed to play a role in the mechanism of ischemic preconditioning.

2.3. End-effectors of ischemic preconditioning

2.3.1. Ion channels

The sarcolemmal K_{ATP} *channel* has been proposed as the final end-effector in ischemic preconditioning [65]. K_{ATP} channel openers mimic and K_{ATP} channel inhibitors abolish ischemic

preconditioning [66-69]. Once activated by PKC, opening of the sarcolemmal K_{ATP} channel shortens the action potential duration and hence, depresses contraction which may protect the heart. However, action potential duration can be dissociated from cardioprotection by preconditioning [70,71]. Moreover, K_{ATP} channel openers and ischemic preconditioning effectively reduce ischemic injury in nonbeating cardiomyocytes, which lack action potentials [72-74]. Alternatively, opening of K_{ATP} channels in the inner mitochondrial membrane by PKC may explain the cardioprotective effects observed with ischemic preconditioning [75-77]. The role of K_{ATP} channels in preconditioning of rabbit and rat hearts [78-80] is controversial but may be related to the type of anesthesia used. However, the observation that expression of recombinant cardiac K_{ATP} channel proteins confers protection against hypoxia/reoxygenation injury in cells [81,82] may indicate that K_{ATP} channel opening may be a valuable tool to pharmacologically protect ischemic myocardium (note: nicorandil, a K_{ATP} channel opener, is now available to treat angina).

Gottlieb et al. [17] and Karwatowska-Prokopczuk et al. [83] suggested that the *vacuolar proton ATPase (VPATPase)* may be a target of PKC in mediating preconditioning. In their hypothesis, preconditioning activates VPATPase-mediated proton efflux (via PKC) resulting in less acidosis and apoptosis [17]. During ischemia, less acidosis by activated VPATPase may reduce Na^+ overload via Na^+/H^+ exchange and consequently Ca^{2+} overload via Na^+/Ca^{2+} exchange [83]. Reduced anaerobic glycolysis and proton production, often observed with preconditioning-induced protection against no-flow ischemia [84], may also contribute to this mechanism.

It has been proposed that ischemic preconditioning is mediated by activation of the *Na^+/H^+ exchanger* and/or the *Na^+-K^+/2Cl^- cotransporter* during global ischemia, reducing proton overload [85,86]. This hypothesis is in sharp contrast to the substantial body of evidence indicating that pharmacological inhibition of Na^+/H^+ exchange during ischemia is protective whereas stimulation of the exchanger is detrimental [86-96]. Preconditioning protection has been dissociated from Na^+/H^+ exchange activity [89,92,93,95]. Moreover, inhibition of Na^+/H^+ exchange appears additive to the protection afforded by ischemic preconditioning [89,95]. Thus, these results argue against the hypothesis that ischemic preconditioning is mediated by modulating Na^+/H^+ exchange activity.

2.3.2. mF_oF_1-ATPase

Within seconds after the onset of ischemia and the cessation of the electrochemical gradient across the inner mitochondrial membrane, the mitochondrial ATP-synthase changes into an ATPase (mF_oF_1-ATPase). Most of the ATP hydrolyzed during global ischemia is due to mF_oF_1-ATPase activity [97]. Preconditioning-induced inhibition of the mF_oF_1-ATPase during ischemia has been proposed to result in an improved energy balance [84]. The few studies conducted this far have obtained results in favour [98] and against [99-101] this hypothesis.

2.3.3. 5'-nucleotidase

Kitakaze's group reported that ischemic preconditioning increases ecto-5'-nucleotidase activity and therefore adenosine production, which protects the heart [102]. They observed more adenosine in coronary sinus blood of preconditioned dog hearts [102]. However, most other studies using microdialysis techniques [103-105] or analysis of coronary effluent of isolated hearts ([105], see also Chapter 6, Figure 4a) showed that adenosine production is actually decreased during long ischemia and not increased. Moreover, ecto-5'-nucleotidase activity is unrelated to infarct size in the canine heart

[106,107]. These observations make it very unlikely that preconditioning is mediated by increased ecto-5'-nucleotidase activity and adenosine production during ischemia.

2.3.4. Carbohydrate metabolism

The role of preconditioning on glycolysis/glycogenolysis during ischemia, as well as its relationship with ischemic injury, is unclear. Anaerobic glycolysis supports cell function by means of ATP production, but accumulated glycolytic end-products (e.g., lactate, and protons originating from ATP hydrolysis [108]) may determine the extent of ischemic damage; consequently, many studies have attempted to relate the beneficial effects of ischemic preconditioning to cardiac glycogen content or glycolytic rates.

Ischemic preconditioning of the heart reduces its preischemic glycogen content and slows glycogen utilization during stop-flow ischemia resulting in less intracellular lactate accumulation [8,29,30,84,99,109-116]. Wolfe et al. [117] correlated the decrease in infarct size caused by preconditioning with glycogen depletion before sustained ischemia and with the attenuation of intracellular acidosis during ischemia. Moreover, loss of the protective effect parallelled the time course of glycogen recovery before sustained ischemia [117] and increasing preconditioning ischemia time resulted in glycogen depletion and infarct-size reduction, both described by an exponential declining curve [114]. The glycogen hypothesis states that reduced preischemic glycogen in preconditioned hearts is protective insofar as it reduces glycolytic rates and consequently glycolytic catabolite accumulation during subsequent prolonged ischemia, despite reduced ATP production. This would lead to less intracellular overload of sodium and calcium [29,30] and preservation of membrane integrity. The advanced technique of ^{13}C-NMR allows to follow myocardial glycogen within one heart throughout the experiment. Both the ^{13}C-NMR studies of Weiss et al. [118] and our results [119] indicate that preconditioning depresses glycogenolysis during ischemia by reducing glycogen phosphorylase activity [118]. However, others observed no relationship between functional recovery after prolonged ischemia and preischemic glycogen levels [120,121], or glycogen depletion during ischemia [122,123].

In contrast to no-flow ischemia, improved uptake and metabolism of glucose by the heart during low-flow ischemia reduces myocardial injury [28,124-132], a notion known as the glucose hypothesis [133]. Furthermore, glycolysis-from-glucose seems more effective than glycogenolysis in protecting hearts against myocardial ischemia [125,134]. The reason for the superiority of glycolysis over glycogenolysis in reducing ischemic injury is not clear either. Compartmentation of the cytoplasm with respect to intermediary metabolism has been suggested by several studies. In porcine carotid artery rings, separate pathways for glycolysis and glycogenolysis exist [135,136]. These studies indicate that under aerobic conditions, glycogenolysis provides substrate for oxidative phosphorylation, which is related to isometric tension development whereas lactate derived from glycolysis is related to the Na^+/K^+-ATPase. However, in situations of decreased oxygen consumption like ischemia [135] and phenytoin treatment [136], mixing of pathways occurs and hence, lactate also originates from glycogen in these conditions. ^{13}C-NMR studies applied to vascular smooth muscle cells reach the same conclusions [137,138]. Although glycolysis and glycogenolysis were both active during aerobic perfusion, no lactate was derived from glycogen [137]; when oxidative metabolism was blocked by cyanide, mixing of both pathways occurred. Provision of the exogenous substrates acetate and pyruvate changed glycolysis and glycogenolysis independently [138], consistent with different regulation and compartmentation of these pathways. This is in line with recent data obtained in aerobic perfused rat hearts [139,140] showing that

glycogen is preferentially oxidized, hardly contributing to lactate production. In heart as in vascular tissue, glycogen contributes significantly to anaerobic ATP production in situations of severe ischemia [28]. Evidence from several studies indicates that glycolysis is preferentially used to support ionic homeostasis in cardiac cells, providing ATP for ATP-dependent ion pumps such as the K_{ATP} channels [141,142], the sarcoplasmatic reticulum Ca^{2+}-ATPase [143], and the Na^+/K^+-ATPase [144]. This may be related to the association of key glycolytic enzymes to ion pumps inside the cell [142,145]. Thus, if the accumulation of the potentially toxic products of anaerobic glycolysis is prevented by sustaining a moderate flow to the heart, sustained glycolysis-from-glucose during ischemia may support ionic homeostasis (read: less Ca^{2+} overload) and thereby reduce ischemic injury. Not many studies examined preconditioning-induced protection against low-flow ischemia. So far, only our studies (Chapters 6 and 7) and that of Janier et al. [146] indicated that preconditioning protection against underperfusion may be mediated by increased glucose uptake during ischemia.

3. Aim and organization of this thesis

3.1. Aim of this thesis

This thesis investigates the mechanism of ischemic preconditioning with respect to adenosine as a trigger and myocardial carbohydrate metabolism as the end-effector of preconditioning protection (Figure 2). We hypothesize that:

(1) Adenosine, released during preconditioning ischemia, triggers ischemic preconditioning by binding to the adenosine A_1 and/or A_3 receptors located on the myocardial membrane;

(2) Stimulation of adenosine receptors finally will result in modification of myocardial carbohydrate metabolism during ischemia. Specifically, we hypothesized that adenosine receptor activation will result in: (i) an increase in myocardial glucose uptake and metabolism, which may be beneficial during low-flow ischemia, and (ii) inhibition of detrimental glycogen breakdown.

In order to answer these questions, we used the isolated Langendorff perfused rat heart as experimental model. This model allowed us to carefully monitor biochemical parameters relevant to our research questions with classical biochemistry and NMR spectroscopy. In addition, we also studied humans undergoing pacing stress testing.

3.2. Organization of this thesis

In Chapter 2, an overview of myocardial energy metabolism is given with special emphasis on nucleotide catabolism giving rise to the formation of adenosine, and myocardial carbohydrate metabolism. In Chapter 3, we review some controversies in the very active field of research of ischemic preconditioning. Chapter 4 deals with the effect of ischemic preconditioning on myocardial carbohydrate metabolism during no-flow ischemia. In Chapter 5, we assessed the effectiveness of acadesine, a drug enhancing endogenous adenosine accumulation, in reducing ischemic injury in humans undergoing pacing stress testing. In Chapter 6, we investigated whether ischemic preconditioning protected against severe low-flow ischemia, a model more relevant to clinical practice. In addition, we assessed the contribution of proglycogen, a stable intermediated in (macro)glycogen synthesis, to glycogenolytic flux

and reduced injury. The hypothesis that ischemic preconditioning is mediated by increased exogenous glucose use during low-flow ischemia, an event triggered by adenosine, is the subject of Chapter 7. In Chapter 8, we investigated whether adenosine A_1 receptor activation is involved in preconditioning of rat hearts. We also assessed whether ischemic preconditioning is mediated by reduced glycogenolysis during ischemia (the glycogen hypothesis). Chapter 9 deals with the effectiveness of adenosine A_1 and A_3 agonists in reducing apoptosis and contractile dysfunction arising from underperfusion. Finally, a general discussion and the main conclusions of the studies presented in this thesis will be given in Chapter 10.

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2

Myocardial energy metabolism

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1. Regulation of myocardial fuel metabolism

In biological systems, free energy is needed to drive exogenous reactions like muscle contraction, active transport of ions and macromolecules, and biosynthesis [1]. Free energy obtained from the oxidation of foodstuffs is transferred to adenosine triphosphate (ATP) in a series of reactions called *energy metabolism* (Figure 1). Thus, ATP is used by the cell as an immediate donor of free energy, thereby sustaining normal homeostasis.

1.1. Normoxia

In normoxic myocytes, ATP utilization and production are tightly coupled. ATP is synthesized for 90% by oxidative phosphorylation in the mitochondria and for 10% by anaerobic glycolysis [1,2]. Depending on factors like the nutritional and exercise state, free fatty acids (FFA; predominantly oleic acid and palmitic acid), the carbohydrates glucose and lactate, and to a lesser extent ketone bodies and amino acids compete as substrates in energy metabolism [3-7].

Regulation of FFA uptake and oxidation [8] is mainly determined by their arterial concentration [9]. Thus, after a high fat meal or during ischemia-induced high catecholamine activity [10], conditions that stimulate lipolysis, plasma FFA levels increase [11] with concomitantly higher rates of oxidation [7]. The uptake of glucose into the cell is regulated by factors like plasma glucose levels and (neuro)humoral activity. Insulin enhances cellular glucose uptake by stimulating the GLUT4 transporter, and the glycolytic enzymes hexokinase (HK) and glycogen synthase [12]. Also, growth hormone, epinephrine, cortisol [2], increased heart work, and tissue hypoxia (see next paragraph) stimulate glucose uptake and utilization. Myocardial carbohydrate and FFA oxidation are inter-related by the so-called glucose-fatty acid cycle [2,13]. High rates of fatty acid oxidation inhibit glucose uptake, lactate uptake, and glucose oxidation [2,13-15] through changes in the levels of: 1) cytosolic citrate (which inhibits phosphofructokinase; PFK), 2) mitochondrial acetyl-CoA and NADH (which regulate pyruvate dehydrogenase; PDH), and 3) cytosolic malonyl-CoA [7,16,17]. Inversely, high circulating levels of glucose and insulin inhibit adipose tissue lipolysis resulting in low plasma FFA levels and removal of the inhibition of glycolysis by high rates of FFA oxidation. Also, glucose may directly inhibit fatty acid oxidation [18] via acetyl-CoA carboxylase and 5'-AMP-activated protein kinase [8,19].

The regulation of myocardial energy metabolism has been dealt with by many authors [3,4,6-8,17]. In the classic biochemical view, metabolic control of the flux through a pathway is exerted at discrete sites called flux-generating steps or regulatory reactions [20,21]. These rate-limiting steps in a linear reaction sequence are non-equilibrium reactions saturated by pathway substrate and subjected to allosteric control. For example, rate limiting steps are the reactions catalyzed by PFK and HK (for the glycolytic pathway), and 2-oxoglutarate dehydrogenase and citrate synthase (for the Krebs cycle) [21,22]. This rather static and dogmatic view of metabolic control (see [23]) has been challenged by the more dynamic 'metabolic control theory' [24-28]. According to this theory, metabolic control of a flux is not determined by unique enzymes but is rather distributed over several steps in many metabolic sequences. The participation of each enzyme in the control of a flux through a pathway is described by the 'flux control coefficient' of that enzyme. Metabolic control analysis applied to normoxically beating isolated rat hearts [29] revealed that glucose uptake and phosphorylation dominated (75%) the control of glycolytic flux. Furthermore, control varied with changing conditions.

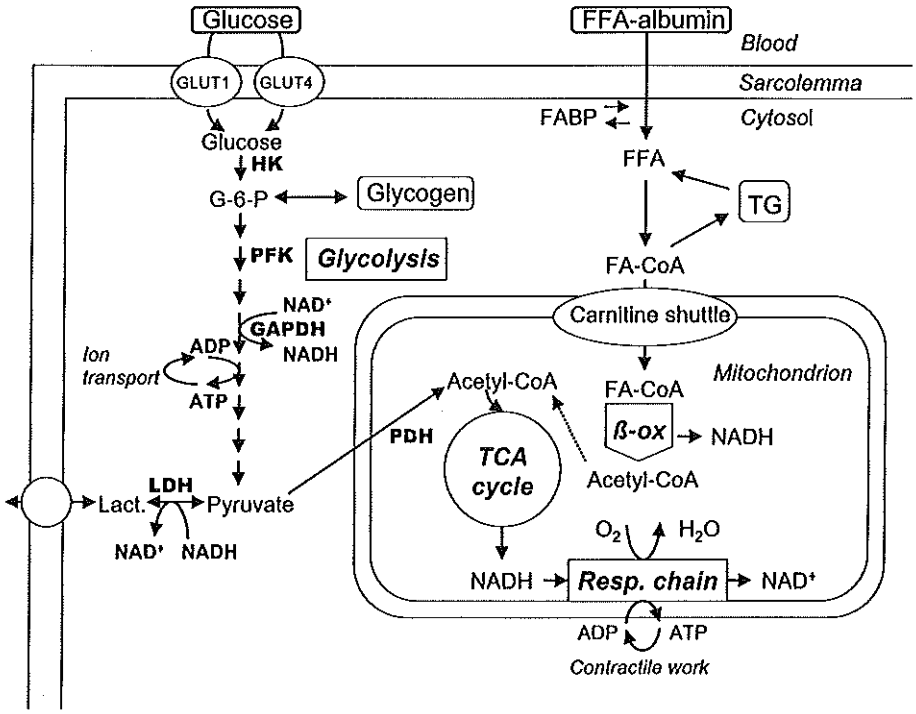

Figure 1. Schematic overview of myocardial energy metabolism. The heart primarily uses glucose and free fatty acids (FFA) as substrates. Myocardial energy metabolism can be divided into three stages: 1) The formation of acetyl-CoA from pyruvate and free fatty acids, 2) the formation of NADH and $FADH_2$ in the tricarboxylic acid cycle (TCA cycle), and 3) the oxidation of NADH and $FADH_2$ in the electron transport chain (respiratory chain), which finally will result in built up of the proton gradient across the inner mitochondrial membrane and subsequent generation of ATP by action of the mitochondrial F_oF_1-ATPase. Pyruvate is formed in the cytosol from the following carbohydrate sources: 1) circulating lactate (lact.), 2) blood glucose (glycolysis) and 3) intracellular glycogen (glycogenolysis). Extracellular glucose is taken up by action of specific glucose transporters (GLUT1 and GLUT4) in the plasma membrane and is directly phosphorylated to glucose 6-phosphate (G-6-P) by hexokinase (HK), which traps glucose into the cell. Ischemia and insulin result in the translocation (and possibly also activation) of GLUT transporters from the cytosol into the sarcolemmal membrane. Glucose 6-phosphate can be used for either glycogen synthesis (via glycogen synthase) or the formation of pyruvate in a series of reactions called glycolysis. An important regulatory step in glycolysis is catalyzed by the enzyme phophofructokinase (PFK). Under anaerobic conditions (ischemia), pyruvate is reduced by NADH to form lactate in a reaction catalyzed by lactate dehydrogenase (LDH). NAD^+, needed in the glycolytic reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase (GAPDH), is generated in the reduction of pyruvate to lactate and sustains the continued operation of glycolysis in anaerobic conditions. Glycogen, a dense and readily mobilized storage form of glucose, is present in the cytosol in the form of granules. Thus, glycogen serves as a reservoir of glucose in conditions of severe ischemia (no-flow ischemia) or sudden changes in work load. Under aerobic conditions, pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase (PDH) in the mitochondrion. It has been proposed that ATP produced in glycolysis is compartmentalized and mainly used to drive ATP-dependent ion pumps supporting membrane integrity. Fatty acids are predominantly supplied by FFA-albumin in blood and intracellular triglyceride (TG) stores. Once inside the cell, fatty acids bind to the heart-type fatty acid-binding proteins (FABP) and are transported to the mitochondrion for conversion to fatty acyl-CoA (FA-CoA) by acyl-CoA synthase. Fatty acyl-CoA is transferred into the mitochondria by the carnitine shuttle, which consists of a complex of enzymes named carnitine palmitoyltransferase 1, carnitine:acylcarnitine translocase, and carnitine palmitoyltransferase 2. In each cycle of the mitochondrial ß-oxidation (ß-ox), long chain acyl-CoA is shortened by 2 carbon atoms to produce acetyl-CoA. Hence, fatty acids can only be oxidized in contrast to glucose residues, which can also be anaerobically degraded. Acetyl-CoA from both carbohydrate and fatty acid sources enters the TCA cycle resulting in the formation of 3 NADH and 1 $FADH_2$, which are reduced in the respiratory chain to form ATP with the use of oxygen. ATP obtained from the oxidation of these substrates is mainly used to deliver contractile work.

1.2. Ischemia

Depending on the degree of residual flow during ischemia, oxidative phosphorylation and Krebs cycle activity are almost completely inhibited [30] due to a lack of oxygen as electron acceptor and subsequent rise in mitochondrial NADH/NAD^+ and $FADH_2$/FAD ratios, respectively. Anaerobic glycolysis is accelerated to deliver ATP [2] due to an increase in the number of GLUT transporters in the sarcolemmal membrane [31,32] and by relief of feed-back inhibition by citrate formed during FFA oxidation. Substrate metabolism during ischemia depends on the degree of residual flow; only exogenous glucose (low-flow ischemia) and endogenous glycogen can be utilized by the severely ischemic heart (Figure 1). The end-products of anaerobic glycolysis are lactate and protons resulting from glycolytic ATP turnover [33] (Figure 1). However, anaerobic ATP production is insufficient to meet the energy demands of the contracting heart and cellular homeostasis is disturbed as ischemia is continued [34]. Thus, myocardial ischemia results in a depletion of high energy phosphates and glycogen stores, an increase in ADP, inorganic phosphate (P_i), and creatine (Cr), and accumulation of lactate and protons [35,36]. If myocardial blood flow to the heart is not restored in time (e.g., by means of thrombolytic therapy), myocardial necrosis (via oncosis or apoptosis) will finally develop.

Glycolytic flux during ischemia may be mainly determined by PFK and, during more severe ischemia, by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) due to accumulation of glycolytic metabolites [2,37]. However, crossover analysis of glycolytic intermediates showed that glycolytic rate during low-flow ischemia is mainly determined by the rate of glucose delivery (substrate supply) and not by enzyme inhibition [38]. Furthermore, although absolute glucose delivery is decreased during ischemia, the capacity to extract glucose is greatly increased [7,38,39].

High rates of FFA oxidation occur during reperfusion following ischemia [8,40,41], diabetes [42] and most ischemic heart diseases [11,43]. Also, glucose intolerance has been observed in patients after cardiac infarction [44]. It has been proposed that shifting substrate utilization away from FFA metabolism and toward glucose metabolism may be an effective strategy to reduce ischemic injury and to treat ischemic heart disease [7,17,39,45-48]. The mechanism has been proposed to involve 1) increased glycolysis-from-glucose during ischemia supporting membrane integrity and ionic homeostasis ([7,39]; see also Chapter 1), 2) increased coupling of glycolysis and glucose oxidation resulting in less production of protons [17,48], and 3) replenishment of moiety-conserved cycles [49]. A shift in energy metabolism can for instance be achieved by provision of glucose-insulin-potassium [49-53] or pharmacological intervention [17,48], a notion known as 'metabolic therapy' or 'metabolic management of ischemic heart disease' [19,49,54-57].

2. Adenine nucleotide metabolism

2.1. Enzymatic machinery

Figure 2 depicts in a schematic way how the high energy phosphate ATP breaks down to purines, including the important regulatory metabolite adenosine. Figure 3 shows the pathways present in heart concerning energy metabolism. During normoxia, ATP is produced from ADP in the mitochondria by oxidative phosphorylation. The triphosphate is transported to the cytoplasm via the adenine nucleotide

translocator, and subsequently shuttled to creatine. Creatine phosphate, in turn, produces ATP, which is needed for contraction. Under physiological conditions the purine-producing enzymes are hardly active. The enzymes involved include 5'-nucleotidases, which convert AMP and IMP to adenosine and inosine, respectively (enzymatic reactions 7, Figure 3), and adenosine deaminase, for the breakdown of adenosine to inosine (reaction 9, Figure 3). We suppose that man lacks cardiac inosine kinase activity, based on our observations in rat heart [58]. We showed that human heart contains purine nucleoside phosphorylase that converts inosine to hypoxanthine (reaction 11), but not its pyrimidine counterpart, responsible for conversion of uridine to uracil [59]. The latter contrasts findings in rat heart [59]. The activity of xanthine oxidoreductase (reactions 12) is minimal in the human heart, but several other species show appreciable activity of this enzyme [58]. Human heart may contain an inactive form of the enzyme (see section 2.2).

We speculate that during normoxia AMP is not dephosphorylated (adenosine pathway), but converted to IMP by AMP deaminase (reaction 4; IMP pathway), or to ADP by adenylate kinase (reactions 2). Thus, the myocytes would conserve their nucleotide pool, save energy for contraction, and maintain the equilibrium between energy supply and energy demand. However, during hypoxia/ischemia and increased heart work, increased P_i inhibits AMP deaminase and hence, the adenosine pathway becomes more important [60,61]. Thus, in normoxic tissue, purine catabolism predominantly follows the IMP pathway, which conserves adenine nucleotide pools via salvage pathways. Switching to the adenosine pathway during ischemia would be advantageous since adenosine acts as a retaliatory metabolite (negative feedback modulator) restoring the imbalance between oxygen demand and supply (see section 3). Moreover, hydrolysis of AMP to adenosine reduces ischemic injury by improving the free energy of ATP hydrolysis (via increased PCr), which might delay or prevent calcium overload [62]. Only a small fraction of adenosine produced during ischemia will be exported out of the cell by way of (facilitated) diffusion, the remainder is directly recycled to AMP in the purine salvage pathways or bound to S-adenosylhomocystein

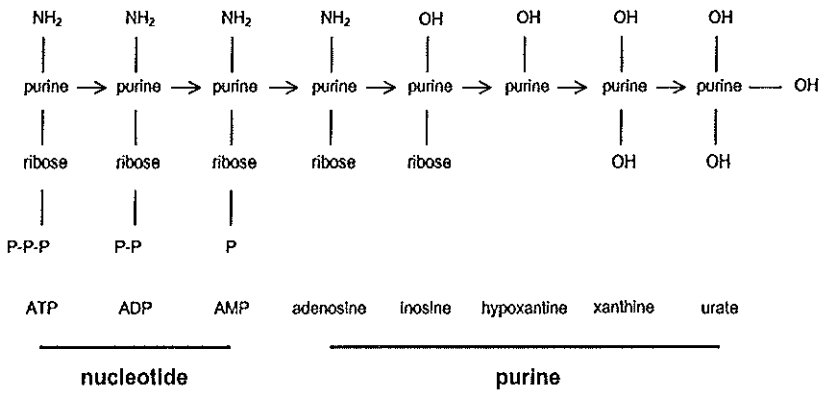

Figure 2. Schematic representation of adenine nucleotides and purines. Because of the energy contained in their phosphate bonds, ATP and ADP are called "high-energy phosphates"; also creatine phosphate falls in this category. Of the compounds depicted, ATP and adenosine seem to be the most important in the heart: The triphosphate fuels the contractile apparatus, whereas adenosine regulates various processes via receptors in the cell membrane. The purine nucleotides ATP, ADP, IMP and AMP are negatively charged and do not readily cross the cell membrane in contrast to the non-phosphorylated purines.

Animal studies show that during aging the catabolic pathways become more prominent [63]. For adenine nucleotide synthesis, the following pathways are available: i) direct phosphorylation of adenosine by adenosine kinase (reaction 8); ii) salvage of purines by hypoxanthine guanine phosphoribosyltransferase (reaction 10); iii) de novo synthesis from small molecules, such as glutamine (see Figure 3); iv) adenine phosphorylation by adenine phosphoribosyltransferase (reaction 3). Phosphorylation of adenosine by adenosine kinase is presumably the fastest. The purine salvage pathway is relatively slow and becomes even slower during aging [64]. De novo synthesis of nucleotides is the slowest and is energetically more costly than salvage synthesis. It is unlikely that adenine salvage contributes significantly to human-heart adenine nucleotide synthesis, because tissue and plasma levels of adenine are very low.

2.2. Cellular distribution of enzymes, involved in energy metabolism

The enzymes involved in purine metabolism are less active in human than in rat heart, but crucial pathways of intracellular adenosine production and rephosphorylation are comparable [65]. The catabolic pathway through adenosine deaminase and nucleoside phosphorylase takes mainly place in vascular endothelium and pericytes, both in humans and various other species [59,65,66]. Furthermore, AMP deaminase capacity is low in human heart and cultured human umbilical vein endothelial cells, and adenosine produced in the latter cells is immediately recycled via adenosine kinase [65,67].

The localization of xanthine oxidoreductase in myocardium is dependent on the animal species studied; the enzyme is present in dogs and rats but absent in rabbits and pigs [58]. Xanthine oxidoreductase seems absent from human myocardium [58,68]. Its localisation in the endothelium is controversial. In human umbilical vein endothelial cells, xanthine oxidoreductase activity is minimal [67] while it is present in capillary endothelial cells and vascular smooth muscle cells of human myocardium [69]. A recent study shows that xanthine oxidoreductase is present not only in the cytoplasm but also on the outer surface of human endothelial and epithelial cells in culture [70] and also is involved in the generation of nitric oxide during ischemia independent of nitric oxide synthase activity [71,72]. Because myocytes do not produce large amounts of hypoxanthine via nucleoside phosphorylase, hypoxanthine from endothelial cells has to be transported to the myocytes before incorporation into IMP by hypoxanthine guanine phosphoribosyltransferase.

Thus, the degradation of purines beyond adenosine occurs mainly in the vascular endothelium [73] and the metabolism to xanthine and urate is species dependent with high xanthine oxidoreductase activity in rat, mouse, and guinea pig and low activity in rabbits and humans [58].

2.3. Subcellular enzyme location

Creatine kinase has a bimodal localization, i.e., mitochondrial and cytoplasmic. Adenylate kinase, which catalyzes the reaction AMP + ATP $\rightleftharpoons$ 2 ADP, is also present in the mitochondria and the cytosolic compartment. AMP dephosphorylation takes place by 5'-nucleotidases in the plasma membrane and the cytoplasm [74]. Two soluble forms exist: the AMP- and IMP-preferring 5'-nucleotidases. The alternative pathway, AMP deamination, is very active in skeletal muscle; however, substantial activity is also present in the supernatant fraction of human- and rat-heart homogenate [75]. In addition, this compartment of the cell contains the other enzymes involved in purine catabolism [75]. Also adenosine

kinase, responsible for rephosphorylation of adenosine, is present in the cytoplasm [75]. Thus, the major part of purine catabolism takes place in the soluble compartment of the cell.

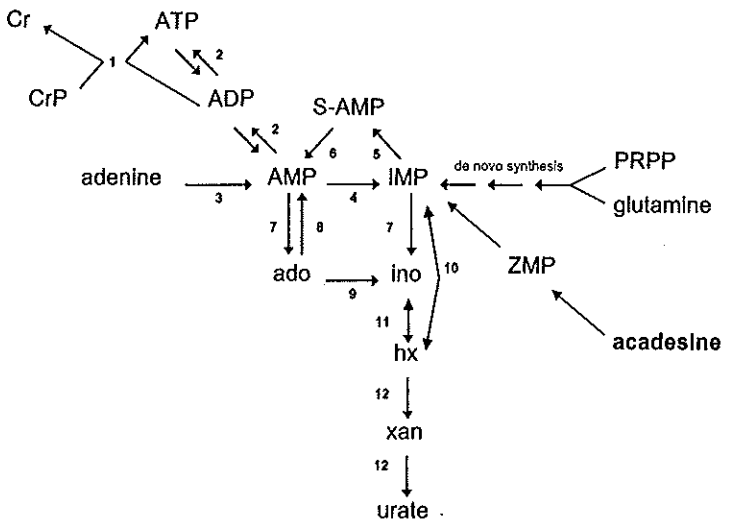

Figure 3. Scheme depicting enzymatic reactions involved in myocardial energy metabolism. During ischemia breakdown becomes predominant. This results in the end product urate (uric acid) in rat heart, and the purine hypoxanthine in human heart. The latter lacks the enzyme necessary for hypoxanthine to urate conversion (xanthine oxidoreductase), but may contain an isozyme of this enzyme reacting with NADH and producing superoxide. 1 = creatine kinase, 2 = adenylate kinase (myokinase), 3 = adenine phosphoribosyltransferase, 4 = AMP deaminase, 5 = adenylosuccinate synthase, 6 = adenylosuccinate lyase, 7 = 5'-nucleotidases, 8 = adenosine kinase, 9 = adenosine deaminase, 10 = hypoxanthine guanine phosphoribosyltransferase, 11 = purine nucleoside phosphorylase, 12 = xanthine oxidoreductase. In addition, various ATP splitting enzymes (ATPases) are responsible for ATP breakdown. Abbreviations: ado, adenosine; Cr, creatine; CrP, creatine phosphate; hx, hypoxanthine; ino, inosine; PRPP, -5-phosphoribosyl-1-pyrophosphate; S-AMP, S-adenylosuccinate; xan, xanthine; ZMP=AICA ribotide. The adenosine raising drug acadesine is converted to ZMP when taken up in cells.

2.4. Enzyme regulation

The size of the cardiac AMP pool depends on the breakdown of the high-energy phosphates ATP and ADP, as well as on the conversion of AMP to adenosine or IMP. Adenine nucleotide breakdown takes place when the supply of ATP cannot meet the tissue demand, for example during ischemia or hypoxia. AMP breakdown is dependent on the activity of the cytosolic enzymes 5'-nucleotidase and AMP deaminase. The AMP-preferring 5'-nucleotidase is activated by ADP, the IMP-preferring form by ADP or ATP; both forms are inhibited by inorganic phosphate. Our earlier work on porcine heart suggests that the myocardial energy charge regulates AMP deaminase. AMP deaminase is also inhibited by inorganic phosphate [60,61]. In rat heart, AMP deaminase activity is several fold higher than 5'-nucleotidase activity [75,76] but the affinity for inorganic phosphate inhibition is lower in the former enzyme [60,61]. This explains why the IMP pathway is dominant during normoxia but is inhibited during ischemia when inorganic phosphate levels rise, which results in adenosine production. Membrane

phospholipids, in addition to ATP, activate cardiac AMP deaminase [77].

2.5. High-energy phosphates and purines as markers of ischemia

De Jong et al. [78] reviewed the use of ATP, phosphocreatine and purine measurements to characterize the metabolic status of the heart. These assays could predict rejection of transplants. In biopsies and plasma samples, high-performance liquid chromatography offers the possibility to estimate these compounds with a high degree of accuracy and sensitivity [79,80]. Nuclear magnetic resonance spectroscopy has the advantage of non-invasive assessment of high-energy phosphates, inorganic phosphate and pH, but this technique is insensitive and expensive. The application of magnetic resonance spectroscopy in clinical cardiology is still in its infancy.

Smolenski et al. have demonstrated release of adenylate degradation products from the human myocardium during open heart surgery [81]; also uridine, a breakdown product of pyrimidine metabolism, accumulated under these conditions [59]. In patients undergoing percutaneous transluminal coronary angioplasty, coronary sinus adenosine is a more sensitive indicator of ischemia than lactate, hypoxanthine, and uric acid [82]. The cumulative release of purines during low-flow ischemia may serve as a marker for the degree of ischemic injury as it is related to the functional recovery upon reperfusion in isolated rat hearts (see Chapters 6 and 9).

3. Adenosine receptors and cardiovascular function

3.1. Adenosine receptor classification and distribution

Adenosine receptors belong to the family of P_1-purinergic receptors [83-85]; they are G-protein coupled receptors with 7 hydrophobic, membrane-spanning domains (Table 1). Adenosine/P_1-purinergic receptors can be further divided into A_1, A_{2A}, A_{2B}, and A_3 receptors according to the IUPHAR Committee on Receptor Nomenclature and Drug Classification [83,84]. A_1 and A_3 receptors mediate inhibition of adenylate cyclase via a guanine nucleotide binding inhibitory protein ($G_{i/o}$) whereas A_2 receptors couple to guanine nucleotide binding stimulatory protein (G_s), which stimulates adenylate cyclase and cAMP formation (Table 1). However, adenosine receptors couple to more signal transduction pathways (Figure 4).

Adenosine receptors are especially abundant in the central nervous system; in lesser numbers, they are found in many other tissues including the heart [85]. Adenosine A_1 receptors are located on cardiomyocytes and vascular smooth muscle cells whereas A_2 receptors are present on the endothelial and vascular smooth muscle cells (Figure 4). The density (B_{max}) of the A_1 receptor is higher in atrial than ventricular tissue [86]. Transcripts of the newly discovered A_3 receptor have been found in rat [87], chick [88], and human [89] hearts. The A_3 receptor is present on ventricular myocytes and not on atrial tissue [90]. Furthermore, in contrast to the A_1 receptor, there is no evidence of the presence of the A_3 receptor in SA and AV nodes [91].

Table 1. Properties of cardiovascular adenosine receptor subtypes

	Subtype		
	A_1	**$A_{2A/B}$**	**A_3**
G protein coupling	G_i/G_o	G_s	G_i/G_o
Effectors	AC↓, PLC↑, I_K↑, I_{Ca}↓, I_{KATP}↑	AC/GC↑, PLC↑, I_{KATP}↑	AC↓, PLC↑, I_{KATP}↑
Molecular weight (kD)	37	45/36	36
K_i-adenosine (μM; rat)	0.1-10	1000-10,000	?
K_i-adenosine (μM; human)	30	?	700
K_i CCPA (μM; rat)	0.4	3900	?
K_i IB-MECA (μM; rat)	54	56	1.1
K_i 8-SPT (μM; rat)	2,600	15,300	≈10,000
Physiological effects	- anti-adrenergic action - ischemic preconditioning - glycolysis↑↓ - negative chronotropy - negative dromotropy - negative inotropy	- vasodilatation - platelet adherence/aggregation to endothelium↓ - adherence activated neutrophils to endothelium and O_2^- release↓	- ischemic preconditioning

AC=adenylate cyclase; GC=guanylate cyclase; PLC=phospholipase C; I_K=inwardly-rectifying potassium channel current; I_{KATP}=ATP-sensitive potassium channel current; I_{Ca}=L-type calcium channel current; G_i/G_o=inhibitory G protein; G_s=stimulatory G protein; CCPA=2-chloro-N^6-cyclopentyladenosine; 8-SPT=8-(*p*-sulfophenyl) theophylline; IB-MECA=N^6-(3-iodobenzyl)-adenosine-5'-*N*-methyl-uronamide.

3.2. Physiological effects of adenosine

The ATP catabolite adenosine is released in response to a decrease in the oxygen supply/demand ratio occurring during ischemia and exercise [92], and by this mechanism signals changes in myocardial energy metabolism [73,93]. The cardiovascular actions of adenosine are aimed at restoring oxygen supply/demand balance ([94]; Table 1) and homeostasis, matching energy consumption (A_1, A_3 effects) with energy delivery (A_2 effects). Adenosine induces vasodilatation, results in negative chronotropic and dromotropic effects, reduces atrial contractility, attenuates the stimulatory actions of catecholamines in ventricular tissue, inhibits norepinephrine release from stimulated sympathetic nerve fibers, and attenuates vascular ischemic injury [73,94-96] (Table 1). Therefore, adenosine has been termed a "retaliatory metabolite" [97,98]. The half-life of adenosine in human blood is 0.6 seconds [99] since it is actively metabolized in endothelial cells and blood platelets. Synthetic adenosine A_1 agonists exhibit substantially longer half-lives in blood dependent on the size of the N^6-substituent [100,101]. In the clinical setting, adenosine is used to terminate supraventricular tachycardia and as a diagnostic tool for the evaluation of various tachyarrhythmias [102]. Furthermore, adenosine is used as a coronary vasodilator during ^{201}Tl-scintigraphy.

3.2.1. Adenosine A_1 and A_3 receptors

In the heart, adenosine A_1-mediated effects, via G_i, can be divided into cAMP-independent (*direct*) effects and cAMP-dependent (*indirect*) effects [94,103-105] (Figure 4). Direct effects, which occur only in atrial tissue, are mediated by $G_{i/o}$-mediated stimulation of the acetylcholine/adenosine-regulated outward potassium current ($I_{KACh/Ado}$) [104] leading to action potential shortening and membrane hyperpolarization in the SA and AV nodes, and atrial tissue. Stimulation of $I_{KACh/Ado}$ by adenosine depresses SA and AV node activity resulting in negative chronotropy and dromotropy, respectively. In atrial myocytes, adenosine A_1 activation results in direct negative inotropic effects [106] via slight inhibition of the nonstimulated, basal inward calcium current (I_{Ca}) and/or via action potential shortening and membrane hyperpolarization [73,107,108]. No direct effects of adenosine occur in ventricular tissues as they lack $I_{KACh/Ado}$ channels. In ventricular and atrial myocytes, indirect effects of adenosine are mediated by depression of catecholamine stimulated I_{Ca} and I_{Ti} (transient inward current) [104] via inhibition of cAMP [109,110]. Thus, in ventricular tissue, adenosine A_1-mediated effects are anti-adrenergic, opposing the positive chronotropic, dromotropic, and inotropic effects of β-adrenergic receptor stimulation. Hence, indirect affects of adenosine can only be demonstrated during β-adrenergic stimulation when cAMP levels are increased [111,112]. A recent study indicated that the anti-adrenegic action of adenosine is more complex and consists of two components [113]. The first short-term and PKC-independent component is only expressed in the presence of elevated interstitial adenosine levels and hence, is reversible upon normalization of these levels. The sustained anti-adrenergic effect of adenosine persists when the adenosine concentration has returned to baseline levels and is induced via activation of the adenosine A_1 receptor and subsequent persistent activation of PKC [113]. Furthermore, adenosine inhibits norepinephrine release from stimulated sympathetic nerve fibers in the heart [114-116]. Like the A_1 receptor, the adenosine A_3 receptor also inhibits adenylate cyclase [87,117].

Other effects of adenosine A_1/A_3 receptors, through G-proteins, include 1) stimulation of phospholipase C resulting in inositol-1,4,5,-triphosphate accumulation and activation of PKC [85,118-123], and 2) activation of atrial and ventricular K_{ATP} channels, either directly [124,125] or indirectly via

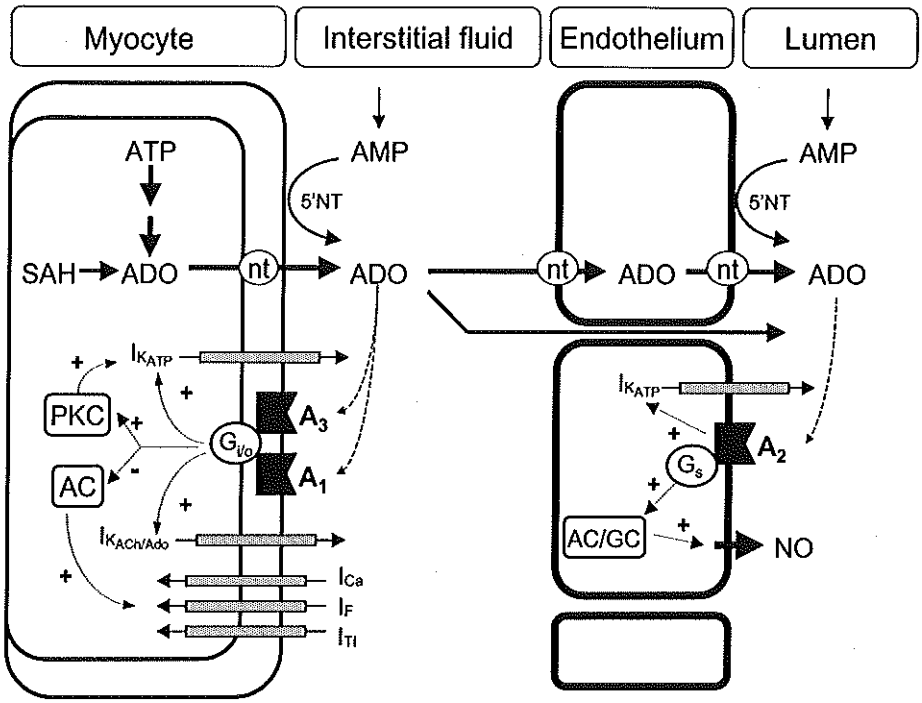

Figure 4. Illustration of the formation of adenosine and receptor-effector coupling system. An imbalance between tissue oxygen demand and supply leads to ATP breakdown and accumulation of adenosine (ADO) within ischemic cardiomyocytes. Adenosine produced from the hydrolysis of S-adenosylhomocysteine (SAH) by action of SAH-hydrolase in the cellular methylation cycle contributes to adenosine production during normoxia [137] but is negligible during ischemia [73]. Adenosine crosses the cell membrane via the nucleoside transporter (nt; facilitated diffusion) to enter the interstitial space. Adenosine is also produced extracellularly from adenine nucleotides released from neurons, cardiac myocytes, endothelial cells, platelets, and neutrophils, by action of ecto-5'-nucleotidases (5'NT) located on the surface membranes of many cells. During ischemia, adenosine derived from myocytes is mainly produced from intracellular AMP breakdown (endo-5'NT) whereas vascular adenosine is mainly formed from extracellular AMP metabolism (ecto-5'NT) [138,139]. The quantity of adenosine produced extracellularly by the endothelium is less than that produced intracellularly in cardiomyocytes [139]. Interstitial adenosine is mainly taken up by endothelial cells via the nucleoside transporter where it is further catabolized; some adenosine may directly reach the lumen of the microvasculature through clefts within the endothelial cell layer.

In the interstitial space, adenosine can bind its A_1 and A_3 receptors located on the myocardial membrane. Adenosine A_2 receptors are located on the vascular endothelium and smooth muscle cells. The endothelium is an active barrier to the equilibration of plasma and interstitial adenosine concentrations due to its high capacity to metabolize adenosine [73,140]. The adenosine gradient between plasma and interstitial compartments (1:8 in normoxic perfused hearts) is almost completely reduced during ischemia [141-144] when adenosine transport and breakdown in endothelial cells are saturated. Most adenosine formed during ischemia originates from the myocardial adenine nucleotide pool [145].

The electrophysiological and contractile effects of stimulation of adenosine A_1 and A_3 receptors by adenosine are mediated via an inhibitory G protein ($G_{i/o}$). In supraventricular tissue, a direct (cAMP-independent) effect of $G_{i/o}$ stimulation by the adenosine-A_1-receptor complex is an increase in the inwardly-rectifying potassium channel current ($I_{KACh/Ado}$) resulting in hyperpolarization and action potential duration shortening. Another direct effect of A_1/A_3 receptors is activation of the ATP-sensitive potassium channel current (I_{KATP}). In atrial and ventricular cells, indirect (cAMP-dependent) or anti-adrenergic effect of adenosine A_1 receptors is attributable to depression of increased cAMP levels and stimulated L-type Ca^{2+} channels (I_{Ca}) and transient inward currents (I_{TI}) by catecholamines (catecholamines increase and adenosine decreases adenylate cyclase [AC] activity). Other effects of adenosine A_1/A_3 receptors are stimulation of phospholipase C, which results in formation inositol-1,4,5,-triphosphate accumulation and activation of protein kinase C (PKC). Adenosine A_2 receptors mediate vasodilatation directly via opening of K_{ATP} channels (A_{2B} subtype) or indirectly (A_{24} subtype) via a stimulatory G-protein (G_s) and release of nitric oxide (NO). I_F, time- and voltage-dependent inward current activated by hyperpolarization (pacemaker current). GC, guanylate cyclase.

PKC [126-129]. Moreover, the cardioprotective effects of adenosine A_1/A_3 receptor stimulation are abolished by K_{ATP} channels blockers [130-135], suggesting a link between the channel and these receptors. The physiological role of the coupling of adenosine receptors with the I_{KATP} is unclear since adenosine activation of the channel may not occur under physiological conditions when ATP levels are high. However, when ATP levels fall during ischemia and the channel closes, K_{ATP} channel opening by adenosine may be involved in adenosine-mediated cardiopotection (see Chapter 1). This probably explains why adenosine minimally affects action potentials in intact, normoxic myocytes [136]. The effect of adenosine on glucose metabolism is controversial (Chapters 3 and 7). Furthermore, both adenosine A_1 and A_3 receptors trigger ischemic preconditioning (Chapters 7-9).

3.2.2. Adenosine A_2 receptor

Besides the well-documented vasodilatatory actions mediated by endothelial and vascular smooth muscle cell adenosine $A_{2A/B}$ receptors [92] in most vascular beds, adenosine has also anti-inflammatory (A_{2A} receptors on neutrophils) and anti-thrombogenic (A_{2A} receptors on platelets) actions, reducing vascular injury during ischemia/reperfusion [94,146-150] (Table 1). Moreover, adenosine is involved in the angiogenic effect observed during ischemia [94,151]. Activation of mast cells is mediated by its adenosine A_{2B} receptors and plays a role in inflammatory and allergic disorders like asthma [85].

It has been proposed that two kinds of adenosine receptors mediate the coronary vasoactivity of adenosine [152,153]. A_{2A} receptors coupled to adenylate cyclase/guanylate cyclase may mediate early vasorelaxation via nitric oxide production [154,155] whereas the other receptor (A_{2B}) induces vasodilatation via direct activation of K_{ATP} channels (Figure 4). Adenosine induces vasoconstriction of the renal glomerular afferent arteriole (anti-diuretic action) and the placental circulation.

3.3. Acadesine, an adenosine regulating agent

The nucleoside analogue acadesine (AICA riboside, 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide), like dipyridamole, belongs to the class of therapeutic drugs called 'adenosine regulating agents' [156,157]. When taken up by cells, acadesine is converted by adenosine kinase to its naturally occurring 5'monophosphate called ZMP or AICA ribotide; ZMP is further metabolized to IMP (Figure 3). Originally, acadesine was advocated as an ATP precursor increasing the replenishment of myocardial adenine nucleotides during postischemic reperfusion, which would result in improved contractile recovery [158]. However, the cardioprotective properties of this compound are unrelated to replenishment of myocardial ATP levels during ischemia/reperfusion [159-165]. Acadesine increased IMP but failed to increase AMP and ATP in these studies, which is probably due to ZMP-mediated inhibition of adenylosuccinate lyase [166] (Figure 3). Alternatively, acadesine may serve as an event-specific and site-specific agent that raises endogenous adenosine levels in ischemic tissues only. The mechanism may involve inhibition of adenosine deaminase and adenosine kinase in (ischemic) tissue where the activities of ATP catabolizing enzymes are very active; furthermore, the acadesine metabolite ZMP inhibits AMP deaminase (see [156,157]). Acadesine does not affect nucleoside transport. Acadesine raises intravascular [164,167] and tissue [163-165] adenosine. The drug shows limited oral bioavailability and has a half life of 1.4 hours in man [156,157].

In different animal models of ischemia, acadesine has been shown to elicit cardioprotection [159,163-165,167-172] although other studies reported no beneficial effects [161,162,173] or, in an early

study, even worsened functional recovery [174] after treatment with this drug. Acadesine improves the infarct-size limiting effects of ischemic preconditioning by lowering the threshold and extending the window of preconditioning protection [175-177]. Some of the negative studies may be explained by the use of relative short periods of (non lethal) ischemia [161], and acadesine dosage [174] and timing of administration [161,173] (for review, see [157]). Cardioprotection by acadesine is abolished in the presence of nonselective blockers of adenosine receptors [164,172]. Thus, cardioprotection by acadesine may be mediated by increased intravascular and interstitial adenosine levels in ischemic tissue. Acadesine extends the window of protection afforded by ischemic preconditioning by an adenosine receptor-dependent mechanism [176,177]. The advantage of acadesine in comparison to other 'adenosine regulating agents' like dipyridamole is its ability to raise adenosine in ischemic tissue only, without hemodynamic side-effects [164]. Acadesine-induced cardioprotection may be merely mediated by increased vascular adenosine (A_2 receptor) instead of interstitial adenosine (A_1/A_3 receptor) [178]. Acadesine acts as an antithrombotic agent by decreasing neutrophil-myocyte adhesion and platelet activation via an adenosine-mediated mechanism [148,179,180].

The efficacy of acadesine in patients undergoing coronary artery bypass surgery has been evaluated in three placebo-controlled, double-blind, multicenter studies [181-183]. In these studies, 0.05 (low-dose group) or 0.1 $mg.kg^{-1}.min^{-1}$ (high-dose group) intravenous acadesine was initiated 15 min before the induction of anesthesia and continued for 7 hours thereafter. In addition, 5 μg/ml acadesine was given as adjunct to the cardioplegic solution. The first small study involved 118 patients at four U.S. academical medical centers [181]. High- and low-dose acadesine did not reduce perioperative myocardial ischemia as judged by electrocardiography, transesophageal Doppler criteria, and creatine-kinase MB (CK-MB) fraction although a trend towards reduced ischemia was observed with acadesine. A larger (n=633) multicenter study in the U.S. [183] also failed to show beneficial effects of high- or low-dose acadesine on the prespecified outcome measures for perioperative myocardial infarction (ECG Q wave, CK-MB elevation, or autopsy evidence). However, post-hoc analysis using more specific measures of myocardial infarction revealed beneficial effects of acadesine in the high-dose group only compared to placebo. An international study (n=821) involving medical centers in Europe and Canada [182] did not find differences for the prespecified primary outcome measures of myocardial infarction between patients treated with high-dose acadesine or placebo. Further stratification of acadesine-treated patients showed a reduced incidence of myocardial infarction in high-risk patients (odds ratio=0.44; P=0.015) but not in low-risk patients (odds ratio=1.12; P=0.665). The studies indicated further that acadesine is safe for patients since no adverse effects (e.g., blood pressure) were observed with administration of the drug. The efficacy of acadesine in other procedures like percutaneous transluminal coronary angioplasty (PTCA) has hardly been examined (see Chapter 5).

4. Myocardial (pro)glycogen

The theory that glycogen consists of two separate forms dates back to 1934, when Willstätter and Rohdewald [184] reported the existence of lyo-glycogen and desmo-glycogen in various mammalial tissues. According to their theory, lyo-glycogen was protein-free and dissolvable in dilute trichloroacetic acid (TCA) whereas desmo-glycogen was protein-bound and TCA insoluble. The desmo-glycogen containing protein precipitant of the TCA treated tissues could be extracted by alkali. More than 25 years

later, Roe et al. [185] reported that the two forms of glycogen were artefacts of the extraction procedure due to inadequate homogenization in cold TCA. In the period between these two reports, evidence has been presented that both subfractions of glycogen behave differently when subjected to changing physiological conditions, a subject reviewed by Stetten and Stetten [186]. In heart muscle and other tissues, fasting [187-190], growth-hormone administration [188-190], epinephrine/norepinephrine injection, insulin administration or adrenalectomy/ hypophysoectomy [187-189], muscle work [191], and thyroxine injections [192] result in changes in macroglycogen (lyo-glycogen) whereas proglycogen (desmo-glycogen) remains more or less constant. When rats were exposed to prolonged anoxia (3.5 h), both macroglycogen and proglycogen fractions decreased, however, the decrease in the latter fraction was less pronounced [193]. In anoxic dog heart, macroglycogen decreased and proglycogen increased [194]. After about 4.5 h, however, proglycogen levels also started to decrease. Furthermore, a link between the resistance to anoxia and the amount of macroglycogen was observed [194].

The notion that glycogen consists in two forms has been take up again [195,196]. Not the absence or presence of protein distinguishes both forms [184], but merely the proportion protein relative to carbohydrate does [197]. Acid-extractable macroglycogen (Mr 10^4 kDa) contains 0.35% by weight of protein and acid-precitable glycogen (Mr 400 kDa) contains 10% by weight of protein. The latter low molecular mass form of macroglycogen is called proglycogen and is a stable intermediate in macroglycogen synthesis [197,198].

An important step in the rediscovery of Willstätter's and Rohdewald's lyo-glycogen and desmo-glycogen [184], and the new insights in how glycogen is synthesized, started with the discovery that glycogen contains covalently bound protein [199,200]. From this time, more knowledge about the biogenesis of glycogen on the protein primer called glycogen in has been obtained, a subject extensively reviewed [195,200-203]. The first step in this process consists of the covalent attachment of glucose to a tyrosine residue (Tyr 194) on the Mr 37 kDa primer glycogenin [196], of which the amino-acid sequence has been determined [204]. The glucose residue is added autocatalytically to Tyr 194 from UDPglucose [195]. Thereafter, glycogen synthase and glycogen in form a 1:1 complex so that the Mg^{2+} dependent autoglycosylation reaction can proceed to form malto-octaosylglycogenin using UDPglucose [195,205-209]. From this point, the combined action of glycogen synthase and branching enzyme, using UDPglucose, lead to the formation of proglycogen [198]. Based on the observation that NH_4^+ mainly blocks macroglycogen synthesis in brain astrocytes, it was concluded that two distinct (glucose 6-phosphate activated) enzymes catalyze the formation of proglycogen and macroglycogen called proglycogen synthase and macroglycogen synthase, respectively [197]. Furthermore, proglycogen synthase seems to be the rate-limiting step in glycogen synthesis which provide further evidence that different processes control the formation and breakdown of macroglycogen and proglycogen. Both the formation of the protein primer glycogen in, which will determine how much glycogen a cell can store, and the regulation of proglycogen synthase, provide new ways to understand carbohydrate metabolism. These new insights also explain the observation done in 1934 [184] that glycogen consists of acid-insoluble (desmo)glycogen and acid-insoluble (lyo)glycogen. Furthermore, observations done in the 50's [186-191,193,194] correspond to recently obtained results [210-212] that glycogen oscillates between macroglycogen and proglycogen whereas breakdown of proglycogen only occurs under more stringent conditions [194,195,211].

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3

Controversies in preconditioning

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Abstract

Preconditioning is an effective means of protecting the heart against prolonged ischemia by pretreating it with a minor insult, and the present paper reviews various controversies in this highly active field of research. In many models, adenosine plays a role by triggering the activation of protein kinase C. It may work in conjunction with other agents, such as bradykinin, but the putative role of noradrenaline is uncertain. Regulation of the enzyme producing adenosine (i.e., 5'-nucleotidase) has been reported during preconditioning but, as its activity does not seem to be associated with infarct size, it is unlikely that the hydrolase plays a pivotal role. Controversial data have been published on the involvement of mitochondrial ATPase, which may be ascribed to the poor time resolution of the experiments described; however, we do not believe that either acidosis or tissue ATP are important factors in triggering preconditioning. The role of glycolysis in the preconditioning effect remains to be firmly established; opposite mechanisms are activated in low-flow and stop-flow protocols. Although species differences regarding preconditioning exist, they seem to be more of a quantitative than a qualitative nature. The phenomenon could be clinically relevant because evidence is accumulating that preconditioning may take place during bypass surgery and coronary angioplasty if longer balloon-occlusion times are used.

1. Introduction

Ischemic preconditioning makes use of a mild stress in order to activate endogenous defence mechanisms and so enable protection from further major stress. All of the protocols described in the literature lead to a first time-window of protection lasting 60-120 min [1]. Originally, Murry et al. [2] used the limitation of infarct size as the criterion establishing the efficacy of an intervention, but subsequent studies have adapted a host of other variables to assess reduction of cell damage, including contractile, morphological, electrophysiological and biochemical parameters. This article reviews various controversies in this highly active field of research.

2. Adenosine and catecholamines

In ischemic preconditioning, endogenous adenosine protects the heart by stimulating the adenosine A_1-receptor. However, comparison between the cardioprotection induced by exogenous adenosine and that induced by preconditioning shows that discrepancies exist regarding the time frame [3] and extent of protection [4]. Goto et al. [5] proposed that a threshold level of protein kinase C (PKC) stimulation must be reached before cardiac protection by ischemic preconditioning takes place (Figure 1), and that adenosine and bradykinin released during preconditioning could play major roles in triggering protection by stimulating the kinase, provided that the activity exceeds the threshold [5]. Others have suggested that components such as norepinephrine [6] play a role in reaching the threshold, and Downey's group has reported that hypoxia also preconditions rabbit myocardium via adenosine and catecholamine release [7]. In line with this observation, ischemic preconditioning failed to limit infarct size in a study of rabbit hearts depleted of norepinephrine [8]. Using a similar approach, Weselchouch

et al. [9] found no evidence that catecholamines are involved in preconditioning in the rat model. In the same species, Hu and Nattel [10] reported that stimulation of α_1-adrenoceptors causes ischemic preconditioning, but recently Moolman et al. [11] have found no evidence of such a mechanism. In agreement with other observations [12], our own data [13] show that short periods of ischemia, such as those used during preconditioning, do not lead to norepinephrine release into the effluent of isolated rabbit hearts. Furthermore, as reported recently by Seyfarth et al. [14], preconditioning suppresses norepinephrine release after a long ischemic period. These authors suggested that transient ischemia may have a protective effect on neural tissue by reducing norepinephrine-induced damage during extended periods of myocardial ischemia. We believe that this is a more likely mechanism than protection induced by high catecholamine levels. Taken together, the above observations suggest that a combination of agents mediate ischemic preconditioning, and further investigation is clearly needed to evaluate the potential interrelated actions of adenosine and norepinephrine.

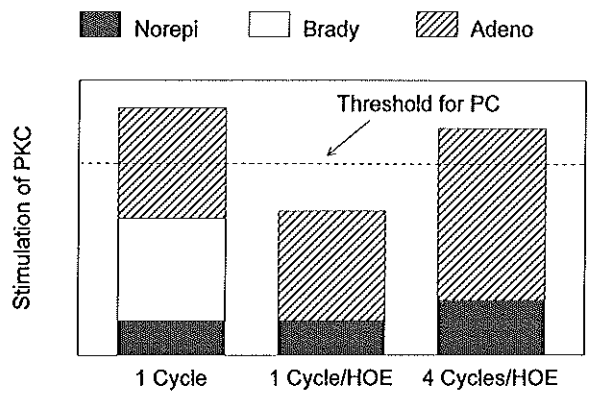

Figure 1. *Hypothetical threshold of protein kinase C (PKC) stimulation that must be reached before cardiac protection takes place by ischemic preconditioning (PC). One cycle of preconditioning produces substantial amounts of adenosine (Adeno) and bradykinin (Brady), in addition to norepinephrine (Norepi), which is enough to simulate PKC. If the second component is absent due to brady B_2-receptor blockade with HOE 140, extra cycles are needed to produce enough agonist for PKC stimulation and preconditioning. (After Goto et al. [5], with permission.)*

3. Hydrolases

3.1. 5'-nucleotidase

After investigating the relationship between adrenoceptors and preconditioning, Kitakaze's group reported that 5'-nucleotidase activity increases during α_1-adrenoceptor stimulation [15]; they also reported increases in 5'-nucleotidase and therefore adenosine production after preconditioning [16]. This latter finding conflicts with results obtained by other groups [13,17]. The older methodology used by Kitakaze et al. to measure phosphatase is aspecific and poorly documented [15,16]; although Przyklenk et al. [18] reported an increase in 5'-nucleotidase activity, they did not find any association with infarct size. It is thus unlikely that 5'-nucleotidase triggers preconditioning in the dog heart.

3.2. Mitochondrial ATPase

Within seconds after the onset of ischemia and the cessation of the electrochemical gradient across the inner mitochondrial membrane, the mitochondrial ATP-synthase changes into an ATPase (mF_1F_o-ATPase). As reviewed by Rouslin [19], inhibition studies with oligomycine show that most of the ATP hydrolyzed during global ischemia is due to mF_1F_o-ATPase activity. The binding of ATPase inhibitor (IF_1) to the ATPase leads to reduced ATP hydrolysis. IF_1 is an unidirectional inhibitor whose binding is reversible upon the resumption of oxidative metabolism. Protonic inhibition of the complex prevents ATP squandering by mF_1F_o-ATPase activity in ischemic heart cells, probably by means of IF_1 binding.

Various groups have examined whether ATPase inhibition may explain the improved energy balance observed in preconditioned hearts (e.g., [2]). In rat hearts perfused according to Langendorff, preconditioning induced greater ATPase inhibition and less high-energy phosphate depletion during sustained global ischemia [20]. However, Vander Heide et al. [21] concluded that altered mF_1F_o-ATPase activity in regionally ischemic dog hearts is not responsible for the energy-sparing effect of ischemic preconditioning. These results are surprising since species with fast heart rates, such as the rat, have less IF_1 inhibitor protein than those with slow heart rates, such as the dog [19]. However, we would like to point out that Vander Heide et al. [21] only measured ATPase activity after the last 5-min reperfusion period of the preconditioning protocol, and so the possibility remains that the inhibition of ATPase in preconditioned hearts became faster or stronger during the sustained ischemic period in their study.

Recent data complicate this issue even further. Kobara et al. [22] observed greater ATPase activity during global ischemia and reperfusion in isolated and preconditioned rat hearts than in controls. Furthermore, the degree of ATP depletion during ischemia was no different between preconditioned and control hearts, whereas ATP levels recovered better during reperfusion in preconditioned hearts. The authors concluded that the preservation of ATPase (and adenine nucleotide translocase) activities may be responsible for the improved restoration of high-energy phosphates.

Although a proper ATP level is crucial for cell homeostasis, it is dissociated from postischemic function [23]. We have no reason to believe that the concentration of ATP is an important factor in eliciting preconditioning.

4. Glycolysis

The role of preconditioning on glycolysis/glycogenolysis during ischemia, as well as its relationship to ischemic injury is controversial. Anaerobic glycolysis supports cell function by means of ATP production, but accumulated glycolytic end products (lactate, sugar and triose phosphates, protons originating from ATP hydrolysis [23]) may determine the extent of ischemic damage. Consequently, many studies have attempted to relate the beneficial effects of ischemic preconditioning to cardiac glycogen content or glycolytic rates.

4.1. Low-flow ischemia

Preconditioning before low-flow ischemia increases ischemic glycolytic flux and decreases

cardiac injury [24]. These observations agree well with the results of studies showing that enhanced glycolysis and maintained glycolytic ATP production by various means lead to reduced damage and improved function upon reperfusion [25,26]. During low-flow hypoxia in rat hearts, glucose prevents the loss of sarcolemmal integrity and contractile function [27].

One may pose the question: Why does anaerobic glycolytic flux protect the myocardium subject to hypoxia/low-flow ischemia? The answer seems to be that the ATP derived from anaerobic glycolysis is preferentially used for membrane-ion pumps and the preservation of cell integrity. During moderate ischemia, anaerobic glycolysis is stimulated (the Pasteur effect) whereas, during severe ischemia, this effect is inhibited by the accumulation of lactate and protons, and glycolysis ceases [28]. During low-flow ischemia, lactate and protons are continuously washed out of the ischemic heart, thus preventing their accumulation in tissue and the consequent inhibition of anaerobic glycolysis. Therefore, if the accumulation of the potentially toxic products of glycolysis is prevented by sustaining a moderate flow to the heart, glycolysis can be maintained and glycolytic ATP may preserve cell-membrane integrity and reduce Ca^{2+}-overload during reperfusion.

We believe that the maintenance of glycolysis during low-flow ischemia prevents myocardial injury [28]. So far, only one study [24] has reported that ischemic preconditioning increases glycolytic flux and reduces ischemic cell damage. More research is necessary to clarify the important relationship between preconditioning and glycolysis during low-flow ischemia.

4.2. Stop-flow ischemia

Unlike low-flow ischemia, ischemic preconditioning causes a decrease in glycolytic flux during stop-flow global [29] and regional [2,30] ischemia in both isolated [29] and in-vivo [2,30] models. Other strategies aiming at decreased glycolytic flux during regional stop-flow ischemia also induce less ischemic injury [31].

Several groups, including Wolfe et al. [30], assume that the beneficial effects of preconditioning are related to the reduced lactate accumulation and proton production that are the result of lower glycolytic rates, despite reduced ATP production. However, Vander Heide et al. [32] dissociated glycogen depletion and the reduction in lactate accumulation and anaerobic glycolytic flux from ischemic damage. Our own NMR studies also demonstrate cardioprotection by preconditioning in isolated rat hearts without any effects on pH [33], leading us to believe that acidosis is not a key factor in either functional recovery or ischemic injury [29].

Ischemic preconditioning of the heart reduces its preischemic glycogen content. Wolfe et al. [30] correlated the decrease in infarct size caused by preconditioning with glycogen depletion before sustained ischemia, and with the attenuation of intracellular acidosis during ischemia. A longer period of preconditioning ischemia leads to glycogen depletion and a reduction in infarct size, which are both described by an exponential declining curve [34]. However, the hypothesis that diminished glycogen stores per se limit glycolysis, catabolite accumulation and cell damage is not supported by other results: Myocardial glycogen loading induced by fasting improves the functional recovery of ischemic rat hearts [35], and, in a retrospective study, the preischemic glycogen content of rat hearts was correlated with the time of day but not postischemic functional recovery [36]. The question remains as to whether reduced glycolytic flux and accumulated glycolytic products mediate or are secondary to preconditioning, but we do not believe that either lactate or proton accumulation is important in this respect.

4.3. Glycolysis and adenosine

Since adenosine has been implicated in ischemic preconditioning, a number of authors have studied its role on glycolytic flux. Various groups [37,38] have shown that by binding to the A_1-receptor, adenosine increases glycolytic flux and induces cardioprotection in isolated rabbit and rat hearts subjected to ischemia and hypoxia, respectively, whereas others have found that it reduces glycolysis and improves functional recovery after ischemia in isolated working rat hearts perfused with glucose and palmitate ([39]; see also [40]). These divergent results could be due to the models studied (low-flow vs. no-flow ischemia), the use of different substrates (glucose vs. glucose/fatty acids), and/or differences between species.

5. Species differences

Although several groups have shown that the rat heart behaves differently from the hearts of other species, the isolated, perfused rat-heart model is quite often used to study preconditioning. It is generally believed that endogenous adenosine does not mediate preconditioning in this species but, as recently pointed out by Headrick [41], the relatively high adenosine levels in rat heart during preconditioning require an increase in (ant)agonist receptor concentration. We refer to the discussion by Przyklenk and Kloner [42] on the species (and model) dependency of the arrhythmic component. The same group [43] could not find any translocation of PKC after ischemic preconditioning, and this failure to reproduce the original observation in rabbit heart [44] may have been due to species differences.

Questions can be raised about the abundant use of inhibitors and activators (often declared to be specific or selective on rather shaky grounds) to probe the proteins involved. The involvement of K_{ATP} channels in the preconditioning of larger animals seems clear but may not be essential in rabbits and rats (for review, see [45]). A pertussis toxin-sensitive G-protein may play a central role in ischemic preconditioning across a broad range of species [10], but major differences may exist in relation to the role of protein kinase C [46]. The involvement of the inhibitory G-protein in the arrhythmic component of preconditioning is also controversial. Nevertheless, some of these discrepancies may be due to the details of the experimental models studied [10].

6. Evidence for preconditioning in human heart

6.1. Preinfarction angina

Ischemic preconditioning clearly exists in many species, and possibly in humans. The phenomenon has been investigated during (retrospective) clinical studies, coronary angioplasty, cardiac surgery, and in vitro. In recent clinical trials, it has been found that preinfarction angina seems to decrease creatine kinase release, in-hospital deaths, arrhythmias, left-ventricular function, and infarct size [42,47]. Ottani et al. [48] used ventriculographically obtained regional wall motion as a means of indirectly determining infarct size and found that this was less in patients with prodromal angina pectoris occurring at rest 24 hours before the infarct than in patients without previous angina, although the two

groups had the same area at risk and in both collaterals were absent. However, as indicated in a recent review by Andreotti et al. [49], controversies still exist that might be explained by the use of thrombolysis, the baseline characteristics of the study population, the presence of multivessel disease, infarcted area, collaterals and the period(s) of angina before infarction. For instance, studies in the period before the use of thrombolytics have shown that preinfarction angina has negative effects [50], a finding that correlates with the observation that preconditioning exerts an effect only if reperfusion takes place within a certain time frame. Andreotti et al. [49] partly explained these discrepancies by the different definitions of preinfarction angina used. Furthermore, in a prospective study, they [49] observed that the infarct size-limiting effect of preinfarction angina was merely related to faster coronary thrombolysis.

6.2. Percutaneous transluminal coronary angioplasty

Deutsch et al. [51] claim that a 90-second coronary occlusion induces preconditioning in humans, basing their conclusions partly on the drop in lactate uptake in 7 out of 12 patients in whom electrophysiological changes during angioplasty were investigated. Jenkins et al. [45] have pointed out that the pretreatment of patients with dipyridamole may potentiate the effect of preconditioning by increasing myocardial adenosine levels.

In a larger patient population (n=28), we measured the myocardial release of lactate and hypoxanthine (an ATP catabolite) immediately after four balloon inflations lasting 60-90 s. The arterio-venous difference in both metabolites did not change significantly as a result of the repeated occlusions (Figure 2). The biochemical markers used provided no evidence for preconditioning during coronary angioplasty [52]. We believe that the angioplasty times of balloon inflations, often lasting less than 2 minutes, are insufficient to induce ischemic preconditioning in humans (see also [45]). In this respect, it is interesting to note that studies using balloon inflations lasting 2 minutes or more (e.g., [53-55]) have demonstrated improved tolerance to myocardial ischemia after repetitive coronary occlusions. The exception seems to be a study described later [56]. Further evidence that ischemic preconditioning occurs during angioplasty and is mediated by adenosine A_1-receptor activation and K_{ATP} channel opening has recently been obtained: Adenosine A_1-receptor blockade by bamiphylline [55] and aminophylline [56], as well as the administration of the selective K_{ATP} channel blocker glibenclamide [54], prevent ischemic preconditioning occurring during repetitive balloon occlusions.

6.3. Coronary artery bypass graft surgery

During both normothermic and hypothermic intermittent aortic crossclamping in coronary artery bypass graft surgery, the release of lactate and inorganic phosphate has been found to be greater during the first than during subsequent reperfusion periods [57]. Yellon's group was the first to report that an ischemic preconditioning protocol slowed the rate of ATP depletion during 10 minutes of crossclamping fibrillation [58], but less ATP depletion in preconditioned hearts gives only indirect evidence of myocardial protection because ischemic cell damage and myocardial function were not reported. It should be noted that the amounts of creatine kinase-MB and lactate released by the ischemic myocardium were no different between the groups, although there was a tendency to lower values in preconditioned hearts. The role of adenosine in ischemic preconditioning has also been studied in the setting of bypass surgery, and it has been found that its presurgery infusion improved intraoperative and

postoperative hemodynamic function and reduced ischemic cell damage [59].

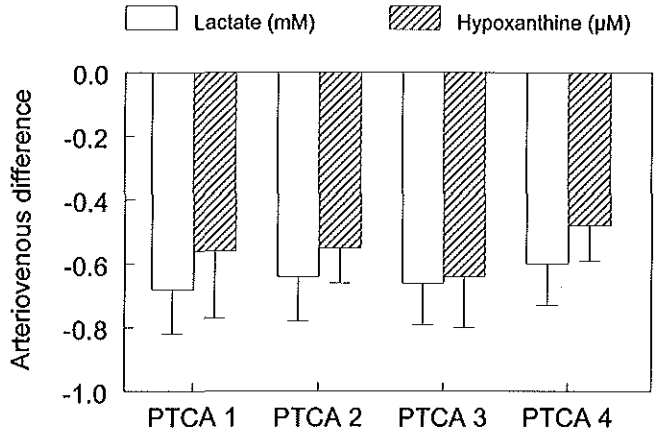

Figure 2. Lactate and hypoxanthine release after coronary angioplasty. In 28 patients undergoing angioplasty of the left anterior descending coronary artery, arterial and coronary sinus blood samples were taken immediately after occlusions lasting 60-90 s. They were rapidly deproteinized with acid and were assayed enzymatically (lactate) or by HPLC (hypoxanthine). Arterio- venous differences are presented means ± S.E.M.

6.4. Isolated trabeculae and cultured myocytes

Yellon's group [60,61] exposed human atrial trabeculae to 90 min of hypoxic substrate-free superfusion, and demonstrated that preconditioning prior to the sustained ischemic period improved functional recovery. This preconditioning effect could be blocked by an adenosine A_1-antagonist, a PKC antagonist, and a K_{ATP} channel blocker. An adenosine A_1-agonist, a K_{ATP} channel opener and a PKC activator mimicked the effect of ischemic preconditioning, whereas the effects of the K_{ATP} channel opener and the PKC activator could be blocked by the K_{ATP} channel blocker glibenclamide. Ikonomidis et al. [62] have demonstrated that the infarct size-limiting effect of ischemic preconditioning also exists in human cultured ventricular cardiomyocytes.

7. Discussion

A number of endogenous compounds are candidate triggers of preconditioning: These are adenosine, bradykinin, and noradrenaline, which may work in conjunction to reach a threshold. Although we doubt the importance of catecholamines in this respect, we would like to point out that all of these metabolites activate intracellular signalling pathways via receptors linked to GTP-binding proteins and phospholipases; protein kinase(s) could be stimulated, and thus lead to the phosphorylation of putative target proteins [5]. As reviewed elsewhere in this issue [46], these include regulatory proteins of the K_{ATP} channels and the expression of heat-shock proteins (for second time window of protection), although,

as outlined earlier, we do not believe that 5'-nucleotidase belongs to this class of possible targets. However, we do consider the possibility that the adenosine released during short periods of ischemia and reperfusion activates PKC, which in turn phosphorylates a glycolytic key enzyme and thus increases glycolytic flux [24,38]. The present data do not allow any firm statement to be made regarding the preconditioning role of (the translocation of) PKC in all species. Although there is growing evidence that the human heart can be preconditioned, there is still a lack of crucial information on the subcellular pathways involved (e.g., with PKC).

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4

Preconditioning in globally ischemic isolated rat hearts: effect on function and metabolic indices of myocardial damage

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Abstract

Objective: We assessed the effects of ischemic preconditioning on heart recovery and metabolic indices of damage following global ischemia and reperfusion, in relationship to post-ischemic lactate release. **Methods:** Three groups of Langendorff rat hearts were studied: 1) A control group of 40 min global ischemia and 45 min reperfusion; 2) preconditioning by 5 min global ischemia and 15 min reperfusion prior to sustained ischemia and reperfusion; 3) Preconditioning by three episodes of brief ischemia-reperfusion prior to sustained ischemia. **Results:** Repetitive episodes of brief ischemia-reperfusion were associated with increased reactive hyperemia, decreased release of purines and prostaglandin 6-keto $F_{1\alpha}$, lower tissue glycogen but no change in lactate washout. After 40 min ischemia, release of lactate was 173±17, 196±6 and 149±9 μmol/g in groups 1, 2 and 3, respectively (P<0.01, group 2 vs. group 3). Preconditioning had no effect on ischemic arrest but had divergent effects on the development and the magnitude of ischemic contracture: delay and attenuation in group 2 but enhanced onset in group 3. Preconditioning provided a dose-dependent protection from the increase in left ventricular end-diastolic pressure, reduced the reperfusion release of purine metabolites and of creatine kinase, but neither improved systolic function nor prevented arrhythmia. 6-Keto $F_{1\alpha}$ production was 87±13, 132±19 and 241±3 5 pmol/g in groups 1, 2, 3, respectively (P<0.01 group 1 vs. group 3). **Conclusions:** We conclude that when subjected to prolonged global ischemia, preconditioned isolated rat hearts develop less post-ischemic contracture, lose less purine nucleosides and creatine kinase activity. In addition, preconditioning leads to increased production of prostacyclin. Differences among preconditioning protocols in lactate production seem to be related to the ischemic contracture development, but may not play an ultimate role in attenuation of myocardial damage or improvement of postischemic recovery.

1. Introduction

Preconditioning the myocardium by brief periods of ischemia-reperfusion confers a substantial protection during a subsequent, prolonged ischemic period. In different experimental models of regional ischemia, beneficial aspects of preconditioning include: attenuation of structural damage [1,2], reduction of infarct size [3,4], prevention of arrhythmia during either ischemia or reperfusion [5-7] and improved functional recovery [8]. Apparently, preconditioning constitutes a process of adaptation to low-energy states, and is indeed present in various models such as globally ischemic [9-12], hypoxic [13] or rapidly paced hearts [14].

Murry et al. [2] proposed a causal relationship between reduced tissue lactate accumulation and the benefits of preconditioning. Yet, there is considerable controversy concerning the role of anaerobic glycolysis in perpetuation versus attenuation of ischemic damage [15] in globally ischemic hearts. The purpose of our study was to assess the effects of ischemic preconditioning on heart recovery and biochemical indices of damage following no-flow global ischemia and reperfusion, in relation to lactate production during the ischemic state.

2. Methods

2.1. Preparation

Fed, male Wistar rats received 500 U heparin i.p. and were anesthetized with i.p. pentobarbital sodium 6 mg/100 g body weight. The hearts were rapidly excised and cooled in ice-cold saline until contraction stopped. Then, hearts were mounted on a Langendorff apparatus and perfused with oxygenated (95% O_2, 5% CO_2), bicarbonate-buffered (pH 7.4), modified Tyrode solution (composition in mmol/l: NaCl 128, KCl 4.7, $CaCl_2$ 1.35, $NaHCO_3$ 20.2, NaH_2P0_4 0.42, $MgCl_2$ 1.05, glucose 10) with a perfusion pressure of 60 mmHg. A stiffened latex balloon of ellipsoid form was specially prepared to suit the dimensions of rat left ventricular cavity in order to minimize the artifacts induced by postischemic contracture on pressure measurements [16]. The balloon was inflated until left ventricular end-diastolic pressure approached 4 mmHg. Systolic pressure of at least 70 mmHg was required for inclusion. The pulmonary artery was cannulated with a 14-gauge catheter for sampling of coronary venous effluent. The O_2 content was determined in aortic and venous samples with a Radiometer ABL3 gas analyser. A bipolar electrogram was obtained between the aortic cannula and a platinum electrode, inserted into the right ventricular epicardium. A pacing electrode was positioned on the right atrial appendage. An overdrive atrial pacing (300-330 bpm) was instituted using a Grass S9 stimulator. The whole system was heated by water jacketing and the heart temperature was strictly maintained in the range 36.5-37.5°C with the aid of an Ellab Teflon thermistor probe, placed in the right atrial cavity. Measurements of coronary flow were obtained volumetrically and with a Skalar extracorporeal electromagnetic flow-meter positioned above the aortic cannula. Coronary flow, heart temperature, electrogram, coronary perfusion pressure and left ventricular pressure with its derivative (dP/dt) were continuously recorded on a Graphtec Linearcorder F WR 3701.

2.2. Protocol

Experiments were carried out in three groups (n = 6-7): 1) An ischemic control group–50 min of baseline perfusion, 40 min no-flow global ischemia and 45 min of reperfusion; 2) A one-cycle ischemic preconditioning group–30 min baseline perfusion, 5 min ischemia with 15 min reperfusion followed by 40 min ischemia and 45 min reperfusion; 3) A repetitive, three cycle, ischemic preconditioning group–20 min baseline perfusion, three rounds of 5 min ischemia followed by 10, 10 and 15 min reperfusion respectively, and then 40 min sustained ischemia with 45 min reperfusion.

The hearts were paced at the same rate during the whole experiment. The time intervals from onset of ischemia to cessation of effective mechanical contracture (developed pressure <0.5 mmHg) and failure of the conductive system were recorded. Functional assessment and measurement of myocardial oxygen consumption were done at baseline, immediately before long-term ischemia and after 30 and 45 min of postischemic reperfusion. Spontaneous heart rate was obtained at baseline and after reperfusion measurements, allowing 3 min recovery from the overdrive suppression. Coronary effluent was collected at baseline, before long-term ischemia and continuously during the various reperfusion periods (at minute 1, 2-3, 4-5, 5-10, 10-15, 15-30, 30-45). At the end of the experiments, the hearts were freeze-clamped, and stored in liquid nitrogen. Additional hearts (n = 5 per group), treated according to protocols 1-3, were freeze-clamped just prior to induction of sustained ischemia, for determination of tissue lactate

and glycogen.

2.3. Biochemical assays

Nucleosides and oxypurines (adenosine, inosine, hypoxanthine, xanthine, urate) were determined in coronary effluent samples by HPLC [17]. Lactate in the effluent was measured enzymatically (Sigma Diagnostics, procedure no. 735). Creatine kinase activity was assayed at 37°C by the N-acetylcysteine activated DGKCh Method (Merck Diagnostica). The stable metabolite of prostacyclin (6-keto $PGF_{1\alpha}$) was determined with the DuPont 3[H]-radioimmunoassay kit. Glycogen in tissue homogenate was determined according to Huijing [18]. One ml homogenate was neutralized with 5M NaOH and used for glycogen determination, with rabbit liver glycogen (Boehringer Mannheim GmbH) as a standard. The homogenate was centrifuged, the clear supernatant fraction was used for lactate determination according to Gutmann and Wahlefeld [19]. Measurement of dry weight was performed as previously described [20].

2.3. Statistics

All biochemical data were calculated per gram dry weight. Data, presented as mean ± S.E.M., were compared by analysis of variance, with repeated measures or with Duncan grouping. Inter-group comparison of postischemic measurements was performed in its absolute form or after correction for individual baseline, as appropriate. Statistical significance was accepted at $P<0.05$.

3. Results

The experimental groups were well balanced in their baseline characteristics, except for a difference in coronary flow between the two preconditioning groups (Table 1).

Table 1. Baseline parameters of the experimental groups

Group	n	Weight (g)	HR (bpm)	PR (bpm)	CF (ml/min/g)	LVEDP (mmHg)	LVDP (mmHg)	$+dP/dt_{max}$ (mmHg/s)	$-dP/dt_{max}$ (mmHg/s)	MVO_2 (ml/min/g)
1	7	245±5	262±17	316±4	62±4	1±1	85±5	2340±120	1560±180	0.67±0.07
2	7	261±5	270±24	307±3	73±5*	0±1	88±5	2400±100	1500±80	0.80±0.06
3	6	250±8	274±24	314±5	57±4	0±1	92±4	2620±80	1660±60	0.70±0.06

HR, spontaneous heart rate; PR, pacing rate; CF, coronary flow (per gram dry weight); LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure; $\pm dP/dt_{max}$, the positive and negative peaks of the first derivative of the left ventricular pressure; MVO_2, myocardial oxygen consumption (per gram dry weight); bpm, beats per minute. *$P<0.05$ vs. group 3.

3.1. Effects of short-term ischemia

The effects of repetitive ischemia on coronary flow (in group 3) are presented in Figure 1. The reflow at the first minute of reperfusion was increased after each consequent cycle while the 'plateau' coronary flow decreased. Preconditioning impaired the cardiac function (Table 2): The average decrease in left ventricular developed pressure following short-term ischemia-reperfusion in preconditioning groups 2 and 3 was 15% and 20%, respectively. The changes in myocardial oxygen consumption were less pronounced. Short-term ischemia resulted in a substantial washout of lactate, prostacyclin and purine metabolites in the coronary effluent, a process taking place within 5 min from the onset of reperfusion. Trace amounts of creatinc kinase were detected in the first minute of reperfusion but not later. The release of prostacyclin, purines metabolites and creatine kinase decreased with repeated ischemia-reperfusion while the release of lactate remained stable (Table 3). Tissue glycogen stores, measured prior to sustained ischemia, were reduced by three, but not by one episode, of short term ischemia-reperfusion. Tissue lactate in the preconditioned hearts was not significantly different from the control (Table 4).

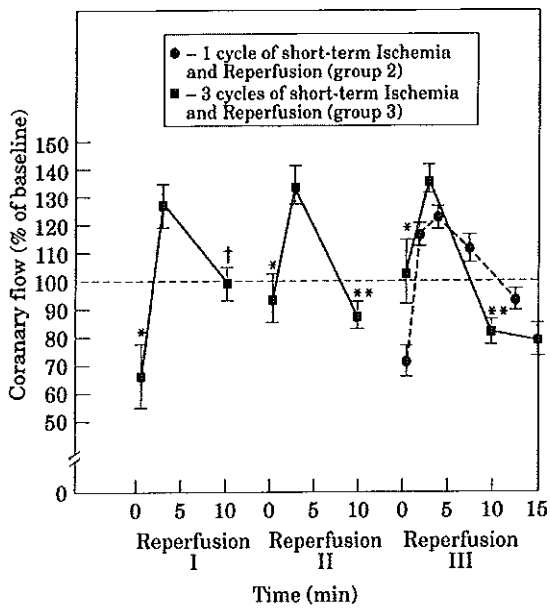

Figure 1. *The effects of repetitive short-term ischemia with reperfusion on coronary flow in Langendorff rat heart. The first minute, flow increased progressively (*P<0.001 in repeated measures ANOVA). The increase in peak hyperemic flow was non-significant, while the coronary flow at 10 min reperfusion progressively decreased in comparison to baseline (†P<0.001). The broken line illustrates the mean coronary flow during reperfusion following a single episode of 5 min ischemia in group 2. Note the similarity to the first reperfusion, but the marked difference from the coronary flow in the third repetitive reperfusion in group 3. The coronary flow in the control group remained at 99±4% of the baseline value (not shown).*

Table 2. Functional parameters before and after 40 min ischemia and reperfusion

Group	n	LVEDP (mmHg)	LVDP (% of baseline)	$+dP/dt_{max}$ (% of baseline)	$-dP/dt_{max}$ (% of baseline)	MVO_2 (% of baseline)
1	7	3±1	98±3	99±4‡	93±4‡	97±3
2	7	2±1	85±3*	81±5	75±4	95±6
3	6	0±1*	80±6†	81±6	73±5	83±6
Post 40 min ischemia and reperfusion						
1	7	20±2	73±8	72±7	72±7	79±3
2	5	13±3	80±8	78±5	76±7	84±11
3	5	8±2§	73±4	77±3	73±4	84±11

LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure; $\pm dP/dt_{max}$, the positive and negative peaks of the first derivative of the left ventricular pressure; MVO_2, myocardial oxygen consumption. Results of ANOVA with Duncan intergroup comparisons: *$P<0.05$; †$P<0.01$ vs. group 1; ‡$P<0.01$ vs. groups 2 and 3; §$P<0.01$ vs. group 1. Note: Two hearts from group 2 and one heart from group 3 did not regain regular rhythm after sustained ischemia.

Table 3. Effects of repeated short-term ischemia and reperfusion on the metabolic markers of ischemic damage

Condition	Purines (μmol/min/g)	(μmol/g)	6-Keto PGF1α (pmol/min/g)	(pmol/g)	Lactate (μmol/min/g)	(μmol/g)	Creatine kinase (U/min/g)
Baseline	0.07±0.01		4.8±1.4		3.7±0.6		1.1±0.1
Rep. Cycle	min 1	Total	min 1	Total	min 1	Total	min 1
First	0.61±0.08	2.36±0.28*	78±16	198±21†	41±4	78±2	2.3±0.2†
Second	0.65±0.04	1.70±0.15	79±19	169±34	54±4	77±8	2.0±0.2
Third	0.57±0.04	1.47±0.13	31±6	101±22	53±5	77±8	1.6±0.5

Values at reperfusion (Rep) constitute the release at the first minute and the total of the first 5 min, except creatine kinase, which was negligible after min 1. Statistical comparisons between reperfusion periods 1-3: *$P<0.02$; †$P<0.05$ (ANOVA for repeated measures). Purines include purine nucleosides and oxypurines (adenosine, inosine, hypoxanthine, xanthine, urate). Data expressed per gram dry weight.

Table 4. Tissue lactate and glycogen prior to sustained ischemia

Group	Lactate (μmol/g)	Glycogen (mg/g)
1	3.3±2.0	18.9±1.0
2	14.3±8.8	16.0±1.7
3	14.8±7.9	6.9±1.4*

Data expressed per gram dry weight. *$P<0.01$ vs. groups 1 and 2. Note the large variation in lactate levels within each group. There were no differences between the groups (ANOVA, $P=0.57$).

3.2. Ischemic arrest and contracture

Electromechanical dissociation often preceded the failure of electrical activation of the ventricles. Preconditioning was not associated with any change in the time of arrest of the contractile or electrical activity (Figure 2). The two protocols of preconditioning had divergent effects on the development and the magnitude of ischemic contracture during sustained ischemia (Figure 3). In group 2, the onset of contracture was delayed and its magnitude diminished significantly. Repetitive preconditioning (group 3) resulted in earlier development of contracture.

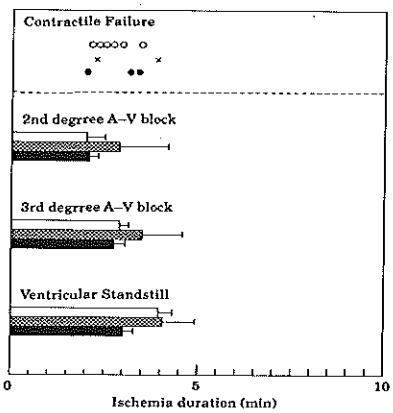

Figure 2. The effect of ischemic preconditioning on the development of ischemic arrest in atrially paced hearts. Open bars, ischemic arrest times (min, mean±S.E.M.) in controls (group 1); hatched bars, a single round of ischemic preconditioning (group 2); solid bars, repeated rounds of preconditioning (group 3). Note that contractile failure preceded electrical arrest (ventricular standstill) only in some of the hearts and therefore individual data are shown (open circles, group 1; x, group 2; solid circles, group 3).

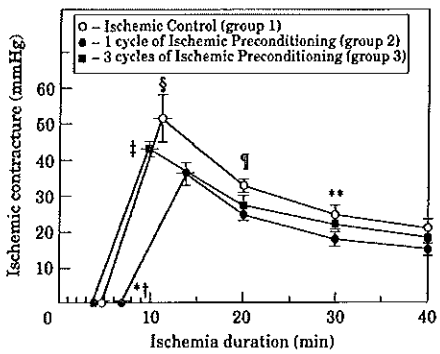

*Figure 3. Effects of ischemic preconditioning on the development and time-course of ischemic contracture in globally ischemic isolated rat heart. The data points along each line represent the time of onset, peak contracture and its magnitude after 20, 30 and 40 min ischemia. *P<0.05, †P<0.01 in ANOVA for time of onset vs. groups 1 and 3, respectively. ‡P<0.05 in ANOVA for time of peak contracture vs. group 2. §P<0.05 for the peak pressure vs. group 2. After the peak, there was a significant decay of pressure with time (P<0.001 in repeated measures ANOVA), a borderline group effect (P=0.067) and a non-significant time group interaction. The 'group effect' arose from a difference between groups 1 and 2 (¶P<0.01, **P<0.05 in one-way ANOVA for contracture at 20, 30 min ischemia, respectively).*

3.3. Reperfusion after long-term ischemia

There was no significant effect of preconditioning on the coronary flow in comparison with controls (Figure 4). The flow at minute 1 was greater in group 2 than in group 3, but afterwards it did not differ among the groups.

Ventricular tachycardia or ventricular fibrillation appeared in all the hearts, approximately within 1 min of reperfusion. Persistent arthythmia was seen in three preconditioned hearts but none of the controls (NS). The duration of transient arrhythmia did not differ between the experimental groups (16.9±3.7, 16.0±4.1 and 9.0± 5.3 min in groups 1, 2 and 3, respectively). The spontaneous rate eventually recovered to 95±8, 88±7, 91±7% of baseline, respectively (P=NS).

Postischemic recovery of function usually stabilized after 30 min of reperfusion. Prolonged arrhythmia delayed the functional recovery, and therefore only measurements at min 45 were accomplished in several hearts. Preconditioning attenuated the increase in left ventricular end-diastolic pressure after prolonged global ischemia. No significant differences were noted in the recovery of the developed pressure or myocardial oxygen consumption (Table 2). The amounts of lactate, prostacyclin, purine metabolites and creatine kinase released following 40 min global ischemia, are compared in Table 5. The metabolite and enzyme release was unrelated to the coronary flow. Lactate release was differently affected by the preconditioning protocols: it was higher in group 2 than in group 3 (both non-significantly different from controls, Figure 5) The time-course of reperfusion release of inosine and adenosine is given in Figure 6, and that of creatine kinase in Figure 7. Preconditioning was clearly associated with reduced loss of purine necleosides and creatine kinase. This effect was more pronounced in the group with repetitive preconditioning (group 3). Prostacyclin metabolite was measured only during minutes 1-5 of reperfusion, since our previous experiments indicated that the washout is nearly complete at that time. The release of 6-keto $PGF_{1\alpha}$ was enhanced by ischemic preconditioning and the effect was related to the number of the preceding short-term episodes of ischemia: the increase was slight after a single episode but marked after repetitive cycles of short-term ischemia-reperfusion (Table 5). The amount of glycogen, remaining in the tissue at the end of the experiment, was low (3.7±0.3 mg/g) in ischemic controls and even lower in preconditioned hearts. We calculated the cumulative amount of lactate and of purine metabolites released after various ischemias during the whole experiment. The amount of purines lost during all periods of ischemia-reperfusion was equal in the three groups. The overall lactate release was inversely related to the residual glycogen (Table 6).

Table 5. Effects of ischemic preconditioning on the washout of metabolites following 40 min global ischemia

Group	Lactate (μmol/g)	6-keto $PGF_{1\alpha}$ (pmol/g)	Purines (μmol/g)	Creatine kinase (U/g)
1	173±17	87±13	12.7±0.8‡	203±44§
2	196±6*	132±19	9.4±0.5	128±22
3	149±9	241±35†	7.6±0.6	111±26

The time periods for calculating the 'postischemic' washout were determined from the time-course of release and were minutes 1-5 for 6-keto $PGF_{1\alpha}$, minutes 1-15 for lactate, minutes 1-30 for purines and the whole reperfusion period for creatine kinase. Purines comprise purine nucleosides and oxypurines (adenosine, inosine, hypoxanthine, xanthine, urate). Data are expressed per gram dry weight. Results of ANOVA with Duncan intergroup comparisons: *P<0.05 . group 3; †P<0.01 vs. group 1; ‡P<0.01 vs. groups 2 and 3; §P<0.05 vs. group 3.

Table 6. Residual tissue glycogen in relation to overall lactate release

Group	Glycogen (mg/g)	Total lactate release (μmol/g)	Total purine release (nmol/g)
1	3.7±0.3*	298±27†	12.8±1.0
2	2.6±0.2	432±8	12.7±0.6
3	2.3±0.3	480±33	12.9±0.5

Total lactate/purine release, sum of the quantities found in effluent following various episodes of short and long-term ischemia. Data expressed per gram dry weight. *$P<0.01$ vs. group 3; †$P<0.01$ vs. groups 2 and 3.

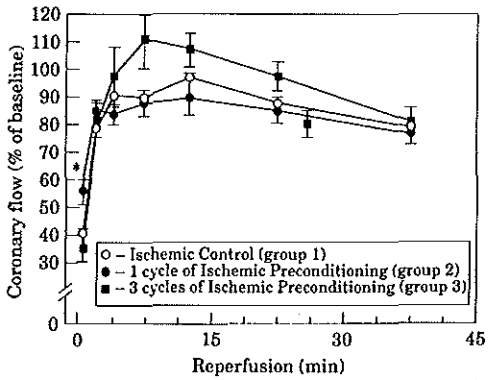

*Figure 4. The effect of ischemic preconditioning on coronary flow at reperfusion following sustained global ischemia in the isolated rat heart. The time points constitute the middle of each period of continuous collection. See Table 1 for baseline values of coronary flow. *$P<0.01$ between groups 2 and 3.*

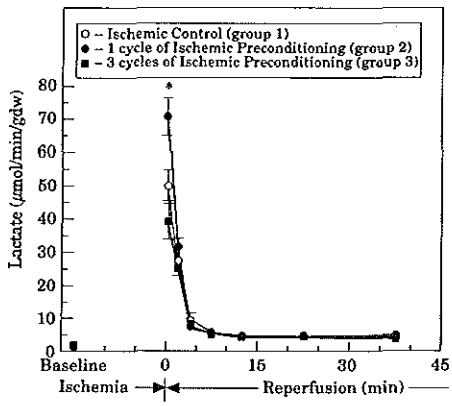

*Figure 5. Release of lactate following 40 min global ischemia: comparison between controls and the ischemic preconditioning groups. The time points identify the middle of each period of continuous collection of the coronary effluent. The release in all groups and intergroup differences were most pronounced in the first minute of reperfusion (group 2 different from 1 and 3; *$P<0.005$ in ANOVA). All differences between the groups disappeared after 3 min of reperfusion. Coronary effluent lactate returned to baseline levels within 10 to 15 min (gdw, gram dry weight).*

4. Discussion

In isolated hearts, preconditioning manifests by reduced infarct size [4] and prevention of arrhythmias during coronary occlusion and reperfusion [7]. Protection in rat hearts, undergoing no-flow global ischemia was shown by Cave and Hearse [9], using preconditioning with 5 min of both ischemia and reperfusion, in the isolated working rat heart model. They found a functional benefit with normothermic global ischemia up to 25 min, and with hypothermic ischemia up to 160 min duration, but no sparing of tissue high energy phosphates. Volovsek et al. [10] preconditioned rat hearts by repetitive episodes of either 2 or 5 min ischemia, thereby improving functional recovery and reducing lactate and enzyme release after 20 min ischemia. Other authors [11,13,21] had similar findings with preconditioning in the Langendorff model, using one or more episodes of 5 min ischemia and 5-10 min reperfusion intervals.

Our results indicate protection by preconditioning manifested by reduction of adenine nucleotide catabolism, reduced leakage of creatine kinase and attenuation of postischemic increase in end-diastolic pressure. However, other components of ischemia-reperfusion damage like systolic function, reperfusion arrhythmias and myocardial oxygen consumption were not significantly affected.

4.1. Coronary flow

Reversible ischemia increases coronary resistance and impairs vasodilator responsiveness [22]. In our study, the hyperemic flow increased with successive bouts of brief ischemia (Figure 1). There was no attenuation of no-reflow following sustained ischemia, as described by Asimakis et al. [11]. In their study, the inner-layer underperfusion ought to be attributed to an excessive end-diastolic pressure in the ischemic controls. We used lower basal balloon pressures. Postischemic diastolic pressures were also lower, permitting a substantially better reflow. We did not find a relationship between the metabolites determined in the coronary effluent and the coronary flow. It may only be postulated that a complex relationship between the products of the cyclooxygenase pathway, adenosine, endothelium-derived (nitric oxide, endothelin) as well as mechanical factors regulate the vascular tone in the preconditioned heart.

4.2. Ischemic arrest

Rapid induction of cardiac arrest prior to the onset of ischemia permits the maintenance of higher energy levels during ischemia, delays the rise in $[Ca^{2+}_{in}]$ and the development of ischemic contracture, and leads to a better recovery upon reperfusion [23,24]. Some authors [2,25] suggested that contractile failure might occur earlier in preconditioned hearts. In our study, preconditioning had no prominent effect on atrioventricular conduction, contractile failure or ventricular electrical arrest. Therefore, early arrest of electrical or mechanical activity is neither necessary, nor does it contribute to the protective effect of preconditioning in globally ischemic isolated rat heart.

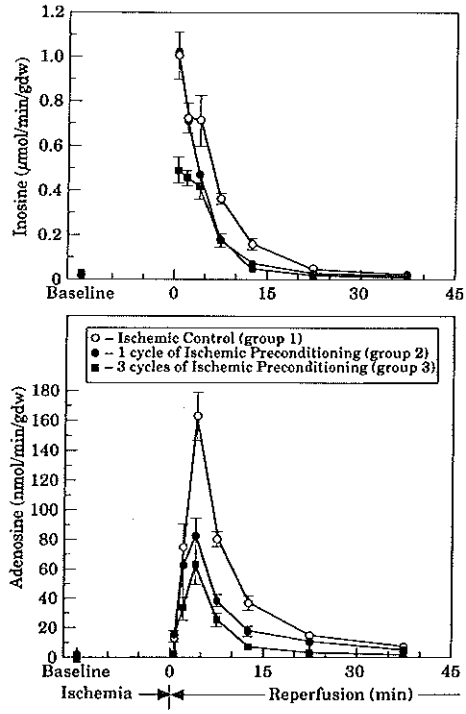

Figure 6. *Release of purine metabolites following 40 min global ischemia: inosine and adenosine. Adenosine was found in the coronary effluent in much smaller quantities than inosine and oxypurines (not shown). Adenosine peaked substantially later than inosine. Preconditioning progressively attenuated purine release (see Table 4 for statistical comparison; gdw, gram dry weight).*

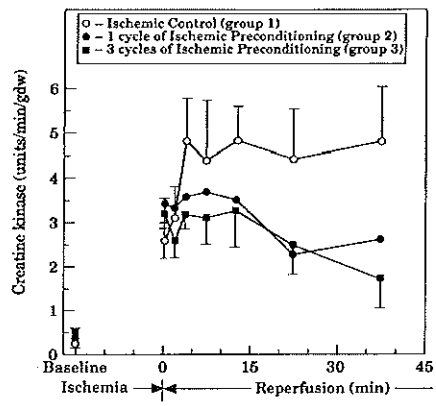

Figure 7. *Release of creatine kinase following 40 min global ischemia. Creatine kinase remained elevated during the whole reperfusion period and showed high variability between the hearts in the time course of release and the total activity lost. The loss of creatine kinase tended to be lesser in preconditioned hearts, achieving statistical significance in group 3 (see Table 4; gdw, gram dry weight).*

4.3. Reperfusion tachyarrhythmia

Reperfusion tachyarrhythmia occurred in all the hearts (following 40 min global ischemia). The lack of the anti-arrhythmic effect of preconditioning in our global ischemic setting is in disagreement with the well-documented antiarrhythmic effect of preconditioning in regionally ischemic hearts [5,7]. The ability of the myocardium to generate arrhythmias at reperfusion is considered to indicate tissue viability, is usually highest with ischemia of 5-20 min and declines afterwards [26]. Reperfusion arrhythmogenesis is related to the size of the ischemic zone: the incidence of ventricular fibrillation increased to 100% when the ischemic region was enlarged to above 45% of the ventricle [27]. Therefore, an antiarrhythmic effect of preconditioning, manifested during regional ischemia or reperfusion, will not necessarily suffice to protect globally ischemic hearts. Our further studies in global ischemia suggest that preconditioning does not attenuate, but alters the temporal relationship of reperfusion tachyarrhythmias [28].

4.4. Cardiac function

The ability of preconditioning to improve postischemic function is a matter of controversy. Regional contractility was not preserved following preconditioning and 60 min ischemia in dogs [29]. A markedly reduced extent of necrosis following long-term global ischemia was not associated with improved post-ischemic functional recovery in isolated rabbit hearts [30]. However, as mentioned above, studies in rats usually demonstrate functional protection by preconditioning. Our experiments utilized atrial overdrive pacing to standardize the heart rate during assessment of contractile performance. Postischemic diastolic dysfunction was attenuated without improvement of systolic contraction. Neither developed pressure, nor spontaneous heart rate, or oxygen consumption were significantly affected (Table 2).

The shorter reperfusion intervals used by other authors in the course of preconditioning could be responsible for their better functional results. Short reperfusion after brief ischemia is associated with a greater degree of stunning than in this study. The energy demand of preischemic myocardium may govern ATP breakdown during subsequent ischemia and thus affect the outcome of preconditioning [31]. In the current study, the recovery of developed pressure was remarkably good. Prolonged ischemic episodes, as used here, damage many of the hearts beyond their ability to recover in the working heart system. The extent of damage is underestimated by measuring the isovolumic pressure development in the non-ejecting Langendorff model of heart perfusion. Because of these limitations, the beneficial effect on systolic function could be inadequately expressed.

4.5. Relationship with ischemic lactate production

Neely and Grotyohann [32] improved ischemic tolerance of isolated rat hearts by anoxic glycogen depletion. However, these findings could not be reproduced in rabbit hearts [33]. The role of glycolysis in attenuating ischemic damage was re-emphasized in studies involving global ischemia [15,24].

In open-chest dogs [2,34] and in isolated rat hearts [10,11], protection after repetitive preconditioning coincided with reduced glycogen stores and diminished lactate accumulation in totally

ischemic myocardium. In contrast, preconditioned isolated rabbit hearts undergoing low-flow ischemia were protected in association with enhanced glycolysis and greater lactate production [12].

In our study, ischemic lactate production was represented by cumulative reperfusion release of lactate. This may underestimate tissue lactate accumulation during ischemia and does not account for glycolysis and lactate metabolism taking place at reperfusion. We believe our approach is valid since: a) Our hearts were not immersed and no lactate loss occured to the surrounding medium; b) It may be assumed that no differences existed between the groups in the ratio of lactate to pyruvate + alanine during global ischemia [12]; c) The time course of lactate release on reperfusion (Figure 5) indicates a washout from the previously ischemic tissue; d) Lactate remaining in tissue after prolonged reperfusion in this model is negligible in comparison to the ischemic levels [35].

Preconditioning per se did not necessarily lead to reduced lactate release (Figure 5 and Table 5). A combination of two factors may explain a somewhat increased lactate washout in group 2: a) Substantial glycogen replenishment occured during the first reperfusion period; b) Although still controversial [36], adenosine released during preconditioning ischemia might be responsible for stimulating the glycolytic flux afterwards [37,38]. Repeated brief ischemia (in group 3) depleted tissue glycogen stores (Table 4) thereby reducing the rate of anaerobic glycolysis during the subsequent prolonged ischemia.

Thus, we compared two protocols of preconditioning, significantly different (and divergent relative to control) in their rate of lactate release. Both preconditioning groups appeared to have beneficial effects on postischemic diastolic pressure and enzyme release. Three rounds of short-term global ischemia-reperfusion provided more protection than a single event. This concurred with treatment-induced depression of myocardial function. However, the beneficial effects of preconditioning on myocardial salvage cannot be explained entirely as a result of reduced oxygen consumption prior to sustained ischemia (Table 2, see also [39,40]). Indeed, "prestunning" seems to decrease myocardial oxygen consumption less than it impairs function [41,42].

These data suggest that the reduction of the ischemic damage in preconditioned hearts is not necessarily dependent on the effect of preconditioning on myocardial energy consumption or ischemic lactate production. Apparently, there was still not as much protection as others have seen. Therefore the present study suggests that it is possible to observe a protective effect of ischemic preconditioning in the absence of a decrease in anaerobic glycolysis, but the magnitude of the protective effect is less than in protocols that reduce energy consumption, lactate production and acid accumulation.

4.6. Ischemic contracture

Ischemic contracture development was markedly different between the preconditioning groups: it was attenuated in group 2 but appeared earlier in group 3 (Figure 2). Earlier onset of contracture was reported in rat hearts preconditioned by 5 min ischemia or hypoxia and 10 or 5 min reperfusion [11,13]. Janier and co-authors [12] reported attenuation of contracture during low-flow ischemia in rabbit hearts preconditioned by 3 min ischemia and 12 min reperfusion.

The onset of contracture is associated with ATP depletion and actin-myosin rigor bond formation [15]. Contracture development in preconditioned hearts may be related to their glycolytic flux. The apparent disagreement between the findings obtained in various studies may be reconciled, if we assume that the effect of repeated ischemia on contracture is mediated by glycolysis, and is dependent on the

model, the number of short-term ischemic episodes and the duration of the reperfusion intervals.

The elevated left ventricular diastolic pressure at later stages of ischemia and at reperfusion, behaves differently from early ischemic contracture. This post-ischemic contracture reflects diastolic functional impairment, generally attributable to cytosolic free Ca^{2+} overload [24,43]. Preconditioning apparently constitutes an effective method for alleviation of ischemia-reperfusion associated calcium overload [21].

4.7. Adenosine and total purine

Adenosine and total purine loss was attenuated in the preconditioned hearts, indicating decreased nucleoside breakdown during sustained ischemia (Figure 6). This confirms previous observations in rabbit hearts [31] and probably indicates a more favorable myocardial energy state following preconditioning [44]. On the other hand, the pattern in rat contrasts the marked increase in adenosine and 5'-nucteotidase activity in preconditioned canine hearts [45]. Several investigators have already shown that preconditioning in rats, in contrast to rabbits and dogs, is not mediated by adenosine [4,46].

4.8. Prostacyclin release

To the best of our knowledge, the effect of preconditioning on the cardiac release of prostacyclin has not yet been reported. Hearts synthesize and release various eicosanoids during and following ischemia, even of short duration [47-49]. Prostacyclin is the main metabolite of arachidonic acid, produced under these circumstances [50]. Early synthesis of prostaglandins by the anoxic/ischemic heart is apparent prior to the development of damage and probably constitutes a compensatory response [51,52]. Endogenous prostaglandins seem to be involved in both reactive hyperemia following brief ischemia [48] and in endothelium-dependent vasoconstriction [53]. The involvement of products of the cyclooxygenase pathway in prevention of ischemic arrhythmias, similarly to exogenous Iloprost, suggests participation of prostacyclin in preconditioning [14]. Other investigators, also using cyclooxygenase inhibitors in preconditioning experiments, could not confirm a role for prostaglandins in limiting infarct size or arrhythmia [54]. The present study (Tables 3 and 5) provides evidence that: a) Cardiac adaptation to repeated short-term ischemia involves a decrease in prostacyclin production; b) The protection during and following prolonged ischemia coincides with an enhanced prostacyclin release. Our data do not prove a link between prostacyclin and the protection afforded by ischemic preconditioning. We believe that the effects of preconditioning on prostaglandin synthesis warrant further investigations, since these mediators may be involved in attenuation of microvascular stunning [55] and other forms of cardioprotection.

4.9. Conclusions

In this model of protracted global ischemia in isolated rat hearts, preconditioning leads to an augmented prostacyclin release after sustained ischemia. Preconditioning is manifested by a decrease in postischemic contracture and biochemical evidence of myocardial salvage, but not necessarily by prevention of arrhythmia or improved systolic contraction. Anaerobic glycolysis appears to be necessary for attenuation of ischemic contracture; however, it may not play an ultimate role in the effect of

preconditioning on myocardial damage or post-ischemic recovery.

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5

Effect of acadesine on myocardial ischemia in patients with coronary artery disease

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Abstract

Objective: Acadesine, an adenosine regulating agent, attenuates the adverse effects of ischemia on ventricular function in animals. This study examined its influence on pacing-induced ischemia in 47 patients undergoing coronary angiography. **Methods:** After 15 min of recovery from control pacing, an infusion of acadesine (5, 10, 20, 50 mg/kg i.v.) was commenced and after a further 15 min the protocol was repeated with the infusion continued. **Results:** At higher doses, minor beneficial effects on ejection fraction and myocardial lactate metabolism were observed. Hemodynamics were unaffected. Systemic lactate rose in relation to acadesine, up to 60% (P<0.001 vs. placebo). **Conclusion:** The data may indicate that acadesine stimulates anaerobic glycolysis in man.

1. Introduction

In experimental models, acadesine (5-amino-4-imidazole carboxamide riboside), an adenosine regulating agent, limits the deleterious effects of ischemia on left ventricular function [1]. Acadesine appears to act by increasing myocardial adenosine concentrations [2], which may then improve regional perfusion, contractility and metabolism [3]. Its actions are unusual in being event- and site-specific. The drug is thought to be only effective in ischemic myocardium, and pharmacologically inert, rendering it a potential therapeutic agent in ischemic heart disease. When studied in man [4], acadesine does not produce the undesirable systemic effects of adenosine.

Although the effects of acadesine in regional as opposed to global myocardial ischemia have not been wholly consistent, acadesine attenuated the decrease in left ventricular wall thickening provoked by atrial pacing in a model of circumflex coronary artery stenosis [5]. Encouraged by the experimental evidence, we investigated the effect of acadesine on left ventricular function, and myocardial lactate metabolism, in relation to pacing stress in coronary patients.

2. Materials and methods

2.1. Patients

The study group was drawn from patients undergoing cardiac catheterization for the investigation of suspected coronary artery disease, with >50% stenosis in at least one coronary artery. Patients with unstable angina, acute myocardial infarction, left main coronary artery stenosis, diabetes mellitus, or chronic renal failure, were excluded.

2.2. Protocol

All medications were discontinued ≥48 h prior to the study. Cardiac catheterization was performed through the femoral artery and vein. Heparin (5000 U) was administered intravenously. Following angiography of the left and right coronary arteries, a dual tip micromanometer pigtail catheter was positioned in the left ventricle and a bipolar coronary sinus catheter was introduced. Baseline left

ventricular angiography was performed and left ventricular and aortic pressures and pressure-derived indices were measured. Atrial pacing commenced at 10 beats/min above the spontaneous heart rate, with increments of 10 beats/min at 2-min intervals. Pacing end-points were a rate of 180 beats/min, angina, or atrioventricular block. Measurements were recorded during maximal pacing and ventricular angiography was repeated immediately on cessation of pacing. After 15 min rest, an i.v. infusion of placebo (0.28% NaCl) or acadesine (5, 10, 20 or 50 mg/kg) was commenced in a randomized, double-blind fashion. After a further 15 min, with acadesine infusion continued, an identical protocol was followed. Coronary sinus and femoral arterial blood samples were taken before, during and after maximal pacing and during recovery.

2.3. Assessment of function and hemodynamics

Global and regional left ventricular function was evaluated as described elsewhere [6]. Left ventricular parameters were measured using the micromanometer catheter [7]. The following indices were computed: Peak left ventricular pressure, its derivatives and the time constants for early relaxation [8].

2.4. Lactate measurement

Blood samples of approx. 1.5 ml were collected and processed as described [9]. In deproteinized samples, lactate was assayed in duplicate with lactate oxidase and peroxidase on a Merck ELAN analyzer.

2.5. Statistical analysis

The effect of acadesine on pacing-induced changes (infusion vs. pre-infusion) was assessed by analysis of variance to detect differences between dosage groups. A paired Student's *t*-test was then applied or linear regression analysis was used to examine the dose-response relationship. Mean values are reported ± S.E.M. Statistical significance was accepted at the 5% level.

3. Results

3.1. Demographics, drug tolerance

The inclusion criteria were met by 47 patients, including eight females, who all provided informed consent. Their median age was 58 years (range: 38-70 years). Acadesine was well tolerated and there were no adverse clinical events.

3.2. Atrial pacing

In all the groups, the maximal heart rates achieved during the second pacing period were similar to those in the first (approx. 150 beats/min).

3.3. Ejection fraction and hemodynamics

The left ventricular ejection fraction before pacing was 63±2%. Atrial pacing induced a small decline in ejection fraction (Table 1). During the second pacing period, following acadesine infusion, this decline was attenuated, significantly so at the 20 mg/kg dose (pacing 2: decrease from 61 to 60% vs. pacing 1: decrease from 63 to 58%, P=0.036; see Table 1). The parameters of left ventricular function were not influenced by acadesine at rest, at maximal pacing (Table 1), or during recovery. Differences observed between the first and second stress tests were <5%.

Table 1. Effect of acadesine on ventricular function and hemodynamics during atrial stress testing

Variable		Acadesine (mg/kg)				
		placebo	5	10	20	50
left ventricular pressure (mmHg)	(1)[a]	127±5	135±5	124±5	152±8	136±8
	(2)	132±6	132±6	123±5	143±8	127±11
LVEDP[b] (mmHg)	(1)	10±3	13±2	9±2	13±2	7±2
	(2)	9±1	12±2	9±2	11±2	10±3
LVdP/dt[b] (mmHg/s)	(1)	1783±153	1728±144	1711±130	1790±127	2010±163
	(2)	1775±161	1700±150	1711±103	1684±85	1841±221
Tau_I (ms)	(1)	45±5	49±5	43±3	52±4	39±2
	(2)	46±4	50±5	43±3	53±4	40±2
ejection fraction (%)	(1, pre)	66±4	57±4	66±4	63±5	65±2
	(1, post)	62±3	54±3	62±5	58±6	63±3
	(2, pre)	66±3	56±3	65±5	61±6	65±2
	(2, post)	63±4	55±4	63±5	60±5	63±3

[a] Measurements were made during first (1 = no acadesine) and second (2 = with acadesine) pacing periods at maximal pacing, except for ejection fraction, where data were obtained before (pre) and immediately after maximal pacing (post). Means ± S.E.M., n=9-10. [b] LVEDP and LVdP/dt = left ventricular end-diastolic pressure and its peak first derivative, respectively.

3.4. Lactate metabolism

In general, arterial and coronary sinus lactate levels were higher in relation to acadesine infusion (Table 2). For instance, the rise in arterial lactate during the post-pacing recovery period varied between the groups (P<0.001, analysis of variance) and lactate levels following the higher doses of acadesine, 20 and 50 mg/kg, were greater than control (both P<0.001). Regression analysis confirmed an incremental effect of acadesine at these doses (20 mg/kg: P=0.026, 50 mg/kg: P=0.01) suggesting dose-dependency.

The arterio-venous lactate data during the first pacing stress test showed that ischemia was induced: Lactate uptake before pacing, 0.13±0.03 mmol/l (n=39), decreased both during (-0.01±0.04 mmol/l) and immediately post-pacing (-0.08±0.06 mmol/l), with return to lactate uptake during recovery (0.09±0.05 mmol/l). Arterio-venous lactate tended to increase during pacing in relation to acadesine, but this did not reach significance (Table 2). However, when the effects of low dose (0-10 mg/kg) and high dose acadesine (20-50 mg/kg) on arteriovenous lactate were compared, there were differences throughout the pacing protocol. The acadesine-induced decline in lactate production was significant during maximal

pacing and recovery. Arteriovenous differences during pacing were: Low dose, 0.09±0.03 mmol/l vs. high dose, 0.17±0.08 mmol/l; P=0.016, n=27 and 12, respectively; those during recovery were: Low dose, 0.06±0.04 mmol/l vs. high dose, 0.23±0.06 mmol/l; P=0.019.

Table 2. Effect of acadesine on blood lactate and arteriovenous differences

Group	Phase	Lactate (mmol/l)					
		- acadesine			+ acadesine		
		arterial	CS[a]	arterial-CS	arterial	CS	arterial-CS
placebo	pre-pacing	0.76±0.09	0.63±0.05	0.13±0.05	0.79±0.07	0.56±0.05	0.23±0.05
(n=9-10)	max. pacing	0.75±0.08	0.76±0.04	0.00±0.07	0.72±0.06	0.64±0.06	0.06±0.05
	post-pacing	0.68±0.06	0.81±0.08	-0.13±0.10	0.76±0.07	0.73±0.08	0.02±0.09
	recovery	0.70±0.08	0.60±0.04	0.11±0.07	0.81±0.07	0.54±0.06	0.27±0.06
5 mg/kg	pre-pacing	0.81±0.11	0.68±0.11	0.13±0.06	0.84±0.12	0.74±0.11	0.11±0.06
(n=10)	max. pacing	0.77±0.09	0.73±0.11	0.04±0.08	0.80±0.10	0.84±0.12	-0.04±0.09
	post-pacing	0.79±0.09	0.85±0.14	-0.06±0.12	0.88±0.12	0.87±0.16	0.01±0.13
	recovery	0.85±0.12	0.73±0.10	0.12±0.08	0.86±0.12	0.76±0.12	0.09±0.09
10 mg/kg	pre-pacing	0.82±0.17	0.52±0.16	0.25±0.08	0.84±0.16	0.49±0.13	0.36±0.11
(n=7-9)	max. pacing	0.77±0.14	0.56±0.16	0.14±0.06	0.79±0.13	0.57±0.14	0.19±0.05
	post-pacing	0.80±0.13	0.58±0.13	0.16±0.08	0.84±0.16	0.56±0.12	0.21±0.12
	recovery	0.84±0.14	0.48±0.11	0.32±0.09	0.91±0.16	0.53±0.16	0.35±0.07
20 mg/kg	pre-pacing	0.80±0.16	0.82±0.16	0.01±0.09	0.95±0.14	0.74±0.16	0.19±0.09
(n=6-8)	max. pacing	0.80±0.14	0.94±0.10	-0.13±0.09	1.03±0.15	1.04±0.18	0.08±0.07
	post-pacing	0.83±0.12	1.16±0.12	-0.30±0.16	1.02±0.13	1.19±0.18	-0.08±0.15
	recovery	0.87±0.13	0.97±0.10	-0.08±0.14	1.12±0.15	1.02±0.18	0.20±0.08
50 mg/kg	pre-pacing	0.67±0.06	0.59±0.05	0.11±0.03	0.84±0.13	0.79±0.11	0.13±0.12
(n=5-7)	max. pacing	0.65±0.08	0.88±0.17	-0.17±0.12	1.10±0.14	1.10±0.14	0.11±0.20
	post-pacing	0.70±0.09	0.87±0.11	-0.06±0.16	1.18±0.13	1.12±0.10	0.20±0.14
	recovery	0.76±0.09	0.87±0.14	-0.07±0.18	1.23±0.11	1.09±0.10	0.25±0.11

After an atrial pacing stress test, the acadesine infusion started and pacing was repeated. Means ± S.E.M. [a] CS = coronary sinus.

4. Discussion

4.1. Cardiac function and metabolism

This is the first report of the effects of acadesine on both left ventricular function and lactate metabolism in relation to myocardial ischemia in man. The decline in left ventricular ejection fraction provoked by pacing stress in patients with coronary artery disease tended to be less in the presence of acadesine (Table 1). This was small, but significant at a dose of 20 mg/kg. Further, myocardial lactate uptake during and after pacing tended to be increased with a high dose as opposed to low dose acadesine (Table 2). The latter is in line with canine data [10]. Left ventricular hemodynamics were not affected

(Table 1), confirming that acadesine was free of negative inotropic actions. The data suggest that acadesine exerted an anti-ischemic effect on the heart, in line with results obtained in patients undergoing coronary artery bypass graft surgery [11].

The 20 mg/kg dose appeared to be associated with a larger effect on left ventricular function than 50 mg/kg (Table 1). Also in animal models of myocardial ischemia, the protective effects of acadesine diminished at higher doses [1,12]. This concentration dependent effect of acadesine remains to be explained. Although the precise cellular events underlying the effects of acadesine and adenosine are as yet undetermined, a G-protein and K_{ATP} channels may be involved. Whether the ribose moiety in acadesine plays a role is unknown; ribose is a cardioprotectant, stimulating adenine nucleotide synthesis [13].

4.2. Peripheral lactate

Systemic lactate levels rose with acadesine, particularly at higher doses (Table 2). The effect of acadesine on arterial and coronary sinus lactate concentrations may be explained by increased glycolysis, in that it is well established that adenosine stimulates glucose uptake [14]. Although reports to the contrary exist [15], many studies show that adenosine promotes glycolysis (e.g., [16]). Species and tissue differences in the capacity to accumulate the phosphorylation product of acadesine and differences in target enzymes sensitive to this product probably determine the effect of the drug on glycolysis [17]. In rat skeletal muscle, acadesine activates glycogen phosphorylase and glycogenolysis [18]. Since skeletal muscle is a major contributor to body mass, it could well be the source of the increases in arterial lactate concentrations seen with acadesine.

4.3. Conclusions

In summary, in this study of pacing-induced ischemia in patients with coronary artery disease and stable angina, we found small protective effects on left ventricular function and myocardial lactate metabolism. We speculate that the rise in systemic lactate is due to acadesine-induced increases in adenosine, which could stimulate glucose uptake and its catabolism.

Acknowledgements

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6

Carbohydrates and purines in underperfused hearts, protected by ischemic preconditioning

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Abstract

Objective: Few, and those controversial, have been published on ischemic preconditioning followed by low-flow ischemia. The aim of this study was to assess whether ischemic preconditioning: 1) confers protection against severe underperfusion, and 2) is mediated by mobilization of proglycogen, resulting in increased anaerobic glycolysis and reduced myocardial injury. **Methods:** Isolated rat hearts were retrogradely perfused and subjected to either 25 min low-flow ischemia (0.6 ml/min) followed by 30 min reperfusion (IC; n=5), or the same protocol preceded by two cycles of 5 min no-flow ischemia and 5 min reperfusion (PC; n=7). Additionally, hearts (n=52) were freeze-clamped at different time points throughout the protocol. **Results:** Preconditioning improved functional recovery (developed force × heart rate in PC hearts: 54 vs. 21% in IC hearts; $P<0.01$) and reduced ischemic damage (cumulative release of creatine kinase during reperfusion: 93 vs. 215 U/g dry wt; $P<0.05$). During ischemia and reperfusion, release of adenosine and the sum of purines was smaller in PC hearts ($P<0.05$), while lactate release was similar in the two groups. PC reduced both macroglycogen and proglycogen by ca. 60% ($P<0.01$) resulting in constant glycogen levels during low-flow ischemia. In contrast, in IC hearts, both fractions decreased by ca. 60% during underperfusion ($P<0.01$). **Conclusions:** These results demonstrate that: 1) ischemic preconditioning reduces injury due to severe flow reduction, and 2) preconditioning reduced glycogenolysis without affecting anaerobic glycolysis, suggesting increased glucose uptake.

1. Introduction

Short periods of ischemia and reperfusion render the heart more tolerant to a subsequent sustained period of ischemia, a phenomenon termed 'ischemic preconditioning' [1]. Most research has focused on preconditioning-induced protection against a period of sustained no-flow ischemia, whereas only a few studies examined low-flow ischemia, probably more relevant as a clinical model. There is ample evidence that ischemic preconditioning protects against no-flow ischemia. However, whether preconditioning confers protection against low-flow ischemia or hypoxia is the subject of controversy: both positive [2,3] and negative results [4,5] have been reported. Residual flow during prolonged ischemia may lead to washout of adenosine from the interstitium and, consequently, loss of protection [4]. This is in line with the observation that adenosine A_1 receptors must be occupied both during preconditioning ischemia (initiation) and prolonged ischemia (mediation) in order to induce protection [6]. To our knowledge, however, the release of potentially protective adenosine during low-flow ischemia in preconditioned hearts has never been studied.

We have recently reviewed the controversial role of glycolysis in ischemic preconditioning [7]. During no-flow ischemia, ischemic preconditioning decreases glycolytic flux, resulting in reduced accumulation of lactate and protons, despite reduced ATP production. On the other hand, an increased glycolytic flux during low-flow ischemia reduces cell injury [8,9]. Using rabbit hearts, Janier et al. [2] attributed the protective effect of ischemic preconditioning on myocardial injury resulting from low-flow ischemia to increased glycolytic flux. Furthermore, a high endogenous glycogen content has been reported to be beneficial [10] or detrimental [11] to hearts subjected to no-flow ischemia. The role of glycogen in preconditioning-induced protection against low-flow ischemia is unclear. Tissue glycogen consists of two forms: classical macroglycogen and 'proglycogen' [12,13]. Proglycogen is a stable

intermediate in macroglycogen synthesis [13,14]. In myocardium, proglycogen levels remain relatively constant whereas macroglycogen forms a more labile fraction, decreasing during hypoxia/ischemia [15,16]. We recently confirmed in rabbit hearts that macroglycogen breakdown stops at proglycogen during continued ischemia [17]. However, intermittent ischemia resulted in a fall in both proglycogen and macroglycogen [17]. Therefore, depending on the physiological situation, glycogen seems to oscillate between macroglycogen and proglycogen, while further breakdown of proglycogen only occurs under special conditions, such as repeated ischemia and reperfusion. Thus, the purpose of this study was to assess the role of anaerobic glycolysis and (pro)glycogen in preconditioning-induced protection against low-flow ischemia. We examined whether ischemic preconditioning protects against severe underperfusion. Since macroglycogen and proglycogen form separate entities with different metabolic activitiy, we examined their response to low-flow ischemia during preconditioning. We hypothesized that ischemic preconditioning mobilizes proglycogen during low-flow ischemia supporting glycogenolytic flux and reducing myocardial injury. The results show that preconditioning confers protection against low-flow ischemia possibly by reducing glycogenolysis and increasing glycolysis during underperfusion. Part of this research has been published in abstract form [18].

2. Materials and Methods

All animals were treated in comformation with the guiding principles in the care and use of animals as approved by the American Physiological Society. The Animal Welfare Committee, Erasmus University Rotterdam, approved the protocol.

2.1. Exclusion criteria

During stabilization, hearts were excluded if they met one of the following criteria: 1) unstable contractile function, 2) coronary flows outside the range of 10-18 ml/min, 3) severe arrhythmias, 4) myocardial temperature outside the range 37 - 39°C.

2.2. Isolated heart preparation

Fed, male Wistar rats (Wag/Rij inbred, weighing 280-330 g) were obtained from Harlan-CPB, Zeist, The Netherlands. They received a commercial rat chow (Hope Farms AM II, Woerden, The Netherlands) and tap water ad libitum. After anesthesia with an intraperitoneal injection of 0.7 ml sodium pentobarbital (Nembutal®, 60 mg/ml) supplemented with 0.1 ml heparin (Thromboliquine®, 5000 I.U./ml), hearts were rapidly excised and arrested in saline (0-4 °C) until beating ceased. Excess tissue was removed, and the hearts were cannulated via the ascending aorta, for retrograde perfusion using a non-recirculating modified Krebs-Henseleit buffer containing (mmol/l): NaCl 118, KCl 5.6, $CaCl_2$ 2.4, $MgCl_2$ 1.2, $NaHCO_3$ 20, Na_2HPO_4 1.2 and D-glucose 10. Before use, the buffer was filtered through a 45-μm porosity filter to remove any particulate matter, and equilibrated with 5% CO_2/95% O_2, to give a pH of about 7.4 at 37°C. Myocardial temperature was kept at 37°C with a water-jacketed heart chamber and buffer reservoir, and regulated with an electric heating coil positioned around the aortic inlet line. The temperature of the outer ventricular wall was monitored with a thermocouple (A-F6, Ellab

A/S, Roedovre, Denmark). Global, no-flow ischemia was induced by clamping the aortic line; low-flow ischemia was achieved using a perfusion pump (MV-MS3, Ismatec, Zurich, Switzerland) operating at a flow rate of 0.6 ml/min.

Coronary flow was measured by timed collection of the pulmonary artery effluent. The hearts were allowed to beat spontaneously. Cardiac contractile function was estimated with a small hook inserted into the apex and connected to a force transducer (F5A-2, Konigsberg Instruments, Pasadena, CA). The heart was pre-loaded with an initial resting tension of 4 g. Systolic tension and diastolic tension were continuously displayed on a paper recorder. Developed tension was calculated as systolic tension minus diastolic tension. Cardiac contractile function was expressed as rate-force product (RFP), the product of developed tension and heart rate. RFP at the end of reperfusion was compared to the preischemic value after the 20-min stabilization period and expressed as percent recovery of RFP. Perfusion pressure was measured by a disposable pressure transducer (Braun Melsungen, Austria) and kept constant at 75 mmHg.

2.3. Experimental protocol

After initial isolation and surgical preparation, all hearts were perfused with modified Krebs-Henseleit buffer and allowed to equilibrate for 20 min. Thereafter, hearts were randomly assigned to one of the following groups: a preconditioning group (PC; n=7) and an ischemic control group (IC; n=5). After stabilization, IC hearts were perfused for an additional 20-min period of normoxic perfusion; PC hearts were assigned to undergo two 5-min episodes of no-flow ischemia, each interrupted by 5 min of reperfusion. Thereafter, PC and IC groups were subjected to 25 min of low-flow ischemia (0.6 ml/min) followed by a reperfusion period of 30 min and freeze-clamping (Figure 1). In addition, PC and IC hearts (n=7 each) were freeze-clamped using Wollenberger clamps pre-cooled with liquid nitrogen before and after sustained ischemia (Figure 1). Furthermore, hearts were freeze-clamped after 1 min (n=5) and 20 min (n=6) of normoxic perfusion. They were weighed and stored in liquid nitrogen until assayed.

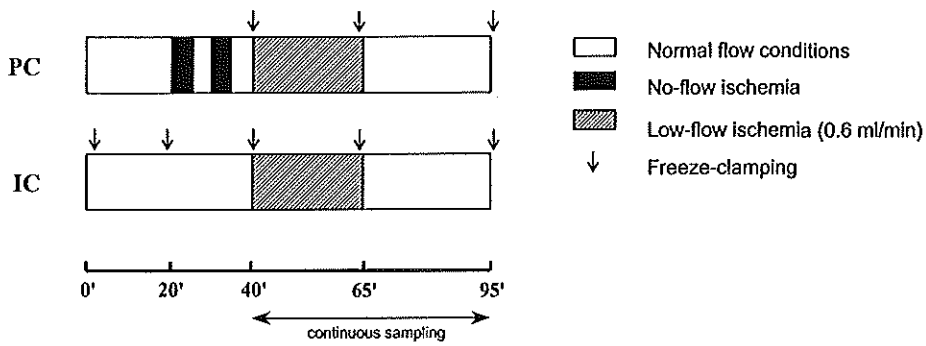

Figure 1. Experimental protocols. After an initial 20 min stabilization period, preconditioned hearts (PC) underwent two cycles of 5-min stop-flow ischemia and reperfusion; ischemic control hearts (IC) were perfused for an additional 20 min. Thereafter, both PC and IC hearts were exposed to 25 min of low-flow ischemia (0.6 ml/min) by use of a perfusion pump, and reperfused for 30 min. In parallel experiments, hearts were freeze-clamped at different time points throughout the protocol as indicated by the arrows. During ischemia and reperfusion, coronary perfusate samples were continuously collected at 1-, 5-, and 10-min intervals, depending on the changes expected. Prior to ischemia, several 1-min samples were taken.

2.4. Biochemical assays

2.4.1. Tissue determinations.

Freeze-clamped hearts were ground under liquid nitrogen using pestle and mortar. A sample was taken for dry weight determination and the remaining part was treated with 4% $HClO_4$ [19]. A part of the homogenate was sonicated for 4 × 20 s on ice and neutralized with 5 M NaOH. After centrifugation (3200 × g) of the remaining part, the precipitate was dissolved in 10 ml 0.1 M KOH; an aliquot of the supernatant fraction was neutralized with 5 M NaOH for glycogen determination, another aliquot (500 μl) was neutralized with 6 M KOH/ 2 M K_2CO_3 for HPLC analysis of high-energy phosphates. Glycogen fractions were determined in the homogenate (total glycogen), the supernatant fraction (macroglycogen), and the pellet fraction (proglycogen). After treatment with amyloglucosidase, glucose was assayed spectrophotometrically according to Huijing [20] and Keppler and Decker [21].

2.4.2. Analysis of coronary effluent and tissue high-energy phosphates.

During ischemia and reperfusion, coronary perfusate samples were continuously collected at 1-, 5-, or 10-min intervals, depending on the changes expected. Prior to ischemia, several 1-min samples were taken. Within 12 h, lactate and creatine kinase in the samples (0-4°C) were determined enzymatically with an Elan auto-analyzer (Eppendorf, Merck, Amsterdam, The Netherlands) according to Sigma procedure #735 (St. Louis, MO, USA). The rest of the samples were stored at -80 °C until further analysis. Purines in coronary perfusate samples and tissue ATP and creatine phosphate (CrP) were determined by reversed phase high-performance liquid chromatography (HPLC) according to Smolenski et al. [22]. Briefly, a C_{18} column (Hypersil ODS 3 μm, 150 × 4.6 mm, Alltech, Deerfield, IL, USA) was employed combined with a C_{18} guard column (Hypersil ODS 5 μm, 7.5 × 4.6 mm). For purines, the system configuration consisted of a Waters 510 pump, a cooled Waters 712 WISP autosampler, a Spectra Focus forward optical scanning detector (Spectra-Physics, San Jose, CA, USA) and a Waters Millenium 2010 data system (Waters, Milford, MA, USA). ATP and CrP were determined using an AS3000 cooled autosampler, a SCM1000 vacuum membrane degasser, a P2000 gradient pump, and PC1000 software (Thermo Separation Products, Riviera Beach, FL, USA) in combination with the above mentioned scanning detector. Peaks were detected at 254 nm (hypoxanthine, xanthine, inosine, adenosine, ATP), at 280 nm (uric acid) and at 214 nm (CrP). Purines, ATP and CrP were identified based on their retention times, co-elution with standards and their 254/280 ratios.

2.5. Statistics

The data are expressed as means ± S.E.M., with n = number of hearts. Hemodynamic and metabolic data were analyzed using two-way repeated measures ANOVA with one repeating factor and one grouping factor. Summary measures were constructed for postischemic release of creatine kinase [23]: for each heart the cumulative enzyme release was determined. Student's unpaired *t*-test was used for comparisons between groups; when data were not normally distributed or groups had unequal variances, the Mann-Whitney rank sum test was used. Values of $P<0.05$ (two-tailed test) were regarded as significant.

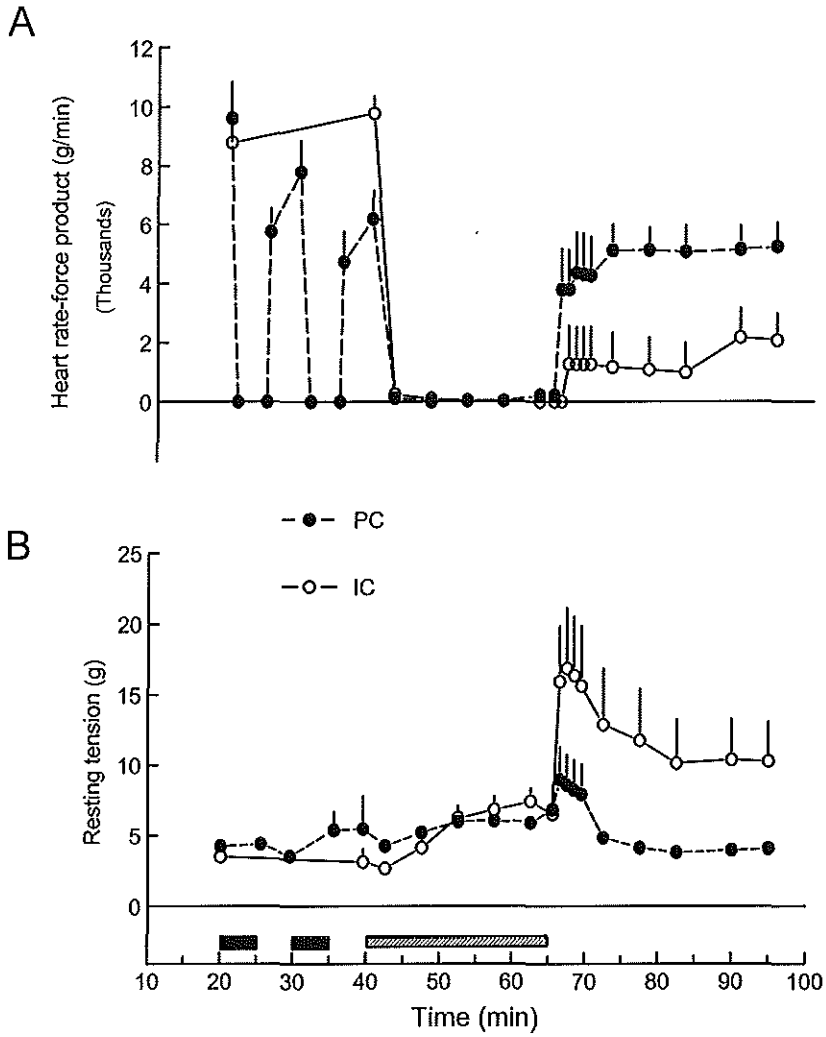

Figure 2. Changes in rate-force product (A) and resting tension (B). After 20 min stabilization, preconditioned hearts (PC) were exposed to two cycles of 5 min no-flow ischemia (solid bars) and reperfusion, whereas control hearts (IC) were perfused for an additional 20-min period. Thereafter, both groups underwent 25 min of low-flow ischemia (0.6 ml/min; hatched bar) and 30 min reperfusion. Preconditioning improved recovery of rate-force product and abolished postischemic contracture ($P<0.05$; repeated measures ANOVA).

3. Results

3.1. Function

Figure 2 presents time-dependent changes in rate-force product and resting tension, reflecting ventricular systolic and diastolic functioning, respectively. During the 20-min stabilization period, RFP, resting tension, heart rate (283±13 beats/min) and coronary flow (13±0.5 ml/min) were similar in PC and IC hearts. With the short periods of preconditioning ischemia, RFP rapidly fell to 0 in the PC group while RFP recovered to 81 and 64% after the first and second episode of ischemia, respectively (Figure 2A). The resting tension of PC hearts was not significantly affected by the short periods of preconditioning ischemia (Figure 2B). After the onset of low-flow ischemia, RFP fell rapidly to 0 in both PC and IC hearts. At the end of sustained ischemia, resting tension had almost doubled in both control and preconditioned hearts. With reperfusion, recovery of RFP was more than double in PC hearts ($P<0.05$ vs. IC). During reperfusion, PC abolished the large increase in resting tension observed in IC hearts ($P<0.05$).

3.2. Tissue Necrosis

Release of creatine kinase in the coronary effluent was used to detect membrane damage due to tissue necrosis. The short periods of preconditioning ischemia resulted in a small release of creatine kinase while little release could be detected in both groups during low-flow ischemia (Figure 3A). Substantial release took place during reperfusion. Cumulative release of creatine kinase during reperfusion was half in PC hearts ($P<0.05$) compared to IC hearts (see inset Figure 3A).

3.3. Metabolite Release

3.3.1. Lactate.

The release of lactate in the coronary effluent was taken as a measure of anaerobic glycolysis both from endogenous (glycogen) and exogenous (glucose) sources. The brief periods of no-flow ischemia resulted in a large efflux of lactate in the PC group (Figure 3B). However, during low-flow ischemia and reperfusion, lactate release was similar in PC and IC hearts.

3.3.2. Purines.

Purines in the coronary effluent reflect ATP catabolism during ischemia and, hence, the energy status of the cell. Release of adenosine and total purines (adenosine + inosine + hypoxanthine + xanthine + urate) is depicted in Figure 4. Similar to lactate release, preconditioning ischemia resulted in a substantial overflow of purines. Release of adenosine (Figure 4A) was more than twofold higher during reperfusion compared to low-flow ischemia in both PC and IC hearts, that of purines (Figure 4B) ca. 1.5-fold. Release of adenosine was more than five times less in PC hearts compared to IC hearts, both during low-flow ischemia ($P<0.05$) and during reperfusion ($P<0.01$). Similarly, preconditioning resulted in 2.5 times less purine release during low-flow ischemia ($P<0.01$) and during reperfusion ($P<0.05$) than control hearts, indicating less myocardial injury [24]. The release of the individual purines followed a pattern similar to that of adenosine.

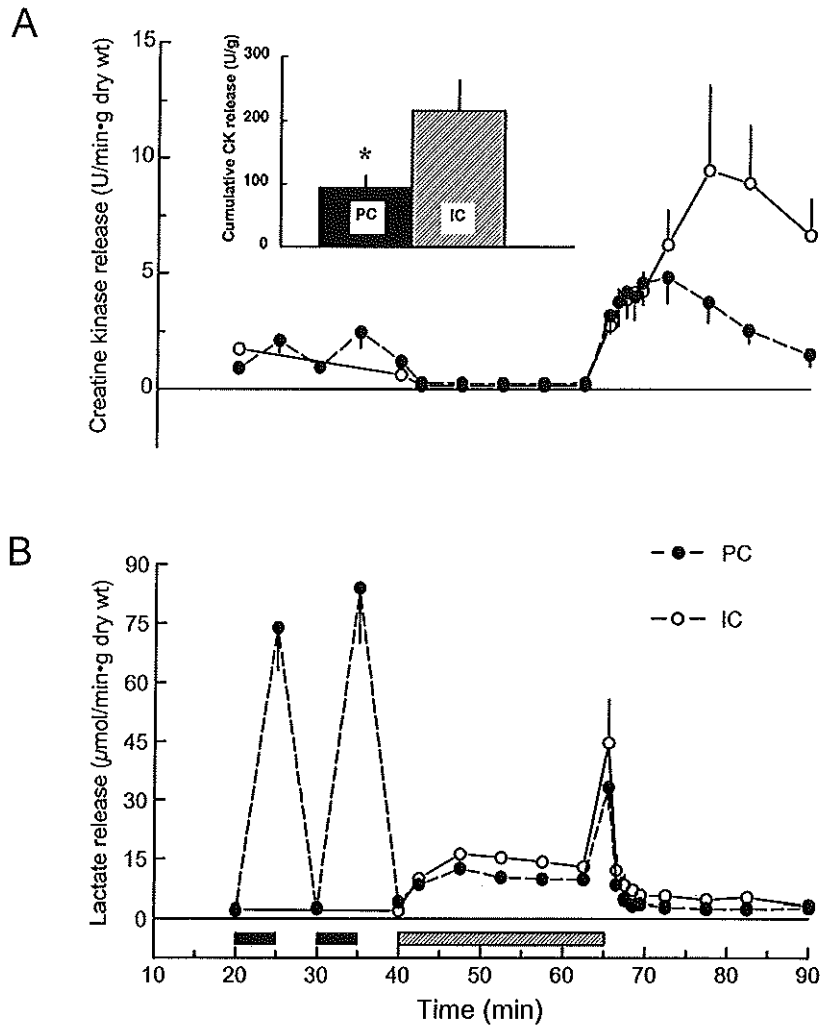

*Figure 3. Release of creatine kinase (A) and lactate (B) in the coronary effluent of ischemic preconditioned (PC) and ischemic control (IC) hearts. See legend to Figure 2 for protocol explanation. Cumulative release of creatine kinase was less in PC hearts compared to IC hearts (inset; *P<0.05). Lactate release during ischemia and reperfusion was similar in PC and IC hearts.*

3.4. Tissue Metabolites

3.4.1. Glycogen.

The contents of proglycogen and macroglycogen as well as total glycogen (proglycogen + macroglycogen) in hearts freeze-clamped at different time points are presented in Figure 5. Preconditioning ischemia depleted both proglycogen and macroglycogen by ca. 60% (Figure 5A). Consequently, preischemic pro- and macroglycogen content in PC hearts was approximately half that in IC hearts (proglycogen: $P<0.01$; macroglycogen: $P<0.05$). No significant breakdown of proglycogen and macroglycogen was observed in PC hearts during low-flow ischemia. In contrast, pro- and macroglycogen decreased ca. 60% in IC hearts during underperfusion. Postischemic pro- and macroglycogen content as well as partial recovery during reperfusion was similar in PC and IC hearts. With reperfusion, there was a similar recovery of proglycogen and macroglycogen in both experimental groups. Total glycogen (Figure 5B) mimicked the pattern of both subfractions: following preconditioning, there was a 60% reduction in total glycogen ($P<0.01$), and no significant glycogenolysis during underperfusion. Preischemic total glycogen content in PC hearts was approximately half that in IC hearts ($P<0.01$). Total glycogen decreased by 62% in IC hearts during low-flow ischemia. Postischemic total glycogen content as well as partial recovery during reperfusion was similar in PC and IC hearts.

3.4.2. High energy phosphates.

ATP levels in freeze-clamped hearts were not statistically different between preconditioned and control hearts pre-ischemia (PC: 12.2±2.9 vs. IC: 9.6±4.0 μmol/g dry weight), post-ischemia (PC: 10.1±1.9 vs. IC: 8.3±3.2 μmol/g dry weight), and after reperfusion (PC: 14.1±3.5 vs. IC: 11.9±4.2 μmol/g dry weight). The same was true for CrP values prior to ischemia (PC: 18.3±2.9 vs. IC: 13.2±4.1 μmol/g dry weight), post-ischemia (PC: 9.8±1.5 vs. IC: 8.8±2.0 μmol/g dry weight), and after reperfusion (PC: 22.2±5.3 vs. IC: 27.9±4.7 μmol/g dry weight).

4. Discussion

This study shows that two successive 5-min bouts of ischemia reduce injury due to severe underperfusion. Preconditioning protection was demonstrated by reduced leakage of creatine kinase, improved recovery of rate-force product, abolishment of the postischemic increase in resting tension, and reduced catabolism of adenine nucleotides. The fact that the bulk of purines was released upon reperfusion indicates that during severe flow reduction, purines are only partly washed out. Preconditioning reduced glycogenolysis without affecting anaerobic glycolysis compared to ischemic control hearts.

4.1. Preconditioning-induced protection against low-flow ischemia

Controversy exists as to whether ischemic preconditioning protects against prolonged low-flow ischemia. The improvement in functional recovery after ischemia observed in preconditioned hearts probably reflects the reduction in lethal cell injury, manifest by reduced release of creatine kinase (cf.

[25]). Reduced myocardial injury after prolonged low-flow ischemia has been demonstrated in ischemic preconditioned rat [3] and rabbit [2] hearts. In contrast to these results, contractile status did not improve in preconditioned rat hearts subjected to normal-flow hypoxia [4] or global, low-flow ischemia [5].

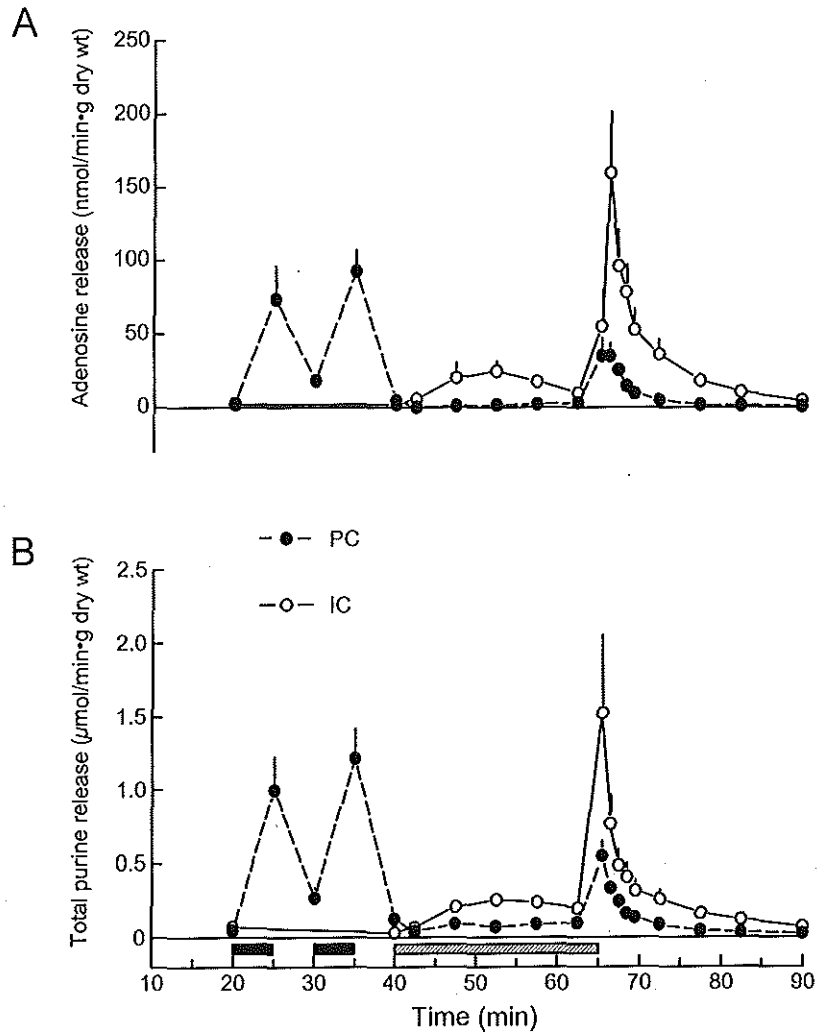

Figure 4. Release of adenosine (A) and total purines (B) in the coronary effluent of ischemic preconditioned (PC) and ischemic control (IC) hearts. See legend to Figure 2 for protocol explanation. PC hearts released substantially less adenosine both during low-flow ischemia ($P<0.05$) and reperfusion ($P<0.01$; repeated measures ANOVA). Release of total purines was also lower in PC hearts (ischemia: $P<0.01$, reperfusion: $P<0.05$; repeated measures ANOVA).

4.2. Tissue high-energy phosphates

ATP and CrP levels in freeze-clamped hearts were not different between preconditioned and control hearts preischemia, postischemia, and after reperfusion. Thus, reduced release of purine compounds in preconditioned hearts was not reflected by tissue ATP levels. Furthermore, a relation between functional recovery and tissue ATP levels (cf. [26]) was not observed in this study.

4.3. Adenosine and ischemic preconditioning

Adenosine both initiates [27] and mediates [6] ischemic preconditioning. This means that adenosine receptors must also be occupied during the prolonged ischemic period to induce the protective effect [6]. Cave et al. [28] obtained similar results in isolated rat cardiomyocytes; they argued that if preconditioning requires the accumulation of a protective substance within the ischemic myocardium, residual flow would prevent the accumulation of such a substance and, hence, protection would be lost.

In normoxic perfused hearts, a ca. 1:8 concentration gradient exists between coronary venous and interstitial adenosine levels [29-32], reflecting the function of the endothelium as an active metabolic barrier. However, the gradient is reduced during adrenergic stimulation [31], hypoxia [29,32] and reductions in coronary flow [30]. In guinea-pig hearts, the venous-to-epicardial adenosine ratio decreases from 1:25 during normal perfusion to 1:3 during ischemia at 10% of baseline flow. This is probably caused by the saturation in the endothelial uptake of adenosine in stressed hearts [29]. Thus, in our study, venous adenosine levels during underperfusion (average values amounted to 0.56 and 3.61 μM in preconditioned and control hearts, respectively) might only slightly underestimate those in the interstitium. Interstitial adenosine levels reflect adenosine A_1 receptor activation, and K_d values of 0.5 nM [33] and 3 nM [34], using different radioligands, have been reported for this receptor in adult rat hearts. Consequently, in our study, interstitial adenosine levels in preconditioned hearts were high enough to occupy the receptor during underperfusion mediating myocardial protection [6]. Therefore, since there could be a critical flow where most endogenous adenosine is washed out rapidly and protection is lost, divergent literature data concerning preconditioning-induced protection against low-flow ischemia could result from differences in the degree of underperfusion. To allow for comparison of data and due to the limited studies on preconditioning protection against low-flow ischemia, we adopted a model used by Perchenet and Kreher [3]. In this model, a modified Krebs-Henseleit buffer relatively high in calcium and a hook inserted into the apex of the heart to measure contractile function are used. Functional recoveries presented in this study correspond to those obtained by Perchenet and Kreher [3].

4.4. Carbohydrate metabolism

Proglycogen synthase seems to be the rate-limiting step in glycogen synthesis [13,14], providing evidence that different processes control the formation and breakdown of macroglycogen and proglycogen. Ischemic stress mainly reduces macroglycogen [15,16,35], while proglycogen is reduced under more special conditions like intermittent ischemia [17] and prolonging ischemia to several hours [36]. We failed to confirm our hypothesis that ischemic preconditioning increased anaerobic glycolysis during low-flow ischemia by mobilizing 'inert' proglycogen. Both fractions decreased in response to

ischemic stress in PC and IC hearts, but with a different timing. In PC hearts, both fractions decreased after the short periods of preconditioning ischemia resulting in virtual cessation of glycogenolytic activity during low-flow ischemia. This is in line with other studies showing glycogen depletion by preconditioning ischemia and reduced glycogenolyis during subsequent prolonged ischemia [11,37,38]. In IC hearts, both glycogen fractions significantly decreased during the low-flow ischemic period. This contrasts our previous observation in rabbit hearts, where proglycogen levels only decreased in response to intermittent ischemia and reperfusion [17]. The fact that macroglycogen levels were already low compared to proglycogen levels prior to sustained ischemia could indicate significant macroglycogen depletion during the stabilization phase. This could be due to the fact that we did not use insulin. Differences between various studies could also be related to species differences in the metabolic activity of both glycogen subfractions.

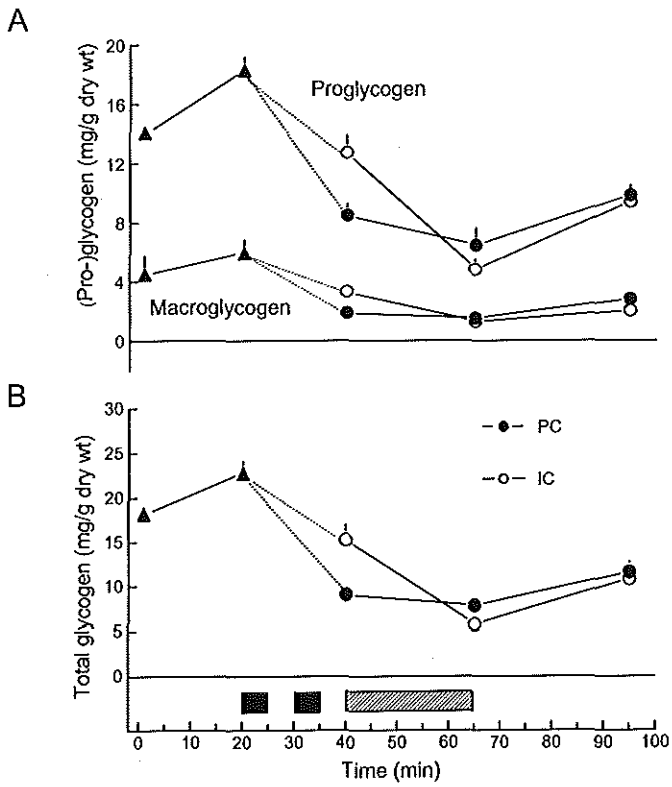

Figure 5. Levels of proglycogen and macroglycogen (A) and total glycogen (B) in freeze-clamped preconditioned (PC), ischemic control (IC), and normoxic perfused hearts (dark triangles). See legend to Figure 2 for protocol explanation. Both macroglycogen and proglycogen fractions of total glycogen decreased in response to ischemic stress in PC and IC hearts. Preconditioning ischemia depleted both fractions resulting in no glycogenolysis during prolonged ischemia. In IC hearts, both macroglycogen and proglycogen decreased during underperfusion. Changes in total glycogen reflected those of its subfractions.

4.5. Lactate and ischemic preconditioning

In our model of low-flow ischemia, we observed no difference in lactate release during ischemia and reperfusion between PC and IC hearts. This contrasts reduced anaerobic glycolysis commonly observed in preconditioned hearts subjected to no-flow ischemia [11,39].

Although lactate release was similar in control and preconditioning hearts, the latter did not show significant glycogenolysis during underperfusion, contrasting control data. Thus, interestingly, this study suggests that ischemic preconditioning-induced protection against low-flow ischemia is associated with reduced glycogenolysis and increased glycolysis-from-glucose during ischemia without altering total glycolytic flux. Cave et al. [4] also observed equal lactate release during hypoxia and reoxygenation between preconditioned and control groups. Janier et al. [2] showed increased lactate release caused by increased glucose uptake in ischemic preconditioned hearts subjected to low-flow hypoxia. Our data strongly suggest that ischemic preconditioning is associated with an increase in exogenous glucose utilization (and reduced glycogenolysis; cf. [40]) rather than by an increase in anaerobic glycolytic flux. A separate study to determine the contribution of glycogen and exogenous glucose to glycolytic flux during low-flow ischemia confirmed that ischemic preconditioning increased exogenous glucose use without increasing total glycolytic flux [41] (see also Chapter 7).

4.6. Glycogen vs. glucose

The above results fit the 'glucose hypothesis' proposed by Opie [42]: increased glycolysis during myocardial ischemia decreases ischemic injury. Similarly, Runnman et al. [43] showed that cardiac dysfunction during hypoxia and reperfusion is related to exogenous glucose utilization, but not total glycolytic flux.

In line with data obtained in aerobic perfused rat hearts [44], Goodwin et al. [45] showed that glycogen is preferentially oxidized in epinephrine stimulated hearts; glycogen hardly contributed to lactate production in this study. However, the relative contribution of glycogen to anaerobic ATP production during low-flow ischemia (0.2 ml/min) amounts to 46% [9].

4.7. Conclusions

This study shows that ischemic preconditioning reduces injury due to a subsequent period of low-flow ischemia (0.6 ml/min). Purine release peaked during reperfusion, and adenosine A_1 receptors were presumably sufficiently occupied during low-flow ischemia to mediate cardioprotection [6]. Our results suggest that preconditioning protects against low-flow ischemia via a shift from endogenous to exogenous glucose utilization without increasing anaerobic glycolysis. In preconditioned hearts, the link between adenosine A_1 receptor activation, the shift to exogenous glucose use, and reduced myocardial injury remains to be determined.

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7

Ischemic preconditioning and glucose metabolism during low-flow ischemia: Role of the adenosine A_1 receptor

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Abstract

Objective: Glycolysis-from-glucose may be more beneficial than glycogenolysis in protecting hearts against ischemia. We tested the hypothesis that ischemic preconditioning is mediated by increased exogenous glucose use during low-flow ischemia, an effect triggered by adenosine A_1 receptor activation. **Methods:** Langendorff rat hearts were subjected to 25 min low-flow ischemia (0.6 ml/min) and 30 min reperfusion. Prior to underperfusion, hearts (n=6 per group) were subjected to two cycles of either preconditioning ischemia (PC), infusion of the adenosine A_1 agonist 2-chloro-N^6-cyclopentyladenosine (CCPA; 0.25 μmol/l), or PC in the presence of the adenosine antagonist 8-(*p*-sulfophenyl)theophylline (SPT; 50 μmol/l). Glycolysis-from-glucose during underperfusion was measured using D-[2-^{3}H]glucose. **Results:** At the end of reperfusion, recovery of rate-force product was enhanced in the PC and CCPA groups (62 and 67% of preischemic value) compared to the ischemic control hearts (IC, 32%; $P<0.05$), whereas protection was abolished in the SPT hearts (20%; $P<0.05$ vs. PC). PC improved total glycolysis-from-glucose during underperfusion by 31% ($P<0.05$ vs. IC); SPT abolished this increase. CCPA reduced total lactate release and glucose uptake during ischemia by 47% and 61%, respectively ($P<0.05$ vs. IC). Abolishment of the preconditioning-associated increase in glucose uptake during underperfusion, by switching to a low glucose buffer, resulted in a loss of functional protection. **Conclusions:** This study strongly suggests that increased exogenous glucose utilization during low-flow ischemia mediates ischemic preconditioning without increasing total anaerobic glycolytic flux. Although adenosine A_1 receptor activation reduces ischemic injury, it does not facilitate the increased glucose uptake observed with ischemic preconditioning, suggesting a different mechanism of protection.

1. Introduction

Opie [1] proposed the 'glucose hypothesis': enhanced uptake and metabolism of glucose during myocardial ischemia delays cellular damage. Many animal studies confirmed this hypothesis by showing that improved uptake and metabolism of exogenous glucose by the underperfused (ischemic) myocardium is associated with reduced diastolic and systolic dysfunction [2-4] and less release of cytosolic marker enzymes [3]. Beneficial effects of glucose-insulin-potassium infusions, stimulating glucose uptake, have been reported in patients after bypass graft surgery [5], after acute myocardial infarction [6,7], or during pacing stress testing [8]. Furthermore, glycolysis-from-glucose seems more effective than glycogenolysis in protecting hearts against myocardial ischemia [2,9]. The latter study showed that protection against the adverse effects of myocardial ischemia is more related to exogenous glucose utilization than to total glycolytic flux (glycolysis *plus* glycogenolysis). The protective effect of increased glucose uptake during ischemia supports the notion that glycolytically derived ATP maintains ionic homeostasis during ischemia and reperfusion [10-12]. The role of carbohydrate metabolism in the phenomenon of 'ischemic preconditioning' [13] is rather controversial [14]. However, the above mentioned results suggest that if ischemic preconditioning increases the rate of glycolysis-from-glucose during low-flow ischemia, this would reduce ischemic injury. We recently showed that preconditioning protection against severe underperfusion is associated with reduced glycogenolysis without affecting anaerobic glycolysis [15].

Triggers, identified to play a role in the mechanism of ischemic preconditioning, include adenosine, bradykinin, and catecholamines. Presumably, adenosine is the most important. Released during preconditioning ischemia, adenosine exerts its effects by binding to the adenosine A_1 receptor located on the myocardial plasma membrane. However, the end-effector involved in the mechanism of preconditioning is unclear. The nucleoside has been shown to influence myocardial carbohydrate metabolism. Adenosine decreases glycolysis during normoxia [16], during low-flow ischemia [17,18], during no-flow ischemia [19,20], and during reperfusion following low-flow [18] or no-flow [16] ischemia. In contrast, increased glycolysis has been reported during normoxia [21] and low-flow ischemia [22]. Thus, adenosine could trigger the changes in carbohydrate metabolism observed in ischemically preconditioned hearts.

We hypothesized that a shift from endogenous carbohydrate to more advantageous exogenous glucose use during low-flow ischemia mediates preconditioning. Furthermore, we speculate that preconditioning effects on carbohydrate metabolism occur via adenosine A_1 receptor activation. Although hardly studied [15], we used a model of preconditioning protection against low-flow ischemia, which we believe is clinically more relevant than the routinely used stop-flow set up. Part of this research has been published in abstract form [23,24].

2. Methods

All animals were treated in conformity with the guiding principles in the care and use of animals as approved by the American Physiological Society. The Animal Welfare Committee, Erasmus University Rotterdam, approved the protocol.

2.1. Exclusion criteria

During stabilization, hearts were excluded if they met one of the following criteria: 1) unstable contractile function, 2) coronary flows outside the range of 9-19 ml/min, 3) severe arrhythmias, 4) myocardial temperature outside the range 37-39°C.

2.2. Isolated heart preparation

Fed, male Wistar rats (Wag/Rij inbred, weighing 280-330 g) were obtained from Harlan-CPB (Zeist, The Netherlands). They received a commercial rat chow (Hope Farms AM II, Woerden, The Netherlands) and tap water ad libitum. After anesthesia with an intraperitoneal injection of 0.6 ml sodium pentobarbital (Nembutal®, 60 mg/ml) supplemented with 0.1 ml heparin (Thromboliquine®, 5000 IU/ml), hearts were rapidly excised and arrested in saline (0°C) until beating ceased. Excess tissue was removed, and the hearts were cannulated within 1 min via the ascending aorta, for retrograde perfusion using a non-recirculating modified Krebs-Henseleit buffer containing (mmol/l): NaCl 118, KCl 5.6, $CaCl_2$ 2.4, $MgCl_2$ 1.2, $NaHCO_3$ 20, Na_2HPO_4 1.2 and D-glucose 10. Insulin (Sigma, St. Louis, MO, USA; from bovine pancreas, 1 U/l) was added to the buffer. Before use, the buffer was filtered through a 45-μm porosity filter to remove any particulate matter, and equilibrated with 5% CO_2/95% O_2, to give a pH of about 7.4 at 37°C. Myocardial temperature was kept at 37°C with a water-jacketed heart chamber and

buffer reservoir, and regulated with an electric heating coil positioned around the aortic inlet line. The temperature of the outer ventricular wall was monitored with a thermocouple (A-F6, Ellab A/S, Roedovre, Denmark). Global, no-flow ischemia was induced by clamping the aortic line; low-flow ischemia was achieved using a perfusion pump (MV-MS3, Ismatec, Zurich, Switzerland) operating at a flow rate of 0.6 ml/min.

Coronary flow was measured by timed collection of the pulmonary artery effluent. The hearts were allowed to beat spontaneously, unless otherwise indicated. Cardiac contractile function of isometrically beating hearts was estimated with a force-transducer (F5A-2, Konigsberg Instruments, Pasadena, Calif., USA) connected to the apex of the heart [2,15]. The heart was pre-loaded with an initial resting tension of 2.5 g. Systolic tension and diastolic tension were continuously displayed on a recorder (Gould signal conditioner and Gould WindoGraf™ recorder, Valley View, OH, USA). Developed tension was calculated as systolic tension *minus* diastolic tension. Cardiac contractile function was expressed as rate-force product (RFP), the product of heart rate and developed tension. RFP at the end of reperfusion was compared to the preischemic value after the 20-min stabilisation period and expressed as percentage recovery of RFP. Perfusion pressure was measured with a disposable pressure transducer (Braun Melsungen, Melsungen, Austria) and kept constant at 65 mmHg.

2.3. Experimental protocol

After initial isolation and surgical preparation, all hearts were perfused with the modified Krebs-Henseleit buffer and allowed to equilibrate for 20 min followed by a 20-min treatment period. Thereafter, hearts were subjected to 25 min of low-flow ischemia (0.6 ml/min) followed by a reperfusion period of 30 min. The 20-min treatment period prior to low-flow ischemia consisted of: 1) normoxic perfusion (IC group; n=6); 2) preconditioning using two 5-min episodes of no-flow ischemia each interrupted by 5 min of reperfusion (PC group; n=6); 3) preconditioning with two 5-min infusions of 0.25 μmol/l of the selective adenosine A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA group; n=6), interspersed by two 5-min periods of drug-free perfusion; 4) PC in the presence of 50 μmol/l of the non-selective adenosine antagonist 8-(*p*-sulfophenyl)theophylline (SPT group; n=6) initiated 2 min prior to transient ischemia; and 5) PC followed by low-flow ischemia where the buffer was switched to one containing low glucose (5 mmol/l) and no insulin (LG group; n=6); during reperfusion the normal high-glucose and insulin containing buffer was used.

In addition, a set of CCPA treated hearts was paced at 350 beats per minute (Grass S9 stimulator, Quincy, MA, USA) to investigate whether preconditioning effects of CCPA were due to negative chronotropic and dromotropic effects of this selective A_1 agonist (CCPAp group; n=6). These hearts were paced throughout the protocol except during ischemia.

2.4. Glycolytic flux and glycogenolysis

The glycolytic flux from glucose during low-flow ischemia was quantitated by measuring ^3H_2O production from D-[2-^{3}H]glucose in the reversible reaction catalyzed by glucose 6-phosphate isomerase [25,26]. Briefly, [2-^{3}H]glucose and ^3H_2O in the coronary effluent samples were separated on 2 × 0.8 cm columns of Dowex 1 (1X2-400; Sigma), equilibrated with potassium tetraborate. Before use, the columns were washed with H_2O. A 0.5 ml sample of the coronary effluent collected during low-flow ischemia was

added to the column and eluted into scintillation vials with 1.2 ml H_2O. The samples, collected in 10 ml scintillation mixture, were counted in a β-counter. For calculating glycolysis, ^3H_2O counts were corrected for the small amount (3%) of [2-^{3}H]glucose not retained by the column. During ischemia, we assumed glucose uptake and phosphorylation to equal the anaerobic glycolytic flux from glucose giving rise to lactate. In fact, in isolated rat hearts, cross-over analysis of glycolytic intermediates [27] showed that the glycolytic flux in low-flow ischemia is determined by the rate of glucose delivery and subsequent transport into the cell and not by enzyme inhibition along the glycolytic pathway as previously suggested [26]. In line with these results, calculation of flux-control coefficients in isolated rat hearts [28] also showed that glucose uptake and phosphorylation dominated the control of glucose flux.

Anaerobic glycogenolysis during low-flow ischemia was estimated from the lactate in excess of that accounted for by glycolytic flux [4]. Lactate washout, glycolysis, and glycogenolysis were expressed as μmol 6-carbon (C_6) units. Total glycogenolysis during 25 min underperfusion was calculated from the total lactate washout (lactate efflux during ischemia + first 2 min of reperfusion) *minus* the total glycolytic flux. We assumed that all glucose taken up during severe underperfusion was converted to lactate. However, a small part of exogenous glucose is oxidized during underperfusion (<3% [4]); hence, this method only slightly overestimates the actual contribution of glycolysis to total lactate production. Incorporation of labelled glucose into the glycogen pool and subsequent release of ^3H_2O is unlikely to occur during underperfusion when substantial glycogen breakdown occurs with little synthesis and amounts less than 5% in normoxic rats [25] and in hypoxic perfused rabbit intraventricular septum [9].

Anaerobic ATP production from both endogenous and exogenous glucose sources was calculated assuming 2 mol ATP per mol glucose taken up, and 3 mol ATP per mol glycosyl units of glycogen broken down.

2.5. Analysis of coronary effluent

During ischemia and reperfusion, coronary perfusate samples were continuously collected at 2-, 3-, 5-, or 10-min intervals, depending on the changes expected. Prior to ischemia, several 1-min samples were taken. Lactate in the samples (0°C) was determined enzymatically with an Elan auto-analyzer (Eppendorf, Merck, Amsterdam, The Netherlands) according to Sigma procedure 735.

2.6. Chemicals

D-[2-^{3}H]glucose (17.0 Ci/mmol) was supplied by Amersham (UK). The drugs 2-chloro-N^6-cyclopentyladenosine and 8-(*p*-sulfophenyl)theophylline were obtained from RBI (Natick, MA, USA). Freshly prepared SPT was directly dissolved in the Krebs-Henseleit buffer. A stock solution of CCPA, dissolved in deionized water, was further diluted in the buffer. Stock solutions of CCPA were discarded after two days storage at 4°C.

2.7. Statistical analysis

The data are expressed as means ± S.E.M., with n = number of hearts. Summary measures were constructed for hemodynamic parameters and carbohydrate fluxes according to published

recommendations [29]: Recovery of rate-force product was expressed as a percentage of baseline value; from the recording of resting tension, the magnitude of peak contracture during ischemia was compared between groups (statistics presented in Figure 1B). The sum of carbohydrate fluxes during low-flow ischemia was calculated as described in the Methods section. One-way analysis of variance with subsequent Student-Newman-Keuls post-hoc tests were used for comparisons between groups. If values were not normally distributed or variances between groups were unequal, Kruskal-Wallis ANOVA on ranks was used. Values of $P<0.05$ (two-tailed test) were regarded as significant.

3. Results

3.1. Contractile function

Figure 1 presents time-dependent changes in rate-force product and resting tension, reflecting ventricular systolic and diastolic functioning, respectively. Rate-force product, resting-tension, and coronary flow were not different between groups after the 20-min stabilization period. Mean coronary flow amounted to 13.5±0.5 ml/min after stabilization. Rate-force product rapidly fell to zero during the short bouts of preconditioning ischemia in the PC, SPT, and LG groups. After transient ischemia, rate-force product recovered to 75% in PC and LG groups and to 65% in SPT hearts. Infusion of CCPA resulted in a 79% decline in myocardial function, mainly due to the negative dromotropic and chronotropic effects of the drug. Rate-force product of all groups of hearts fell to 0 within 5 min after the onset of low-flow ischemia. Low-flow ischemia amounted to 4% of baseline flow. Recovery of rate-force product expressed as a percentage of baseline (Figure 2) was improved in PC and CCPA hearts (62±6% and 67±7%, respectively) compared to control hearts (32±9%; $P<0.05$). SPT and LG abolished preconditioning protection (20±12% and 30±7% recovery; $P<0.05$ vs. PC). Pacing (CCPAp) prevented the decline in rate-force product during CCPA infusion but did not diminish the protection by CCPA against prolonged ischemia (68±5%; $P<0.05$ vs. IC; Figures 1 and 2).

No functional recovery was observed (0%) in a set of ischemic control hearts supplied with low glucose (5 mM) during underperfusion (IC-low glucose group; n=4, data not shown).

Prior to underperfusion, resting tension was not significantly affected by any of the interventions. During low-flow ischemia, resting tension gradually increased towards a maximum at 25 min of ischemia (indicated as peak ischemic contracture, statistics in Figure 1B) except in CCPA and CCPAp hearts where contracture was suppressed. Peak ischemic contracture was lower in CCPA (0.2±0.2 g) hearts compared to PC (6.0±0.7 g), IC (6.0±1.4 g), SPT (3.6±1.3 g), and LG (9.7±0.9 g) groups ($P<0.05$). Peak ischemic contracture was also lower in CCPAp hearts (1.7±0.5 g; $P<0.05$ vs. PC, IC, LG). It was similar in PC, IC, and SPT hearts. LG resulted in a greater peak contracture compared to all other groups ($P<0.05$).

3.2. Lactate

The release of lactate in the coronary effluent was taken as a measure of anaerobic glycolysis both from exogenous (glucose) and endogenous (glycogen) sources. In PC, SPT and LG hearts, the short

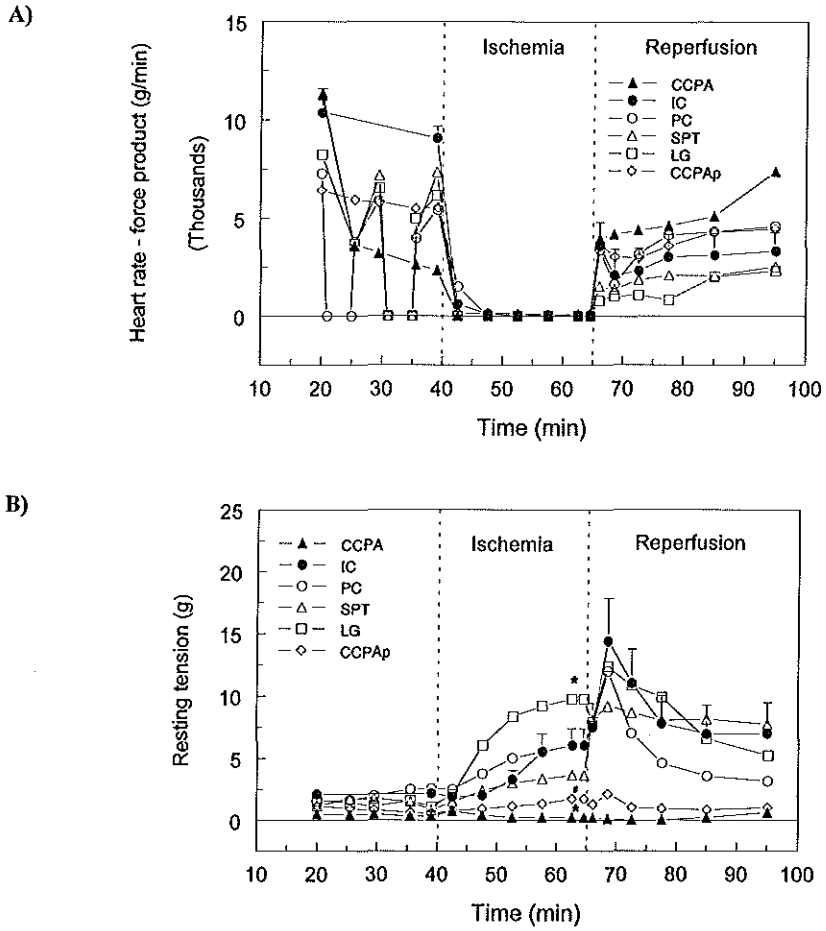

*Figure 1. Changes in rate-force product (A) and resting tension (B). After 20 min stabilization, hearts were preconditioned with two 5-min periods of the following interventions interspersed by two 5-min periods of normal perfusion: no-flow ischemia (PC), infusion of 0.25 µmol/l of the selective adenosine A_1 agonist 2-chloro-N^6-cyclopentyladenosine (CCPA), or PC in the presence 50 µmol/l of the non-selective adenosine antagonist 8-(p-sulfophenyl)theophylline (SPT), given 2 min prior to transient ischemia. Control hearts (IC) underwent an extra 20-min period of normoxic perfusion following stabilization. A group of CCPA hearts was paced to prevent the fall in rate-force product during drug infusion (CCPAp). Thereafter, all hearts were exposed to 25 min of low-flow ischemia (0.6 ml/min) and 30 min of reperfusion. In one group of PC hearts during low-flow ischemia, the perfusion buffer was switched at the start of ischemia to one containing low glucose (5 mmol/l) and no insulin (LG). Pretreatment with ischemia or CCPA improved recovery of rate-force product compared to control hearts whereas protection was abolished in LG and SPT hearts. Peak ischemic contracture was significantly lower in CCPA hearts compared to controls whereas contracture was increased in LG hearts. Figure 1B: *P<0.05 vs. all other groups; #P<0.05 vs. PC, IC, and LG (one-way ANOVA). Means ± S.E.M. (n=6). For clarity reasons, error bars are only presented for IC hearts; error bars for the other groups were the same order of magnitude as those in control hearts. Summary statistics error bars for rate-force product and resting tension are given in Figure 2 and in the results section, respectively.*

bouts of no-flow ischemia induced a large efflux of lactate (Figure 3). Lactate release during ischemia and reperfusion was similar in the PC, IC, SPT, and LG groups; CCPA pretreatment resulted in reduced lactate efflux during underperfusion and reperfusion. The sum of lactate released during low-flow ischemia and reperfusion is depicted in Figure 4. Total lactate released during low-flow ischemia was not different between groups except for CCPA which reduced lactate release by 47% ($P<0.05$ vs. all groups). Reduced lactate release during underperfusion was also observed in paced CCPA hearts (Figure 3). Hence, less lactate production in CCPA pre-treated hearts was not due to reduced preischemic contractility (see Figure 1A).

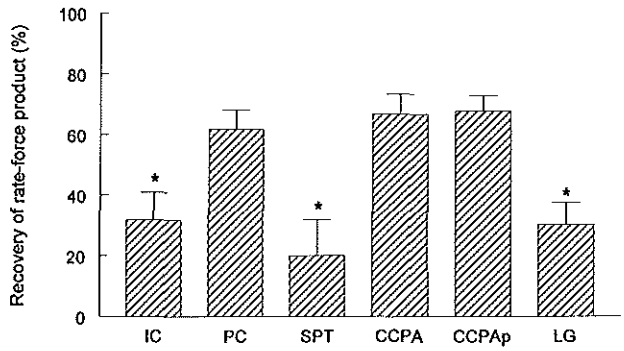

*Figure 2. Summary of the recovery of rate-force product obtained from the time-dependent changes in myocardial function depicted in Figure 1A. See legend to Figure 1 for protocol explanation. An extra group of CCPA-treated hearts was paced throughout the experiment (CCPAp; n=6) except during low-flow ischemia. Recovery of rate-force product expressed as a percentage of baseline value was significantly improved in PC, CCPA, and CCPAp hearts compared to controls. The supply of low glucose during underperfusion or SPT during pretreatment to ischemic preconditioned hearts abolished protection completely. *P<0.05 vs. PC, CCPA, and CCPAp (one-way ANOVA).*

3.3. Glycolysis-from-glucose and glycogenolysis

Glucose uptake and phosphorylation was monitored using [2-^{3}H]glucose present in the perfusion medium during low-flow ischemia. Anaerobic glycolysis-from-glucose during underperfusion (Figure 5) reached a plateau after about 15 min of ischemia (at 52.5 min). Figure 4 presents the sum of glycolysis-from-glucose during ischemia. PC increased glycolysis from exogenous glucose by 31% ($P<0.05$ vs. IC) whereas CCPA reduced this flux by 61% ($P<0.05$ vs. IC). Increased total glycolysis in PC hearts was mainly due to increases during the first 15 min of ischemia whereas CCPA reduced glycolysis throughout the ischemic period (Figure 5). Total glycogen breakdown during low-flow ischemia was estimated from the lactate in excess of that accounted for by glycolytic flux-from-glucose (Figure 4). Estimated glycogen breakdown was not different between groups but tended to be lower in PC hearts.

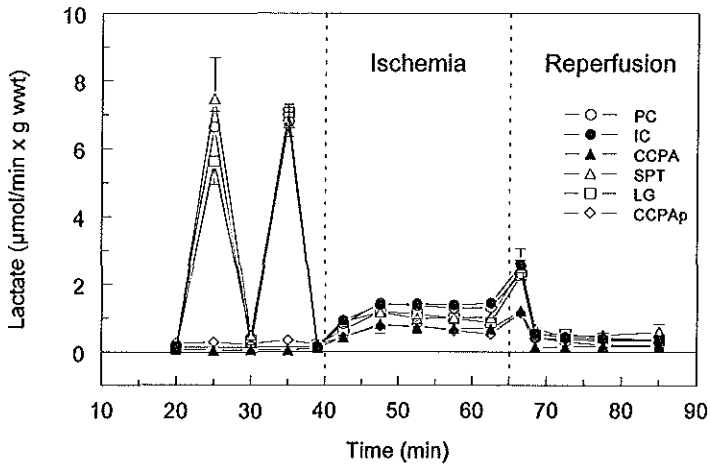

Figure 3. Release of lactate in the coronary effluent. See legend to Figure 1 for protocol explanation. Lactate release during low-flow ischemia and reperfusion was similar in the PC, IC, SPT and LG groups. However, CCPA decreased lactate release during ischemia and reperfusion. For CCPAp hearts, n=3.

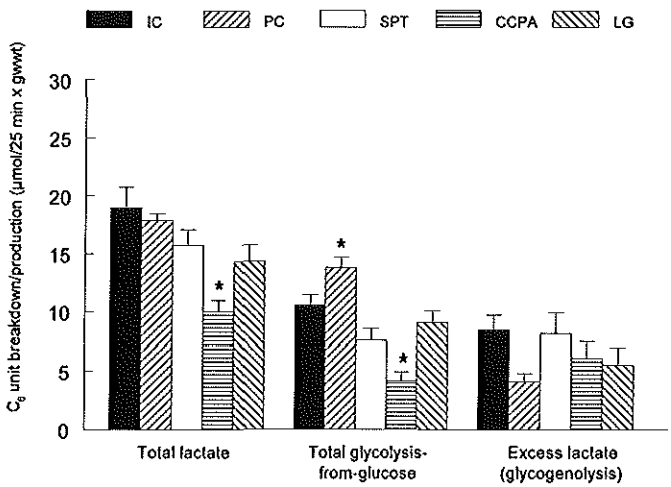

*Figure 4. Total lactate release, total glycolysis-from-glucose, and estimated total glycogen breakdown during 25 min of underperfusion. Total lactate release reflects total anaerobic glycolysis from both endogenous and exogenous glucose sources and was calculated based on the data in Figure 3. Total glycolysis-from-glucose was obtained from the data in Figure 5. Total glycogen breakdown was estimated from the lactate in excess of that accounted for by glycolytic flux. See legend to Figure 1 for protocol explanation. Total lactate release was not different between groups except for CCPA-treated hearts, which depressed lactate release by 47% compared to control hearts. PC increased and CCPA decreased glycolysis from exogenous glucose compared to controls. *P<0.05 vs. all other groups (one-way ANOVA).*

3.4. ATP production

Total ATP production during 25 min low-flow ischemia from the anaerobic breakdown of glucose and glycogen (Figure 6) was calculated based on the total fluxes from glucose and glycogen during ischemia presented in Figure 4. PC increased ATP production from exogenous glucose by 31% compared to controls ($P<0.05$) whereas CCPA reduced glycolytic ATP by 61% ($P<0.05$ vs. IC). ATP production from endogenous glycogen during ischemia was similar in all experimental groups.

4. Discussion

4.1. Carbohydrate metabolism during ischemia

Values of glucose utilization and calculated glycogen breakdown (Figure 4) in this study correspond to those obtained in a study using a similar level of underperfusion [4]. Furthermore, the present results using direct quantitation of glucose flux (Figure 5) and indirect calculation of glycogen breakdown confirm a previous study of our group that assessed glycogen in freeze-clamped hearts [15]. In that study, glycogen breakdown was reduced in preconditioned hearts during underperfusion despite similar lactate production, suggesting increased glycolysis-from-glucose. Although not significant in the present study, calculated glycogen breakdown was less than half in PC hearts compared to IC.

Ischemic preconditioning increased glycolysis early during ischemia compared to controls (Figure 5). This corresponds to the observation of Runnman et al. [9] that the cardioprotective effects of increased glucose flux occurred early during hypoxia. To exclude the possibility that increased glycolysis-from-glucose during underperfusion is just an epiphenomenon of ischemic preconditioning, we reduced glycolysis-from-glucose during ischemia in preconditioned hearts by switching to a buffer with lower glucose and zero insulin (note: glucose supply to the underperfused ischemic heart is the rate-limiting step in glycolytic flux from glucose [27]). This resulted in a reduction of glycolysis to values similar to the control group. The fact that these hearts did not show improved contractile recovery upon reperfusion with normal buffer suggests that ischemic preconditioning-induced protection against underperfusion is mediated by increased glycolytic flux early during ischemia. A study in isolated rabbit hearts reached similar conclusions [30]. Furthermore, we observed no functional recovery (0%) in a set of ischemic control hearts supplied with low glucose (5 mM) during underperfusion (IC-low glucose group; data not shown). Thus, this confirms the hypothesis that 1) glycolytic flux controls myocardial viability during underperfusion (please, compare IC vs. IC-low glucose and PC vs. LG), and 2) suggests that ischemic preconditioning is mediated by increased glycolysis during low-flow ischemia (please, compare PC vs. IC and LG vs. IC-low glucose). Beneficial effects of increased glycolytic flux by various means during underperfusion/hypoxia have been well documented in animal experiments [2-4] and also in clinical trials [7,31]. Vanoverschelde et al. [32] observed a linear relation between glucose uptake during ischemia and functional recovery after reperfusion in isolated rabbit hearts. We add to these observations that the mechanism of protection by ischemic preconditioning against low-flow ischemia may involve increased glucose uptake.

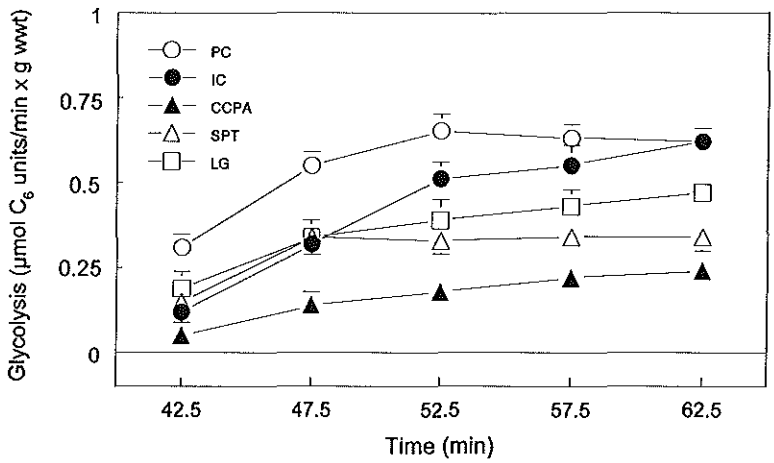

Figure 5. Glycolytic flux (from glucose) during underperfusion as estimated from the release of tritiated water from labelled glucose. See legend to Figure 1 for protocol explanation. Glycolysis-from-glucose was increased in PC hearts during the first 15 min of ischemia compared to control hearts. This increase was abolished in the SPT and LG groups. CCPA treatment resulted in depressed glucose uptake throughout the period of underperfusion. Same time scale as other figures.

In our study, total anaerobic glycolysis (lactate production) was similar in preconditioned and control hearts (Figure 3) in line with other reports [15,33]. This contrasts with no-flow ischemic models of ischemic preconditioning where lactate production is reduced [34]. However, in the latter ^{13}C-NMR study, glucose utilization actually increased in preconditioned hearts during the first five min of ischemia despite reduced glycogenolysis and lactate production.

Contrary to our expectations, pretreatment with CCPA protected contractile function despite reduced lactate production (Figure 3) and reduced glycolysis-from-glucose during underperfusion (Figure 5). This was not due to the negative chronotropic effect associated with CCPA infusion since less ischemic lactate production was also observed in paced CCPA hearts (Figure 3). Other studies described similar effects of adenosine infusion on lactate production [18-20] and on glycolysis-from-glucose [18] during low-flow or no-flow ischemia. However, increased lactate production [35] and glycolysis-from-glucose [22] with adenosine treatment have also been observed during low-flow ischemia. These discrepancies on the effects of adenosine on glycolysis during ischemia may be related to the preischemic metabolic status of the heart [16]. Thus, although glucose uptake may mediate ischemic preconditioning in this study, the beneficial effect of CCPA on mechanical recovery was associated with depressed glucose flux. This could indicate that the observed increase in glucose uptake in ischemic preconditioned hearts is not mediated by adenosine A_1 receptor activation. However, care should be taken since opposite effects of the adenosine A_1 agonist N^6-cyclohexyladenosine and adenosine itself on glycolysis have been reported [16]. In that study, adenosine pretreatment decreases glycolysis whereas adenosine combined with brief ischemia increases glycolysis. Moreover, N^6-cyclohexyladenosine, a compound similar to CCPA, also decreases glycolysis, in line with the results presented in this report [16]. Thus, it is possible that adenosine released during brief ischemia mediates the increase in

glycolysis-from-glucose during underperfusion in this study (Figure 5). The fact that the effects of ischemic preconditioning on mechanical recovery and increased glucose uptake during ischemia were abolished when the nonspecific adenosine antagonist SPT was present during the pretreatment supports this notion. Paradoxically, in the present study, CCPA induced pronounced cardioprotective effects (no ischemic contracture and improved functional recovery) despite depressed glycolysis-from-glucose. We do not have an suitable explanation for this observation. Myocardial protection against ischemia and reperfusion induced by pretreatment with ischemia or CCPA may involve different mechanisms. Ischemic preconditioning is a highly redundant phenomenon: it can take place by many alternative routes of which increasing glycolysis-from-glucose during low-flow ischemia may be one.

4.2. Recovery of function

Most ischemic preconditioning experiments have examined no-flow ischemia after pretreatment and consistently shown increased protection. In clinical practice, it is more likely that low-flow conditions will prevail, due to partial coronary occlusion and/or collaterals present. However, ischemic preconditioning-induced protection against low-flow ischemia is rather controversial. The few studies conducted so far have obtained both positive [15,30,36-38] and negative results [33]. In line with a recent report of our group [15], ischemic preconditioning protected against contractile dysfunctioning arising from severe underperfusion in the present study (Figures 1 and 2). We showed before that preconditioning also reduces irreversible injury after low-flow ischemia [15]. Furthermore, ischemic preconditioning exists in species having collateral flow. We [15] and others [33] have suggested that there could be a critical flow above which preconditioning effects are lost.

Pharmacological preconditioning with CCPA, a selective adenosine A_1 agonist, effectively mimicked ischemic preconditioning by improving contractile recovery after low-flow ischemia whereas protection was abolished in hearts ischemically preconditioned in the presence of the non-selective adenosine antagonist SPT. We showed before, using the same drugs, that the adenosine A_1 receptor is involved in protection by ischemic preconditioning in a no-flow ischemic model [39]. This sharply contrasts reports that adenosine is not involved in ischemic preconditioning of the rat heart (for reviews, see [40,41]). The protective effect of pretreatment with CCPA was not due to negative chronotropic and dromotropic effects of the drug since pacing, which kept myocardial contractility constant during drug infusion, did not abolish protection by CCPA. Moreover, the different metabolic response in CCPA-treated hearts is unlikely to be the cause of incomplete washout prior to ischemia and consequent negative chronotropic effects during ischemia since the time from onset ischemia to complete contractile arrest was not statistically different between groups (IC: 7.2±4.1 min; PC: 3.5±0.5 min; LG: 5.3±1.7 min; CCPA: 4.1±1.2 min; SPT: 2.4±0.4 min; CCPAp: 7.6±3.9 min). Furthermore, in a previous study [39], we showed that the effects of CCPA are also independent of changes in coronary flow since in that study flow was held constant. CCPA infusion did not result in negative inotropic effects (see CCPAp group in Figure 1A). Adenosine A_1 receptors have been shown to couple to K_{ATP} channels via G-proteins in membrane patches of rat ventricular myocytes [42], which could lead to negative inotropic effects. However, in intact guinea pig ventricular myocytes, adenosine does not affect potassium membrane current [43] in line with our observation that CCPA induced negative chronotropic/dromotropic effects without affecting the inotropic state (Figure 1A). With some exceptions, most rat-heart studies using adenosine or adenosine antagonists have failed to mimic or abolish preconditioning, respectively.

However, the relatively high adenosine levels in this species may require increased antagonist concentrations [44]. The fact that the endothelium forms an active barrier for the transport of adenosine to the interstitium and the especially high activities of adenosine degrading enzymes in the rat heart may limit the efficacy of exogenous adenosine administration. In this respect, it is interesting to note that studies using adenosine A_1 agonists, like the one used in this investigation, have obtained positive results in rat hearts [44,45].

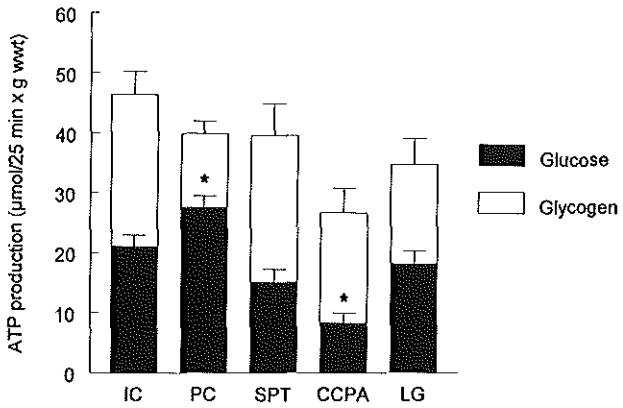

*Figure 6. ATP production during 25 min low-flow ischemia from both anaerobic breakdown of exogenous glucose (black bars) and endogenous glycogen (open bars). Values were calculated from the total glycolytic flux and the estimated total glycogen breakdown during underperfusion as depicted in Figure 4. See legend to Figure 1 for protocol explanation. PC increased and CCPA decreased ATP production from exogenous glucose; ATP production from glycogen during underperfusion was not different between groups. *P<0.05 vs. all other groups (one-way ANOVA).*

4.3. Possible mechanisms of action

The cause of the increase in exogenous glucose utilization in preconditioned hearts was not the subject of this study. However, transient ischemia may result in translocation of heart GLUT4 glucose transporters [46]. The fact that preconditioning increased glucose uptake especially during early ischemia in this study could indicate that glucose transporters were already/more rapidly translocated to the sarcolemma due to the short antecedent ischemia. However, this explanation seems unlikely since in the presence of insulin, glucose uptake is not a rate-limiting step in glucose metabolism [28]. Alternatively, preconditioning may affect key glycolytic enzymes inhibiting glycogenolysis [34] and stimulating glycolysis. Moreover, the reason for the superiority of glycolysis over glycogenolysis in reducing ischemic injury is unclear, but sustained glycolysis-from-glucose during ischemia may support ionic homeostasis and thereby reduce ischemic injury [10-12].

In summary, this study strongly suggests that ischemic preconditioning is mediated by enhanced utilization of exogenous glucose during low-flow ischemia without increasing total anaerobic glycolytic flux. Adenosine A_1 receptor stimulation attenuated ischemic injury in rat hearts but did not mediate the

increase in glucose utilization observed in ischemically preconditioned hearts. Therefore, ischemic and pharmacological preconditioning may involve different pathways.

Acknowledgements

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8

Role of adenosine and glycogen in ischemic preconditioning of rat hearts

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Submitted

Abstract

Objective: Adenosine is important in ischemic preconditioning in many species, but its role in the rat heart has been questioned. Furthermore, cardioprotection by ischemic preconditioning is suggested to be mediated by glycogen depletion, resulting in attenuation of glycolytic catabolite accumulation and the development of intracellular acidosis during sustained ischemia (the glycogen hypothesis). We tested whether ischemic preconditioning is mediated by reduced glycogenolysis during ischemia, an event triggered by adenosine A_1 receptor (AA_1R) activation. **Methods:** Hearts (n=40) were studied with ^{31}P- and ^{13}C-NMR spectroscopy, using the Langendorff perfusion technique (5.5 mM [1-^{13}C]-glucose, 10 U/l insulin). They were subjected to either ischemic preconditioning (4 cycles of 2 min ischemia and 3 min reperfusion, PC), PC in the presence of 50 μM adenosine antagonist, 8-(*p*-sulfophenyl)-theophylline (SPT), or intermittent infusion of 0.25 μM AA_1R agonist, 2-chloro-N^6-cyclopentyladenosine (CCPA). **Results:** Administration of CCPA and SPT neither affected pH nor high-energy phosphates. Recovery of heart rate × pressure product was improved in hearts treated with preconditioning (33±13%) or CCPA (58±14%) compared with the SPT and ischemic control groups, which both failed to recover (P<0.05). CCPA administration induced a 58% increase in preischemic ^{13}C-glycogen (P<0.05 vs. all groups). In the PC and SPT groups, ^{13}C-glycogen decreased by 25 and 47%, respectively (P<0.05) due to the short bouts of ischemia, resulting in lower preischemic glycogen compared to ischemic control and CCPA hearts (P<0.05). The rate of ^{13}C-glycogen utilization during the first 15 min of ischemia (in μmol/min.g wwt) was similar in IC (0.42±0.03), PC (0.30±0.04), and CCPA (0.38±0.05) hearts, but was reduced in SPT hearts (0.24±0.05; P<0.05). The changes in ^{13}C-glycogen were similar to those determined in freeze-clamped hearts. **Conclusions:** This study strongly suggests that in rat hearts, adenosine is involved in ischemic preconditioning. However, protection is unrelated to preischemic glycogen levels and glycogenolysis during ischemia.

1. Introduction

Ischemic preconditioning [1] occurs in many animal species, and most likely also in humans [2]. Many substances like adenosine, bradykinin, and catecholamines released during preconditioning ischemia may trigger protection [3]. Of these substances, adenosine is probably the most important one. Adenosine triggers and mediates preconditioning by stimulating the adenosine A_1 receptor [4], located mainly on myocytes. Recently, also the adenosine A_3 receptor has been implicated in preconditioning protection [5-7]. Evidence of adenosine-mediated preconditioning has been obtained in most animal species including rabbits [4,7], dogs [8], mice [9,10], chick [11,12], and pigs [13,14], and recently also in humans [6,15,16]. However, the role of adenosine in mediating cardioprotection in rat hearts is controversial and both results against [17-20] or in support [21,22] of a role of adenosine in preconditioning of rat hearts have been published. This study examined whether the adenosine A_1 receptor is involved in protection induced by ischemic preconditioning in the rat heart.

Glycogen depletion by preconditioning ischemia results in less accumulation of glycolytic end-products during ischemia; it has been proposed to mediate protection [23]. Moreover, loss of the protective effect parallelled the time course of glycogen recovery before sustained ischemia [23] and increased preconditioning ischemia time resulted in glycogen depletion and infarct-size reduction, both

described by an exponential declining curve [24]. Both data supporting [25] or rejecting [26-28] this glycogen hypothesis have been published. The study of Weiss et al. [29] and our results [30], which both used the advanced technique of ^{13}C-NMR to follow myocardial glycogen within one heart throughout the experiment, indicate that preconditioning depresses glycogenolysis during ischemia. Interestingly, adenosine has been shown to influence carbohydrate metabolism during normoxia [31-33], low-flow ischemia [34-36], and no-flow ischemia [37,38]. Thus, this study examined whether ischemic preconditioning is mediated by reduced preischemic glycogen and glycogenolysis during ischemia, an event triggered by adenosine A_1 receptor (AA_1R) activation. We used both the selective adenosine A_1 agonist 2-chloro-N^6-cyclopentyladenosine and the non-selective adenosine antagonist 8-(*p*-sulfophenyl)-theophylline in isolated perfused rat hearts to verify whether we could mimic or abolish ischemic preconditioning, respectively. High energy phosphates and myocardial glycogen were measured with ^{31}P- and ^{13}C-NMR, respectively. This study shows that adenosine is involved in preconditioning of rat hearts and that neither preischemic glycogen nor glycogen depletion during ischemia are related to preconditioning-induced cardioprotection. Part of this research has been published in abstract form [30].

2. Materials and Methods

2.1. Materials

The following ^{13}C-enriched materials were used: [1-^{13}C]glucose, [2-^{13}C]ribose (99%, Cambridge Isotope laboratories, Inc.). The synthetic drugs 2-chloro-N^6-cyclopentyladenosine (CCPA) and 8-(*p*-sulfophenyl)-theophylline (SPT) were obtained from RBI (Natick, MA, USA).

2.2. Heart perfusion

Fed, male Sprague-Dawley rats of 250-300 g were stunned and bled. The heart was rapidly isolated and arrested in ice-cold perfusion fluid. The aorta was cannulated and the heart was Langendorff-perfused at constant pressure (70 mm Hg) for 20 min in order to allow coronary flow to be measured. This value (9-11 ml/min) was applied to the subsequent 10 min constant flow perfusion period. The perfusion medium was a modified Krebs-Henseleit solution (composition in mmol/l): NaCl 137; KCl 5.4; $MgCl_2$ 1.2; $CaCl_2$ 1.8; NaH_2PO_4 0.46; $NaHCO_3$ 12; α-D-glucose 5.5 (or [1-^{13}C]glucose), with 10 U/l insulin and saturated at 37°C with O_2-CO_2 (95-5%); pH 7.4. To minimize temperature changes during ischemia, the hearts were immersed in 37°C perfusate, controlled by a Bruker temperature controller accessory. Myocardial function (heart rate [HR], developed pressure [dP], and rate-pressure product [RPP]) was assessed using a pressure transducer connected to an intraventricular balloon filled to give an end-diastolic pressure of 4-8 mm Hg.

2.3. ^{31}P and ^{13}C NMR spectroscopy

The heart was inserted in a broad-band 20-mm probe of a Bruker AMX 500 wide-bore NMR spectrometer operating at 11.74 T. Field homogeneity was optimized by shimming the water proton signal using the decoupling coil. ^{31}P NMR spectra were obtained at 202.4 MHz with a 60° pulse of 20

μsec, and a 2-sec delay using blocks of 60-90-150 transients corresponding to 2-3-5 min of accumulation time. Relative metabolite quantification was obtained as already reported [39,40] and the results expressed as a percentage of the baseline value (100%). Intracellular pH was calculated from the chemical shift (δ) of Pi relative to phosphocreatine (PCr) using the equation pH = 6.77 - log (δ -5.78)/(3.27 - δ). Zero ppm was assigned to PCr. The validity of this equation has been checked for our experimental condition [41].

^{1}H-decoupled ^{13}C spectra were obtained at 125.72 MHz using the MLEV-16 sequence. Free induction decays were collected in 2K data points and zero-filled to 8K, using blocks of 100-150-250 transients corresponding to 2-3-5 min of accumulation time. The pulse width was 23 μsec (60°) and the recycling time 1.1 sec. A line broadening of 25 Hz was introduced before Fourier transformation. The chemical shifts are reported in relation to tetramethylsilane, using β-C1 glucose at 97 ppm as the internal reference standard. A capillary tube filled with a solution containing 100 mmol/l [2-^{13}C]ribose was used as the external standard. Metabolite concentrations were measured by integrating the areas under the individual peaks and were expressed as a percentage of the baseline value (100%) at 30 min of stabilization. Corrections from the nuclear Overhauser effect and partial saturation effects were performed. Absolute concentrations were calculated using the 100 mmol/l [2-^{13}C]ribose solution.

2.4. Experimental protocol

The protocol is shown in Figure 1. The hearts were randomly assigned to four groups: IC , PC, CCPA and SPT, which all underwent 30 min stop-flow ischemia and 30 min reperfusion. Pretreatment consisted of (for ^{31}P-NMR and ^{13}C-NMR: n=4-6 per group):

1) Group IC, ischemic control: 42 min stabilization.
2) Group PC, ischemic preconditioned: 30 min stabilization, 4 cycles of 2 min ischemia and 3 min reperfusion.
3) Group CCPA, adenosine A_1 agonist treated: 30 min stabilization, four cycles of 2 min CCPA (0.25 μM) and 3 min normal perfusion.
4) Group SPT, adenosine antagonist treated: 30 min stabilization, 4 cycles of 2 min ischemia and 3 min reperfusion. SPT (50 μM) was added to the perfusion medium 2 min before and during intermittent ischemia-reperfusion.

The hearts were weighed at the end of the experiment. Reperfusion was performed at constant pressure (70 mm Hg) for 10 min and continued at constant flow. 5.5 mM [1-^{13}C]-glucose was present throughout the protocol in ^{13}C-NMR experiments.

2.5. Biochemical analysis

Total glycogen in freeze-clamped hearts was determined as previously described [42].

2.6. Statistical analysis

The values are expressed as means ± S.E.M. ANOVA and Student-Newman-Keuls *t*-test were used for evaluating their statistical significance. P values <0.05 were considered statistically significant. NS, not statistically significant.

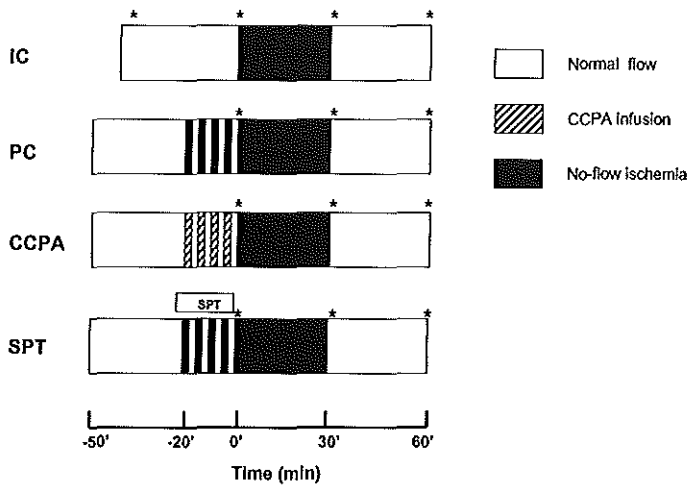

*Figure 1. Experimental protocols. All hearts were subjected to 30 min no-flow ischemia and 30 min reperfusion. Hearts (n=4-6 per group in ^{31}P- and ^{13}C-NMR experiments) were preconditioned with either four cycles of 2 min ischemia and 3 min reperfusion (PC group) or four 2-min infusions of 0.25 μM adenosine A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA group), followed by 3 min drug-free perfusion. In one group of ischemic preconditioned hearts, 50 μM of the adenosine antagonist 8-(p-sulfophenyl)theophylline (SPT group) was present in the perfusion medium 2 min before and during intermittent ischemia. In ^{13}C-NMR experiments, 5.5 mM [1-^{13}C]-glucose was present in the perfusion buffer throughout the protocol. Because PC hearts were perfused with labeled glucose during preconditioning, the ischemic control hearts (IC group) received a 42 min stabilization period to ensure that hearts received equal amounts of labeled glucose before ischemia. In parallel experiments, hearts were freeze-clamped at different time-points throughout the protocol (n=3-5 per group; indicated with *.)*

3. Results

3.1. Contractile function

Figure 2 presents the time-dependent changes in rate-pressure product, a reflection of ventricular systolic functioning. With the short periods of intermittent ischemia in the PC and SPT groups, RPP rapidly fell to near zero values. RPP recovered to 90% in the PC hearts and to 100% in the SPT hearts (NS vs. IC) after the fourth 2-min period of ischemia. The first 2-min infusion period of CCPA induced a 47% decrease in contractility which did not recover during the first 3-min period of CCPA-free perfusion. The subsequent cycles of CCPA infusion further decreased RPP to a preischemic value of 23% (P<0.05 vs. PC, SPT, CCPA). With the onset of prolonged no-flow ischemia, RPP fell to zero within 5 min in all groups. Upon reperfusion after long ischemia, functional recovery was significantly improved in PC (33±13%) and CCPA (58±14%) hearts compared to IC (0±0%; P<0.05). SPT abolished preconditioning protection (0.4±0.4%; P<0.05 vs. PC).

3.2. Glycogen

The time-dependent changes in ^{13}C-glycogen are presented in Figure 3a. The presence of 10 U/l insulin in the perfusion medium induced a marked increase in total glycogen with the concurrent incorporation of labeled glucose residues, as monitored in the ^{13}C spectra by the growth of the signal at 100.6 ppm. The ^{13}C-glycogen signal leveled off after 30 min of normoxic perfusion. Short intermittent ischemia

resulted in a 25% decrease in ^{13}C-glycogen in PC hearts (P<0.05 vs. IC). This decrease was not abolished by SPT; preischemic glycogen was 53% of the value after stabilization in SPT hearts (P<0.05 vs. IC). CCPA induced a 58% increase in ^{13}C-glycogen resulting in significantly higher preischemic glycogen levels compared to IC hearts (P<0.05 vs. IC). Changes in ^{13}C-glycogen reflected those of total glycogen determined in freeze-clamped hearts during parallel experiments (Figure 3b): Short intermittent ischemia decreased total glycogen by 40% in PC hearts and by 25% in SPT hearts whereas CCPA infusion increased total glycogen by 65%. ^{13}C-glycogen utilization was delayed during the first 2-5 min of prolonged ischemia in PC and CCPA groups (Figure 3a), this delay was not blocked by SPT. ^{13}C-glycogen utilization during the first 15 min of prolonged ischemia (in μmol/min.g wwt) was similar in PC (0.30±0.04), IC (0.42±0.03), and CCPA (0.38±0.05) hearts, but was lower in SPT hearts (0.24±0.05; P<0.05 vs. IC; Figure 3a). At the end of ischemia, ^{13}C-glycogen was almost completely exhausted in PC, IC and SPT groups, whereas it was still 64% of the value after stabilization in CCPA treated hearts. In CCPA hearts, freeze-clamped at the end of sustained ischemia, total glycogen was 87% of the 2-min stabilization value (Figure 3b). Upon reperfusion, only PC and CCPA hearts resumed ^{13}C-glycogen synthesis compared to IC and SPT groups (P<0.05; Figure 3a). This was partly reflected in total glycogen values determined after reperfusion (Figure 3b): glycogen was higher in PC hearts compared to all other groups (P<0.05).

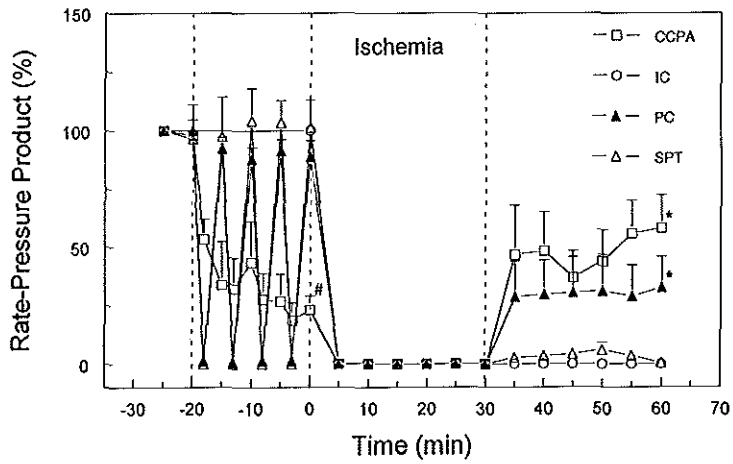

*Figure 2. Changes in rate-pressure product. Recovery of rate-force product at the end of reperfusion following ischemia was improved in CCPA and PC hearts compared to SPT and IC groups. #P<0.05 v IC, PC, SPT; *P<0.05 v IC, SPT. For abbreviations and protocol, see Figure 1.*

3.3. High energy phosphates

Intermittent ischemia and reperfusion induced cyclic variations in PCr and P_i in PC and SPT groups (Figures 4 b and c, respectively). However, pre-ischemic PCr was not different between groups; pre-ischemic P_i was higher in SPT hearts (P<0.05 vs. IC, PC, CCPA). ATP levels (Figure 4a) and pH (Figure 4d) were not affected by any of the treatments prior to ischemia.

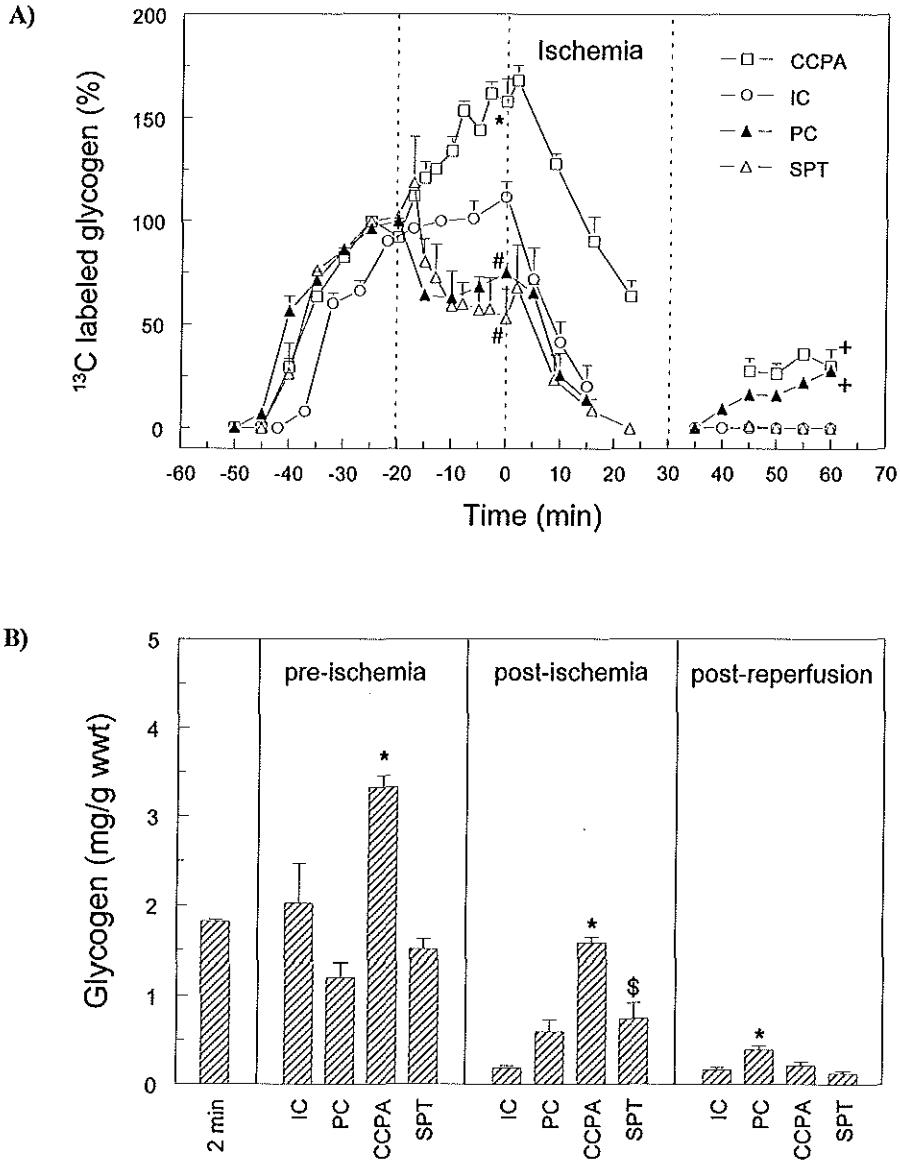

*Figure 3. ^{13}C-labeling and mobilization of glycogen (A) and total glycogen in hearts freeze-clamped at different time-points throughout the protocol (B). Preischemic glycogen was unrelated to functional recovery. 2 min = 2 min normoxic perfusion (B); *P<0.05 v IC, PC, SPT; #P<0.05 v CCPA, IC; †P<0.05 v IC, SPT; $P<0.05 v IC. For abbreviations and protocol, see Figure 1.*

The fall in ATP during prolonged ischemia was similar in all groups (Figure 4a); at the end of ischemia, ATP levels were depleted in all hearts. During early ischemia, PCr levels decreased more slowly in CCPA hearts ($P<0.05$ vs. IC) although PCr depletion was similar in all groups at the end of ischemia. During ischemia, P_i rose similarly in all groups. Ischemic acidification was similar in all groups but tended to be less in IC hearts.

Upon reperfusion, recovery of ATP, PCr, and pH was similar in all groups. At the end of reperfusion, recovery of P_i was worse in PC hearts ($P<0.05$ vs. IC).

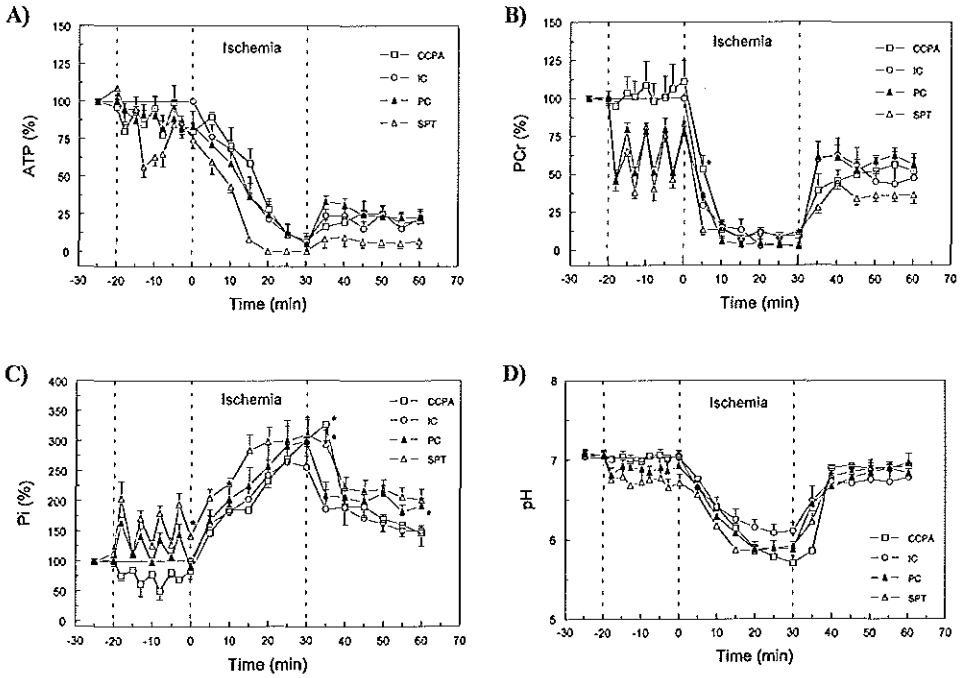

*Figure 4. Changes in ATP (A), creatine phosphate (PCr; B), inorganic phosphate (P_i; C), and pH (D). *P<0.05 vs. IC. For abbreviations and protocol, see Figure 1.*

4. Discussion

4.1. Adenosine is involved in preconditioning of rat hearts

In this study we show that ischemic preconditioning of rat hearts can be mimicked by pre-treatment with the selective adenosine A_1 agonist CCPA (Figure 2). CCPA treatment resulted in marked negative chronotropic and dromotropic effects, which resulted in a reduction in preischemic contractility (Figure 2). However, we showed earlier [43] (see also Chapter 7) that these contractile side-effects of CCPA were

unrelated to its cardioprotective effects since cardioprotection was also observed in paced CCPA treated hearts. Furthermore, ischemic preconditioning could be abolished by the non-selective adenosine antagonist SPT. From these observations we can conclude that adenosine is involved in preconditioning of rat hearts. This is in contrast to several studies that showed no involvement of adenosine in ischemic preconditioning of rat hearts (see introduction). There could be several reasons for this discrepancy. First, interstitial adenosine is much higher in the rat than for instance the rabbit, which may require higher adenosine antagonist concentrations [21]. Second, the high activity of adenosine degrading enzymes in the rat coronary endothelium [44], the barrier function of the endothelium for the transport of adenosine to the interstitium[45], and the short half-life of adenosine in blood [46] may lead to suboptimal activation of the adenosine A_1 receptor located on the myocardial membrane. Interestingly, studies using selective adenosine agonists, which exhibit substantially longer half-lives than adenosine [47], could mimic ischemic preconditioning [21,48]. Third, the use of selective adenosine A_1 antagonists or non-selective xanthine derivatives like SPT, which weakly bind the adenosine A_3 receptor in rat hearts [49,50], do not abolish the adenosine A_3 receptor mediated component of ischemic preconditioning.

4.2. Role of glycogen in ischemic preconditioning

In this study, we tested whether ischemic preconditioning is mediated by reducing preischemic glycogen and glycogenolysis during ischemia, resulting in attenuation of glycolytic catabolite accumulation and the development of intracellular acidosis, a notion known as the 'glycogen hypothesis of ischemic preconditioning' [23,51]. Furthermore, we assessed whether reduced glycogen depletion during ischemia is mediated by adenosine A_1 receptor activation, an important trigger of ischemic preconditioning. The results of our study show that preischemic glycogen is unrelated to preconditioning protection since both high (CCPA group) and low (PC group) preischemic glycogen (Figure 3) are associated with improved functional recovery (Figure 2). A comparison of the PC and IC hearts shows that glycogenolysis during early ischemia (first 5 min) was attenuated in PC hearts, in line with other reports [25,27,29,51]. CCPA also reduced glycogenolysis during early ischemia (Figure 3). However, although SPT abolished preconditioning protection, it did not abolish reduced glycogen utilization during the first 5 min of ischemia (Figure 3). ^{13}C-glycogen utilization during the first 15 min of sustained ischemia was not different between PC, IC, and CCPA groups, and was even reduced in SPT hearts (Figure 3a). Analysis of glycogen in freeze-clamped hearts (Figure 3b) showed that total glycogen depletion during 30 min ischemia was reduced in PC (0.61 mg/g wwt) and SPT (0.77 mg/g wwt) hearts compared to IC (1.84 mg/g wwt) and CCPA (1.75 mg/g wwt) hearts. Thus, preischemic glycogen and ischemic glycogenolysis can be dissociated from the degree of protection. Moreover also against the glycogen hypothesis is our observation that preischemic glycogen is not necessarily related to the rate of its depletion during ischemia. Other studies also have failed to observe a relation between functional recovery after prolonged ischemia and preischemic glycogen levels [26-28] or glycogen depletion during ischemia [27]. Furthermore, we did not find any differences between groups in the degree of acidosis during ischemia (Figure 4d) in line with the glycogen data. This contrasts many studies showing reduced accumulation of glycolytic end-products (lactate, protons, sugar phosphates) in preconditioned hearts during ischemia [26,28,29,51-53]. Soares et al. [28] and Schaefer et al. [54] showed that although preischemic glycogen content relates to pH during ischemia, it is unrelated to functional recovery upon reperfusion. Moreover, several groups [55,56] were also unable to find a relation between preconditioning protection and reduced acidosis during ischemia in line with the present study. We also found the rate of high-energy phosphates depletion (Figures 4a and b)

during ischemia to be similar in all groups. In conclusion, the results of this study provide evidence that preconditioning protection against sustained ischemia is not mediated by 1) reduced glycogen utilization and consequent attenuation of glycolytic catabolite accumulation, and 2) reduced rate of high-energy phosphates depletion (slowing of energy metabolism) as originally proposed by Murry et al. [51]. Hence, both correlations merely represent epiphenomena of ischemic preconditioning. However, we did not calculate the phosphorylation potential. It is possible that the phosphorylation potential was improved in preconditioned hearts in our study as has been previously shown [56].

Rather to our surprise, CCPA infusion increased glycogen synthesis resulting in higher preischemic tissue glycogen content compared to the other groups (Figures 3a and b). This may be due to stimulation of glycogen synthesis and inhibition of glycogen breakdown by the drug. Finegan et al. [32] showed that both adenosine and N^6-cyclohexyladenosine, a compound similar to CCPA, reduce glycolysis during normoxia. Due to the high preischemic glycogen levels in CCPA hearts, glycogen was not severely depleted during ischemia in CCPA hearts: the postischemic glycogen content was still 87% of the stabilization value in freeze-clamped hearts while it was much lower in the other groups (Figure 3b). Cross et al. [57] showed that although preischemic glycogen is unrelated to functional recovery after ischemia, glycogen depletion and cessation of glycolytic ATP production during ischemia is detrimental to the heart. These authors hypothesized that with inhibition of glycolytic ATP production, the Na^+/K^+-ATPase can no longer function and continued Na^+/H^+ exchange will finally lead to Na^+ and Ca^{2+} overload, and myocardial injury. Thus, one of the mechanisms of CCPA-induced cardioprotection may be prevention of glycogen depletion and cessation of glycolytic flux during ischemia.

Not many studies have examined the effect of adenosine preconditioning directly on ischemic glycogenolysis. Adenosine prior to no-flow ischemia reduced ischemic lactate accumulation [37]. We recently showed that CCPA pretreatment inhibited anaerobic glycolysis and glycolysis-from-glucose during low-flow ischemia [43] (see also Chapter 7), in line with other reports [34,58]. In ischemic dog hearts, adenosine did not significantly affect the rate of glycogen depletion as determined in transmural biopsies [38]. In the present study, CCPA mimicked PC in the initial delay (first 5 min) in ischemic glycogenolysis (Figure 3a). However, delayed glycogenolysis in preconditioned hearts was not blocked by the adenosine antagonists SPT (Figure 3a). Moreover, overall glycogen depletion during 30 min no-flow ischemia was higher in CCPA and IC hearts compared to SPT and PC groups (Figures 3a and b).

In conclusion, adenosine mediates protection by ischemic preconditioning in rat hearts. However, glycogen depletion prior to ischemia and reduced glycogenolysis during ischemia are not causually related to preconditioning protection.

Acknowledgements

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9

Preconditioning of rat hearts by adenosine A_1 or A_3 receptor activation

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Submitted

Abstract

Objective: Apart from infarct-size reduction and improved functional recovery, ischemic preconditioning (IPC) has been recently shown also to reduce apoptotic cell death in rat hearts. Moreover, the role of adenosine receptors in IPC in this species is controversial. Our study in rat hearts, examined whether activation of adenosine A_1 (AA_1R) or A_3 (AA_3R) receptors improved functional recovery and reduced apoptosis resulting from low-flow ischemia. **Methods:** Prior to 30 min low-flow ischemia (0.6 ml/min; 6% of baseline flow), Langendorff rat hearts were preconditioned with two 5-min cycles of a) ischemia (PC; n=7), b) infusion of 250 nM AA_1R agonist 2-chloro-N^6-cyclopentyladenosine (CCPA; n=6), or c) infusion of 50 nM AA_3R agonist N^6-(3-iodobenzyl)-adenosine-5'-*N*-methyl-uronamide (IB-MECA; n=8). **Results:** IB-MECA did not affect contractile function whereas CCPA infusion reduced the rate-force product by 83%. Recovery of function was improved in PC (71±3%), CCPA (68±6%) and IB-MECA (68±4%) groups compared to control hearts (46±5%; $P<0.05$). Cumulative release of total purines during ischemia/reperfusion was approx. 50% lower in PC, CCPA and IB-MECA groups compared to controls ($P<0.05$) and was significantly correlated to the percentage functional recovery ($R^2=0.55$; $P<0.05$). The number of cytosolic histone-associated-DNA fragments, a hallmark of apoptosis and measured by ELISA, was small and not different between groups after 30 min reperfusion. However, CCPA (0.6±0.1 absorbance units) and MECA (0.7±0.1 units; $P<0.05$ vs. PC) decreased apoptosis after 150 min reperfusion compared to PC (1.4±0.3 units) and control (1.2±0.1 units) hearts. Lactate production was reduced by 54% in CCPA-treated hearts ($P<0.05$ vs. controls). **Conclusions:** This study shows that adenosine triggers protection of function in preconditioned rat hearts via both the A_1 and A_3 receptor. In clinical practice, pharmacological stimulation of AA_3R may be advantageous over AA_1R activation due to a lack of contractile side-effects. In contrast to ischemic preconditioning, pharmacological stimulation of adenosine A_1 or A_3 receptors reduced apoptosis. Furthermore, total purine release may serve as a marker of the degree of functional protection.

1. Introduction

Ischemic preconditioning refers to the paradoxical mechanism that short, pre-emptive periods of ischemia protect the heart from a subsequent period of prolonged ischemia [1]. This phenomenon seems to occur in all vertebrates [2] including humans [3,4]. Of the humoral factors released during its induction that may trigger the event (cf. [5]), adenosine is one of the most important. In most animal species and humans [6-9], adenosine's cardioprotective effects are mediated via adenosine A_1 receptors located on the myocardial membrane. The recently discovered adenosine A_3 receptor has also been implicated in preconditioning of chick [10-12], rabbit [13-19], and human [9] hearts. However, adenosine mediated cardioprotection is believed to play no role in rat hearts (e.g., [20,21]). In contrast, we [22] and others [23,24] observed beneficial effects of adenosine A_1 receptor stimulation in this species. To the best of our knowledge, no study has examined before the role of the adenosine A_3 receptor in preconditioning of rat hearts.

Ischemia/reperfusion injury includes both apoptotic and necrotic myocyte cell death [25-28]. In contrast to necrosis, apoptosis is an active energy-consuming process orchestrated by a genetic program. Apoptotic cell death is often called programmed cell death to distinguish it from necrotic or accidental

cell death (oncosis). Apart from reduced ventricular arrhythmias, infarct size, and contractile dysfunctioning, ischemic preconditioning has also been shown to reduce apoptosis in vitro [29] and in vivo [30]. No data exist as to whether ischemic preconditioning effects on apoptosis are also mimicked by stimulation of adenosine A_1/A_3 receptors prior to ischemia. Furthermore, the relative contribution of apoptosis and necrosis to cell death following low-flow ischemia and reperfusion in isolated hearts is poorly described. Thus, this study examined the effectiveness of both adenosine A_1 and A_3 receptor stimulation in reducing contractile dysfunction and apoptosis in rat hearts after low-flow ischemia and reperfusion. This study shows for the first time that the adenosine A_3 receptor, in addition to the A_1 receptor, is involved in preconditioning of rat hearts. Although the degree of apoptosis was low, pharmacological preconditioning reduced its occurrence.

2. Methods

All animals were treated in conformation with the guiding principles in the care and use of animals as approved by the American Physiological Society. The Animal Welfare Committee, Erasmus University Rotterdam, approved the protocol.

2.1. Exclusion criteria

During stabilization, hearts were excluded if they met one of the following criteria: 1) unstable contractile function, 2) coronary flows outside the range of 9-19 ml/min, 3) severe arrhythmias, 4) myocardial temperature outside the range 37-39°C.

2.2. Isolated heart preparation

Fed, male Wistar rats (Wag/Rij inbred, weighing 280-330 g) were obtained from Harlan-CPB (Zeist, The Netherlands). They received a commercial rat chow (Hope Farms AM II, Woerden, The Netherlands) and tap water ad libitum. After anesthesia with an intraperitoneal injection of 0.6 ml sodium pentobarbital (Nembutal®, 60 mg/ml) supplemented with 0.1 ml heparin (Thromboliquine®, 5000 IU/ml), hearts were rapidly excised and arrested in saline (0°C) until beating ceased. Excess tissue was removed, and the hearts were cannulated within 1 min via the ascending aorta, for retrograde perfusion using a non-recirculating Krebs-Henseleit buffer containing (in mmol/l): NaCl 118, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and D-glucose 11. Insulin (Sigma, St. Louis, MO, USA; 1 U/l) was added to the buffer. Before use, the buffer was filtered through a 45-μm porosity filter to remove any particulate matter, and equilibrated with 5% CO_2/95% O_2, to give a pH of about 7.4 at 37°C. Myocardial temperature was kept at 37°C with a water-jacketed heart chamber and buffer reservoir, and regulated with an electric heating coil positioned around the aortic inlet line. The temperature of the outer ventricular wall was monitored with a thermocouple (A-F6, Ellab A/S, Roedovre, Denmark). Global, no-flow ischemia was induced by clamping the aortic line; low-flow ischemia was achieved using a perfusion pump (MV-MS3, Ismatec, Zurich, Switzerland) operating at a flow rate of 0.6 ml/min.

Coronary flow was measured by timed collection of the pulmonary artery effluent. The hearts were allowed to beat spontaneously. Cardiac contractile function was estimated with a force transducer

(LVS-50GA, Kyowa Electronic Instruments, Tokyo, Japan) connected to the apex of the heart [22,31]. The heart was pre-loaded with an initial resting tension of 2 g. Systolic tension and diastolic tension were continuously displayed on a recorder (Gould signal conditioner and Gould WindoGraf™ recorder, Valley View, OH, USA). Developed tension was calculated as systolic tension *minus* diastolic tension. Cardiac contractile function was expressed as rate-force product (RFP), the product of heart rate and developed tension. RFP was expressed as a percentage of baseline function (20 min stabilization). Perfusion pressure was measured with a disposable pressure transducer (Braun Melsungen, Melsungen, Austria) and kept constant at 65 mmHg.

2.3. Experimental Protocol

After initial isolation and surgical preparation, all hearts were perfused with the modified Krebs-Henseleit buffer and allowed to equilibrate for 20 min followed by a 20-min treatment period. Thereafter, hearts were subjected to 30 min of low-flow ischemia (0.6 ml/min) followed by a reperfusion period of 30 min. The 20-min treatment period prior to low-flow ischemia consisted of (Figure 1): 1) normoxic perfusion (IC group; n=7); 2) preconditioning using two 5-min episodes of no-flow ischemia each interrupted by 5 min of reperfusion (PC group; n=7); 3) preconditioning with two 5-min infusions of 250 nM of the selective adenosine A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA group; n=6), interspersed by two 5-min periods of drug-free perfusion; and 4) preconditioning with two 5-min infusions of 50 nM of the selective adenosine A_3 receptor agonist N^6-(3-iodobenzyl)-adenosine-5'-*N*-methyl-uronamide (IB-MECA; n=8), interspersed by two 5-min periods of drug-free perfusion. The used concentrations of CCPA and IB-MECA were based on their binding activities for (brain) adenosine receptors as indicated by the supplier (CCPA: K_iA_1 = 0.4 μM vs. K_iA_2 = 3900 μM; IB-MECA: K_iA_3 = 1.1 nM vs. K_iA_1/A_2 = 50-60 nM). After 30 min reperfusion, a part of the heart was flash frozen in liquid nitrogen. In a parallel study, hearts were also freeze-clamped after 150 min reperfusion for determination of the degree of apoptosis (n=4 per group).

2.4. Analysis of coronary effluent

During ischemia and reperfusion, coronary perfusate samples were continuously collected at 2-, 3-, 5-, or 10-min intervals, depending on the changes expected. Prior to ischemia, several 1-min samples were taken. Lactate in the samples, kept at 0°C, was determined enzymatically with an Elan auto-analyzer (Eppendorf, Merck, Amsterdam, The Netherlands) according to Sigma procedure 735. The remainder of the samples was stored at -80°C until further analysis. Purines in coronary perfusate samples were determined by reversed phase high-performance liquid chromatography (HPLC) according to Smolenski et al. [32]. Briefly, a C_{18} column (Hypersil ODS 3 μm, 150 × 4.6 mm, Alltech, Deerfield, Ill., USA) was employed combined with a C_{18} guard column (Hypersil ODS 5 μm, 7.5 × 4.6 mm). The system configuration consisted of an AS3000 cooled autosampler, an SCM1000 vacuum membrane degasser, a P2000 gradient pump, a Spectra Focus forward optical scanning detector, and PC1000 software (Spectra-Physics, San Jose, CA, USA). Peaks were detected at 254 nm (hypoxanthine, xanthine, inosine, adenosine) and at 280 nm (uric acid). Perfusate purines were identified based on their co-elution with standards, retention times, and their 254/280 ratios.

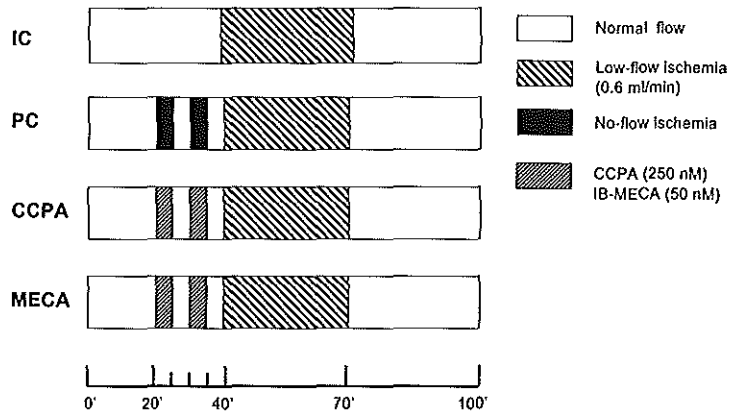

Figure 1. Diagram showing the different experimental protocols. Each experiment started with a 20-min stabilization period followed by a 20-min treatment period. Thereafter, hearts were subjected to 30 min of low-flow ischemia (0.6 ml/min) followed by a reperfusion period of 30 min. The 20-min treatment period prior to low-flow ischemia consisted of: 1) normoxic perfusion (IC group); 2) preconditioning using two 5-min episodes of no-flow ischemia each interrupted by 5 min of reperfusion (PC group); 3) preconditioning with two 5-min infusions of adenosine A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA group), interspersed by two 5-min periods of drug-free perfusion; 4) preconditioning with two 5-min infusions of adenosine A_3 agonist N^6-(3-iodobenzyl)-adenosine-5'-N-methyl-uronamide (IB-MECA group), interspersed by two 5-min periods of drug-free perfusion.

2.5. Sandwich enzyme immunoassay

Quantization of DNA fragmentation into cytosolic mononucleosomes and oligonucleosomes was done with an ELISA kit (Cell Death Detection ELISA^{PLUS}; Boehringer Mannheim, Germany). This test quantifies histone-associated DNA fragments in a sandwich-enzyme-immunoassay using mouse monoclonal antibodies directed against DNA and histones, respectively. At the end of reperfusion, atria were removed and ventricles were frozen in liquid nitrogen and stored at -55°C. Hearts were ground under liquid nitrogen using pestle and mortar. From the total homogenate, 50 mg was added to 800 μl lysis buffer supplied with the kit and incubated for 30 min at room temperature (ca. 20°C). After incubation, the homogenate was centrifuged at 13000g for 20 min. The supernatant fraction was further diluted 12-fold in phosphate-buffered saline (in mmol/l: NaCl 137, KCl 2.7, Na_2HPO_4 8.1, KH_2PO_4 1.5; pH 7.4) and used as antigen in the ELISA which was performed according to the manufacturer's instructions. Duplicate values of the double absorbance measurements (405 - 492 nm) were averaged from which the negative control (incubation buffer in stead of sample solution) was substracted.

2.6. Chemicals

CCPA and IB-MECA were obtained from RBI (Natick, Mass., USA). Stock solutions (100 μM) of CCPA in deionized water and IB-MECA in dimethylsulfoxide were diluted 400 and 2000 times, respectively, in the perfusion medium. Stock solutions were kept at -55°C.

2.7. Statistical analysis

The data are expressed as means ± S.E.M., with n = number of hearts. Summary measures were constructed for contractile parameters, lactate release, and total purine release [33]. Recovery of rate-force product was expressed as a percentage of baseline value. The sum of lactate and total purines produced during ischemia was calculated as the cumulative release in the venous effluent during 30 min underperfusion and the first 5 min of reperfusion. One-way or two-way analysis of variance with subsequent Student-Newman-Keuls post-hoc tests were used for comparisons between groups. If values were not normally distributed or variances between groups were unequal, Kruskal-Wallis ANOVA on ranks was used. Values of $P<0.05$ (two-tailed test) were regarded as significant.

3. Results

3.1. Contractile function and coronary flow

After 20 min stabilization, there were no differences between groups in rate-force product (mean and SEM of all groups: 4510±224 g/min) or coronary flow rate (10±1 ml/min). Infusion of IB-MECA did not significantly affect preischemic rate-force product (Figure 2) but increased coronary flow up to 70 % (Figure 3). In contrast, CCPA reduced rate-force product by 83% (Figure 2) mainly due to a reduction in heart rate; coronary flow increased during the first infusion (76%) but was reduced 20 % vs. pre-drug value during the second infusion (Figure 3). In the PC group, rate-force product rapidly fell to zero during transient ischemia and recovered to 79% after the second period of reperfusion (Figure 2). Contractile function fell to 0 within 5 min in all groups at the start of prolonged ischemia. After 30 min reperfusion, rate-force product was improved in PC (71±3%), CCPA (68±6%), and MECA (68±4%) groups compared to controls (46±5%; $P<0.05$). Early during reperfusion after prolonged ischemia, the extent of reactive hyperemia was similar in all groups (Figure 3). Coronary flow recovered to normal values after 30 min reperfusion (coronary flow data for 100 min normoxic perfusion not shown) and was not different between groups. Resting tension did not significantly change throughout the protocol in any of the groups.

3.2. Metabolite release

The appearance of purines in the coronary effluent reflects ATP catabolism during ischemia and, hence, the energy status of the cell. Figure 4 depicts the release of total purines (adenosine + inosine + hypoxanthine + xanthine + urate) throughout the protocol. Cumulative release of total purines (in nmol/g wwt) during long ischemia and reperfusion was approx. 50% lower in PC (672±184), CCPA (486±165) and IB-MECA (690±109) hearts compared to controls (1330±184; $P<0.05$).

Lactate released in the coronary effluent was taken as measure of anaerobic glycolysis from both endogenous glycogen and exogenous glucose sources. Lactate produced during prolonged underperfusion was not different between IC, PC, and IB-MECA groups (Figure 5). The sum of lactate released during underperfusion and reperfusion (in μmol/g wwt) was not different between IC (44.5±4.4), PC (40.3±4.5), and IB-MECA (36.6±5.7) groups but was halved in CCPA treated hearts (19.4±2.3; $P<0.05$ vs. all other groups).

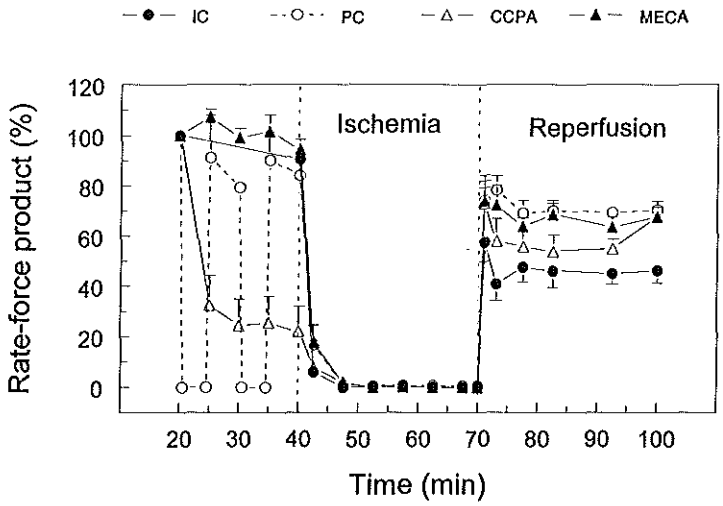

Figure 2. Changes in rate-force product. In contrast to CCPA, IB-MECA did not affect preischemic contractility. Recovery of rate-force product at the end of reperfusion was significantly improved in PC, CCPA and IB-MECA hearts compared to IC, the controls (P<0.05). For abbreviations and protocol, see Figure 1.

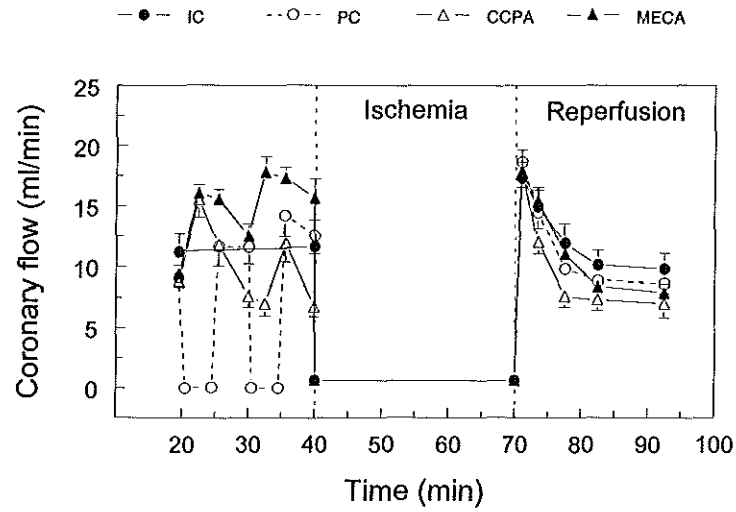

Figure 3. Changes in coronary flow. IB-MECA infusion resulted in increased coronary flow. Compared to baseline, coronary flow was increased during the first CCPA infusion but was reduced during the second. During the 20 min-period of stabilization and during recovery following ischemia, coronary flow was the same between groups. For abbreviations and protocol, see Figure 1.

3.3. Relationship between functional recovery and cumulative purine release during ischemia

To determine whether purine release during ischemia can serve as a marker for the degree of ischemic injury, we correlated recovery of function at the end of reperfusion with the cumulative release of total purines during prolonged ischemia (Figure 6). There was a significant inverse correlation between both variables (R^2=0.55; P<0.05).

3.4. Apoptosis

The number of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes), a hallmark of apoptosis, was determined by ELISA. Fragmentation of DNA was similar in PC (0.9±0.1 arbitrary absorbance units), CCPA (0.6±0.1 units), IB-MECA (0.8±0.1 units), and IC (0.7±0.1 units) hearts after 30 min reperfusion (Figure 7). Apoptosis was higher after 150 min reperfusion compared to 30 min reperfusion (P=0.001). Pharmacological preconditioning with CCPA (0.6±0.1) and IB-MECA (0.7±0.1; P<0.05 vs. PC) decreased apoptosis compared to PC (1.4±0.3) and IC (1.2±0.1). The degree of apoptosis appears to be small in hearts subjected to low-flow ischemia and reperfusion since fragmentation in control hearts perfused normoxically for 2 min (NC (2 min); 0.2 units; n=1) or 100 min (NC (100 min); 0.4±0.1 units; n=2) were only 2-4 times lower compared to hearts subjected to ischemia and reperfusion.

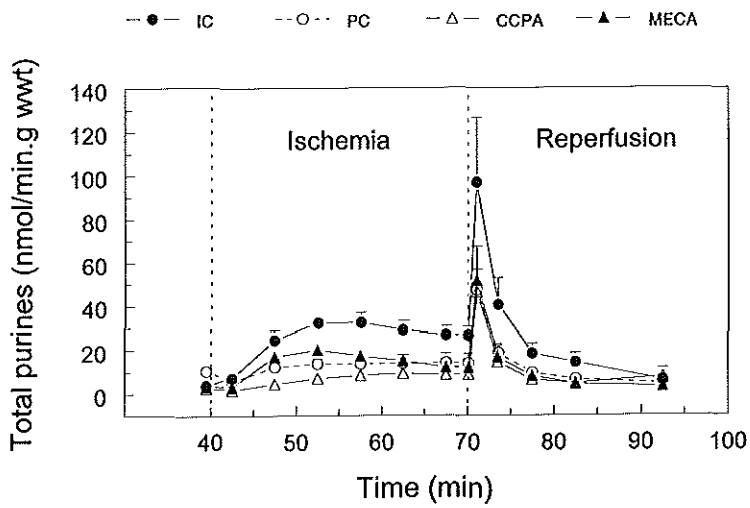

Figure 4. Release of total purines (adenosine + inosine + hypoxanthine + xanthine + urate) in the coronary effluent. Both pharmacological and ischemic preconditioning reduced the cumulative amount of total purines released during ischemia and reperfusion compared to control hearts (P<0.05). For abbreviations and protocol, see Figure 1.

4. Discussion

4.1. Role of the adenosine receptor in preconditioning of rat hearts

This is the first study indicating that adenosine A_3 receptors are also involved in protection by ischemic preconditioning in rat hearts. Furthermore, in this study we confirm earlier observations of our group [34] that CCPA, a selective adenosine A_1 agonist, reduces ischemic injury in rat hearts. Infusion of the selective adenosine A_3 agonist IB-MECA prior to ischemia, resulted in a similar degree of myocardial protection to adenosine A_1 receptor stimulation with CCPA (Figure 2), in line with published reports [9,12,16]. The absence of any contractile side-effects during infusion of 50 nM IB-MECA indicates that this dose did not activate the adenosine A_1 receptor. Thus, both adenosine A_1 and A_3 receptors trigger preconditioning protection by endogenous adenosine. However, Hill et al. [19] recently concluded that the adenosine component of *ischemic* preconditioning is preferentially mediated by the A_1 receptor since adenosine binds this receptor with a greater affinity than the A_3 receptor in rabbit hearts (K_iA_1 = 28 nM; K_iA_3 = 532 nM). Whether both receptors are maximally activated in ischemic preconditioning of rat hearts is unclear since the affinity of adenosine for the adenosine A_3 receptor has not been determined yet in this species. However, higher interstitial adenosine levels during preconditioning ischemia (~7 μM) have been reported in the rat than in other species [23] which makes it likely that both adenosine A_1 and A_3 receptors are maximally activated during ischemic preconditioning, co-operating in its induction [15].

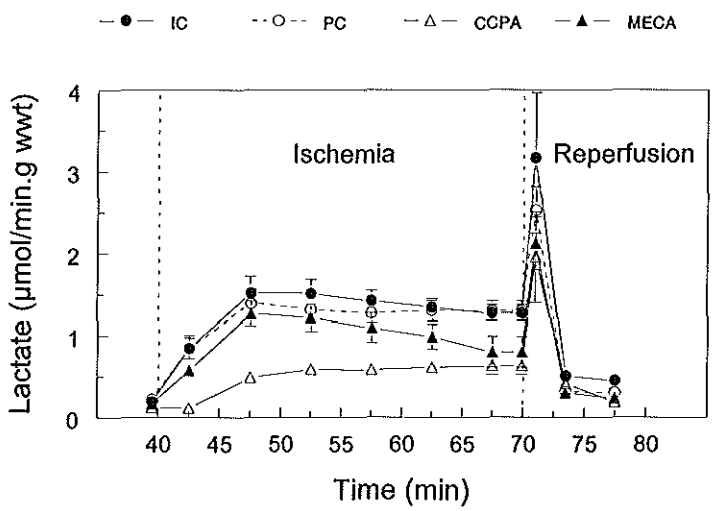

Figure 5. Release of lactate in the coronary effluent. Lactate release during prolonged ischemia and reperfusion was similar in the IC, PC and IB-MECA groups but was significantly reduced in CCPA hearts ($P<0.05$). For abbreviations and protocol, see Figure 1.

The observation in the present study that both adenosine A_1 and A_3 receptors trigger preconditioning protection in rat hearts is in contrast to other studies that were unable to mimic or abolish ischemic preconditioning with adenosine and adenosine antagonists, respectively [20,21]. The inability to abolish ischemic preconditioning with adenosine antagonists could be related to the fact that 1) interstitial adenosine levels during preconditioning are very high in the rat compared to other species which may require increased antagonist concentration [23], and 2) the use of selective A_1 antagonists, which do not abolish the A_3 mediated component of ischemic preconditioning. We showed before that 50 μM 8-sulfophenyltheophylline, a non-selective adenosine antagonist, abolished protection by ischemic preconditioning in rat hearts ([34]; see also Chapter 8). Administration of exogenous adenosine in rats may be limited by the instability of this compound and the high activities of adenosine-degrading enzymes in the coronary endothelium, making the coronary endothelium an active barrier for adenosine transport. This is probably the reason why only studies using selective agonists of the A_1 receptor, instead of the natural ligand adenosine, have demonstrated protection against ischemic injury [23,24,35].

Infusion of IB-MECA did not affect contractility in contrast to the large (83%) decrease observed with administration of CCPA (Figure 2), in line with data obtained in conscious rabbits [18]. Earlier, we showed that the cardioprotective effects of adenosine A_1 receptor activation are unrelated to the negative chronotropic and dromotropic side-effects of these drugs [34]. Activation of the adenosine A_3 receptor induces hypotension by mast cell degranulation in rats in vivo [18,36]. In humans and dogs, however, mast cell activation is probably mediated by the adenosine A_{2b} receptor, not the A_3 receptor [37,38]. In contrast, the A_3 receptor of these species has anti-inflammatory effects by inhibiting eosinophil migration (chemotaxis) [38,39], neutrophil degranulation [40], and TNFα release by macrophages [41]. Thus, although both adenosine A_1 and A_3 receptor activation protect against ischemic injury, agonists of the latter may be more promising as cardioprotective agents in the clinical setting due to a lack of hemodynamic side effects, anti-inflammatory effects, and a more sustained duration of protection than A_1 agonists [12]. Furthermore, selective adenosine A_3 agonists have been reported to protect against both infarction and stunning [18] in contrast to ischemic preconditioning, which only reduces infarct-size. This may be a major advantage of selective A_3 agonists since myocardial stunning is often a greater problem after bypass surgery than infarction, which has a low incidence with current surgical techniques.

4.2. Purine metabolism

Based on the observation that ischemic preconditioning reduces interstitial purine accumulation during regional ischemia in dog hearts, Van Wylen [42] suggested that ischemic preconditioning improves energy balance and consequently reduces ATP hydrolysis during ischemia. We previously also observed reduced purine release in the coronary effluent of preconditioned rat hearts during low-flow ischemia [22]. In the present study we extend this finding, demonstrating that also hearts protected by adenosine A_1 and A_3 agonists show reduced purine release during ischemia and reperfusion (Figure 4). Goto et al. [43] concluded that decreased purine release is an epiphenomenon of ischemic preconditioning and cannot be relied upon to serve as a marker of protection by preconditioning. However, these authors only examined purine release during short preconditioning ischemia and not during long ischemia. The present study strongly suggests that reduced myocardial purine production during ischemia is correlated to the functional recovery at the end of reperfusion, serving as a marker

for the degree of ischemic injury (Figure 6).

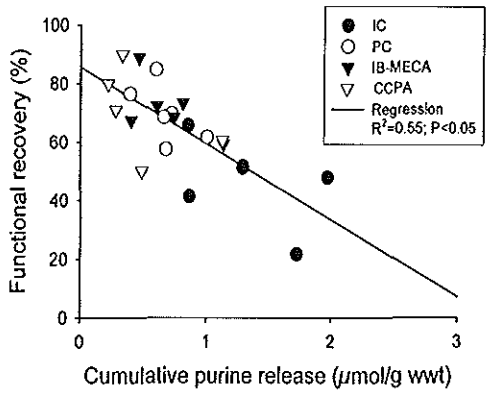

Figure 6. *Negative correlation between % functional recovery at the end of reperfusion and the cumulative amount of total purines (adenosine + inosine + hypoxanthine + xanthine + urate) released during prolonged ischemia and reperfusion. For abbreviations and protocol, see Figure 1.*

4.3. Carbohydrate metabolism

This study shows that CCPA decreases lactate production during underperfusion (Figure 5) compared to the other groups, confirming earlier findings [34,44]. Paced CCPA hearts also showed reduced lactate production during low-flow ischemia (data not shown). Thus, reduced contractility caused by CCPA infusion (Figure 2) was unrelated to less lactate production during subsequent ischemia. We previously suggested that ischemic preconditioning is mediated by increased glucose uptake during underperfusion without increasing total anaerobic glycolytic flux and that CCPA-induced protection may involve a different mechanism as it decreased glycolysis [34]. Cardioprotection induced by adenosine A_1 agonists has been suggested to involve reduced proton production from glucose metabolism reducing Ca^{2+} overload [24]. To our knowledge, this is the first report on the effect of adenosine A_3 receptor stimulation on anaerobic glycolysis. Infusion of IB-MECA prior to underperfusion did not result in reduced anaerobic glycolysis during ischemia as observed with CCPA.

4.4. Apoptosis

Necrosis and apoptosis are two distinct forms of lethal cell injury resulting from ischemia [25-28]. This is the first study reporting the effects of adenosine analogs on the degree of apoptosis occurring during ischemia and reperfusion. Although ischemic preconditioning improved contractile functioning after ischemia/reperfusion, it did not reduce the degree of apoptosis compared to ischemic control hearts (Figure 7). This is in contrasts to a study in rat hearts in vivo [30] in which both infarct size and apoptosis decreased after ischemic preconditioning. Although we did not assess infarct size in the present study,

we showed before that ischemic preconditioning reduces necrosis after 25 min low-flow ischemia as assessed by reduced leakage of creatine kinase [22]. Moreover, less purine release in preconditioned hearts (Figure 4) also reflects reduced ischemic injury. Thus, we believe that improved functional recovery in preconditioned hearts in the present study for a large part results from a reduction in lethal cell injury. In contrast to ischemic preconditioning, both adenosine A_1 and A_3 agonists decreased apoptosis. Thus, our study provides evidence for the first time that adenosine analogs may reduce both apoptosis and necrosis (oncosis). In our study, the degree of apoptosis after low-flow ischemia and reperfusion appears low since values were only 2-4 times higher than in rat hearts perfused normoxically (NC 2 and 100 min; Figure 7). This could be related to the severity of ischemia. In regional ischemic rat hearts in vivo, the degree of apoptosis in ischemic control hearts [30], determined with the same commercial ELISA kit as used in the present study, was ca. 15 times higher in the ischemic vs. the nonischemic region. Therefore, the inability of preconditioning protocols to reduce apoptosis in our study may relate to its low incidence after low-flow ischemia and reperfusion. Future studies need to clarify the contribution of apoptosis and necrosis to various forms and degrees of ischemic injury.

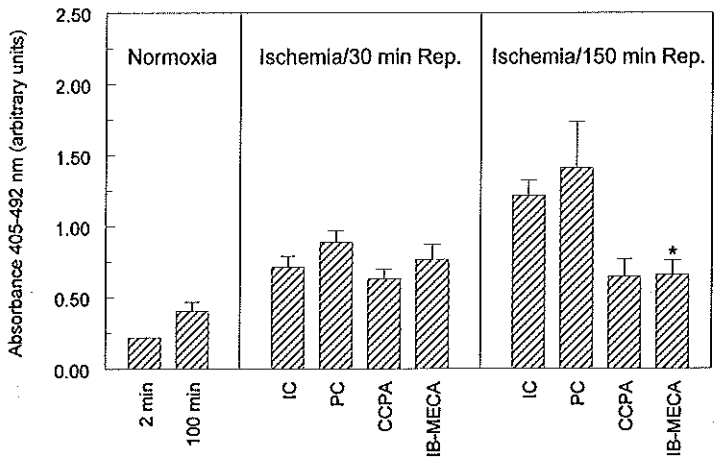

*Figure 7. Number of cytosolic histone-associated DNA fragments, a hallmark of apoptosis, measured by ELISA (absorbance: A405 – A492 nm) after 30 min and 150 min of reperfusion. The degree of apoptosis is low; pharmacological preconditioning decreased apoptosis. NC (2 min)=2 min of normoxic perfusion only; NC (100 min)=100 min of normoxic perfusion; *P<0.05 vs. PC. For other abbreviations and protocol, see Figure 1.*

4.5. Validity of the model

Whether we may extrapolate our observation concerning cardioprotection by adenosine A_3 receptor activation in the isolated rat heart to the human situation is unclear. Adenosine A_3 receptors have been reported to be present in rat [45], rabbit [17], chick [10], and human [46] hearts. However, the rat may not be the model of choice to study the cardioprotective properties of the adenosine A_3 receptor, due to the high divergence from other species, including humans [47]. The fact that both adenosine A_1

[9,48,49] and A_3 [9] receptors have been implicated in preconditioning of human [8,47,48] and animal hearts indicates that myocardial protection triggered by adenosine receptor activation is a central feature in all species. Our study clearly demonstrates that the rat heart forms no exception to this rule, contrasting other suggestions [20,21]. Thus, although species differences with regard to the adenosine-mediated component of ischemic preconditioning exist, they seem to be more of a quantitative than a qualitative nature.

4.6. Conclusion

The present study demonstrates that adenosine A_1 and A_3 receptors are implicated in preconditioning of rat hearts. Furthermore, purine release may be used as a marker of the degree of ischemic injury. The degree of apoptosis was low in hearts subjected to 30 min underperfusion and reperfusion and was reduced by pharmacological preconditioning. Adenosine A_3 agonists may represent a new, potentially useful therapeutic class of agents for providing cardioprotection as they lack cardiovascular side effects associated with A_1 receptor activation.

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10

General discussion and conclusions

Partly based on:

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With the discovery of ischemic preconditioning in 1986 by Murry and coworkers [1,2], many studies have been conducted to elucidate the mechanism of its protective action. An overview of the current status of preconditioning research is given in Chapter 1.

This thesis examines the protective effect of ischemic preconditioning in relation to adenosine as the trigger and myocardial carbohydrate metabolism as the end-effector of the event. We hypothesized that:

(1) Adenosine, released during preconditioning ischemia, triggers ischemic preconditioning by binding to the adenosine A_1 and/or A_3 receptors located on the myocardial membrane;

(2) Stimulation of adenosine receptors finally will result in modification of myocardial carbohydrate metabolism during ischemia. Specifically, we hypothesized that adenosine receptor activation will result in: (i) an increase in myocardial glucose uptake and metabolism which may be beneficial during low-flow ischemia, and (ii) inhibition of detrimental glycogen breakdown.

1. Ischemic preconditioning

1.1. Preconditioning protection against no-flow and low-flow ischemia

In this thesis, we demonstrate that both ischemic and pharmacological preconditioning reduce contractile dysfunctioning and irreversible injury arising from both no-flow (Chapters 4 and 8) and severe low-flow ischemia (Chapters 6, 7, and 9). Most ischemic preconditioning experiments have focussed on zero-flow after the pretreatment. There is no doubt that ischemic preconditioning protects against no-flow ischemia in many species; however, few studies have examined low-flow ischemia. In clinical practice, it is more likely that low-flow conditions will prevail, due to partial coronary occlusion and/or collaterals present. Indeed, sophisticated analyses of residual flow to the infarcted territory in patients with acute myocardial infarction demonstrated this region is affected by low-flow, not no-flow ischemia [3,4]. Also, during cross-clamping of the aorta in coronary artery bypass graft surgery, the induced global ischemia is not total but rather of the low-flow type due to residual flow from extracardiac sources [5].

Preconditioning-induced protection against low-flow ischemia is rather controversial. Some authors [6,7] suggest that ischemic preconditioning may not be able to protect against injury arising from underperfusion/hypoxia. However, other studies, in line with our own results, showed that ischemic preconditioning reduced injury resulting from severe underperfusion [8-11]. Adenosine A_1 receptors, the most popular candidate of mediating preconditioning protection, must be occupied both during preconditioning ischemia (initiation) and prolonged ischemia (mediation) to induce the protective effect [12]. Therefore, residual flow during prolonged ischemia may lead to washout of adenosine from the interstitium and consequently loss of protection. Against this argument is our observation that coronary venous adenosine levels during low-flow ischemia were in the μM range (mean values amounted to 0.56 μM and 3.61 μM in preconditioned and control hearts, respectively; see Chapter 6) whereas the K_d value of adenosine A_1 receptor activation lies in the nM range [13,14]. Coronary venous adenosine levels during underperfusion reflect interstitial adenosine levels because the concentration gradient between both compartments present during normoxia is almost completely reduced during underperfusion due to the saturation in the endothelial uptake of adenosine in stressed hearts [15-17]. A recent study using

the microdialysis technique showed that interstitial adenosine levels during no-flow ischemia were similar to those in the coronary venous effluent during early reperfusion [18]. Thus, interstitial adenosine levels (a reflection of adenosine A_1 receptor activation) during severe underperfusion are probably high enough to occupy the adenosine A_1 receptor, mediating preconditioning protection [12]. Against the washout hypothesis is also the observation that ischemic preconditioning exists in species having collateral flow [1]. It is possible that there could be a critical flow above which preconditioning effects are lost. It is important to keep in mind that fundamental differences exist between no-flow and low-flow ischemia, especially with respect to carbohydrate metabolism (see section 3). An important difference between both forms of ischemia is that during underperfusion washout of metabolic end-products may occur as we have shown in Chapter 6.

In the experimental model of acute hibernation, prolonged hypoperfusion is preceded by short no-flow ischemia without reperfusion [19,20]. This is akin to the human situation of hibernating myocardium where hypoperfusion is preceded by an acute ischemic insult. Because ischemic preconditioning not necessarily requires intermittent reperfusion [21,22], preconditioning and hibernation may be linked.

1.2. Presumed redundant pathways in preconditioning

Although there is ample evidence that preconditioning can take place by many alternative (receptor) routes [23], it is surprising that inhibition of one pathway often abolishes preconditioning completely. Goto et al. [23] proposed that protein kinase C (PKC) must be stimulated beyond a threshold level before cardiac protection by ischemic preconditioning takes place. In this model, adenosine, bradykinin and norepinephrine released during preconditioning ischemia trigger protection by stimulating PKC, provided that the threshold activity of the kinase is exceeded. Blockade of a single of these 'receptor routes' results in the abolition of ischemic preconditioning, as has been shown by many studies (see, e.g., Goto et al. [23]). In this thesis, we demonstrated that 8-SPT completely blocked the protective effect of ischemic preconditioning (Chapters 7 and 8). This contrasts sharply with the redundancy of vasodilator systems in the coronary circulation. Thus, in chronically instrumented dogs, neither adenosine receptor blockade with 8-phenyltheophylline [24] nor the K_{ATP} channel blocker glibenclamide [25] prevented exercise-induced increases in coronary blood flow, when either blocker was administered alone. In contrast, when both vasodilator mechanisms were blocked simultaneously [26], the increase in coronary blood flow was markedly attenuated, indicating that endogenous adenosine and K_{ATP} channels play a synergistic role in maintaining coronary vasodilation in exercising dogs. Thus, when a single vasodilator mechanism is blocked, another mechanism can act to compensate and mediate the active coronary hyperemia during exercise.

1.3. Does preconditioning protect against necrosis and apoptosis?

In line with the first report on the infarct-size limiting effect of ischemic preconditioning in dogs [1] (see also Chapter 1, Figure 1), we demonstrated that both ischemic and pharmacological preconditioning improved contractile function in isolated rat hearts subjected to ischemia. Improved functional recovery in these hearts most likely reflects reduced infarct-size since release of CK, a 'marker' enzyme of myocardial necrosis, was also reduced in these hearts (Chapter 6, Figure 3a).

Moreover, total purine release, a marker of the degree of ischemic injury, was also lower in ischemically or pharmacologically preconditioned hearts (Chapter 6, Figure 4; Chapter 9, Figures 4 and 6). Other studies have indicated that reduced contractile dysfunctioning in ischemically preconditioned hearts indeed reflects reduced infarct-size rather than reduced stunning [27-29].

Recently, it was discovered that cell loss during ischemia/reperfusion not only consists of necrosis but also apoptosis [30-33]. In contrast to necrosis, apoptosis is an active energy-consuming process orchestrated by a genetic program not leading to an inflammatory reaction and formation of scar tissue. Apoptosis counterbalances cell proliferation and is involved in the remodeling of tissue in fetal development (organogenesis) and the removal of unwanted or damaged cells. Deregulated apoptosis due to an imbalance between survival factors and death promoting factors plays a role in the pathogenesis of many diseases [34-36]. Apoptosis can be inhibited (e.g., in cancer, autoimmune diseases, and viral infections) or excessive (e.g., in aids and neurodegenarative diseases) and is also involved in cardiovascular diseases like heart failure, ischemic heart disease, and atherosclerosis [34]. Necrosis (accidental cell death or oncosis) and apoptosis (programmed cell death) most likely are two independent and distinct pathways of ischemia-induced cell loss [30,37]. The relative contribution of apoptosis and necrosis to cell death following ischemia and reperfusion is poorly described.

In Chapter 9, we demonstrate that the degree of apoptosis was low after 30 min of low-flow ischemia and 30 or 150 min of reperfusion (Figure 7). Furthermore, ischemic preconditioning did not reduce apoptosis in contrast to earlier observations in vitro [38] and in vivo [39]. Whether pharmacological 'preconditioning mimetics' like K_{ATP}-channel openers and adenosine analogs also reduce apoptosis is hardly studied. The results presented in Chapter 9 (Figure 7) indicate that adenosine analogs might be more effective than ischemic preconditioning in reducing apoptosis.

2. Does adenosine trigger ischemic preconditioning?

2.1. Adenosine receptors are involved in ischemic preconditioning

In this thesis, we demonstrate that adenosine triggers ischemic preconditioning of rat hearts. Adenosine exerts its cardioprotective action by binding to the adenosine A_1 and A_3 receptors located on the myocardial plasma membrane. Thus, pretreating hearts with either the A_1-selective agonist CCPA (Chapters 7-9) or the A_3-selective agonist IB-MECA (Chapter 9) mimicked protection by ischemic preconditioning. Moreover, the non-selective adenosine antagonist 8-SPT could abolish the protective effect of ischemic preconditioning (Chapters 7 and 8). The evidence for the involvement of adenosine, acting on either A_1 or A_3 receptors, in ischemic preconditioning is overwhelming and is highly conserved between species since adenosine receptor-mediated cardioprotection has also been observed in dogs [1,40,41], pigs [21,42,43], rabbits [44-51], chick [52-54], and humans [55-59]. Moreover, transgenic overexpression of the adenosine A_1 receptor in mice [60,61] or overexpression of the adenosine A_1 and A_3 receptors in chick myocytes [62] reduces ischemic injury.

In Chapter 5, the effectiveness of the adenosine regulating agent acadesine, used as a preconditioning agent, was examined in patients undergoing pacing-induced ischemia. No beneficial effects of this drug were observed on ventricular function and hemodynamics (Chapter 5, Table 1). Our observations are in line with several clinical trials on the effectiveness of acadesine administrated to

patients just prior to and during coronary artery bypass surgery [63-65]. The limited success of this drug to protect patients against the deleterious effects of ischemia may have several reasons. First, acadesine might only raise vascular adenosine during ischemia and not interstitial adenosine [66]. The pronounced cardioprotective properties of preconditioning are related to adenosine A_1 receptor activation, which is located on the myocardial membrane and activated by interstitial adenosine (see Chapter 2, Figure 4). Second, since acadesine raises adenosine in ischemic tissues only, the severity of ischemia during coronary artery bypass surgery [63-65] or maximal pacing (Chapter 5) may be too low to increase adenosine and to protect the heart. This may explain the observation that acadesine only was effective in high-risk patients undergoing coronary artery bypass surgery [64].

In Chapter 9, we show that selective agonism of either the adenosine A_1 or A_3 receptor resulted in a similar degree of myocardial protection (Figure 2), in line with published reports [48,54,56]. However, from the results presented in that chapter, we cannot draw any conclusions concerning the relative contribution of the adenosine A_1 and A_3 receptor in the protective effect of *ischemic* preconditioning. A recent study in rabbit hearts [51] concluded that the adenosine component of ischemic preconditioning is preferentially mediated by the A_1 receptor since adenosine binds to this receptor with a greater affinity than to the A_3 receptor (K_iA_1 = 28 nM; K_iA_3 = 532 nM). However, most likely adenosine A_1 and A_3 receptors are both maximally activated during ischemic preconditioning co-operating in its induction [47] since interstitial adenosine levels during preconditioning ischemia as well as during prolonged ischemia reach values in the micromolar range in dogs [67], rabbits and rats [18,68].

CCPA infusion induced profound negative chronotropic effects resulting in a 79% decline in pre-ischemic rate-force product (Chapter 7, Figure 1). We demonstrated that CCPA-induced cardioprotection is unrelated to its negative chronotropic and dromotropic effects (Chapter 7) as well as to changes in coronary flow (Chapter 8) during infusion of the drug. Cardioprotection by pretreatment with the adenosine A_3 receptor agonist IB-MECA did not lead to hemodynamic side-effects (Chapter 9, Figure 2). Hence, this class of agents may be more beneficial for use in patients.

In Chapters 6 (Figure 4) and 9 (Figures 4 and 6), we demonstrate that ischemic preconditioning reduces the release of adenosine and total purines during underperfusion in line with other reports measuring interstitial adenosine [18,67]. Reduced purine release during ischemia in preconditioned hearts was thought to reflect reduced ATP catabolism [2]. However, no differences in ATP levels between preconditioned and control hearts were observed in freeze-clamped hearts (Chapter 6) or in hearts subjected to ^{31}P-NMR (Chapter 8, Figure 4a; see also [18]). The analysis of a relation between ATP catabolism in the ischemic heart and the amount of purines released may be hampered by the fact that small reductions in ATP may lead to a large release of purines.

2.2. Does adenosine play a role in preconditioning of rat hearts?

In Chapters 7-9, we showed that adenosine A_1 and A_3 receptors are involved in ischemic preconditioning of rat hearts. Pharmacological stimulation of these receptors prior to ischemia with selective agonists directed at the A_1 (CCPA) or A_3 receptor (IB-MECA) mimicked ischemic preconditioning. Also, protection was abolished in hearts ischemically preconditioned in the presence of the non-selective adenosine antagonist 8-SPT. Thus, we confirm observations in other species that adenosine is one of the most important triggers of ischemic preconditioning (see previous paragraph). However, the role of adenosine in preconditioning of rat hearts is controversial in contrast to all other

animal species studied. With some exceptions, most studies using adenosine or adenosine antagonists have failed to mimic or abolish preconditioning protection in the rat heart, respectively (see Tables 1 and 2). This paradox may have several reasons. First, interstitial adenosine levels during preconditioning are high in the rat compared to other species (e.g., rat ~7 μM vs. rabbit ~2 μM), which may require increased antagonist concentration [68]. Headrick [68] showed that 10 μM 8-SPT effectively abolished preconditioning protection in rabbits but not in rats. In the latter species, preconditioning protection could be abolished when the 8-SPT dose was increased to 50 μM. We confirmed this observation (Chapters 7 and 8). Second, administration of exogenous adenosine in rats may be limited by the instability of this compound [69] and the high activities of adenosine-degrading enzymes in the rat coronary endothelium [70], making the coronary endothelium an active barrier for the transport of adenosine to the interstitium [71]. This is probably the reason why mostly studies using selective agonists of the A_1 receptor, in stead of the natural ligand adenosine, have demonstrated protection against ischemic injury in this species (Table 1). Note, the half-life of adenosine in human blood is very short (0.6 seconds [69]) compared to that of synthetic adenosine A_1 agonists, which exhibit substantially longer half-lives dependent on the size of the N^6-substituent (e.g., cyclopentyladenosine: 15 min; cyclohexyladenosine: 250 min [72,73]). Third, the inability of adenosine antagonists to block ischemic preconditioning in rat hearts may reside in the use of selective A_1 antagonists, which do not abolish the A_3 mediated component of ischemic preconditioning. Moreover, rat and rabbit A_3 receptors weakly bind xanthine derivatives like 8-SPT compared with human and sheep A_3 receptors [74]. This makes it likely that in the rat, the use of non-selective xanthine-based adenosine antagonists like 8-SPT do not completely block the adenosine A_3-mediated part of protection by ischemic preconditioning. Of interest is also the observation that in human pediatric myocytes there is a U-shaped dose response relationship between cellular damage after simulated ischemia and the concentration adenosine in the preincubation media [57]: only 10 and 50 μM adenosine showed a significant protective effect against cellular damage while both higher and lower doses did not confer any protection. In conclusion, species differences in the adenosine-mediated component of ischemic preconditioning seem to be more of a quantitative than a qualitative nature.

3. Myocardial carbohydrate metabolism: final end-effector of ischemic preconditioning?

Alhough it is without doubt that preconditioning is receptor-mediated (see previous section), with a subcellular cascade of G-proteins, phospholipases and protein kinases, the end effector is still elusive [23]. Of the humoral factors released during preconditioning ischemia that may trigger the event (cf. Chapter 1, Figure 2; Chapter 3, Figure 1; see also [23]), adenosine is one of the most important. These triggers are all coupled to G-proteins. It has been proposed that preconditioning through these G-protein coupled receptors results in activation of PKC. Subsequent studies have shown that inhibition or stimulation of PKC could abolish or mimic the protective effect, respectively. Once activated, PKC may phosphorylate many target proteins. It is still unknown which end effector(s) is (are) involved in ischemic preconditioning. Besides K_{ATP} channels, PKC may phosphorylate an unknown key-regulating enzyme of glycolysis.

Table 1. Preconditioning with adenosine receptor agonists and myocardial protection in rat hearts

Reference	Model	ADO agonist	Administration (A) of drug & reperfusion (R) before Ischemia (min)	Ischemia (I) and reperfusion (R) (min)	Effect measure	Result
[75]	Isolated heart	ADO (100 μM)	30A, no reperfusion	30I - 45R	TIC	Mimics*
[76]	Isolated heart, regional I	CCPA (0.5 μg)	5A - 10R	30I - 120R	IS	Mimics*
[77,78]	Isolated heart	ADO (100 μM) CHA (0.25 μM)	10A, no reperfusion	30I - 45R	FR	Mimics* Mimics*
[79]	In situ, regional I	ADO (1.5 mg)	5A - 10R	90I - 320R	IS	Failed to mimic
[80]	Isolated heart	ADO (20 μM)	12A, no reperfusion	30I - 10R	FR;TIC	Mimics*
[81]	Isolated heart	ADO (10 μM) ADO (50 μM) PIA (10^{-7} M)	5A - 5R	40I - 30R	FR	Failed to mimic Exacerbates** Exacerbates**
[82]	Working heart	ADO (10 μM) ADO (50 μM) ADO (100 μM)	5A - 5R	20I - 35R	FR;CK	Failed to mimic Failed to mimic Failed to mimic
[83]	Isolated heart	ADO (20 μM)	??	30I - 20R	FR	Mimics
[84]	Adult myocytes	ADO (100 μM) CCPA (100 nM)	15A, no reperfusion	180I	CI	Mimics*# Mimics*#
[85]	Isolated heart	ADO (100 μM)	??	30I - 45R	FR	Mimics
[86]	Isolated heart, regional I	ADO (10 μM)	5A - 5R	10I - 10R	VT, VF	Failed to mimic*
[68]	Isolated heart	CHA (0.5 μM)	6A - 10R	30I - 30R	FR	Mimics
[87]	Isolated ventricle	ADO (125 μM)	5A - 10R	30H - 60R	FR	Mimics
[88]	Isolated heart	ADO (125 μM)	2A - 10R	20I - 40R	FR CK	Mimics* Failed to mimic*
[89]	Isolated heart, regional I	PIA (10^{-10} - $5*10^{-8}$ M)	10A, no reperfusion also during I	30I	VT VF VPB	Failed to mimic* Mimics* ($\geq 10^{-9}$ M) Mimics* ($\geq 5*10^{-9}$ M)
[90]	Working heart	CHA (0.5 μM)	? (prior to ischemia)	30I - 30R	FR	Mimics*

ADO=adenosine; CCPA=2-Chloro-N^6-cyclopentyladenosine; PIA=N^6-(2-phenylisopropyl)-adenosine; I=ischemia; H=hypoxia; R=reperfusion; IS=infarct-size; FR=functional recovery; TIC=time-to-onset-of-ischemic contracture; VT=ventricular tachycardia; VF=ventricular fibrillation; VPB=ventricular premature beat; CK, creatine kinase release upon reperfusion; CI=cellular injury; CHA=N^6-cyclohexyladenosine; *=compared to controls (no preconditioning group present); **=lower FR compared to controls; #=effect not as large as ischemic preconditioning.

Table 2. Adenosine receptor antagonists and ischemic preconditioning in rat hearts

Reference	Model	ADO antagonist	Administration (preconditioning protocol [ischemia (I)-reperfusion (R)])	Ischemia (I) and reperfusion (R) (min)	Effect measure	Result
[75]	Isolated heart	BW-A1433U (10 μM)	+ ADO (100 μM), 30' before I + PIA (1 μM), 30' before I	30I - 45R	TIC	Abolishes*
[76]	In situ, regional I	PD 115,199 (3 mg/kg) i.v.	5' before PC (3[5I-5R])	30I - 120R	IS	Failed to abolish
[77]	Isolated heart	DPCPX (5 μM)	+ 100 μM ADO 10' before I	30I - 45R	FR	Abolishes ADO protection
[79]	In situ, regional I	8-SPT (10 mg/kg) bolus	2 * 6' before PC (3[3I-5R]), 15' later	90I - 360R	IS	Failed to abolish
[91]	Isolated heart	BW-A1433U (10 μM) CPDPX (5 μM)	during PC (4[5I-5R])	30I - 20R	FR	Abolishes Failed to abolish
[81]	Isolated heart	BW-A1433U (10 μM)	before and during PC [5I-5R]	40I - 30R	FR	Failed to abolish
[92]	Isolated heart	BW-A1433U (10 μM) CPDPX (5 μM)	before PC [5I -10R] before hypoxic PC [5I-10R]	30I - 30R 30H - 30R	FR	Failed to abolish Failed to abolish
[82]	Working heart	8-SPT (10 μM)	throughout experiment [5I-5R]	20I - 35R	FR; CK	Failed to abolish
[83]	Isolated heart	BW-A1433U (10 μM) CPDPX (5 μM)	during PC (4[5I-5R])	30I - 20R	FR	Abolishes Failed to abolish
[84]	Adult myocytes	8-SPT (100 μM)	+ 100 μM ADO 15' before and during I	180I	CI	Abolishes
[93]	Isolated heart, regional I	8-SPT (100 μM) + α_1-antagonist	before, during PC (3[5I-5R], and first 5 min of regional ischemia	120I - 120R	IS	Failed to abolish
[85]	Isolated heart	DPCPX (5 μM)	+ 100 μM ADO before I	30I - 45R	FR	Abolishes
[94]	In situ, regional I	8-PT (10 mg/kg) i.v.	10' before PC (2I - 3R)	5I - 10R	VT, VF	Failed to abolish
[68]	Isolated heart	8-SPT (10 μM) 8-SPT (50 μM)	during PC (6I -10R)	30I - 30R	FR	Failed to abolish Abolishes
[87]	Isolated ventricle	8-SPT (50 μM)	3' before and during ADO-preconditioning (5'A - 10'R)	30H - 60R	FR	Abolishes ADO protection
[89]	Isolated heart,regional I	DPCPX (10^{-8} M)	+ PIA (10^{-8}M), 10' before I	30I	VT, VF, VPB	Abolishes

PC=preconditioned; I=ischemia; H=hypoxia; R=reperfusion; FR=functional recovery; VT=ventricular tachycardia; VF=ventricular fibrillation; VPB=ventricular premature beat; CK, creatine kinase release upon reperfusion; TIC=time-to-onset-of-ischemic contracture; 8-SPT=8-(*p*-sulfophenyl) theophylline; 8-PT=8-phenyltheophylline; PD115,199=*N*-(2-(dimethylamino)ethyl)-*N*-methyl-4-(2,3,6,7-tetrahydra-2,6-dioxo-1,3-dipropyl-1-purin-8-yl) benzosulfonamide; BW-A1433U=paraphenyl carboxyl substituted derivative of 1.3-dipropyl-8-phenylxanthine; DPCPX=8-cyclopentyl-1,3-dipropylxanthine.

3.1. The glycogen hypothesis: Is preconditioning mediated by preischemic glycogen depletion and reduced glycolytic catabolite accumulation during ischemia?

In this thesis, we show that irrespective of the type of ischemia studied (no-flow or low-flow), ischemic preconditioning reduces 1) preischemic glycogen and 2) glycogenolysis during subsequent prolonged ischemia (Chapter 6, Figure 5b; Chapter 7, Figure 4; Chapter 8, Figure 3a). Preischemic glycogen depletion is dependent on the preconditioning regimen used: 2 cycles of 5 min stop-flow ischemia and reperfusion reduced total glycogen by 60% (Chapter 6, Figure 5b) whereas 4 cycles of 2 min stop-flow ischemia and 3 min reperfusion reduced total glycogen by 40% (Chapter 8, Figure 3b). These observations are in line with other studies showing that ischemic preconditioning reduces glycogen breakdown during no-flow ischemia, therefore attenuating the accumulation of glycolytic end-products (e.g. hexose 6-phosphates, lactate) and the development of intracellular acidosis [2,95,96]. The glycogen hypothesis states that reduced pre-ischemic glycogen in preconditioned hearts is protective insofar as it reduces glycolytic catabolite accumulation during ischemia, despite reduced ATP production [97-99]. However, several lines of evidence are against this hypothesis. First, pH during ischemia is unrelated to preconditioning protection. Although many studies showed reduced acidosis in preconditioned hearts [97,99-102], we demonstrated in Chapter 8 (Figure 4d) using ^{31}P-NMR that there is no relation between pH during ischemia and protection by preconditioning, in line with other reports [18,96,103-105]. Second, glycogen loading prior to ischemia reduces ischemic injury [106,107]. Third, the pre-ischemic glycogen content is correlated with the time of day but not post-ischemic functional recovery [108]. Fourth, although Weiss et al. [95] suggested that depressed glycogenolysis is responsible for the protective effect of ischemic preconditioning, others observed no relationship between functional recovery after prolonged ischemia and pre-ischemic glycogen levels [104] or glycogen depletion during ischemia [109]. We demonstrated in Chapter 8 that reduced ischemic injury in hearts pharmacologically preconditioned with CCPA was associated with the highest preischemic glycogen levels (Figures 3a and b). Furthermore, we observed no relation between glycogen depletion during ischemia and functional recovery in the various groups studied. In conclusion, reduced glycogen utilization and consequent reduced glycolytic catabolite accumulation during ischemia merely represent epiphenomena of ischemic preconditioning.

3.2. Ischemic preconditioning is mediated by increased glycolysis-from-glucose during low-flow ischemia without increasing total glycolytic flux

In contrast to no-flow ischemia [2,95], total anaerobic glycolysis (read: lactate production) during low-flow ischemia is similar in preconditioned and control hearts (Chapter 6, Figure 3b; Chapter 7, Figure 3; Chapter 9, Figure 5). Cave et al. [6] also observed equal lactate release during hypoxia and reoxygenation between preconditioned and control hearts. Since preconditioning reduced glycogenolysis during ischemia (see previous section), we argued that ischemic preconditioning protects against underperfusion by inducing a shift from endogenous to exogenous glucose utilization, without increasing total glycolytic flux. We examined this hypothesis more closely in Chapter 7 by measuring glucose uptake during low-flow ischemia by the detrition rate of [2-^{3}H]glucose. As hypothesized, preconditioning increased glycolysis-from-glucose during underperfusion by 31% (Chapter 7, Figures 4 and 5). Similarly, Janier et al. [8] showed both increased anaerobic glycolysis and glucose uptake in isolated rabbit hearts

subjected to low-flow hypoxia. Another interesting observation of our research was that increased glucose uptake in preconditioned hearts mainly occurred during the first 10 min of ischemia; glucose uptake was ca. twice higher in preconditioned hearts during early ischemia (Chapter 7, Figure 5). Similarly, Runnman et al. [110] showed the importance of enhanced glucose utilization and reduced glycogenolysis in hypoxic rabbit ventricle without increasing total glycolytic flux. These authors also demonstrated that elevated glucose during hypoxia (read: increased glycolysis-from-glucose) was more beneficial during early than late hypoxia. To exclude the possibility that increased glucose uptake during low-flow ischemia is an epiphenomenon of ischemic preconditioning, we lowered the glucose concentration during underperfusion from 10 to 5 mM and omitted the insulin. This would result in reduced glycolysis-from-glucose since delivery of glucose (substrate supply) is the rate-limiting step of glycolysis in low-flow ischemia and not the extent of glycolytic enzyme inhibition [111,112], as we showed in Figure 5 of Chapter 7. Compared to normal glucose, the % functional recovery was proportionally decreased in preconditioned (from 62 to 30%) and control (from 32 to 0%) hearts supplied low glucose. From this we can conclude that 1) ischemic preconditioning is mediated by increased glycolysis-from-glucose during underperfusion, and 2) glycolytic flux controls the extent of damage during low-flow ischemia. Our conclusions fit the 'glucose hypothesis' proposed by Opie [113-116] and an extensive body of literature data [111,117-122] that enhanced glycolysis-from-glucose delays cell necrosis. Also, glycolysis is more effective than glycogenolysis in reducing ischemic injury [110,120]. It may appear clear that preconditioning-mediated increased glycolytic flux can only be beneficial in situations of low-flow ischemia.

The reasons for the protective effect of 'glucose for the heart' [122] may be related to the compartmentation of ATP in the cell. Glycolytically derived ATP supports ionic homeostasis during ischemia and reperfusion [123-126] preventing Ca^{2+} overload and ischemic injury.

3.3. Proglycogen

Low molecular weight proglycogen is a stable intermediate in the synthesis of classical macroglycogen [127]. Ischemia mainly reduces macroglycogen whereas proglycogen is reduced under more special situations like intermittent ischemia [128]. In Chapter 6, we tested the hypothesis that preconditioning increased anaerobic glycolysis during low-flow ischemia by mobilizing "inert" proglycogen. This hypothesis was rejected based on the observations that 1) anaerobic glycolysis was not increased during ischemia in preconditioned hearts (Chapter 6, Figure 3b), and 2) both subfractions of glycogen decreased in response to ischemia (Figure 5a) in contrast to previous observations in rabbits [128]. Thus, both subfractions behaved similarly as the total glycogen fraction (macroglycogen + proglycogen). We showed that the proglycogen fraction of total glycogen is larger than the macroglycogen fraction (Figure 5a) in line with a recent report [129]. These authors showed that at higher total glycogen concentrations, the relative contribution of macroglycogen increases but never equals the proglycogen fraction. The physiological role of proglycogen still has to be determined.

3.4. Are preconditioning-induced changes in carbohydrate metabolism mediated by adenosine?

The hypothesis that preconditioning effects on carbohydrate metabolism during underperfusion are mediated by the adenosine A_1 receptor is investigated in Chapter 7. Paradoxically, although increased

glucose uptake mediated ischemic preconditioning in this study (see section 3.2), reduced contractile dysfunctioning in CCPA pretreated hearts (Figures 1 and 2) was associated with a 47% reduction in lactate production (Figures 3 and 4) and a 61% reduction in glucose uptake (Figures 4 and 5). These observations were confirmed in Chapter 9 (Figures 2 and 5). From this one might conclude that ischemic preconditioning-induced effects on carbohydrate metabolism during underperfusion are not mediated by the adenosine A_1 receptor. However, the effect of adenosine on glycolysis during ischemia is related to the pre-ischemic metabolic status of the heart [130]. In that study, N^6-cyclohexyladenosine, a compound similar to CCPA, also reduced glycolysis, in line with our study whereas adenosine had the opposite effect [130]. Thus, adenosine released during preconditioning *ischemia* may differently affect glycolysis compared to pharmacological preconditioning of hearts by *normoxic* infusion of adenosine (analogs). The non-selective adenosine antagonist 8-SPT both blocked glycolysis-from-glucose and preconditioning protection in our study (Chapter 7; Figures 1, 2, 4, and 5), which supports the notion that ischemic preconditioning, via adenosine, is mediated by increased glycolysis-from-glucose. We cannot explain the paradoxical observation that CCPA induces protection despite depressed glucose uptake (Chapter 7, Figure 5) and anaerobic glycolysis (Chapter 7, Figure 3; Chapter 9, Figure 5). CCPA may induce cadioprotection by a different mechanism, and might be just another example of the great redundancy in routes by which ischemic preconditioning can take place (see also discussion section 1.2).

4. Clinical relevance

4.1. Is ischemic preconditioning an "healthy animal heart phenomenon"?

Most studies have investigated the cellular basis of ischemic preconditioning in young, healthy animal hearts [131]. Since ischemic heart disease is often associated with hypertension, hypercholesterolemia, heart failure, increased age, and diabetes, one might ask whether studies in young healthy animal hearts can be extrapolated to the diseased (human) heart. Ischemic preconditioning may not occur in aged hearts [132,133] due to a decrease in noradrenalin release during transient ischemia [132] and/or a decrease in adenosine receptor function [134]. Hypercholesterolemia but not atherosclerosis blocks preconditioning protection [131]. Preconditioning of hearts from animals with diabetes, a risk factor for ischemic heart disease, is controversial: both positive [11,135,136] and negative [137,138] results have been published. This discrepancy in animal studies may be due to differences in 1) the type of ischemia used (no-flow vs. low-flow), 2) the duration and severity of the diabetic state, and 3) the substrates used [139]. Thus, hearts from animals with more severe and prolonged diabetes, supplied glucose and fatty acids, and/or subjected to low-flow ischemia are more prone to ischemic injury [139]. In contrast, mildly diabetic hearts supplied only glucose and subjected to no-flow ischemia are less sensitive to ischemic injury [139,140]. This may be related to the high rates of fatty acid oxidation and inhibition of glycolytic pathways in diabetic animals, which may be beneficial during no-flow ischemia but are detrimental during low-flow ischemia (see also section 3.3). Clinically, the diabetic heart is more sensitive to ischemic injury since diabetes mellitus is associated with increased cardiovascular mortality. Isolated human right atrial trabeculae from diabetes mellitus patients undergoing coronary artery bypass graft surgery (CABG) can be ischemically preconditioned [141]. In Chapter 3, we discussed the evidence for the existence of ischemic preconditioning in humans. Evidence

that the human heart can be preconditioned has been obtained 1) in human trabeculae and cultured myocytes, 2) during coronary angioplasty, 3) in patients with preinfarction angina, 4) in patients with 'warm-up' or 'walk-through' angina, and 5) during cardiac surgery (see also [27,142,143]). Preconditioning in the form of two cycles of brief aortic cross-clamping prior to CABG [144,145] or aortic and mitral valve replacement surgery [146] provides the most direct evidence of preconditioning the human myocardium. Thus, it seems reasonable to assume that the human heart can be preconditioned. Moreover, the mechanism of preconditioning protection in human hearts is similar to that in animal hearts. Thus, preconditioning of human trabeculae [56,58,141,147-149] and human myocytes [57,150,151] involves adenosine A_1/A_3 receptor activation, α-1-adrenoreceptor activation, protein kinase C, and opening of K_{ATP} channels. However, whether preconditioning is of benefit to all patients is questionable and the application of preconditioning in humans needs further research. For instance, diabetic patients taking oral sulfonylurea hypoglycemic drugs are at increased risk of cardiovascular mortality and are not susceptible to ischemic preconditioning in contrast to diabetic patients not taking this type of agents [141]. This may be related to the non-specific action of sulfonylurea drugs, which apart from inhibition of pancreatic K_{ATP} channels also blunt the K_{ATP} channel-dependent component of ischemic preconditioning in the heart [152,153].

4.2. Adenosine

Administration of adenosine to patients prior to CABG [55] or percutaneous transluminal coronary angioplasty (PTCA) [59] reduces myocardial injury. Although not strictly pharmacological *preconditioning*, adenosine given following aortic cross-clamping as well as just prior to removal of the cross-clamp [154] or adenosine added as adjunct to blood cardioplegia [155] resulted in improved post-operative cardiac function and less use of inotropic support, respectively, in humans undergoing CABG. Furthermore, adenosine is safe to administer during CABG [155,156]. Thus, pharmacological preconditioning with adenosine may provide a safe and effective strategy to attenuate ischemic injury in patients undergoing heart surgery. Preconditioning with adenosine seems more advantageous than ischemic preconditioning since 1) brief ischemia can induce arrhythmias, and 2) ischemic preconditioning does not reduce stunning in contrast to adenosine [155]. The efficacy of selective adenosine A_1/A_3 agonists as pharmacotherapy in humans needs to be tested. The use of selective adenosine A_3 analogs may be useful in the clinical setting due to the absence of hemodynamic side-effects.

Preconditioning requires pretreatment to be effective and extends the development of infarction with ca. 1-2 hours. Therefore, it can be easily applied in the setting of planned ischemia such as in cardiac surgery. However, acute myocardial infarction due to rupture of atherosclerotic plaques is a sudden and unpredictable event. Whether patients at risk for acute myocardial infarction can be kept in a continued protected state using prophylactic treatment with pharmacological preconditioning mimetics like adenosine or K_{ATP} channel openers (e.g., nicorandil), needs further investigation and provides a major challenge. In animals, tolerance develops and protection is lost in response to multiple ischemic episodes [157] or continued administration of adenosine A_1 agonists [158,159]. Thus, tolerance to chronic adenosine administration, probably due to downregulation/desensitization of the receptor, forms a major problem for the pharmacotherapeutic exploitation of adenosine in patients at risk for myocardial infarction.

In animal models, adenosine given at reperfusion after myocardial infarction reduces infarct size [160] probably via adenosine A_2-mediated effects on the coronary vasculature (prevention of no-reflow phenomenon, anti-inflammatory and anti-thrombotic actions). Whether adenosine in conjunction with reperfusion strategies can prevent 'reperfusion injury' is currently under investigation in two Phase II clinical trials (AMISTAD and ALIVE trials) [160].

4.3. Carbohydrate metabolism

In this thesis, we showed that increased glycolysis-from-glucose during low-flow ischemia mediates ischemic preconditioning. This fits the notion that glucose is beneficial for the ischemic heart [113-116,122,161-165]. However, ischemic preconditioning forms a rather difficult strategy to increase glucose use and to decrease fatty acid oxidation in the ischemic heart. Because myocardial ischemia in humans is of the low-flow type [3,4], where glucose supply is the rate-limiting step in glycolysis-from-glucose [112], it may be simpler to treat the ischemic heart with glucose. Thus, the concept of 'metabolic therapy' or 'metabolic management of ischemic heart disease'[162,165-167] is based on shifting substrate utilization away from detrimental free fatty acid metabolism and toward beneficial glucose metabolism by provision of glucose-insulin-potassium (GIK) or pharmacological intervention. A meta-analysis [168,169] on the effect of GIK therapy in patients with acute myocardial infarction showed a significant mortality reduction (28% to 48%, depending on the subgroup). The included trials were all conducted in the prethrombolytic era. One might argue that there is no need for such a metabolic therapy since the degree of mortality reduction with GIK is comparable to that currently achieved with thrombolytic therapy (GUSTO11b investigators, [170]). However, a recent clinical trial revealed a relative mortality reduction of 66% in patients treated with GIK and reperfusion strategies as compared to patients who received reperfusion strategies alone [171,172]. Similarly, The Diabetes Mellitus Insulin-Glucose Infusion in Acute Myocardial Infarction (DIGAMI) trial [173] showed a 29% to 58% (depending on the subgroup) reduction in mortality in patients treated with insulin and glucose. Thus, GIK initiated as soon as possible after suspected acute myocardial infarction (e.g., in the ambulance) may extend the time window in which successful salvage is possible with thrombolytic therapy or primary angioplasty.

5. Conclusions

With reference to the aims of this thesis, the following conclusions can be drawn:

- Ischemic preconditioning protects against the deleterious effects of no-flow and low-flow ischemia.
- Both adenosine A_1 and A_3 receptors are involved in ischemic preconditioning of rat hearts.
- Preconditioning protection can be dissociated from reduced ischemic glycogenolysis and attenuated glycolytic catabolite accumulation.
- Increased glycolysis-from-glucose during early low-flow ischemia mediates ischemic preconditioning.
- Whether increased glucose uptake in preconditioned hearts is mediated by the adenosine A_1

receptor is unclear. Cardioprotection by pre-treatment with a selective adenosine A_1 agonist may involve a different mechanism compared to ischemic preconditioning.

6. Recommendations for future studies

- A randomized clinical trial on the efficacy of adenosine (agonists) administrated prior to and during coronary artery bypass surgery or percutaneous coronary angioplasty should be initiated.
- Further studies should examine the role of the adenosine A_3 receptor in cardioprotection and the underlying signal transduction pathways.
- Strategies to circumvent tachyphylaxis after chronic treatment with adenosine (analogs) should be explored for the benefit of patients at risk for myocardial infarction.
- The controversial effects of adenosine (analogs) on glycolysis during ischemia need further investigation, especially in relation to the preischemic metabolic status of the heart.
- The possible compartmentation of glycolytic ATP and glycogenolytic ATP should be further explored. Is it possible that glycolytic ATP is beneficial and glycogenolytic ATP is detrimental to the ischemic myocardium?

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Summary

Prolonged and sustained myocardial ischemia due to (partial) coronary artery occlusion induces cell death and tissue infarction if the heart is not reperfused within time. In 1986, the paradoxical observation was done that brief periods of (nonlethal) ischemia and reperfusion render the heart more tolerant to a subsequent sustained period of ischemia, a phenomenon termed 'ischemic preconditioning'. The delay of infarct size development produced by ischemic preconditioning is the most powerful endogenous form of cardioprotection described thus far.

This thesis investigates the mechanism of ischemic preconditioning with respect to adenosine as a trigger and myocardial carbohydrate metabolism as the end-effector of preconditioning protection. We hypothesized that adenosine, released during brief preconditioning ischemia, induces cardioprotection by changing myocardial carbohydrate metabolism during sustained ischemia. In order to answer these questions, we used the isolated Langendorff perfused rat heart as experimental model. This model allowed us to carefully monitor biochemical parameters relevant to our research questions with classical biochemistry and NMR spectroscopy. In addition, we also studied humans undergoing pacing stress testing.

In *Chapter 1*, an overview of the current knowledge of the mechanism of ischemic preconditioning is given. The mechanism of ischemic preconditioning includes triggers, mediators, and end-effectors. Binding of substances, released during brief ischemia, like adenosine, bradykinin, and opioids to their G-protein-coupled receptors constitute the trigger for entrance to the preconditioned state. Stimulation of these receptors leads to activation of protein kinase C, tyrosine kinases, and mitogen activated protein kinases, which mediate the preconditioning response. Possible end-effectors activated by these kinases include the ATP-dependent potassium channel and enzymes of myocardial carbohydrate metabolism. In *Chapter 2*, the myocardial energy metabolism is reviewed with special emphasis on myocardial nucleotide catabolism giving rise to the formation of adenosine, and carbohydrate metabolism. Moreover, the physiological role of the various adenosine receptor subtypes is discussed in this chapter. *Chapter 3* reviews some of the controversies in the field of ischemic preconditioning. Discrepancies between no-flow and low-flow ischemia with respect to carbohydrate metabolism and species differences are discussed. Moreover, evidence for the existence of preconditioning in human hearts is presented.

The relation between cardioprotection by preconditioning and myocardial lactate production during no-flow ischemia is addressed in *Chapter 4*. We tested the hypothesis that reduced lactate production during no-flow ischemia is causally related to ischemic preconditioning. The results show that preconditioning protects against no-flow ischemia as manifested by reduced leakage of the 'marker' enzyme creatine kinase, reduced adenine nucleotide catabolism, and attenuation of the postischemic increase in end-diastolic pressure; in contrast, preconditioning did not reduce arrhythmias. Furthermore, there was no relation between lactate production during no-flow ischemia and protection by preconditioning.

In *Chapter 5*, we tested whether acadesine, a drug enhancing endogenous adenosine production, reduced ischemic injury in patients with coronary artery disease and stable angina undergoing pacing-

induced ischemia. Acadesine given as an 'preconditioning agent' prior to and during pacing stress testing resulted in minor anti-ischemic effects in terms of attenuation of the decline in ejection fraction and reduced production of lactate during pacing stress. The limited success of this drug may be related to the fact that the severity of ischemia in these type of patients may be too low to increase adenosine because acadesine raises adenosine only in ischemic tissues. Furthermore, infusion of acadesine increased systemic lactate, which may be due to an acadesine-induced stimulation of glucose uptake and catabolism.

There is ample evidence that ischemic preconditioning protects against no-flow ischemia. However, whether preconditioning confers protection against low-flow ischemia is subject of controversy. Because adenosine A_1 receptors must be occupied both during preconditioning ischemia (initiation) and prolonged ischemia (mediation) in order to induce protection, residual flow during prolonged ischemia may lead to washout of adenosine from the interstitium and, consequently, loss of protection. Whether ischemic preconditioning protects against low-flow ischemia (0.6% of baseline flow), a model more relevant to clinical practice, was investigated in *Chapter 6.* Moreover, we speculated that preconditioning mobilizes proglycogen, a stable intermediate in macroglycogen synthesis, supporting glycogenolytic flux and reducing ischemic injury. Results show that ischemic preconditioning in the form of two cycles of 5 min ischemia and 5 min reperfusion reduces ischemic injury during subsequent prolonged low-flow ischemia evidenced by improved functional recovery, less postischemic contracture development, and reduced release of creatine kinase and purines. Venous and interstitial adenosine levels during underperfusion were probably high enough to occupy the receptor mediating myocardial protection in preconditioned hearts. Preconditioning did not differently affect the proglycogen fraction compared to control hearts. Moreover, in line with what was observed in Chapter 5, lactate release was unrelated to ischemic preconditioning. Compared to control hearts, preconditioning reduced glycogenolysis during ischema while lactate release was similar, suggesting that preconditioning induces protection against low-flow ischemia via a shift from endogenous to exogenous glucose utilization, without increasing anaerobic glycolysis.

Based on the results presented in Chapter 6, we hypothesized in *Chapter 7* that ischemic preconditioning is mediated by increased glucose use during low-flow ischemia, an effect triggered by adenosine A_1 receptor activation. We quantitated glycolysis-from-glucose during low-flow ischemia using the stable radioisotope D-[2-^{3}H]glucose. Compared to controls hearts, ischemic preconditioning improved functional recovery by 94% and increased glucose uptake almost twofold during early low-flow ischemia whereas lactate release was unaffected. Blockade of glucose uptake during underperfusion abolished preconditioning protection completely. A non-selective antagonist at adenosine receptors blocked both the increase in glucose uptake and the improved functional recovery, observed in preconditioned hearts. Pharmacological preconditioning with an adenosine A_1 agonist improved functional recovery by 110% but reduced lactate release and glucose uptake during ischemia by 47% and 61%, respectively. Thus, increased glycolysis-from-glucose during low-flow ischemia mediates ischemic preconditioning without increasing anaerobic glycolytic flux. Although adenosine A_1 receptor activation reduces ischemic injury, it does not facilitates the increased glucose uptake observed with ischemic preconditioning, suggesting a different mechanism of protection.

In *Chapter 8*, we tested the hypothesis that ischemic preconditioning is mediated by reduced glycogen depletion and less accumulation of glycolytic end-products during no-flow ischemia (the glycogen hypothesis). Furthermore, we hypothesized that reduced glycogen utilization in preconditioned hearts is mediated by adenosine A_1 receptor activation during brief ischemia. We used ^{13}C-NMR to follow myocardial glycogen within one heart throughout the experiment. The results show that in rat hearts, ischemic preconditioning is mediated by adenosine A_1 receptor activation because preconditioning protection could be mimicked with a selective adenosine A_1 agonist and abolished with a non-selective adenosine antagonist. However, glycogen depletion prior to ischemia and reduced glycogenolysis during ischemia are not causually related to preconditioning protection.

Ischemia/reperfusion injury includes both apoptotic and necrotic myocyte cell death. Apart from infarct size reduction and improved functional recovery, ischemic preconditioning has been recently shown also to reduce apoptosis. *Chapter 9* addressed the question whether activation of adenosine A_1 and A_3 receptors prior to ischemia improved functional recovery and reduced apoptosis resulting from low-flow ischemia. This study shows that adenosine triggers protection of function in preconditioned hearts via both the adenosine A_1 and A_3 receptor. Infusion of an adenosine A_3 agonist prior to ischemia did not affect hemodynamics in contrast to the adenosine A_1 agonist. Thus, adenosine A_3 agonists may represent a new, potentially useful therapeutic class of agents providing cardioprotection. Pharmacological stimulation of adenosine A_1 and A_3 receptors, but not ischemic preconditioning, reduced apoptosis.

Conclusions

With reference to the aims of this thesis, the following conclusions can be drawn:

- Ischemic preconditioning protects against the deleterious effects of no-flow and low-flow ischemia.
- Both adenosine A_1 and A_3 receptors are involved in ischemic preconditioning of rat hearts.
- Preconditioning protection can be dissociated from reduced ischemic glycogenolysis and attenuated glycolytic catabolite accumulation.
- Increased glycolysis-from-glucose during early low-flow ischemia mediates ischemic preconditioning.
- Whether increased glucose uptake in preconditioned hearts is mediated by the adenosine A_1 receptor is unclear. Cardioprotection by pre-treatment with a selective adenosine A_1 agonist may involve a different mechanism compared to ischemic preconditioning.

Samenvatting

Onvoldoende doorbloeding van de hartspier (ischemie) leidt tot celschade en na verloop van tijd zelfs tot celdood. Ischemie, dat optreedt tijdens 'Dotter' procedures, 'bypass' operaties, angina pectoris en bij atherosclerotische laesies (hartinfarct), vormt daarom een bedreiging voor het herstel van de patiënt. Echter, kortdurende periodes van ischemie-reperfusie beschermen paradoxaal genoeg het hart tegen een daaropvolgende langere periode van zuurstofgebrek. Dit fenomeen, ischemische preconditionering genaamd, is één van de krachtigste methoden om het hart tegen ischemie te beschermen. Het in dit proefschrift beschreven onderzoek richt zich op de rol die adenosine, een stof die vrijkomt tijdens ischemie, en de koolhydraatstofwisseling spelen in het mechanisme van preconditionering. De hypothese werd getoetst of adenosine, via de adenosine A_1 en A_3 receptoren, het cardiale koolhydraatmetabolisme tijdens langdurige ischemie beïnvloedt en daarmee de cardioprotectieve werking van ischemische preconditionering teweeg brengt. Om deze hypothese te toetsen hebben we gebruik gemaakt van het geïsoleerde, doorstroomde rattenhart volgens Langendorff. Dit model maakt het mogelijk om biochemische en fysiologische parameters, die relevant zijn voor dit onderzoek, te bestuderen door gebruik te maken van klassieke biochemische en kernspinresonantie (NMR) technieken. Ook werd een onderzoek verricht in hartpatiënten, met een mogelijke vernauwing van één van de kransslagaderen, waarin ischemie werd opgewekt door het hart elektrisch te stimuleren ('pacen').

In *Hoofstuk 1* wordt de huidige kennis over het mechanisme van preconditionering samengevat. Het mechanisme van ischemische preconditionering omvat 'triggers', 'mediators' en de uiteindelijke 'end-effectors'. Tijdens de korte periodes van ischemie en reperfusie komen stoffen uit het hart vrij, zoals adenosine (een afbraakproduct van ATP), bradykinine en opioïden. Deze stoffen binden aan hun eigen specifieke G-eiwit gekoppelde receptoren in de hartmembraan. Stimulatie van deze receptoren leidt tot activering van bepaalde signaaltransductie-eiwitten (kinases) in de hartcellen, waaronder eiwit kinase C en tyrosine kinases. Het is nog niet geheel duidelijk welke 'end-effectors' door deze kinases worden geactiveerd om het beschermende effect van preconditionering te bewerkstelligen. Mogelijke kandidaten betreffen het ATP-afhankelijke kaliumkanaal (K_{ATP} kanaal) en/of bepaalde enzymen van het koolhydraatmetabolisme.

Hoofdstuk 2 behandelt het myocardiale energiemetabolisme. De nadruk in dit hoofdstuk ligt op de cardiale koolhydraatstofwisseling en het katabolisme van adenine nucleotiden tijdens ischemie, dat aanleiding geeft tot de vorming van de regulerende stof adenosine. Verder worden in dit hoofdstuk de verschillende typen cardiale adenosine receptoren en hun fysiologische functie nader besproken.

In *Hoofdstuk 3* worden controverses in de literatuur naar het onderzoek van ischemische preconditionering samengevat. Er bestaan discrepanties tussen experimentele modellen die (een deel van) het hart volledig ('stop-flow') of gedeeltelijk ('low-flow') ischemisch maken in het effect dat preconditionering heeft op het koolhydraatmetabolisme tijdens ischemie. Ook de verschillen tussen diersoorten en het bewijs voor het optreden van preconditionering in mensenharten worden in dit hoofdstuk besproken.

De mogelijk causale relatie tussen een verminderde lactaatproductie tijdens 'stop-flow' ischemie enerzijds en bescherming van het hart via ischemische preconditionering anderzijds, werd onderzocht in *Hoofdstuk 4*. De resultaten tonen aan dat ischemische preconditionering een effectieve manier is om

het hart tegen stop-flow ischemie te beschermen wat blijkt uit een verminderde afgifte van het 'marker' enzym creatine kinase tijdens reperfusie, een verminderde depletie van adenine nucleotiden tijdens ischemie en een geringere stijging van de diastolische druk tijdens reperfusie. Echter, de incidentie van aritmieën was niet gereduceerd in gepreconditioneerde harten. Er bestond geen relatie tussen lactaat productie tijdens ischemie en cardioprotectie via preconditionering.

In *Hoofdstuk 5* werd getest of acadesine, een drug die de adenosine concentratie in hartweefsel zou doen stijgen, een anti-ischemische werking heeft in hartpatiënten, waarin ischemie wordt opgewekt door het hart te 'pacen'. Toediening van acadesine (preconditionering) leidde tot een geringe bescherming tegen ischemie in termen van een verminderde daling van de hartfunctie en minder lactaatproductie door het hart. Deze teleurstellende resultaten zijn mogelijk te wijten aan de geringe mate van ischemie die tijdens 'pacen' wordt opgeroepen in deze groep van patiënten. Acadesine zorgt namelijk alleen voor een stijging van de adenosinespiegels in ischemisch weefsel. Een opmerkelijke bevinding was dat acadesine de systemische lactaatspiegels deed stijgen. Dit is mogelijk te wijten aan een stimulatie van de glycolyse in skeletspieren door de drug.

Er is overtuigend bewijs dat ischemische preconditionering beschermt tegen 'stop-flow' ischemie in proefdieren. Of preconditionering ook beschermt tegen 'low-flow' ischemie is controversieel. Om het hart te beschermen moeten adenosine A_1 receptoren, die gelokaliseerd zijn op de hartmembraan, zowel tijdens de korte periodes van ischemie als tijdens de lange periode van ischemie geactiveerd zijn. Het is daarom goed mogelijk dat tijdens 'low-flow' ischemie, adenosine wordt uitgewassen uit de interstitiële ruimte en zo continue activering van de receptor onmogelijk maakt en bescherming verloren gaat. In *Hoofdstuk 6* toetsten we of ischemische preconditionering inderdaad beschermt tegen 'low-flow' ischemie (0.6% residuale doorbloeding). Dit is van belang omdat ischemie die optreedt bij mensen vaak van het type 'low-flow' is door partiële vernauwingen in kransslagaders of door het grote aantal collateralen. De hypothese werd getoetst of ischemische preconditionering wordt veroorzaakt door stimulatie van de anaërobe glycolyse via mobilisatie van het normaal inerte proglycogeen, een stabiele intermediair in de synthese van macroglycogeen. Ischemische preconditionering resulteerde in een goede bescherming tegen 'low-flow' ischemie. Gepreconditioneerde harten vertoonden een beter herstel van de hartfunctie en gaven minder creatine kinase en purines af. De adenosineconcentratie in de veneuze circulatie tijdens 'low-flow' ischemie was hoog genoeg om de adenosine A_1 receptoren te activeren en preconditionering te bewerkstelligen. Lactaatproductie tijdens ischemie was niet verschillend tussen controle en gepreconditioneerde harten. Aangezien ischemische preconditionering leidde tot een remming van de glycogenolyse tijdens ischemie, suggereerde dit dat de glucose opname in gepreconditioneerde harten was toegenomen. Ischemische preconditionering leidde echter niet tot een andere mobilisatie van de proglycogeen fractie in vergelijking met controle harten.

Gebaseerd op de resultaten gepresenteerd in Hoofdstuk 6 wierpen we in *Hoofdstuk 7* de hypothese op dat ischemische preconditionering, via activering van de adenosine A_1 receptor, wordt veroorzaakt door de glucose opname tijdens 'low-flow' ischemie te bevorderen. We kwantificeerden de cardiale glucose opname tijdens ischemie door gebruik te maken van radioactief gelabeld glucose (D-[2-^{3}H]glucose). Ischemische preconditionering leidde tot een 94% beter herstel van de hartfunctie na ischemie vergeleken met controle harten. De glycolyse was bijna twee maal hoger in

gepreconditioneerde harten tijdens de eerste 10 min van 'low-flow' ischemie, terwijl lactaatproductie niet verschillend was tussen gepreconditioneerde en controle harten. Remming van de glucose opname tijdens ischemie in gepreconditioneerde harten deed de beschermende werking teniet. Wanneer een niet-selectieve remmer van de adenosine receptoren werd gegeven tijdens preconditonering, werden zowel de toegenomen glucose opname als de cardioprotectieve eigenschappen van preconditionering geblokkeerd. Farmacologische preconditionering met een selectieve adenosine A_1 agonist, resulteerde in een 110% beter functieherstel na ischemie, 47% minder lactaatproductie en een 61% lagere glucose opname tijdens ischemie ten opzichte van de controle groep. In conclusie, ischemische preconditioning beschermt het hart door een toename van de glucose extractie tijdens ischemie zonder de totale anaërobe glycolyse te stimuleren. Paradoxaal genoeg beschermt stimulatie van de adenosine A_1 receptoren het hart tegen ischemie terwijl de glucose extractie en de totale anaërobe glycolyse sterk geremd is, wat kan duiden op een ander mechanisme van bescherming.

De centrale vraag in *Hoofdstuk 8* was of ischemische preconditionering wordt veroorzaakt door minder glycogeen afbraak tijdens 'no-flow' ischemie die leidt tot een geringere ophoping van glycolytische metabolieten (de zogenaamde glycogeenhypothese van preconditionering). Daarbij wilden we bezien of een gereduceerde glycogeen afbraak in gepreconditioneerde harten werd veroorzaakt door stimulatie van de adenosine A_1 receptor. Met behulp van ^{13}C-NMR konden we veranderingen in de glycogeenspiegels van elk hart gedurende het hele experiment volgen. Uit de resultaten blijkt dat stimulatie van de adenosine A_1 receptor in de hartmembraan, het rattenhart beschermt tegen langdurige ischemie: toediening van een selectieve adenosine A_1 agonist voor ischemie bootste de beschermende werking van preconditionering na, terwijl een niet-selectieve adenosine antagonist de cardioprotectieve werking van preceonditionering blokkeerde. Echter, depletie van glycogeen door preconditionering en een remming van de glycogenolyse tijdens ischemie zijn epifenomenen van cardioprotectie door preconditionering.

Ischemie en reperfusie leidt tot celdood door zowel acute celdood (oncose) en geprogrammeerde celdood (apoptose). Ischemische preconditionering is een effectieve strategie om de mate van acute celdood en contractiele disfunctie, die het gevolg zijn van ischemie, te reduceren. Zeer recentelijk heeft men aangetoond dat preconditionering ook de mate van apoptose, die optreedt tijdens ischemie-reperfusie, kan reduceren. In *Hoofdstuk 9* vroegen we ons af of farmacologische stimulatie van de cardiale adenosine A_1 receptor en de recent ontdekte A_3 receptor voorafgaand aan 'low-flow' ischemie, leidt tot beter functieherstel en minder apoptose. Het bleek dat een adenosine A_1 en een adenosine A_3 agonist in gelijke mate bescherming boden tegen de contractiele disfunctie die optreedt tijdens ischemie-reperfusie. Stimulatie van de adenosine A_3 receptor leidde echter niet tot hemodynamische bijwerkingen in tegenstelling tot adenosine A_1 stimulatie. Adenosine A_3 agonisten vormen daarom een interessante, nieuwe klasse van cardioprotectieve drugs. Farmacalogische maar niet ischemische preconditionering resulteerde in minder apoptose na 2.5 uur reperfusie in vergelijking met controle harten.

Op basis van de doelstellingen van dit onderzoek kunnen de volgende conclusies getrokken worden:

- Ischemische preconditionering beschermt tegen zowel 'no-flow' als 'low-flow' ischemie.

- Zowel de adenosine A_1 als adenosine A_3 receptoren in de hartmembraan zijn betrokken bij het mechanisme van ischemische preconditionering.
- Ischemische preconditionering wordt niet veroorzaakt door een reductie in de glycogeenspiegels voor ischemie en een gereduceerde glycogenolyse tijdens ischemie.
- Ischemische preconditionering wordt veroorzaakt door een toegenomen glucose opname tijdens vroege ischemie.
- Of een toegenomen glucose opname in gepreconditioneerde harten wordt veroorzaakt door stimulatie van de adenosine A_1 receptor is onduidelijk. Bescherming van het hart tegen ischemie door toediening van een adenosine A_1 agonist (farmacologische preconditionering) loopt mogelijk via een ander mechanisme als ischemische preconditionering.

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Curriculum vitae

Robert de Jonge werd geboren op 10 november 1968 te Bussum. In 1988 behaalde hij het VWO diploma aan het Willem de Zwijger College in Bussum. In datzelfde jaar begon hij met de studie Bewegingswetenschappen aan de Vrije Universiteit in Amsterdam waar hij zich specialiseerde in de richtingen Inspanningsfysiologie, Biochemie en Sportpsychologie. Tijdens zijn studie deed hij een biochemische stage (isolatie van mitochondriaal ATPase) bij het E.C. Slater Instituut (Faculteit Scheikunde) van de Universiteit van Amsterdam, onder begeleiding van dr. J.A. Berden. In de zomer van 1993 was hij betrokken bij een veldonderzoek in Santa Cruz (Bolivia) naar het effect van ondervoeding en hypoxie op de lichamelijke gezondheid van Boliviaanse kinderen. Aansluitend op dit onderzoek studeerde hij een jaar aan de Faculteit der Geneeskunde in Clermont-Ferrand (Frankrijk) op de afdeling Fysiologie (prof. dr. J. Coudert). Gedurende dit jaar werkte hij de resultaten, die in Bolivia verzameld werden, uit en volgde hij ook een post-academische opleiding in de Fysiologie (prof. dr. J.R. Lacour) met als specialisatie hypoxie (prof. dr. J. Coudert). Het examen van de post-academische opleiding in Frankrijk en het doctoraalexamen Bewegingswetenschappen werden respectievelijk in september en oktober 1994 behaald. In april 1994 begon hij als assistent in opleiding (AIO) bij het Cardiochemisch Laboratorium van de afdeling Experimentele Cardiologie (hoofd prof. dr. P.D. Verdouw) van het Thoraxcentrum in Rotterdam. Gedurende vier jaar heeft hij zich bezig gehouden met onderzoek naar de rol die adenosine en het koolhydraatmetabolisme spelen in het mechanisme van ischemische preconditionering. Meerder keren bezocht hij de Universiteit van Milaan waar hij samenwerkte met de afdelingen Organische Chemie (dr. S. Bradamante) en Farmacologie (prof. dr. F. Piccinini). Het in dit proefschrift beschreven onderzoek stond onder leiding van dr. J.W. de Jong (hoofd Cardiochemisch Laboratorium) en werd gesubsidieerd door de Nederlandse Hartstichting.

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