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Understanding myocardial ischemic preconditioning, and the implications for a role of adenosine catabolism[1]

Review based on the doctoral thesis

"Myocardial energy metabolism in ischemic preconditioning, role of adenosine catabolism"

Mohammad Kavianipour

Department of public health and clinical medicine, Umea university, Umea, Sweden

INTRODUCTION

Ischemic heart disease constitutes a collection of clinical conditions which affect the functional state of the heart by means of blood supply and can in its severest form culminate in acute myocardial ischemia/infarction. Acute myocardial infarction is a major cause of death in most Western countries. Early reperfusion is the primary goal in any strategy aiming at maintaining viability and protecting the heart against acute myocardial ischemia. Despite considerable progress in reperfusion therapy (*i.e.* angioplasty/thrombolysis), early reperfusion is not always possible or practicable, *i.e.* in evolving myocardial infarction with attempted thrombolysis or in cardiac transplantation with extended storage times. This problem has given rise to the concept of cardioprotection in which a variety of interventions are used to slow the rate of progression of ischemic injury so that less irreversible injury occurs and more tissue is available when reperfusion is finally accomplished. In this respect, a pharmacological approach has been shown successful in the laboratory but with a few exceptions (β-blockade- and Glucose insulin treatment) such interventions have failed in the clinical setting. Hence, there is considerable interest in exploiting the ability of the heart to use endogenous adaptive mechanisms such as the ischemic preconditioning phenomenon. Today, much is known about the signalling pathways involved in IP however, the details of the final steps leading to cardioprotection, remain elusive. Elucidating these unknown mechanisms may aid in developing new treatment strategies for controlling ischemic injury.

Historic background

In the mid 80s an American research group was interested to learn more about the pathophysiology of ischemic myocardial injury. Reimer *et al.* (1) had observed that prolonged ischemia (40 min) resulted in marked decrease of ATP levels and subse-

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quent myocardial necrosis. However they wanted to find out whether brief episodes of ischemia, each reversible by itself, could cause cumulative metabolic derangements leading to myocardial necrosis. It was also desirable to know whether it was the accumulation of toxic catabolites or the declining ATP levels that caused myocardial injury. In parallel with a previous report demonstrating preservation of nucleotide levels following repetitive episodes of ischemia (2), using a canine model of myocardial infarction they designed a study consisting of four 10-min periods of ischemia (compromising altogether 40 min of ischemia) interspaced with short periods of reperfusion – allowing wash out of catabolites. They found that the ATP levels decreased following the first ischemic bout but were preserved after the following ischemic periods. This was in contrast with the progressive loss of ATP observed in the control group which were treated with a single 40 min long ischemia (1). The investigators suspected the existence of an adaptation mechanism induced by ischemia. Their suspicions were materialized in the follow-up experiment as the developed infarct size following a 40 min ischemia was distinctly smaller when the 40 min insult was preceded by four repetitive short cycles of ischemia and reperfusion (3). The observed cardioprotection was independent from confounding factors such as collateral blood flow and hemodynamic variables. Murry *et al.* referred to this protective phenomenon as "ischemic preconditioning"[1] (IP) (3). Since then IP has been demonstrated to induce cardioprotection (against subsequent ischemia) in virtually all tested models.

IP a phenomenon merely in laboratory animals?

Over the years our knowledge concerning IP has grown considerably.[2] IP has proven to be one of the most potent means of protecting the myocardium against ischemic injury in experimental models. Ethical considerations have confined the possibilities of studying IP in humans. Nonetheless, evidence for existence of the IP phenomenon in man has been gathered from a variety of clinical and experimental observations.

The warm-up angina[3] is ascribed as a phenomenon occurring in patients with ischemic heart disease undergoing an exercise-test, who experience signs of ischemic chest pain during initial exercise but can perform the same task without noticeable discomfort after a few minutes of rest. Traditionally the recruitment of collateral blood flow was considered as the main contributing factor to the warm-up angina phenomenon (4). However, recently it was proposed that the warm-up angina also occurred in absence of collateral recruitment (5), implicating IP as a possible explanatory mechanism (6, 7). Moreover, balloon inflations during angioplasty have been exploited as a model of IP in humans. Patients undergoing angioplasty showed

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[1] Also known as classic preconditioning or early IP.
[2] The search term "ischemic preconditioning" [ALL FIELDS] yielded over 2100 published articles in MEDLINE in August 2002.
[3] Also referred to as first effort, first hole, or walk through angina.

improvement of ischemic parameters (*i.e.* chest pain, ST segment elevation, and lactate release) following an initial balloon inflation that at least lasted for 60 to 90 seconds (8, 9). These effects were shown to be unrelated to collateral recruitment (10, 11). Although such beneficial effects were not observed by all (12), the effects of first balloon inflation have been shown to be influenced by pharmacological modifiers of the IP phenomenon, thus suggesting involvement of IP (13, 14). Furthermore, pre-infarct angina was proposed to be a model of human IP (15, 16). It was suggested that ischemic chest pain might be a sign of accumulation of IP triggering substances (reviewed in 5). Retrospective and prospective studies have shown a beneficial role of pre-infarct angina for survival outcome (17, 18). In addition, intermittent cross clamping of the aorta during coronary artery bypass surgery has been used as a model of IP by Yellon *et al.* who were able to show a preservation of ATP levels in the myocardium following this procedure (19). Also, small atrial trabeculae harvested during cardiac surgery (20) and cultured human adult cardiomyocytes (21) have been shown to be protected from ischemic injury after IP treatment. Altogether these data suggest that the IP phenomenon does occur in man. Understanding of the involved mechanisms may help to extend the current therapeutic window in ischemic heart disease.

Mechanisms of IP

It was early speculated that the IP phenomenon was receptor-mediated and initiated by the local release of triggering substances. Adenosine binding to adenosine A_1 receptors was the first triggering mechanism to be recognised (22). Stimulation of myocardial adenosine A_1 receptors mimicked while the inhibition of these receptors abolished the protective effect of IP in rabbits (22). Today a number of other triggering mechanisms have been identified, including: adenosine via A_3 receptors (23), opioids via delta-opioid receptors (24), bradykinin via B_2 receptors (25), angiotensin II via AT_1-receptors (26), catecholamine via alpha $_1$-receptors (27), endothelin 1 via type 1 receptors (28), and acetylcholine via M_2 receptors (29). Nitric oxide (NO) was also demonstrated to be involved in the IP response (reviewed in 30). It was suggested that triggering IP by ischemia or any of the aforementioned triggers resulted in an NO-surge which mediated cardioprotection by activation of the protein kinase C (PKC) system, vasodilation, induction of heat stress proteins, and anti arrhythmic effects (reviewed in 31). Moreover, the release of free radicals have been shown to play a role in IP as introduction of free radical scavengers blocked, while infusion of hypoxanthine and xanthine oxidase (to form a free radical generating system) mimicked the protective effect of IP (32). It was observed that the block of protection observed by inhibiting one or more of the aforementioned triggering mechanisms could be restored by increasing the number of ischemia and reperfusion cycles used in the IP protocol (25, 32). This finding together with the observed redundancy in the receptor mechanisms that trigger IP were the background for the introduction of a threshold theory concerning IP (33). Accordingly, the cardioprotective effect of IP was the result of the cumulative actions of all trigger-mechanis-

ms reaching a threshold for protection. Thus, inhibition of one trigger mechanism would require increased stimulus by the remaining triggers in order to reach the stipulated threshold. In addition, there seems to exist a species-related difference in the predominant triggering mechanism. In the rat, adrenergic and opioid signalling seems dominant, whilst bradykinin and adenosine signalling is more important in rabbit myocardium (reviewed in 34).

All receptor systems involved in IP couple to and activate G-proteins and phopholipases (35), which in turn result in diacylglycerol release. Diacylglycerol activates the PKC system. Activation of PKC by agonists and its inhibition by antagonists mimicked, and blocked the protective effect of IP, respectively (36, 37). However, the importance of the activation of the PKC system for the IP phenomenon in large animal models (dog and pig) has not been conclusive.

It was observed that inhibition of the PKC system failed to block the cardioprotection offered by IP when a PKC-antagonist was given during the IP phase while the same antagonist completely blocked the protection when given during the prolonged ischemia (38). This raised the question of how receptor activation during the IP phase was linked to PKC activation during the prolonged ischemic insult. An explanation was offered by Liu et al. (39) who suggested that translocation of PKC from the cytosol to the sarcolemma would account for the temporal discrepancy between receptor activation and kinase activity and thus explain the "memory" of the IP phenomenon. This line of reasoning has been confirmed by others (40, 41).

Moreover, experimental evidence suggested that tyrosine kinase (also activated by diacylglycerol) mediated the protective effect offered by IP (42, 43). The activity of tyrosine kinase can be in parallel to or downstream the activation of the PKC system (43). An increased activity of the mitogen-activated protein kinases (MAPKs)[4] was also observed following IP (42, 44). Stimulation of MAPKs activity (via JNK and p38) mimicked (43, 45), while inhibition of their activity blocked the protective effect of IP (46). Similar to PKC activation, the modulation of tyrosine kinase and MAPK systems was effective only when performed during the prolonged ischemic insult.

The final step leading to cardio-protection induced by IP is not known. So far a number of hypotheses have been put forward. However, none has been able to offer a conclusive explanation. In recent years the K_{ATP} – channel[5] has emerged as the most probable end-effector in IP, as K_{ATP} – channel openers mimicked and K_{ATP} –

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[4] Discovered first in 1988 the MAPKs constitute a superfamily of proteins with a conserved amino acid sequence (The-X-Tyr) within their activation loop. Phosphorylation of both Thr and Tyr results in activation of these kinases. MAPKs are involved in signal transduction in the cell. Two subgroups of MAPKs have been shown to be activated by cellular stress namely, cJun N-terminal kinase (JNK), and p38/reactivating kinases (p38).

[5] The K_{ATP}-channel was first described by Noma (47) in cardiac ventricular myocytes. These channels are inhibited by physiologic levels of ATP and as ATP falls channel open probability increases allowing exodus of K^+. Other modulators of K_{ATP} activity include, pH, fatty acids, NO, SH-redox state, various nucleotides, and G-proteins (reviewed in 14).

channel inhibitors abolished the protective effect of IP (48, 49). Initially it was believed that opening of K_{ATP} – channels mediated cardio-protection via shortening of the (myocardial) action potential duration and subsequent cardioplegia (reviewed in 50). However later studies demonstrated that cardioprotection afforded by IP was dissociated from action potential duration (51, 52). Moreover it was suggested that mitochondrial (rather than sacrolemmal) K_{ATP} – channels were involved in mediating cardioprotection (53, 54). Although the role of K_{ATP} – channels in meditating the effects of IP is indisputable it is still unclear how the opening of these channels result in cardioprotection. Experimental data suggest that opening of K_{ATP} –channels lead to a net flux of K^+ into the mitochondrial matrix and subsequent, membrane depolarisation, and matrix swelling. It will also result in enhanced respiration, and reduced calcium overload (reviewed in 55).

Gottlieb *et al.* (56) suggested that the vacuolar proton ATPase (VPATPase) may be a target of PKC in mediating IP. According to their hypothesis IP activated VPATPase-mediates proton efflux (via PKC). Hence, during ischemia, less acidosis induced by VPATPase may reduce Na^+ overload via Na^+/H^+ exchange and consequently, Ca^{2+} overload via Na^+/Ca^{2+} exchange. Altogether this will result in less acidosis, apoptosis, and subsequent cardioprotection. Further investigations are needed to elucidate the biologic significance of VPATPase activity in IP. In addition, mitochondrial F_1–F_0 ATPsynthase has been implicated as a possible end effector of the IP phenomenon (57). Under normoxic conditions F_1–F_0 ATPsynthase acts as a major source of ATP production by phosphorylating ADP in presence of orthophosphate (P_i). The energy required for this phosphorylation is derived from the electrochemical proton gradient across the mitochondrial inner membrane which is generated by the actions of the electron transport chain. It was observed that during ischemia, F_1–F_0 ATPsynthase catalysed the formation of ADP and P_i from ATP, resulting in ATP wastage (58). Thus the activity of this enzyme may be deleterious to the heart during ischemia. Vourinen et al. (59) reported an inhibition of mitochondrial F_1–F_0 ATPsynthase activity following IP. They suggested that the inhibition of the ATPsynthase might account for sparing of high energy-phosphates and thus improvement of the energy state of the myocardium following IP. However, later studies showed that the protective effect of IP was not dependent on F_1–F_0 ATPsynthase activity (60, 61). A comprehensive summary of known mechanisms involved in the IP phenomenon is presented in Fig. 1.

Late IP

It was early observed that the protective effect of IP lasted for merely 2–3 h (3, 62). However, Kuzuya *et al.* (63) reported the reoccurrence of tolerance against ischemia approximately 24h after the initial IP stimulus in a dog model, suggesting the existence of a second phase of protection. Later studies confirmed this and such a second phase of protection which was referred to as second window of protection (SWOP) or late IP (64, 65). The cardioprotective effect of late IP differed substantially from that afforded by classic IP. Whereas the protective effect of the latter was

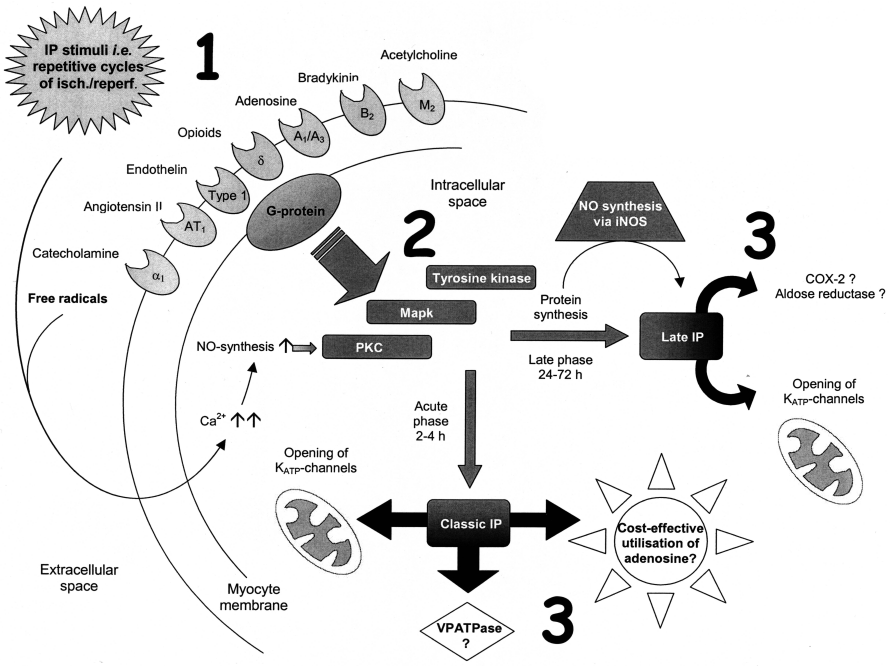

Fig. 1. Schematic overview of the mechanisms involved in the ischemic preconditioning (IP) phenomenon. (1), triggering mechanisms. (2), mediators of the signalling cascade. (3), end-effectors providing protection. Abbreviations: Mapk, Mitogen activated protein kinase; PKC, protein kinase C; NO, nitric oxide; iNOS, inducible NO-synthethase; COX-2, cyclooxygenase-2, VPATPase, vacuolar proton ATPase.

immediate, lasted for 2–3 h, and mainly protected against ischemia-induced necrosis, late IP has been shown to be protective against both ischemia-induced necrosis as well as stunning. Moreover, it occurs after approximately 24 h and lasts up to 72 h (66–68). In analogy with classic IP, studies of late IP have highlighted the role of triggering mechanisms, mediators and end-effectors (Fig. 1). A_1 and A_3 adenosine receptors, NO, reactive oxygen species, and opioid receptors have all been implicated in the mechanism of late IP (reviewed in 69). Moreover, PKC, tyrosine kinase, and MAPK:s were shown to be mediators of the protective effects (70–72). It was demonstrated that protein synthesis (and not merely activation of pre-existing proteins) was necessary for manifestation of the protective effect of late IP (73). Hence, heat stress proteins,[6] were proposed to be involved in the cardioprotective mechanisms of late IP as myocardial HSP70 content increased 24 h after IP treatment (76, 77). However, later studies have not been able to show a correlation between HSP70 increase and protection against myocardial infarction following IP (78, 79).

[6] Heat stress proteins (HSP) are overexpressed in response to heat stress or ischemia. It was observed that transgenic mice overexpressing HSP 70 were more resistant to ischemia induced myocardial injury (74–75).

Inhibition of all three NO synthase (NOS) isoforms (endothelial [eNOS], inducible [iNOS], and neuronal [nNOS]) before the IP stimulus abolished the cardioprotective effect of late IP (80) and maintained iNOS activity was shown to be important for cardioprotection 24 h after the PC stimulus (81, 82). These findings together with the data supporting the involvement of NO in activation of IP suggested a dual role of NO, first acting as a trigger (via eNOS and/or nNOS) and subsequently as a mediator (via iNOS) of protection in the IP phenomenon (69, 83).

A number of studies have identified the opening of K_{ATP} – channels as an end-effector, mediating the infarct-sparing effects of late IP (84–88). The opening of these channels was associated with the infarct-sparing effect of late IP but not found necessary for the protection against stunning (89). Moreover, Shinmura *et al.* using the rabbit heart model demonstrated that the expression of cyclo oxygenase-2 (COX-2) (90) and aldose reductase[7] (91) was upregulated 24h after an initial IP stimulus. Inhibition of COX-2 or aldose reductase activity abrogated the protective effect of late IP, suggesting the involvement of these enzymes in the overall mechanisms of late IP.

Remote IP

Przyklenk *et al.* (92) were the first to demonstrate that IP stimulus in one region of the heart protected another remote, virgin region from subsequent sustained coronary artery occlusion. The concept of remote IP was born. Cardioprotection mediated via remote IP has been observed after inducing ischemia in kidney (93), intestine (93), and skeletal muscle (94). Little is known about the overall mechanisms of remote IP. However, adenosine (95), and bradykinin (96) have been implicated in the signalling pathways. Furthermore, the concept of IP has been successfully demonstrated in other organs than the heart (97–99).

IP and the diseased heart

The bulk of our knowledge concerning IP is based on studies of healthy subjects. The few studies focusing on the efficacy of IP in the diseased heart have produced conflicting results. Speechly *et al.* (100) were able to demonstrate preserved efficacy of IP in a rat model of hypertension and hypertrophy, which was confirmed by others (101–104). Moreover, the presence of a critical coronary stenosis did not abolish the protective effect of IP in pigs (105). However, Szilvassy *et al.* (106) using a model of pacing induced ischemia in the rabbit heart reported the loss of the beneficial effects of IP in hypercholesterolemic subjects. This was also demonstrated in the rat (107). Ischemic heart disease is associated with a more severe prognosis in diabetic patients (108, 109). Interestingly, patients with non-insulin-dependent diabetes mellitus experience a higher cardiovascular mortality rate than patients with insulin-dependent diabetes mellitus

[7] Aldose reductase catalyses the metabolism of glucose to sorbitol and takes part in detoxification of lipid aldehydes derived by reactive oxygen species. The activity of aldose reductase is upregulated following exposure to oxidative or osmotic stress, cytokines and NO.

(110). Recently the use of the oral antidiabetic agent glibenclamide was reported to be associated with increased risk of in-hospital mortality among diabetic patients undergoing coronary angioplasty for acute myocardial infarction (111). Glibenclamide exerts its actions by inhibiting K_{ATP} – channels, which raises the question whether treatment with oral hypoglycaemic agents or diabetes per se interferes with the protective effect of IP. We have shown that glibenclamide inclusion did not alter myocardial energy metabolism in normal porcine hearts subjected to IP (112). Tosaki *et al.* (113) reported that streptozotocin-induced diabetic rat hearts could not be protected by IP. In addition, the protective effect of a single ischemia/reperfusion cycle was abrogated by streptozotocin treatment (114). Amplifying the IP stimulus to 3 ischemia/reperfusion cycles circumvented the effect of streptozotocin. Moreover, IP did not limit infarct size in diabetic canine hearts (115). However, Cleveland *et al.* reported that human right atrial trabeculae extracts with insulin-treated diabetes were responsive to an IP stimulus whereas tissues subjected to long term treatment with oral antidiabetic agents, were not (116). Furthermore, the beneficial effects of prodromal angina (limited infarct size, enhanced recovery of LV function and improved survival) were not observed in non-insulin-treated diabetic patients (117). Treatment of type II diabetics with glibenclamide blocked the warm up phenomenon (118) and prevented the improvement of hemodynamic variables normally observed following the first balloon inflation during PTCA (119). The evidence reviewed above suggests that IP may protect the myocardium in insulin-treated diabetics, but its protective effects are abrogated by concurrent use of oral hypoglycaemic agents such as glibenclamide, which block the K_{ATP} – channel.

Studying the effect of subject's age on the cardioprotection of IP has recently received attention. A number of experimental studies (all using the rat model) have reported an age-related decrease/loss of the protective effect of IP (120–123). In contrast Przyklenk *et al.* did not observe diminished cardioprotection in aged rabbits (124). Recent studies in humans have added to the uncertainty in this matter. Tomai *et al.* (125) reported that the beneficial effects of IP (related to first balloon inflation) were preserved in the elderly undergoing angioplasty. However, Lee *et al.* (126) using a 120-second coronary occlusion prior to coronary artery bypass surgery as a model of IP, did not observe an improvement of the ischemic burden following IP as assessed by ST-segment shift, chest pain score, and myocardial lactate extraction ratios in an elderly group (age $\geq$ 65 years; mean age, 71 $\pm$ 3 years) as compared to the control group (age $\leq$ 55 years; mean age, 45 $\pm$ 5 years). This was confirmed by others (127). Moreover, it was observed that the warm up phenomenon was lost in the elderly with coronary artery disease (128) and that exercise training could reinstate beneficial effects of IP in these patients (129). The interpretation of data reviewed above is hampered by the lack of a robust model of IP in humans. Additional studies are needed to further elucidate the impact of age on the protective effect of IP.

The IP protocol and factors influenced by IP

Originally, IP protocols consisted of repetitive episodes of ischemia and reperfusion. It was observed that at least a single ischemic episode of three min was neces-

sary to trigger the IP response (130, 131), whereas more than four ischemia/reperfusion cycles were deleterious to the myocardium (132, 133). The time span of protection in classic IP is about 2–4 h (134, 135).

There is some uncertainty regarding the necessity of a reperfusion period after the IP stimulus and before sustained ischemia. Fifteen min of partial (50%) coronary artery occlusion did not protect the myocardium from a subsequent 60 min no-flow ischemic insult in the dog (136). However, 30 min of partial (70%) coronary artery occlusion reduced infarct size induced by a 60 min of coronary occlusion (*n.b.* without intermittent reperfusion) in pigs (137). In addition, a 10 min no-flow ischemic episode preceding an 80 min long low-flow ischemic period was cardioprotective compared to 90 min low-flow ischemia (138).

It was reported that regional myocardial blood flow is reduced by IP stimulus (139, 140). Moreover, IP was found to protect the heart against reperfusion-induced coronary endothelial dysfunction (141, 142). In addition IP reduced the production of malon dialdehyde, an indirect marker of free radical formation, in isolated perfused rat hearts (143).

Our experimental findings

Using the microdialysis technique in an *in vivo* porcine model, we have extensively mapped the dynamic changes of interstitial energy related metabolites (lactate, pyruvate, adenosine, inosine, hypoxanthine, guanosine, adenine, and xanthine) during IP (4 x 10min ischemia + 20 min reperfusion) (144, 145). A distinct pattern of fluctuation was observed for these metabolites. Interstitial lactate levels (expectedly) increased and decreased during each ischemia/reperfusion cycle of the IP protocol. The peak levels of lactate were similar during all four ischemia/reperfusion cycles. Additional increase of lactate was observed during the following sustained ischemia, however these lactate levels were significantly lower than the peak levels of lactate observed during ischemia in non-preconditioned animals, Fig. 2 top. The interstitial levels of adenosine and other purines were also influenced by ischemia (increased levels) and reperfusion (decreased levels). However, in contrast to lactate, adenosine (and also inosine and hypoxanthine) levels displayed a successive die-away curve pattern following IP (see Fig. 2 bottom). Thus, successively lower peak levels of adenosine were observed following each ischemia/reperfusion cycle. These findings were also observed by others (146–149). It was suggested that the die away curve pattern of adenosine was due to diminished nucleotide decay induced by IP (146). However, Kitakaze *et al.* (150–152) using the dog and rabbit model demonstrated an enhanced 5'-nucleotidase activity following IP. 5'-Nucleotidase is the key enzyme responsible for hydrolysis of AMP into adenosine and Pi (see also section 3.2). These findings were confirmed by others (153). The described discrepancy between the increased adenosine production (evidenced by increased 5'-nucleotidase activity) and the successively lower adenosine levels observed in the interstitial space following IP is not fully accounted for. We propose that this discrepancy in adenosine production *vs.* availability may be due to a cost-effective metabolic

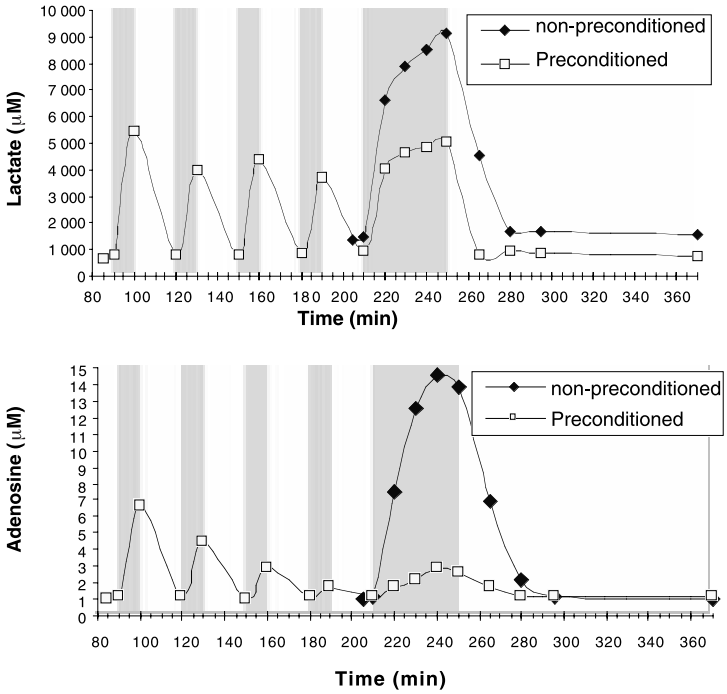

Fig. 2. Representative microdialysis data from ischemic tissue of preconditioned and non-preconditioned subjects. Typical curve-patterns of lactate (top) and adenosine (bottom) are demonstrated. In the preconditioned subjects the interstitial concentration of lactate markedly increased during ischemia (shaded background) and quickly returned to normal values during reperfusion (white background) whereas the interstitial concentration of adenosine, in contrast to lactate concentration, presented a die away curve-pattern for every successive episode of ischemia.

utilisation of adenosine by the preconditioned myocardium (see below). Such cost-effective metabolism may at least partly explain the cardioprotective effect of IP. The above hypothesis was put on test in the consecutive studies comprising the current reviewed thesis. Data were collected using the microdialysis technique and tissue biopsies in a porcine heart-model.

Adenosine in the cardiovascular system

Adenosine a catabolite of adenine nucleotides is a ubiquitous biological compound found in every cell of the human body. Adenosine exerts a number of physiologic actions which are mediated through at least three subclasses of receptors (Table 1).

In the myocardium, adenosine is a product of cell energy metabolism being produced from AMP by cytosolic- and/or ecto 5'-nucleotidase (154) or through hydrolysis of S-adenosylhomocysteine (SAH) by SAH-hydrolase. Hydrolysis of SAH is the major source of adenosine during normoxia. The cellular concentration of adenosine is minute under normoxia but increases markedly following stress *i. e.* ischemia. This is primarily due to increased 5'-nucleotidase activity which by removing

Table 1. Overview of three adenosine receptor subclasses and their main effects on the cardiovascular and extra-cardiac systems.

Receptor-type	Cardiovascular effects	Extra-cardiac effects
A_1	Attenuation of the inotropic effects of β-adrenergic receptor stimulation. Increased chemotaxis of leukocytes. Inhibition of renin and catecholamine release. Modulation of Na^+-Ca^{2+} exchange.	Bronchoconstriction. Reduced HCL secretion in gastric mucosa. Reduced lipolysis, stimulated glucose uptake and increased insulin sensitivity in adipose tissue. Modulation of germ cell production. Modulation of pituitary hormone release.
A_2	Increased nitric oxide production. Relaxation of smooth muscles. Inhibition of cytokine and O_2-derived free radical production. Inhibition of platelet aggregation.	Bronchodilation. Inhibition of respiration in medulla oblongata. Immunosuppression. Modulation of germ cell production. Modulation of pituitary hormone release.
A_3	Increased histamine release.	Bronchoconstriction. Modulation of germ cell production.

AMP shifts the equilibrium of the adenylate kinase reaction in favour of ATP restoration (adenylate kinase catalyses the dismutation reaction between two ADP giving rise to one ATP and one AMP). Although adenosine can be rephosphorylated to AMP by adenosine kinase at the expense of ATP, it does not for obvious reasons seem to be the preferred pathway in the ischemic myocardium (155, 156). Newly-formed adenosine crosses the myocyte membrane by simple diffusion and accumulates in the interstitial fluid. From there it escapes to the intravascular compartment by paracellular washout or it is taken up by endothelial cells, red blood cells, and pericytes by nucleoside carriers. Inside these cells adenosine is further metabolised to inosine, hypoxanthine, xanthine, uric acid, and allantoin. However, as will be discussed later, adenosine may also be metabolised inside the myocyte as part of a cost-effective energy utilisation.

Adenosine, besides being directly converted to inosine can participate in a number of other pathways. Fig. 3 gives a schematic presentation of adenosine metabolism. Routes of adenosine metabolism include a) conversion directly to AMP by adenosine kinase requiring ATP. b) Conversion to AMP via IMP. This is also an energy requiring reaction c) Conversion to GMP via IMP. GMP can further be converted to guanosine and guanine, producing ribose-1-phosphate. GMP exerts negative feed back on its own production. d) Conversion to adenine and ribose 1-phosphate in presence of orthophosphate. d) Hydrolysis to inosine via IMP. This route seems to be the main pathway of adenosine metabolism under normoxic conditions (157).

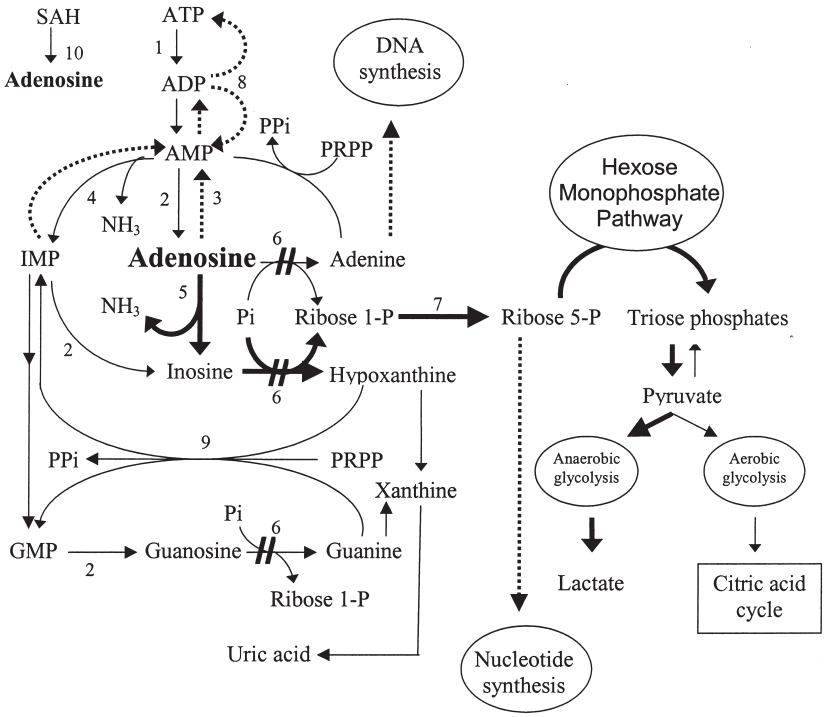

Fig. 3. Schematic illustration of the pertinent steps in adenosine metabolism. Abbreviations: 1: ATPase; 2. ecto-/endo 5' nucleotidase; 3: adenosine kinase; 4: AMP deaminase; 5: adenosine deaminase; 6: purine nucleoside phosphorylase; 7: ribose 1,5 phosphomutase; 8: adenylate kinase; 9: hypoxanthine guanine phosphoribosyltransferase; 10: S-Adenosyl homocysteine hydrolase; (.....): Energy requiring reaction; (//) the site of inhibition in our studies. According to the proposed hypothesis (indicated by thicker lines) adenosine is primarily converted to inosine. Inosine in turn is catalysed by purine nucleoside phosphorylase yielding hypoxanthine and ribose-1-phosphate. The latter can be converted to ribose-5-phosphate which is an intermediate metabolite of the hexose monophosphate pathway. Hence the ribose moiety of adenosine can be utilised to produce pyruvate and eventually lactate and ATP *n.b.* without any initial ATP investment.

Attenuating purine levels during IP and the role of adenosine catabolism

Attenuating purine levels (described above) are characteristic findings during IP [158–162, see also Fig. 2]. Goto *et al.* [163] using isolated rabbit hearts found that adenosine levels in coronary effluent collected during IP were unaffected by adenosine receptor inhibition during IP (which abolished the protective effect of IP) or by adenosine/ischemic preconditioning. They concluded that the attenuating purine production during subsequent ischemia following IP was unrelated to the mechanism of protection [163]. However, these investigators did not measure purine levels during prolonged ischemia. It is possible that adenosine receptor inhibition may alter the profile of adenosine metabolism during prolonged ischemia. Moreover, estimation of purine levels from coronary effluents may be biased, as endothelial cells and erythrocytes are known for their ability to influence blood purine levels

[164]. Mortimer *et al.* [162] using an *in vivo* heart-model in rabbit observed that the progressive attenuation of interstitial purine levels occurring during two consecutive ischemia/reperfusion cycles persisted even if the time space between the two ischemic episodes was extended to 180 min. This time frame is considered to be longer than the duration of the infarct-limiting effect of IP in the rabbit [165]. Hence, attenuating purine levels following IP may be dissociated from the mechanisms of protection of IP (at least in the rabbit). Nonetheless, a number of studies have demonstrated an increased 5'-nuclcleotidase activity following IP [166–172], which would imply an increased production of adenosine and its catabolites. As mentioned earlier, the described difference between the increased adenosine production (evidenced by increased 5'-nucleotidase activity) and the successively lower adenosine levels observed in the interstitial space following IP may have a common explanation. Accordingly, IP activates a metabolic pathway favouring metabolic utilisation of adenosine towards pyruvate and ATP production (Fig 3). Thus newly-formed adenosine by 5'-nucleotidase is deaminated to form inosine and ammonia in stoichiometric amounts. Inosine is further degraded to hypoxanthine by purine nucleoside phosphorylase,[8] in the presence of Pi, thereby forming ribose-1-phosphate, [173]. Ribose-1-phosphate can then be transformed to ribose-5-phosphate in a phosphoribomutase reaction. Ribose-5-phosphate is an intermediate of the hexose monophosphate pathway, operative also under anaerobic conditions. Hence the ribose moiety of adenosine can be utilised to generate pyruvate and ultimately ATP, *n.b.* without any initial ATP investment. This pathway communicates with the glycolytic pathway ending up in pyruvate and lactate [174]. The above mentioned formation of ammonia is favourable in this context, as it can act as a proton acceptor thereby counteracting lactacidosis. Utilisation of adenosine in this way may explain the successive die-away pattern of adenosine seen in consecutive IP cycles and at least in part be responsible for the protective effect of IP. Evidence in support of this line of resoning was found in our studies through following observations:

- Addition of adenosine via microdialysis probes enhanced the interstitial release of inosine, hypoxanthine and lactate in the myocardium of IP subjects during prolonged ischemia. This finding did not occur in non-preconditioned subjects. Similar addition of deoxyadenosine a non-metabolizable[9] adenosine receptor-agonist, did not evoke the same metabolic response (175).

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[8] PNP is a trimer of 90,000 daltons, and is found in the cytoplasm of a variety of organisms, both prokaryotic and eukaryotic. Mammalian PNP (EC 2.4.2.1) catalyses the reversible conversion of ribonucleoside and 2'-deoxyribonucleoside in presence of P_i to the corresponding bases and ribose-1-phosphate or 2'-deoxyribose-1-phosphate, respectively. Inherited deficiency of PNP is associated with severe T-cell immune deficiency (but normal B cell function). Although there seems to exist uncertainty regarding the cellular origin of PNP in myocardium [176, 177], significant activities of PNP have been demonstrated in rat cardiomyocytes [178]. The importance of this enzyme in the preconditioned ischemic myocardium is unknown.

[9] Deoxyadenosine can be metabolized in the cell, but it can not be catabolised into inosine, hypoxanthine and lactate.

- Inclusion of 8'-aminoguanosine (a competitive inhibitor of PNP) decreased interstitial hypoxanthine release and increased the release of taurine as a token of increased cellular injury[10] in the ischemic IP myocardium. Addition of inosine (a natural substrate of PNP) reverted these changes. The changes evoked by 8'-aminoguanosine were due to a competitive inhibition of PNP and not due to unspecific toxicity.
- The tissue levels of adenine nucleotide breakdown products (ANBP) of IP subjects consisted of proportionally more hypoxanthine during prolonged ischemia, suggesting a modified handling of ANBP in the preconditioned myocardium. Moreover, the ratio of tissue levels of inosine/hypoxanthine (used as an indicator of PNP activity) was significantly smaller in the IP groups, suggesting an early activation of PNP by IP (since inosine is one of the substrates and hypoxanthine is one of the two reaction products).

Concluding remarks

The IP phenomenon is a powerful cardioprotective mechanism. Current evidence suggest that the IP phenomenon also exists in man. Understanding the mechanisms of IP offers new opputunities to improve the therapeutic window in ischemic desease. Today, much is known about the signalling pathways involved in IP, but the underlying mechanisms of cardioprotection remain elusive. Opening of K_{ATP}-Channels, VPATPase- and cyclooxygenase-2 activity seem relavant in the overall scheme.

The IP phenomenon is associated with attenuating purine levels which is not accounted for. We propose that the attenuating purine levels may be due to a (cost-effective) metabolic utilisation of these compounds, induced by IP. Such cost-effective utilisation involves the deamination of adenosine to inosine. Inosine is further catalysed (in presence of Pi) to hypoxanthine and ribose-1-phosphate. Ribose-1-phosphate can be converted to ribose-5-phosphate in a phosphoribomutase reaction. Ribose-5-phosphate is an intermediate of the hexose monophosphate pathway also operative under anaerobic conditions. Hence the ribose moiety of adenosine can be utilised to generate pyruvate and ultimately ATP (*via* lactate formation) *n.b.* without any initial ATP investment. Such cost-effective adenosine utilisation offers an explanation to the aforementioned attenuating purine levels and may at least partly explain the cardioprotective effect of IP. Current experimental data are in favour of this line of resoning, however additional studie are waarented to further elucidate the importance of the proposed metabolic pathway in the overall mechanism of protection offered by IP.

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[10] Regarding the use of taurine as a marker for ischemic myocardial injury please refer to (179, 180) and discussion in (181).

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Correspondence to: Mohammad Kavianipour
Department of public health and clinical medicine
Umea University
SE-901 87 Umea, Sweden
Email: ropison@hotmail.com