Mechanistic and Therapeutic Aspects of Ischemic Myocardial Preconditioning

Mechanistische en therapeutische aspecten van ischemische myocardiale preconditionering

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In loving memory of my father Liem Ing-Hay who once taught me about strength, courage and dignity.

"More matured from loss and defeat, I now realize you were right about everything……especially about me"

"Thank you for giving me direction dad"

For my mother Tan Lay-Nien

People should study to improve themselves,
not to impress others

-adapted from Confucius-

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Introduction

Background

Despite significant therapeutic advances at the end of the 20th century, cardiovascular disease accounted for almost half of all deaths in modern society and one fourth in the developing world.[1,2] By the year 2020, cardiovascular disease is estimated to have surpassed infectious disease and to become the world's leading cause of death and disability.[3,4] In the industrialized world, cardiovascular disease is responsible for approximately 45% of overall mortality of which 50% is due to acute myocardial infarction.[2] The proportion of cardiovascular deaths in the industrialized world is expected to show a modest decline to 42% of total death by the year 2020.[2] In the developing world however, 23% of total mortality was due to cardiovascular disease in 1990. By 2020 this figure will increase to 34% of total mortality, accounting for approximately 75% of all cardiovascular deaths worldwide.[2]

In the middle of the 20th century both arrhythmias and pump failure were the major causes for both high early and late in-hospital mortality. In the early 1960s the development of the coronary care unit with continuous electrocardiographic monitoring and external defibrillation significantly decreased early arrhythmic deaths by acute myocardial infarction thereby reducing early mortality by 50%. Substantial use of thrombolytic therapy, aspirin, β-blockers and coronary angioplasty further improved mortality and survival rates of acute myocardial infarction.[5]

Due to a reduced mortality from acute myocardial infarction, left ventricular remodeling and heart failure will become a major problem in cardiovascular disease. In the mid 20th century the major causes of heart failure were rheumatic fever and hypertension. With the application of antibiotics, valve repair surgery and the improved therapy of hypertension, the most important cause of heart failure was shifted to myocardial ischemia and infarction. As a consequence of the improved survival of acute myocardial infarction, ventricular remodeling associated with large infarct size is currently the major cause of chronic congestive heart failure. Development of new therapeutic strategies to control and prevent ischemic heart disease and limit infarct size in order to mitigate development of remodeling and heart failure remains therefore a major challenge in cardiovascular therapy.[6]

Many investigators have studied myocardial ischemia and searched for means of reducing it in order to limit cellular injury. In 1935, Tennant and Wiggers observed that dyskinetic contraction of the ischemic myocardium appeared within seconds after occlusion of a major coronary branch. When the occlusion lasted less then 2 minutes, normal myocardial contraction returned upon reperfusion. But when the occlusion lasted longer, dyskinetic contraction continued.[7] In 1941, Blumgart and co-workers further observed in the dog that severe infarction occurred after coronary occlusions for 40 minutes or longer. However, occlusions of 5-20 minutes did not result in infarction. With occlusions of intermediate duration, the extent of necrosis was dependent on the time to reperfusion.[8] Many years later, during the late 1960s and early 1970s Braunwald en colleagues demonstrated that the extent and severity of ischemic injury could be extended by increasing myocardial oxygen demands. But more importantly, ischemic injury could be reduced by lowering oxygen demands by β-adrenergic blockers.[9-11] However, many other studies were not able to reproduce these findings.[12] In the mid 1970s Reimer and Jennings documented in the dog the presence of a subepicardial zone of ischemic but viable myocardium which is available for pharmacological or surgical salvage for at least three hours following coronary occlusion.[13] Since timely reperfusion was the only way to limit damage during evolving myocardial infarction, in the

mid 1970s and early 1980s several investigators focused on developing techniques for myocardial reperfusion in patients. With the exception of reperfusion therapy, much basic and clinical research efforts in the search for means to delay or prevent myocardial ischemic injury yielded disappointing results. Early attempts at tissue salvage with calcium antagonists and ß-blockers showed a large number of both promising and disappointing results.[12,14]

However, in 1986 Murry and coworkers[15] made a seemingly paradoxical observation that relined interest in pharmacological infarct size reduction. In dogs, Murry et al. exposed the myocardium to a "preconditioning protocol" with repetitive short periods of regional ischemia. Surprisingly, they found that this protocol induced resistance to a subsequent long-lasting ischemic period. Infarct size after 40 minutes of ischemia was limited from 29% without preconditioning to 7% with preconditioning. The investigators concluded that they had discovered a new phenomenon of cardioprotection that was initiated by ischemia. Ever since its first report, many efforts of investigation on this phenomenon has allowed a better understanding into myocardial ischemia and several ischemic syndromes. However, the exact mechanism of ischemic preconditioning is not fully elucidated and many aspects of its molecular cellular process are still incompletely understood. Moreover, therapeutical application is very promising but has so far been very slow. In order to better understand this phenomenon and to facilitate its translation from the experimental laboratory to the clinical setting, this thesis will discuss some of the mechanistic and therapeutic aspects of the most powerful endogenous cardioprotection against ischemia, the phenomenon termed *"ischemic preconditioning"*.

Myocardial ischemia

Definition

Ischemia is characterized by an imbalance between myocardial oxygen supply and demand. In most situations this imbalance is due to a reduction of blood flow and oxygen supply secondary to a coronary artery obstruction caused by vasospasm or intra-coronary platelet aggregation and thrombus formation at the site of an atherosclerotic lesion. Myocardial ischemia can be defined as an imbalance between the supply of oxygenated blood and the oxygen requirements of the myocardium leading to disturbance of cardiac function.[16] Complete abolition of blood flow to a myocardial region is termed "total ischemia". When an imbalance between supply and demand results from increased myocardial metabolic requirements even though coronary blood flow is maintained we refer to "demand ischemia". The term 'regional ischemia" is used when myocardial ischemia is confined to a region of the heart. If the entire heart becomes ischemic as occurs during systemic hypotension or aortic cross-clamping necessitated by various cardiac surgical procedures, the term "global ischemia" is employed.

Consequences of myocardial ischemia

In order to retain normal function and to furnish the energy demands of the contracting muscle, the heart strongly depends on a sufficient oxygen and substrate supply. The energy that is required is derived from high-energy phosphates such as adenosine triphosphate (ATP), mainly produced by oxidative phosphorylation in the mitochondria. Subsequent to ischemia, oxidative phosphorylation is thought to stop within only 8 seconds after coronary occlusion leading to inhibited contractile function and anaerobic metabolism i.e. ATP production by

anaerobic glycolysis.[17] End products of anaerobic glycolysis are protons and lactate from glycolytic turnover.[18]

Total coronary artery occlusion can elicit three responses of myocardial function depending upon the duration of ischemia. First, ischemia lasting less than 2 minutes does not result in any irreversible myocardial damage and both cardiac metabolism and contractile function return to baseline during reperfusion.[7] Secondly, an occlusion lasting approximately between 2- and 20-min results in reversible damage characterized by a temporary impaired contractile function during restored normal myocardial blood flow with no evidence of cell death. During the 1970s Heyndrickx et al. first described this phenomenon of prolonged contractile dysfunction of fully reperfused viable myocardium.[19,20] which was later termed "*myocardial stunning*" by Braunwald and Kloner.[21,22] In conscious dogs, contractile dysfunction after 15 min of coronary occlusion varies from less than 1 hour to several days.[23] Finally, when coronary blood flow is not restored and ischemia persists for more than 20 min, cardiomyocytes become irreversibly damaged leading to cell death either by apoptosis or necrosis. Myocardial cell death by ischemia is defined as "*myocardial infarction*".

Determinants of myocardial infarct size

A major determinant of infarct size is *the time during which the myocardium is deprived from blood flow.* Experiments in pigs have indicated that by 60 minutes occlusion the transmural extent of the infarct was 87%, increasing to 96% by 90 minutes and to maximum infarction at 24 hours.[24]

In rats however, a full transmural myocardial infarction develops within 2 hours.[25] In reviewing such interspecies differences Schaper and co-workers pointed out that infarct size depends upon the presence of *collateral blood flow.*[26] In rabbits, pigs and rats, collateral flow is virtually absent resulting in complete infarction after 1-1.5 hours ischemia. However, in guinea pigs in which collateral circulation is well developed, occlusion of a major coronary branch does not result in myocardial necrosis. Infarct size progression in humans is more gradual due to the presence of moderate collateral flow,[26] two hours of ischemia results in 50% infarction of the area at risk, whereas infarction after 6 hours is complete (fig. 1).[27] Next to duration of ischemia and collateral blood flow, an important determinant on the variability in infarct size is the *myocardial area at risk* zone. In dogs, the amount of necrosis after 40 min proximal coronary circumflex occlusion correlated closely with the size of the occluded coronary bed.[28] Therefore infarct size is typically expressed as percentage of area at risk zone.

The role of myocardial oxygen demand at the onset of occlusion is controversial. In dogs a positive correlation has been reported between infarct size and myocardial oxygen demand estimated by the product of heart rate and left ventricular systolic pressure.[29,30] However, these studies used anesthetics that highly affected heart rate and ventricular pressure. An increase in heart rate can decrease collateral blood flow and redistribute collateral flow away from subendocardium, while an increase in blood pressure can increase collateral flow by increasing collateral driving pressure.[31] In species with minimal collateral flow, infarct size is not significantly correlated with the rate-pressure product.[32] However, the myocardium ceases to contract early after onset of occlusion so that energy utilization is confined mainly to maintenance of ionic homeostasis and basal metabolism. Under these conditions, the rate-pressure may no longer reflect the energy demands of ischemic myocardium.

Finally, another important factor that contributes to infarct size variance is *temperature*. Hypothermia with temperatures below 30° C has shown to reduce infarct size.[33,34] Heart rate is decreased by hypothermia, however the decrease of myocardial oxygen demand does not explain the correlation between infarct size and temperature.[35-37] Proposed mechanisms responsible for protection by hypothermia include acidosis and maintenance of ion homeostasis during ischemia and reperfusion. Furthermore, a reduction of basal metabolic rates leading to slowing of high-energy phosphate depletion may be responsible.

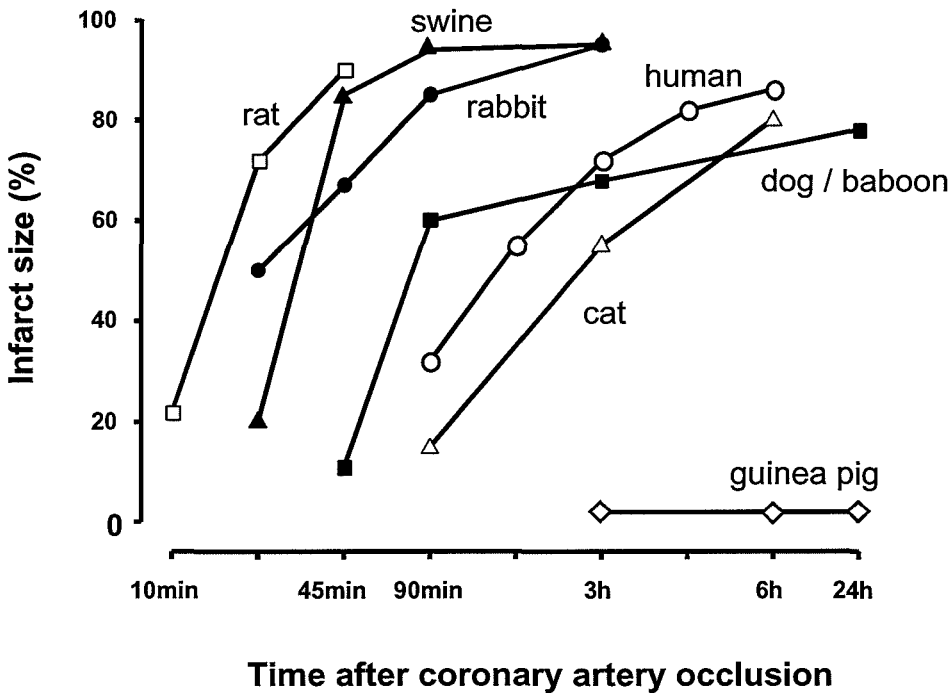


***Figure 1** Development of irreversible myocardial damage during total coronary occlusion in different species. (modified from Schaper et al[26] and Arnold et al[27])*

Reperfusion and reperfusion injury

The concept of limiting infarct size was first developed based on studies involving human autopsies showing that infarct size was significantly smaller than the myocardial region supplied by the occluded coronary artery. In the early 1970s Braunwald and co- workers reported that ischemic injury could be limited by lowering myocardial O_2 demands by beta adrenergic blockade.[9-11] Early experimental studies occasionally showed an infarct size limiting effect by a drug, but others were often unable to reproduce these observations.[38] To date, early coronary reperfusion is clinically the most effective means to limit the size of a myocardial infarct. To salvage ischemic or hypoxic myocardium, reinstatement of coronary flow is necessary. Timely reperfusion will limit cardiomyocyte damage and decrease cardiac mortality and morbidity. Current reperfusion therapy includes thrombolysis and percutaneous coronary intervention (PTCA), which are standard treatment in impending acute myocardial infarction.

Reperfusion itself may paradoxically lead to additional tissue injury beyond that generated by ischemia alone, a phenomenon termed *reperfusion injury*. Rosenkranz and Buckberg have provided a general definition of this term: *"Reperfusion injury refers to those metabolic, functional and structural consequences of restoring coronary flow that can be avoided or reversed by modification of the conditions of reperfusion.*[39] The cellular damage that results from reperfusion can be reversible or irreversible, depending on the length of the ischemic insult. When reperfusion is initiated approximately within 20-min after the onset of ischemia, the resulting myocardial injury is reversible and is characterized by functional impairment of contractility that may persist for a variable period but eventually recovers completely. As mentioned earlier, this phenomenon is known as *myocardial stunning.*[20] Another manifestation of reversible reperfusion injury after less prolonged ischemia is the occurrence of *reperfusion arrhythmias*. In anesthetized dogs, ventricular fibrillation within the first few seconds of reperfusion is most likely to occur when the entire ischemic region is still viable (and excitable) and is progressively less likely to occur as the amount of potentially salvageable myocardium decreases by cell death.[40] For any particular duration of ischemia, the major determinants of reflow ventricular fibrillation are the severity of ischemia, the size of the area at risk and the heart rate.[41] Ventricular tachyarrhythmias after myocardial revascularization are considered to be a benign transient event. In the clinical setting however, it appears to increase the risk of future life-threatening events in patients.[42] Sustained ventricular tachycardia after coronary artery bypass grafting is uncommon but considered as a serious complication with an unsatisfactory prognosis.[43]

Myocardial reperfusion, which is initiated after a duration of ischemia longer than 20-min, results in irreversible myocardial injury or cell death. Tissue necrosis by reperfusion injury may occur within minutes of reperfusion and is additive to cell death due to the ischemic event itself.[44] Moreover, the extent of cell death during reperfusion appears to be related to the duration of the ischemic event.[44] Increasing evidence indicates that lethal reperfusion possibly consists of two forms of cell death namely *necrosis* and *apoptosis*. Several studies strongly support the existence of reperfusion-induced necrosis.[45,46] Moreover, it has been reported that the apoptotic process (programmed cell death) is initiated upon onset of ischemia but becomes markedly enhanced during reperfusion.[47] Finally, when a coronary artery is occluded sufficiently long, detrimental changes occur in the cardiac capillaries and arterioles.[48] After release of occlusion, blood flow to the ischemic tissue may still be impeded, a phenomenon known as *"no reflow"*. In a study by Kloner et al.,[49] dogs were subjected either to 40- or 90 minutes of coronary artery occlusion. When the coronary occlusion was restored after 40-min, blood flow to the ischemic myocardium was completely restored. In contrast, when blood flow was restored after 90-min occlusion, there was only partial restoration of blood flow to the myocardial tissue. It appears that the longer ischemia lasts, the more likely the "no-reflow" phenomenon is to occur. Further examination of the no-reflow areas revealed significant capillary damage in the form of swollen endothelium, intraluminal platelets and fibrine thrombi. These microcirculatory changes in combination with interstitial edema could compress the capillaries and may therefore be responsible for the no-reflow phenomenon.[48]

At least three major pathways have been proposed as possible mechanisms of lethal reperfusion injury. First, cellular acidosis could contribute to a *calcium overload* via sodium/hydrogen exchange and calcium/sodium exchange.[50] When irreversibly injured myocytes are reperfused, the development of contraction band necrosis is well known to be associated with a major calcium overload resulting in mitochondrial calcification.[51] In this

setting, a major calcium overload is the most likely cause of myofibrillar hypercontraction, and could contribute to rupture of the sarcolemma. Moreover, cellular calcium overload, particularly in association with oxidative stress, is followed by a mitochondrial calcium uptake which may trigger pathological states that lead to cell death.[52] Nevertheless, the importance of a modest calcium overload during ischemia and early reperfusion as a cause of myocardial cell injury remains a subject of debate. Second, ischemia is accompanied by an intracellular osmotic load of accumulated catabolites that could cause *cell swelling* when reperfusion provides an effectively unlimited supply of plasma water. This osmotic swelling coupled with an already weakened membrane, could result in membrane rupture.[53] Finally, *oxygen derived free radicals* such as superoxide anion, hydroxyl radicals and peroxynitrite may injure the sarcolemma.[54] Bolli and colleagues demonstrated that free radicals produced within the first few minutes of reflow play a significant role in myocardial stunning.[55] Free radicals can be generated by several sources such as the mitochondrial respiratory, xanthine oxidase, metabolism of arachidonic acid, oxidation of catecholamines and the NADPH oxidase of activated neutrophils. Reperfusion injury by free radicals can be induced by a reaction with polyunsaturated fatty acids, which results in the formation of lipid peroxides and hydroperoxides that damages the sarcolemma and impairs the function of several membrane-bound enzyme systems. It remains however unclear whether free radicals contribute to lethal reperfusion injury.

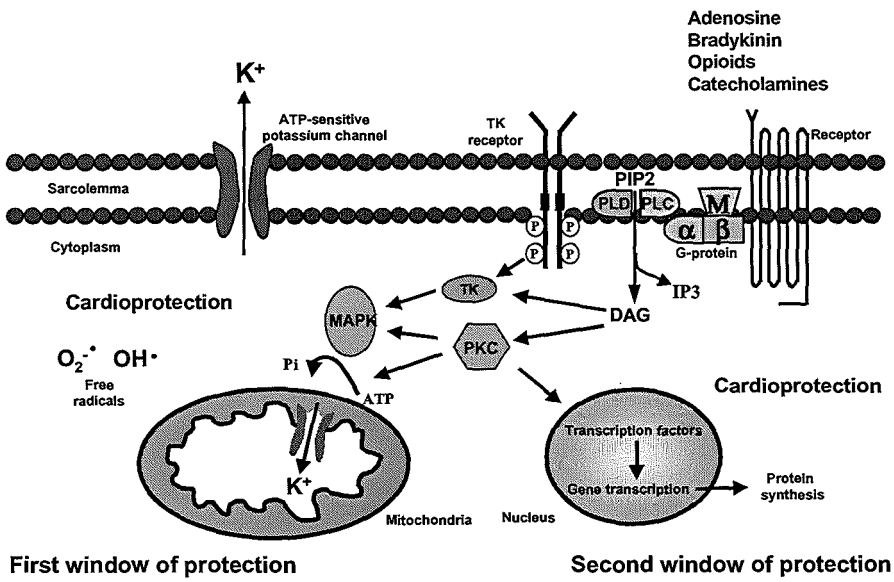
Cardiac adaptation to ischemia: Ischemic Myocardial Preconditioning.

Much basic and clinical effort has been put into the quest for means to delay or prevent myocardial ischemic injury. Potential application of such therapy includes (i) preservation of myocardium during cardiac surgical procedures when transient periods of myocardial ischemia can occur during a substantial part of the procedure, and (ii) prevention or limitation of myocardial infarct size. During the 1970s and early 1980s considerable efforts in limiting myocardial infarct size had little success with the exception of reperfusion therapy.[38]

However, in 1979, Verdouw et al[56] studied the effect of repeated local ischemia and reperfusion in pigs on myocardial metabolism and ventricular performance and observed that lactate and inosine release were much smaller during the second coronary occlusion period than during the first. Capitalizing on this apparent metabolic adaptation of the heart to repeated ischemic stress, Murry et al.[15] first demonstrated in 1986 that repeated brief episodes of non-lethal ischemic myocardial stress elicit an increased resistance to infarction caused by a subsequent prolonged ischemic insult. Abundant evidence showed that this phenomenon known as "ischemic preconditioning" is the most powerful endogenous protective mechanism against ischemic-reperfusion injury in animals. An enormous amount of research over the last 17 years has documented this phenomenon in all species including the pig,[57] the rabbit[58] and the rat.[59,60] Most importantly, substantial evidence suggests that ischemic preconditioning occurs in human myocardium.[61-65] Remarkable observations in the laboratory of this protective phenomenon have made extrapolation to the cardiology clinic or the operating room very attractive. However, since acute myocardial infarction in patients has usually already occurred when reaching the emergency room, application of preconditioning as a therapeutic strategy has so far been very prudent.

Definition of ischemic myocardial preconditioning

In the in vivo open chest dog, Murry and co-workers observed that infarct size after 40-min of coronary occlusion was reduced by 75% when preceded by 5-min episodes of coronary occlusion interspersed by 5-min reperfusion.[15] Thus, despite a longer cumulative duration of coronary occlusion, infarct size in preconditioned canine myocardium averaged only one fourth of that seen in controls. *In the strict sense, ischemic preconditioning refers to the phenomenon that one or more brief non-lethal ischemic episode(s) initiates a cellular myocardial adaptation process that limits myocardial injury produced by a prolonged period of coronary artery occlusion and reperfusion.* As a result, reduction of infarct size is regarded as "gold standard" of ischemic preconditioning.[66]



Characteristics of ischemic preconditioning

Figure 2 Schematic illustration of intracellular signal transduction pathway during ischemic preconditioning leading to an early "first window of protection" and a late "second window of protection". PIP2 indicates phosphatidyl inositol diphosphate; DAG, diacyl glycerol; PLC, phospholipase C; PLD, phospholipase D; IP3, inositol triphosphate; TK, tyrosine kinase; PKC, protein kinase C; MAPK, mitogen-activated kinase (modified from Cohen et al[66])

Ischemic preconditioning is not organ specific and has been demonstrated in several other organs including the kidney,[67] brain,[68] skeletal muscle,[69] liver,[70,71] lung[72] and small intestine[73]. However, in these reports, endpoints were not always infarct size.

Cardioprotection by an ischemic stimulus manifests itself in two different time windows. The first window of protection known as *classic preconditioning* appears a few minutes after initiation of the stimulus and disappears within approximately two hours.[74-76] Classic preconditioning is believed not to involve protein synthesis. The second window of protection known as *delayed preconditioning* reappears approximately 24 hours after the stimulus lasting for about 72-96 hours and requires transcription and protein synthesis. Both windows of protection partly share the same intracellular transduction pathway in which G-linked sarcolemmal receptors, protein kinase C and ATP-sensitive K^+- channels play a key role (Fig. 2).

Ischemic preconditioning refers to delaying infarct development during ischemia. It is important to point out that the evolution of cell death is only delayed but not prevented. After prolonged (i.e. exceeding two hours) or permanent occlusion, infarct size in preconditioned myocardium is equally severe to non-preconditioned myocardium. Thus, cardioprotection by classic preconditioning is limited to a *time-window of protection* (fig. 3).
A possible explanation for the delay of cell death (either by necrosis or apoptosis) is that ischemic preconditioning also reduces the energy demand during the early phase of a subsequent ischemic episode thereby resulting in less accumulation of glycolytic catabolites and slowing down of ATP depletion. The mechanism by which a decreased anaerobic glycolysis exactly determines the extent of ischemic injury remains unclear.

Another possible characteristic of preconditioning is the reduction in ischemia-reperfusion arrhythmias in rats[77-79] and rabbits.[80,81] In dogs however, results have been conflicting.[79,82] The situation is further complicated in the pig model in which brief antecedent ischemia appears to be proarrhythmic.[83] Nevertheless, there is evidence which demonstrate an antiarrhythmic protection in humans.[84-86]

Finally, ischemic preconditioning also reduces postischemic contractile dysfunction[87,88] which is most likely reflected by reduced infarct size[38,89] since it does not attenuate myocardial stunning.[90,91,92] Focusing on the heart as the target to protect, reduction of infarct size will remain as "the gold standard" of protection.

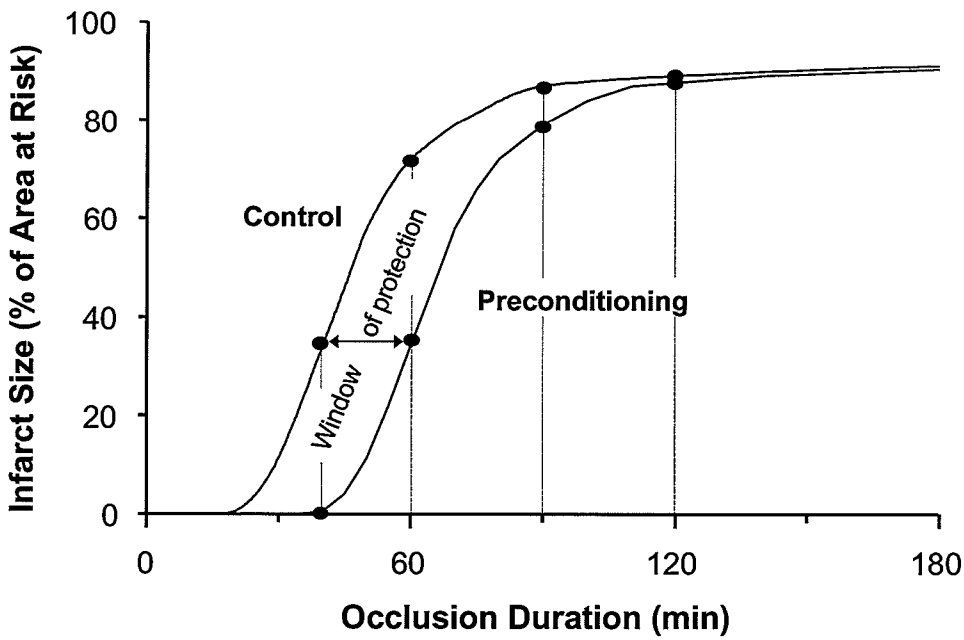


Figure 3 *Schematic illustration of the time window of protection showing that ischemic preconditioning results in a rightward shift of the infarct size – time relation indicating a delay in development of cell death. Infarct size by prolonged coronary occlusions exceeding two hours, is equally severe to non-preconditioned myocardium.*

***Ischemic- and non-ischemic myocardial preconditioning* stimuli**

In the aforementioned study by Murry et al.,[15] ischemic preconditioning was elicited by abrupt coronary occlusion and reperfusion in normal non pathologic arteries which does not reflect the clinical setting. However, Ovize et al.[93] demonstrated that partial stenosis causing 50% coronary flow reduction for 15-min, was able to confer cardioprotection. In that study reperfusion between the ischemic stimulus and the prolonged total coronary occlusion was a necessity to obtain infarct size reduction. Using the Harris two-stage model, Koning et al.[94] observed in the pig that cardioprotection can be initiated without intermittent reperfusion provided that the ischemic stimulus is sufficiently severe. Thus a 70% coronary flow reduction lasting for 30-min immediately followed by a 60-min total occlusion resulted in infarct size reduction. However, moderate flow reduction of 30% did not show any protective effect. Finally, Ito et al. reported cardioprotection after gradual progressive coronary flow reduction.[95] These findings clearly suggest that the severity and/or duration of the flow reduction play a pivotal role in triggering the protective effect.

In addition to brief myocardial ischemia, several other non-ischemic triggers have been shown to initiate myocardial preconditioning. For instance, acute left ventricular volume overload leading to *ventricular stretch* protects the myocardium against a subsequent prolonged ischemia. Stretch-induced cardioprotection evolves most likely through protein kinase C and opening of ATP-sensitive K^+ channels.[96] In addition, Koning et al. increased heart rate up to 200 bpm in the porcine heart by electrical *ventricular pacing* which was also able to induce protection against myocardial ischemia. Pacing lasted for 30-min without developing ischemia. Cardioprotection was completely abrogated by pretreatment with the ATP-sensitive K^+ blocker glibenclamide suggesting a K^+ channel involved mechanism.[97] Apart from changes in collateral flow and protein kinase C activation, preconditioning was mimicked by 5 brief episodes of tachycardia in the canine, which was mediated by adenosine. Finally, *heat stress* by a 15-min period of 42° C hyperthermia has also been shown to trigger myocardial preconditioning via opening of ATP-sensitive K^+ channels. Thus, the mechanism by which non-ischemic stimuli elicit cardioprotection is very similar to the mechanism of ischemic stimuli.

Many investigators have attempted to unravel the mechanism of this protective phenomenon for pharmacological exploitation in the clinical setting. Agents that activate components involved in the mechanism of ischemic preconditioning can also initiate cardioprotection after oral or intravenous administration. As part of unraveling the mechanism, several *pharmacological agents* like adenosine,[98,99] bradykinin,[100] opioids[101] norepinephrine,[102] protein kinase C activators[103,104] and ATP-sensitive K^+ channel openers[105,106] have demonstrated a protective effect when administered before sustained ischemia which is confirmed in all species used for experimental study.

Remote Preconditioning

In the above-mentioned studies, both the ischemic stimulus and the sustained ischemia were applied to the same myocardial region. Therefore, Przyklenk et al. investigated the concept whether a brief ischemic stimulus applied in one coronary vascular bed protects adjacent naïve myocardium from subsequent sustained ischemia.[107] In this study, dogs were assigned to undergo 4 episodes of 5-min occlusions of the circumflex coronary artery followed by one hour of prolonged ischemia by occlusion of the left anterior descending artery. Indeed, infarct size was significantly reduced when compared to sustained occlusion in the non-

preconditioned group. This concept of cardioprotection at a distance, further referred to as *intra-cardiac remote preconditioning* can be explained by the activation of stretch-activated channels, since severe myocardial dysfunction leads to stretch of the adjacent non-ischemia myocardium.[96] However, it is also possible that the protection could be due to the transfer of protective factor(s) via collaterals connecting both territories.[108] Other possible proposed means of transport are diffusion[109] and communication via cardiac gap-junctions. Some reports suggest that gap-junction communication plays a role in conventional preconditioning.[110,111] Most interestingly, the second messenger IP3 which has been shown to be increased during classical ischemic preconditioning is also known to traverse gap-junctions.[112]

Since Przyklenk et al. were able to elicit cardioprotection in adjacent myocardium via remote coronary artery occlusion, other investigators extended the concept of preconditioning at a distance by applying the brief ischemic stimulus into a remote organ or vascular bed. This concept of *inter-organ remote preconditioning* was first suggested by a preliminary study of McClanahan and colleagues who reported that cardioprotection in the in vivo rabbit model by a transient 10-min occlusion of the left renal artery was similar in reducing infarct size to preconditioning by 5-min of brief coronary artery occlusion.[113] Subsequently, Gho et al.[114] showed in the anesthetized rat model in which body core temperature was maintained constant, that remote preconditioning by brief 15-min mesenteric artery occlusion effectively limited infarct size caused by a 60-min sustained coronary occlusion. This inter-organ preconditioning of the heart has been further confirmed for the small intestine,[115-119] kidney[120-122] and skeletal muscle.[123] Nevertheless, not all remote organs might be able to precondition the heart at a distance since de Zeeuw et al.[124] did not find any infarct size reduction after coronary artery occlusion when preceded by global cerebral ischemia. Although very limited, several reports demonstrate involvement of a delayed phase via endogenous calcitonin-gene-related peptide in a nitric oxide dependent pathway[125] and the activation of capsaicin-sensitive sensory nerves.[115]

The precise mechanism underlying inter-organ preconditioning is still incompletely understood. In the study by Gho et al. cardioprotection was abolished when the animals were pretreated with the ganglion blocker hexamethonium, indicating that the protective mechanism is transported via a neurogenic pathway rather than humoral factors. Another fundamental issue is the importance of intervening reperfusion of the remote organ after the ischemic and prior to the coronary artery occlusion. In the same study, Gho et al. observed that permanent mesenteric artery occlusion without interspersing reperfusion failed to limit infarct size to a subsequent 60-min coronary occlusion suggesting that reperfusion of the small intestine was mandatory, presumably to wash-out mediators from the remote organ to activate the neurogenic pathway. However, the specific mediators remain poorly characterized. There is emerging evidence that inter-organ preconditioning is triggered by the release of adenosine, bradykinin and/or endogenous opioids from the remote organ. Thus, recent studies suggest that cardiac preconditioning by brief renal and intestinal ischemia may be achieved via adenosine receptors,[120,121] activation/translocation of PKC,[118,119] and opening of mitochondrial and/or sarcolemmal K_{ATP} channels.[120] Recent data on hind-limb ischemia by infra-renal aortic occlusion showed involvement of the δ1-opioid receptor as well as PKC activation, but in contrast to small intestinal ischemia, cardiac preconditioning by brief hind-limb ischemia may be transmitted by a humoral pathway rather than a neurogenic pathway.[126] If humoral factors are able to elicit remote preconditioning, it may be possible to transfer the protection at a very great distance via collection and transfusion of coronary effluent. Dickson et al.[127,128] therefore explored in the isolated rabbit heart whether coronary effluent released during 3 brief ischemic

preconditioning stimuli of global ischemia when reoxygenated and administered to naïve donor acceptor hearts can trigger cardioprotection. Indeed, Dickson observed that the acceptor hearts exhibited infarct size limitation after receiving donor coronary effluent released during preconditioning stimuli when compared to the donor control group. The mechanism responsible for *transferred remote preconditioning*, most likely humoral factors, remains incompletely understood. Both adenosine and/or norepinephrine have been proposed as the responsible humoral factors.[128]

Mechanism of the signal transduction pathway

Since ischemic preconditioning exerts a very powerful anti-infarct effect, elucidation and manipulation of the signal transduction pathway could lead to development of anti-infarct therapy. By gaining substantial understanding of how cells can protect themselves against ischemic injury we may be able to develop agents capable of directly limiting cellular injury. Ever since its first report by Murry et al. in 1986, many efforts have attempted to unravel the mechanism behind the transduction pathway of preconditioning including *triggers* and *mediators* in the early phase, and potential *end-effectors* in the late phase of ischemic preconditioning.[129] Triggers may be important during preconditioning ischemia and reperfusion. Mediators may be important during the prolonged index ischemia. As aforementioned, mechanisms of the first window of protection and the second window are not entirely similar. This thesis will mainly focus on classic preconditioning i.e. the first window of protection.

Trigger phase

Protection by preconditioning is receptor mediated. Several humoral factors released during preconditioning ischemia initiate the cardioprotective effect by binding to their respective receptors. These metabolites and by products are *adenosine,*[130,131] *bradykinine,*[100,132,133] *opioids,*[134-136] *acetylcholine,*[137] *norepinephrine,*[138,139] *angiotensine,*[140] *endothelin*[141,142] and *free radicals.*[143,144] All receptors are linked to inhibitory G-proteins and *phospholipases C and/or D*. Phospholipase C catalyzes the hydrolyses membrane inositol-containing phospholipids into inositol triphosphate and *diacylglycerol* which stimulates the translocation and activation of *protein kinase C*. Many investigators have reported that adenosine plays the major key role in triggering the preconditioning response, but it is hypothesized that bradykinin, opioids and free radicals share their triggering role in addition to adenosine reaching a threshold together.[66]

The *role of nitric oxide* in ischemic preconditioning has not yet been precisely clarified, but it has been reported that blockade of nitric oxide synthase attenuates the increased functional recovery in ischemia/reperfusion injury in rats.[145,146] However, several studies in Bolli's laboratory demonstrated that nitric oxide seems to be of major importance in the second window of protection.[147]

The *flux of calcium* through L-type calcium channels has been in dispute as a potential trigger. Atrial muscle from patients with blockade of L-type calcium channels failed to be preconditioned by ischemia.[148] Nevertheless, the calcium antagonist nisoldipine failed to abrogate cardioprotection in the anesthetized pig.[149]

The importance of each receptor may depend on the species and the induced stimulus. An increase of interstitial adenosine concentration occurs in rats,[150] rabbits,[151] dogs,[152] and pigs.[133] Blockade of A_1- and A_3- but not A_2-receptors abolishes the infarct size limitation.[130,153,154] In the rat however, involvement of adenosine in ischemic preconditioning is still unclear. Kuzmin et al. demonstrated an increase of interstitial adenosine levels during regional ischemia and reperfusion in the rat.[150] In contrast, several studies using adenosine-receptor antagonists were not able to abrogate preconditioning in rats.[60,155] A study by Li and Kloner, failed to block cardioprotection with the non-selective adenosine receptor antagonist 8-SPT by a triple 3-min ischemic stimulus.[156] Therefore Ganote and Armstrong recently concluded that there is now general agreement that, in contrast to other species used for experimental research, adenosine does not play a role in myocardial infarct size limitation in rats.[157] Bradykinin, clearly demonstrates an anti-infarct effect. The increase in the interstitial bradykinin concentration occurs earlier than the interstitial adenosine levels in pigs.[133] It is therefore a major trigger during ischemic stimuli of shorter durations while during more prolonged ischemic stimuli adenosine is more important.[100,133]

During hypoxia or ischemia-reperfusion, mitochondrial respiration and oxidative phosphorylation are gradually uncoupled resulting in an increase of free radical production.[158] Free radicals not only damage cardiac myocytes[159] but also act as triggers of ischemic preconditioning. Infusion of N-2-mercaptoglycerine an antioxidant or dimethylthiourea a radical scavenger both block the protection by ischemic preconditioning.[143,144] It is still elusive how exactly free radicals can trigger a preconditioning effect but they are known to activate G-proteins,[160] protein kinases[144] and ATP-sensitive potassium channels.[161]

Mediator phase

Studies in rabbits by Ytrehus et al.[103] and rats by Mitchell et al.[104] simultaneously concluded that *protein kinase C activation* is central to the protection by ischemic preconditioning. Activation of protein kinase C is physiologically mediated by liberation of diacylglycerol, which binds to the regulatory subunit of protein kinase C. In rabbits, blockade of protein kinase C alone with chelerythrine completely abolishes the infarct size reduction triggered by an ischemic stimulus.[162] But in rats, dogs and pigs the results of pharmacological blockade are still controversial.[163-165] In the pig, the selective protein kinase C inhibitor staurosporine was not able to block myocardial preconditioning.[165] Ping et al.[166] identified 11 protein kinase C isozymes in the rabbit heart. Using specific antibodies for each isozyme she demonstrated that 4 cycles of reperfusion interspersed by 6-min reperfusion caused *translocation* of only two isozymes namely ε and η suggesting it was one of these two isozymes that contributed to cardioprotection.[163] It is now generally accepted that isozyme translocation of protein kinase C either to the sarcolemma or the surface of mitochondria, does occur during ischemic preconditioning further conducting the signal transduction pathway by a phosphorylation cascade.

Events downstream have currently been the main focus of investigation. In the rabbit, administration of tyrosine kinase antagonists such as genistein or lavendustin A aborted ischemic preconditioning's protection in the rabbit.[167] Since both genistein and lavendustin A can block the protection induced by PMA, a direct activator of protein kinase C, the involved *tyrosine kinase* is most likely downstream from protein kinase C.[167] In rabbits, protein kinase C and tyrosine kinase appears to be in series. In other species however, tyrosine kinase may also act in a parallel pathway bypassing protein kinase C. Vahlhaus and coworkers failed to

abrogate cardioprotection from ischemic preconditioning with an antagonist of protein kinase C or tyrosine kinase alone. Yet, combined blockade with both genistein and staurosporin completely eliminated the protection, suggesting the existence of a parallel pathway next to protein kinase C containing at least on tyrosine kinase residue. This parallel pathway may be similar in rats and dogs.[168,169] In rats, multiple cycles of preconditioning can overcome the abrogation of protection by protein kinase C blockers.[168]

Another potential downstream target in addition to protein kinase C and tyrosine kinase are the *mitogen-activated protein kinase (MAPK)*. Each subfamily: *ERK*, *JNK* and *p38* have been suggested to play a major part in the preconditioning transductionpathway.[170,171] The MAP-kinase family is activated by dual phosphorylation of a serine or threonine residue by a MAP kinase kinase and can translocate into the nucleus.[170] The MAP kinase kinase appears to be the tyrosine kinase in the signaling transduction pathway.[172] Most attention has focused on p38 MAP kinase. The selective inhibitor SB203580 can block the preconditioning effect in isolated rabbit cardiomyocytes suggesting p38 mediates its protection.[172] However, in isolated rat hearts[173] and pigs in vivo,[174] blockade of p38 does not affect the infarct limiting effect by ischemic preconditioning. Following ischemic preconditioning, phophorylation of p38 during index ischemia has been reported to be increased in isolated rat- and rabbit hearts, unaltered in pig hearts in vivo and even decreased in dog hearts. Explanations for the controversial findings might be due to the different isoforms of p38 in different species.

Potential end effectors

Although many investigators have speculated on the elusive end-effector, to date this is still an ongoing debate. A possible mechanism for the infarct size limitation by ischemic preconditioning is the reduction of energy demand by slowing down ATP consumption during index ischemia.[175] In addition, a reduced accumulation of ischemic catabolites such as lactate resulting in an decreased intracellular acidification reduction has been reported.[176] This reflects a *reduced anaerobic glycolysis.*

Whether *protein synthesis* is required to mediate the first window of protection in ischemic preconditioning is controversial. A transcriptional blockade seems not have any affect,[177] but a blockade at the translational level was able to abolish preconditioning.[178,179] Furthermore, Xiao and Allen have suggested that the *sodium-proton exchanger* could be the end-effector. They noted that the sodium-proton exchanger was blocked during reperfusion when rat hearts were preconditioned.[180] In rabbits, blockade of the sodium-proton exchanger limited infarct size to a similar extent as an ischemic stimulus.[181] Both several kinase inhibitors and ATP-sensitive potassium channel blockers were able to abrogate this protection suggesting that the sodium-channel exchanger must be operational at a distant point in the signal transduction pathway.

Prolonged ischemia can cause cytoskeletal disruption.[182] The small *heat shock protein HSP27* is known to regulate actin filament organization and stabilizes actin filaments during oxidative stress.[183] In isolated cardiac rat myocytes, overexpression of HSP27 confers protection to ischemia.[184] It appears that redistribution of heat shock proteins from the cytosol to the sarcomere followed by alteration in the stability of the cytoskeleton is important for triggering cardioprotection by ischemic preconditioning.[185,186]

During normal conditions the osmolarity of bodyfluid and cellular osmotic balance is maintained by matching the osmotic pull of proteins and nucleotides within the cell and by sodium outside the cell. During ischemia, ATP breaks down to AMP and inorganic phosphates

hereby tripling the osmotic pull leading to osmotic imbalance with cellular swelling of the ischemic cells. In preconditioned rat and pig hearts, the extent of myocardial edema is reduced along with infarct size.[187,188] *Chloride channels* are involved in volume regulation opening the possibility that opening of swelling induced chloride channels are responsible for the protection of ischemic preconditioning.[189] Furthermore, inhibition of the *sodium-hydrogen exchanger* may also prevent cell swelling. Finally, *connexin 43* concentration, which regulates cell volume, may be maintained by preconditioning since loss of connexin 43 occurs during myocardial ischemia.[190] All the above-proposed end-effectors however, resulted in confounding results leading to speculation rather than clear conclusions.

Gross and Auchampach were the first to propose that ATP-sensitive potassium channels mediate the effect of ischemic preconditioning based on pharmacological studies. In canine hearts ATP-sensitive channel openers like cromakalim and bimakalim mimicked cardioprotection whereas channel blockers, such as glibenclamide and 5-hydroxydecanoate abort the protection.[191] Two distinct ATP-sensitive potassium channels have been identified in cardiomyocytes: the sarcolemmal and mitochondrial channels each with a distinct pharmacological profile. Since Gross used glibenclamide blocking both channels, it was originally assumed that *sarcolemmal ATP-sensitive potassium channel opening* was the end-effector by shortening of the action potential duration which can possibly lead to a lower metabolic demand and less Ca^{2+} overload. Subsequently, Grover and coworkers demonstrated that ATP-sensitive potassium channel opening by either BMS-180448 and cromakalim were also able to exert cardioprotection independent of any action potential shortening.[192,193] The class III antiarrhythmic agent dofetilide abolished action potential shortening without affecting the protection of preconditioning.[194] Additionally, HMR-1883 which selectively closes sarcolemmal potassium channels had no affect on infarct size limitation in preconditioned rabbit hearts.[195] Both Marban and Grover groups simultaneously provided convincing evidence that it was indeed not the surface potassium channel, but the *mitochondrial ATP-sensitive potassium channel* that was involved in the protection.[196-198] They found that the selective mitochondrial potassium channel opener diazoxide was as cardioprotective as ischemic preconditioning without affecting the sarcolemmal potassium channels. 5-hydroxydecanoate reversed this protection. For a while it was generally accepted that the mitochondrial ATP-sensitive potassium channels were the end-effectors. Nevertheless, it has been difficult to understand how opening of mitochondrial ATP-sensitive potassium channels exerts cardioprotection, therefore many investigators are currently questioning whether these channels are indeed end-effectors rather than mediators or even triggers.

Role of mitochondria in cardioprotection

Mitochondrial function during normal conditions

Under normal conditions, myocardial oxygen supply and expenditure are tightly coupled, from the point of energy production in the inner membrane of the mitochondria to the point of energy utilization within cellular sites. Reducing equivalents are generated from sequential breakdown of substrates and transferred in the form of NADH and FADH2. Oxygen serves as the ultimate electron recipient in the electron transport chain, and the overall reaction results in 1.5 moles of ATP per mole of NADH and 2.5 moles of ATP per mole of FADH2 when transferred to O_2. It is within the inner membrane of the mitochondria that the biochemical reactions of oxidative phosphorylation result in the transfer of electrons from

reducing agents, by virtue of differences in oxidation-reduction potentials. As electrons are transferred between complex I and complex IV, protons are translocated across the inner membrane of the mitochondria and a proton gradient is established. A total of 10 protons are "pumped" across the membrane in the transfer of two electrons from NADH to O_2. It is by this series of proton pumps that the matrix becomes more alkaline relative to the external surface of the inner membrane, creating an electrochemical gradient ($-\Delta\Psi$). This electrochemical energy is sufficient to drive ATP synthesis from ADP in complex V (fig. 4).

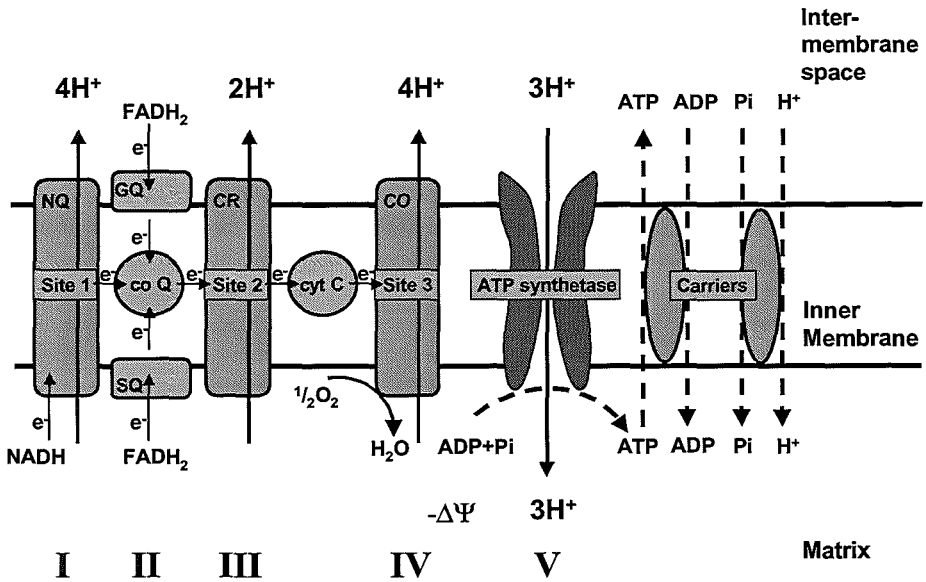


Figure 4 Overview of the complexes I-V and pathways of electron transfer in mitochondrial electron transport chain. NQ indicates NADH-co-Q- reductase; GQ, glycerol-3-P co-enzyme Q reductase; co Q, co-enzyme Q; SQ, Succinate-co-enzyme Q reductase; CR, co-enzyme Q-cytochrome c reductase; CR, Cytochrome c; CO, cytochrome c oxydase

Mitochondria and ischemic preconditioning

The preservation of mitochondrial function in ischemic preconditioning has been well recognized by the observation that the rate of ATP loss was reduced during the subsequent prolonged period of ischemia versus control hearts.[175] Similarly, a study by Fryer et al.[199] demonstrated that isolated mitochondria from the area at risk of preconditioned hearts had higher rates of ATP synthesis compared to those of hearts subjected to prolonged ischemia without ischemic preconditioning stimulus. This preservation of mitochondrial function was abrogated by 5-hydroxydecanoate, supporting an improved ATP production as a common feature of ischemic preconditioning and mitochondrial ATP-sensitive potassium channel opening. In addition, ischemic preconditioning has been shown to suppress mitochondrial cytochrome-c release and apoptosis during ischemia-reperfusion.[200] Most recently Baines et al. demonstrated that PKCε translocates to the surface of the mitochondria[201] and can form physical interaction with the mitochondrial transition pore[202] which are also important clues that the mitochondria play a critical role in the infarct-sparing effects of preconditioning.

Mitochondrial ATP-sensitive potassium channels

No previous study had defined the timing for the opening of mitochondrial ATP-sensitive potassium channels. Therefore, Pain et al.[203] determined the critical timing for these channels to open in order to protect the heart. Infarct size limitation by either diazoxide or an ischemic stimulus were both abolished by free radical scavenging or potassium channel blockade during the trigger phase, whereas when administered during the mediator phase (i.e. index ischemia) cardioprotection was still maintained. Pain subsequently proposed that opening of ATP-sensitive potassium channels during either preconditioning ischemia or diazoxide infusion generates free radicals, which then trigger cardioprotection most likely by activating several kinase proteins. The protective role of free radicals has also been striking in the role of various agonists activating cell surface receptors involved in the signaling cascade. Acetylcholine when added to chick cardiomyocytes exerted a burst of free radical production which was blocked by both myxothiazol, an inhibitor of mitochondrial electron transport, and by 5-hydroxydecanoate.[204] The investigators suggested that acetylcholine leads to opening of mitochondrial ATP-sensitive potassium channels immediately followed by the release of free radicals which was further confirmed in intact hearts.[205-207] In addition, bradykinin, opioids and phenylephrine all appear to trigger ischemic preconditioning via a pathway that requires opening of mitochondrial ATP-sensitive potassium channels and release of free radicals.[205] But in contrast to other agonists, the triggering action of adenosine seems to be independent of opening potassium channels and release of free radicals. Cohen et al.[205] showed that the protective action of exogenous adenosine was not affected by either mercapto propionyl glycerine or 5-hydroxydecanoate. It appears that all agonists are not equivalent and may work via separate pathways in order to exert myocardial preconditioning (fig. 5).

The hypothesis that the mitochondrial ATP-sensitive potassium channels play a major role in both the first window- and the second window of protection has been well supported by an accumulating body of evidence.[208] However, to date the mechanism in exerting cardioprotection has been incompletely understood. It seems paradoxical that the opening of an energy dissipating cation conductance on the mitochondrial inner membrane would be beneficial. Tight coupling between proton pumping and ATP production requires a relatively impermeable inner membrane and therefore opening of mitochondrial ATP sensitive potassium channels most likely lead to changes in mitochondrial function that compensate the loss of energy attributable to uncoupling. At least three mechanistic hypotheses have been proposed to explain the protective effect of mitochondrial ATP-sensitive potassium channel opening: *(i) release of free radicals.* Mitochondria are a source of free radical production because of leakage from the electron transport chain. Free radicals have been associated with cellular damage during ischemia and reperfusion.[209] On the other hand, free radicals have also been demonstrated to trigger ischemic preconditioning.[144] Therefore, opening of mitochondrial ATP-sensitive potassium channels could possibly enhance free radical production during early ischemia to trigger cardioprotection, or it could inhibit release during reperfusion. *(ii) Reduction of mitochondrial Ca^{2+} overload.* During ischemia or reperfusion, mitochondrial Ca^{2+} overload may be slowed down by depolarization of the mitochondrial membrane potential. In isolated mitochondria, ATP-sensitive potassium channel opening by diazoxide depolarized the mitochondrial baseline resting membrane potential by 15 to 20 mV which results in a decreased driving force for Ca^{2+} entry.[210] *(iii) Mitochondrial matrix swelling leading to an optimized energy production.* Opening of mitochondrial ATP-sensitive potassium channels induces an influx of potassium, which is accompanied by the movement of diffusible weak

acids in order to maintain electro neutrality and water movement attributable to osmotic forces. The netto result of this process is matrix swelling.[211] Matrix swelling has been suggested to improve mitochondrial function by activating fatty acid oxidation, respiration and ATP production.[212] In addition, it has also been theorized that that optimal efficiency of oxidative phosphorylation is achieved when mitochondria are partially uncoupled which may be induced by mitochondrial uncoupling proteins and opening of ATP-sensitive potassium channels.[213,214]

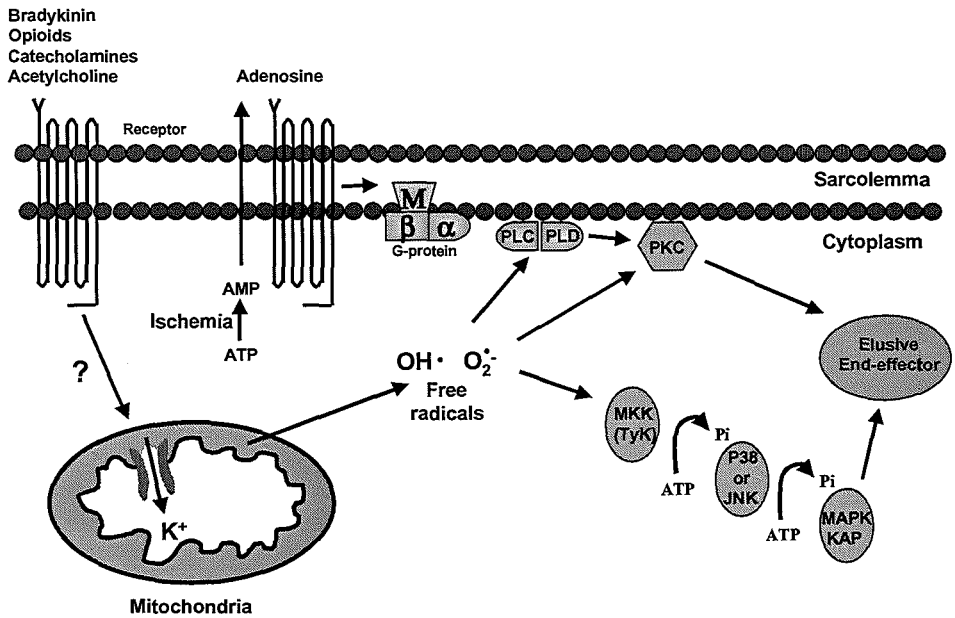


Figure 5 Hypothetical model for separate signal transduction pathways of pharmacological preconditioning. It appears that bradykinin, actylcholine and opioids somehow interact with mitochondria to produce free radicals that then go on to initiate a kinase cascade. However, adenosine seems to induce preconditioning independent of free radicals and mitochondrial ATP-sensitive potassium channels and may therefore activate phospholipases and protein kinase C. PLC indicates phospholipase C; PLD, phospholipase D; PKC, protein kinase C; TK, tyrosine kinase; JNK, c-Jun N-terminal kinase; MAPKAPK2, mitogen-activated protein kinase 2 (modified from Cohen et al[205])

Tolerance to multiple episodes of ischemic preconditioning

Pharmacological exploitation of ischemic preconditioning to limit myocardial infarct size in the clinical setting appears highly attractive. However, the therapeutical application may be limited by the development of tolerance to cardioprotection by repetitive brief ischemic stimuli. Most studies have demonstrated that repeated bouts of brief ischemic stimuli do not yield a cumulative cardioprotective effect.[215,216] Contrary to providing additive protection, there may be an apparent loss in efficacy of preconditioning with multiple cycles of ischemic stimuli. Thus in a conscious rabbit model, Cohen and coworkers[217] demonstrated that myocardial protection to prolonged ischemia by a single ischemic stimulus of 5-min coronary artery occlusion is waned when preceded by 40 to 65 5-min occlusions over a three to four day period.[217] The study by Cohen did not discriminate between the first window of protection and the second window since induction period lasted for three days. However, development of

tolerance has been demonstrated to occur for the first window of protection. In the rabbit, six to eight repeated bouts of brief 5-min ischemia has been reported to result in a loss of efficacy.[218] The study by Cohen further showed that if the multiple ischemic stimuli were followed by a 3-day rest interval, cardioprotection could be re-established.[217] To date, the mechanism underlying development of tolerance to repetitive ischemic stimuli is poorly understood. Several studies either in rabbits or pigs suggested progressive loss of adenosine production[219] and /or reduced adenosine receptor responsiveness.[220]

Aim and outline of the thesis

To obtain a better understanding of myocardial ischemia-reperfusion injury and to advance therapeutical application of cardiac adaptation to ischemia, this thesis investigates several mechanistic aspects of ischemic preconditioning with respect to triggers, mediators and possible end-effectors.

Adenosine is a well established trigger of ischemic preconditioning which is released during ischemia as a breakdown product of ATP. At least four adenosine receptor subtypes have been identified: A_1-, A_{2a}-, A_{2b}- and A_3- receptors.[221] Both the A_1- and the A_3- receptor subtypes are believed to trigger myocardial preconditioning.[131,153,222] The cardioprotective role of adenosine has been confirmed in all species used for experimental investigation.[38] However, based on several studies using selective adenosine receptor antagonists which failed to block ischemic preconditioning, its role in rats is still controversial.[155,156] In these studies the duration of the ischemic stimuli were 3-5 min. Interestingly, Schulz et al.[133] demonstrated in the porcine heart that adenosine plays a significant role after 10-min of coronary artery occlusion. Therefore in *chapter 2* we studied the role of adenosine in ischemic preconditioning in rats with respect to the duration of the ischemic stimulus.

There are current indications that signal transduction pathway(s) exclusive of ATP sensitive potassium channel opening are able to induce ischemic preconditioning.[205,223] In contrast to bradykinin, acetylcholine, opioids and phenylephrine, it appears that adenosine exerts myocardial preconditioning via a separate transduction pathway which does not involve opening of ATP sensitive potassium channels and release of free radicals.[205] Since there are signs that separate pathways might co-exist upon triggering ischemic preconditioning, in *chapter 3* we study whether an adenosine-dependent ischemic preconditioning stimulus exerts cardioprotection without opening ATP sensitive potassium channels and release of free radical, which are proximal to tyrosine kinase and protein kinase C. In addition, we further investigate whether an adenosine-independent ischemic stimulus. Moreover, it has recently been proposed that slight mitochondrial uncoupling may be a key event during myocardial protection.[224] The aforementioned in vitro studies have demonstrated that not only ischemia, but also mitochondrial ATP-sensitive potassium channel opening resulted in slight uncoupling of mitochondria and cytoprotection. Additionally, superoxide has been reported to activate mitochondrial uncoupling proteins.[225] Therefore in chapter 3, we further studied whether the adenosine dependent and/or the adenosine independent ischemic stimulus lead to an uncoupled state of mitochondria.

Adenosine may also be involved in inter-organ remote preconditioning of the heart. Takaoka and co workers[121] demonstrated that adenosine concentrations were 10 times higher after renal ischemia, implying that myocardial adenosine receptor stimulation might be involved. Based on the observation by Gho et al. that remote preconditioning is induced via

activation of a neurogenic pathway upon reperfusion, several investigators suggested that adenosine released in the ischemic kidney stimulate afferent nerves in order to protect the myocardium.[120,205] In addition, both bradykinin and opioids have been shown to be involved in remote preconditioning. Nevertheless, the site(s) of action of these mediators has so far never been studied. Consequently in *chapter 4* we investigated the involvement of adenosine receptors, and which of its subtypes, in myocardial protection by brief intestinal ischemia. Furthermore, we determined the location(s), i.e. myocardium and/or small intestine, of the involved adenosine receptors.

Pharmacological exploitation of ischemic preconditioning to limit infarct size in a clinical setting is very promising. However, its therapeutical application may be limited by the development of tolerance to cardioprotection by repetitive brief ischemic stimuli. In isolated rabbit hearts it was shown that prolonged infusion with an A_1-adenosine receptor agonist results in desensitization of its protective effect which is accompanied with a loss of protection from ischemic preconditioning. In addition, Hashimi et al. concluded that tolerance to ischemic preconditioning after prolonged infusion of adenosine must be due to down regulation or desensitization of A_1 -adenosine receptors since both G_i and PKC components of the preconditioning pathways were still intact. Therefore it appears that tachyphylaxis may develop when many ischemic preconditioning stimuli are induced. In contrast to the loss of cardioprotection by Preconditioning after prolonged infusion with adenosine, the mechanism behind tolerance to ischemic preconditioning followed by multiple ischemic episodes has to date never been elucidated. In *chapter 5* we therefore elucidate the phenomenon of tolerance to ischemic preconditioning in greater detail.

Protein tyrosine phosphatase is known to trigger programmed cell death named apoptosis. Furthermore, apoptosis may play a pivotal role during myocardial ischemia-reperfusion injury. Vanadate is an ortho-molecule that inhibits tyrosine phosphatases resulting in an enhanced tyrosine residue phosphorylation. We therefore study in *chapter 6* whether the tyrosine phosphatase inhibitor vanadate induces cardioprotection to ischemia-reperfusion injury.

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Role of adenosine in ischemic preconditioning in rats depends critically on the duration of the stimulus and involves both A_1 and A_3 receptors.

Role of adenosine in ischemic preconditioning in rats depends critically on the duration of the stimulus and involves both A_1 and A_3 receptors

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Summary

Objectives: There is currently general agreement that adenosine is not involved in ischemic preconditioning (IP) in rat hearts. We hypothesized that the failure to show a role for adenosine is due to the use of brief preconditioning stimuli, and therefore investigated whether adenosine is involved when longer stimuli are employed and which receptor subtypes are involved.

Methods and results: Infarct size (IS) was determined in anesthetized rats after 180 min of reperfusion (REP) following a 60-min coronary artery occlusion (CAO). IS was 69±2% of the risk area in control rats and 45±2% ($P<0.05$) following IP by a single 15-min CAO. The non-selective adenosine receptor antagonist SPT, which itself had no effect on IS (74±1%), blunted the protection by IP (IS =57±2%, $P<0.05$) in a dose of 2x5 mg/kg IV, and abolished the protection (IS=70±1%) at 2x25 mg/kg IV. Following IP by 3 cycles of 3-min CAO and 3-min REP, IS was 24±6% ($P<0.05$), which was not affected by SPT in doses of 2x10 and 2x25 mg/kg IV. The A_3 antagonist MRS-1191 (3.3 mg/kg, intraperitoneally), which itself did not affect IS (70±2%), blunted the protection by IP with a 15-min CAO (IS=54±2%, $P<0.05$). When 2x5 mg/kg SPT (a dose selective for A_1-receptors, as it did not affect the protection by the A_3 selective agonist IB-MECA, 51±3%) and MRS 1191 were combined the protection by IP was abolished (IS=67±2%).

Conclusions: Involvement of adenosine in IP in rats depends critically on the duration of the stimulus. Thus, whereas adenosine was not involved when stimuli of 3-min duration were employed, activation of both A_1 and A_3 receptors contributed when a stimulus of 15-min was used.

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Introduction

The cardioprotective role of adenosine has been firmly established in all species in which this has been investigated. Adenosine has also been shown to be one of the mediators involved in the protection afforded by ischemic preconditioning (IP). However, based on numerous studies including those in which the selective adenosine A_1-receptor antagonist PD 115,199 and the non-selective antagonist SPT failed to block IP,[1-3] Ganote and Armstrong[4] conclude in a recent issue of this journal that there is now general agreement that adenosine does not play a role in the myocardial infarct size (IS) limitation by IP in rats. In these studies[1-3] the duration of the (multiple) IP stimuli was 3-5 min. Interestingly, Schulz et al.[5] have shown that intracoronary adenosine deaminase was ineffective in attenuating IS limitation by a 3-min coronary artery occlusion (CAO), but abolished the cardioprotection by a 10-min CAO. We therefore hypothesized that adenosine could play a role in IP in rats when preconditioning stimuli of longer duration are used. Consequently, we investigated the role of adenosine in IP by a 15-min CAO, a stimulus which we have previously shown to protect the rat heart,[6,7] and repeated the protocol of three cycles of 3-min CAO and 3-min REP used by Li and Kloner,[2] to exclude that differences in results were caused by differences in breed, sex and experimental procedures. Our results not only confirmed the findings by Li and Kloner[2] but also demonstrated that a high dose of SPT completely blocked the cardioprotection by a single 15-min CAO stimulus. Because SPT is a non-selective adenosine receptor antagonist, we subsequently investigated which of the adenosine receptor subtypes are involved in IP. This is of interest, because evidence is accumulating that not only selective A_1 but also selective A_3 agonists confer cardioprotection in a variety of animal models. For instance, the selective A_3 agonists APNEA and IB-MECA limit myocardial injury to a similar degree as IP in isolated rabbit cardiomyocytes[8], and in isolated[9] and *in vivo*[10] rabbit hearts. Since the affinity of adenosine for the A_3 receptor is 10-100 less than for the A_1 receptor, it has been questioned whether A_3 receptor activation is involved in the protection afforded by IP.[11] Studies on the role of A_3 receptors in IP *in vivo* have been hampered by lack of selective A_3 receptor antagonists.[4] However, MRS-1191 has recently been described as a highly selective A_3 receptor antagonist.[4,11] Consequently, we studied the contributions of A_1 and A_3 receptor subtypes to the protection by IP in the *in vivo* rat heart.

Methods

Experiments were performed in ad libitum fed male Wistar rats (~300 g) in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication 86-23, revised 1985) and with approval of the Animal Care Committee of the University.

Drugs

The following agents were used: the non-selective adenosine receptor antagonist 8-p-sulfophenyltheophylline (SPT); the selective A_1 agonist 2-chloro-N^6-cyclopentyladenosine (CCPA); the selective A_3 receptor agonist N-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA); the selective A_3 receptor antagonist MRS-1191 (3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4,-[±]-dihydro-pyridine-3,5-decarboxylase); the histamine H_1 antagonist mepyramine (Mep), and N,N-dimethylacetamide (DMAC) in which MRS-1191 was dissolved.

Surgical and experimental procedures

Pentobarbital-anesthetized (60 mg/kg) rats were intubated for positive pressure ventilation (Harvard rodent ventilator) with room air.[6,7] Through the carotid artery a PE-50 catheter was positioned in the thoracic aorta for measurement of arterial blood pressure and heart rate. In the inferior caval vein a PE-50 catheter was placed for infusion of Haemaccel (Behringwerke) to compensate for blood loss during surgery. After thoracotomy, via the left third intercostal space, the pericardium was opened and a silk 6-0 suture was looped under the left anterior descending coronary artery for later CAO. A catheter was positioned in the abdominal cavity to allow intraperitoneal administration of pentobarbital for maintenance of anesthesia. Rectal temperature was continuously measured and maintained at 36.5-37.5°C.[6,7] To prevent local heat loss from the thorax, the thoracotomy site was covered with aluminum foil. After completion of surgery, a 30-min stabilisation period was allowed before experimental protocols were carried out.

Rats that fibrillated were allowed to complete the protocol, provided that conversion to normal sinus rhythm occurred spontaneously within 1 min, or that defibrillation by gently thumping on the thorax or with a 9V battery was successful within 2 min after onset of fibrillation. Occlusion and reperfusion were visually verified.[6,7]

Experimental groups

Fig. 1 depicts the experimental groups in which IS was determined after 180-min REP following a 60-min CAO. Area at risk (AR) and infarct area (IA) were determined using Trypan blue and nitro-blue-tetrazolium staining (Sigma Chemical).[6,7] IS was expressed as IA/AR.

Protocol I: Does adenosine contribute to ischemic preconditioning in the rat heart?

One group underwent a 25-min sham period prior to the 60-min CAO followed by 180-min of REP (Control). To determine whether adenosine contributed to IP when stimuli of longer duration are used, we preconditioned three groups with a 15-min CAO followed by 10-min of REP. These groups received either no treatment (PC15), or were pretreated with intravenous (IV) SPT in doses of 2x5 mg/kg (PC15+10SPT) or 2x25 mg/kg (PC15+50SPT), respectively. To study the effects of SPT on IS per se, one group was pretreated with 2x25 mg/kg SPT IV prior to the 60-min CAO (Control+50SPT). To exclude that confounding factors such as breed (Sprague-Dawley[2] versus Wistar), sex (females[2] versus males) or experimental procedures (90-min CAO[2] versus 60-min CAO) contributed to differences between our observations and those by Li and Kloner,[2] we also determined in two groups whether 3 cycles of 3-min CAO followed by 3-min REP (PC3), protected the myocardium against infarction produced by a 60-min CAO and whether 2x10 mg/kg SPT (PC3+SPT20), the dose of SPT used by Li & Kloner,[2] or the high dose of 2x25 mg/kg SPT affected this cardioprotection.

Protocol II: Is A_3 receptor stimulation cardioprotective and does SPT in the doses used possess A_3-receptor antagonistic properties in the rat heart?

In two groups the effect of A_3 receptor stimulation on IS was studied by administration of IB-MECA in doses of 33 μg/kg (IB-MECA33) or 100 μg/kg (IB-MECA100), prior to the 60-min CAO and compared to the Control group from Protocol I to which two animals were added (Fig. 1). Since IB-MECA releases histamine from mast cells in rats,[10] another group was pretreated with 5 mg/kg Mep prior to IB-MECA and the 60-min CAO (IB-MECA100+Mep),

and compared to a group that received only 5 mg/kg Mep prior to the 60-min CAO (Control+Mep). To investigate whether SPT possesses A_3 receptor antagonistic properties, we pretreated rats with 2x5 mg/kg SPT prior to administration of 33 µg/kg IB-MECA (10SPT+IB-MECA33) or 100 µg/kg IB-MECA with Mep (10SPT+Mep+IB-MECA100) and studied the effect of 2x25 mg/kg SPT on the cardioprotection by 100 mg/kg IB-MECA in the presence of Mep (IB-MECA100+Mep+50SPT).

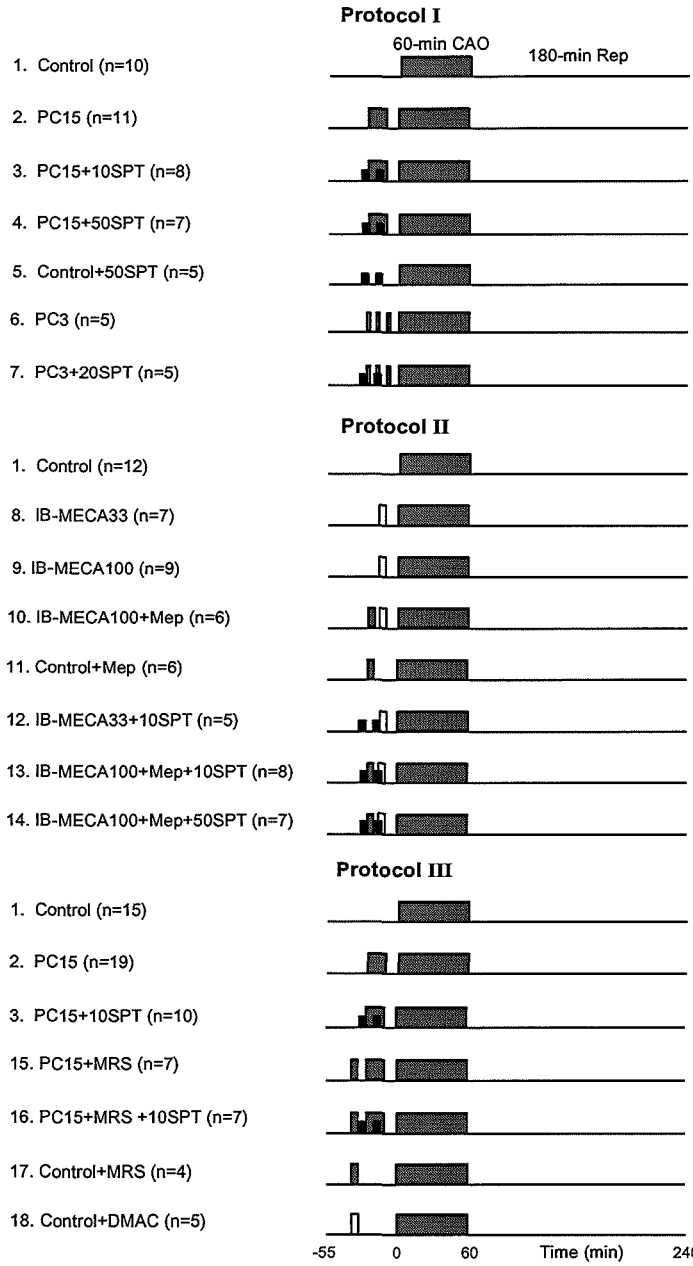

Figure 1 *Overview of the study protocols. Numbers in parenthesis indicate number of animals that completed the protocol.*

Protocol III: Are A_3-receptors involved in ischemic preconditioning in the rat heart?

Two groups received the 15-min CAO IP stimulus and 3.3 mg/kg MRS-1191 (intraperitoneally) without (PC15+MRS) or with 2x5 mg/kg SPT (PC15+MRS+10SPT). The results of these two groups were compared to the Control, PC15 and PC15+10SPT groups from Protocol I, to which a number of new animals were added (see Fig. 1). To exclude an effect of MRS-1191 or its solvent DMAC on IS per se, one group received 3.3 mg/kg MRS-1191 intraperitoneally (Control+MRS) and another group received the solvent DMAC (Control+DMAC) prior to the 60-min CAO.

MRS-1191 has been reported to be a highly selective A_3 antagonist, but *in vivo* experience with this compound is limited. Therefore, to exclude that A_1 antagonistic properties contributed to the actions of MRS-1191, we studied the bradycardic reponses to the selective A_1 agonist CCPA after treatment with MRS-1191 (n=6) or its solvent DMAC (n=5). Two consecutive doses (50 and 100 μg/kg, IV) of CCPA were administered. The second dose was given when heart rate had reached a stable level after administration of the first dose.

Data analysis and presentation

IS was analyzed by one-way ANOVA followed by Dunnett's test. Hemodynamic variables were compared by two-way ANOVA for repeated measures followed by the paired or unpaired *t* test. Statistical significance was accepted when $P<0.05$. Data are presented as mean±S.E.M.

Results

Mortality

Eleven rats were excluded because of sustained ventricular fibrillation or pump failure (2 in Control, 1 in PC15, 1 in PC15+10SPT, 1 in PC3+20SPT, 1 in PC3+50SPT, 2 in IB-MECA33, and 3 in Control+DMAC.

Infarct size - Area at risk

There were no differences ($P=0.61$) in AR (34±1%) between any of the experimental groups.

Infarct size - Effect of duration of preconditioning stimuli on the involvement of adenosine

Fig. 2 shows that IP by a 15-min CAO limited IS to 49±3% versus 70±2% in Control. SPT in a dose of 2x5 mg/kg attenuated the cardioprotection, while a 5 times higher dose, which by itself had no effect on IS, abolished the protection. Fig. 2 also shows that IP by 3-min CAO's limited IS to 24±6% and that SPT, not only in a dose of 2x10 mg/kg (confirming the observations by Li and Kloner[2]), but also in the high dose of 2x25 mg/kg, did not attenuate the cardioprotection (18±3%).

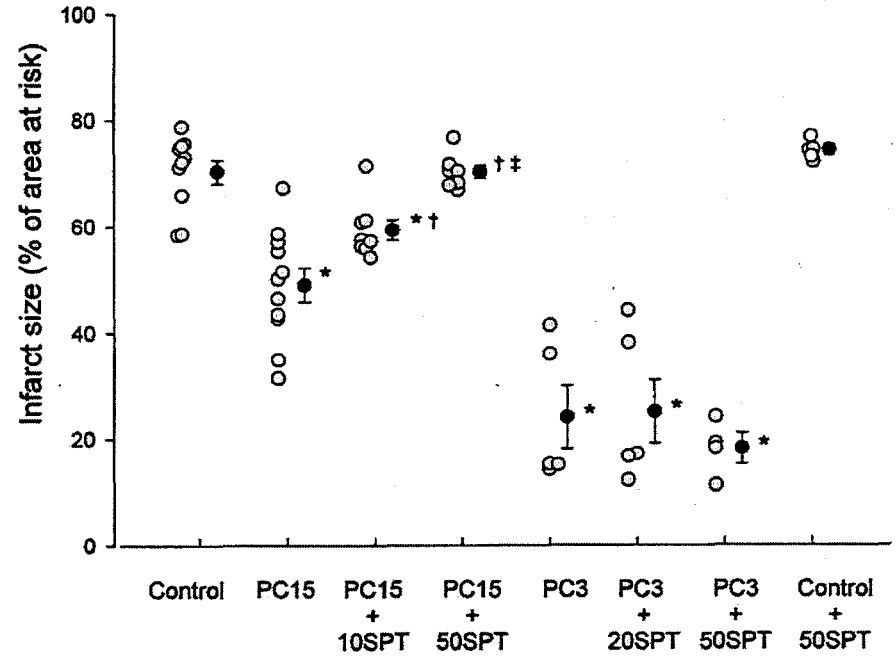

Figure. 2 *PC3 and PC15 (although less) limit infarct size. Note that only the protection by PC15 is attenuated by SPT. *P<0.05 vs Control; †P<0.05 vs IP; ‡P<0.05 vs IP+10SPT.*

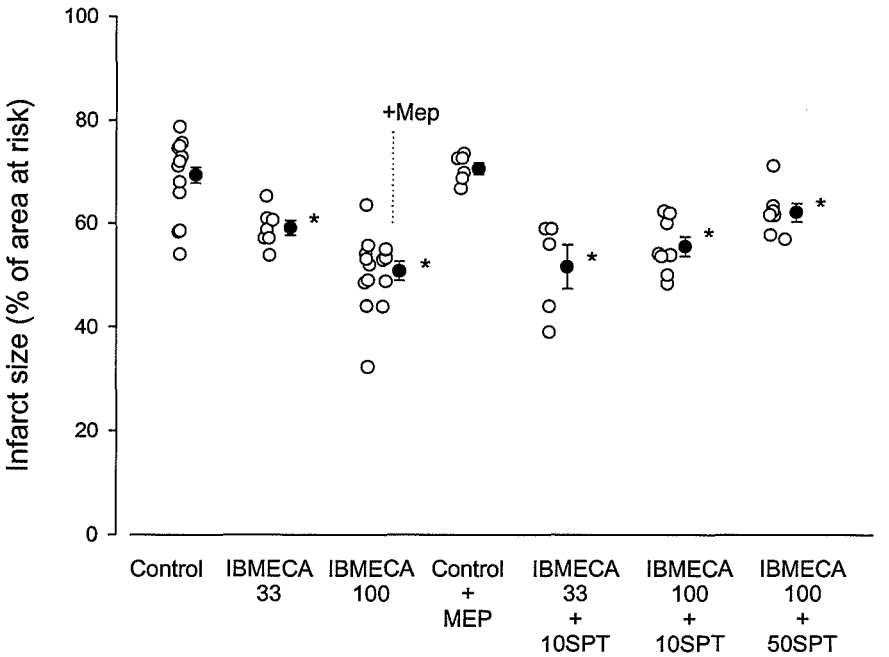

Figure 3 *Protection by IB-MECA is not affected by the low dose of SPT indicating that this dose is selective for A_1 receptors. In contrast, the high dose blunts the protection by IB-MECA100, indicating that this dose possesses A_3 antagonistic properties. * P<0.05 vs Control; †P<0.05 vs IB-MECA100.*

Infarct size - Selectivity of SPT and IB-MECA for A_1 and A_3 receptor subtypes

Pretreatment with IB-MECA limited IS to 59±1% and 51±3% at doses of 33 and 100 μg/kg, respectively (both $P<0.05$, Fig. 3). Mep blunted the IB-MECA-induced hypotension by 35%, but had no effect on IS limitation, indicating that histamine release did not contribute to the cardioprotection by IB-MECA. IB-MECA, in either dose, had no effect on heart rate (Table), which indicates that IB-MECA, in the doses used, was selective for the A_3 receptor. The low dose of SPT had no effect on the cardioprotection by either dose of IB-MECA, indicating that the low dose of SPT had no A_3 antagonistic effects. In contrast, the high dose of SPT blunted the cardioprotection by IB-MECA100 (62±2%, $P<0.05$), suggesting that SPT possesses A_3 antagonistic properties at this dose. For this reason, we employed the A_1 selective low dose of SPT in combination with MRS-1191 to study the role of A_1 and A_3 subtypes in IP in protocol III.

Infarct size - Adenosine subtypes involved in ischemic preconditioning

Cardioprotection by IP was reduced by approximately 50%, when animals were pretreated with either 3.3 mg/kg MRS-1191 or the low dose of SPT (Fig. 4). Combining SPT and MRS-1191 completely abolished the cardioprotection by IP.

To establish that the actions of MRS-1191 were indeed caused by A_3 antagonism, we studied the bradycardic response to the selective A_1 agonist CCPA in the absence and presence of MRS-1191. In the animals pretreated with DMAC, heart rate was lowered by 183±22 and 261±21 bpm by the 50 and 100 μg/kg dose of CCPA, respectively (Fig. 5). After pretreatment with 3.3 mg/kg MRS-1191, the CCPA-induced heart rate reductions (191±23 and 275±11 bpm, respectively) were not different from those in the presence of DMAC, indicating that at the

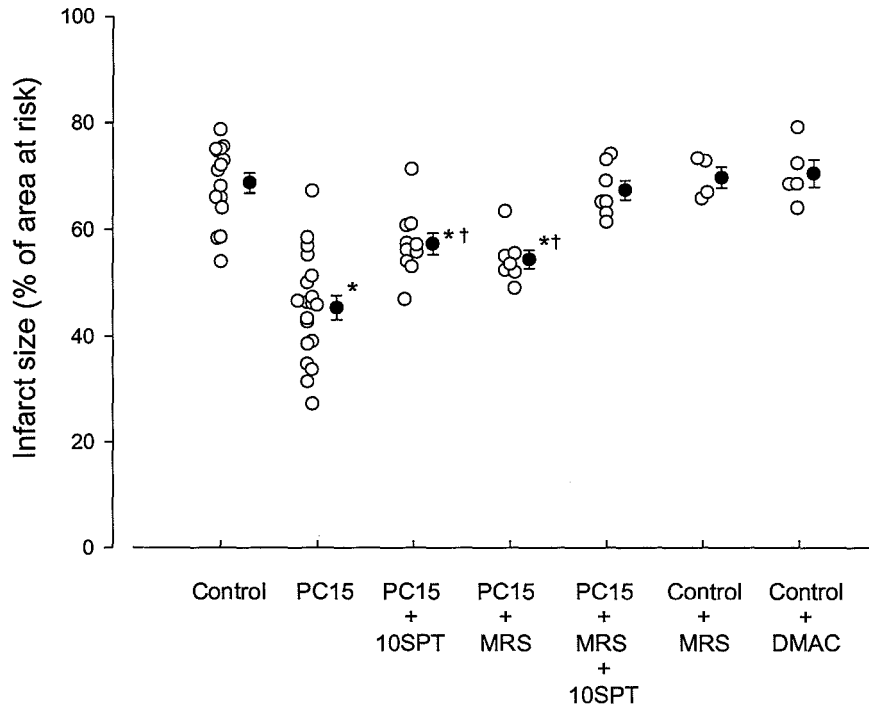

***Figure.** 4 Infarct size limitation by PC15 is attenuated by the low dose of SPT as well as MRS-1191, and abolished by combined pretreatment with the A_1 and A_3 antagonists. *$P<0.05$ vs Control; †$P<0.05$ vs IP; ‡$P<0.05$ vs IP+10SPT and IP+MRS.*

dose used, MRS-1191 had no A_1 antagonistic properties. In contrast, 3.3 mg/kg MRS-1191 produced a high degree of A_3 blockade as it attenuated the hypotension induced by 100 μg/kg IB-MECA by 70% (from 40±4 mmHg to 12±3 mmHg, n=7, $P<0.05$).

Heart rate and arterial blood pressure during 60-min CAO and 180-min REP

In all groups of Protocol I arterial blood pressure had decreased at the end of the 60-min CAO and did not recover during reperfusion (Table). Heart rate remained virtually unchanged, although it decreased slightly (10-15 %) in some, but not all, groups treated with SPT.

In the animals pretreated with IB-MECA, mean arterial pressure was lower than in the other groups of animals at the start of the 60-min CAO, which is most likely the reason for the smaller decrease in mean arterial pressure in these animals during the 60-min CAO, while heart rate remained virtually unchanged. In these animals, heart rate decreased slightly (5-10%) during 180-min REP. Mepyramine produced a 10% decrease in heart rate and blood pressure, but had no effect on blood pressure and heart rate response to the 60-min CAO and 180-min REP in any group.

Animals pretreated with MRS-1191 behaved similarly as the other groups of animals during the 60-min CAO and 180-min REP procedure. In these animals heart rate increased 10-15% after administration of MRS-1191, which was mostly likely due to its solvent.

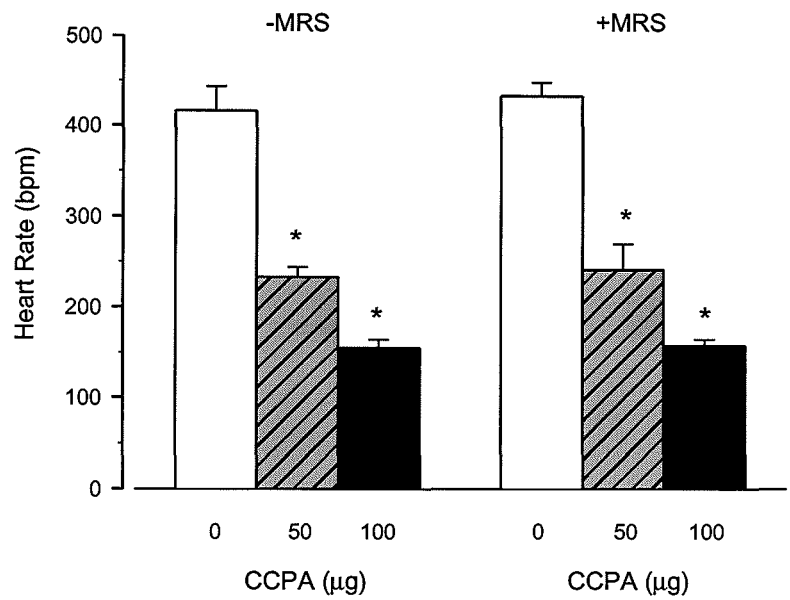

Figure 5 *Bradycardic responses to CCPA are not modified by MRS-1191, implying that at this dose MRS-1191 is devoid of A_1 antagonistic properties. *P<0.05 vs 0 μg/kg CCPA.*

Table
Heart rate and arterial blood pressure

	Baseline	60-min CAO		180-min REP
		Before	End	End
Control (n=15)				
HR	363 ± 10	362 ± 10	365 ± 9	363 ± 12
MAP	101 ± 5	100 ± 4	90 ± 4*†	83 ± 4*†
PC 15 (n=19)				
HR	360 ± 9	361 ± 6	362 ± 7	369 ± 7
MAP	107 ± 3	100 ± 3	95 ± 3*	87 ± 4*†
PC 15 + 10 SPT (n=10)				
HR	362 ± 7	364 ± 9	354 ± 11	331 ± 9*†
MAP	102 ± 5	101 ± 7	83 ± 5*†	70 ± 5*†
PC 15 + 50 SPT (n=7)				
HR	369 ± 10	360 ± 10	345 ± 10	348 ± 10
MAP	119 ± 6	111 ± 6	96 ± 4*†	86 ± 5*†
PC3 (n=5)				
HR	414 ± 17	417 ± 18	431 ± 16	435 ± 11
MAP	80 ± 3	86 ± 6	79 ± 4	70 ± 7
PC3 + 20 SPT (n=5)				
HR	400 ± 23	395 ± 9	410 ± 14	397 ± 18
MAP	88 ± 8	86 ± 11	75 ± 8	60 ± 10*†
PC3 + 50 SPT (n=4)				
HR	396 ± 27	421 ± 44	392 ± 37	424 ± 9
MAP	89 ± 11	110 ± 7	90 ± 6	88 ± 12
Control + 50 SPT (n=5)				
HR	381 ± 16	382 ± 18	345 ± 13*	326 ± 8*
MAP	98 ± 4	133 ± 5*	86 ± 8	71 ± 9*
IB-MECA 33 (n=7)				
HR	341 ± 9	332 ± 14	330 ± 12	293 ± 7*†
MAP	107 ± 6	89 ± 5*	77 ± 4*	73 ± 7*
IB-MECA 100 (n=9)				
HR	374 ± 13	367 ± 13	357 ± 17	331 ± 15*†
MAP	113 ± 7	66 ± 6*	71 ± 8*	67 ± 7*
IB-MECA 100 + Mep (n=6)				
HR	419 ± 6	369 ± 12*	368 ± 10*	351 ± 8*
MAP	114 ± 6	82 ± 7*	77 ± 6*	79 ± 3*
Control + Mep (n=6)				
HR	410 ± 13	371 ± 11*	366 ± 8*	360 ± 14*
MAP	121 ± 8	108 ± 6	102 ± 5*	85 ± 7*†
IB-MECA 33 + 10 SPT (n=5)				
HR	429 ± 10	431 ± 19	398 ± 29	370 ± 18*†
MAP	83 ± 4	74 ± 7	66 ± 6	43 ± 3*†
IB-MECA 100 + Mep + 10 SPT (n=8)				
HR	403 ± 10	366 ± 14*	340 ± 11*	336 ± 9*†
MAP	124 ± 5	67 ± 5*	72 ± 7*	72 ± 7*
IB-MECA 100 + Mep + 50 SPT *(n=7)*				
HR	390 ± 9	367 ± 14	341 ± 16*	316 ± 10*†
MAP	125 ± 8	86 ± 4	72 ± 7	65 ± 6
PC 15 + MRS (n=7)				
HR	356 ± 11	419 ± 9*	407 ± 5*	402 ± 5*
MAP	109 ± 3	99 ± 3*	88 ± 4*†	83 ± 4*†
PC 15 + MRS + 10 SPT (n=7)				
HR	341 ± 9	404 ± 6*	408 ± 9*	400 ± 16*
MAP	112 ± 4	116 ± 5	101 ± 5†	82 ± 6*†
Control + MRS (n=4)				
HR	328 ± 10	363 ± 10*	375 ± 7*	388 ± 15*
MAP	97 ± 6	103 ± 3	96 ± 4	84 ± 3
Control + DMAC (n=5)				
HR	371 ± 13	413 ± 14*	421 ± 6*	422 ± 4*
MAP	115 ± 3	104 ± 4	99 ± 3*	81 ± 5*

Data are mean±S.E.M.; *P<0.05 vs Baseline; †P<0.05 vs before CAO.

Discussion

Major findings in the present study in the rat heart were that (*i*) the non-selective adenosine receptor antagonist SPT had no effect on cardioprotection produced by 3 cycles of 3-min CAO and 3-min REP, confirming observations by Li and Kloner [2], but attenuated (low dose) and abolished (high dose) the protection afforded by a single 15-min CAO and 10-min REP; (*ii*) The selective A_3 receptor agonist IB-MECA limited infarct size dose-dependently, which was not affected by pretreatment with the low dose, but was blunted by the high dose of SPT, and (*iii*) the selective A_3 receptor antagonist MRS-1191 attenuated IP and together with the low dose of SPT abolished the protection by IP.

Involvement of adenosine in IP in the rat heart

Since the original observation by Downey and co-workers[12] in an *in vivo* rabbit model, that the adenosine receptor blockers SPT and PD115,199 were able to abolish cardioprotection afforded by ischemic preconditioning, the role of adenosine in ischemic preconditioning has been confirmed in other species such as dogs[13] and swine.[14,15] However, the evaluation of the contribution of adenosine to IS limitation by IP has led to negative results in the rat, although several studies have shown that pretreatment with exogenous adenosine can be cardioprotective in this species.[4] Capitalizing on observations by Schulz et al.[5] we hypothesized that the failure to establish the role of adenosine in the aforementioned studies in rats[4] could be due to the fact that the duration of the IP stimulus may have been too short. In the present study we not only confirmed the findings by Li and Kloner[2] that protection afforded by 3 cycles of 3-min CAO and 3-min REP was not amenable to adenosine receptor blockade with SPT in a dose of 2x10 mg/kg,[2] but also in the high dose of 2x25 mg/kg which antagonized both A_1 and A_3 receptors. However, when a preconditioning stimulus of longer duration was used (15-min CAO), the protection was blunted by a dose of SPT that was half (2x5 mg/kg) and abolished by a dose that was 2.5 times (2x25 mg/kg) that used by these investigators. These observations imply that adenosine receptor activation is involved in the protection by IP, provided that the duration of the preconditioning stimulus is sufficiently long.

Headrick[16] reported that in interstitial fluid collected between 1 and 6 min of a 6-min of global normothermic ischemia in isolated hearts, myocardial interstitial adenosine concentrations increased from 0.25 to 6.8 μM in rats and only from 0.33 to 2.0 μM in rabbits. The K_i value of adenosine for the A_1 receptor is 10-100 nM in rats and 28 nM in rabbits.[4,11] These findings of higher interstitial adenosine levels together with a similar affinity for A_1 receptors in rat and rabbit hearts are difficult to reconcile with a contribution of adenosine to IP by brief stimuli in the rabbit, but not the rat heart.[4] Since Headrick[16] used a 6-min CAO, the different results obtained in rats and rabbits can only be explained by an early increase in adenosine in the rabbits, whereas in rats, similar to pigs,[5] interstitial adenosine levels do not increase during the initial 3 min of occlusion.

Adenosine subtype receptor activation and cardioprotection

SPT is a non-selective adenosine receptor antagonist which is only five-fold more potent for A_1 receptors than for A_3 receptors.[11] Consequently, the SPT-induced dose-dependent attenuation of the cardioprotection by IP may therefore have been the result of adenosine A_1 as well as A_3 antagonism. The cardioprotection by the selective A_3 agonist IB-MECA, demonstrates the presence of cardioprotective A_3 receptors in the rat heart, similar to the rabbit heart.[9,10] However, SPT in a dose of 2x5 mg/kg did not modify the cardioprotection by IB-

MECA in a dose of 33 and 100 μg/kg, which indicates that this low dose of SPT was selective for A_1 receptors. Therefore, the attenuation of cardioprotection by IP with the low dose of SPT can be attributed to A_1 blockade. In contrast, the high dose of SPT blunted the protection by IB-MECA, indicating that SPT possesses A_3 antagonistic properties at this dose. These findings suggest that the abolition of protection by IP with the high dose of SPT may have been caused by combined A_1 and A_3 blockade. Alternatively, the abolition of the protection by IP by the high dose of SPT may also have been the result of a higher degree of A_1 blockade. Consequently, additional studies using the selective A_3 antagonist MRS-1191 were pivotal. We observed that at a dose of 3.3 mg/kg MRS-1191 produced a high degree of A_3 blockade without any A_1 blockade, confirming its reported 28-fold selectivity for A_3 over A_1 receptors in the rat heart.[11] Single treatment with either MRS-1191 or the A_1 selective low dose of SPT attenuated IS limitation by the 15-min CAO by approximately 50%, while the combination of these two agents completely abolished the cardioprotection by IP. These findings indicate that adenosine A_1 and A_3 receptors both contribute to cardioprotection by the 15-min CAO preconditioning stimulus.

Conclusions and therapeutic implications

The present study demonstrates for the first time that adenosine is involved in ischemic preconditioning in rat hearts *in vivo* but only when the duration of the stimulus is sufficiently long. The study also shows that *in vivo* that A_3 receptors contribute to ischemic preconditioning. It is not yet clear whether A_3 receptors also contribute to IP in species other than the rat in which interstitial adenosine levels may rise to higher levels than in other species, during IP stimuli of >5-min.[16] Since endogenous adenosine has a 10-100 fold higher affinity for the A_1 receptors over A_3 receptors, it is possible that in other species the A_3 receptor may not contribute to the same extent. Interestingly, the protection by *hypoxic* preconditioning against hypoxia-induced damage in isolated rabbit cardiomyocytes could only be partially blocked by the adenosine A_1 selective antagonist DPCDX, and required a combination of DPCPX with either the adenosine A_1/A_3 antagonist BWA1433 or with SPT, for complete blockade, suggesting that also in the rabbit heart both adenosine A_1 and A_3 receptors might contribute to *ischemic* preconditioning *in vivo*.[17]

Stimulation of A_3 receptors on mast cells produces histamine release in rats but not in rabbits or humans.[10,11] Consequently, in the latter species A_3 receptor stimulation does not result in hemodynamic alterations,[10] contrasting with the bradycardia and hypotension that results from A_1 and A_2 receptor stimulation, which suggests that A_3 receptor agonists are of potential interest for clinical application as cardioprotective agents. However, Lee and Emala[18] reported that pretreating the kidney with IB-MECA added to the loss of renal function produced by renal ischemia. Although the authors used a ten-fold higher dose of IB-MECA than in the present study, the observation that A_3 receptor blockade with MRS-1191 enhanced the protection by IP in the kidney suggests that also A_3 receptor stimulation by endogenous adenosine levels during brief renal ischemia exerts a deleterious effect in the kidney. Consequently, systemic treatment of myocardial ischemia with A_3 receptor agonists would appear premature.

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3

Ischemic Preconditioning Stimuli can Activate Different Signal Transduction Pathways but Converge into Mitochondrial Uncoupling

Summary

In the present study we tested *in vivo* the hypothesis that different signal transduction pathways contribute to ischemic preconditioning which converge in eliciting mitochondrial uncoupling. A single 15-min coronary artery occlusion elicited cardioprotection in the *in vivo* rat heart which was triggered by adenosine receptor stimulation, followed by PKC and tyrosine kinase activation and then K^+_{ATP} channel opening. In contrast, the cardioprotection by 3 cycles of 3-min coronary artery occlusions involved the release of reactive oxygen species followed by PKC and tyrosine kinase activation. Both stimuli resulted in mitochondrial uncoupling. Blockade of each signal transduction pathway was paralleled by concomitant blunting of uncoupling, which is consistent with the concept that mitochondrial uncoupling contributes to infarct size reduction. In conclusion, while ischemic preconditioning can activate different signal transduction pathways, mitochondrial uncoupling appears to a unifying step in the signal transduction pathway that results in cardioprotection.

Introduction

The original studies investigating the mechanism of ischemic preconditioning (IPC) did not consider that the signal transduction pathways leading to the infarct size limitation might depend on the employed preconditioning stimulus. Recent evidence, however, indicates that activation of the signal transduction pathway depends critically on the IPC stimulus. Thus, in swine adenosine was not involved in the infarct size limitation produced by a single 3-min coronary artery occlusion (CAO), but contributed to the cardioprotection by a single 10-min CAO.[1] Similarly, in the in vivo rat heart adenosine receptor stimulation mediates cardioprotection by a single 15-min CAO (1CAO15),[2] whereas a triple 3-min CAO interspersed by 5-min of reperfusion (3CAO3) did not require adenosine receptor stimulation.[2,3] Moreover, Cohen *et al.*[4] reported that cardioprotection in the rabbit produced by bradykinin and acetylcholine, but not adenosine, involved K^+_{ATP} channel activation and production of reactive oxygen species (ROS). However, while these studies suggest that different signal transduction pathways can be activated by varying stimuli, the question remains whether and where these different pathways converge into a common (end-)point. In human Girardi cells and murine skeletal myotubes, Minners et al.[5,6] recently demonstrated that not only simulated ischemia, but also adenosine and the K^+_{ATP} channel opener diazoxide resulted in mild mitochondrial uncoupling and cytoprotection. Furthermore, a low dose of the mitochondrial uncoupler dinitrophenol (DNP), afforded protection in isolated rat cardiomyocytes[7] and the isolated rat heart.[5]

In view of these observations, the aim of the present study was two-fold. First, we explored the signaling pathways of the adenosine-independent (3CAO3) and adenosine-dependent (1CAO15) preconditioning stimuli to test the hypothesis that the signal transduction pathway of 3CAO3 but not 1CAO15 involves release of ROS and activation of K^+_{ATP} channel in the *in vivo* rat heart.[2] The second aim of the study was to test the hypothesis that the signal transduction pathways of these two stimuli converge into mitochondrial uncoupling.

Methods

Experiments were performed in male Wistar rats (300-380 g) in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication 86-23, revised 1985) and with approval of the Erasmus University Rotterdam Care Committee.

Surgical and Experimental Procedures

Pentobarbital-anesthetized (60 mg/kg) rats were intubated for positive pressure ventilation with oxygen-enriched room air. Through the carotid artery a PE-50 catheter was positioned in the thoracic aorta for measurement of arterial blood pressure and heart rate. In the inferior caval vein a PE-50 catheter was placed for infusion of Haemaccel (Behringwerke) to compensate for blood loss during surgery, and for drug infusion during the experiments. After thoracotomy, via the left third intercostal space, the pericardium was opened and a silk 6-0 suture was looped under the left anterior descending coronary artery for later CAO. A catheter was positioned in the abdominal cavity to allow intraperitoneal administration of pentobarbital for maintenance of anesthesia. Rectal temperature was continuously measured and maintained at 36.5-37.5 °C. To prevent local heat loss from the thorax, the thoracotomy site was covered with aluminum foil. After completion of surgery, a 30-min stabilisation period was allowed before experimental protocols were carried out.

Rats that fibrillated were allowed to complete the protocol, provided that conversion to normal sinus rhythm occurred spontaneously within 1 min, or that defibrillation by gently thumping on the thorax or with a 9V battery was successful within 2 min after onset of fibrillation. Occlusion and reperfusion were visually verified.

Experimental protocols

Infarct size

All animals, in which a myocardial infarction was produced, underwent a 60-min CAO followed by 120 min of reperfusion (Fig. 1). Then, area at risk (AR) and infarct area (IA) were determined using Trypan blue and nitro-blue-tetrazolium staining.[2] In view of the proportionality of the area at risk and infarct area in the rat heart, infarct size was expressed as IA/AR.[8,9]

Preconditioning stimuli

Preceding the 60-min CAO, animals underwent either a 25 min sham period, a 15-min CAO followed by 10-min of reperfusion (1CAO15), or three cycles of 3-min CAO interspersed by 5-min of reperfusion (3CAO3; Fig. 1).

Components of signal transduction pathway

To study the involvement of ROS in the two preconditioning stimuli, several groups of rats that underwent the sham or either one of the preconditioning protocols, were pretreated with a continuous intravenous (IV) infusion of 1 mg/kg/min of the free radical scavenger n-(-2-mercaptopropionyl)glycine (MPG, Figure 1). To study the involvement of K^+_{ATP}-channels in the two PC stimuli, various groups of animals were intravenously pretreated with K^+_{ATP} channel inhibitor glibenclamide (3 mg/kg), the mitochondrial K^+_{ATP} channel inhibitor 5-hydroxydecanoic acid (5-HD, 20 mg/kg + 20 mg/kg/h), or the sarcolemmal K^+_{ATP} channel inhibitor HMR-1098 (6 mg/kg). Finally, to study the contribution of TyK and PKC, several groups of animals were pretreated intravenously with the TyK inhibitor genistein (2 x 5 mg/kg), the PKC inhibitor chelerythrine (5 mg/kg), or their combination.

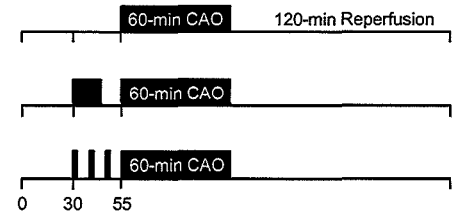
Order of involvement of components of signal transduction pathway

To study the sequence of adenosine receptor stimulation, activation of TyK and PKC, generation of ROS and activation of K^+_{ATP} channels in 1CAO15 and 3CAO3, several groups of animals received either adenosine (ADO, 200 mg/kg infused IV over 15 min), the mitochondrial K^+_{ATP} opener diazoxide (10 mg/kg), or the mitochondrial free radical generating compound menadione (37.5 mg/kg).[10]

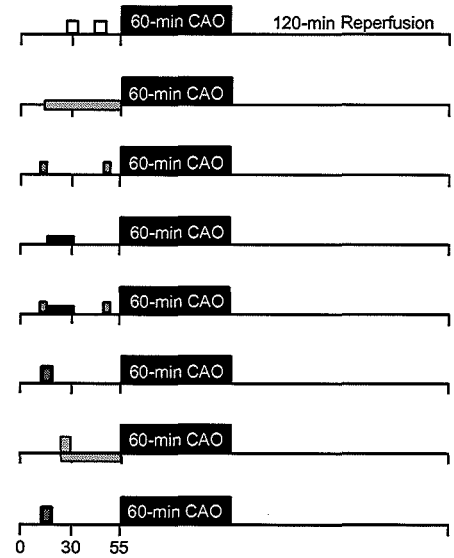
Mitochondrial function

To study the role of mitochondrial ROS production and mitochondrial uncoupling in the cardioprotection by the various cardioprotective stimuli, groups of rats were subjected to sham procedure, 3CAO3, 1CAO15 (Fig. 2). In several of these groups, animals were pretreated with glibenclamide or MPG. At a time point corresponding with the onset of the 60-min CAO, the preconditioned LAD area was dissected out and mitochondria were isolated. In isolated mitochondria, O_2 consumption (nmoles/min/mg protein) was measured using standard Clark electrodes in state 2 (using succinate as a complex II substrate) and in state 3 (succinate + ADP). Respiratory control index was computed as state 3 / state 2. In our hands, isolated mitochondria display a respiratory control index greater than 4 in the presence of complex I substrates malate/glutamate.

Adenosine depedent- and independent ischemic stimuli



Involvement of mediators



Sequence of signal transduction

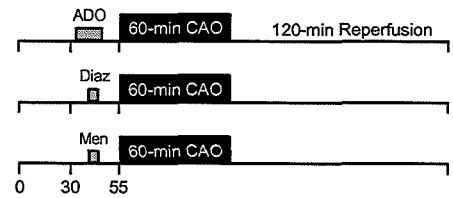


Figure 1 Experimental protocols in the infarct-size studies. Shown are the preconditioning protocols and the protocols for administering the various pharmacological agents to inhibit or stimulate the signal transduction pathways.

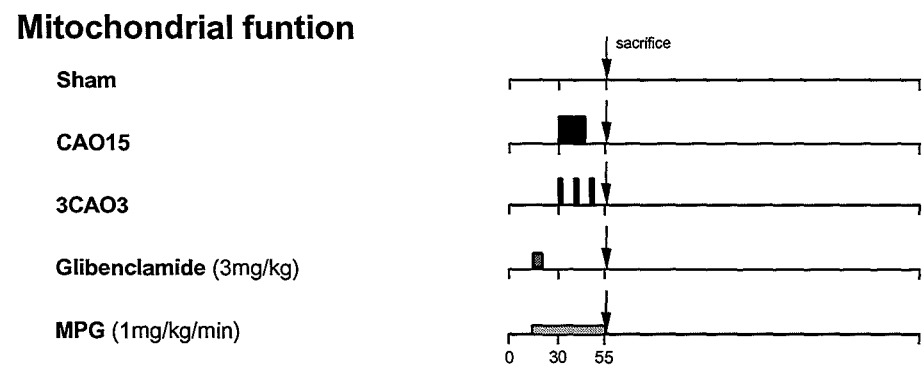


Figure 2 Experimental protocols in the mitochondrial function studies. Shown are the preconditioning protocols and the protocols for administering the various pharmacological agents to inhibit or stimulate the signal transduction pathways.

Data Analysis and Presentation

IS was analyzed by one-way ANOVA followed by Student-Newman-Keuls test. Hemodynamic variables were compared by two-way ANOVA for repeated measures followed by Dunnett's test. Statistical significance was accepted when $P<0.05$. Data are presented as mean±SEM.

Drugs

HMR-1098 was a generous gift from Dr. Gögelein (Aventis Pharma Deutschland GmbH). All other compounds were purchased from Sigma. Fresh drug solutions were prepared daily.

Results

Mortality

Five rats in the myocardial infarction studies were excluded due intractable ventricular fibrillation (no more than one rat per group).

Hemodynamics

There were no significant differences between baseline heart rate (365±3 bpm) and mean arterial blood pressure (101±1 mmHg) in the various experimental groups. Similar to previous reports,[2,11] there was no significant correlation between the rate-pressure product of the individual animals at the onset of the 60-min CAO and their corresponding infarct size (r2= 0.001; P=0.66).

Infarct Size

Preconditioning stimuli.

Infarct-size produced by a 60-min CAO (69±2%) was limited by 1CAO15 and 3CAO3 to 45±3% and 28±4%, respectively (Fig. 3).

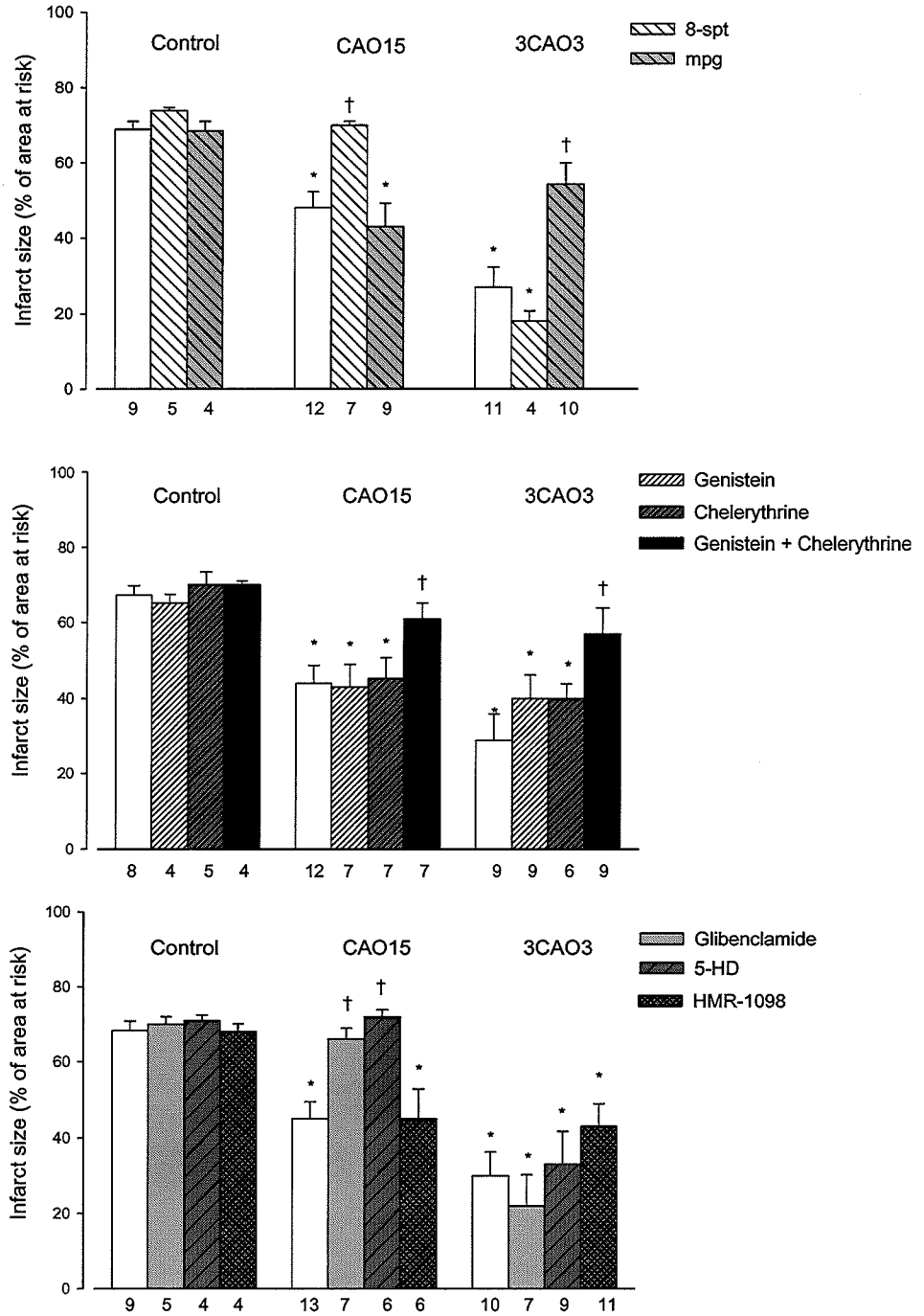


Figure 3 *Components of signal transduction pathways in the two preconditioning stimuli. Shown are the effects of the inhibitors of the various components in the signal transduction pathway.* *$P<0.05$ *vs corresponding Sham;* †$P<0.05$ *vs corresponding untreated 1CAO15 or 3CAO3*

Components of signal transduction pathway

Adenosine receptor blockade with 8-SPT abolished the cardioprotection by 1CAO15, but had no effect on the protection by 3CAO3 (Fig. 3; historic data[2]). Conversely, the ROS scavenger MPG had no effect on the protection by 1CAO15 but blunted the protection by 3CAO3. Neither PKC blockade with chelerytrine, nor TyK blockade with genistein had any effect on cardioprotection by 1CAO15 when administered alone, whereas combined administration blunted the cardioprotection by 1CAO15. Also, genistein and chelerythrine each tended to blunt the cardioprotection by 3CAO3 ($P>0.05$), but only combined administration significantly attenuated the cardioprotection by 3CAO3. The mitochondrial K^+_{ATP}-channel inhibitor 5-HD and the general K^+_{ATP}-channel inhibitor glibenclamide, but not the sarcolemmal K^+_{ATP}-channel inhibitor HMR-1098, abolished the protection by 1CAO15. In contrast, none of these K^+_{ATP}-channel inhibitors affected IPC by 3CAO3, suggesting that its cardioprotection does not require opening of K^+_{ATP}-channels.

Order of involvement of components of signal transduction pathway

Either glibenclamide or combined chelerythrine and genistein abolished cardioprotection by adenosine (Fig. 4). In contrast, the protection by the mitochondrial K^+_{ATP}-channel opener diazoxide was not affected by chelerythrine and genistein. These findings suggest that in 1CAO15 adenosine activates TyK and PKC which then leads to opening of mitochondrial K^+_{ATP} channels. Finally, the infarct size limitation by menadione was abolished by combined administration of genistein and chelerythrine, consistent with the concept that in 3CAO3 ROS lead to activation of TyK and PKC.

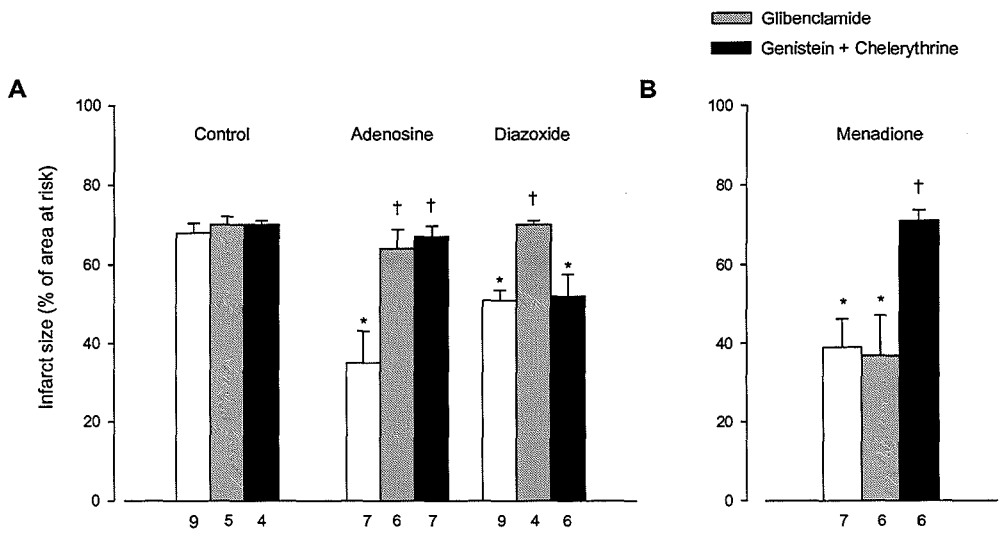


Figure 4 *Mitochondrial respiratory control index (RCI) in mitochondria subjected to sham procedure or either 1CAO15 or 3CAO3. *P<0.05 vs Sham; †P<0.05 vs 1CAO15 or 3CAO3*

Mitochondrial function studies

Both IPC stimuli 1CAO15 and 3CAO3 resulted in mitochondrial uncoupling (Fig. 5). The uncoupling produced by 1CAO15 was blocked by in vivo administration of glibenclamide but not MPG, while the reverse was true for 3CAO3. The respiratory control index (RCI) was significantly correlated with infarct size (Fig. 6).

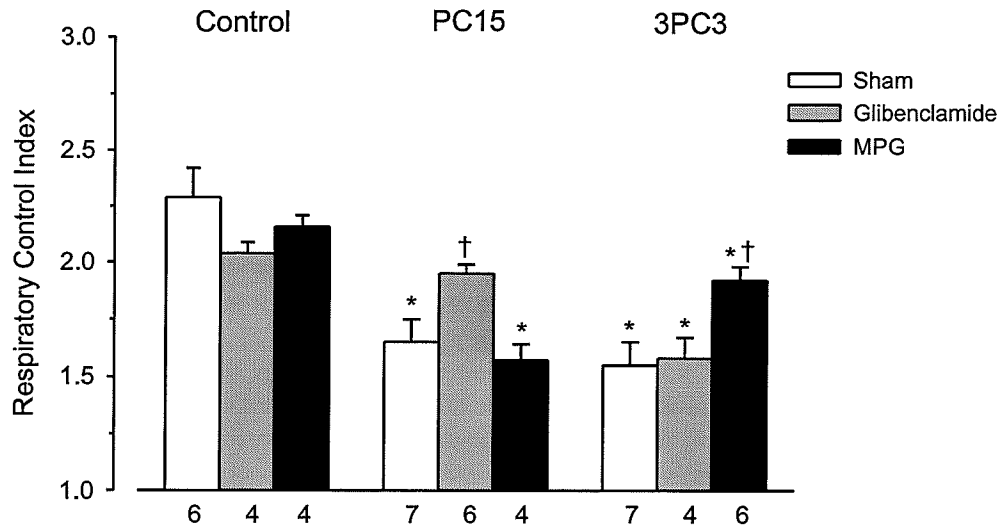


Figure 5 *Relation between mitochondrial respiratory control index (RCI) and myocardial infarct size. Shown are mean±SEM for all experimental groups displayed in Fig. 5.*

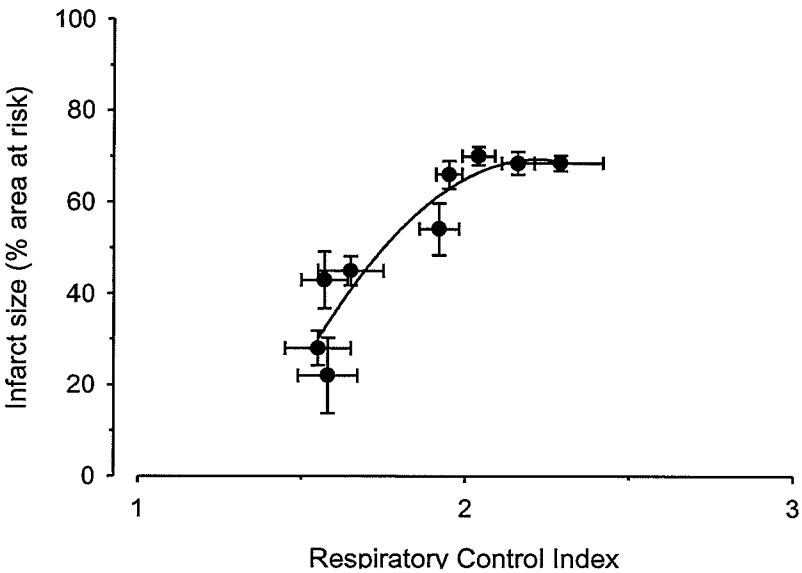


Figure 6 *Relation between mitochondrial respiratory control index (RCI) and myocardial infarct size. Shown are mean±SEM for all experimental groups displayed in Fig. 5.*

Discussion

The major findings of the present study are that: (*i*) 1CAO15 elicits protection via adenosine receptor stimulation, TyK and PKC and subsequently activation of mitochondrial K^+_{ATP} channels, without any involvement of ROS; (*ii*) 3CAO3 elicits protection involving the release of ROS which subsequently activate TyK and PKC, without any involvement of K^+_{ATP} channel opening; (*iii*) Despite employing different signal transduction pathways, both stimuli resulted in mitochondrial uncoupling. (*iv*) Blockade of the signal transduction pathways abolished not only their infarct size reduction, but also the stimulus-induced mitochondrial uncoupling. The implications of these findings will be discussed in detail.

The existence of different ischemic preconditioning stimuli was first demonstrated by Schulz et al.[1] who reported in the porcine heart that intracoronary adenosine deaminase was ineffective in attenuating IS limitation by a 3-min CAO, but abolished the cardioprotection by a 10-min CAO. Subsequently, we demonstrated in the in vivo rat heart that preconditioning by 3CAO3 did not involve adenosine, whereas in 11CAO15 adenosine plays a pivotal role.[2] Moreover, Cohen et al. demonstrated in the intact rabbit heart that pharmacological cardioprotection by bradykinin and opioids, but not by adenosine involved K^+_{ATP} channel activation and release of ROS.[4] In accordance with the latter findings, we observed in the *in vivo* rat heart that the adenosine-dependent stimulus 1CAO15 did not involve production of ROS, but found in contrast, that the adenosine-dependent stimulus involved activation of mitochondrial K^+_{ATP} channels.

Several studies have reported an important contribution of ROS in triggering IPC, as infusion of pro-oxidants has been shown to induce cardioprotection in rabbit hearts,[12] whereas radical scavengers can block IPC.[13,14] Moreover, in hypoxic chick cardiomyocytes an early increase of ROS during the IPC period was shown by VandenHoek et al.[15] Xanthine oxidase reaction have been suggested as a possible source of ROS in order to open K^+_{ATP} channels.[16] During myocardial preconditioning, the mitochondrial electron transport chain at site III may be the source of ROS.[17] The mechanistic link between opening of of mitochondrial K^+_{ATP} channels and release of ROS remains poorly understood. Several reports have shown that opening of mitochondrial K^+_{ATP} channels leads to release of ROS.[18-20] However, in contrast to these findings, several other reports have shown that ROS are able to open the mitochondrial K^+_{ATP} channels.[16,21] Zhang et al., reported in mitochondria from bovine ventricular myocardium that ROS, possibly from xanthine oxidase reaction, can lead to opening of mitochondrial K^+_{ATP} channels.[21]. Finally, Lebuffe et al.[22] recently concluded that ROS can trigger preconditioning by causing activation of the K^+_{ATP} channel, which then induces generation of ROS and nitric oxide that are required for preconditioning protection. In contrast to other G-protein linked receptor agonists, adenosine has been reported to trigger IPC independently of mitochondrial K_{ATP} and generation of ROS.[4] The present study shows that IS-limitation by 3CAO3, which could not be blocked by K_{ATP} inhibition, was markedly blunted by free radical scavenging, suggesting an adenosine-independent ischemic preconditioning stimulus in which generation of ROS play a key role without any involvement of mito K_{ATP} activation. In contrast, IS-reduction by 1CAO15 was completely abolished by K_{ATP} inhibition, but was not affected by the free radical scavenger MPG, demonstrating an adenosine dependent ischemia stimulus, which does not involve generation of ROS. Since preconditioning by 3CAO3 was not abrogated by glibenclamide, we exclude mitochondrial K^+_{ATP} channels as a source of ROS. Moreover, cardioprotection by menadione which generates mitochondrial ROS, could not be abrogated by glibenclamide demonstrating an insignificant

role of K^+_{ATP} channel activation. Conversely, cardioprotection by 1CAO15 was not affected by MPG implying multiple short bursts of ischemia-reperfusion augment generation of ROS. It is still not clear in what way ROS can precondition myocardium, but they are known to activate G-proteins and protein kinases.[23] Both P38 MAPkinase and TyK can be activated, whereas PKC seems to be independent of radical signaling.[14]

Early studies indicated that cardioprotection by a single 10-min CAO in the pig could be abolished by glibenclamide, implicating a critical role for K^+_{ATP} channel opening in IPC in swine.[24,25] In contrast, Schwartz et al.[26] recently reported that glibenclamide failed to block cardioprotection after preconditioning by two 5-min ischemic preconditioning stimuli. The latter authors did not consider different preconditioning stimuli as a cause for the different results regarding the role of K^+_{ATP} channels. However, the results from the present study clearly suggest that the different observations in pigs may have been due to multiple brief (2CAO5) versus long (1CAO10) ischaemia stimuli. Thus, it appears that opening of K^+_{ATP} channels in IPC may depend on the preconditioning stimulus.

Preconditioning by either 1CAO15 or 3CAO3 was abolished when PKC and TyK were inhibited. These findings not only confirm a major contribution for both PKC and TyK in ischemic preconditioning either by single- or multiple stimuli,[27-32] but also demonstrate their activation either without K_{ATP} opening in 3CAO3, or ROS in 11CAO15. Similar to the findings of Valhaus et al,[33] cardioprotection by both preconditioning stimuli was only abolished by simultaneous inhibition. Single inhibition of either PKC or TyK did not affect IS-reduction implying their parallel activation. Since IS-reduction by diazoxide was unaffected by PKC- and TyK-inhibition, mitochondrial K^+_{ATP} opening in 1CAO15 most likely occurs downstream as an end-effector.[34] In addition, cardioprotection by menadione was completely abolished by combined treatment with chelerythrine and genistein. Thus, during preconditioning by 3CAO3, both PKC and TyK are most likely activated in parallel pathways after generation of ROS.

The present study clearly demonstrates that the adenosine-dependent preconditioning stimulus 1CAO15 involves mitochondrial K^+_{ATP} channels but not ROS, whereas in the adenosine-independent preconditioning stimulus 3CAO3, ROS play a pivotal role but does not involve K^+_{ATP} channel activation demonstrating potent cardioprotection by two separate pathways. However, despite their markedly different signaling pathways, both stimuli resulted in mitochondrial uncoupling, as reflected in the decrease of RCI. *In vitro* evidence is accumulating that mild uncoupling of mitochondria may increase cardiomyocytes survival during sustained ischaemia and reperfusion. It cannot be determined from the present study how the two stimuli produced uncoupling, but there is evidence that both opening of K^+_{ATP} channels in the inner mitochondrial membrane and ROS can cause mitochondrial uncoupling.[35-37] Conversely, the exact mechanism by which uncoupling protects against cardiomyocytes death is also incompletely understood and may involve reduced mitochondrial matrix calcium overload,[38] mitochondrial swelling,[37,39] and reduced oxidative stress[40] during prolonged ischaemia and reperfusion and possibly augmentation of glucose uptake.[41]

In conclusion we clearly demonstrate the diversity of IPC stimuli regarding the involvement of adenosine, activation of K^+_{ATP} channels and the generation of ROS. Furthermore, while different IPC stimuli can induce cardioprotection by separate pathways, the protective signaling pathway converges into mitochondrial uncoupling.

Acknowledgements

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4

On the sites of action of adenosine in interorgan preconditioning of the heart

On the sites of action of adenosine in interorgan preconditioning of the heart

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Summary

The mechanism underlying interorgan preconditioning of the heart remains elusive, although a role for adenosine and activation of a neurogenic pathway have been postulated. We tested in rats the hypothesis that adenosine released by the remote ischemic organ stimulates local afferent nerves, which leads to activation of myocardial adenosine receptors. Preconditioning with a 15-min mesenteric artery occlusion (MAO15) reduced infarct size produced by a 60-min coronary artery occlusion (60-min CAO) from 68±2% to 49±4% ($P<0.05$). Pretreatment with ganglion blocker hexamethonium or 8-(p-sulfophenyl)theophylline (8-SPT) abolished the protection by MAO15. Intramesenteric artery (but not intraportal vein) infusion of adenosine 10 μg/min was as cardioprotective as MAO15, which was also abolished by hexamethonium. Whereas administration of hexamethonium at 5 min reperfusion following MAO15 had no effect, 8-SPT at 5 min reperfusion abolished the protection. Permanent reocclusion of the mesenteric artery before the 60-min CAO enhanced the cardioprotection by MAO15 (30±5%) but all protection was abolished when 8-SPT was administered after reocclusion of the mesenteric artery. Together these findings demonstrate the involvement of myocardial adenosine receptors. In conclusion, locally released adenosine during small intestinal ischemia, stimulates afferent nerves in the mesenteric bed during early reperfusion, initiating a neurogenic pathway that leads to activation of myocardial adenosine receptors.

(Am J Physiol Heart Circ Physiol 283:H29-H37)

Introduction

Ischemic preconditioning is not organ specific as it has not only been demonstrated for the heart,[1] but also for the kidneys, liver, brain, skeletal muscle and the lung.[2] Przyklenk et al.[3] expanded the concept of ischemic preconditioning from intraregional to interregional myocardial protection by showing that a brief coronary artery occlusion (CAO) not only preconditioned the myocardium nourished by that coronary artery but also protected the adjacent virgin myocardium. Gho et al.[4] subsequently showed that 15 min of small intestinal or renal ischemia preceding a 60-min CAO by 10 min was also capable of limiting myocardial infarct size. This interorgan preconditioning (IOPC, remote myocardial preconditioning; preconditioning at a distance) of the heart by preceding transient ischemia in remote organs has been confirmed for the small intestine,[5,6] kidney[7-9] and skeletal muscle.[10] However, not all remote organs may be able to protect the myocardium as De Zeeuw et al.[11] did not find any infarct size limitation when a 60-min CAO was preceded by global cerebral ischemia. Although not yet extensively studied, IOPC of the heart also appears to involve a delayed phase.[5,6]

The mechanism underlying classical ischemic myocardial preconditioning is still incompletely understood, but there is now consensus that it involves the release of a number of local mediators such as adenosine, norepinephrine and bradykinin during the preconditioning stimulus, which, most likely via different signal transduction pathways,[12] activate the mitochondrial K^+_{ATP} channels.[13] The mechanism underlying interorgan preconditioning is less clear. Gho et al.[4] showed the involvement of a neurogenic pathway in IOPC by preceding small intestinal ischemia, as pretreatment with the ganglion blocker hexamethonium abolished the cardioprotection. In that study it was also shown that reperfusion of the occluded mesenteric artery, responsible for small intestinal ischemia, was mandatory (an observation confirmed for the renal bed[9]). The latter suggests that activation of the neurogenic pathway occurs upon reperfusion[4] or that reperfusion facilitates the transfer of hormonal preconditioning factors from the ischemic organ to the heart.[14] Bradykinin[15] and adenosine[8,9] may also be involved in IOPC of the heart but the site(s) of action of these mediators, i.e. the ischemic organ or the heart or both, has not been investigated. Takaoka et al.[9] suggested that myocardial adenosine receptors might be involved because the adenosine concentrations in the carotid artery were ten times higher after renal ischemia than after regional myocardial ischemia. However, based on the hypotension following renal artery reperfusion, Pell et al.[8] proposed that adenosine release from the kidney was insufficient to produce cardioprotection via the circulation and suggested, based on the observations by Gho et al.,[4] that adenosine released in the ischemic kidney stimulated the afferent renal nerves and thereby protected the myocardium. The latter hypothesis was recently confirmed by Ding et al.[7] in anesthetized rabbits.

To further elucidate the mechanism of IOPC, we not only investigated whether activation of adenosine receptors (and which of its subtypes) is involved in the protection by small intestinal ischemia, but we also determined the location(s) (myocardium and/or small intestine) of the adenosine receptors involved. Studies were performed in anesthetized rats. Until recently it has been assumed that adenosine, is not involved in ischemic preconditioning in the rat,[16,17] despite its capability to limit infarct size in this species.[18] We have recently shown, however, that adenosine is involved classic ischemic preconditioning in the rat, but that its role depends critically on the duration of the preconditioning stimulus.[19] Capitalizing on our earlier observation that hexamethonium abolished the cardioprotection by small intestinal

ischemia,[4] we hypothesized that adenosine released in the small intestine during small intestinal ischemia stimulates afferent nerves within the mesenteric bed, which via a neurogenic pathway leads to activation of myocardial adenosine receptors prior to the coronary artery occlusion, thereby preconditioning the heart. This hypothesis also implies that once the myocardial adenosine receptors have been activated, blockade of the neurogenic pathway, which occurs upstream from activation of the myocardial adenosine receptors will not abolish the cardioprotection by small intestinal ischemia.

Methods

Experiments were performed in 228 ad libitum fed male Wistar rats (280-360 g) in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 86-23, revised 1985) and with approval of the Erasmus University Rotterdam Animal Care Committee.

Experimental procedures

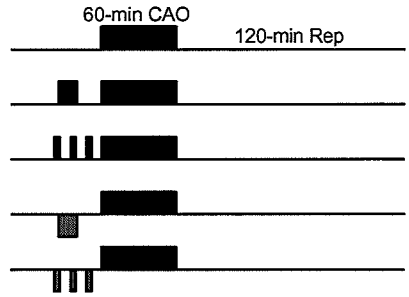
Pentobarbital anesthetized (60 mg/kg, ip) rats were intubated (PE-240) for positive pressure ventilation (Harvard rodent ventilator) with room air.[4,19,20] The thoracic aorta was cannulated via the carotid artery with a PE-50 catheter for measurement of arterial blood pressure and computation of heart rate. A catheter was positioned in the femoral vein for infusion of Haemaccel (Behringwerke) to maintain fluid balance. After thoracotomy via the left third intercostal space and opening of the pericardium, a silk 6-0 suture was looped under the coronary artery for later occlusion. Following laparotomy, the anterior mesenteric artery was dissected free and looped by a loose suture to allow later mesenteric artery occlusion with an atraumatic clamp. Pentobarbital was suffused in the abdominal cavity to maintain anesthesia. Rectal temperature was continuously measured with an electronic thermometer (Electromedics Inc.) and was maintained at 36.5-37.5 °C.[4,19,20]

Rats that fibrillated during occlusion or reperfusion were allowed to complete the protocol when conversion to normal sinus rhythm occurred spontaneously within 1 min, or when resuscitation by gently thumping on the thorax or defibrillation with a modified battery of 9V was successful within 2 min after onset of fibrillation. Occlusion and reperfusion were visually verified by appearance and disappearance of myocardial cyanosis.

Experimental design

After surgery, a 30-min stabilization period was allowed before the start of the experimental protocol. All animals were subjected to a 60-min CAO followed by 120 min of reperfusion, after which area at risk (AR) and infarct area (IA) were determined using trypan blue and nitro-blue-tetrazolium staining.[4,19,20] Infarct size (IS) was defined as 100 x IA/AR (%).

Protocol I: Choice of interorgan preconditioning stimulus



- Sham (n=15, historic data)[19]
- CAO15 (n=19, historic data)[19]
- 3-CAO3 (n=5, historic data)[19]
- MAO15 (n=10, historic data)[4]

1. 3-MAO3 (n=4)

Protocol II: Involvement of adenosine receptors

2. Sham (n=12)
3. MAO15 (n=12)
4. 50SPT + Sham (n=5)
5. 50SPT + MAO15 (n=8)
6. 10SPT + Sham (n=4)
7. 10SPT + MAO15 (n=9)
8. MRS + Sham (n=4)
9. MRS + MAO15 (n=8)
10. 10SPT + MRS + MAO15 (n=5)

Protocol III: Involvement of small intestinal adenosine receptors

2. Sham (n=12)
11. Saline IMA (n=5)
12. ADO IMA (n=8)
13. Hex + ADO IMA (n=4)
14. ADO IV (n=5)
15. ADO IPV (n=5)

Figure 1 *Schematic overview of experimental protocols I, II and III. For details see text.*

Protocol I: Choice of IOPC stimulus

Using the identical protocol of Li and Kloner[14] we recently observed,[19] that 3 cycles of 3-min CAO interspersed by 5 min of reperfusion (3-CAO3) provided a greater degree of myocardial protection than a 15-min CAO (CAO15, Fig. 1). Therefore, we first investigated the cardioprotection afforded by IOPC elicited by 3 cycles of 3-min MAO interspersed by 5 min of reperfusion (3-MAO3, group 1), and compared it to the protection afforded by a single 15-min mesenteric artery occlusion followed by 10 min of reperfusion (MAO15, historic data.[4] Because, 3-MAO3 failed to afford cardioprotection (see results), we selected MAO15 as the IOPC stimulus.

Protocol II: Involvement of adenosine receptor stimulation in IOPC

To investigate the role of adenosine in IOPC, four groups of rats were studied (Fig. 1), in which the effect of a single MAO15 on infarct size by 60-min CAO was determined in the absence (Sham, group 2; MAO15, group 3) and presence (50SPT+Sham, group 4; 50SPT+MAO15, group 5) of a non-selective dose (2 x 25 mg/kg iv, 50SPT) of the adenosine receptor antagonist 8-SPT [8-(p-sulfophenyl)theophylline]. Since we established the involvement of adenosine in this model of IOPC (see results), we further investigated whether the A_1- and A_3-receptor subtypes are involved. For this purpose, Sham and IOPC animals were pretreated with either an A_1-selective dose (2 x 5 mg/kg iv) of 8-SPT (10SPT, groups 6 and 7) or an A_3-selective dose (3.3 mg/kg iv) of the adenosine receptor antagonist MRS 1191 (MRS, groups 8 and 9).[19] Finally, IOPC animals were pretreated with a combination of 10SPT and MRS (group 10).

Protocol III: Effect of small intestinal adenosine receptor stimulation on myocardial infarct size

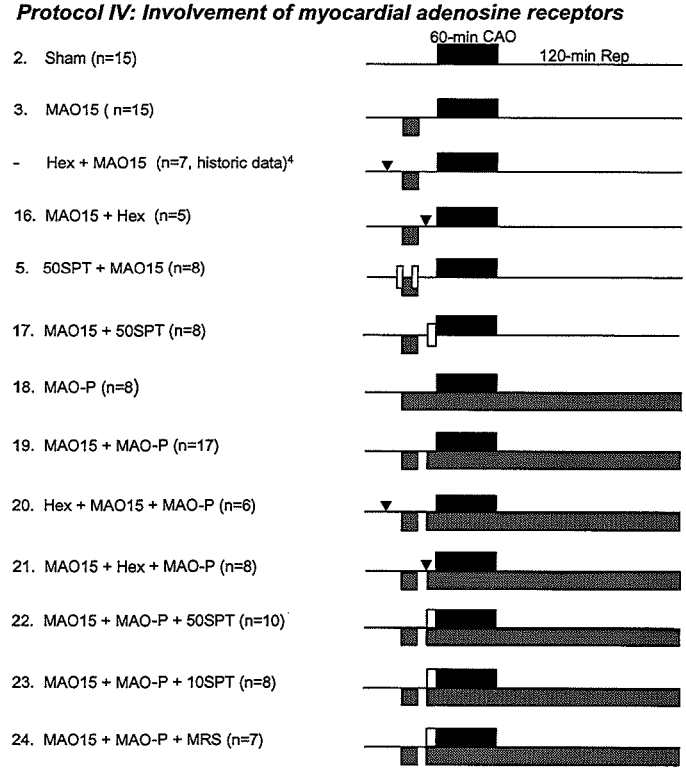
To investigate whether activation of adenosine receptors in the small intestine contributes to IOPC, we employed intramesenteric artery infusions of vehicle (saline 50 µl/min, group 11) and adenosine (10 µg/min, ADO IMA, group 12). This dose of adenosine has been shown to stimulate intestinal afferent nerves.[21] The involvement of the neurogenic pathway in the cardioprotection by intramesenteric adenosine was verified by pretreating animals with hexamethonium (Hex+ADO IMA, group 13). To exclude that the intramesenteric artery infusion of adenosine protected the myocardium by direct stimulation of myocardial adenosine receptors (after recirculation), we also infused this dose of adenosine intravenously (ADO IV, group 14). Finally, to exclude that stimulation of the afferent nerves in the liver contributed to the cardioprotection, we also studied the effect of infusion of 10 µg/min adenosine into the portal vein (ADO IPV, group 15).

Protocol IV: Involvement of myocardial adenosine receptor stimulation in IOPC

It is well established that adenosine receptor blockade between the preconditioning stimulus and the sustained CAO abolishes the cardioprotection in ischemic myocardial preconditioning.[22] We therefore followed two approaches to study the involvement of myocardial adenosine receptors in IOPC. Firstly, we established that the neurogenic pathway (required for IOPC of the heart Hex+MAO15, see reference[4] was no longer required for IOPC 5 min after mesenteric artery reperfusion (MAO15+Hex, group 16), indicating that the neurogenic pathway had already activated cardiac adenosine receptors. Subsequently, we determined the role of myocardial adenosine receptors by administering 50SPT at 5 min of mesenteric artery reperfusion (MAO15+50SPT, group 17). The second approach involved

reocclusion of the mesenteric artery 5 min prior to the 60-min CAO in order to prevent that the subsequently administered 8-SPT would, we also occluded in a number of animals the collaterals in the mesenteric vascular bed reach the small intestine. To exclude that 8-SPT would reach the small intestine via collaterals [23]. We first confirmed that a permanent MAO (MAO-P, group 18) was not cardioprotective,[4] and then studied IOPC produced by MAO15+MAO-P (group 19). These experiments revealed a potentation of the cardioprotection by MAO15+MAO-P (see results). To establish whether this potentiated cardioprotection also involved the neurogenic pathway, we determined the effects of treatment with hexamethonium administered prior to MAO15 (Hex+MAO15+MAO-P, group 20) and more importantly when administered after 5-min of mesenteric reperfusion just prior to MAO-P (MAO15+Hex+MAO-P, group 21). We then studied the effect of 8-SPT administered after re-occlusion of the mesenteric artery (MAO15+MAO-P+50SPT, group 22) to obtain further evidence for the involvement of myocardial adenosine receptor activation in IOPC. Finally, we studied the role of myocardial A_1 (MAO15+MAO-P+10SPT, group 23) and A_3 (MAO15+MAO-P+MRS, group 24) adenosine receptors in IOPC.

Protocol IV: Involvement of myocardial adenosine receptors



Small intestinal collateral blood flow

Megison et al.[23] reported that after mesenteric artery occlusion in rats, small intestinal collateral blood flow amounted 17±6% of basal flow, an after subsequent occlusion of collateral blood vessel amounted 2±1% of basal flow. To determine small intestinal blood flow in our model, radioactive microspheres were injected in 4 additional rats at baseline, after

Figure 2 *Schematic overview of experimental protocol IV. For details see text.*

mesenteric artery occlusion and after additional occlusion of collateral vessels (i.e. arcades between right colic artery and ileocolic artery and between jejunal branches just proximal and distal to the point of the superior mesenteric artery occlusion.[23]

Data analysis and presentation

Infarct size was analyzed by one-way analysis of variance for all groups, followed by one-way analysis of variance within each protocol and Student-Newman-Keuls test. Hemodynamic variables were compared by two-way (time and treatment) analysis of variance for repeated measures followed by the paired or unpaired t-test with Bonferroni correction for multiple comparisons. Statistical significance was accepted when $P<0.05$. Data are mean±SEM.

Results

Mortality

Of the 224 rats that entered the IOPC study 30 animals were excluded, because of sustained ventricular fibrillation during the 60-min CAO or cardiac pump failure. Because the excluded rats were equally distributed over the various groups, the exclusion did not cause a bias towards any intervention. In 13 animals the area at risk was less than 15% of total left ventricular mass and were therefore also excluded. Data are presented for 181 animals.

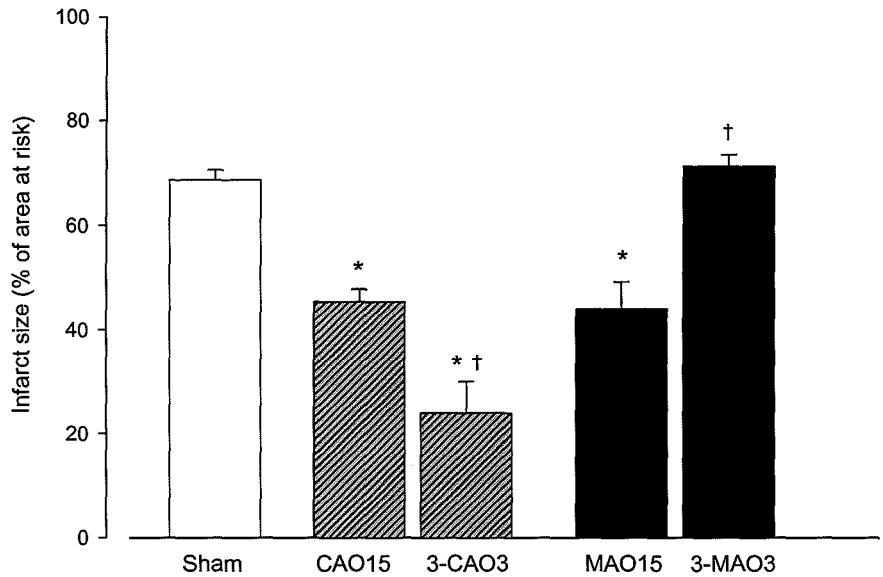


Figure 3 *Cardioprotection by classical and interorgan preconditioning (IOPC). Note that whereas 3-CAO3 was more cardioprotective than CAO15 in classical preconditioning, 3-MAO3 was ineffective in IOPC. Historic data have been presented for Sham, CAO15 and 3-CAO3, and for MAO15.* *$P<0.05$ *vs Sham;* †$P<0.05$ *vs corresponding 15-min stimulus.*

Area at risk

There were no differences between the area at risk of the experimental groups (30.5±0.7%, n=181, $P=0.16$).

Infarct size

Protocol I: Choice of IOPC stimulus

Figure 3 shows that MAO15 and CAO15 afford similar cardioprotection. However, in contrast to 3-CAO3, 3-MAO3 failed to protect the myocardium. Consequently, MAO15 was selected as the stimulus for IOPC.

Protocol II: Involvement of adenosine receptor stimulation in IOPC

Pretreatment with a non-selective dose of 8-SPT (50SPT) abolished the cardioprotection by MAO15 (Fig. 4). The A_1-selective dose of 8-SPT (10SPT) and the A_3-selective dose of MRS 1191 each attenuated the cardioprotection by MAO15. Combined pretreatment with the A_1- and A_3-selective doses of these antagonists (10SPT + MRS) abolished the cardioprotection.

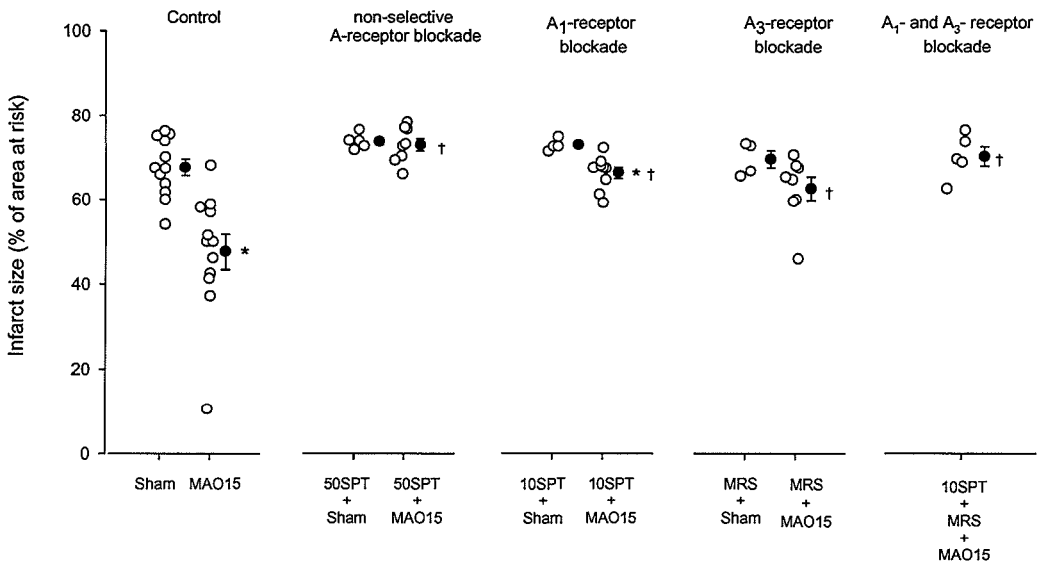


Figure 4 *Involvement of adenosine receptor stimulation and of A_1 and A_3-receptor subtypes in IOPC by MAO15.* *P<0.05 *vs corresponding Sham;* †P<0.05 *vs MAO15.15-min stimulus.*

Protocol III: Effect of small intestinal adenosine receptor stimulation on myocardial infarct size

Intramesenteric artery infusion of 10 μg/min adenosine (ADO IMA), but not of its vehicle, limited infarct size (IS=47±4%) to the same extent as MAO15 (Fig. 5). Pretreatment

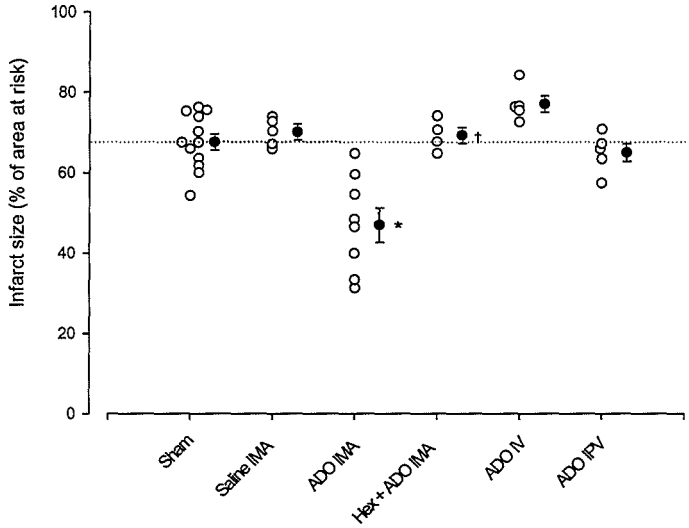


Figure 5 Involvement of small intestinal adenosine receptor stimulation in IOPC. Intramesenteric artery infusion of 10 μg/min adenosine (ADO IMA) limited infarct size to the same extent as MAO15 (see Figure 4). The protection by ADO IMA is under neurogenic control as pretreatment with hexamethonium (Hex+ADO IMA) abolished the protection and intravenous adenosine infusion (ADO IV) was ineffective. Stimulation of adenosine receptors in the liver by spillover of adenosine during ADO IMA does not contribute to the protection by ADO IMA, as adenosine infusion into the portal vein (ADO IPV) was ineffective. *P<0.05 *vs Sham.* †P<0.05 *Hex+ADO IMA vs ADO IMA.*

with hexamethonium abolished the cardioprotection by ADO IMA (Hex+ADO IMA), implying involvement of a neurogenic pathway in the cardioprotection by locally administered adenosine. This is also confirmed by the lack of effect of intravenous adenosine infusion (ADO IV), excluding that recirculation of adenosine during ADO IMA stimulated cardiac adenosine receptors directly. Finally, infusion of adenosine into the portal vein (ADO IPV) did not limit myocardial infarct size, excluding that spillover of adenosine during ADO IMA stimulated adenosine receptors in the liver and contributed to the cardioprotection by ADO IMA.

Protocol IV: Involvement of myocardial adenosine receptor stimulation in IOPC

Figure 6 shows that whereas pretreatment with hexamethonium abolished IOPC (Hex+MAO15, IS=74±2%), hexamethonium did not abrogate IOPC when administered at 5 min of mesenteric artery reperfusion (MAO15+Hex, IS=54±2%). In contrast, both pretreatment (50SPT+MAO15, IS=73±2%) and treatment at 5 min of mesenteric reperfusion with 8-SPT (MAO15+50SPT, IS=67±3%) abolished IOPC. Figure 6 also confirms that permanent occlusion of the MAO (MAO-P) did not confer significant cardioprotection (IS=63±2%). However, MAO-P potentiated the cardioprotection protection by MAO15 as IS was only 30±5% after MAO15+MAO-P vs 49±4% after MAO15 (P<0.05). This enhanced protection was also mediated via a neurogenic pathway as (i) pretreatment with hexamethonium (Hex+MAO15+MAO-P) completely abrogated all cardioprotection and (ii) posttreatment, selectively abolished the potentation leaving the protection by MAO15 unperturbed (MAO15+MAO-P+Hex). Administration of 8-SPT after the mesenteric artery was reoccluded following MAO15 (MAO15+MAO-P+50SPT) entirely abolished the protection. In

several of these experiments, the collaterals of the mesenteric vascular bed were also occluded to minimize the adenosine antagonist from entering the mesenteric bed via these collaterals and blocking the adenosine receptors in the small intestine (Fig. 6). Both selective A_1 and A_3 receptor blockade blunted the cardioprotection by MAO15+MAO-P as IS was 61±2% after 10SPT and 53±3% after MRS, respectively.

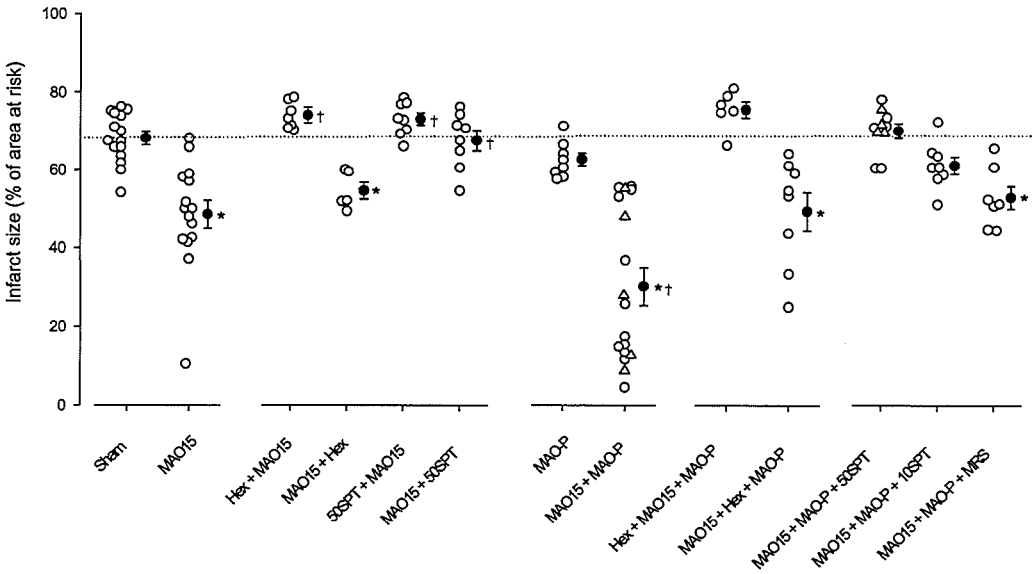


Figure 6 *Involvement of myocardial adenosine receptor stimulation in IOPC by MAO15. Notice that, whereas posttreatment with hexamethonium (MAO15+Hex) had no effect on IOPC, post-treatment with 8-SPT (MAO15+50SPT) abolished IOPC. To prevent that 8-SPT would reach the adenosine receptors in the small intestine, 8-SPT (50 mg/kg iv) was administered after the mesenteric artery was permanently occluded (MAO-P) following the 15-min MAO (MAO15+MAO-P+50SPT). The figure shows that MAO-P potentiated the cardioprotection by MAO15 and that this enhanced protection was also under neurogenic control (MAO15+Hex+MAO-P). IOPC involved myocardial adenosine receptor stimulation as cardioprotection was abolished in MAO15+MAO-P+50SPT. Infarct size was not different in animals in which the small intestine collateral vessels were occluded (animals indicated by triangles).* *P<0.05 *vs Sham;* †P<0.05 *vs MAO15;* ‡P<0.05 *vs MAO15+MAO-P.*

Heart rate and blood pressure

Baseline heart rate and mean arterial blood pressure for all 181 animals were 381±3 bpm and 93±2 mmHg, with minimal differences between the experimental groups with the exception of 50SPT+MAO15 in which baseline mean arterial blood pressure was 135±4 mmHg. Similar to earlier reports,[20] there was no correlation between the product of heart rate and mean aortic pressure of the individual animals at the onset of the 60-min CAO and their infarct size (linear regression: r^2= 0.01; P= 0.88).

Small intestinal collateral blood flow

Following occlusion of the mesenteric artery small intestinal blood flow was reduced to 5±2% (range 0-10%) of baseline. Subsequent ligation of the collateral vessels further reduced flow to 0.7±0.7% (range 0-2%) of baseline, confirming previous observations.[23]

Discussion

The major findings of the present study are: (*i*) In contrast to classical ischemic myocardial preconditioning, in which 3-CAO3 provided greater cardioprotection than CAO15, 3-MAO3 was unable to limit myocardial infarct size. (*ii*) Pretreatment with a non-selective dose of the adenosine receptor antagonist 8-SPT abolished the cardioprotection by MAO15, while pretreatment with an A_1-selective dose of 8-SPT or the A_3-selective antagonist MRS1191 attenuated cardioprotection by MAO15. (*iii*) Intramesenteric artery infusion of a dose of adenosine, that was ineffective when infused into either the portal or inferior caval vein, mimics the IOPC by MAO15 which was also abolished by pretreatment with hexamethonium. (*iv*) Hexamethonium abolished IOPC by MAO15, but only when administered before the mesenteric artery occlusion, and not when administered after 5-min of mesenteric artery reperfusion. In contrast, 8-SPT abolished IOPC also when administered after 5-min of mesenteric artery reperfusion. (*v*) Whereas a permanent mesenteric artery occlusion was not cardioprotective by itself, it potentiated the protection by MAO15; this enhanced protection was also abolished by ganglion blockade. (*vi*) After the myocardium was preconditioned by MAO15, administration of 8-SPT, at a time point that the mesenteric artery was permanently reoccluded to prevent 8-SPT to reach the small intestine, abolished all cardioprotection IOPC.

IOPC stimulus

In our original study,[4] MAO15 was as effective as a CAO15 in limiting myocardial infarct size produced by a subsequent 60-min CAO. Because 3-CAO3 afforded greater protection than CAO15,[19] we investigated whether 3-MAO3 was also more effective than MAO15 in eliciting cardioprotection. Our data showed that these multiple brief MAO's were unable to precondition the myocardium. In this respect it is of interest, that Tang et al.[5] recently showed that a single 10-min episode of small intestinal ischemia was equally effective in producing early and delayed (24-72 h) IOPC. The latter could also be elicited by 6 cycles of 4-min small intestinal ischemia and 4 min of reperfusion.[6] Although in this latter study,[6] only the delayed preconditioning phase was investigated, it cannot be excluded that a larger number and/or longer duration than the 3-min periods of small intestinal ischemia used in the present study might have provided an effective stimulus for IOPC.

That MAO15 does not yet provide optimal protection was shown when we reoccluded the mesenteric artery permanently after the MAO15. Thus, while MAO-P alone was not cardioprotective, we observed that MAO-P potentiated the cardioprotection by MAO15. The reason for the activation of the neurogenic pathway during MAO-P by the preceding of MAO15 is not clear. However, it is likely that the MAO15 not only preconditioned the heart but also the small intestine itself possibly via adenosine, calcitonin-gene related peptide or endogenous opioids.[24,25] It may then be postulated that, while the virgin small intestine is unable to activate the neurogenic pathway during mesenteric artery occlusion and requires reperfusion (10), the preconditioned small intestine is capable of activating the neurogenic pathway during occlusion and thereby enhances the protection by the MAO15. Future studies, involving measurement of interstitial adenosine concentrations in the small intestine and discharge rate of the small intestinal afferent nerves, are needed to test this hypothesis.

Involvement of adenosine receptor stimulation

The cardioprotection by small intestinal ischemia was completely prevented by pretreating rats with a high non-selective dose of 8-SPT (50 mg/kg), implying that adenosine is at least one of the mediators leading to cardioprotection in this model of IOPC. The present study also reveals that, at least with the currently used stimulus, both the A_1 and A_3 receptor subtypes contribute. A role for adenosine has also been suggested for the IOPC by renal ischemia,[7-9] but in none of these studies the site of location or the subtype of the involved adenosine receptors was investigated.

Myocardial adenosine receptors

Because in Protocol II the adenosine receptor antagonists were administered intravenously and prior to both MAO15 and 60-min CAO, the data in Fig. 4 do not reveal the site of action of adenosine. In order to investigate whether the myocardial adenosine receptors were involved, we administered 8-SPT (*i*) after 5 min of mesenteric reperfusion at a time when the cardioprotective mechanism no longer required continued activation of the neurogenic pathway, and (*ii*) after the mesenteric artery had been reoccluded following MAO15 to prevent that 8-SPT would reach the small intestine. In several animals, we additionally ligated the collaterals in the mesenteric vascular bed[23] to exclude that 8-SPT would still reach the small intestine via these vessels during MAO-P in sufficient amounts to prevent activation of the neurogenic pathway. In this situation (MAO15+MAO-P+50SPT), 8-SPT abolished all cardioprotection by IOPC (and not only the potentation by MAO-P), implying that the *myocardial* adenosine receptors must be involved in the mechanism underlying this phenomenon. The myocardial adenosine receptors involved are of both of the A_1 and A_3 subtypes. This observation is in agreement with earlier studies in which a role for both receptor subtypes was established when CAO15 was used to precondition the myocardium.[19]

Small intestinal adenosine receptors

Intramesenteric infusion of 10 μg/min of adenosine limited myocardial infarct size to the same extent as MAO15. Moreover, because infusion of the same dose of adenosine in the portal vein or inferior caval vein did not protect the myocardium, the adenosine must have acted in the small intestine. The action of locally administered adenosine was abolished when rats were pretreated with hexamethonium, which implies that the neurogenic pathway was also involved in this model of pharmacological IOPC. The intramesenteric artery infusion rate of adenosine was chosen such that only the adenosine receptors in the small intestine were activated. However, we do not know how the adenosine concentrations in the small intestine achieved by this route of administration compare to the adenosine concentrations that are achieved during MAO15. It cannot be excluded that in the portal vein higher concentrations were reached upon reperfusion of the ischemic small intestine than during the direct infusion of adenosine into the portal vein. If true, these higher concentrations might have been sufficient to stimulate afferent nerves in the liver and thereby have contributed to the cardioprotection by small intestine ischemia. That the concentrations during intramesenteric adenosine infusion and MAO15 were similar is suggested by the observation that the protection by the intramesenteric artery infusion of adenosine was very similar to the protection by MAO15.

To definitively demonstrate the involvement of adenosine receptor stimulation in the small intestine in IOPC requires blockade these receptors, while leaving the myocardial receptors unaffected. In view of the half-life of the antagonists used, which would result in recirculation and concomitant adenosine receptor blockade in the heart, this is technically not

feasible. Nevertheless, the present observations are consistent with the hypothesis that in IOPC by small intestinal ischemia, locally released adenosine triggers afferent nerves which in turn lead to stimulation of myocardial adenosine receptors.

The findings in the present study do not exclude that mediators other than adenosine may also contribute to IOPC similar to classical ischemic myocardial preconditioning. For example, Schoemaker and Van Heijningen[15] showed, without determining the site(s) of action, that the cardioprotection by transient small intestinal ischemia could be mimicked by intramesenteric artery infusion of bradykinin and abolished by pretreatment with the bradykinin antagonist HOE-140. Similarly, a role for calcitonin-gene related peptide, of which the release can be modulated by adenosine,[26] has been implicated in the cardioprotection by small intestinal ischemia.[5,6]

Conclusions

Small intestinal ischemia results in local release of adenosine that activates a neurogenic pathway during the first few minutes of mesenteric artery reperfusion, which then leads to stimulation of A_1 and A_3 adenosine receptors in the heart prior to the coronary artery occlusion,[4] thereby preconditioning the heart. Adenosine is currently under investigation for its purported ischemic reperfusion-damage limiting effects.[27] The results of the present study suggest that the cardioprotective effects of intravenous adenosine may not only be due to direct effects in the heart but could in part result from adenosine receptor activation in remote tissues which via neurogenic pathways increase interstitial adenosine levels in the heart.[27] Such a role for adenosine complies with a paraphrase of the statement by Ribeiro[28]: "adenosine in its 'obsession' to protect cells from insults uses as many receptor systems in as many tissues as possible in order to exert what seems to be the 'destiny' of this nucleoside: protection of the cardiac myocytes".

Acknowledgements

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5

Does tolerance to classic ischemic preconditioning cause cross-tolerance to other preconditioning stimuli?

Summary

Objectives
To test the hypothesis that development of tolerance to the first window of cardioprotection by ischemic preconditioning does not cause cross-tolerance to preconditioning stimuli that employ a different signaling pathway.
Background
The mechanism of myocardial tolerance to preconditioning remains incompletely understood, but may involve loss of adenosine production, reduced adenosine receptor responsiveness or modifications in downstream signaling pathways. However, not all preconditioning stimuli use signaling pathways involving adenosine.
Methods
Anesthetized rats underwent classical, remote or pharmacological preconditioning of the heart. Size of the myocardial infarction, produced by a 60-min coronary artery occlusion (CAO) was determined using tetrazolium staining. Myocardial interstitial adenosine concentrations were measured using microdialysis.
Results
Preconditioning by either a single 15-min CAO (CAO15) or by two CAO15 resulted in a potent cardioprotection against a subsequent 60-min CAO. However, preconditioning with six CAO15 did not limit infarct size, demonstrating that the myocardium had become tolerant to this stimulus when repetitively applied. During consecutive 15-min CAO, interstitial adenosine levels did not increase after the second CAO. In this tolerant myocardium, cardioprotection by exogenous adenosine was unperturbed, suggesting that development of tolerance is not due to blunting of adenosine receptor responsiveness or a signal transduction pathway downstream of the adenosine receptor. In corroboration with these findings, we observed that inter-organ preconditioning by small intestinal ischemia or preconditioning by an adenosine-independent classic preconditioning stimulus consisting of 3 cycles of 3-min CAO still produced protection. Finally, multiple episodes of 15-min exogenous adenosine infusions resulted in blunting of the infarct size limiting effects, indicating that responsiveness of adenosine receptors (or downstream components) can be attenuated.
Conclusions
The *in vivo* rat heart is susceptible to the induction of tolerance to preconditioning by repetitive stimulation with brief periods of ischemia. Although repeated adenosine infusions can also blunt adenosine's cardioprotective effects, the tolerance to ischemic preconditioning was most likely due to a loss of adenosine production, as cross-tolerance to preconditioning by exogenous adenosine, remote and a classic but adenosine-independent stimulus did not occur.

Introduction

Ischemic preconditioning, the phenomenon whereby brief periods of ischemia and reperfusion render the heart more resistant to infarction by a subsequent prolonged period of ischemia,[1] has been identified as the most powerful means of endogenous cardioprotection to irreversible cell injury.[2] Although the mechanism underlying ischemic preconditioning may involve activation of adenosine receptors, intravenous infusion of adenosine can enhance cardioprotection, even when hearts are already preconditioned by brief episodes of ischemia.[3] Abundant evidence has been presented that ischemic preconditioning also occurs in man.[4-6] Nevertheless, the demonstration of infarct size limitation by brief anginal episodes in patients with an acute myocardial infarction or the benefit of pharmacological exploitation of ischemic preconditioning by administration of adenosine is still not convincing.[7-9] One of the reasons may be the development of tolerance to cardioprotection by repetitive applying the same stimulus, has been described for both the first and second window of protection.[10-12] Hence, without a detailed knowledge of the number and type of preconditioning stimuli (anginal episodes) preceding a myocardial infarction it will be next to impossible to classify hearts as preconditioned or tolerant to ischemic preconditioning stimuli.

It has become apparent that not all preconditioning stimuli activate the same signaling pathway to exert their cardioprotective action.[13-16] For instance, we have shown that in the rat the cardioprotection by a 15-min coronary artery occlusion (CAO15) involves activation of adenosine receptors ultimately leading to opening of K^+_{ATP} channels, whereas in the more potent cardioprotection by 3 cycles of 3-min coronary artery occlusion interspersed by 5-min of reperfusion (3CAO3) neither adenosine nor the K^+_{ATP} channel play a role.[16] It is currently unknown whether tolerance to an ischemic preconditioning stimulus also implies tolerance to a cardioprotective stimulus that employs a different signal transduction pathway. The myocardium can also be preconditioned by brief ischemia in non-cardiac tissue such as the small intestine, kidneys and skeletal muscle,[17-20] which, at least for the small intestine involves a neurogenic pathway.[17,18] However, whether cross-tolerance occurs between a classical ischemic preconditioning stimulus and a remote myocardial preconditioning stimulus has not been investigated. The purpose of the first part of the present study was therefore to investigate whether tolerance to a particular ischemic preconditioning stimulus (adenosine-dependent CAO15) also affects the cardioprotection by a classical ischemic preconditioning stimulus that involves a different mechanism (adenosine-independent 3CAO3) and that by a non-cardiac ischemia stimulus (15-min mesenteric artery occlusion, MAO15) in the rat.

The mechanism underlying tolerance to ischemic preconditioning remains unclear. However, studies in rabbits and pigs indicate that refractoriness and tolerance to protection or may involve a progressive loss of adenosine production[21] and/or reduced adenosine receptor responsiveness.[22] Since we observed in the first part of the study that the ischemia-induced increase in myocardial interstitial adenosine concentrations was no longer significant during the development of tolerance, we hypothesized that administration of exogenous adenosine could still be cardioprotective.

Chronic administration of adenosine receptor agonists produces tolerance to the cardioprotection by adenosine as well as ischemic preconditioning,[22] which may involve a defect at the A_1 receptor.[23] In contrast, the involvement of adenosine receptor desensitization in the development of tolerance to the first window of cardioprotection by adenosine has not been investigated. However, Iliodromitis et al.,[11] proposed that the time course of development of tolerance to the first window of protection by ischemic preconditioning (<2 hours) was too fast

to be explained by alterations in the number of adenosine receptors or responsiveness to adenosine.[24] To assess whether adenosine receptor responsiveness can be attenuated during the first window of protection, we additionally investigated whether repetitive 15-min adenosine infusions produce tolerance to the cardioprotection by adenosine.

Methods

Experiments were performed in ad libitum fed male Wistar rats (300-380 g, n=158) in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication 86-23, revised 1996) and with approval of the Erasmus University Rotterdam Animal Care Committee

Surgical and Experimental Procedures

Pentobarbital-anesthetized (60 mg/kg) rats were intubated for positive pressure ventilation with oxygen-enriched room air. Through the carotid artery a PE-50 catheter was positioned in the thoracic aorta for measurement of arterial blood pressure and heart rate.[18,25] In the inferior caval vein a PE-50 catheter was placed for infusion of Haemaccel (Behringwerke) to compensate for blood loss during surgery, and for drug infusion during the experiments. After thoracotomy, via the left third intercostal space, the pericardium was opened and a silk 6-0 suture was looped under the left anterior descending coronary artery for later coronary artery occlusion. A catheter was positioned in the abdominal cavity to allow intraperitoneal administration of pentobarbital for maintenance of anesthesia. Rectal temperature was continuously measured and maintained at 36.5-37.5° C.[18,25] To prevent local heat loss from the thorax, the thoracotomy site was covered with aluminum foil. After completion of surgery, a 30-min stabilisation period was allowed before experimental protocols were carried out. Rats that fibrillated were allowed to complete the protocol, provided that conversion to normal sinus rhythm occurred spontaneously within 1 min, or that defibrillation by gently thumping on the thorax or with a 9V battery was successful within 2 min after onset of fibrillation. Occlusion and reperfusion were visually verified.

To measure myocardial interstitial adenosine levels during the development of tolerance, a CMA/20 microdialysis probe (Carnegie Medicine AB, Stockholm, Sweden; membrane 4 mm x 0.5 mm, cut-off: 20 kD) was implanted into myocardial area at risk of 5 rats.[26] After 90 min of stabilization, baseline measurements were obtained and rats were subjected to four 15-min episodes of coronary artery occlusion. Samples were collected during each 15-min period of myocardial ischemia at a rate of 2 µl/min. At the conclusion of each experiment probe recovery was determined *ex vivo* using a solution containing 100 µM adenosine, and found to be 14±1%. All samples were stored at –50° C for later analysis. The adenosine concentration in dialysate samples was determined by reversed phase high-performance liquid chromatography.[27]

Experimental design

Rat hearts were preconditioned with either one or multiple 15-min coronary artery occlusions separated by 15 min of reperfusion (nCAO15, adenosine-dependent stimuli), a sequence of 3 coronary artery occlusions of 3 min interspersed by 5 min of reperfusion (3CAO3, adenosine-independent stimulus) or 2 mesenteric artery occlusions of 15 min separated by 15 min of reperfusion (2MAO15, remote myocardial preconditioning stimulus). Pharmacological cardioprotection was produced by either one or multiple 15-min intravenous

infusions of 200 μg/min adenosine separated by 15 min (nADO). Myocardial infarcts were produced by a 60-min CAO (index ischemia) and infarct size (IS) was determined after 120-min of reperfusion.[28] The area at risk and infarct area were determined using Trypan blue and nitro-blue-tetrazolium staining, respectively.[15,17,18,25] Infarct size was expressed as the ratio of infarct area and the area at risk.

Development of tolerance to classical ischemic preconditioning by an adenosine-dependent stimulus

In a control group of rats, infarct size was determined after 120 min of reperfusion following the 60-min index ischemia. Subsequently, we determined the duration of the first window of protection by the adenosine-independent stimulus single CAO15 (1CAO15) by varying the duration of the reperfusion period between the preconditioning stimulus and the 60-min index ischemia from 10 min to 180 min. Other groups of rats were subjected to either two or six episodes of CAO15 (2CAO15 and 6CAO15, respectively) preceding the 60-min CAO. The reperfusion period between the last CAO15 and the 60-min CAO lasted 10 min. To determine the course of the myocardial interstitial adenosine concentration during development of tolerance to ischemic preconditioning, a group of ten rats underwent only four episodes of CAO15 without the 60-min period of index ischemia (4CAO15). In separate rats we determined the irreversible damage produced by 4CAO15.

Cross-tolerance between adenosine-dependent and adenosine-independent classical ischemic myocardial preconditioning

To investigate whether the adenosine-independent preconditioning stimulus 3CAO3 can still produce cardioprotection after myocardium has become tolerant to an adenosine-dependent stimulus, we replaced the fifth and sixth CAO15 by sham periods (4CAO15+2Sham) or by 3CAO3 (4CAO15+3CAO3).

Cross-tolerance between classical ischemic myocardial preconditioning and remote myocardial preconditioning

We recently reported that a single MAO15 limits myocardial infarct size by local release of endogenous myocardial adenosine.[17] Consequently, we investigated whether intramyocardial release of adenosine by remote preconditioning can still produce protection of myocardium that has become tolerant to ischemic myocardial preconditioning by 4CAO15 or that cross-tolerance to protection by MAO15 had also developed. For this purpose, we replaced the fifth and sixth CAO15 by two episodes of MAO15 (4CAO15+2MAO15).

Effect of exogenous adenosine on myocardium tolerant to an adenosine-dependent ischemic preconditioning stimulus

To determine whether an exogenous adenosine infusion will reinstate protection in myocardium that has become tolerant to ischemic preconditioning by 4CAO15, we replaced the fifth and sixth CAO15 by two episodes of ADO15 (4CAO15+2ADO15).

Development of tolerance to cardioprotection by exogenous adenosine

To investigate whether multiple infusions of adenosine also affected cardioprotection, three groups of rats received either one, two or six adenosine infusions (1ADO15, 2ADO15 and 6ADO15, respectively) before index ischemia.

Data Analysis and Presentation

Infarct size was analyzed by one-way ANOVA followed by Student-Newman-Keuls test. Hemodynamic variables were compared by two-way ANOVA for repeated measures followed by Dunnett's test. Statistical significance was accepted when $p<0.05$. Data are presented as mean±SEM.

Results

Mortality and exclusions

Of the 158 rats that entered the study, 18 rats were excluded because of sustained ventricular fibrillation during CAO or pump failure. Seven rats were excluded due to an area at risk <15% of the left ventricle.

Heart rate and arterial blood pressure

Baseline heart rate and mean arterial blood pressure for all 106 animals were 353±5 bpm and 96±2 mmHg, with no significant differences in heart rate ($p=0.17$) and mean arterial blood pressure ($p=0.24$) between the experimental groups. Similar to previous reports, there was no significant linear correlation between the heart rate-pressure product at the onset of the 60-min CAO and their corresponding infarct size (linear regression: $r^2 = 0.001$; $p=0.78$).

Area at risk

There were no differences in area at risk (33±1% of the left ventricle; $p=0.15$) between the experimental groups.

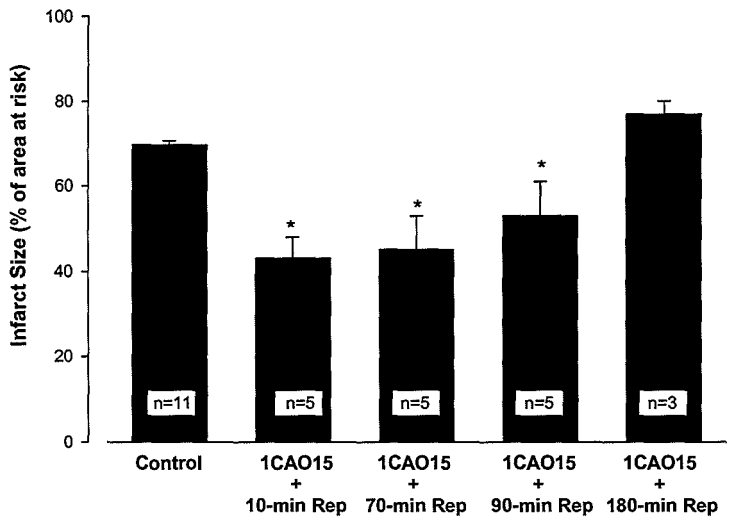

Figure 1 *Time window of protection by 1CAO15. *p<0.05 versus Control.*

Infarct size

Figure 1 shows that the cardioprotection by 1CAO15 was unmitigated after 70 min but was lost after 90 min of reperfusion. 1CAO15 and 2CAO15 limited infarct size to 43±5% and to 37±6%, respectively, compared to 69±2% in control rats ($p<0.05$; Fig. 2). In contrast, 6CAO15 failed to elicit cardioprotection (68±1%), demonstrating development of tolerance.

The loss of cardioprotection was not caused by the development of irreversible damage during the period that the myocardium became resistant to the classical ischemic preconditioning stimuli as infarct size was 10±4 % after 4CAO15.

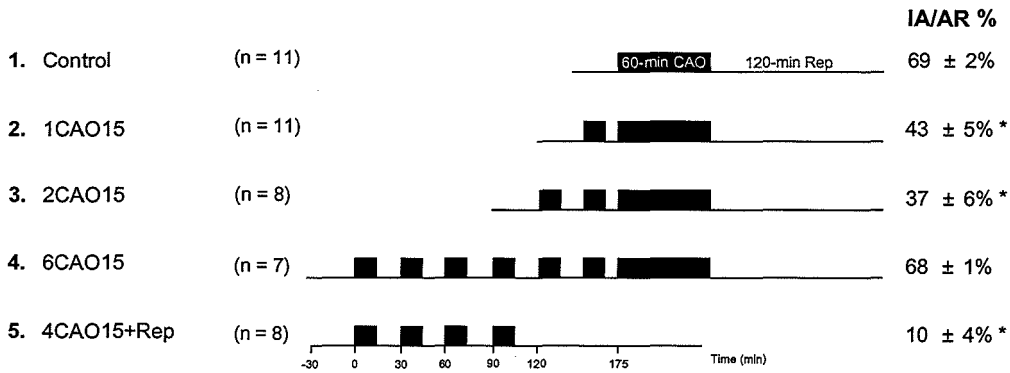

*Figure 2 Loss of cardioprotection with multiple CAO15. *p<0.05 versus control; †p<0.05 versus 1CAO15; ‡p<0.05 versus 2CAO15.*

Ninety min after insertion of the microdialysis probe, adenosine concentrations had reached a stable level. Baseline levels, measured 120-min after probe-insertion, were 3.8±0.6 μM. During the first and second CAO15 the average interstitial adenosine concentrations increased to 27.6±9.8 and 6.4±1.0 μM, respectively (both p<0.05 vs baseline), but during the third and fourth CAO15 the adenosine concentrations were no longer different from baseline (Fig. 3).

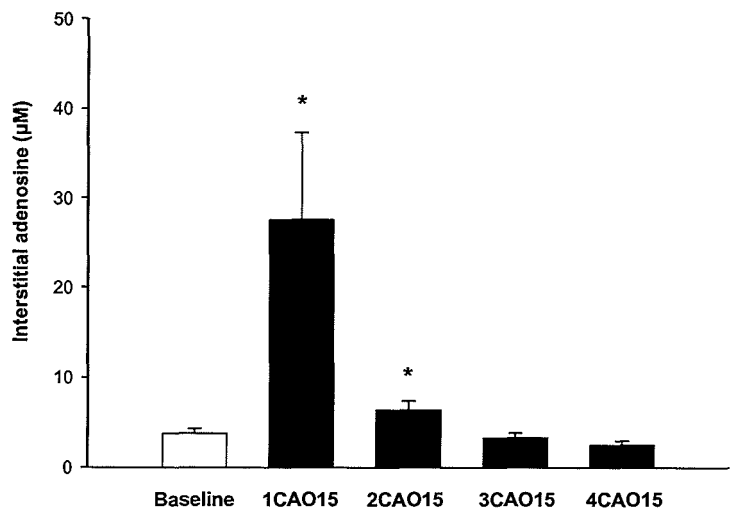

*Figure 3 Interstitial adenosine concentrations during 4CAO15. Adenosine concentrations represent averages for each 15-min period. *p<0.05 versus baseline.*

Cross-tolerance between adenosine-dependent and adenosine-independent classical ischemic myocardial preconditioning stimuli

After 4CAO15 followed by a sham period (4CAO15+2Sham), infarct size (69±2%) was not different from that of the control group (69±2%, Fig. 4) or the 6CAO15 (68±1%, Fig. 1). When 4CAO15 preceded the adenosine-independent 3CAO3 ischemic preconditioning stimulus, limitation of infarct size was still present, although less than the limitation produced by 3CAO3 alone (Fig. 4). Taking into account that after 4CAO15 alone infarct size was 10±4%, it can be calculated that 44±9% of the area at risk that was viable at the onset of 3CAO3 became infarcted. These findings indicate that at a time when the myocardium has become tolerant to the protection produced by an adenosine-dependent stimulus protection, albeit less, can still be elicited by an adenosine-independent classical ischemic preconditioning stimulus.

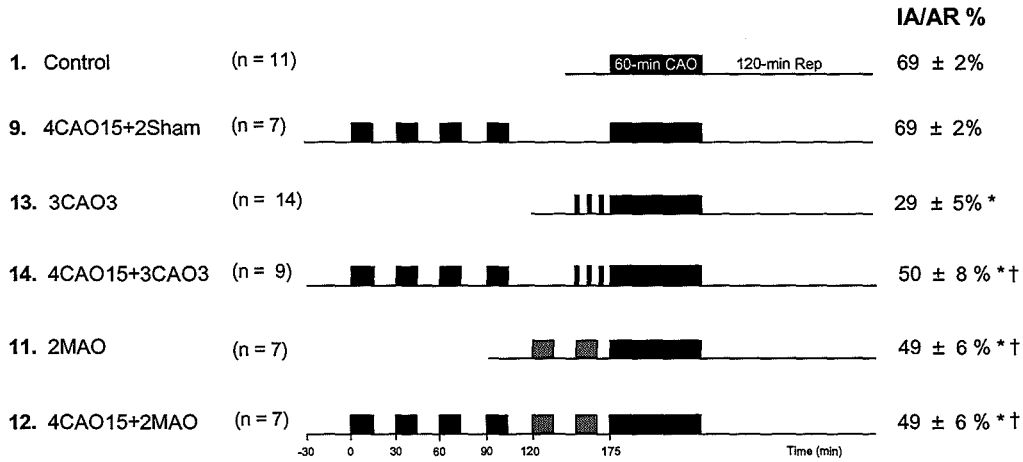

*Figure 4 Cardioprotection by 3CAO3 and by 2MAO15 is still present in myocardium that has become tolerant by 4CAO15 to the protection by 2CAO15. *p<0.05 versus Control; †p<0.05 versus 4CAO15+2Sham; ‡p<0.05 3CAO3 versus 4CAO15+3CAO3.*

Cross-tolerance to classical ischemic myocardial preconditioning and remote myocardial preconditioning

Remote myocardial preconditioning by 2MAO15 limited infarct size to 49±6% versus 69±2% in control rats ($p<0.05$; Fig. 4). When 4CAO15 preceded the 2MAO15 (4CAO15+2MAO15), infarct size was limited to 49±4% (43±7% of the area at risk viable at the onset of 2MAO15) which was not different from the cardioprotection by 2MAO15.

Effect of exogenous adenosine on myocardium tolerant to classical ischemic preconditioning by an adenosine-dependent stimulus

When 4CAO15 was followed by 2ADO15, infarct size was limited to 40±4% (33±6% of the viable area at risk; both $p<0.05$ vs 4CAO15+2Sham), which was not different from the infarct size limitation produced by 2ADO15 alone. These findings indicate that exogenous adenosine can still produce cardioprotection at a time when the myocardium has become tolerant to the protection by the adenosine-dependent stimulus 2CAO15.

Development of tolerance to cardioprotection by exogenous adenosine

1ADO15 and 2ADO15 limited infarct size to 26±7% and 35±8%, respectively, while after 6ADO15 infarct size was 50±6% ($p<0.05$ vs 1ADO15) compared to 69±2% in the control group (Fig. 6). These data demonstrate that repetitive adenosine infusions can induce, at least partial, tolerance to the pharmacological cardioprotection by adenosine.

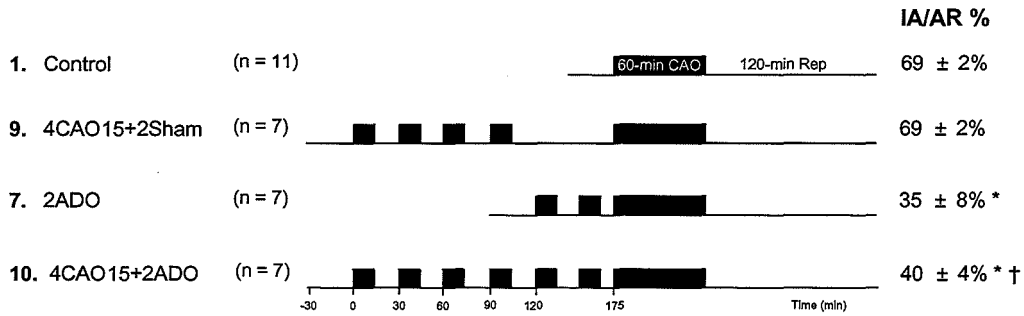

Figure 5 *Cardioprotection by 2ADO15 is maintained in myocardium that has become tolerant by 4CAO15 to the protection by 2CAO15. *p<0.05 versus control; †p<0.05 versus 6CAO15.*

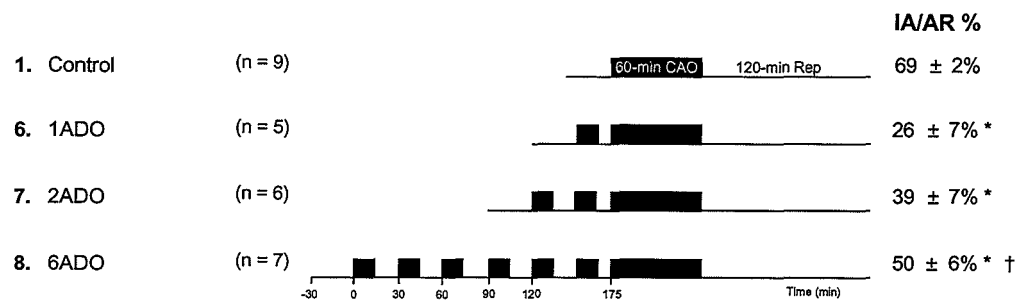

Figure 6 *Progressive loss of cardioprotection with increasing number of ADO15. *p<0.05 versus control; †p<0.05 versus 1ADO15; ‡p<0.05 versus 2ADO15.*

Discussion

The major findings of this study can be summarized as follows: (*i*) Ischemic preconditioning by 2CAO15 resulted in potent cardioprotection to subsequent 60-min index ischemia. However, 6CAO15 did not limit infarct size, demonstrating that the myocardium had become tolerant to the protection by 2CAO15 after four episodes of CAO15 were applied. (*ii*) Interstitial adenosine levels were progressively reduced during repetitive episodes of CAO15. (*iii*) After tolerance to preconditioning had been produced by 4CAO15, cardioprotection could still be obtained with the adenosine-independent classic preconditioning stimulus 3CAO3 and by remote preconditioning with 2MAO15. (*iv*) Similarly, when tolerance had occurred cardioprotection could still be produced by 2ADO15, suggesting that development of tolerance to ischemic preconditioning was not due to blunting of the myocardial responsiveness to adenosine. (*v*) After 4ADO15, the protection by 2ADO15 was attenuated, indicating that myocardial responsiveness to adenosine can be blunted. The implications of these findings will be discussed.

Refractoriness and tolerance to classical ischemic myocardial preconditioning

Refractoriness to ischemic preconditioning was first described by Sack et al.,[12] who preconditioned pigs by 2CAO10 interspersed by 30 min of reperfusion. They observed that cardioprotection was lost when the duration of the reperfusion period between the second CAO10 and the index ischemia was extended from 30 min to 60 min. Interestingly, when after 60 min the 2CAO10 stimulus was again applied, preconditioning could not be recaptured. This so called myocardial "refractoriness" to cardioprotection by brief episodes of ischemia was later shown to be the result of progressive loss of adenosine production during multiple brief ischemic episodes.[21] In contrast, in rabbits[29] and rats[30] the early phase of preconditioning can be reinstated by applying the same stimulus when protection from the first period has waned indicating that, unlike the pig, *true refractoriness* does not occur in rodent species.[31] However, multiple repeated brief coronary artery occlusions preceding a sustained coronary artery occlusion can result in *tolerance* to protection in the rabbit heart, in the first as well as the second window of protection.[11] The present study demonstrates that multiple brief coronary artery occlusions can also elicit tolerance to ischemic preconditioning in the rat heart.

Mechanism of tolerance to cardioprotection

The mechanism of development of tolerance in the rabbit heart is presently unknown. However, based on the mechanism of myocardial refractoriness in the porcine heart, as well as the prominent role of adenosine in preconditioning in both rabbits and pigs, a role for progressive loss of adenosine production during repeated occlusions also appears a likely explanation for tolerance in the rabbit heart. Alternatively, a reduced adenosine receptor responsiveness could also contribute. For example, in rabbit hearts, cardioprotection from ischemic preconditioning was lost after 72 hours of administration of the adenosine A_1 agonist CCPA in a high dose of 100μg/kg/h.[22,23] The latter effect may depend on the dose-regimen of CCPA, since a lower dose of 40 μg/kg administered at 48 hr intervals over 10 days did not induce tolerance.[32]

The mechanism underlying the development of tolerance in the rat heart has not been fully elucidated. Although in this species, a role for adenosine in ischemic preconditioning has been questioned,[33] we recently showed that not only classical preconditioning by CAO15,[15] but also remote preconditioning by MAO15[17] involves the activation of myocardial adenosine receptors. Consequently, we hypothesized that tolerance could be due to a progressive exhaustion of adenosine production during 4CAO15, and/or a blunted myocardial responsiveness to adenosine, stemming from adenosine receptor exposure to repeated elevations of intramyocardial adenosine levels. Using microdialysis, we observed that interstitial adenosine increased only during the first two episodes of CAO15 but was not different from baseline during the third and fourth CAO15. These observations are in agreement with observations regarding refractory myocardium in the pig heart,[21] and suggest that both myocardial tolerance and refractoriness are caused by exhaustion of intracellular adenosine pools. Alternatively, attenuation of myocardial responsiveness to adenosine could have contributed to tolerance, as this has been described in rabbits for the second window of protection produced by a high dose of the adenosine A_1 receptor agonist CCPA.[22] However, based on the fast time course of tolerance development, other investigators have questioned the probability of involvement of adenosine responsiveness in the development of tolerance to the first window of protection.[11,24] In the present study multiple infusions of intravenous adenosine (administered over a period of 2 hours) induced blunting of the protective effect of adenosine, indicating that a reduction of responsiveness to adenosine can already occur during within the

first window of protection. From the present study we cannot determine at what level (i.e. which subtype of adenosine receptor or their downstream signaling pathways) tolerance was induced, but there is evidence that chronic adenosine administration can cause desensitization of the A_1 receptor.[22,23] We have shown earlier that in the rat heart not only A_1- but also A_3 receptors are involved in ischemic preconditioning,[15] and can therefore not exclude that a reduced responsiveness to A_3 receptor stimulation may also have contributed to the development of tolerance in the rat heart.

Although exogenous adenosine was capable of inducing myocardial tolerance to its own protection, a loss in responsiveness of adenosine receptor or the downstream signaling pathway did not contribute to the development of tolerance by 4CAO15. Thus, the cardioprotection by 2ADO15 was not affected after pretreating hearts with 4CAO15, which is in agreement with data regarding refractoriness in swine.[12] Cross-tolerance did also not occur to remote preconditioning of the heart by mesenteric ischemia, as 2MAO15 was capable of producing a preconditioned state in myocardium that had become tolerant to the protection by 2CAO15. We have previously shown that MAO15 elicits protection in the heart via activation of a neurogenic pathway that ultimately leads to adenosine receptor stimulation in the heart.[17] The present findings suggest that 2MAO15 may have protected the heart via release of adenosine from a source that was not exhausted by 4CAO15. Finally, tolerance by 4CAO15 produced partial cross-tolerance to the classical, but adenosine-independent, preconditioning stimulus 3CAO3. This observation is difficult to explain in view of the unmitigated protection to remote and pharmacologic stimuli, as well as the different signal transduction pathway involved in 3CAO3 versus CAO15. Future studies, involving other mediators such as bradykinin,[13-16] are required to determine the biochemical basis for this partial cross-tolerance to other classical preconditioning stimuli. Taken together, the observations that exogenous adenosine, release of endogenous adenosine via remote preconditioning and classical preconditioning via an adenosine-independent stimulus were all still capable of protecting myocardium that had become fully tolerant to the protection by 2CAO15, indicates that adenosine receptor responsiveness and the various downstream signaling pathways were still intact.

Clinical relevance

The results of this study show that tolerance to the first window of cardioprotection by ischemic preconditioning can occur within hours when the same stimulus is repeatedly applied. However, stimuli that operate through a different signaling pathway may still be effective. In studies evaluating the effect of pre-infarction angina on infarct size in patients this has to be taken into account as pooling of all pre-infarction angina data might result in a mix of data from preconditioned hearts and hearts that have become tolerant to ischemic preconditioning. The observation that administration of exogenous adenosine was still protective in hearts that had become tolerant to ischemic preconditioning, suggests that in patients with unstable angina administration of pharmacological agents that mimic preconditioning can still afford cardioprotection. However, observations that repeated administration of adenosine or selective agonists can induce (at least partial) tolerance, warrants future studies to determine whether chronic administration of cardioprotective agents that act downstream of the adenosine receptor, such as K^+_{ATP} channel openers, also induce tolerance.

Acknowledgements

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6

Attenuation of myocardial reperfusion injury by the tyrosine phosphatase inhibitor bis(maltolato)-oxovanadium involves the opening of K^+_{ATP} channels

Attenuation of myocardial reperfusion injury by the tyrosine phosphatase inhibitor bis(maltolato)-oxovanadium involves the opening of K^+_{ATP} channels

David A. Liem, Coen C. Gho, Ben C. Gho, Shahla Kazim, Pieter D. Verdouw and Dirk J. Duncker

Summary

Objectives

To test the hypothesis that the organic vanadate compound bis(maltolato)-oxovanadium (BMOV) limits myocardial infarct size by attenuating reperfusion injury and to investigate the underlying mechanism.

Background

Vanadate has been shown to inhibit tyrosine phosphatase, leading to an increased tyrosine phosphorylation state. The latter has been demonstrated to be involved in the signal transduction pathway of ischemic preconditioning, the most potent endogenous mechanism to limit myocardial infarct size. Furthermore, there is evidence that phosphatase inhibition may be cardioprotective when given late after the onset of ischemia, but the mechanism of protection is unknown.

Methods

Myocardial infarction was produced in 94 anesthetized rats by a 60-min coronary artery occlusion and infarct size was determined histochemically after 180 min of reperfusion.

Results

Intravenous infusion of BMOV in doses of 3.3, 7.5 and 15 mg/kg iv decreased infarct size dose-dependently from 70±2% of the area at risk in vehicle-treated rats down to 41±5% ($P<0.05$ vs control), when administered prior to occlusion. Administration of the low dose just prior to reperfusion was ineffective, but administration of the higher doses was equally cardioprotective as compared to administration before occlusion. The cardioprotection by BMOV was abolished by the tyrosine kinase inhibitor genistein and by the K^+_{ATP} channel blocker glibenclamide, but was not affected by the ganglion blocker hexamethonium.

Conclusions

We conclude that BMOV (*i*) afforded significant cardioprotection; (*ii*) exerted its benefits principally by limiting reperfusion injury, and (*iii*) appeared to act directly on the heart by increasing tyrosine phosphorylation and opening of K^+_{ATP} channels.

(Submitted)

Introduction

An increase in tyrosine residue phosphorylation via increased tyrosine kinase activity has been implicated in the signal transduction pathway of cardioprotection produced by ischemic preconditioning,[1-3] which is the most potent endogenous mechanism to limit myocardial infarct size. There is evidence that increased tyrosine residue phosphorylation, produced by a shift in the balance between tyrosine kinase and tyrosine phosphatase, increases cell survival.[4-6] Vanadate enhances tyrosine residue phosphorylation by inhibition of tyrosine phosphatase.[7,8] Consequently, we postulated that vanadate might limit myocardial infarct size. To test this hypothesis, we administered different doses of bis(maltolato)-oxovanadium (BMOV) to anesthetized rats before a coronary artery was occluded for 60 min and reperfused for 3 hours. Interestingly, Armstrong et al.[9] reported that serine threonine phosphatase inhibitors were highly effective in protecting isolated cardiomyocytes subjected to ischemia, when administered late (75 min) after onset of ischemia. Since those findings suggest that these phosphatase inhibitors do not require administration prior to the onset of ischemia, we also evaluated whether limitation of reperfusion injury contributed to the infarct size limitation by administration of BMOV just before reperfusion. To establish whether the limitation of infarct size indeed involved an increase in tyrosine phosphorylation, we investigated the effect of BMOV on infarct size in the presence of tyrosine kinase inhibition. K^+_{ATP} channels are downstream targets or tyrosine kinase in the signalling pathway of ischemic preconditioning.[1,2] Hence, we determined the involvement of K^+_{ATP} channel opening in the protection by BMOV. Finally, a brief period of ischemia in a remote organ can precondition the myocardium by stimulation of afferent nerves in the remote ischemic organ that results in activation of a neurogenic pathway[10] ultimately leading to myocardial adenosine release.[11] To study whether activation of a neurogenic pathway by BMOV also contributed to the cardioprotection, we determined the effect of BMOV on infarct size in the presence of the ganglion blocker hexamethonium.

Material and methods

Experiments were performed in ad libitum fed male Wistar rats (~300 g) in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication 86-23, revised 1996) and with approval of the Animal Care Committee of the University.

Surgical and experimental procedures. Pentobarbital-anesthetized (60 mg/kg) rats were intubated for positive pressure ventilation (Servo ventilator) with oxygen enriched room air.[12,13] Through the carotid artery a PE-50 catheter was positioned in the thoracic aorta for measurement of arterial blood pressure and heart rate. In the inferior caval vein a PE-50 catheter was placed for infusion of physiological saline to maintain fluid balance. Following thoracotomy, via the left third intercostal space, the pericardium was opened and a silk 6-0 suture was looped under the left anterior descending coronary artery for later coronary artery occlusion (CAO). A catheter was positioned in the abdominal cavity to allow intraperitoneal administration of pentobarbital for maintenance of anesthesia. Rectal temperature was continuously measured and maintained at 36.5-37.5° C.[14] To prevent local heat loss from the thorax, the thoracotomy site was covered with aluminium foil. After completion of surgery, a 30-min stabilisation period was allowed before experimental protocols were carried out.

Rats that fibrillated were allowed to complete the protocol, provided that conversion to normal sinus rhythm occurred spontaneously within 1 min, or that defibrillation by gently thumping on the thorax or with a 9V battery was successful within 2 min after onset of fibrillation. Occlusion and reperfusion were visually verified.[10,12-14]

Experimental protocols

All rats were subjected to index ischemia consisting of a 60-min CAO followed by 180 min of reperfusion. At the end of reperfusion the left anterior descending coronary artery was reoccluded and the area at risk determined with negative trypan blue staining, after which the heart was excised and infarct size determined with negative nitro-blue-tetrazolium staining.[10,12-14]

To determine whether BMOV had any effect on infarct size 3 doses of BMOV (3.3 mg/kg, 7.5 mg/kg and 15 mg/kg) or its vehicle (up to 2.5 ml phosphate buffered saline (PBS)) were administered over 10 min, starting 20 min before the 60-min CAO. We subsequently investigated whether attenuation of reperfusion injury contributed to the limitation of infarct size. Since the former experiments established a dose-dependent limitation of infarct size by BMOV, the same doses of BMOV or its vehicle were again administered over 10 min, but now starting 10 min prior to reperfusion.

To establish whether the limitation of infarct size/reperfusion injury by BMOV required an increased state in tyrosine phosphorylation during reperfusion, we investigated whether the cardioprotection by BMOV, in a dose of 7.5 mg/kg iv, administered either prior to occlusion or prior to reperfusion was affected by the presence of the tyrosine kinase inhibitor genistein. Genistein was administered intravenously in a dose of 10 mg/kg over 5 min, starting 15 min prior to reperfusion. To investigate the involvement of K^+_{ATP} channel opening in the protection by BMOV, we determined whether the K^+_{ATP} channel blocker glibenclamide affected the cardioprotection by BMOV (7.5 mg/kg) administered prior to reperfusion. Glibenclamide was administered in two doses of 3 mg/kg each and infused over a 5 min period, the first infusion starting 20 min prior to occlusion and the second infusion starting at 45 min after the onset of occlusion. Finally, we investigated the involvement of activation of a neurogenic pathway in the protection by BMOV by studying the effect of BMOV (7.5 mg/kg, prior to reperfusion) in the presence of the ganglion blocker hexamethonium (20 mg/kg iv) administered over 15 min starting 35 min after the onset of occlusion. We and others have previously shown that genistein (2,12,15), glibenclamide[12,16] and hexamethonium[10,11] *per se* do not affect myocardial infarct size.

Data analysis and presentation

Infarct size was analysed by one-way ANOVA followed by Dunnett's test. Hemodynamic variables were compared by two-way ANOVA for repeated measures followed by paired or unpaired t testing. Statistical significance was accepted when $P<0.05$. Data are presented as mean±SEM.

Drugs. BMOV (GHO-1®, GHO-Pharma, Maastricht, The Netherlands) was dissolved in 1 ml (3.3 and 7.5 mg/kg) or 2.5 ml (15 mg/kg) phosphate buffered saline (PBS, modified Sörensen). Genistein (10 mg/kg, Sigma Chemicals) was dissolved in 0.3 ml of 95% ethanol and alkamuls EL-620 (a generous gift from Rhodia) to which 0.3 ml physiologic saline was added. Glibenclamide (3 mg/kg, Sigma Chemicals) was dissolved in 1 ml of deionized H_2O at a pH of 10. Hexamethonium (20 mg/kg, Sigma Chemicals) was dissolved in 1 ml physiologic saline. Fresh drug solutions were prepared on each day.

Results

Exclusion criteria. Of the 107 rats that entered the study, 10 rats were excluded because of sustained ventricular fibrillation (no more than 3 rats in one group) and 3 rats were excluded because the area at risk comprised less than 10% of the left ventricular mass.

Infarct size

There were no differences (P=0.71) between the areas at risk of the various experimental groups (41±1%, n=94). Infarct size, which was 70±2% in vehicle-treated rats, was limited in a dose-dependent manner to 41±5% by administration of BMOV prior to the 60-min CAO ($P<0.05$; Fig. 1, left panel). When administered just prior to reperfusion, BMOV in the dose of 3.3 mg/kg was ineffective, but the doses of 7.5 and 15 mg/kg were equally cardioprotective as the comparative doses when administered before the 60-min CAO (Fig. 1, right panel).

The cardioprotection by BMOV in a dose of 7.5 mg/kg was abolished when rats were treated with genistein, independent of whether BMOV was administered prior to the 60-min CAO (Fig. 2, left panel) or the 180-min reperfusion period (Fig. 2, right panel). The cardioprotection by BMOV, administered prior to reperfusion, was also abolished by glibenclamide but not by hexamethonium (Fig. 3).

Heart rate and arterial blood pressure

There were no significant baseline differences in heart rate (P=0.24) and mean arterial blood pressure (P=0.35) between any of the experimental groups (Table). In the PBS-treated groups, heart rate and mean aortic blood pressure remained virtually unchanged throughout the 60-min CAO. During the subsequent 180-min reperfusion period, blood pressure slightly decreased and heart rate slightly increased. BMOV, administered either prior to occlusion or prior to reperfusion, produced transient and dose-dependent increases in mean arterial pressure (37±4 mmHg), which were accompanied by decreases in heart rate. Apart from these transient effects the hemodynamic responses tot occlusion and reperfusion were not different from the responses in the vehicle-treated animals.

Pretreatment with genistein markedly attenuated the pressor response induced by 7.5 mg/kg BMOV (14±5 mmHg compared to 37±4 mmHg, P<0.05; Table). In contrast, the BMOV-induced increase in blood pressure was not altered by pretreatment with either glibenclamide (28±10 mmHg) or hexamethonium (53±12 mmHg).

Infarct size limitation by pre-occlusion treatment with BMOV was not related to alterations in the product of heart rate and mean arterial blood pressure at the onset of occlusion (Fig. 4, left panel), which is in line with previous observations that infarct size is not correlated with oxygen demand at the onset of occlusion.[10,14,17,18] In addition, the cardioprotection by BMOV administered prior to reperfusion was also not correlated with the rate-pressure product at the onset of reperfusion (Fig. 4, right panel).

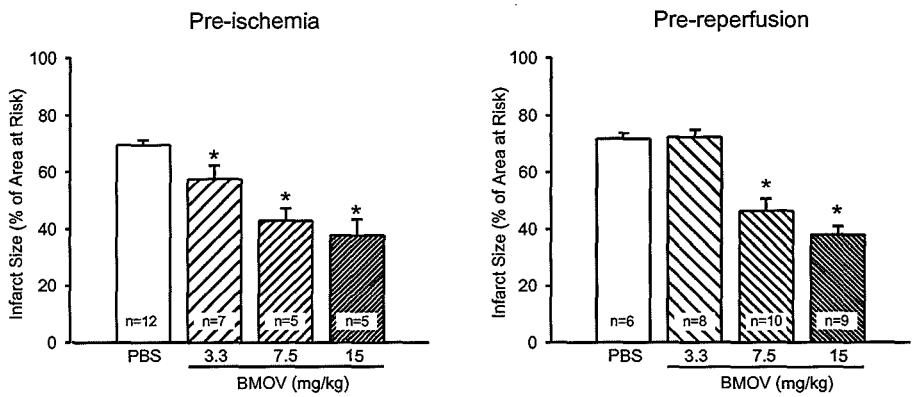

Figure 1 *Effects of BMOV administered prior to ischemia (left panel) or reperfusion (right panel) on myocardial infarct size produced by a 60-min CAO. *P<0.05 vs corresponding vehicle (PBS); †P<0.05 BMOV pre-reperfusion vs BMOV pre-CAO.*

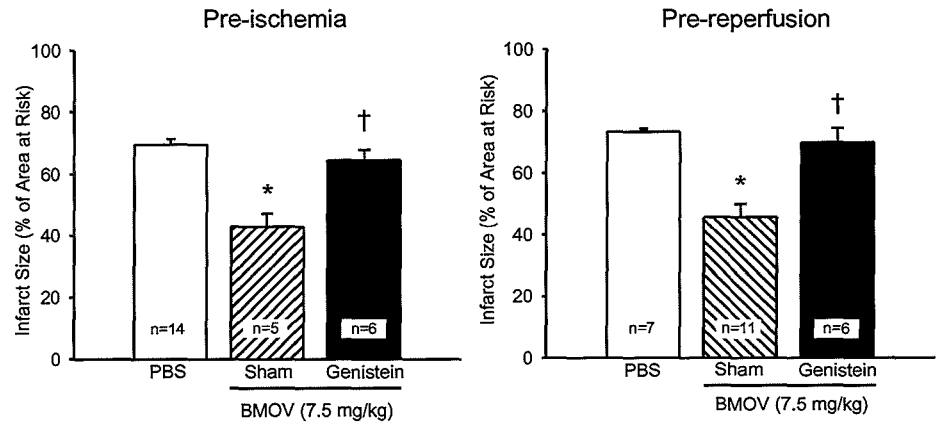

Figure 2 *Effects of the tyrosine-kinase inhibitor genistein (10 mg/kg) iv, administered 15 min prior to reperfusion, on the cardioprotection by intravenous administration of 7.5 mg/kg BMOV administered either prior to ischemia (left panel) or prior to reperfusion (right panel). *P<0.05 vs corresponding vehicle (PBS); †P<0.05 vs BMOV.*

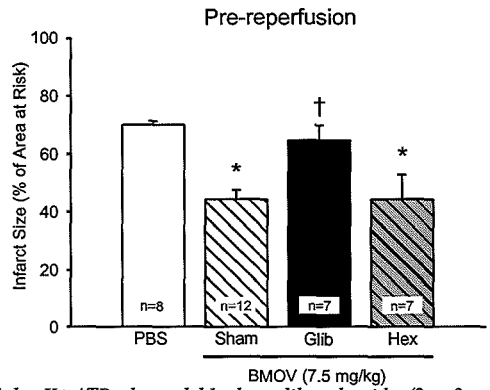

Figure 3 *Effects of the K+ATP channel blocker glibenclamide (2 x 3 mg/kg iv) or the ganglion blocker hexamemethonium (20 mg/kg iv) on the cardioprotection by 7.5 mg/kg BMOV intravenously administered prior to reperfusion. *P<0.05 vs vehicle (PBS); †P<0.05 vs BMOV.*

Table Heart rate and arterial blood pressure

		Pre-Coronary Artery Occlusion (min)			Coronary Artery Occlusion (min)			Reperfusion (min)			
		-25	-10	-1	30	50	60	15	60	120	180
1.	**PBS pre-CAO (n=14)**										
	HR	360±8	357±9	359±8	368±8	368±8	367±8	369±9	379±13	390±11*†	397±9*†
	MAP	95±3	100±3	98±2	95±3	95±3	96±3	90±4	92±3	86±4	91±4
2.	**BMOV 3.3 pre-CAO (n=7)**										
	HR	385±12	297±16*	367±15	380±14	378±17	379±17	382±18	396±15*†	398±12*†	407±9*†
	MAP	99±3	123±4*	100±3	92±5	94±4	95±3	89±5	93±5	88±6	87±4
3.	**BMOV 7.5 pre-CAO (n=5)**										
	HR	347±11	251±15*†	310±15	362±7†	359±12†	362±14†	370±14†	370±16†	393±17*†	391±10*†
	MAP	91±1	134±5*	100±5	101±5	102±3	96±1	93±3	91±4	88±3	80±6†
4.	**BMOV 15 pre-CAO (n=5)**										
	HR	340±9	298±11*†	334±11	358±9	360±8	346±14	360±8	375±6†	409±13*†	400±17*†
	MAP	94±3	138±10*†	102±10	87±12	84±10†	84±10†	83±7†	85±9†	84±6†	72±11*†
5.	**PBS pre-REP (n=8)**										
	HR	374±7	342±10*	338±6*	344±11*	341±7*	341±7*	349±8*	355±9*	373±13†	383±8†
	MAP	97±2	99±3	97±3	95±4	96±5	100±5	94±4	92±5	89±7	89±3
6.	**BMOV 3.3 pre-REP (n=8)**										
	HR	387±11	367±11	361±10	361±5	363±4	340±11*	371±9	371±8	382±11	404±11†
	MAP	99±5	95±3	84±4*	84±6*	92±4	106±5†‡	94±6	83±7*	76±6*	78±7*
7.	**BMOV 7.5 pre-REP (n=12)**										
	HR	371±7	335±7*	331±9*	340±11*	335±11*	286±8*†‡	333±10*	333±12*	361±11†	363±19†
	MAP	104±2	98±2	97±2	96±3	98±3	134±4*†‡	97±2	91±6	83±3*	84±3*
8.	**BMOV 15 pre-REP (n=9)**										
	HR	367±6	338±6*	333±3*	339±5*	339±5	274±5*†‡	314±7*	344±9*	357±6†	379±4†
	MAP	101±3	97±3	95±2	97±3	102±2	135±5*†‡	96± 3	93±4*	83±2*†	88±4*†
9.	**BMOV 7.5 + Genistein (n=6)**										
	HR	351±16	249±9*	295±10*	330±10	300±13*	355±9†	357±12†	345±9†	332±20	348±15†
	MAP	108±2	161±3*†	115±5	95±5†	75±8*†	101±5†	88± 4*†	86±5*†	89±7†	83±9*†
10.	**Genistein + BMOV7.5 (n=6)**										
	HR	375±13	353±14	333±9*	369±12	356±18	330±6	358±26	395±15†	402±15†	387±17†
	MAP	101±5	100±3	106±2	88±4†	91±8	105±8†	86±6†	83±2†	94±6	81±5†
11.	**Glibenclamide + BMOV 7.5 (n=7)**										
	HR	341±14	343±8	349±11	345±10	351±11	281±20*†‡	328±12	373±15	372±15	385±17*†
	MAP	104±7	124±3	126±2	104±5	117±4	145±8*‡	105±7	98±6†	87±10†	94±7†
12.	**Hexamethonium + BMOV 7.5 (n=7)**										
	HR	363±16	365±13	367±13	365±10	334±10†	298±14*†‡	327±9*†	351±13	357±14	378±11
	MAP	103±6	101±4	105±3	100±1	71±4*†	124±14*‡	83±3*†	86±3	84±4†	87±3

HR= heart rate (bpm); mean arterial blood pressure (mmHg); -10 min of occlusion corresponds to the end of infusion of vehicle. Data are mean±SEM; *$P<0.05$ vs Baseline; †$P<0.05$ vs pre-CAO (-1 min); ‡$P<0.05$ 60 min CAO vs 50 min CAO.

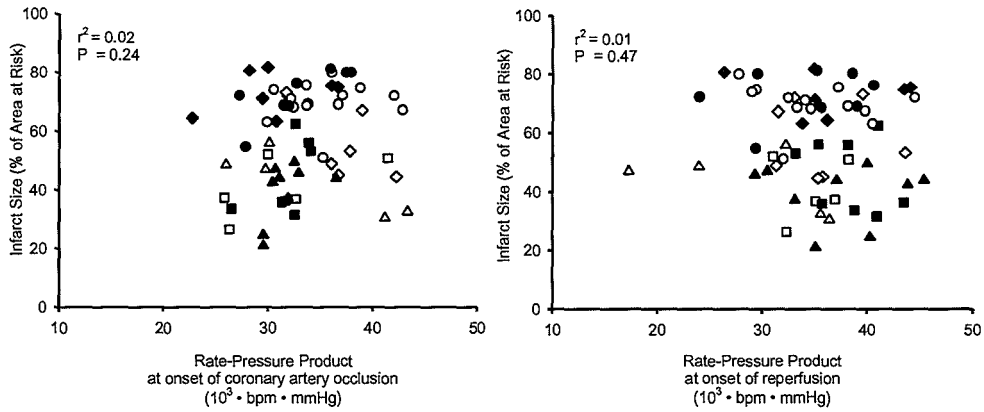

Figure 4 *Lack of relation between the rate-pressure product at the onset coronary artery occlusion (left panel) and the onset of reperfusion (right panel) and myocardial infarct size in rats receiving PBS or BMOV prior to occlusion (open symbols) or prior to reperfusion (solid symbols). Circles denote PBS, squares denote 3.3 mg/kg BMOV, while upward and downward triangles denote 7.5 and 15 mg/kg BMOV, respectively.*

Discussion

The major findings in the present study in the *in vivo* rat heart are that (*i*) pretreatment with BMOV limited myocardial infarct size in a dose-dependent manner, (*ii*) at sufficiently high doses, BMOV was equally cardioprotective when administered prior to reperfusion as compared to administration prior to coronary artery occlusion, (*iii*) tyrosine kinase inhibition and K^+_{ATP} channel blockade abolished the cardioprotection by BMOV, and (*iv*) ganglion blockade had no effect on BMOV's cardioprotection. The implications of these findings will be discussed below.

Importance of dosage and timing of administration of BMOV

Previous *in vitro* studies have indicated that vanadate possesses cardioprotective properties. For example, vanadate limited acidosis and lactate accumulation during global ischemia in isolated buffer perfused rat hearts, although post-ischemic recovery of left ventricular-developed pressure was only minimally improved.[19] These data are difficult to interpret, because vanadate was administered in a high dose of 40 μM that produced marked cardiodepression at baseline, as reflected in the more than 50% reduction in left ventricular-developed pressure.[19] Furthermore, ischemia lasted up to 15 min, which in buffer-perfused rodent hearts may already result in significant necrosis.[20] Consequently, without measurement of infarct size, no distinction can be made between a vanadate-induced attenuation of reversible contractile dysfunction (stunning) versus limitation of myocardial infarct size.

In rabbits with pressure-overload induced left ventricular hypertrophy, vanadate was administered for a period of 3-4 weeks in a dose of 7.5 mg/kg PO per day dissolved in drinking water. When hearts were subsequently isolated and buffer perfused, glycolytic flux in the hypertrophied compared to normal hearts was depressed but glucose transport was enhanced[21]. Furthermore, during a 40 min period of global ischemia, these vanadate-treated hypertrophic hearts showed reduced lactate release and improved post-ischemic recovery of left ventricular-developed pressure compared to vehicle- treated hypertrophied rabbit hearts.[21] However, since

the duration of ischemia exceeded 15-20 min, again no distinction can be made between a vanadate-induced attenuation of reversible contractile dysfunction (stunning) versus limitation of myocardial infarct size.[22]

The present *in vivo* study is the first to demonstrate that BMOV, in a dose-dependent manner, limits myocardial infarct size in vivo. At a low dose of 3.3 mg/kg only pre-ischemia treatment was effective in limiting infarct size, suggesting either that the compound exerts principally anti-ischemic actions or that tissue concentrations were too low at the onset of reperfusion. Rats lack a significant collateral circulation in the coronary vascular bed, so that administration of BMOV may not have reached the jeopardized myocardium in sufficient concentrations prior to the onset of reperfusion. However, at sufficiently high doses, BMOV was equally effective when administered prior to reperfusion as compared to administration prior to coronary artery occlusion. Taken together these findings suggest that BMOV exerts its effects principally during reperfusion, with minimal effect on ischemia-related injury, but that sufficiently high concentrations need to be present in the blood at the onset of reperfusion. It is of interest to note that in the same rat model the degree of cardioprotection by the highest dose of BMOV, i.e. a reduction in infarct size from 70±2% to 40±3%, is similar to the protection afforded by a adenosine-dependent ischemic preconditioning stimulus consisting of a single 15-min coronary artery occlusion (from 70±2% to 49±3%) but less than the protection afforded by the adenosine-independent stimulus consisting of three cycles of 3-min occlusion and 5 min of reperfusion (from 70±2% to 24±6%).[13]

Mechanism of cardioprotection by BMOV

The cardioprotective effect of vanadate has been proposed to be in part mediated by its apparent free radical scavenging properties. Thus, vanadate has been reported to inhibit the generation of superoxide produced by 5 min of perfusion with a mixture of xanthine and xanthine oxidase resulting in a blunting of the superoxide-induced loss of sarcolemmal Ca^{2+}-pump activity and Na^{+}-dependent Ca^{2+}-uptake in isolated rat hearts.[23] In contrast, under certain conditions *in vitro*, vanadate has been shown to be capable of generating free radicals.[24] However, this does not account for all its actions, because other investigators failed to observe any action of reductants or antioxidants on vanadate-induced expression of actin and c-Ha-ras.[25] Importantly, the role of reactive oxygen species in lethal reperfusion injury is still poorly understood, as studies on efficacy of scavengers of reactive oxygen species against reperfusion injury have been highly equivocal.[26,27]

In the present study, the tyrosine kinase-inhibitor genistein markedly attenuated the cardioprotection by BMOV even when BMOV was administered before ischemia and genistein was administered after 45 min of ischemia (i.e. just before reperfusion). These findings reflect the strong dependency of BMOV on an intact tyrosine kinase activity during reperfusion, and indicate the importance of tyrosine phosphorylation status in the pathogenesis of ischemia-reperfusion damage. An increased phosphorylation of tyrosine residues has been proposed to afford protection against ischemia-reperfusion damage via a number of subcellular actions. First, vanadate exerts insulin-like effects including enhanced stimulation of glucose transport and oxidation in the isolated rat heart, which might be due to its tyrosine phosphatase inhibitory actions.[21] Interestingly, recent clinical trials indicate that a combination of glucose and insulin might increase the salvage of cardiomyocytes during early reperfusion.[28] The mechanism by which enhanced glucose utilization produces protection might be related to increased ATP production at the site of the sarcolemma (and perhaps the mitochondria) during the first few min of reperfusion (at a time when mitochondria have not yet resumed ATP

production) thereby maintaining ion homeostasis and chaperoning the vulnerable cardiomyocytes into a phase in which the mitochondria resume ATP generation.[29] Another mechanism by which an increase in tyrosine phosphorylation may exert cardioprotection could involve in opening of K^+_{ATP} channels. Thus, ischemic preconditioning has been shown to involve activation of tyrosine kinase and opening of K^+_{ATP} channels.[2,3] In accordance with the concept that tyrosine kinase can activate K^+_{ATP} channels, we observed that the cardioprotection by BMOV was abolished by pretreatment with the K^+_{ATP} channel blocker glibenclamide.

We have previously shown that a brief episode of intestinal ischemia can elicit remote preconditioning of the heart via a neurogenic pathway that induces protection via myocardial adenosine release and consequent receptor stimulation in the rat heart.[10,11] Because BMOV, when administered intravenously after a total coronary artery occlusion, cannot easily reach the jeopardized myocardium before reperfusion has been reinstated (rats lack a significant coronary collateral circulation), we hypothesized that activation of a neurogenic pathway could have contributed to the protective actions of BMOV. However, in the present study we observed that the ganglion blocker hexamethonium had no effect on the cardioprotection by BMOV, suggesting that a neurogenic pathway is not involved in the cardioprotection by BMOV.

Clinical relevance

Ischemic heart disease, in particular myocardial infarction, has become the most important cause of heart failure in developed countries. Early restoration of blood flow to jeopardized ischemic myocardium is compulsory for salvaging as many cardiomyocytes as possible from cell death. Hence, reperfusion through thrombolysis or percutaneous coronary angioplasty is standard treatment in patients with an impending acute myocardial infarction. Despite its necessity, several investigators[28,30-33] have suggested that reperfusion causes irreversible myocardial damage by itself, beyond that inflicted by ischemia alone. This so-called *"lethal reperfusion injury"* implies the death of cardiomyocytes, which are still viable at the onset of reperfusion, as a direct result of sequela initiated by reperfusion itself. The ultimate consequence is an extension of myocardial infarction. Since myocardial infarct size is a strong predictor of left ventricular dysfunction and mortality,[34,35] research efforts have been aimed at developing pharmacological therapies to limit infarct size.[27,36,37] However, most if not all of the cardioprotective agents developed to date require administration prior to the onset of the period of sustained ischemia in order to be effective. In patients that encounter a myocardial infarction as the first symptom of ischemic heart disease, pharmacotherapy can only be applied after a coronary artery has occluded. Hence, there is a need for agents that are protective even when given after the onset of ischemia or just before reperfusion. To our knowledge, BMOV when administered in a sufficiently high dose is the first compound to be equally cardioprotective when administered prior to reperfusion as compared to administration before ischemia.

Conclusions

The present study in an *in vivo* myocardial infarction model demonstrates that BMOV limits myocardial infarct size, principally by attenuating reperfusion-injury. The effect of BMOV does not depend on activation of a neurogenic pathway and suggests that BMOV acts directly on the heart. The observation that the tyrosine kinase inhibitor genistein abolished the cardioprotection by BMOV is consistent with the compounds' purported tyrosine phosphatase inhibiting properties, which resulted in opening of K^+_{ATP} channels. These findings also elude to

the importance of the status of phosphorylation of tyrosine residues and K^+_{ATP} channels in the pathogenesis of reperfusion injury.

Acknowledgements
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7

Summary, general discussion and future perspectives

In this thesis are presented the results of our studies on the mechanism of ischemic preconditioning in the rat heart in vivo. In contrast to all other species used for experimental study, the major role of adenosine in triggering ischemic preconditioning has never been confirmed in rat hearts. We have therefore studied the role of adenosine by stimulating the adenosine A_1- and A_3-receptors in triggering classic ischemic preconditioning (Chapter 2). We further studied whether different ischemic stimuli, i.e. a single 15-min ischemic stimulus or a triple 3-min ischemic stimulus, activate separate intracellular pathways involving adenosine receptors, free radicals, multiple kinases and activation of ATP-sensitive potassium channels. In both stimuli, we have demonstrated a pivotal role of uncoupled mitochondria as a unifying point (Chapter 3). Adenosine has been suggested to play a major role in inter-organ preconditioning. Therefore, we investigated the role of adenosine and its sites of action in inter-organ preconditioning by brief occlusion of the mesenteric artery (Chapter 4). Since the development of tolerance to cardioprotection by repeatedly applied preconditioning stimuli may interfere with therapeutical applications, we have further elucidated the underlying mechanism and search for means to circumvent development of tolerance (Chapter 5). Finally, we have shown that ischemia-reperfusion injury by 60-min coronary occlusion can be limited by administration of a tyrosine phosphatase inhibitor just prior to reperfusion (Chapter 6).

Adenosine receptors and classical ischemic preconditioning in the rat heart

The pivotal role of adenosine in eliciting preconditioning has been firmly established in most species used in experimental studies. However, in the rat heart its role had never been demonstrated and it was therefore concluded that adenosine does not play a role in myocardial infarct size limitation by ischemic preconditioning in rats.[1] In previous studies in the rat heart, the duration of the ischemic stimuli never lasted longer than 3-5 minutes.[2-4] Interestingly, Schulz et al. have shown in pigs that intracoronary infused adenosine deaminase was ineffective in attenuating infarct size limitation by a 3-min ischemic stimulus, but abolished the cardioprotection by a 10-min ischemic stimulus.[5] Capitalizing on these previous studies, we hypothesized that adenosine could play a role in ischemic preconditioning in rats when ischemic stimuli of longer duration are employed. In chapter 2 we therefore investigated the role of adenosine in a stimulus consisting of a 15-min coronary artery occlusion which has been demonstrated to elicit potent cardioprotection in the rat heart.[6] Pretreatment with non-selective adenosine receptor blockade (with 8-SPT) abolished the cardioprotective effect of the 15-min ischemic stimulus whereas cardioprotection by three cycles of 3-min coronary artery occlusion was not affected. Using selective A_1 (low dose of 8-SPT) and selective A_3 (MRS-1191) adenosine receptor blockade, we demonstrated that A_1 and A_3 receptors contribute equally to the cardioprotection by 15-min coronary artery occlusion. These findings indicate that adenosine receptors trigger ischemic preconditioning in the rat heart when the ischemic stimulus is sufficiently long.

In conclusion, the involvement of adenosine (and its receptors) in eliciting ischemic preconditioning is not species dependent, but depends critically on the duration of the ischemic preconditioning stimulus.

Mitochondrial uncoupling as a common (end)point of different cardioprotective signaling pathways

Classic myocardial ischemic preconditioning has traditionally been considered to trigger a single transduction pathway involving the stimulation of G-protein linked receptors (i.e. by adenosine, bradykinin and catecholamines), and activation of protein kinase C resulting in opening of ATP sensitive potassium channels. As aforementioned, Schulz et al recently demonstrated that different ischemic stimuli may coexist since adenosine was not involved in preconditioning by a 3-min coronary artery occlusion but contributed to the cardioprotection by a 10-min coronary artery occlusion.[5] In addition, Cohen et al reported in the rabbit heart, that in contrast to bradykinin, acetylcholine, opioids and phenylephrine, adenosine exerts myocardial preconditioning via a separate transduction pathway which does not involve opening of ATP sensitive potassium channels and the release of free radicals.[7] In chapter 3 we demonstrate that infarct size limitation by the adenosine-dependent 15-min coronary artery occlusion was not affected by the free radical scavenger mercapto-propionyl-glycerine, whereas combined pretreatment with both the tyrosine kinase inhibitor genistein and the protein kinase C inhibitor chelerythrine abolished infarct size limitation. Moreover, the mitochondrial ATP-sensitive potassium channel blocker 5-hydroxydecanoate also abolished cardioprotection by 15-min occlusion. Finally, we demonstrated that cardioprotection by adenosine infusion was abolished by genistein and chelerythrine, whereas cardioprotection by the selective mitochondrial ATP-sensitive potassium channel opener diazoxide was not affected. Thus in the rat heart, it appears that preconditioning by a single 15-min coronary artery occlusion elicits protection via adenosine, tyrosine kinase and protein kinase C and subsequently activation of mitochondrial ATP-sensitive potassium channels.

Conversely, infarct size limitation by the adenosine independent triple 3-min ischemic stimulus was abrogated by either mercapto-propionyl-glycerine, genistein or chelerythrine hereby implicating a pivotal role for free radicals, tyrosine kinase and protein kinase C. But, in contrast to the single 15-min ischemic stimulus, both pretreatment with 5-hydroxydecanoate or the non-selective ATP-sensitive potassium channel blocker glibenclamide had no effect on cardioprotection by the triple 3-min ischemic stimulus. To further study the role of free radicals and the sequence in the signal transduction pathway, we intravenously infused menadione wich generates mitochondrial free radicals by complex III inhibition. Indeed, menadione infusion limited infarct size to a subsequent coronary artery occlusion which was completely blocked by inhibiton of both tyrosine kinase and proteine kinase C, but unaffected by glibenclamide. It appears that preconditioning with a triple 3-min occlusion leads to release of free radicals, subsequently activating tyrosine kinase and protein kinase C without involving either adenosine or ATP-sensitive potassium channel opening.

The preservation of myocardial energy demand in ischemic preconditioning has been demonstrated by the observation that the rate of ATP loss was reduced during the subsequent prolonged period of ischemia versus control hearts.[8] Moreover, opening of mitochondrial ATP-sensitive potassium channels appears to play a major role in the signal transduction pathway in ischemic preconditioning.[9] Therefore, after inducing the preconditioning stimulus in the in vivo rat heart, mitochondria from the area at risk zone were isolated to determine oxygen consumption in state 2 (succinate) and state 3 (succinate+ADP). Subsequently, the respiratory control index (RCI) was calculated from an oxygen change in state 3 over state 2. Respiratory control index was decreased by both the single 15-min stimulus and the triple 3-min stimulus versus sham treatment demonstrating an uncoupled state of the mitochondria. Most interestingly, both glibenclamide and mercapto-propionyl-glycerine not only abolished the

cardioprotection by the single 15-min and the triple 3-min preconditioning stimuli, respectively, but also prevented the decrease in the respiratory control index. It appears that either an adenosine dependent signal transduction pathway, subsequently opening mitochondrial ATP-sensitive potassium channels, or an adenosine independent signal transduction pathway, generating free radicals, both result in uncoupled mitochondria.
In conclusion, different ischemic stimuli can activate different cardioprotective signal transduction pathways both leading to mitochondrial uncoupling as a common (end)point.

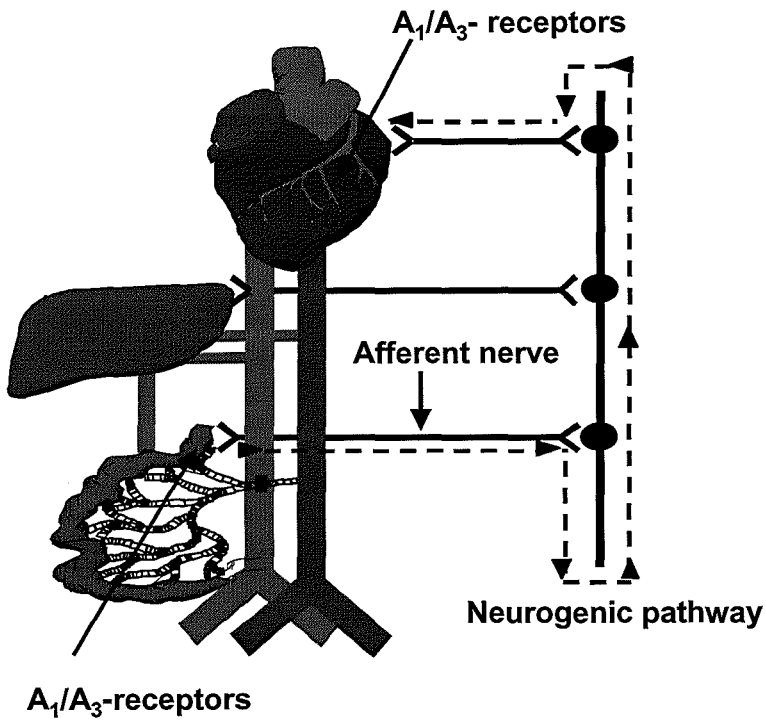

Figure 1 *Adenosine released during small intestinal ischemia activates a neurogenic pathway, that preconditions the heart via myocardial A_1- and A_3 receptors.*

Adenosine receptors and inter-organ preconditioning at a distance

Gho et al. previously demonstrated that 15-min of small intestinal ischemia followed by 10-min reperfusion was also capable of limiting infarct size produced by a subsequent prolonged coronary artery occlusion.[10] The authors observed that this phenomenon of inter organ preconditioning of the heart is induced via activation of a neurogenic pathway upon reperfusion. Based on these observations, Pell et al, implicated that adenosine released in the ischemic remote organ stimulated afferent nerves leading to myocardial protection.[11] In this *chapter 4* we investigated whether activation of adenosine receptors are involved in the protection by small intestinal ischemia, and we determined the locations of the adenosine receptors i.e. myocardial and/or small intestinal. Both pretreatment with an A_1-receptor selective low dose of 8-SPT and MRS attenuated infarct size limitation by interorgan

preconditioning with 15-min mesenteric artery occlusion. When 8-SPT and MRS were combined, the protection was abolished implying the involvement of both A_1- and A_3-receptors in myocardial preconditioning by 15-min intestinal ischemia. In addition, infusion of adenosine into the mesenteric artery limited infarct size by a subsequent 60-min coronary artery occlusion, which was abolished by pretreatment with the ganglion blocker hexamethonium. Moreover, a similar dose of adenosine, infused either into the portal vein or into the inferior caval vein, did not trigger cardioprotection which demonstrates that adenosine can elicit myocardial protection without directly reaching the heart via stimulation of small intestinal adenosine receptors and a neurogenic pathway. Finally, we demonstrated that hexamethonium when infused 5-min after the ischemic stimulus in the remote organ, did not affect cardioprotection suggesting that at this time point the neurogenic pathway is already activated. In contrast, infusion of a non-selective dose of 8-SPT also 5-min after the preconditioning stimulus, completely abolished infarct size limitation.

In conclusion, locally released adenosine during small intestinal ischemia stimulates afferent nerves in the mesenteric bed during reperfusion initiating a neurogenic pathway that leads to activation of myocardial adenosine receptors. (fig. 1) These findings suggest that adenosine and possible other cardioprotective agents can induce cardioprotection against ischemia without reaching the myocardial area at risk.

Tolerance to multiple ischemic preconditioning stimuli

Pharmacological exploitation of ischemic preconditioning to limit infarct size in the clinical setting is very promising. However, the therapeutical application may be limited by the development of tolerance to cardioprotection by repetitive brief ischemic stimuli. In conscious rabbits, Cohen et al[12] demonstrated that myocardial protection to prolonged ischemia by a single ischemic stimulus of 5-min coronary artery occlusion waned when preceded by forty to sixty-five 5-min occlusions over a three to four day period, but could be reinstated after an ischemia-free interval. The mechanism of myocardial tolerance to ischemic preconditioning remains incompletely understood, but it may involve loss of adenosine production, a reduced adenosine receptor responsiveness or modifications in downstream signaling pathways. In *chapter 5* we showed that classic preconditioning by either one or two 15-min classic ischemic preconditioning stimuli potently limited infarct size produced by a subsequent 60-min coronary artery occlusion. In contrast, ischemic preconditioning by six 15-min ischemic stimuli did not afford infarct size limitation demonstrating for the first time, that rats become tolerant to preconditioning after multiple ischemic stimuli. We first measured interstitial adenosine levels during repeated 15-min coronary occlusions by using a microdialysis probe. Adenosine levels were highly increased during the first ischemic episode whereas adenosine levels during the fourth episode were back to baseline level. We further investigated the occurance of reduced adenosine receptor responsiveness by preconditioning with multiple episodes of exogenous adenosine. Six episodes of 15-min adenosine infusions resulted in a blunted cardioprotective effect compared to one or two episodes implying a decreased responsiveness of adenosine receptors or down stream components. However, myocardium in which tolerance had developed after four episodes of 15-min coronary artery occlusions, two 15-min episodes of adenosine infusion was equally cardioprotective as two adenosine episodes by itself. This implies that tolerance after four episodes of classic preconditioning is not due to a reduced adenosine receptor responsiveness. Additionally, we demonstrated in myocardium that had become tolerant to four episodes of 15-min ischemia, that an alternative preconditioning stimulus was still able to elicit cardioprotection. Two episodes of interorgan preconditioning

stimuli, i.e. two 15-min of mesenteric artery occlusions, still limited infarct size to a subsequent 60-min coronary artery occlusion. Moreover, the adenosine-independent triple 3-min ischemic preconditioning stimulus was also still cardioprotective although slightly less.
In conclusion, the development of tolerance to classic ischemic preconditioning is due to a loss of adenosine production and not to a blunted receptor responsiveness. Moreover, tolerance does not lead to cross-tolerance to an alternative preconditioning stimulus.

Inhibition of tyrosine phosphatase limits myocardial reperfusion injury.

An increased tyrosine residue phosphorylation via increased tyrosine kinase activity has been implicated in the signal transduction pathway of cardioprotection produced by ischemic preconditioning.[13,14] There is evidence that increased tyrosine residue phosphorylation increases cell survival. Moreover, a recent study reported that phosphatase inhibition can be highly effective in protecting isolated cardiomyocytes when administered late after onset of ischemia.[15] Therefore in *chapter 6* we tested whether myocardial reperfusion injury can be limited by inhibition of tyrosine phosphatase. For this purpose, the tyrosine phosphatase inhibitor bis(maltolato)-oxovanadium (BMOV), was infused either before a 60-min coronary occlusion or prior to onset of reperfusion. When infused before occlusion, BMOV dose dependently limited infarc size by 60-min coronary occlusion. Infusion of the tyrosine kinase inhibitor genistein 10 minutes before onset of reperfusion, completely abrogated infarct size reduction implying that the infarct size limiting effect by BMOV is due to reperfusion injury in a tyrosine kinase dependent way. Indeed, when BMOV was infused 10 minutes prior to reperfusion, infarct size limitation was equal to treatment with BMOV before occlusion. Since ATP-sensitive potassium channels are downstream targets of tyrosine kinase we tested the protective effects of BMOV during ATP-sensitive potassium channel inhibition with glibenclamide. Subsequently, glibenclamide completely abrogated infarct limitation by BMOV, demonstrating a pivotal role of ATP-sensitive potassium channels. Because BMOV was infused during late coronary artery occlusion, it may not have reached myocardial area at risk zone. In view of our results in chapter 4, we further investigated whether BMOV elicited cardioprotection at a distance via a neurogenic pathway similar to inter-organ preconditioning. However, the ganglion blocker hexamethonium had no effect on cardioprotection, so it appears that BMOV acted directly on the heart.
In conclusion, inhibition of tyrosine phosphatase can protect against myocardial reperfusion injury via opening of ATP-sensitive potassium channels.

Determinants of infarct size

Within each preconditioning group, infarct size showed wide variability which was less in the control group. Variability in infarct size can be ascribed to variations in several known determinants of infarct size such as body temperature,[16] systemic hemodynamics, occlusion time,[17] collateral blood flow[18] and size of area at risk.[19,20] However, all experimental groups, either preconditioned or in control, were subjected to exactly the same index ischemia period. Myocardial temperature was carefully kept constant between 36.5 – 37.5 °C which excludes temperature as a possible factor for variability in infarct size. Moreover, in each study, heart rate-pressure product prior to onset of the 60-min coronary artery occlusion did not show any effect on infarct size, demonstrating that the variability in infarct size in the preconditioning groups is not due to hemodynamic changes or myocardial oxygen demands before occlusion. In dogs, infarct size variability is due to variable collateral blood flow, whereas rats are known

to have an insignificant collateral circulation.[21] Therefore in rats, it is not necessary to incorporate collateral circulation as a covariate in infarct size. Since area at risk size is a major determent of left ventricular necrosis, infarct size is generally expressed as a percentage of area at risk. Nevertheless, the size of the area at risk has been shown to affect the degree of cardioprotection by ischemic preconditioning in dogs,[22] rats[23] and pigs.[24] In these studies, the infarct size limiting effect of ischemic preconditioning shows a positive correlation with the size of area at risk, being most pronounced when area at risk is small. In contrast, infarct size in control hearts appeared independent of changes in area at risk. We have therefore examined whether the area at risk in our in vivo rat model has an affect on infarct size limitation by either the 15-min coronary occlusion, the triple 3-min occlusion and the 15-min mesenteric artery occlusion. In the control group, infarct area as a percentage of left ventricular mass showed a highly linear relation with area at risk as a percentage of left ventricular mass (figure 2A).

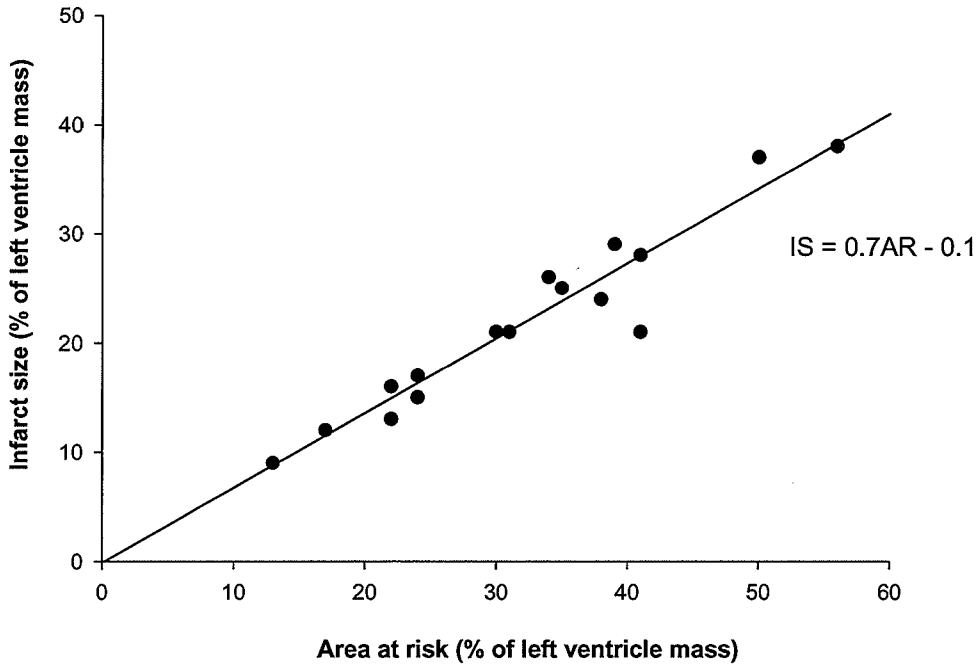

***Figure 2A** Relationship between the infarct size and the area at risk both presented as percentage of the left ventricular mass for the control group. The linear regression line shows a very strong relation. (r^2=0.92, P<0.001) Intercept is almost completely through the origin, suggesting a low collateral circulation in rats.*

For dogs, it has been shown that the linear relation between infarct size and area at risk in control hearts has a positive intercept on the area at risk axis which can be ascribed to a well developed collateral blood flow. In our in vivo rat heart, the linear relation in control hearts, intercepted almost completely through the axis with a very slight positive intercept suggesting a low collateral circulation in the rat heart. However, in pigs which are known to have limited collateral circulation, infarct size also showed a positive intersep with area at risk.[26] Since infarct size variations are limited in the control group, it is unlikely that wide infarct size variations in each preconditioning stimulus are due to collateral blood flow in our in vivo rat model. Additionally, in species like rabbits and pigs, the collateral flow to the area at risk is

negligible which results in less infarct size variability. To further investigate infarct size limiting effect of our ischemic preconditioning stimuli in relation to area at risk size, we plotted infarct size as a percentage of area at risk versus area at risk as a percentage of left ventricle mass (figure 2B to 2D). Confirming previous studies in dogs, pigs[24] and rats,[23] control infarction showed no correlation. However, in contrast to the aforementioned studies in which the infarct size limitation of ischemic preconditioning depends on the size of area at risk, all ischemic preconditioning stimuli showed no correlation with area at risk size. Thus, the wide variability in all our ischemic preconditioning groups is most likely not due to the variations in area at risk size since there is no correlation with infarct size limitation.

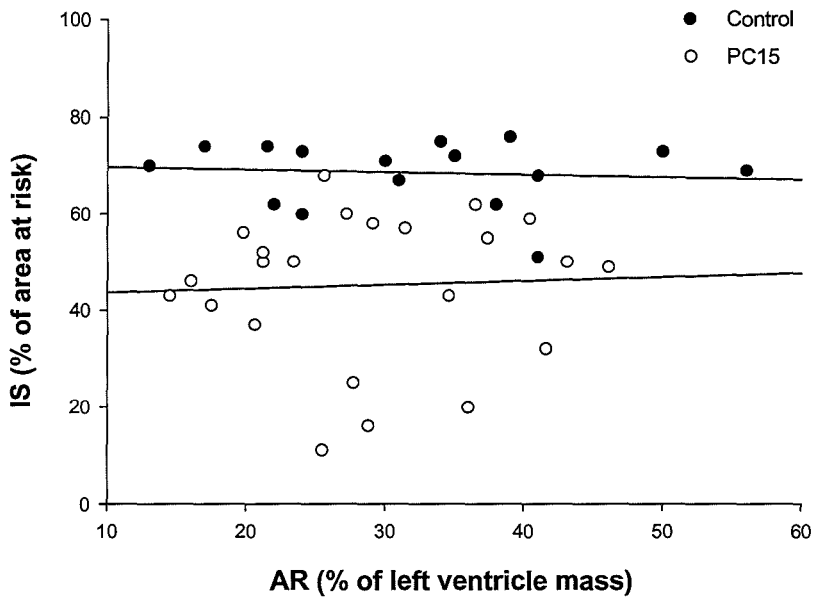

Figure 2B *Scatterplot of size of area at risk as a percentage of ventricular weight vs infarct size as a percentage of area at risk. No correlation was found between variables in the control group. (r^2 =0.01, P=0.81) In contrast to former reports in dogs, pigs and rats, no correlation was found in preconditioning by 15-min coronary occlusion. (r^2 = 0.002, P=0.84)*

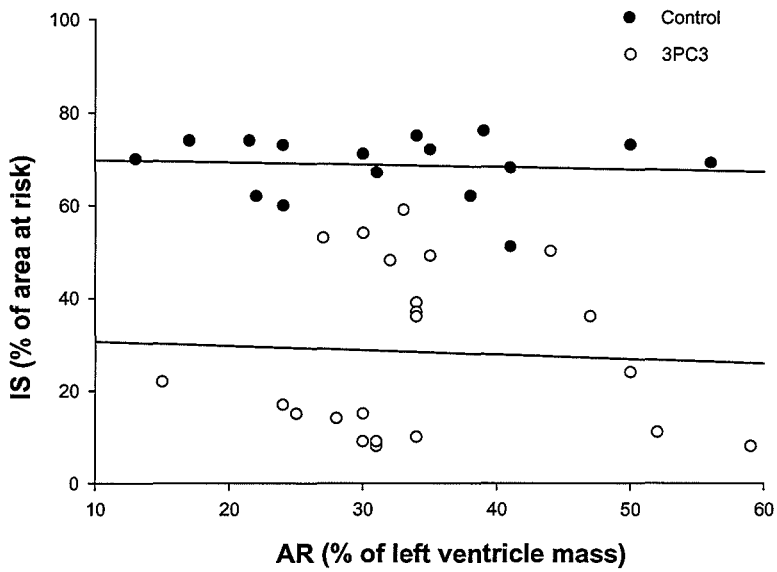

Figure 2C Scatterplot of size of area at risk as a percentage of ventricular weight vs infarct size as a percentage of area at risk. No correlation was found between variables in ischemic preconditioning by triple 3-min coronary occlusion (r^2 =0.01, P=0.81)

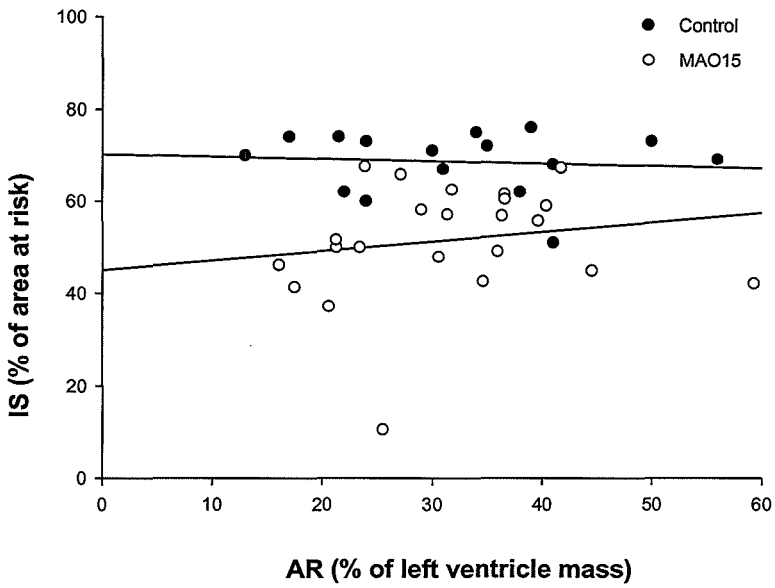

Figure 2D Scatterplot of size of area at risk as a percentage of ventricular weight vs infarct size as a percentage of area at risk. No correlation was found between variables in ischemic preconditioning by 15-min mesenteric artery occlusion (r^2 =0.03, P=0.5)

Over the past three years, standard deviation in each preconditioning group remained constant when an experiment was randomly added. Therefore it can be concluded that mutual infarct size variation within each preconditioning group can be ascribed to a physiological phenomenon. The evolution of myocardial infarction over time, follows a sigmoidal course. Ischemic preconditioning results in a rightward shift indicating a delay in cell death (figure 3). After 60-min coronary occlusion, infarct size development in the rat heart is nearly complete as the slope of the curve is almost horizontal. However, in the preconditioning curve, the curve of infarct size development is stil very steep at 60-min coronary occlusion indicating a fast rate of cellular death at this time point. Thus in our in vivo rat model, it appears that after ischemic preconditioning, wide variability in infarct size can be ascribed to the fast rate of cellular death at 60-min coronary occlusion.

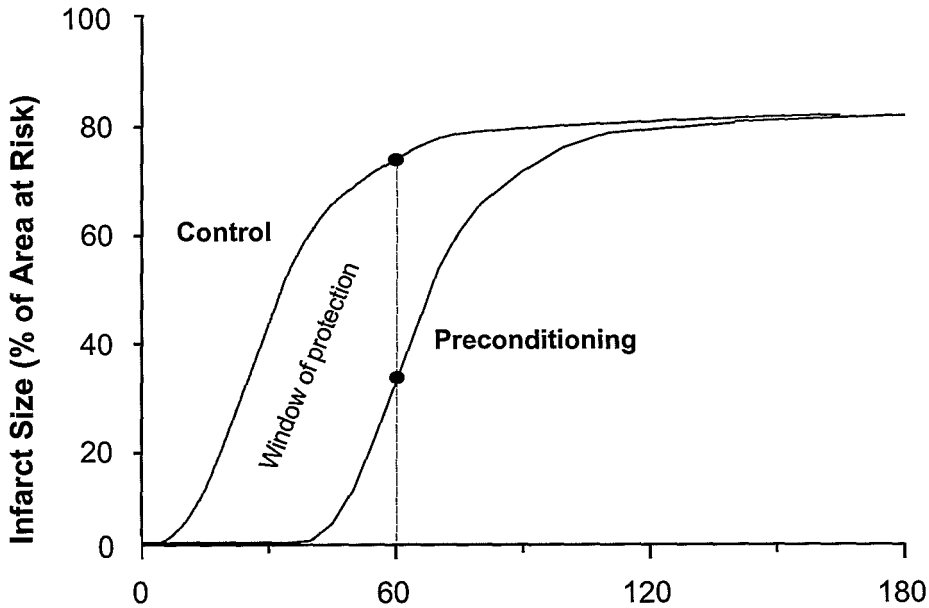

Figure 3 Schematic presentation of infarct size development by sustained coronary artery occlusion. At 60-min occlusion, the slope of the curve in the preconditioning group is much steeper versus the control group demonstrating an increased rate of cellular death at this time point.

Suggestions for future research

Intense research have yielded a vast amount of insight into the characteristics and mechanism of ischemic preconditioning. Moreover, several intracellular mechanisms responsible for the cardioprotection achieved with brief antecedent ischemia has been identified in virtually every species tested including human myocardium. Mechanistic studies have elucidated the underlying pathway(s) from the initial receptors to the incompletely understood end-effectors that are associated with this adaptation against prolonged ischemia. Subsequently, we not only learned many aspects of cellular physiology and molecular biology, but we have also identified a rational framework of pharmacological agents that mimic ischemic preconditioning. However, before the principles of ischemic preconditioning can be

added to the therapeutical arena, many aspects and questions need to be answered in greater detail.

Mechanisms of the second window of protection by separate transduction pathways

In this thesis we have shown that different ischemic preconditioning stimuli i.e. either an adenosine dependent stimulus by 15-min coronary artery occlusion or an adenosine independent stimulus by a triple 3-min coronary artery occlsusion, activate separate transduction pathways. In addition, the triple 3-min preconditioning stimulus limited infarct size by a subsequent coronary occlusion to a greater extent, also implying a separate mechanism. Ischemic preconditioning involves two phases of cardioprotection namely the first window of protection and a delayed second window of protection.[27] This thesis was focused on studying the first window of protection. Since all ischemic preconditioning stimuli confer delayed cardioprotection approximately 24-hours after the stimulus via transcription factors and protein synthesis, it would be interesting to elucidate the mechanisms of both ischemic stimuli (i.e. the single 15-min versus the triple 3-min stimulus) in the second window of protection, and to assess whether separate transduction pathways exert similar cardioprotection in the delayed phase. Moreover, separate transduction pathways may lead to synthesis of different proteins.

The role of uncoupled mitochondria in myocardial protection against ischemia

To date, the end-point of ischemic preconditioning has been in dispute. Three years ago, at the start of this thesis, the mitochondrial ATP-sensitive potassium channels were considered as potential end-effectors.[28] However, they have currently been proposed as mediators[29] and even as trigger.[30] A recent study by Schwartz et al even demonstrated cardioprotection by a preconditioning stimulus without the opening of ATP-sensitive potassium channels.[31] In our in vivo rat model, it appears that both the single 15-min preconditioning stimulus and the triple 3-min stimulus after activating separate pathways, unify into slightly uncoupled mitochondria. In chapter 3, both diazoxide and menadione have shown a strong infarct size limiting effect when infused before a 60-min coronary artery occlusion. The next step is to further investigate whether opening mitochondrial ATP-sensitive potassium channels or generation of free radicals respectively with diazoxide and menadione in vivo, lead to mitochondrial uncoupling. Other important targets to elucidate cardioprotection in relation to mitochondrial uncoupling are the uncoupling proteins which have been suggested to be activated by superoxide.[32] Uncoupling proteins are a family of mitochondrial membrane proteins that are known to uncouple electron transport from ATP production.[33]

Finally, a very important potential target for further investigation is the mitochondrial transition pore which is a protein complex between the inner and outer mitochondrial membranes. Pore opening as a result of calcium overload and oxidative stress is followed by the release of pro-apoptotic proteins such as cytochrome-c.[34,35] Preconditioning has been shown to reduce cytochrome-c release. Moreover, Baines et al has recently reported that protein kinase Cε can directly interact with and inhibit the mitochondrial transition pore. Therefore, further elucidation of the mitochondrial transition pore in both the single 15-min and the triple 3-min ischemic may yield a better understanding of the mitochondrial role in cardioprotection.

Inter-organ preconditioning of the heart

Despite accumulating evidence regarding the existence of inter-organ preconditioning, its exact mechanism remains incompletely understood. In contrast to classic preconditioning, the temporal profile of inter-organ preconditioning is unknown. In our rat model, a triple 3-min mesenteric artery occlusion did not confer cardioprotection whereas a triple 3-min coronary artery occlusion showed a strong infarct size limitation. Therefore, the minimal duration of preconditioning ischemia and its required reflow phase needs further investigation since it may not be similar to classic preconditioning. In addition, it also remains interesting to further study other possible charasteristics of inter-organ preconditioning such as the mechanism of the second window of protection.[36]

In chapter 4 we demonstrated a pivotal role for the mesenteric release of adenosine which is responsible for the activation of a neurogenic pathway. Also bradykinine has been reported to elicit cardioprotection at a distance. Similarly, a role for calcitonin gene related peptide, of which the release the release can be modulated by adenosine has been implicated in cardioprotection by small intestinal ischemia.[36] Therefore, further studies are required to elucidate other humoral factors and their sites of action in inter-organ preconditioning.

Finally, in chapter 3 we have demonstrated that protection by classical ischemic preconditioning likely involves mitochondrial uncoupling which may play a pivotal role in inducing myocardial protection against ischemia. Similarly, it would be of interest to further investigate in our in vivo rat model whether a small intestinal ischemic preconditioning stimulus also involves mitochondrial uncoupling.

Development of tolerance to classic ischemic preconditioning?

Four episodes of 15-min coronary artery occlusion lead to development of tolerance in which infarct size limitation is abrogated. However, we have demonstrated that this does not lead to cross-tolerance for alternative preconditioning stimuli such as exogenous adenosine, mesenteric artery occlusions and a triple 3-min coronary artery occlusion. In addition, we could further study the development of tolerance by multiple episodes of the triple 3-min coronary occlusions or 15-min mesenteric artery occlusion and the occurance of cross-tolerance for alternative stimuli.

Finally, since mitochondrial uncoupling may be a unifying endpoint in ischemic preconditioning and therefore be responsible for the mechanism behind cardioprotection against ischemia, it would be interesting to further investigate whether mitochondrial uncoupling is also lost after development of tolerance by multiple ischemic preconditioning stimuli.

Tyrosine kinase and myocardial reperfusion injury

As aforementioned, mitochondrial permeability transition has been shown to be a critical event in both necrotic and apoptotic cell death.[34,35] The exact nature of mitochondrial permeability transition in relation to apoptosis and/or necrosis is yet to be discovered. Protein tyrosine phosphatase is known to trigger apoptosis.[37] It has been suggested that apoptosis plays a pivotal role during myocardial reperfusion injury which may be triggered via pro-apoptotic proteins released from the mitochondrial transition pore. Moreover, protein kinase Cε has recently been demonstrated to inhibit the transition pore.[38] Therefore, a further goal of investigation would be the interaction of tyrosine kinase with the mitochondrial transition pore.

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Nederlandse samenvatting

In dit proefschrift staan de resultaten van de studies van het Laboratorium voor Experimentele Cardiologie naar het mechanisme van ischemische preconditionering in genarcotiseerde ratten. De rol van adenosine in ischemische preconditionering is, in tegenstelling tot de andere proefdier modellen, nooit aangetoond in het in vivo rattenhart. Daarom hebben wij de rol van adenosine A_1- en A_3-receptoren bij het induceren van klassieke ischemisch preconditionering bestudeerd (hoofdstuk 2). Hierop volgend hebben wij onderzocht of verschillende ischemische preconditionerings stimuli (een 15-min durende coronaire occlusie, of 3 cycli van 3-min coronaire occlusie) verschillende transductie paden induceren met betrekking tot adenosine-receptoren, vrije radicalen, kinase eiwitten en ATP-gevoelige kaliumkanalen. Wij hebben aangetoond dat ontkoppelde mitochondrieen een belangrijke rol spelen in beide ischemische preconditionerings prikkels (hoofdstuk 3). Er zijn studies die suggereren dat adenosine een belangrijke rol speelt bij inter-orgaan preconditionering. Daarom hebben wij onderzocht of adenosine een rol speelt bij inter-orgaan preconditionering door een 15-min durende occlusie van de arterie mesenterica superior. Verder hebben wij gekeken op welke lokatie (d.w.z. in het mesenterium, in de lever of in het myocardium) adenosine een rol speelt in dit phenomeen (hoofdstuk 4). Omdat de ontwikkelling van tolerantie voor cardioprotectie na herhaalde ischemische preconditionerings prikkels de therapeutische applicatie kan vertragen, hebben verder gekeken naar mogelijk heden om deze tolerantie te kunnen omzeilen (hoofdstuk 5). Tenslotte hebben wij aangetoond dat reperfusie-schade na een 60-min durende coronaire occlusie kan worden gereduceerd door infusie met een tyrosine phosphatase remmer vlak voor de reperfusie (hoofdstuk 6).

Adenosine receptoren en klassieke ischemische preconditionering in het rattenhart

Bij de meeste diersoorten is de rol van adenosine in ischemische preconditionering duidelijk aangetoond. Echter, in het rattehart heeft men de rol van adenosine nooit kunnen aantonen waarna men concludeerde dat deze in de rat geen rol zou spelen bij infarct schade beperking door ischemische preconditionering. In eerdere studies in de rat, heeft men altijd preconditionerings prikkels van 3-5 minuten gebruikt. Echter, Schulz et al. heeft in genarcotiseerde varkens aangetoond dat intra-coronair geinfundeerde adenosine deaminase niet in staat was de infarct schade beperking te blokkeren door een 3-min ischemische preconditionerings prikkel maar wel door een 10-min ischemische preconditionerings prikkel. Met het oog op deze studies hebben wij daarom als hypothese gesteld dat adenosine in de rat wel een rol speelt bij ischemische preconditionerings prikkels van langere duur. In hoofdstuk 2 hebben wij daarom de rol van adenosine bestudeerd bij een prikkel van 15-min coronaire occlusie welke in de rat leidt tot potente infarct schade beperking. Voorbehandeling met de non-selectieve adenosine receptor blokker 8-SPT blokkeerde het infarct beperkend effect van de 15-min durende preconditionerings prikkel. Echter, het infarct schade beperkend effect van drie cycli van 3-min coronaire occlusie werd met dezelfde dosering niet geblokkeerd. Door middel van een A_1 selectieve- (met een lage dosis 8-SPT) en een A_3-selectieve blokker, hebben wij aangetoond dat zowel A_1- en A_3-receptoren een gelijkwaardige rol spelen bij een ischemische preconditionerings prikkel van 15-min coronaire occlusie. Onze bevindingen suggereren dat adenosine receptoren ischemische preconditionering induceren in het rattenhart wanneer de ischemische preconditionerings prikkel voldoende lang is.

Concluderend kan gesteld worden dat de rol van adenosine (en adenosine receptoren) niet afhankelijk is van de diersoort, maar van de duur van de ischemische preconditionerings prikkel.

Mitochondriale ontkoppeling als een gemeenschappelijk (eind)punt van verschillende cardioprotectieve signaal transductie paden

Het wordt algemeen verondersteld dat klassieke ischemische preconditionering geactiveerd wordt via een enkele signaal transductie pad met betrekking tot G-eiwit gelinkte receptoren (voor adenosine, bradykinine, en catecholamines), activatie van proteine kinase C, en opening van ATP-gevoelige kalium kanalen. De eerder genoemde studie van Schulz et al. toonde aan dat verschillende ischemische preconditionerings stimuli naast elkaar kunnen bestaan aangezien adenosine niet betrokken is bij een 3-min coronaire occlusie, maar wel bij een 10-min coronaire occlusie. Hierop volgend, heeft Cohen et al. aangetoond in het konijnen hart dat, in tegenstelling tot bradykinine, acetylcholine, opiaten en catecholamines, adenosine myocardiale preconditionering induceert via een alternatieve transductie pad zonder generatie van vrije radicalen en ATP gevoelige kalium kanalen. In hoofdstuk 3 tonen wij aan dat de infarct schade beperking van de adenosine afhankelijke ischemische preconditionerings stimulus na 15-min coronaire occlusie niet kan worden gebokkeerd door de vrije radicalen scavenger MPG. Voorbehandeling met zowel de tyrosine kinase blokker genisteine en de proteine kinase C blokker chelerythrine blokkeerde de infarct schade beperking volledig. Ook de mitochondriale ATP-gevoelige kalium kanaal blokker 5-HD was in staat de cardioprotectie van een 15-min coronaire occlusie te blokkeren. Ten slotte hebben wij aangetoond dat cardioprotectie na adenosine infusie wordt geblokkeerd door voorbehandeling met zowel genistein en chelerythrine terwijl de cardioprotectie van de mitochondriale kalium kanaal opener diazoxide niet kon worden geblokkeerd. In de ratten hart blijkt preconditionering met een 15-min coronaire occlusie geinduceerd te worden via adenosine, tyrosine kinase en proteine kinase C hetgeen resulteert in opening van mitochondrial ATP-gevoelige kalium kanalen.

Hierop volgend hebben wij aangetoond dat het cardioprotectieve effect van de adenosine-onafhankelijke preconditionerings prikkel van drie cycli van 3-min coronaire occlusie kan worden geblokkeerd door zowel MPG, genistein en chelerythrine. Echter, 5-HD was, in tegenstelling tot de 15-min prikkel, niet in staat het cardioprotectieve effect te blokkeren. Om de verdere rol van vrije radicalen in het signaal transductie pad hebben wij de mitochondriale radicalen generator menadione geinfundeerd. Infusie van Menadione blijkt inderdaad in staat te zijn infarct schade te beperken tegen een hierop volgend 60-min coronaire occlusie. De cardioprotectie van menadione kon volledig worden geblokkeerd door genistein en chelerythrine maar niet door de non-selectieve ATP-gevoelige kalium kanaal blokker glibenclamide. Preconditionering na drie cycli van 3-min coronaire occlusie leidt blijkbaar tot generatie van vrije radicalen gevolgd door activatie van tyrosine kinase en proteine kinase C, zonder activatie van ATP-gevoelige kalium kanalen.

Gedurende een lange periode van ischemie, is het myocardiale verbruik van ATP sterk vermindert na ischemische preconditionering in vergelijking met controle harten. Voor verdere studie hebben wij na toediening van een ischemische preconditionerings prikkel in het in vivo ratten hart de mitochondrieen van het risico gebied geisoleerd en gekeken naar de zuurstof consumptie in state 2 (succinaat) en state 3 (succinate + ADP). Hieruit werd de respiratoire controle index (RCI) bepaald in state 3 over state 2. Zowel in de 15-min preconditionerings stimulus als in de 3 keer 3-min preconditionerings stimulus was de RCI licht gedaald in vergelijking met de controle groep. Na beide preconditinerings prikkels zijn de mitochondrien dus licht ontkoppeld. Glibenclamide en MPG blokkeerden de RCI dalingen respectivelijk in de 15-min occlusie en de drie keer 3-min occlusie. Blijkbaar resulteren zowel een adenosine-afhankelijke preconditionerings prikkel (met ATP-gevoelige mitochondriale kalium kanaal

opening), en een adenosine-onafhankelijke preconditionerings prikkel (met generatie van vrije radicalen), tot licht ontkoppelde mitochondrieen.
Concluderend kan gesteld worden dat verschillende ischemische preconditionerings stimuli, verschillende cardioprotectieve transductie paden induceren. De signaal transductiepaden komen samen op het niveau van mitochondriale ontkoppeling.

Adenosine receptoren en inter-orgaan preconditionering op afstand

Een studie door Gho et al. heeft eerder aangetoond dat een 15-min durende occlusie van de arterie mesenterica superior gevolged door 10-min reperfusie ook in staat is het hart te beschermen tegen ischemie. Dit phenomeen van inter-orgaan preconditionering van het hart bleek te berusten op activatie van een neurogeen pad tijdens de reperfusie periode. Op basis van deze observatie, hebben andere onderzoekers aangetoond dat adenosine, vrijgekomen in een orgaan op afstand, afferente zenuwen prikkelt hetgeen leidt tot myocardiale bescherming tegen ischemie. In hoofdstuk 4 werd bestudeerd of adenosine receptoren betrokken zijn bij inter-orgaan preconditionering na een 15-min durende occlusie van de darm arterie. Vervolgens werd gekeken naar de lokalisatie van deze receptoren d.w.z. mesenteriaal en/of cardiaal. Zowel een A_1 receptor selctieve dosis van 8-SPT als de A_3-antagonist MRS blokkeerden het infarct-reducerend effect van 15-min darm arterie occlusie. Infusie van adenosine in de arterie mesenterica bleek de infarct –schade van een hierop volgend 60-min coronaire occlusie te reduceren hetgeen kon worden geblokkeerd door de ganglion blokker hexamethonium. Een gelijke dosis adenosine welke in de vena porta werd geinfundeerd bleek geen cardioprotectief effect te induceren. Adenosine blijkt dus cardioprotectie te kunnen induceren via stimulering van mesenteriale adenosine receptoren gevolgd door activatie van een neurogeen pad. Aangezien hexamethonium op 5-min reperfusie na de preconditionerings prikkel geen effect had op het cardioprotectieve effect terwijl 8-SPT dit effect volledig blokkeerde, kunnen wij concluderen dat ook myocardiale adenosine receptoren zijn betrokken bij inter-orgaan preconditionering van het hart.
Concluderend kan worden gesteld dat adenosine dat vrij komt tijdens een kortdurende darm-arterie occlusie, afferente zenuwen kan stimuleren in het mesenterium tijdens reperfusie waarop een neurogeen pad wordt geactiveerd, hetgeen wederom leidt tot activatie van myocardiale adenosine receptoren. Deze bevindingen suggereren dat adenosine, en mogelijk ook andere cardioprotectieve agentia, cardioprotectie kunnen induceren zonder dat deze het risicogebied bereiken.

Tolerantie voor preconditionering na multiple ischemische preconditionerings stimuli

Farmacologische exploitatie van ischemische preconditionering lijkt zeer veel belovend. Echter, de therapeutische applicatie zou wel eens belemmerd kunnen worden door de ontwikkeling van tolerantie voor cardioprotectie na herhaalde ischemische prikkels. In het konijnhart is aangetoond dat in tegenstelling tot een enkele 5-min ischemische preconditionerings prikkel, veertig tot vijfenzestig 5-min ischemische preconditionerings prikkels niet leiden tot cardioprotectie. Het mechanisme van myocardiale tolerantie voor ischemische preconditionering is nog steeds moeilijk te omvatten. Het onderliggend mechanisme kan een verminderde adenosine produktie omvatten, een verminderde receptor activiteit, of andere componenten van het signaal transductie pad. In hoofdstuk 5 hebben wij aangetoond dat zowel een enkele, als twee episoden van 15-min klassieke ischemische preconditionerings prikkels in staat waren om de infarct schade veroorzaakt door 60-min durende coronair occlusie te beperken. Echter, ischemische preconditionering door zes

episoden van 15-min coronaire occlusies bleek geen cardioprotecie te induceren. Hiemee werd voor het eerst in rattenharten aangetoond dat tolerantie kan optreden na herhaalde ischemische preconditionerings stimuli. Eerst werd de interstitiele adenosine concentratie gemeten tijdens herhaalde 15-min coronaire occlusies d.m.v. de microdialyse techniek. Tijdens de eerste 15-min occlusie was de adenosine concentratie duidelijk verhoogd, terwijl deze bij de vierde 15-min occlusie op baseline niveau bleven. Zes episoden van adenosine infusies resulteerden in een licht verminderde cardioprotectie in vergelijking met een- of twee episoden van adenosine infusies, hetgeen suggereert dat er een verminderde respons optreedt van adenosine receptoren of verder op gelegen componenten in het signaal transductie pad. In myocardium waarin tolerantie is opgetreden na vier episoden van 15-min coronaire occlusie, bleken twee hierop volgende episoden van adenosine infusie een gelijke mate van cardioprotectie te induceren t.o.v twee enkele episoden van adenosine infusie. Dit suggereert dat het optreden van tolerantie na vier episoden van 15-min coronaire occlusie niet het gevolg is van een verminderde adenosine receptor gevoeligheid. Zowel inter-orgaan ischemische preconditionering, als drie cycli van 3-min coronair occlusies waren nog in staat om cardioprotectie te induceren.
Concluderend kunnen wij stellen dat het optreden van tolerantie voor klassieke ischemische preconditionering het gevolg is van een verlies van adenosine productie, en niet het gevolg van een verminderde receptor gevoeligheid. Tevens leidt tolerantie voor klassieke ischemische preconditionering niet tot tolerantie voor een alternatieve preconditionerings prikkel.

Inhibitie van tyrosine fosfatase leidt tot beperking van myocardiale reperfusie schade

Verondersteld wordt dat een verminderde tyrosine forforylering, hetgeen leidt tot een verhoogde tyrosine kinase activiteit, een belangrijke rol speelt in het mechanisme van ischemische preconditionering. Er zijn aanwijzingen dat een toegenomen tyrosine fosforylering leidt tot een betere cel overleving bij ischemie. Recent is aangetoond dat inhibitie van tyrosine fosfatase in een late fase tijdens ischemie nog altijd kan leiden tot cardioprotectie tegen ischemie in geisoleerde cardiomyocyten. In hoofdstuk 6 hebben wij onderzocht of myocardiale reperfusie schade kan worden beperkt door inhibitie van tyrosine fosfatase. Hiervoor werd de tyrosine fosfatase inhibitor bis(maltolato)-oxovandium (BMOV) gebruikt. BMOV werd voor een 60-min coronaire occlusie, en vlak voor de reperfusie geinfundeerd. Bij infusie vlak voor occlusie, bleek er een dosis afhankelijke infarct schade beperking op te treden. Wanneer hierbij de tyrosine kinase blokker genistein vlak voor reperfusie werd geinfundeerd, bleek de cardioprotectie volledig te worden opgeheven. Het blijkt derhalve dat BMOV myocardiale reperfusie schade beperkt op basis van een tyrosine kinase afhankelijke mechanisme. Wanneer BMOV 10-min voor de start van reperfusie werd geinfundeerd, bleek de infarct schade beperking gelijk te zijn t.o.v. behandeling met BMOV voor de 60-min occlusie. Aangezien ATP-gevoelige kalium kanalen downstream zijn gelocaliseerd van tyrosine kinase, hebben wij derhalve de effecten van BMOV bestudeerd tijdens inhibitie van ATP-gevoelige kalium kanalen. Het cardioprotectieve effect van BMOV bleek volledig te worden geblokkeerd door behandeling met glibenclamide hetgeen een belangrijke rol voor ATP-gevoelige kalium kanalen suggereert. Aangezien BMOV in de late fase van de coronaire occlusie werd geinfundeerd, is het mogelijk dat deze cardioprotectie induceert zonder dat deze het risico gebied bereikt. Derhalve hebben wij onderzocht of het effect van BMOV te blokkeren is met voorbehandeling met hexamethonium. Deze bleek echter geen invloed te hebben op het cardioprotectieve effect van BMOV hetgeen suggereert dat deze direkt werkt op het hart.
Concluderend kunnen wij stellen dat inhibitie van tyrosine fosfatase myocardiale reperfusie schade aanzienlijk kan beperken via tyrosine kinase en ATP-gevoelige kaliumkanalen.

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Time to move on.

Curriculum vitae

David Liem was born on the 5th of December 1973 in Tilburg. After one year of medical school at the University of Antwerp in Belgium, he continued his education in September 1993 at the Erasmus University Rotterdam. In February 1997 he started a twelve-month period as a student-research assistant at the Laboratory of Experimental Cardiology. After graduating as a medical doctor in April 2000, he started as a PhD-candidate at the Laboratory of Experimental Cardiology under the intense guidance of Professor Piet Verdouw and Professor Dirk Duncker. On the 4th of November 2001, he finished the New York Marathon after "approximately" four hours. He was beaten by the Ethiopian runner Tesfaye Jifar who won the marathon in two hours and seven minutes. In July and August 2003 he worked as an assistant at the Department of Cardio-Thoracic Surgery at the Academic Medical Center Amsterdam. In September 2003 he will start as a post-doctoral fellow in Los Angeles at the Division of Cardiology, David Geffen School of Medicine at UCLA.

List of publications

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