Protection Against Myocardial Ischemia and Reperfusion

Preconditioning, postconditioning and hibernation

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Olivier Christiaan Manintveld
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Promotiecommissie

Promotoren:	Prof. dr. D.J.G.M. Duncker
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	Prof. dr. A. van der Laarse

What we do in life echoes on in eternity (Maximus Decimus Meridius).

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Chapter 1

General introduction and outline of the thesis

Epidemiology

Ischemic heart disease is currently the leading cause of morbidity and mortality in the industrialized world and is expected to become the leading cause of death world wide by the year 2020 when it will surpass infectious diseases.[1, 2] In 2006 42,522 people died of cardiovascular disease (CVD) in the Netherlands. This translates into an average of 116 people per day that die due to CVD. When looking at all cause mortality one in every three persons dies due to CVD. Ischemic heart disease is the main component of this group with 12,491 deaths, while 9,976 deaths are due to cerebrovascular disease. Together they are responsible for 52% of the deaths in CVD. Around 70% of the people with ischemic heart disease die due to an acute myocardial infarction (AMI).[3] The impact of CVD on health care is reflected by the number of invasive interventions: the number of percutaneous coronary interventions (PCI) is still increasing (33,678 in 2006), while the number of open heart surgeries (coronary artery bypass grafting or CABG) is stable over the past 5 years (around 15,000).[3]

After myocardial infarction, the surviving myocardium undergoes a complex sequence of cardiac remodeling, which may have a beneficial effect on cardiovascular performance in the short-term, but which become detrimental in the long-term and ultimately causes heart failure. In experimental studies, the degree of deleterious remodeling is highly affected by the size of the infarct.[4] Accordingly, clinical reports indicate that infarct size estimated by peak plasma creatine kinase activity is an independent predictor of left ventricular remodeling and the subsequent development and severity of heart failure.[5] In view of the important role myocardial infarct size plays in the etiology of heart failure, limiting infarct size is an important strategy to reduce the incidence and severity of heart failure. Furthermore, infarct limitation is an important therapeutic goal since it is also related to other factors such as severity of ventricular arrhythmias, mortality and loss of productivity. Hence, it is of utmost importance to salvage as much myocardium as possible by initiating reperfusion as rapidly as possible, either by PCI or CABG, in addition to other therapeutic strategies that target the remodeling process (e.g. angiotensin converting enzyme inhibitors).[6]

This thesis will mainly focus on some of the mechanisms involved in adaptation to ischemia and reperfusion in the myocardium in order to minimize cell death and hence limit infarct size.

Consequences of ischemia and reperfusion

Ischemia is defined as insufficient supply of oxygenated blood to the heart that is due to obstruction of the inflow of arterial blood by the narrowing of coronary arteries by spasm or disease. Prolonged ischemia can result in myocardial dysfunction. In an experimental setting an abrupt coronary artery occlusion (CAO) is performed by closure of a suture, which is

looped around the left anterior descending coronary artery, to initiate ischemia. This suture can be released to initiate reperfusion. Intravascular balloon occlusion is another manner to produce CAO. Both models allow strict control over duration of ischemia and reperfusion. Ischemia has various effects on function, metabolism and structure of the myocardium. The following paragraphs describe several stages of functional impairment of increasing severity: acute myocardial stunning, hibernation and myocardial infarction.

Acute myocardial stunning

Acute myocardial ischemia rapidly impairs contractile function,[7] which can persist for several hours but eventually fully recovers, provided that the episode is not too long.[8] Heyndrickx *et al*.[8] were the first to demonstrate that myocardial ischemia produced by a 5- or 15-min episode of CAO resulted in a prolonged deficit in regional contractile function in dogs, which returned to normal after 6 hours and 24 hours, respectively. This phenomenon is now known as myocardial stunning.[9, 10] Myocardial stunning can also occur in a variety of clinical settings; following exercise in the presence of a flow-limiting stenosis;[11, 12] following a brief period of coronary spasm in a patient with angina;[12] following global ischemia after cardiopulmonary bypass; in combination with subendocardial infarction, where the overlying subepicardium may be stunned for days to weeks.[13] Even angioplasty can cause stunning.[13] The underlying mechanism of acute myocardial stunning and its treatment are beyond the scope of this thesis and will not be further discussed. For a detailed overview the reader is referred to a recent review.[14]

Hibernation

Over 30 years ago, surgeons and clinicians noticed that chronic myocardial dysfunction, present before coronary bypass, often improved after revascularization.[15-17] This phenomenon was termed hibernation, a term borrowed from zoology and implies an adaptive reduction of energy expenditure through reduced activity in a situation of reduced energy supply. Rahimtoola[18] was the first to propose that myocardial hibernation was a condition in which a prolonged subacute or chronic state of myocardial ischemia results in a new equilibrium in which myocardial necrosis is not present because myocardial metabolism and function are both reduced to match a concomitant reduction in coronary blood flow due to the presence of a moderate to severe flow-limiting stenosis. This is referred to as the "smart heart" hypothesis.[18] Later, an alternate mechanism was proposed: the "repetitive stunning" hypothesis states that myocardium is subjected to repeated episodes of ischemia reperfusion which causes stunning that eventually creates a sustained depression of contractile function.[19] The essential features of hibernating myocardium are that metabolism and contractility are reduced in response to a reduction in coronary blood flow, but that reperfusion can restore contractile function back to normal. However, hibernating myocardium can still exhibit alterations of cellular metabolism, myocardial structure, myocardial perfusion and subendocardial flow

reserve.[20] Morphological changes in myocardial hibernation are characterized by signs of atrophy, apoptosis, degeneration and possibly dedifferentiation.[21] However, remote control myocardium can also exhibit important alterations,[22, 23] In patients with coronary artery disease, repeated episodes of ischemia may lead to cumulative stunning that could lead to the development of chronic post-ischemic left ventricular dysfunction.[24] Importantly, it has been shown that the magnitude of improvement in left ventricular ejection fraction after revascularization is directly related to the extent of hibernating myocardium.[25] The presence and extent of viability is also significantly related to the improvement of heart failure symptoms and quality of life after revascularization.[25] The phenomenon of myocardial hibernation is focus of research in Chapter 6 of this thesis.

Myocardial infarction

Ischemia (without residual flow in the infarct-related artery and without collateral flow) lasting more than 20 min leads to irreversible tissue injury. In 1960, Jennings *et al*.[26] described the deleterious changes observed after reperfusion of ischemic myocardium. They reported cell swelling, contracture of myofibrils, disruption of sarcolemma, and calcium phosphate granules within mitochondria;[26] all factors associated with necrosis, which is known to induce an inflammatory response.[27] At the time of their discovery, Jennings *et al*.[26] suggested that reperfusion hastened the demise of cells that were already injured by ischemia. Reperfusion was not thought to induce cell death in cells that were viable at the end of ischemia. The post-ischemic events and their physiological manifestations were collectively termed "reperfusion injury". Since then, the concept of lethal reperfusion injury as an independent mediator of cell death, distinct from ischemic injury, has been hotly debated. The uncertainty relates to the fact that we are unable to accurately assess *in situ* the progress of necrosis during the transition from myocardial ischemia to reperfusion.[28] The most convincing evidence of the existence of lethal reperfusion injury as a distinct mediator of cardiomyocyte death is to show that myocardial infarct size can be reduced by an intervention used at the beginning of myocardial reperfusion. Other factors that can be influenced by limiting reperfusion injury include dysfunction of the area at risk, endothelial dysfunction, metabolic defects, microvascular blood flow defect, necrosis and apoptosis.[28]

Cardiomyocyte apoptosis has gained notoriety as an important source of cell loss, both in ischemic[29-33] and heart failure syndromes.[34-37] Apoptosis, in contrast to necrosis, is a form of programmed cell death that is characterized by cellular shrinkage, condensation of chromatin and nuclei, non-random DNA fragmentation, formation of apoptotic bodies, and persistence of intact cellular organelles such as mitochondria and cell membranes, without inciting an inflammatory response.[38] Ongoing research is looking into the critical events in cell death, both necrotic and apoptotic.

Mitochondria: gatekeepers of life and death

The mitochondrion is the powerhouse of the body, being responsible for >95% of the adenosine 5'-triphosphate (ATP) generation in most cell types. Thus, it is not surprising that, besides intracellular calcium regulation and oxygen sensing, initiation of cell death by apoptosis or necrosis is regulated by the mitochondria.[39] Mitochondrial ATP production is a highly sophisticated and regulated process. Substrate is provided by oxidation of glucose (glycolysis), fats, and amino acids from which acetyl coenzyme A is produced within the mitochondrion. This is incorporated within the Krebs cycle, generating reducing equivalents in the form of nicotinamide adenine dinucleotide and flavin adenine dinucleotide, which then provide electrons to complex I and II, respectively, of the electron transport chain.[39] As electrons are passed down the chain to complexes III and IV, protons move across the inner mitochondrial membrane to generate a proton gradient that can drive ATP synthase to generate ATP from adenosine 5'-diphosphate (ADP).[39] Ischemia causes a rapid decline in creatine phosphate and increased content of intracellular phosphate. Glycolysis is increased, leading to the accumulation of harmful metabolic byproducts such as lactate and hydrogen ions. In the absence of oxygen, mitochondrial ATP levels rapidly fall due to the arrest of oxidative phosphorylation as well as due to an increase in consumption of high energy phosphates by ATP synthase by the reverse catalytic reaction of ATP to ADP.[39] If ischemia continues indefinitely, myocyte ATP sources are reduced to a critical level and intracellular sodium and calcium are increased, with the ultimate progression to necrosis.[39] Although mitochondrial changes in structure and respiratory function in response to ischemia and reperfusion have been documented for over half a century, the last decade has brought understanding of the decisive role played by mitochondria in determining cell fate during and after cellular stress. It is known that an elevated mitochondrial membrane potential is associated with an enhanced formation of reactive oxygen species (ROS),[40, 41] while a small decrease in mitochondrial membrane potential (i.e. mild mitochondrial uncoupling) very effectively prevent ROS formation without seriously compromising cellular energetics. This occurs due to a limitation of the life span of reduced electron transport chain intermediates capable of generating ROS, in addition to a decrease in local oxygen tensions.[40, 42-44] The prevention of mitochondrial ROS release can avoid cytotoxic effect of ROS generated in excess, including oxidation of proteins, DNA and lipids.

A key component of mitochondrial response to stress is the formation of the mitochondrial permeability transition pore (MPTP), a non-specific, multimeric pore structure spanning the mitochondrial inner and outer membranes.[45, 46] The precise structure of the MPTP is still unknown. MPTP serves as a high-conductance, non-selective voltage-dependent channel which is closed under normal physiological conditions leaving the inner membrane impermeable to most solutes. Due to cellular stress the MPTP can open in the inner membrane resulting in the loss of membrane impermeability and rapid collapse of the mitochondrial

membrane potential. The factors that determine whether and when the MPTP opens during myocardial ischemia/reperfusion and evidence for MPTP opening contributing to ischemia/reperfusion injury have been studied extensively during the last twenty years.[47, 48] Current opinion is that conditions during early reperfusion, but not during ischemia, favor the formation of the open pore and that inhibition of pore opening in reperfusion protects against cell death. Evidence favors MPTP opening causes swelling and uncoupling of mitochondria, which unrestrained, leads to cell death by necrosis,[49] although it has been proposed that either apoptosis or necrosis might be precipitated, depending on the extent of MPTP opening.[48] Although molecular events during ischemia determine whether MPTP opens during reperfusion, this new view invokes the idea that specific manipulation during reperfusion of conditions that inhibit MPTP opening offers the potential to attenuate cell death through reperfusion-specific cardioprotective strategies.

Therapy of ischemia and reperfusion damage

Reperfusion

Ischemia ultimately leads to cell death if not resolved by reperfusion. Many investigators have studied myocardial ischemia and searched for means to reduce it in order to limit cellular injury. Early studies by Maroko *et al*.[50] introduced the concept of myocardial salvage from ischemic injury. These studies attempted to lower the energy demand of the heart to reduce overall severity of the energy supply/demand mismatch during ischemia. However, that approach proved unsuccessful in the absence of reperfusion. Subsequent studies laid the foundation for early reperfusion as the definitive approach to treat AMI.[51] Specifically, Reimer *et al*.[51] described the time course of infarction resulting from coronary artery occlusion as an advancing "wave front" whereby the necrotic edges extend toward the epicardial surface with increasing durations of coronary artery occlusion. The implication was that the advancing necrotic wave front within the area at risk could be limited by restoring reperfusion as early as possible after onset of ischemia. In patients with an AMI it has been shown that AMI was largely an occlusive event of sudden onset, due to thrombus formation in the affected coronary artery.[52] This has set the stage for therapies aimed at resolving the thrombus or "thrombolysis" as an approach to restore flow to the ischemic myocardium. Two years after the first thrombolytic therapy study,[53] Rentrop *et al*.[54] were the first to disrupt a coronary artery thrombus in an acutely occluded artery using a guide wire passed through a catheter, the first PCI. Since then PCI and CABG have become the standard treatment for AMI. Despite the necessity to initiate reperfusion in order to salvage myocardial tissue, reperfusion itself has the potential to cause further myocardial damage. This is referred to as "reperfusion injury".[55] Further modification of reperfusion conditions could thus limit myocardial infarct size.

Ischemic preconditioning

Characteristics of ischemic preconditioning

The first report that the heart can adapt to ischemic stress was demonstrated in a porcine model of repeated myocardial ischemia. Lactate release and ATP breakdown proved to be significantly less during the second of two identical episodes of 15 min coronary blood flow reduction, which were separated by 30 min of complete reperfusion.[56] A few years later the responses of ischemic acute renal failure to additional ischemic events was described.[57] Accordingly, the phenomenon of ischemic preconditioning (IPC) was described.[58] Briefly, in anesthetized dogs IPC by four repetitive 5 minute periods of regional ischemia and reperfusion induced a powerful protection against the following period of index ischemia. Infarct size after 40 min of ischemia and 4 days of reperfusion was limited by IPC from 29±4% to 7±2% of the area at risk. The recognition of IPC since 1986 has proven to be the most significant development in the quest to identify rational approaches to limit infarct size. The protective effect of IPC was independent of changes in transmural myocardial blood flow. It was proposed that the effect was the result of rapid metabolic adaptation of the ischemic myocardium. The wide reproducibility of this phenomenon using a variety of preconditioning protocols in a number of species and experimental preparations and with a number of endpoints in protection rapidly led to IPC being established as a "golden standard" by which other strategies are compared.[59] An abundance of research effort (over 3000 papers cite the work of Murry *et al*.[58]) has identified a number of molecular mechanisms pertinent to cell death and cytoprotection that form the basis of contemporary experimental approaches. Various investigators have recognized that the cardioprotective potential of IPC is transient,[60, 61] which has lead to the distinction of a first and second window of protection. While "classic" or "early" preconditioning is effective up to 2 hours after its stimulus (i.e. first window of protection), "delayed" or "late" preconditioning is effective between 24-72 hours after the preconditioning stimulus (i.e. second window of protection).[62, 63] Whereas early IPC relies on post-translational modification of proteins that are already present, late IPC requires transcription and protein synthesis to exhibit its protective effect.[62, 63]

With IPC the preconditioned myocardium has been ischemic before that area at risk enters the index ischemia. However, this is not a prerequisite since remote intra-organ IPC, as well as remote IPC in a distant organ have also been shown to protect the myocardium. Remote intra-organ IPC was first described by Przyklenk *et al*.,[64] who showed that by applying brief circumflex coronary occlusions in canine myocardium induced protection preceding prolonged ischemia in remote myocardium supplied by the left anterior descending coronary artery. In our laboratory, it has subsequently been shown that brief ischemia in remote organs, such as the intestines and the kidney, preceding a sustained CAO is also cardioprotective.[65] A neurogenic pathway was shown to be involved in the protection of remote IPC in mesenteric artery occlusion, but this was not investigated in the kidney.[65] Locally released adenosine has been

shown to be an important mediator in the protection of the neurogenic pathway involved in remote IPC by mesenteric artery occlusion.[66] In addition to these findings proteomic research suggests that a humoral mediator smaller than eight kDa or a neurogenic pathway is involved in remote IPC in the kidney.[67] Also skeletal muscle has been investigated as a remote stimulus of preconditioning.[68] Birnbaum *et al*.[68] induced remote IPC by 30 min of skeletal muscle ischemia in a rabbit model by means of a flow reduction of ~60% of the femoral artery combined with electric stimulation of the gastrocnemius muscle. Subsequently the animals were exposed to a 30-min CAO followed by four hours of reperfusion. Infarct sizes were considerably smaller in the animals that had received skeletal muscle preconditioning.[68] This model is clinically very relevant due to the occurrence of peripheral vascular disease in humans. Although peripheral vascular disease may not cause any problems in rest, it might be that walking leads to ischemia in the legs due to insufficient blood supply. Ischemic pain causes the patient to stop walking after which blood supply in rest is once more sufficient. This can cause a preconditioning effect.

Some studies have demonstrated that a large number of repeated bouts of brief ischemic stimuli do not confer cumulative cardioprotection.[69, 70] In fact, Cohen *et al*.[69] have shown that 40 to 65 5-min CAO periods can result in loss of cardioprotection. However, after 3 days of reperfusion cardioprotection could be re-instated by an IPC stimulus.[69] Subsequently, Iliodromitis *et al*.[70] showed that six to eight repeated bouts of 5-min CAO resulted in a loss of cardioprotection. The mechanism underlying the development of tolerance to repetitive ischemic stimuli is still poorly understood, but has been suggested to involve a progressive loss of adenosine production[71] and/or reduced adenosine receptor responsiveness.[72]

Several groups of investigators have shown that stimuli that do not cause ischemia in either the target or a remote organ can also protect the myocardium. For instance, ventricular stretch,[73] ventricular pacing,[74] heat stress,[75] cytokines[76] and physical exercise[77] have all been found to be cardioprotective through similar signaling pathways as IPC.[78] The molecular adaptation that underlies the potent but short-lived effect of classic preconditioning is not fully understood and immensely complex. It is highly likely that multiple signal transduction pathways converge on mitochondria to exert their protective effect.

Mechanism of ischemic preconditioning

The mechanisms responsible for cardioprotection with IPC have been the topic of numerous studies during the past 20 years. When considering the signaling cascade, triggers and mediators that ultimately converge on end-effectors, can be differentiated. Triggers are released during the short preconditioning ischemia and exert their activity only during this period, whereas end-effectors are solely active during the prolonged index ischemia and, more importantly, just after the lethal ischemia. It has been proposed that a threshold for IPC exists in which different receptor subtypes contribute to the cardioprotection by IPC.[79] If one receptor type is blocked, then activation is dependent on the remaining receptors

and more ischemic stimulation is required to raise levels of the other receptor agonists to compensate for the absence of activation of the blocked receptor.[79] Thus, a single receptor antagonist does not totally block the ability to precondition the heart, but rather raises its threshold. In the following paragraphs we will discuss some of the components, which have been discovered to be of importance in the protection afforded by preconditioning.

Adenosine, Bradykinin and Opioids. The first and probably most important trigger of IPC that was identified was adenosine. Myocardial interstitial adenosine concentrations rapidly rise during ischemia.[80] Liu *et al*.[81] were the first to observe that a preconditioned state is triggered by stimulation of G_i-coupled adenosine A_1 receptors during the preconditioning phase, since an adenosine receptor antagonist, given prior to sustained ischemia, was able to abolish the protective effect of IPC. Furthermore, they also showed that infusion of adenosine or an A_1 adenosine receptor agonist was as effective in protecting the myocardium as IPC. This report suggested that adenosine was a strong candidate as an agent for pharmacological preconditioning. After reviewing the existing literature, Ganote & Armstrong[82] concluded that adenosine is not involved in IPC in the rat heart. However, while in this species, adenosine receptor stimulation is indeed not required for cardioprotection by a triple 3-min CAO interspersed by 5-min of reperfusion (3CAO3),[83, 84] it is mandatory for cardioprotection by a single 15-min CAO (1CAO15).[84] Two other endogenously released trigger substances, bradykinin[85] and opioids,[86] were subsequently found to be involved in the protective effect of IPC. Inhibition of any one of these three receptors blocked IPC's protection from a single preconditioning cycle.

Protein Kinase C. Ytrehus *et al*.[87] found that inhibition of protein kinase C (PKC) abolishes the limitation of infarct size by pre-treatment with adenosine or by IPC, suggesting that PKC plays a pivotal role in the cardioprotective effect of preconditioning. It was the first kinase to be identified in preconditioning's signal transduction pathways. The question arose as to which PKC isoform might be exerting this effect. Ping *et al*.[88] found that preconditioning was associated with a translocation of PKCε and PKCη from the cytosol to the particulate fraction in the myocytes. Subsequent research has shown that the ε-isoform of PKC was the one conferring protection[89, 90] and that the δ-isoform is actually detrimental.[90] Several studies have shown that PKC activation is mediated by activation of phosphatidylinositol-3-kinase (PI3K),[91, 92] which is an important upstream signaling molecule that is part of the reperfusion injury salvage kinase (RISK) pathway (reviewed by Hausenloy *et al*.[93]).

RISK pathway. Both PI3K and mitogen-activated protein extracellular signal regulated kinases 1 and 2 (MEK1/2)-p42/44 (ERK1/2) kinases have been shown to be important components of reperfusion anti-apoptosis.[94] Together they are components of the RISK pathway.[93] The PI3K-Akt signaling cascade conveys extracellular signals elicited by growth factors and hormones to intracellular signaling pathways implicated in cell growth, cell survival and cell migration (reviewed by Manning & Cantly[95]). Tong *et al*.[91] were the first study to link activation of the PI3K-Akt pathway with cardioprotection by IPC by demonstrating that abolishing Akt phosphorylation by administering a pharmacological inhibitor of PI3K during the IPC

phase abrogated the cardioprotective effects of IPC. This finding has been confirmed and subsequent studies have further delineated other essential components of the PI3K-Akt pathway.[96-98] It was shown that there was a biphasic response in Akt and ERK1/2 phosphorylation, with the first phase directly after the IPC stimulus and the second phase at reperfusion after the lethal ischemic insult.[99] Importantly, both phases of Akt activation are required to mediate IPC-induced cardioprotection, as inhibiting either phase of activation abolishes the infarct-limiting effect of IPC.[96] The requirement of Akt activation at the time of reperfusion in IPC treated hearts has been confirmed in subsequent studies.[100-102]

Other protein kinases. There are several other protein kinases involved in IPC, but these are outside the scope of this thesis and will not be discussed further. These other protein kinases include ERK1/2, JNK1/2, JAK-STAT, p38MAPK and the tyrosine kinases Src and Lck. For detailed information reviews by Armstrong[103] and Hausenloy & Yellon[104, 105] are suggested.

Mitochondrial K^+_{ATP} channel. Several hundred studies have focused on opening of the mitochondrial (mito) K^+_{ATP} channel as a central mechanism in IPC.[106-109] However, the selectivity of compounds for mitoK^+_{ATP} channels remains an issue to be resolved by further investigation. A significant recent finding is the discovery of a macro-molecular complex in the inner membrane of the mitochondria with mitoK^+_{ATP} channel activity.[110] The pharmacological overlap between succinate dehydrogenase (SDH) and mitoK^+_{ATP} channels led to the hypothesis that SDH and mitoK^+_{ATP} channels interact with each other, both physically and functionally.[110] Nevertheless, the identity of the pore-forming unit of the channel remains to be determined. Recent evidence has linked mitoK^+_{ATP} channels activation to the inhibition of apoptosis and the putative components of the mitochondrial permeability transition pore, suggesting the possibility of a direct functional connection between these processes.[111, 112]

Reactive Oxygen Species. The opening of mitoK^+_{ATP} channels results in an influx of potassium that causes swelling of the mitochondria and this is thought to somehow lead to production of ROS. It was found that diazoxide, a mitoK^+_{ATP} channel opener, confers its protection through ROS,[113] a known trigger of the preconditioned state and that IPC's protection could be mimicked by exposure to ROS in the absence of ischemia and that it was PKC-dependent.[114, 115] It was also found that a ROS scavenger could block protection from IPC.[114, 115] The finding that ROS act as second messengers was contradictory to the popular belief that ROS only contributed to reperfusion injury. In fact, we now know that ROS act as second messengers for redox signaling in multiple systems. One important target of redox signaling is PKC.[116] It appears that the ROS-dependent as well as adenosine-dependent pathways converge on PKC. Nonetheless, the source of ROS in preconditioning has yet to be identified. It is known that inhibition of site III of the electron transport chain with myxothiazol prevents the ROS burst and any maneuver that brings potassium into the mitochondria will produce it.[117] Interestingly, connexin 43 is involved in the mechanism as connexin-deficient hearts cannot be preconditioned and cells from those hearts produced much less ROS in response to the

mitoK$^+_{ATP}$ channel opener, diazoxide.[118] Those cells did produce ROS normally when potassium entry was initiated by an ionophore suggesting involvement of the mitoK$^+_{ATP}$ channel.[118]

Nitric Oxide. In IPC bradykinin, opioids and adenosine are intermediates that can release nitric oxide (NO). In particular adenosine can act on endothelial cells and cardiomyocytes activating endothelial NO synthase (eNOS).[119-122] Also the protective effect of NO was attributed to induce the opening of mitoK$^+_{ATP}$ channels either via PKC or directly.[123, 124] More recently the guanylyl cyclase-protein kinase G (CG-PKG) pathway was found to be involved in the release of NO. PKG is suggested to induce the opening of mitoK$^+_{ATP}$ channels, followed by the production of ROS, which in turn would be responsible for the activation of PKC.[125] A number of studies showed that inhibition of NOS during IPC abolishes its protective effects.[125-127] It is postulated that NO and superoxide (O_2^-) combine to generate peroxynitrite (NOO^-), which is a trigger of an intracellular signal transduction cascade that leads to attenuation of NO, O_2^- and NOO^- generation in the preconditioned heart during a subsequent period of ischemia and reperfusion.[126] Furthermore, the protection elicited by the NO-GC-cyclic GMP pathway has also been attributed to the inhibition of the MPTP.[128, 129] In the pathway leading to myocardial protection, NO can be produced by the activation of eNOS by PI3K-Akt, initiating the reperfusion injury salvage kinase (RISK) pathway.[130] Among endogenous ligands which can trigger PI3K-Akt activation bradykinin, opioids and adenosine play a role.[131]

Mitochondrial Permeability Transition Pore. Mild uncoupling has been implicated in the protection by IPC,[132, 133] which is supported by observations in isolated ventricular myocytes, that the uncoupling produced by the uncoupler dinitrophenol (DNP) protected the mitochondria against calcium overload and rigor following metabolic inhibition with reperfusion.[134] Also, in the isolated rat heart, DNP reduced infarct size produced by global total ischemia and reperfusion.[135] An interesting hypothesis has been proposed that a stress-resistant phenotype is caused by a modest degree of uncoupling within the inner membrane of the mitochondria from preconditioned hearts, so that during prolonged ischemia and reoxygenation, ROS production is attenuated.[40, 132, 133] MPTP opening causes swelling and uncoupling of mitochondria, which unrestrained, leads to cell death by necrosis.[49] Although it has been proposed that either apoptosis or necrosis might be precipitated, depending on the extent of MPTP opening.[48] Fluorescence microscopy to measure MPTP opening in isolated cardiomyocytes has confirmed that the pore opens under conditions of simulated ischemia and reperfusion.[112] It might be expected that the pore would also open after prolonged ischemia and some have reported this to be the case.[136, 137] However, these studies relied on cytochrome c release as a measure of MPTP opening, but it is known that this can occur independently of the MPTP as a result of Bax translocation to mitochondria during ischemia.[138] By contrast, others have shown that MPTP opening does not occur during ischemia, but during reperfusion.[139, 140] Many studies have reported the cardioprotective effect of IPC and that this involves inhibition of MPTP opening.[112, 128, 141, 142] Some studies have implicated mitochondrial protein phosphorylation to exert this effect, but none have been able to identify any that mediate

cardioprotection. However, very recently it was reported that inhibition of MPTP opening by IPC is probably mediated by a reduction of oxidative stress rather than mitochondrial protein phosphorylation.[143] Indeed, Clarke *et al*.[143] reported that IPC gave no detectable changes in mitochondrial protein phosphorylation. Mitochondria isolated from IPC treated hearts during reperfusion exhibited less protein carbonylation, a measure of protein oxidation and a surrogate marker of mitochondrial oxidative stress,[142] whereas no effect of IPC was observed in pre-ischemic hearts.

How all these signal transduction pathways mediate protection and ultimately reduce infarct size is currently unknown. Suggested mechanisms include maintenance of mitochondrial ATP generation, reduced mitochondrial calcium accumulation, reduced generation of oxidative stress, attenuated apoptotic signaling and inhibition of MPTP opening.[144]

With increasing emphasis on the pivotal role of limitation of reperfusion injury in the infarct size limitation by IPC, several studies explored whether interventions during reperfusion, rather than before ischemia, could also limit infarct size. Since ischemic events are seldom predictable, interventions at the time of reperfusion are more suited to most clinical scenarios. Thus, all signaling molecules identified in IPC have been tested in experimental models, not only given pre-ischemia, but also pre-reperfusion.

Ischemic postconditioning

Characteristics of ischemic postconditioning

In the absence of reperfusion, no intervention is able to limit infarct development, and it is clear that reperfusion is mandatory for tissue survival. Reperfusion and revascularization therapies in AMI have the primary aim to salvage viable tissue, which may be reversibly injured within the ischemic risk zone, thus limiting the extent of tissue necrosis. In AMI, both early and late mortality are closely related to the duration of unrelieved coronary occlusion. This philosophy of prompt reperfusion/revascularization in AMI is defined in the axiom: "time is muscle and muscle is life"[145] Despite the unquestioned need for reperfusion to prevent ischemic necrosis, in the past five years there has been accumulating experimental evidence that reperfusion *per se* is associated with the paradoxal activation of lethal signals that accumulate in necrosis and apoptosis.

Long before postconditioning (POC) was described gradual, staged or stutter reperfusion were investigated as targets for protection at the onset of reperfusion. In 1986, Okamoto *et al*.[146] first described that early temporary, gentle reperfusion limits the post-ischemic damage, that occurs with sudden, complete revascularization in dogs. Other studies in dogs confirmed the benefit of gradual reperfusion on reduced myocardial infarct size and preserved myocardial endothelial function.[147, 148] Similarly it was shown in pigs that controlled reperfusion lessens end-diastolic wall thickness and reduces myocardial calcium deposition compared to uncontrolled reperfusion.[149] Furthermore, it was shown in isolated guinea pigs

hearts that gradual reperfusion improved functional markers.[150] Despite these promising results and the attractive potential as a clinical protective strategy, the mechanism of gradual reperfusion was never clearly realized. In 2003, Zhao *et al*.[151] were the first to describe in dogs an algorithm of POC, which consists of repetitive ischemia applied during early reperfusion attenuating reperfusion injury. Infarct size after a 60-min CAO and 3 hours of reperfusion was reduced from 25±3% to 14±2% of the area at risk, which was equal to that of IPC (15±2% of the area at risk). Both POC and gradual reperfusion target the onset of reperfusion and lessen myocardial injury after ischemia. In fact, POC has not proven to be substantially different from gradual or staged reperfusion.[152] POC may simply describe an easy and effective algorithm of gradual or staged reperfusion.[153] Indeed, the failure to effectively implement the results from the older studies might be due to the difference in algorithm, since it was shown in rats that in order for POC to be protective it should be initiated no later than one minute after re-establishing reperfusion.[154]

The protection of POC has been shown to be effective in several animal models, both *in vitro* and *in vivo* and has even been shown to be effective in small human trials (reviewed by Vinten-Johansen).[155] Cardioprotection of POC is limited when occlusion duration becomes too long[156] and the extent of protection might differ between males and females.[157] Renal artery occlusion and release just before coronary artery reperfusion also provides myocardial infarct size reduction showing that a remote postconditioning phenomenon also exists.[158] Since Heusch[153] commented that POC resembled the protection seen in earlier studies in which the heart was perfused with either low flow or reduced pH, Cohen *et al*.[159] have shown that maintaining a low pH during the first minutes of reperfusion duplicated POC's protection. This indicates that also non-ischemic stimuli at reperfusion can cause cardioprotection.

Mechanism of ischemic postconditioning

Since POC has only been described recently the investigation of the mechanism underlying POC is still at an early stage. It is clear that the protection afforded by POC is reproducible and is observed also in isolated buffer-perfused hearts indicating that there is no substantial role of blood-borne factors.[152, 160]

Adenosine, Bradykinin and Opioids. Yang *et al*.[160] concluded that the protection of POC is dependent on adenosine receptors when the non-selective adenosine receptor antagonist 8-(p-sulfophenyl) theophylline blocked POC's infarct-sparing effect. Follow-up research has shown that different subtypes are involved in different animal models. Specifically, the protection of POC in rabbits is dependent on the adenosine A_{2B} receptor,[161] while in rats it is dependent on adenosine A_{2A} and A_3 receptors.[162] However, in the latter study the use of ZM241385 to block adenosine A_{2A} receptors might have also blocked adenosine A_{2B} receptors, since the affinity of rat receptors for ZM241385 is similar to that of cloned human adenosine receptors.[163] Several reports have shown that bradykinin administered at the onset of reperfusion reduced infarct size by activating the RISK pathway and NO production. Subsequently, Penna *et al*.[164] have

shown that endogenous bradykinin, and it's downstream pathway elements (NOS, PKG and ROS), is an essential component in POC's protection. Specifically, both POC and intermittent bradykinin infusion resulted in cardioprotection that could be blocked by a bradykinin B_2 receptor antagonist (HOE140 or WIN64388) given for only three minutes at the onset of reperfusion. The same effect was seen when a NOS inhibitor (L-NAME) or ROS scavengers (NAC or MPG) were given for three minutes at the onset of reperfusion. Interestingly, continuous infusion of bradykinin for three minutes at the onset of reperfusion did not result in protection. Kin *et al*.[165] provided evidence for the fact that endogenous opioids are involved in POC, since the non-selective opioid receptor antagonist naloxone was able to completely abrogate the protective effect of POC. Utilizing more specific antagonists they showed that δ and κ opioid subtype receptors are instrumental in POC's protection.[165] Subsequently, Zatta *et al*.[166] have shown that POC preserves myocardial opioid content and involves both δ and μ opioid subtype receptors.

Protein Kinase C. Recently, it was reported that the effect of POC could be blocked by administration of the non-selective PKC antagonist chelerythrine five minutes before the onset of reperfusion.[161, 167, 168] Also, the PKCε antagonist KIE1-1 was able to reverse the effects of POC.[167] Indeed, PKCε is associated with cardioprotection, potentially by inhibiting MPTP opening.[169] On the other hand, PKCδ is involved in the pathogenesis of myocardial reperfusion injury after ischemia-reperfusion[90, 170] by increased O_2^- generation, mitochondrial dysfunction and the release of cytochrome c and downstream pro-apoptotic factors.[171, 172] Inhibition of PKCδ by rottlerin five minutes before reperfusion reduced infarct size, but did not further reduce the infarct size reduction achieved with POC.[167] POC increased total cell homogenate levels of phosphorylated PKCε relative to the decreased levels observed after ischemia reperfusion, suggesting a translocation site other than the mitochondrion, while phosphorylated levels and mitochondrial translocation of PKCδ were reduced.[167] These data suggest that POC's protection is dependent on PKC signaling, increasing the cardioprotective component of endogenous PKCε while simultaneously decreasing the cardio-destructive component of PKCδ. A signaling role of PKC is consistent with the involvement of G-protein coupled receptor activators of PKC, specifically adenosine[162] and opioids.

RISK pathway. Tsang *et al*.[130] were the first to report that there is involvement of the RISK pathway after POC. In that study, POC activated Akt and downstream eNOS, while pharmacologically inhibition of PI3K by either wortmannin or LY294002 in POC-treated hearts abrogated cardioprotection.[130] In addition to activation of the PI3K-Akt pathway, POC has also been shown to activate MEK1/2-ERK1/2, since it was found that the ERK1/2 inhibitor PD98059 was able to abolish the cardioprotective effect of POC.[160] The role of the RISK pathway in POC has been confirmed in several other studies utilizing different models with and without comorbidities.[160, 173-175] However, not all studies have confirmed the role of a PI3K-Akt pathway in POC-treated hearts.[176] Interestingly, in one study in *in vivo* pig hearts no cardioprotection of POC was present, although Akt activation was apparent.[177] However, in another study it

was shown that the dissociation of Akt activation and POC-induced cardioprotection may be related to the algorithm used for POC since increasing the amount of ischemic insult in the POC algorithm could summon cardioprotection.[178] This suggests that there might be a threshold for Akt activation that is required to confer the cardioprotective effects of POC.

Other protein kinases. There are several other protein kinases involved in POC, but these are outside the scope of this thesis and will not be discussed further. These other protein kinases include ERK1/2, JNK1/2, JAK-STAT and p38MAPK. However, there are some differences in the detail between the signaling components recruited at the time of myocardial reperfusion when compared to POC. For detailed information reviews by Hausenloy & Yellon[104, 105] are suggested.

Mitochondrial K^+_{ATP} channel. Support for a role of mitoK^+_{ATP} channels in POC was provided by several groups by reversing the cardioprotective effects of POC with both glibenclamide, a non-selective K^+_{ATP} channel inhibitor, and 5-hydroxydecanoate, a selective mitoK^+_{ATP} channel blocker.[130, 179] Additional support was provided by the fact that CGX-1051, a mitoK^+_{ATP} channel activator reduced infarct size when given 5 min prior to reperfusion.[180] Furthermore, intermittent administrated diazoxide, a mitoK^+_{ATP} channel opener, for 3 min at reperfusion, has also shown to be cardioprotective, while this effect was not seen when diazoxide was given continuously during these 3 min.[164] These data suggest that POC's protection is dependent on mitoK^+_{ATP} channel opening at the time of reperfusion.

Reactive Oxygen Species. Initially described as the "oxygen paradox" by Hearse *et al*.[181] a robust generation of ROS has been shown to occur at reperfusion in myocardium[182, 183] and isolated mitochondria.[184] In addition, direct administration of ROS in concentrations approximating those observed at reperfusion causes injury to myocardium.[185] ROS can play a dual role by the fact that it can cause cellular injury, but it can also be a signaling molecule. Sun *et al*.[186] have shown *in vitro* that hypoxic POC in cultured neonatal rat cardiomyocytes reduces ROS generation, which was associated with attenuated cytosolic and mitochondrial calcium loading and reduced cell death. These results have been confirmed in several *in vivo* models,[151, 154, 187] including longer durations of reperfusion.[188] These data would suggest that POC reduces oxidant-induced injury. However, Penna *et al*.[168] demonstrated in isolated rat hearts that the scavenger N-acetyl cysteine (NAC) given at reperfusion abrogated the infarct sparing effect of POC, while delaying administration of NAC, until after the POC algorithm was applied, did not abolish POC's protection. These data suggest that the mechanism of cardioprotection by POC may involve not only a reduction of oxidant induced injury, but also preservation of ROS-related signaling pathways. Finally, it was shown that POC may also preserve anti-oxidant reserves in previously ischemic myocardium, since POC can attenuate the oxidation of gluthathione, the major intracellular anti-oxidant system.[184] This indicates a reduction in ROS burden may not only reduce direct tissue injury, but may preserve endogenous anti-oxidant levels in the affected myocardium as well.

Nitric Oxide. Tsang *et al*.[130] have provided compelling evidence that POC causes a marked increase in eNOS phosphorylation, which is Akt-dependent since PI3K-Akt inhibitors wortmannin and LY294002 blocked not only protection but also eNOS phosphorylation.[93, 130] Others have also provided evidence that indicates the protection provided by POC is abolished by L-NAME,[160, 179] thus implicating NOS isoforms as important elements of the signal transduction cascade of POC. NO, generated downstream of PI3K-Akt, appears to be a key signaling element of the RISK pathway since many classes of agents are capable of protecting the myocardium at reperfusion. These include adrenomedullin, bradykinin and insulin (reviewed by Hausenloy & Yellon).[104] Furthermore, simvastatin, given intravenously to rats just before reperfusion, reduced infarct size with a concomitant increase in myocardial PI3K, Akt and eNOS phosphorylation.[189] In addition, the cardioprotective effect of simvastatin was blunted by L-NAME, suggesting that endogenous NO production is important in it's protection.[189] Another statin, atorvastatin was also cardioprotective when given at the onset of reperfusion and it's effect was shown to be wortmannin-sensitive and was lost in $eNOS^{-/-}$ mice.[190, 191] Finally, the rho kinase inhibitors fasudil and Y27632, administered at reperfusion, are able to limit infarct size in a rat isolated rat heart model subjected to ischemia reperfusion.[192] This protection could be reversed by wortmannin and L-NAME, confirming a protective mechanism of PI3K-Akt and NOS.[192]

Mitochondrial Permeability Transition Pore. The MPTP is a critical mediator of cell death in the setting of ischemia and reperfusion as MPTP opening has been shown to occur at the time of reperfusion[49, 139] and inhibiting MPTP opening specifically at the time of reperfusion with cyclosporin-A and sanglifehrin-A is cardioprotective.[193-195] MPTP opening is also modulated by POC, underlining the importance of the MPTP as a mediator of myocardial reperfusion injur.[173, 196, 197] Argaud *et al*.[196] have shown that isolated rabbit heart mitochondria, which had been postconditioned *in situ*, display increased resistance to calcium-induced MPTP opening, suggesting that POC protects the myocardium by inhibiting MPTP opening. This finding was supported by Zhao *et al*.[197] who showed that MPTP opening was inhibited by hypoxic POC in H9c2 cells and that this effect was downstream to Akt as well as mitoK^{+}_{ATP} channel opening. Bopassa *et al*.[173] also confirmed the dependency of POC on PI3K since wortmannin and LY294002 mitigated the positive effects of POC. The mechanism through which POC prevents opening of the MPTP at time of myocardial reperfusion is still unclear and warrants further research. It might very well be related to the kinases involved in the RISK pathway looking at the results mentioned above.

Surprisingly, the very short period of intervention that defines POC activates a complex and growing array of molecular pathways, one of which is the RISK pathway.[104, 131] The RISK pathway likely is proximal to eNOS,[104] the activation of which has been found to be important to cardioprotection by POC,[179] and involves two targets that have been shown to be pivotal to POC's cardioprotection; the mitoK^{+}_{ATP} channel[164, 179] and the MPTP.[196, 198] Inhibition of the RISK pathway has, in most studies, abrogated the effect by POC.[130, 160]

Outline of the thesis

The search for the exact mechanism behind both IPC and POC continues to identify the signaling pathways and targets involved in its protection in order to create better pre- and postconditioning mimetics, which can potentially be used in clinical scenarios. In this thesis the results of our studies on pre- and postconditioning in rats, as well as hibernation in pigs, are presented.

A frequently overlooked factor in studies addressing the mechanism of IPC is the nature of the IPC stimulus that leads to cardioprotection. Several studies clearly show that adenosine-dependent and –independent pathways exist and that the employed pathway depends on the IPC stimulus.[82-84, 199] Although different signal pathways are activated by different IPC stimuli, the question remains whether these different pathways ultimately converge into a common cardioprotective phenotype. Mitochondria have been proposed to be an important endpoint in cardioprotection by preconditioning.[92] In **Chapter 2** we tested, *in vivo*, the hypothesis that the signal transduction pathways of 1CAO15 and 3CAO3 converge at the level of the mitochondria and studied the signal transduction pathways involved in these different IPC stimuli.

Widespread interest in the cardioprotective properties of adenosine was fueled by compelling evidence from Downey and co-workers that adenosine plays a pivotal role in the phenomenon of IPC.[81] Support for their hypothesis was provided by numerous studies demonstrating that the favorable effect of IPC could be mimicked by treatment with adenosine receptor agonists given in lieu of brief IPC ischemia and, conversely, abrogated by adenosine receptor antagonists given during the IPC stimulus.[78, 125] However, the mechanism of protection by intravascularly administered adenosine (i.e. exogenous adenosine) is still incompletely understood, and doubt has been expressed as to whether exogenous adenosine and endogenous adenosine released during IPC employ the same signaling pathways.[80, 200-202] In contrast to IPC, access of adenosine into the interstitial compartment is impeded by the active metabolic barrier function of the endothelium,[203, 204] which may explain why intravenous adenosine failed to decrease infarct size in some[81, 83, 205] though not all studies,[80, 206] whereas high intracoronary doses[200, 201, 207, 208] or co-infusion with dipyridamole[209] afforded cardioprotection. In **Chapter 3** we used microdialysis to determine whether myocardial interstitial adenosine levels were similarly affected during IPC by 1CAO15 or adenosine infusion (ADO) in a dose that was equally effective in limiting myocardial infarct size as IPC by 1CAO15. Since we observed that myocardial interstitial adenosine levels remained unchanged during ADO, but increased during 1CAO15, we subsequently investigated the role of NO in the cardioprotection by ADO.

There is evidence that both pharmacological and IPC stimuli can protect the myocardium by a number of different pathways (see Chapter 2 and 3).[84, 199, 210] Studies addressing the signal

transduction pathways involved in the cardioprotection by IPC focus increasingly on the role of the pro-survival kinases including the PI3K–Akt–NOS pathway and ERK1/2 (i.e. RISK pathway).[131] However, not all stimuli appear to depend on the RISK pathway for cardioprotection, as infarct size limitation by infusion of tumor necrosis factor-α was mediated via the signal transducer activator of transcription-3 (STAT-3) but not the RISK pathway.[100] Endogenous release of adenosine during IPC has been proposed to afford cardioprotection via increased activity of the RISK pathway during early reperfusion.[101] Consequently, in **Chapter 4** we hypothesized that the adenosine-dependent (but ROS-independent) stimulus 1CAO15, but not the adenosine-independent (but ROS-dependent) stimulus 3CAO3, involves activation of the RISK pathway. Conversely, since ROS have been identified as possible mediators of the activation by IPC of the JAK/STAT pathway[211-213] and the AMPK pathway,[214, 215] we hypothesized that 3CAO3, but not 1CAO15, results in activation of these pathways. Furthermore, because cardioprotection by intravascular adenosine in the isolated rabbit heart was recently shown to be PI3K-independent,[216] we hypothesized that the RISK pathway is not involved in pharmacological preconditioning by intravenous infusion of exogenous adenosine in the *in vivo* rat heart.

Clinical studies on infarct size limitation by brief anginal episodes preceding AMI are ambiguous,[217-223] which has been attributed to a loss of cardioprotection by IPC in the ageing[224-226] or pathological[227-229] heart. Another confounding factor could be the development of tolerance to IPC, i.e. the loss of cardioprotection when the same preconditioning stimulus is repetitively applied.[61, 69, 70] In **Chapter 5** we set up a model of tolerance to repeated application of an IPC stimulus (4CAO15) and investigated whether tolerance that develops when the same IPC stimulus is applied repetitively also implies tolerance to a stimulus that employs a different signal transduction pathway (e.g. 3CAO3, ADO (see Chapter 2-4) or remote preconditioning by mesenteric artery occlusion). Capitalizing on the observations that in pigs progressive loss of adenosine production rendered myocardium tolerant to protection by 10-min CAOs, but still responsive to exogenous adenosine,[71] we also investigated whether loss of adenosine release also contributes to the development of tolerance in the rat heart and whether exogenous adenosine still induces protection once tolerance has developed.

Myocardium can respond to a chronic decrease in perfusion of moderate severity by achieving a state of down regulation of metabolic demand matched to the available oxygen delivery, termed hibernating myocardium.[18] The cellular mechanisms utilized in the transition from acute ischemia to chronic ischemia, yet viable state, remain incompletely understood. There is emerging evidence that the mitochondria can be primed into a "stress-resistant state", so that cell death is reduced following prolonged, severe ischemia and reperfusion (see Chapter 2 and 4). Within chronically ischemic myocardium, the mechanisms by which the mitochondria can adapt against repetitive oxidant stress are unclear. In **Chapter 6** we

looked at the mitochondria isolated from chronic ischemic myocardial tissue and explored the potential mechanisms underlying this protection, in particular mitochondrial uncoupling and attenuated maximal superoxide production.

An increase in tyrosine residue phosphorylation via increased tyrosine kinase activity has been implicated in the signal transduction pathway of cardioprotection by IPC.[78, 230, 231] There is evidence that increased tyrosine residue phosphorylation, produced by a shift in the balance between tyrosine kinase and tyrosine phosphatase, increases white blood cell survival by inhibiting apoptosis.[232-234] Vanadate enhances tyrosine residue phosphorylation by inhibition of tyrosine phosphatase,[235, 236] suggesting that vanadate may be of therapeutic benefit in myocardial infarction, which may involve both apoptosis and necrosis.[237, 238] In support of this concept, Armstrong *et al*.[239] reported that serine threonine phosphatase inhibitors are highly effective in protecting isolated cardiomyocytes subjected to ischemia (without reperfusion), even when administered late (75 min) after onset of ischemia, suggesting that vanadate may not require administration prior to the onset of ischemia and might also act against reperfusion injury. In **Chapter 7** we examined the effect of the tyrosine phosphatase inhibitor vanadate, administered pre-ischemia and pre-reperfusion, and what signaling pathways in its protection are involved.

Recently, Zhao *et al*.[151] reported that abrupt reperfusion after a sustained CAO can limit irreversible damage provided that this was interrupted by a number of brief periods of coronary artery re-occlusion and abrupt reperfusion started within 30 sec after the initial abrupt reperfusion (i.e. postconditioning or POC). Subsequent studies confirmed this original observation and aimed to define the optimal algorithm of POC and revealed that, in order to be effective, the first re-occlusion has to be applied within one min after the onset of reperfusion.[154] Increasing the number of re-occlusion/reperfusion cycles beyond four cycles does not confer greater protection.[154, 162, 179] Nevertheless, the optimal duration of the re-occlusion and reperfusion periods of POC is currently unknown, but most likely differ between animal species. While POC with three 30 sec periods of abrupt CAO starting 30 sec after the initial reperfusion, and interspersed by 30 sec of abrupt reperfusion limited infarct size produced by a 60-min CAO in the dog,[151, 187] it failed to afford cardioprotection against a 30-min CAO in the rat.[152] On basis of this single observation the authors suggested that briefer periods (i.e. 10-15s) of re-occlusion and reperfusion are required in smaller than in larger animals, in which 30s cycles are effective,[152] but conclusive evidence for this hypothesis is lacking. For instance, in discordance with this concept is a recent study by Schwartz and Lagranha[240] who showed in swine that 3 cycles of 30s of re-occlusion and reperfusion failed to limit infarct size produced by a 30-min CAO. Since POC with the 30s algorithm was effective against a 60-min CAO[151, 187] but not against a 30-min CAO,[152] we hypothesized that the duration of the index ischemia also plays a major role in determining the effect of the POC stimulus. Consequently,

the aim in **Chapter 8** was to investigate the influence of index ischemia duration (ranging from 15- to 120-min) on the protective effect of POC. We subsequently investigated how the duration of index ischemia affected the role of potential mechanisms reported to be involved in the cardioprotection by POC such as the activation of the PI3K-Akt-eNOS pathway and the production of ROS to explain our findings.

Because IPC and remote preconditioning have to be applied before the onset of ischemia, in the case of AMI ischemic POC upon reinstatement of reperfusion appears to be a more logical approach for intervention. However, POC may then be applied on myocardium that is already preconditioned or on myocardium that has become unresponsive to IPC because of numerous short periods of ischemia preceding the infarction (see Chapter 5). The effect of POC on preconditioned myocardium has been addressed in a limited number of studies,[130, 179, 187] but the results are ambiguous possibly due to animal species or anesthesia regimen. Additionally, IPC can be achieved by stimuli that operate via different signal transduction pathways (see Chapter 2 and 4), while the effect of POC on infarct size depends on the duration of index ischemia and under certain conditions might even be detrimental (see Chapter 8). Consequently, in **Chapter 9** the first aim of the was to determine the interaction between IPC and POC taking into account both the type of preconditioning stimulus as well as the duration of index ischemia. In addition, we investigated whether myocardium that had become tolerant to preconditioning had also become resistant to the protection by POC. Finally, in view of the proposed role of NO in the signal transduction of both IPC[241, 242] and POC,[130, 179, 243] we investigated the obligatory role of NO in the cardioprotection by IPC and POC alone and in combination.

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Chapter 2

Ischemic preconditioning modulates mitochondrial respiration, irrespective of the employed signal transduction pathway

David A Liem*, Olivier C Manintveld*, Kees Schoonderwoerd, Edward O McFalls, Andre Heinen, Pieter D Verdouw, Wim Sluiter and Dirk J Duncker.

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** Both authors have contributed equally to this work.*

Abstract

We tested in the *in vivo* rat heart the hypothesis that although ischemic preconditioning can employ different signal transduction pathways, these pathways ultimately converge at the level of the mitochondrial respiratory chain. Infarct size produced by a 60-min coronary artery occlusion (69±2% of the area at risk) was limited by a preceding 15-min coronary occlusion (45±6%). Cardioprotection by this stimulus was triggered by adenosine receptor stimulation, followed by protein kinase C and tyrosine kinase activation and then mitochondrial K^+_{ATP}-channel opening. In contrast, cardioprotection by 3 cycles of 3-min coronary occlusions (infarct size 28±4% of the area at risk) involved the release of reactive oxygen species, followed by protein kinase C and tyrosine kinase activation, but was independent of adenosine receptor stimulation and K^+_{ATP}-channel activation. However, both pathways decreased respiratory control index (RCI, state-3/state-2, using succinate as complex-II substrate) from 3.1±0.2 in mitochondria from sham-treated hearts to 2.4±0.2 and 2.5±0.1 in hearts subjected to a single 15-min and triple 3-min coronary occlusions, respectively (both $P<0.05$). The decreases in RCI were due to an increase in state-2 respiration, while state-3 respiration was unchanged. Abolition of cardioprotection by blockade of either signal transduction pathway was paralleled by a concomitant abolition of mitochondrial uncoupling. These observations are consistent with the concept that mild mitochondrial uncoupling contributes to infarct size reduction by various ischemic preconditioning stimuli, despite using different signal transduction pathways. In conclusion, in the *in vivo* rat heart different IPC stimuli can activate highly different signal transduction pathways, which appear to converge at the level of the mitochondria where they increase state-2 respiration.

Introduction

A frequently overlooked factor in studies addressing the mechanism of ischemic preconditioning (IPC), is the nature of the IPC stimulus that leads to cardioprotection. For example, adenosine does not contribute to the infarct size (IS) limitation induced by a 3-min coronary artery occlusion (CAO) in swine, whereas it contributes to the protection induced by a 10-min CAO.[1] After reviewing the existing literature, Ganote and Armstrong[2] concluded that adenosine is not involved in IPC in the rat heart. However, while in this species, adenosine receptor stimulation is indeed not required for cardioprotection by a triple 3-min CAO interspersed by 5-min of reperfusion (3CAO3),[3, 4] it is mandatory for cardioprotection by a single 15-min CAO (1CAO15).[4] Furthermore, in the rabbit the cardioprotection by adenosine, in contrast to bradykinin and opioids, does not involve K^+_{ATP}-channel opening and production of reactive oxygen species (ROS).[5] These studies clearly show that adenosine-dependent and -independent pathways exist and that the employed pathway depends on the IPC stimulus.

Although different signal transduction pathways are activated by different stimuli, the question remains whether these different pathways ultimately converge into a common cardioprotective phenotype. *In vitro* evidence suggests that the mitochondria might represent such a common target of various signal transduction pathways.[6] For example, a mild degree of uncoupling may represent a common characteristic of stress-resistant mitochondria within *in vitro* preconditioned myocardium.[6-9] In support of this concept, cytoprotection of human Girardi cells and murine skeletal myotubes can be induced by both simulated ischemia and administration of adenosine or the K^+_{ATP}-channel opener diazoxide and in all cases the protective state is characterized by a mild degree of mitochondrial uncoupling.[10] In addition, a low dose of the mitochondrial uncoupler dinitrophenol is cardioprotective in isolated rat cardiomyocytes[11] as well as in the isolated rat heart.[12, 13]

In light of these considerations we tested, *in vivo,* the hypothesis that the signal transduction pathways of 1CAO15 and 3CAO3 converge at the level of the mitochondria. For this purpose we investigated in detail the signal transduction pathways of these two stimuli and investigated their effects on mitochondrial respiration.

Materials and Methods

Experiments were performed in 357 male Wistar rats (300-380 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 Erasmus MC Animal Care Committee.

Surgical and Experimental Procedures

Pentobarbital-anesthetized (60 mg/kg) rats were intubated for positive pressure ventilation with oxygen-enriched room air.[3,4] 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 vena cava, a PE-50 catheter was placed for infusion of drugs, and Haemaccel (Behringwerke) to maintain fluid-balance. After thoracotomy, 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. The thoracotomy site was covered with aluminum foil to prevent local heat loss. After completion of surgery, a 30-min stabilization 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 was established within 2 min after onset of fibrillation.

Experimental Protocols

Infarct Size. All rats, in which a myocardial infarction was produced, underwent a 60-min CAO followed by 120 min of reperfusion (Fig. 1). Then, area at risk and infarct area were determined using trypan blue and nitro-blue-tetrazolium staining.[3,4] Infarct size (IS) was expressed as infarct area/area at risk x 100%.

Preconditioning Stimuli. Preceding the 60-min CAO, animals underwent a 25-min sham period, or preconditioning by either 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. 1A).

Signal Transduction Pathways. To study the involvement of adenosine, ROS, tyrosine kinase and protein kinase C in the cardioprotection by the two preconditioning stimuli, sham- and preconditioned rats were pretreated intravenously with the adenosine receptor antagonist 8-S-phenyltheophylline (8-SPT, 2 x 25 mg/kg),[4] a continuous infusion of 1 mg/kg/min of the free radical scavenger mercapto-propionyl-glycine (MPG),[5] the tyrosine kinase inhibitor genistein (2 x 5 mg/kg)[14,15] or the protein kinase C inhibitor chelerythrine (5 mg/kg),[15] respectively (Fig. 1B). Finally, to study the contribution of K^+_{ATP}-channel opening, rats were pretreated intravenously with either the mixed K^+_{ATP}-channel inhibitor glibenclamide (3 mg/kg),[14,16] the mitochondrial K^+_{ATP}-channel (mitoK^+_{ATP}-channel) inhibitor 5-hydroxydecanoic acid (5-HD, 20 mg/kg + 20 mg/kg/h),[17] or the sarcolemmal K^+_{ATP}-channel (sarcK^+_{ATP}-channel) inhibitor HMR-1098 (6 mg/kg).[17]

Sequence of Signals in the Signal Transduction Pathway. To determine the sequence of adenosine receptor stimulation, activation of tyrosine kinase and protein kinase C, generation of ROS and activation of K^+_{ATP}-channels in 1CAO15 and 3CAO3, additional rats received intravenous infusion of either adenosine (ADO, 10 mg/kg over 15 min), the mitoK^+_{ATP}-channel

opener diazoxide [10 mg/kg over 5 min][17] or the ROS generating compound menadione [37.5 mg/kg over 5 min; Fig. 1C].[18]

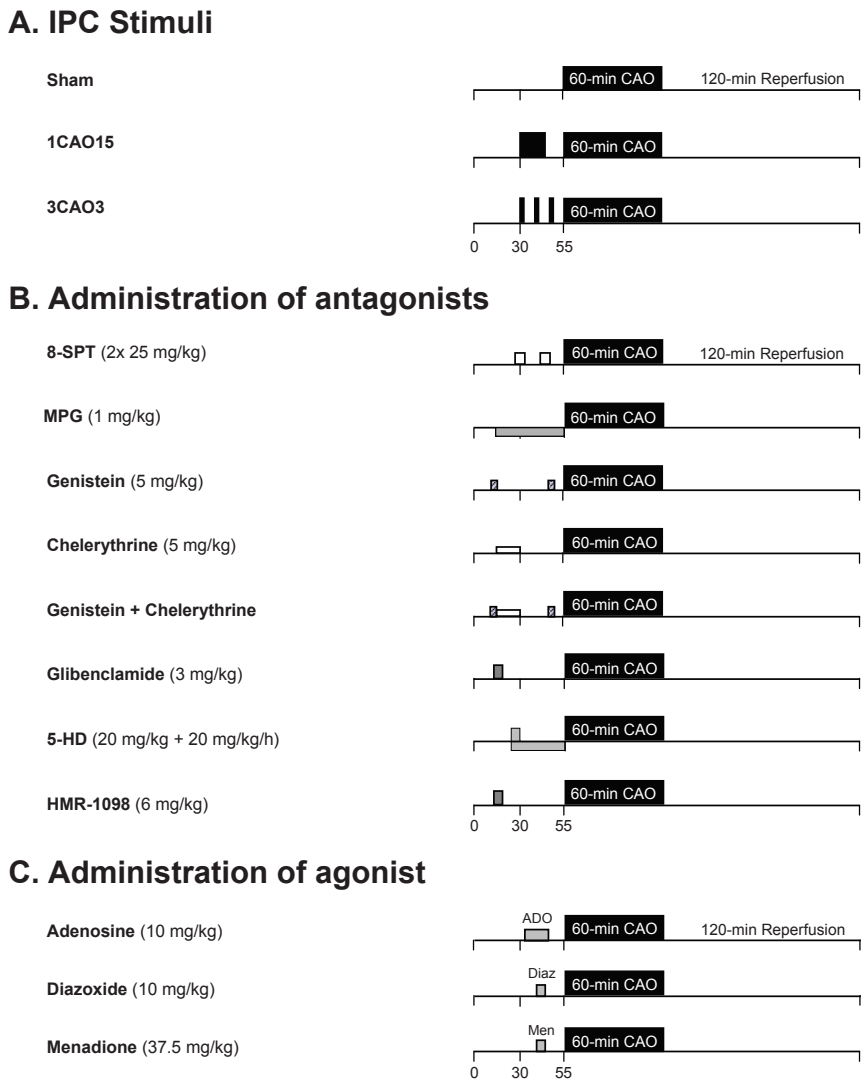

Fig. 1. Shown are the preconditioning protocols (panel A) and the protocols for administering the various pharmacological antagonists (panel B) and agonists (panel C). In the mitochondrial respiration experiments, mitochondria were harvested at a time point corresponding with the onset of the 60-min CAO.

Microdialysis

Myocardial interstitial adenosine levels were measured using a CMA/20 microdialysis probe (Carnegie Medicine AB), which was implanted into the myocardium to become preconditioned.[19] Samples were collected during the entire preconditioning protocol (including the reperfusion phase) at a rate of 2 µl/min. At the conclusion of each experiment, probe recovery was determined *ex vivo* using the sampling rate of 2 µl/min and a solution containing 100 µM adenosine. Samples were stored at -50° C. Adenosine concentrations were determined by reversed phase high-performance liquid chromatography.[20] Interstitial adenosine levels were computed using the recovery of each individual probe (14±1%).

Mitochondrial Respiration

Mitochondrial respiration was studied in rats subjected to sham procedure, 3CAO3 or 1CAO15, under control conditions, or after pretreatment with MPG or glibenclamide, exactly as described above under "Signal Transduction Pathways". At the time point corresponding with the onset of the 60-min CAO, hearts were quickly excised, the left anterior descending area was dissected out and placed in ice cold mitochondrial isolation buffer, pH 7.15, containing 50 mM sucrose, 200 mM mannitol, 1 mM EGTA, 5 mM KH_2PO_4, 5 mM MOPS and 0.1 % fatty acid free BSA, and minced. Fresh mitochondrial isolation buffer mitochondrial isolation buffer was added and tissue separation was carried out in a glass homogenizer with a teflon pestle. Homogenates were centrifuged (Biofuge fresco eppendorf centrifuge, Heraeus, the Netherlands, 750 g at 4°C for 10 min) to pellet the cellular debris and mitochondria were collected from the supernatant. The supernatant was centrifuged and washed twice (8,000 g for 15 min), and mitochondria were collected and protein concentrations determined. The isolation procedure did not include the use of nagarse and the samples contained therefore principally subsarcolemmal mitochondria.[21]

The mitochondria were subsequently suspended in mitochondrial respiration buffer, pH 7.15, comprised of 110 mM sucrose, 0.5 mM EGTA, 3 mM $MgCl_2$, 70 mM KCl, 10 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES and 0.1% fatty acid free BSA. The oxygen consumption rate (flux; nmol/min/mg protein) was measured at 30° C by high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria), in state-2 (10 mM succinate in the absence of ADP) and in state-3 (10 mM succinate + 1 mM ADP). Respiratory control index (RCI) was calculated as state-3/state-2. Since barbiturate anesthesia have been proposed to inhibit complex-I activity,[22, 23] we elected to perform all mitochondrial studies using the complex-II substrate succinate. To prevent retrograde flux of electrons via complex-I, measurements were performed in the presence of the complex-I inhibitor rotenone.[8, 24]

Drugs

Except for HMR-1098 (a gift from Dr. Gögelein, Aventis Pharma), all drugs were purchased from Sigma.

Data Analysis and Presentation

IS and mitochondrial respiration was analyzed by 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

Five out of 264 rats that entered the infarct studies were excluded due to intractable ventricular fibrillation (≤1 rat/group). Eleven out of 76 rats that entered the mitochondrial respiration studies were excluded due to technical failure (≤3 rats/group).

Hemodynamics

Baseline heart rate was 372±3 bpm and mean arterial blood pressure was 105±1 mm Hg in the various experimental groups. 8-SPT and glibenclamide caused increases in mean arterial pressure (respectively 32±8 and 20±4 mm Hg), with no change in heart rate. Conversely, adenosine and diazoxide caused decreases in mean arterial pressure (respectively 34±11 and 17±3 mm Hg), with no change in heart rate. None of the other drugs had a significant effect on blood pressure or heart rate. There was no correlation between the rate-pressure product of the individual animals at the onset of the 60-min CAO and IS (r^2= 0.001; P=0.56).

Effect of Ischemic Preconditioning on Infarct Size

Following the 60-min CAO, IS was 68±2% in sham animals, which was limited to 48±4% by 1CAO15 and to 27±5% by 3CAO3 (both $P<0.05$ versus sham; Fig. 2A).

Signal Transduction Pathways. 8-SPT abolished the cardioprotection by 1CAO15 (IS=70±1%), but not by 3CAO3 (IS=28±7%; Fig. 2A). Interstitial adenosine levels increased from 3.7±0.7 µmol/l at baseline to 26.4±9.8 µmol/l during IPC with 1CAO15 (n=9, $P<0.05$), but were unaltered during 3CAO3 (3.2±0.6 µmol/l at baseline versus 7.5±2.8 µmol/l during 3CAO3; n=8, P=0.11). Conversely, MPG had no effect on the protection by 1CAO15 (IS=43±6%), but attenuated the protection by 3CAO3 (IS=54±6%; Fig. 2A).

Neither chelerythrine nor genistein had any effect on the cardioprotection by 1CAO15, but their combination blunted the cardioprotection by 1CAO15 (IS=59±5%; Fig. 2B). Similarly, only the combination of genistein and chelerythrine significantly attenuated the cardioprotection by 3CAO3 (IS=57±7%).

Both glibenclamide (IS=66±3%) and 5-HD (IS=72±2%) abolished the protection by 1CAO15, but the selective sarcK^+_{ATP}-channel inhibitor HMR-1098 had no effect (IS=45±8%; Fig. 2C). In contrast, none of these inhibitors affected the cardioprotection by 3CAO3.

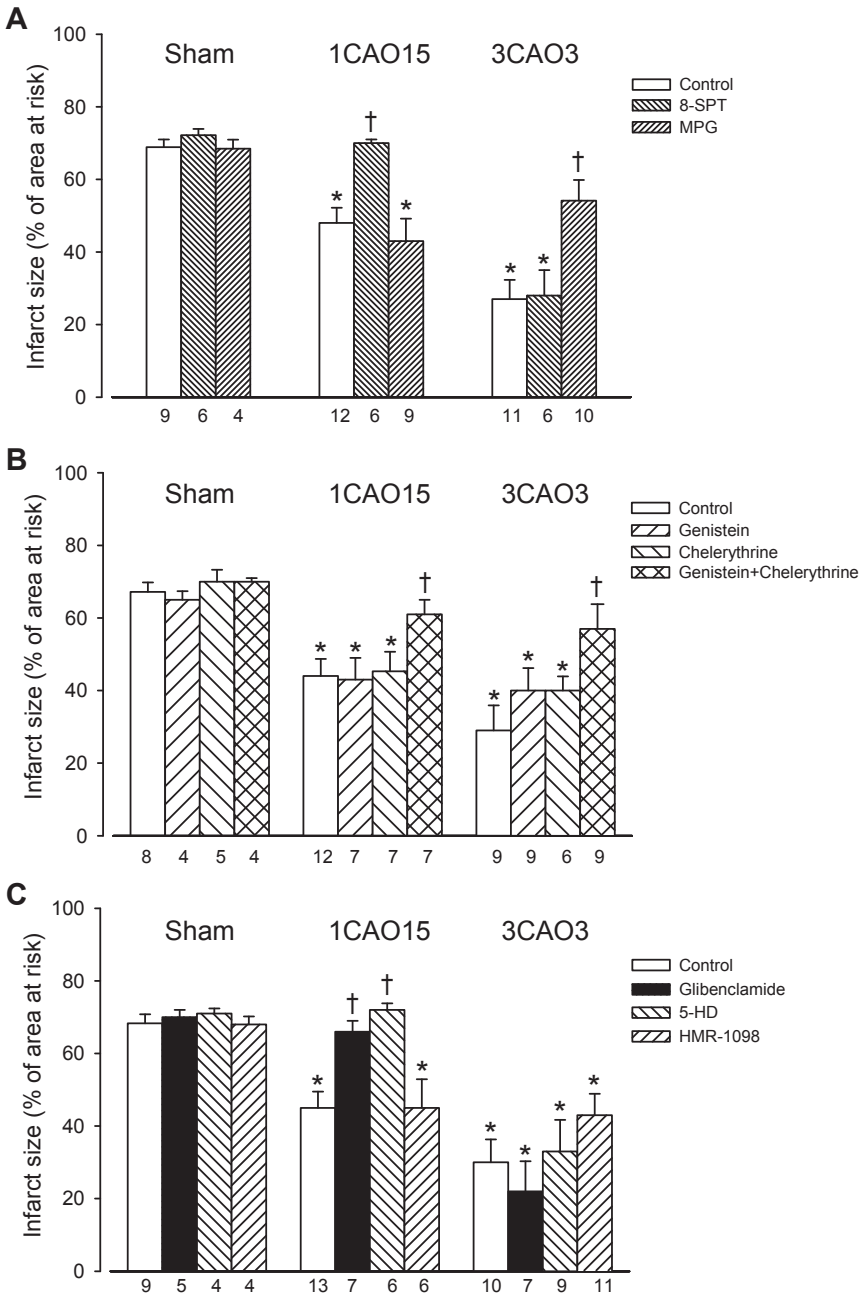

Fig. 2. The effects of inhibition of adenosine and reactive oxygen species (panel A), inhibition of tyrosine kinase and protein kinase C (panel B) and blockade of mitochondrial and sarcolemmal K^+_{ATP} channel opening (panel C) were studied to determine the component of the signal transduction pathways involved in the protection by 1CAO15 and 3CAO3. Data are mean±SEM. The number of animals is indicated below each bar. *P<0.05 vs corresponding Sham; †P<0.05 vs corresponding untreated 1CAO15 or 3CAO3.

Sequence of Signals in the Signal Transduction Pathways. Both glibenclamide and the combination of chelerythrine and genistein abolished cardioprotection by adenosine (Fig. 3A), whereas the protection by diazoxide (IS=51±7%) was not affected by chelerythrine and genistein. Finally, the cardioprotection by menadione (IS=39±7%) was not affected by glibenclamide, but was abolished by combining genistein and chelerythrine administration.

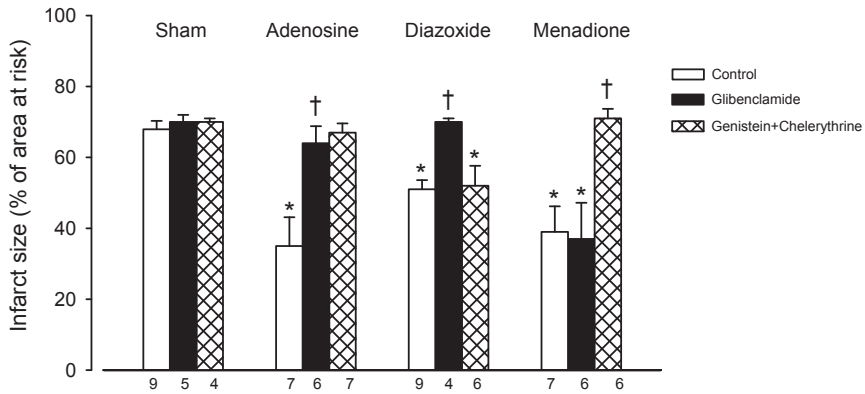

Fig. 3. The order of involvement of the various components is shown for the signal transduction pathways employed by 1CAO15 and 3CAO3. Depicted are the effects of glibenclamide and combined treatment with genistein+chelerythrine on the cardioprotection by adenosine, diazoxide and menadione. Data are mean±SEM. The number of animals is indicated below each bar. **P*<0.05 vs corresponding Sham; †*P*<0.05 vs corresponding Control.

Mitochondrial Respiration

Independent of the employed stimulus, IPC resulted in a decrease of RCI that was the result of increase in state-2 respiration, as state-3 respiration was maintained (Table 1 and Fig. 4). Glibenclamide abolished the decrease in RCI produced by 1CAO15, but had no effect on the decrease by 3CAO3. Conversely, MPG had no effect on the decrease in RCI by 1CAO15 but abolished the decrease by 3CAO3 (Fig. 4). Infarct size correlated well with state-2 respiration and with RCI (Fig. 5).

Table 1. Mitochondrial respiration.

		Rate of state-2 respiration (nmol O_2/min/mg protein)			Rate of state-3 respiration (nmol O_2/min/mg protein)		
Group		Sham	1CAO15	3CAO3	Sham	1CAO15	3CAO3
Drug	Control	50±7	90±15*	75±8*	147±17	209±38	185±28
	Glib	45±6	47±10†	72±10*	137±14	136±26	166±20
	MPG	42±6	80±16*	47±10†	137±13	195±41	148±37

State-2 respiration using succinate; state-3 respiration using succinate+ADP; Glib = glibenclamide; MPG = mercapto-propionyl-glycine; Data are mean±SEM. The number of animals per group is 6-10 (see Fig. 4). **P*<0.05 vs corresponding Sham; †*P*<0.05 vs corresponding Control.

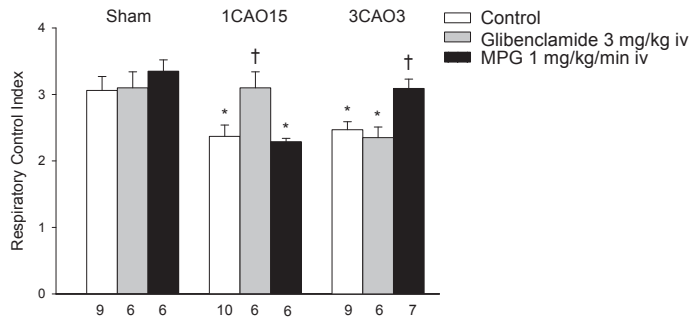

Fig. 4. Respiratory control index (RCI = state-3/state-2) of mitochondria and state-3 and state-2 from hearts subjected *in vivo* to sham procedure or IPC by 1CAO15 or 3CAO3 with vehicle (open bars), glibenclamide (black bars) or n-(-2-mercaptopropionyl)glycine (grey bars). Data are mean±SEM. The number of animals is indicated below each bar. **P*<0.05 vs corresponding Sham; †*P*<0.05 vs corresponding Control.

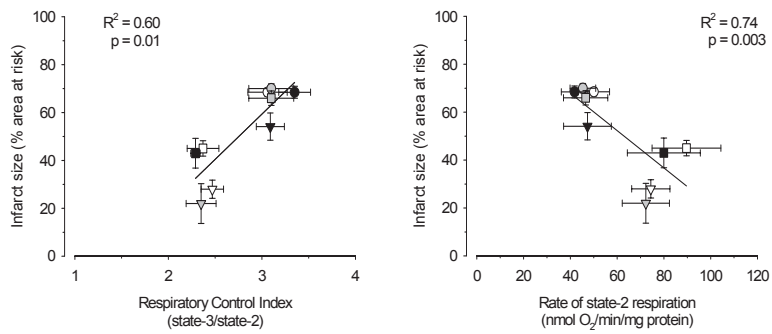

Fig. 5. Left panel shows relation between respiratory control index (RCI= state-3/state-2) of mitochondria and infarct sizes from hearts subjected *in vivo* to sham procedure (circles) or IPC by 1CAO15 (squares) or 3CAO3 (triangles) with vehicle (white), glibenclamide (black) or n-(-2-mercaptopropionyl)glycine (grey). The right panel shows the relation of state-2 respiration of mitochondria versus infarct sizes in these same groups. Data are mean±SEM.

Discussion

The major findings of the present study in the *in vivo* rat heart are: (*i*) preconditioning by 1CAO15 involves adenosine receptor stimulation which, via protein kinase C and tyrosine kinase signaling, results in opening of mitoK$^+_{ATP}$-channels; (*ii*) preconditioning by 3CAO3 involves the release of ROS which subsequently activate tyrosine kinase and protein kinase C, but does not depend on opening of mitoK$^+_{ATP}$-channels; (*iii*) both 1CAO15 and 3CAO3, despite using different signal transduction pathways, converge at the level of the mitochondria by increasing state-2 respiration and hence RCI.

Stimulus-Dependency of Signal Transduction Pathways.

Adenosine. We confirmed that in the rat, cardioprotection by 1CAO15, but not by 3CAO3, was prevented by adenosine receptor blockade.[4] A novel observation is that interstitial adenosine levels increased 7-fold during 1CAO15, whereas only marginal increases were observed during 3CAO3. The latter was apparently too low for sufficient adenosine receptor stimulation. These findings support observations by Schulz *et al*.[1] who reported that in the porcine heart a single 3-min CAO did not affect interstitial adenosine levels, while adenosine deaminase did not affect its IS-limitation. However, a single 10-min CAO led to marked increases in interstitial adenosine levels, while its cardioprotection was attenuated by adenosine deaminase. Hence, a rather long IPC stimulus appears required to elevate interstitial adenosine levels sufficiently to produce cardioprotection via adenosine receptor stimulation.

K_{+ATP}-channels. In the rat, similar to other species,[25-27] the cardioprotection by exogenous adenosine is mediated by K^+_{ATP}-channels. The cardioprotection by endogenous adenosine also involves opening of K^+_{ATP}-channels, as the cardioprotection by the adenosine-dependent stimulus 1CAO15 is prevented by glibenclamide. The observation that both the non-selective K^+_{ATP}-channel blocker glibenclamide and the mitoK^+_{ATP}-channel blocker 5-HD (but not the sarcK^+_{ATP}-channel blocker HMR-1098) blocked the protection by 1CAO15 implicates a role for mitoK^+_{ATP}-channels in this IPC stimulus.[28] This is further supported by the finding that diazoxide limited IS, which was abolished by glibenclamide, suggesting that the protection by diazoxide was mediated via mitoK^+_{ATP}-channels and not via a non-specific mitochondrial effect.[28]

Conversely, IS-limitation by the adenosine-independent IPC stimulus 3CAO3 was unaffected by K^+_{ATP}-channel blockade. Although K^+_{ATP}-channels are believed to play a central role in IPC,[28, 29] a few studies failed to confirm the involvement of K^+_{ATP}-channels. For example, in the rat heart 5-HD blocked cardioprotection by a single 5-min CAO,[16] but was ineffective against four cycles of 5-min CAO.[30] Similarly in the pig heart, IPC by a single 10-min CAO was abolished by glibenclamide,[31, 32] whereas 5-HD (in a dose that blocked diazoxide-induced cardioprotection) did not affect IPC by two 5-min CAOs.[33] Our findings suggest that these equivocal results were caused by the use of different IPC stimuli, although a contribution of other experimental factors (e.g. blood versus buffer perfusion, type of K^+_{ATP}-channel antagonist) cannot be entirely excluded.

Reactive Oxygen Species. Since ROS levels increase during IPC and free radical scavengers can prevent IPC, there is broad support for a role of ROS in IPC.[6, 29] However, not all IPC stimuli require ROS for the induction of cardioprotection. For instance, in anesthetized rabbits[34, 35] and rats,[36] MPG prevented cardioprotection by a single 5-min CAO, whereas protection by three[36] or four[34, 35] 5-min CAOs was unmitigated. In the present study, cardioprotection by the adenosine-independent stimulus 3CAO3, but not that by the adenosine-dependent 1CAO15, was blocked by MPG. Although the involvement of ROS in the protection by 3CAO3 and not

by 1CAO15 seems at odds with the aforementioned studies, our findings are in agreement with earlier observations that adenosine-mediated cardioprotection does not require ROS.[5]

The source of ROS produced by 3CAO3 could be several-fold. For example, ROS can be generated in rat cardiomyocytes by xanthine oxidase during the breakdown of hypoxanthine and xanthine, formed during ischemia, into uric acid.[29, 35] Thus, while the small increase in adenosine during 3CAO3 was insufficient to result in significant adenosine receptor stimulation, it may have provided sufficient substrate for xanthine oxidase to result in ROS production. However, in additional experiments the xanthine oxidase inhibitor allopurinol (20 mg/kg iv)[37], which by itself had no effect on infarct size produced by CAO60 (IS of 62±3% during control versus 60±3% with allopurinol), had also no effect on cardioprotection afforded by 3CAO3 (IS of 45±3% during control vs 39±1% with allopurinol). These findings do not support a role for adenosine catabolism and xanthine oxidase as the source of the ROS involved in the cardioprotection by 3CAO3.

There is substantial evidence that the mitochondrial electron transport chain is a predominant source of ROS during IPC,[28, 29] either originating at the level of complex-III[38] or by mitoK^+_{ATP}-channel opening.[39, 40] However, the relation between the mitoK^+_{ATP}-channels and ROS remains incompletely understood. Several studies suggest that mitoK^+_{ATP}-channel opening triggers the release of ROS,[39, 40] but others indicate that ROS are responsible for mitoK^+_{ATP}-channel opening.[41, 42] Lebuffe *et al*.[43] proposed a role of ROS-induced ROS generation in IPC, i.e. ROS trigger IPC by K^+_{ATP}-channel opening, subsequently leading to generation of additional ROS. We observed that the cardioprotection by 3CAO3 was not blocked by glibenclamide, making it highly unlikely that ROS either originated from mitoK^+_{ATP}-channel opening or resulted in mitoK^+_{ATP}-channel opening. This is further corroborated by the observation that the cardioprotection induced by menadione, a stimulus of mitochondrial ROS production at complex-III, could also not be abolished by glibenclamide. Taken together, these findings demonstrate that for ROS-mediated cardioprotection in the rat heart *in vivo*, opening of K^+_{ATP}-channels is not obligatory.

Protein Kinase Signaling. The cardioprotection by 1CAO15 and 3CAO3 were abolished with protein kinase C and tyrosine kinase inhibitors, confirming the involvement of protein kinase C and tyrosine kinase signaling in IPC.[15, 29, 44-49] Similar to others[46, 47, 50] we found that cardioprotection by both IPC stimuli was only abolished by combined protein kinase C and tyrosine kinase inhibition, whereas inhibition of protein kinase C or tyrosine kinase alone had no significant effect. This suggests the presence of parallel kinase signaling pathways whereby one kinase compensates when another kinase is blocked.[46, 47, 50] Since cardioprotection by diazoxide was unaltered by protein kinase C and tyrosine kinase inhibition, mitoK^+_{ATP}-channel opening by 1CAO15 lies downstream of the kinase signaling pathway. Furthermore, combining chelerythrine and genistein treatment abolished cardioprotection by menadione, suggesting that both protein kinase C and tyrosine kinase are the target of ROS.

Interestingly, both 1CAO15 and 3CAO3 required protein kinase C and tyrosine kinase signaling. However, the downstream targets of these kinases differed for both IPC stimuli as 1CAO15, but not 3CAO3, resulted in mitoK^+_{ATP}-channel opening. Although the present study does not explain these observations, it is now recognized that localized changes in signaling occur in cardioprotection.[6] Thus, compartmentalization of intracellular signaling may explain our finding that G-receptor-triggered activity of protein kinase C and tyrosine kinase results in mitoK^+_{ATP}-channel opening, while ROS-mediated kinase activity targets a yet unknown downstream effector.

Convergence of Signal Transduction Pathways: Mitochondrial Respiration.

Despite their markedly different signaling pathways, both 1CAO15 and 3CAO3 resulted in a reduction in RCI, which was the result of an increase in state-2 respiration, consistent with mild mitochondrial uncoupling. The importance of mitochondrial uncoupling for the protection by both IPC stimuli is suggested by the observation that the various antagonists abolished not only cardioprotection but also the mitochondrial uncoupling, as reflected in the correlation between infarct size and RCI. How these signaling pathways result in uncoupling cannot be determined from the present study, but it is likely that opening of mitoK_{ATP}-channels located in the inner mitochondrial membrane contributed to the mitochondrial uncoupling by 1CAO15.[6, 7, 9, 10, 28] The mechanism of uncoupling produced by 3CAO3 is not clear, but there is evidence that other ion channels in the inner mitochondrial membrane could have similar effects, including K_{Ca}-channels.[28] Alternatively, ROS have been shown to cause mitochondrial uncoupling via stimulation of uncoupling proteins,[51] by activation of the RISK pathway,[52] or by transient opening of the mitochondrial permeability transition pore (MPTP).[12, 13] Future studies need to address the involvement of mitoK_{Ca}-channels, uncoupling proteins and MPTP in the cardioprotection by 3CAO3.

A potential limitation of the present study is that the methods used to assess mitochondrial function required mitochondrial isolation. Although this has the advantage of allowing detailed analysis of mitochondrial function, it cannot be excluded that the isolation procedure per se affected mitochondrial function, and hence that the observed uncoupling in the isolated mitochondria may not necessarily reflect the presence of uncoupling being present in the preconditioned, intact myocardium prior to the induction of the test period of ischemia.[53] It could therefore be that the observed mitochondrial uncoupling in the isolated mitochondria is a marker of a protective change rather than playing a causal role in the protection. Conversely, mitochondrial uncoupling itself has been shown to be cardioprotective, as a low dose of the mitochondrial uncoupler dinitrophenol is cardioprotective in isolated rat cardiomyocytes[11] as well as in the isolated rat heart.[12, 13] These observations suggest that uncoupling is not merely a marker, but rather a mediator, of cardioprotection produced by IPC.

The exact mechanism by which mitochondrial uncoupling can protect cardiomyocytes against ischemia-reperfusion damage is incompletely understood, but may include reduced mitochondrial matrix calcium overload[54] and mitochondrial swelling leading to preserved energy production during prolonged ischemia and reperfusion (for recent reviews see references[55, 56]). Another mechanism could involve a reduction in pathological oxidative stress, thereby preventing sustained opening of the MPTP and the consequent massive release of cytochrome c into the cytosol during sustained ischemia-reperfusion.[6, 7, 9] The effect of uncoupling could be mediated via a lowering of the inner membrane potential leading to an increased flux of electrons through the respiratory chain. The increased electron flux oxidizes the respiratory chain, thereby leading to reduced ROS formation.[6, 7, 9] Future studies are required to determine the mechanism via which the IPC-induced increase in state-2 leads to cardioprotection in our *in vivo* rat model of myocardial infarction.

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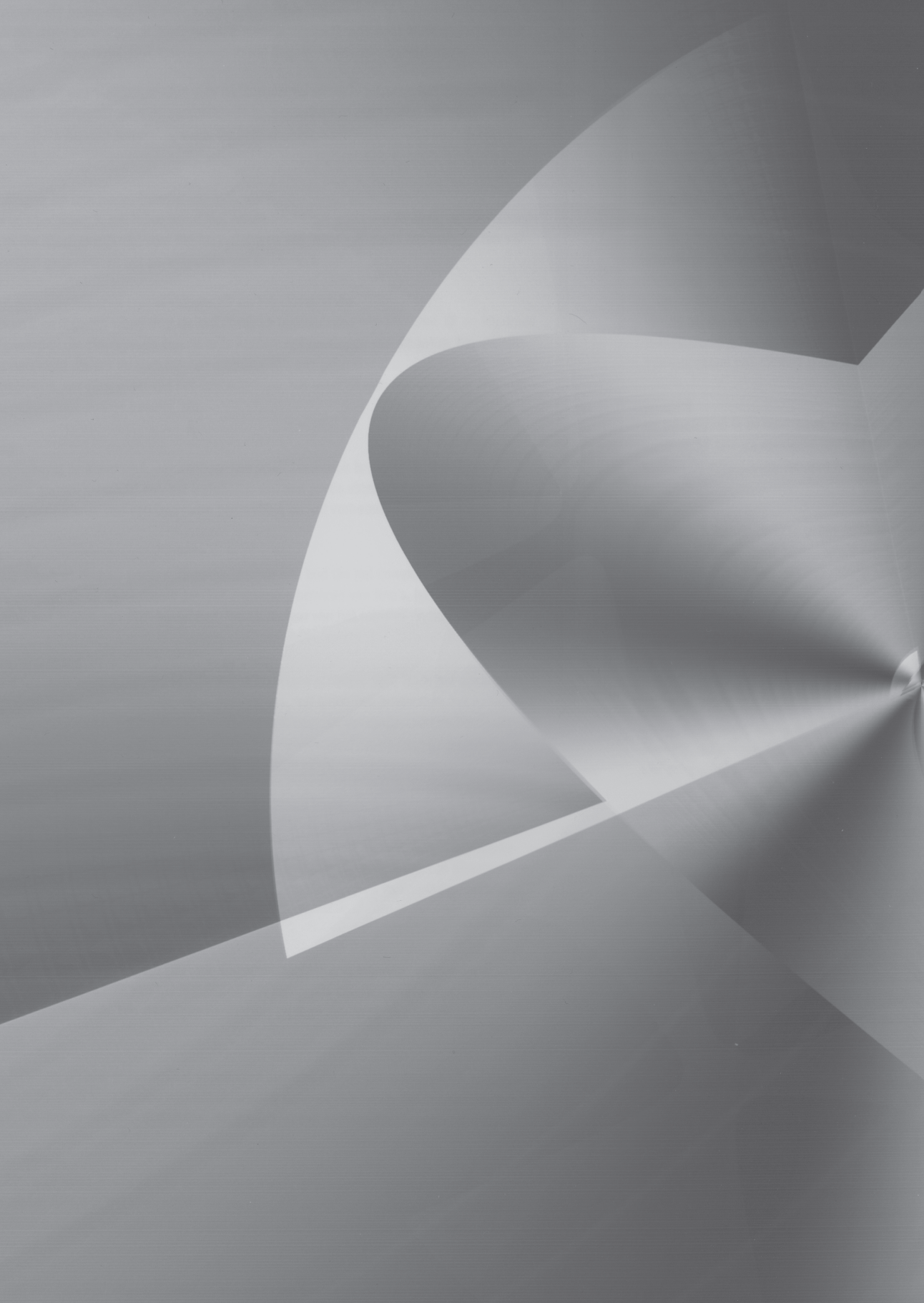

Chapter 3

Intravenous adenosine protects the myocardium primarily by activation of a neurogenic pathway

Olivier C Manintveld, Maaike te Lintel Hekkert, Elisabeth Keijzer, Pieter D Verdouw and Dirk J Duncker.

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Abstract

1 Endogenous adenosine is a trigger for ischemic myocardial preconditioning (IPC). Although intravascular administration of adenosine has been used to further unravel the mechanism of protection by IPC, it is questionable whether adenosine and IPC employ the same signaling pathways to exert cardioprotection.

2 We therefore investigated whether the active metabolic barrier of the endothelium prevents an increase in myocardial interstitial adenosine concentrations by intravenous adenosine, using microdialysis, and investigated the role of NO and activation of a neurogenic pathway in the cardioprotection by adenosine.

3 In pentobarbital-anaesthetised rats area at risk and infarct size (IS) were determined 120 min after a 60-min coronary artery occlusion (CAO), using trypan blue and nitro-blue-tetrazolium staining, respectively.

4 IPC with a single 15-min CAO and a 15-min adenosine infusion (ADO, 200μg/min iv) limited IS to the same extent (IS=41±6% and IS=40±4%, respectively) compared to control rats (IS=63±3%, both $P<0.05$). However, IPC increased myocardial interstitial adenosine levels seven-fold from 4.3±0.7 to 27.1±10.0 μM ($P<0.05$), while ADO had no effect on interstitial adenosine (4.1±1.2 μM), or any of the other purines.

5 The NO synthase inhibitor N-nitro-L-arginine (LNNA), which did not affect IS (IS=62±3%), attenuated the protection by ADO (IS=56±3%; $P<0.05$ vs ADO, P=NS vs LNNA). The ganglion blocker hexamethonium, which had also no effect on IS (IS=66±3%), blunted the protection by ADO (IS=55±4%; $P<0.05$ vs ADO and vs hexamethonium).

6 These observations demonstrate that cardioprotection by ADO is dependent on NO, and is primarily mediated by activation of a neurogenic pathway.

Introduction

Adenosine has been identified as one of the triggers of ischemic myocardial preconditioning (IPC), based on the capability of adenosine receptor antagonists to abolish and adenosine receptor agonists to mimic the cardioprotection by IPC.[1-3] However, the mechanism of protection by intravascularly administered adenosine is still incompletely understood, and doubt has been expressed as to whether adenosine and endogenous adenosine released during IPC employ the same signaling pathways.[4-7] For instance, several groups of investigators have shown that myocardial interstitial adenosine levels increase during the brief ischemic episodes that are employed to precondition the myocardium,[8-10] and the increased adenosine level has been proposed as a primary determinant of the degree of cardioprotection by IPC.[11] In contrast to IPC, access of adenosine into the interstitial compartment is impeded by the active metabolic barrier function of the endothelium,[3, 12] which may explain why intravenous adenosine failed to decrease infarct size in some[13-15] though not all studies,[6, 16] whereas high intracoronary doses[4, 5, 17, 18] or co-infusion with dipyridamole[19] afforded cardioprotection. Furthermore, although several studies indicate that adenosine can reach the interstitium,[6, 7] other investigators observed that intra-arterial infusion of adenosine into the forearm of healthy human volunteers only showed an increase in the interstitial adenosine levels of the forearm in the presence of the nucleoside transporter blocker dipyridamole,[20] lending further support to the concept of the barrier function of the endothelium for adenosine.[3, 18] In line with this notion, several groups of investigators have shown that the cardiovascular effects of adenosine involve, at least in part, the release of endothelium-derived substances, including nitric oxide (NO) and prostanoids,[21, 22] although this is not a ubiquitous finding.[23] Equally important, there is substantial, although somewhat conflicting evidence suggesting a role for NO in the second window of protection by adenosine, while very little is known about the role of NO in the first window of cardioprotection by adenosine.[24]

Adenosine administered via the intravenous route does not only reach the myocardium but other organs as well. This is noteworthy, because we have previously shown that an intramesenteric artery infusion of adenosine (in a dose that did not afford cardioprotection when infused into the portal vein or intravenously), mimics remote ischemic myocardial preconditioning[25-27] by activating a neurogenic pathway.[25, 26] These observations suggest that actions at extracardiac sites could contribute to the limitation of myocardial infarct size by adenosine. Furthermore, in view of earlier findings in our laboratory that blockade of the neurogenic pathway by the ganglion blocker hexamethonium does not modify the cardioprotection by IPC,[25] the latter would imply that IPC and adenosine use distinctly different mechanisms to exert cardioprotection.

In light of these considerations, we used microdialysis to determine whether myocardial interstitial adenosine levels were similarly affected during IPC or adenosine in a dose that was equally effective in limiting myocardial infarct size as IPC.[28] Since we observed that myo-

cardial interstitial adenosine levels remained unchanged during adenosine, but increased during IPC, we subsequently investigated the role of NO in the first window of cardioprotection by adenosine. Finally, we addressed the putative contribution of extracardiac sites to the cardioprotection by adenosine.

Methods

Experiments were performed in ad libitum fed male Wistar rats (300-380 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 Erasmus University Rotterdam Animal Care Committee.

Surgical Procedures

Pentobarbital-anesthetized (60 mg/kg ip) 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 (Hoechst) 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.[25, 29]

Microdialysis

In 17 rats a CMA/20 microdialysis probe (Carnegie Medicine AB, Stockholm, Sweden; membrane 4 mm x 0,5 mm, cut-off: 20 kD) was implanted in the area perfused by the left anterior descending coronary artery, to determine myocardial interstitial adenosine levels.[28, 30] The probe was inserted tangentially to the epicardial surface and positioned in the left ventricular midwall; the proper probe position was confirmed at the end of each experiment. Dialysate samples were collected (with an 8 min delay to correct for 16.1 µl dead space of the probe and the distal tubing) at 15 min intervals a rate of 2 µl/min (total volume of each sample was 30 µl). At the conclusion of each experiment adenosine recovery of the probe was determined *ex vivo* using a solution containing 100 µM adenosine, and found to be 15±1%. All samples were stored at -50°C for later analysis.

Experimental protocols

Cardioprotection by adenosine and IPC. In the animals that were included in the infarct studies, a 30-min stabilization period was allowed before experimental protocols were carried

out. Infarct size was determined after a 60-min CAO followed up by 120 min of reperfusion. Nine rats underwent the 60-min CAO (Control), while seven animals were preconditioned by a 15-min CAO followed by 10 min of reperfusion prior to the 60-min CAO (Figure 1A). This IPC stimulus has been shown to precondition the myocardium via an adenosine-dependent signalling pathway.[2] Seven rats received a 15-min infusion of intravenous adenosine (ADO) in a dose (200 µg/min iv) that produced a similar degree of cardioprotection as IPC with a 15-min CAO.[28]

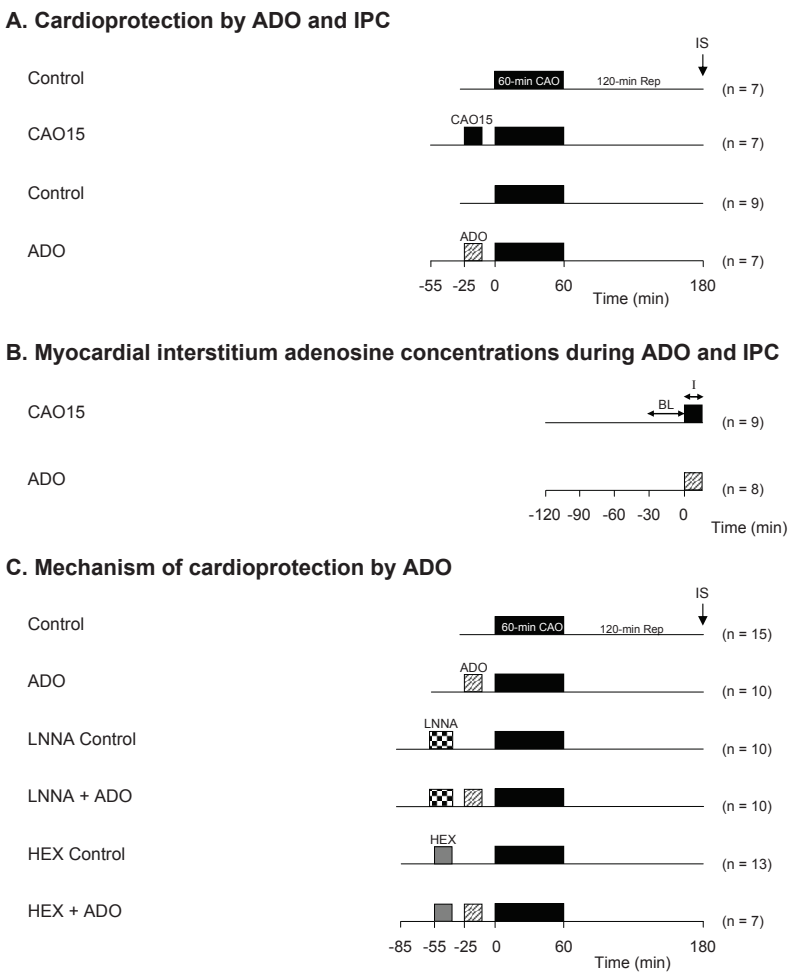

Figure 1 Experimental protocol in which the effects of IPC by 15-min CAO and of 15-min ADO (200 µg/min iv) on myocardial infarct size (Protocol A) and myocardial interstitial adenosine concentrations (Protocol B) were studied. In protocol C we studied the effects of NO synthase inhibition with N-nitro-L-arginine (LNNA, 25 mg/kg iv) and ganglion blockade with hexamethonium (20 mg/kg iv) on cardioprotection by ADO. IS = infarct size; BL = baseline; I = intervention (ADO or IPC).

Myocardial interstitial adenosine levels during ADO and IPC. In the 17 rats, in which myocardial dialysis was performed, baseline measurements were obtained 90 min after insertion of the microdialysis probe (Figure 1B). Subsequently, rats were subjected to either a 15-min CAO (n=9), or a 15-min intravenous infusion of 200 µg/min of adenosine (n=8).

Mechanism of cardioprotection by ADO. To investigate the involvement of endothelial NO synthase in the protection by ADO, rats were pretreated with the NO synthase inhibitor N-nitro-L-arginine (LNNA, 25 mg/kg intravenously infused over a 20-min period; Figure 1C). To investigate whether activation of a neurogenic pathway was involved, rats were pretreated with the ganglion-blocker hexamethonium (20 mg/kg intravenously infused over a 15-min period). Appropriate controls were added where necessary. All drugs were purchased from Sigma Chemical Co.

Rats that encountered ventricular fibrillation during CAO or reperfusion were allowed to complete the experimental protocol, provided that conversion to normal sinus rhythm occurred spontaneously within 1 min or that defibrillation via gently thumping on the thorax was successful within 2 min after onset of fibrillation. Occlusion and reperfusion were visually verified.

Infarct size analysis

Infarct size was determined as previously described.[25, 29] Briefly, after 120 min of reperfusion the LAD was re-occluded, immediately followed by intravenous infusion of 10 ml trypan blue (0.4%, Sigma Chemical Co) into the femoral vein to stain the normally perfused myocardium dark blue and delineate the non-stained area at risk. Subsequently, hearts were excised, rinsed in cold NaCl 0.9%, and cut into slices of 2 mm thickness from apex to base. From each slice the right ventricle was removed and the left ventricular area at risk (non-stained) was dissected from the remaining left ventricular tissue. The area at risk was then incubated for 10 min in 37°C nitro-blue-tetrazolium (Sigma Chemical Co; 1 mg/ml Sorensen buffer, pH 7.4), which stains viable tissue purple but leaves infarcted tissue unstained. After the infarcted area was isolated from the non-infarcted area, the different areas of the left ventricle were dried and weighed separately. Myocardial infarct size (IS) was computed as infarcted area expressed as a percentage of the area at risk (AR).

HPLC analysis of purine concentrations

The adenosine, inosine, hypoxanthine, xanthine and uric acid concentrations in the dialysate samples were determined by reversed-phase high-performance liquid chromatography as described by Smolenski *et al.*.[31] In brief, adenosine and its metabolites were determined by reversed-phase high-performance liquid chromatography using a C_{18} column (Hypersil ODS 3 µm, 150 x 4.6 mm, Alltech, Deerfield, IL, USA) combined with a C_{18} guard column (Hypersil ODS 5 µm, 7.5 x 4.6 mm). We used an AS 3000 cooled autosampler, a SCM 1000 vacuum membrane degasser, a P2000 gradient pump, a 50 µl sample loop and PC 1000 software from

Thermo Separation Products, Riviera Beach, FL, USA) in combination with a Spectra Focus forward optical scanning detector (Spectra-Physics, San Jose, CA, USA). Peaks were detected (and concentrations determined) at 254 nm (hypoxanthine, xanthine, inosine and adenosine) and at 280 nm (uric acid). Purines were identified based on an external standards, retention times and the ratios of the areas under the curve at 254 and 280 nm.[31]

Data analysis and presentation

Infarct data were analysed using one-way analysis of variance followed by post-hoc testing using Student-Newman-Keuls Method. Hemodynamic variables were compared using two-way analysis of variance for repeated measures followed by post-hoc testing using Student-Newman-Keuls Method. Purine data were analysed using the paired t-test. Statistical significance was accepted when $P<0.05$. Data are presented as mean±S.E.M.

Results

Mortality and exclusions

Of the 71 rats that entered the infarction protocol, four rats were excluded because of pump failure during the 60-min. Several rats fibrillated during the 60-min CAO period (no more than 3 rats per group), but were successfully reverted to sinus rhythm and completed the experimental protocol. Infarct size was not different in rats that fibrillated and were thus included in the final analysis. Finally, one rat was excluded due to technical failure and one rat due to an AR<10% of the left ventricle.

Heart rate and arterial blood pressure

Baseline heart rate and mean arterial blood pressure for all animals were 351±3 bpm and 99±1 mmHg, with no differences in heart rate ($P=0.55$) and mean arterial blood pressure ($P=0.11$) between the experimental groups. ADO produced a small decrease in heart rate (5.4±2.5%) while decreasing mean arterial blood pressure by up to 41±3% (both $P<0.05$) at the end of the infusion (Table 1). After discontinuation of ADO, both heart rate and arterial pressure recovered to baseline values well before the onset of the 60-min CAO. Infusion of LNNA caused a marked pressor response, as arterial pressure increased by up to 37±3% ($P<0.05$), which was accompanied by an 11±2% decrease in heart rate. These changes were sustained until the onset of the 60-min CAO. Administration of hexamethonium produced decreases in both heart rate (11±3%) and arterial pressure (33±4%), which had recovered partly at the onset of the 60-min CAO. LNNA and hexamethonium did not blunt the hemodynamic responses to ADO.

Table 1. Heart rate and arterial blood pressure

		n	Baseline	Control/ADO		Coronary Artery Occlusion		Reperfusion
				Pre (-25 min)	End (-10 min)	Pre (-1 min)	End (60 min)	End (120 min)
1.	Control	15						
	HR		345 ± 8	346 ± 10	348 ± 11	348 ± 12	365 ± 12*	394 ± 12*
	MAP		91 ± 3	93 ± 2	98 ± 3	96 ± 3	92 ± 3	79 ± 4*
2.	ADO	10						
	HR		361 ± 9	358 ± 7	337 ± 6*†	368 ± 9	368 ± 12	385 ± 13
	MAP		104 ± 5	106 ± 5	63 ± 3*†	118 ± 4*	106 ± 4	93 ± 6*
3.	LNNA Control	10						
	HR		338 ± 6	301 ± 6*	304 ± 7	299 ± 6*	319 ± 8	311 ± 17
	MAP		101 ± 2	138 ± 4*	145 ± 3*	140 ± 3*	107 ± 7	72 ± 6*
4.	LNNA + ADO	10						
	HR		348 ± 6	316 ± 6*	335 ± 8†	325 ± 6*	323 ± 8*	324 ± 14*
	MAP		103 ± 3	150 ± 4*	81 ± 5*†	151 ± 3*	117 ± 7	74 ± 9*
5.	HEX Control	13						
	HR		353 ± 8	312 ± 6*	318 ± 8*†	327 ± 8	343 ± 11	377 ± 13*
	MAP		100 ± 5	67 ± 2*	83 ± 2*†	89 ± 4	95 ± 4	96 ± 5
6.	HEX + ADO	7						
	HR		346 ± 11	311 ± 8*	307 ± 5*	340 ± 12	349 ± 9	399 ± 11*
	MAP		93 ± 4	70 ± 3*	49 ± 1*†	89 ± 4	96 ± 4	89 ± 5

HR = heart rate (b.p.m.); MAP = mean aortic pressure; Data are mean±s.e.m.; *P<0.05 vs Baseline; †P<0.05 End Control/Adenosine vs Pre Control/Adenosine.

Cardioprotection and myocardial interstitial adenosine concentrations

IPC with a 15-min CAO and a 15-min ADO produced similar marked reductions in IS (Figure 2A). However, while IPC produced marked increments in myocardial interstitial adenosine levels from 4.3±0.7 μM at baseline to 27.1±10.0 μM during the 15-min CAO (P<0.05), as well as increases in dialysate concentrations of the other purines (Table 2), ADO had no effect on myocardial interstitial adenosine levels (4.1±1.2 μM; Figure 2B), or on dialysate concentrations of any of the other purines.

Table 2. Dialysate concentrations of purines

		Concentration (μM)	
		Baseline	Intervention
IPC (n = 9)	Adenosine	0.6 ± 0.1	4.7 ± 1.9*
	Inosine	3.0 ± 0.4	9.4 ± 2.7*
	Hypoxanthine	0.3 ± 0.1	3.9 ± 1.3*
	Xanthine	0.7 ± 0.2	3.6 ± 0.8*
	Uric Acid	5.0 ± 0.5	5.9 ± 0.5
	Total purines	9.6 ± 0.9	27.5 ± 6.8*
ADO (n = 8)	Adenosine	0.9 ± 0.2	0.7 ± 0.1
	Inosine	2.8 ± 0.3	2.7 ± 0.2
	Hypoxanthine	0.2 ± 0.1	0.3 ± 0.1
	Xanthine	0.6 ± 0.1	0.6 ± 0.1
	Uric Acid	5.9 ± 0.4	6.9 ± 0.4
	Total purines	10.5 ± 0.8	11.4 ± 0.8

Data are mean±s.e.m; *P<0.05 vs corresponding Baseline

Mechanism of protection by ADO

There were no differences (P=0.32) between the area at risk of the various experimental groups (Table 3). In agreement with earlier reports in rats,[28, 29] rabbits[32] and pigs[33], we observed no significant linear correlation between the rate-pressure product at the onset of the 60-min CAO and the corresponding IS (linear regression: $r^2 = 0.02$; P=0.31). LNNA, which had no significant effect on IS by itself (IS=62±3% vs IS=66±2% in sham treated control rats), virtually abolished the protection by ADO (Figure 3). Pretreatment with hexamethonium, which did also not effect IS (IS=66±3%) by itself, attenuated the amount of protection by ADO by 65% (Figure 3).

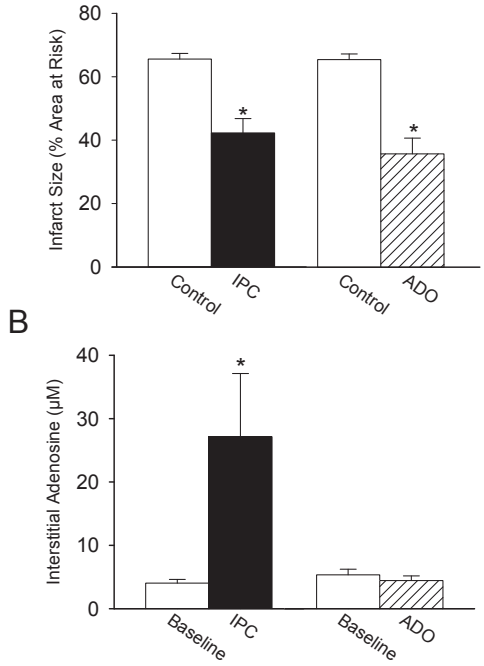

Figure 2 Panel A displays the protective effect of IPC (n=7) and ADO (n=7) compared to control rats that underwent only the 60-min CAO (n=9). Panel B displays the increase in myocardial interstitial adenosine concentrations from baseline produced by IPC (n=9) but not by ADO (n=8). **P*<0.05 vs corresponding Control or Baseline.

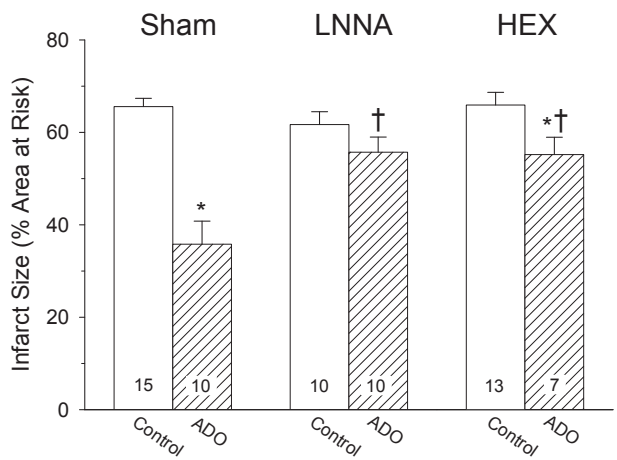

Figure 3 Infarct size in control rats and in rats receiving ADO, without (Sham) or after NO-synthase blockade (LNNA) or ganglion blockade (Hexamethonium, HEX). Infarct size is expressed as percentage of the area at risk. The number of animals in each group is shown within the bars. **P*<0.05 vs corresponding Control; †*P*<0.05 vs corresponding Sham.

Table 3. Area at risk and infarct area

	n	*AR (% LV)*	*IA (%LV)*
Control	15	38±2	24±2
IPC	12	32±3	13±2*
ADO	10	32±3	12±2*
LNNA Control	10	38±3	23±1
LNNA + ADO	10	39±3	22±2†
HEX Control	13	38±2	26±2
HEX + ADO	7	36±4	20±3†

AR = area at risk; LV = left ventricle; IA = infarct area
Data are mean±s.e.m.
**P*<0.05 vs corresponding Control
† *P*<0.05 vs ADO

Dicussion

The mechanism by which IPC protects the myocardium has been the topic of numerous studies since the first description of the phenomenon by Murry *et al*.[34] in the expectation that knowledge of the mechanism would permit the development of pharmacological exploitation in the clinical setting.[35, 36] The discovery that activation of adenosine receptors is one of the triggers of IPC has led to the investigation of the usefulness of adenosine in the treatment of a coronary artery stenosis by elective percutaneous coronary intervention,[37, 38] as adjuvant to thrombolysis[39] or percutaneous coronary intervention[40] in myocardial infarction and as adjuvant to the cardioplegic solution during cardiac surgery.[41]

Adenosine has been shown to be a trigger of IPC in all animal species studied. However, based on several studies including those in which the selective adenosine A_1-receptor antagonist PD115199 and the non-selective antagonist SPT failed to block IPC,[14, 42] Ganote & Armstrong[43] concluded that adenosine does not play a role in the myocardial IS limitation by IPC in rats. Importantly, in these studies[14, 42] the duration of the multiple IPC stimuli was 3-5 min. We subsequently confirmed the observations by Li & Kloner[14] that the cardioprotection by a triple 3-min CAO did not depend on intact adenosine receptors, but in contrast, that cardioprotection by a single 15-min CAO was completely abolished by the adenosine receptor antagonist 8-SPT.[2] Thus, similar to the porcine heart,[44] in the rat heart the role of adenosine in IPC depends critically on the type of IPC stimulus.

The mechanism of protection by intravascular adenosine is still incompletely understood, but a large number of studies indicate that it may differ from that of endogenous adenosine in IPC.[4-7] For example, while myocardial interstitial adenosine levels increase during IPC [8-10], access of intravascular adenosine into the interstitial compartment is impeded by the active metabolic barrier function of the endothelium.[3, 12] This barrier function may explain why in some studies, though not all,[6, 16] intravascular adenosine failed to decrease infarct size,[14, 15, 19] or increase interstitial adenosine concentrations,[20] unless the adenosine transport inhibitor dipyridamole was co-administered.[20] In contrast, studies employing high doses of intra-arterial adenosine observed cardioprotection,[4, 5, 13, 18] and increases in interstitial adenosine concentrations.[7, 17, 18] In the present study, we observed that ADO produced a marked reduction in IS, which contrasts with Li & Kloner[14] who reported a lack of cardioprotection by adenosine in the rat heart *in situ*. These divergent findings are difficult to explain but could be related to differences in the employed anesthesia. Thus, the signaling pathway involved in IPC has been shown to differ in ketamine-xylazine vs pentobarbital anesthesia.[45] In addition, differences in rat strain (Sprague-Dawley vs Wistar) and gender (female vs male), the dose and duration of intravenous adenosine infusion (1.5 mg administered over 5 min vs 3 mg administered over 15 min) and CAO duration (90 min vs 60 min) may also have contributed to the different outcomes.

Interestingly, we observed that while ADO produced marked cardioprotection, it failed to increase myocardial interstitial adenosine concentration. These findings are at variance with the increases in myocardial interstitial adenosine concentrations produced by intravenous adenosine, in a dose that produced a degree of cardioprotection in the rabbit,[6] that was comparable to the cardioprotection observed in the present study. Failure to detect an increase in interstitial adenosine does not appear to be due to increased adenosine catabolism in the rat heart, because concentrations of the adenosine metabolites remained similarly unchanged (Table 2). It could also be argued that the probe recovery was too low to detect changes in adenosine concentrations. The recovery rate of our microdialysis fibers was 15±1%, which is considerably lower than that reported in other studies (64-66%).[6, 18] However, the lower recovery in the present study is at least in part due to the higher dialysate flow rate (2 µl/min compared to 0.75 µl/min in the studies by Lasley *et al.*),[6, 18] which is inversely related to recovery percentage of the probe.[46] Furthermore, we readily detected marked increases in adenosine and other purine concentrations during total coronary artery occlusion, that are comparable to the increases observed in the rabbit heart.[6] An alternative explanation could be that adenosine produced an increase in coronary blood flow that caused enhanced adenosine washout, thereby masking a small increase in interstitial adenosine concentrations.[17] Although this would not explain the increase in interstitial adenosine that was observed in the rabbit heart,[6] we cannot entirely exclude that this effect may have increased importance in the *in situ* rat heart, in which we observed relatively high interstitial adenosine concentrations under baseline conditions.

The observation in the present study that ADO did not result in elevated myocardial interstitial adenosine levels, suggests that adenosine remained principally confined to the intravascular compartment. In support of that concept, there is evidence to suggest that the cardiovascular effects of adenosine involve, at least in part, the release of endothelium-derived substances, including NO and prostanoids.[21, 22] Furthermore, there is, albeit somewhat controversial, evidence that NO plays a role during the second window of protection.[24] Since the involvement of NO in the early phase of protection by adenosine has not been previously studied, we investigated the role of NO in the early phase of protection by ADO. In the presence of LNNA, ADO no longer afforded cardioprotection, which in conjunction with the lack of increase in myocardial interstitial adenosine levels, could be interpreted to suggest that ADO affords cardioprotection via (coronary) endothelium-derived NO. However, from our *in vivo* experiments we cannot determine the site of NO production by ADO. For example, recent evidence suggests that adenosine may not only stimulate eNOS in the endothelium, but also in cardiomyocytes.[47] Moreover, we cannot exclude that interstitial adenosine concentrations may have increased in tissues other than the heart, which would implicate the involvement of NO production at sites other than the endothelium, e.g. downstream of the neurogenic pathway. Another limitation is that LNNA is a non-specific NO synthase inhibitor, and hence we cannot exclude that isoforms other than eNOS are involved in the cardioprotection by ADO. Future studies, using microdialysis in other organs and using selective inhibitors of the various NOS isoforms are required to address these important issues.

Recently, the concept of IPC has been expanded to include remote preconditioning, the phenomenon that a brief period of ischemia in an organ or tissue not only elicits a local preconditioning effect, but also provides protection against prolonged ischemia in virgin tissue and organs at a distance.[25, 27] For instance, Gho *et al*.[25] have shown that a brief episode of intestinal ischemia produced by a 15-min mesenteric artery occlusion limited myocardial IS produced by a subsequent 60-min CAO. Remote preconditioning was mimicked by a low dose of intramesenteric adenosine infusion, but not by infusion of the same dose into the portal vein.[26] Both cardioprotection by remote preconditioning and intramesenteric adenosine infusion were abolished by ganglion blockade, implying the involvement of a neurogenic pathway. In light of these considerations, we investigated whether an action at extracardiac sites contributed to the protection by ADO. The observation that hexamethonium, which does not modify the protection by IPC with a 15-min CAO[25] attenuated the protection by 65%, indicates that the ADO-induced cardioprotection originates, at least in part, at extra-cardiac sites where it initiates cardioprotection via activation of a neurogenic pathway. The design of the study does not permit to draw any conclusion about the location of these extracardiac sites. The small intestine is a prime candidate, considering our earlier observations with the intramesenteric artery infusion of adenosine,[26] but other organs such as the kidney may also be involved (see Przyklenk *et al*.[27]). There is evidence that remote preconditioning by skeletal muscle ischemia may not depend on a neurogenic pathway.[48, 49] Hence, we cannot simply

ascribe the residual protection by ADO that was not amenable to ganglion blockade to direct intracardiac action of ADO, as we cannot exclude that a humoral factor released from skeletal muscle may also have contributed.

The dose of adenosine that produced the cardioprotection caused a 44±4 mm Hg decrease in mean arterial blood pressure and it might be argued that it is therefore not clinically relevant. It must be kept in mind, however, that sodium pentobarbital was used to anesthetize the animals and that this anesthetic regimen suppresses baroreceptor-mediated reflexes[50] and thereby exaggerates the hypotension.[51] Indeed, we observed a small decrease in heart rate during ADO, consistent with observations by Li & Kloner,[14] suggesting the absence of significant baroreflex activity. In the present study, it should also be considered that ADO was administered to animals with a normal endothelial function and it cannot be excluded that ADO would increase the interstitial adenosine levels when administered to animals with endothelial dysfunction. Because this may be of clinical relevance, we also administered the same dose of ADO to rats that had been exposed to four sequences of 15-min CAO. In this model, that causes 10% of the area at risk to become infarcted[28] and which is likely associated with endothelial dysfunction,[52] we also did not find a rise in interstitial adenosine levels (2.1±0.5 μM before vs 2.0±0.4 μM during ADO).

In conclusion, the findings in the present study demonstrate that the early phase of cardioprotection by ADO: (*i*) is not associated with a detectable increase in myocardial interstitial purine concentrations, (*ii*) depends critically on NO production, and (*iii*) involves the activation of a neurogenic pathway. These findings indicate that ADO administered as adjunct therapy to reperfusion treatment in patients with a pending myocardial infarction may not require access to the jeopardized myocardium, but rather may initiate cardioprotection at remote extracardiac sites.

Acknowledgements

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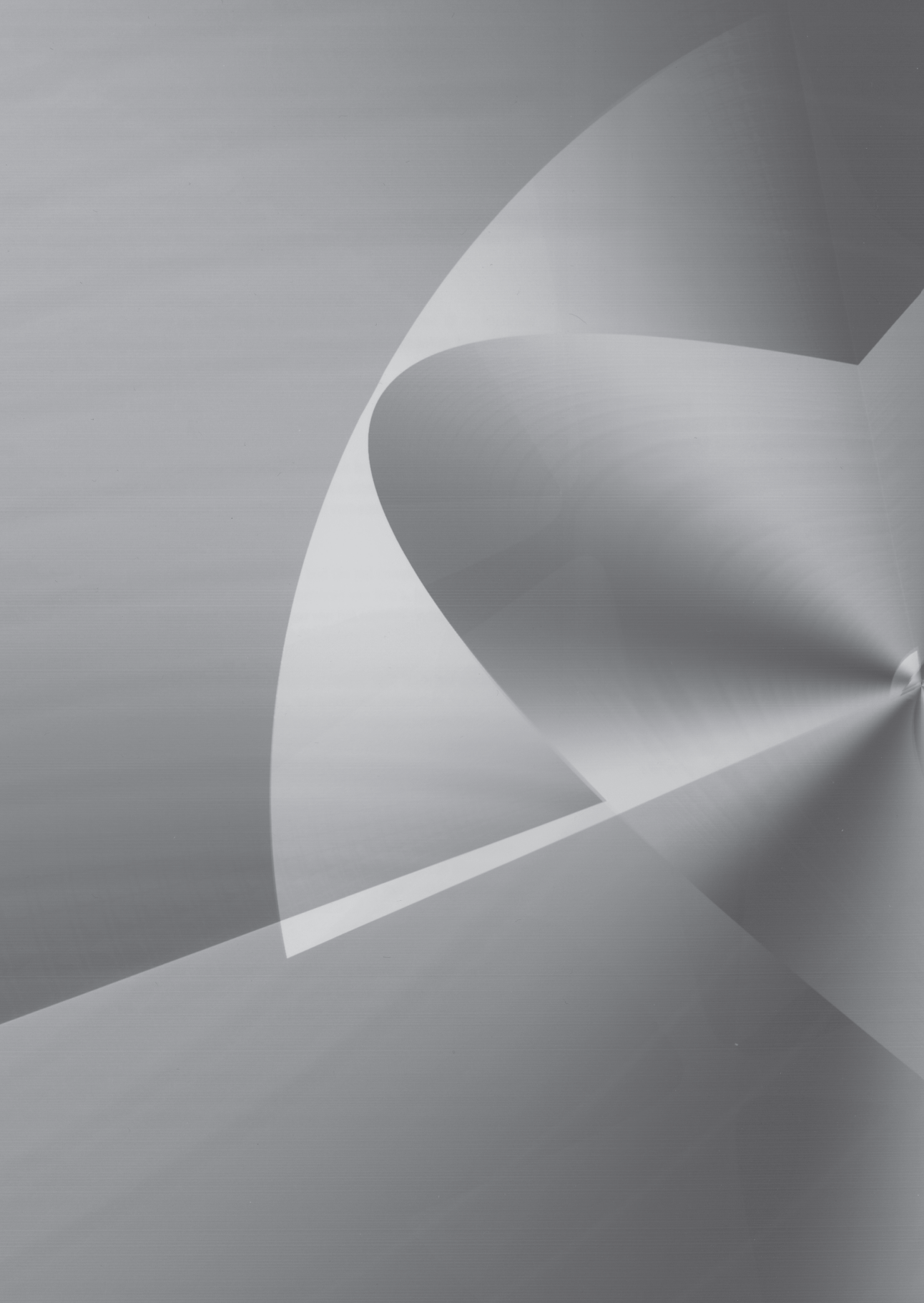

Chapter 4

Involvement of the Reperfusion Injury Salvage Kinase pathway in Preconditioning Depends Critically on the Preconditioning Stimulus

Olivier C Manintveld, Wim Sluiter, Dick H Dekkers, Maaike te Lintel Hekkert, Pieter D Verdouw, Jos M Lamers and Dirk J Duncker.

Submitted

Abstract

Different preconditioning stimuli can activate divergent signaling pathways. In rats, adenosine-independent pathways (triple 3-min CAO, 3CAO3) and adenosine-dependent pathways (15-min coronary artery occlusion, 1CAO15) exist, both ultimately converging at the level of the mitochondrial respiratory chain. Furthermore, while 3CAO3, 1CAO15 and exogenous adenosine (ADO) are equally cardioprotective, only 1CAO15 increases interstitial myocardial adenosine levels. The Reperfusion Injury Salvage Kinase (RISK) pathway has been implicated in ischemic preconditioning, but not all preconditioning stimuli activate this pathway. Consequently, we evaluated in anesthetized rats the effects of three distinctly different preconditioning stimuli (3CAO3, 1CAO15 or ADO) on mitochondrial respiration and infarct size, and investigated the signaling pathways involved with a special emphasis on the RISK pathway. All three stimuli increased state-2 respiration (using succinate as complex-II substrate) thereby decreasing the respiratory control index, which was accompanied by a limitation of infarct size produced by a 60-min CAO. NO synthase inhibition abolished the mitochondrial effects and the cardioprotection by 3CAO3, 1CAO15 or ADO. In contrast, the PI3 kinase inhibitor wortmannin blocked protection by 1CAO15, but did not affect protection by 3CAO3 or ADO. Western blotting confirmed that the RISK pathway was activated by 1CAO15, but not by 3CAO3 or ADO. The latter two stimuli also failed to activate the JAK/STAT or the AMPK pathway. Although the three cardioprotective stimuli 3CAO3, 1CAO15 or ADO, afford cardioprotection via NO-mediated modulation of mitochondrial respiration, only the endogenous adenosine-dependent 1CAO15 exerts its protection via the RISK pathway.

Introduction

Despite numerous efforts over the past twenty years, the mechanism of ischemic preconditioning (IPC) remains incompletely understood. A frequently overlooked factor in studies addressing the mechanism of IPC is that the employed signal transduction pathway depends on the IPC stimulus.[1, 2] Indeed, we observed in *in vivo* studies in the rat that a triple 3-min coronary artery occlusion (CAO) interspersed by 5-min of reperfusion (3CAO3) did not elevate of interstitial myocardial adenosine levels, while its cardioprotection was not affected by either adenosine receptor or K^+_{ATP} channel blockade, but was attenuated by the reactive oxygen species (ROS) scavenger mercapto-propionyl-glycine.[3, 4] In contrast, a single 15-min CAO (1CAO15) resulted in markedly elevated interstitial myocardial adenosine concentrations and stimulation of adenosine receptors and K^+_{ATP} channels.[3, 4] However, despite these markedly different signaling pathways, both IPC stimuli resulted in mild mitochondrial uncoupling,[3] suggesting that although the signaling pathways that mediate the cardioprotection by various IPC stimuli may differ, the mitochondria play a critical role effectuating protection by all IPC stimuli. Furthermore, we also demonstrated that intravenous infusion of adenosine also afforded cardioprotection via K^+_{ATP} channel activation, yet did not result in detectable elevations of interstitial adenosine.[5] Furthermore, the cardioprotection by exogenous adenosine, but not that by 1CAO15, was blunted by the ganglion blocker hexamethonium,[5] indicating that cardioprotection by exogenous adenosine, but not by endogenous adenosine, was partly produced via activation of a neurogenic pathway, consistent with the findings by Liem *et al*.[6] These observations lend further support to the concept that activation of a particular signal transduction pathway depends critically on characteristics of the preconditioning stimulus.[1-4]

Studies addressing the signal transduction pathways involved in the cardioprotection by IPC focus increasingly on the role of the pro-survival kinases including the phosphatidylinositol 3-kinase–Akt–endothelial NO synthase (PI3K–Akt–eNOS) pathway and the extracellular signal-related kinase (ERK1/2).[7] This is not surprising as these components of the Reperfusion Injury Salvage Kinase (RISK) pathway can be activated and contribute to the cardioprotection by various IPC stimuli.[7] Furthermore, several cardiovascular drugs and hormones, including statins, erythropoietin and insulin, limit infarct size in animal models by activating the RISK pathway.[8] However, not all stimuli employ the RISK pathway for cardioprotection, as cardioprotection by infusion of tumor necrosis factor-α is mediated via the signal transducer activator of transcription-3 (STAT3) but not the RISK pathway.[9]

In light of evidence that both pharmacological and IPC stimuli can protect the myocardium by different pathways,[1-5] we set out to investigate the role of the PI3K–Akt–eNOS pathway in the cardioprotection by the three aforementioned preconditioning stimuli in the *in vivo* rat heart. Since endogenous release of adenosine during IPC has been proposed to afford cardioprotection via increased activity of the RISK pathway during early reperfusion,[10] we hypothesized that the adenosine-dependent (but ROS-independent) stimulus 1CAO15, but

not the adenosine-independent (but ROS-dependent) stimulus 3CAO3 involves activation of the PI3K–Akt–eNOS pathway. Conversely, the IPC-induced activation of the Janus kinase/STAT (JAK/STAT) pathway[11, 12] and possibly also adenosine 5'-monophosphate-activated protein kinase (AMPK)[13] has been shown to depend on ROS signaling. Consequently, we hypothesized that 3CAO3, but not 1CAO15, activates the JAK/STAT and AMPK pathway. Finally, because in the isolated rabbit heart cardioprotection by intravascular adenosine has been reported to be PI3K-independent,[14] we hypothesized that in the *in vivo* rat heart the PI3K–Akt–eNOS pathway is not involved in pharmacological preconditioning by intravenous infusion of exogenous adenosine.

Materials and Methods

Experiments were performed in male Wistar rats (300-400 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 Erasmus MC Animal Care Committee.

Surgical and Experimental Procedures. Pentobarbital-anesthetized (60 mg/kg) rats were intubated for positive pressure ventilation with oxygen-enriched room air.[5, 15] A PE-50 catheter was inserted in the carotid artery and positioned in the thoracic aorta for measurement of arterial blood pressure and heart rate. In the inferior vena cava, a PE-50 catheter was placed for infusion of drugs, and Haemaccel (Behringwerke) to maintain fluid-balance. After thoracotomy, the pericardium was opened and a silk 6-0 suture was looped under the left anterior descending (LAD) 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.[16] Following completion of surgery, a 30-min stabilization 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 was established within 2 min after onset of fibrillation.

Infarct size. Infarct size (IS) was determined after 120 min of reperfusion following a 60-min CAO (Fig. 1). Area at risk and infarct area were determined using Trypan blue and nitro-blue-tetrazolium staining.[16] IS was expressed as infarct area / area at risk (x 100%).

Preceding the 60-min CAO, animals underwent a 25-min sham period, or IPC by either three cycles of 3-min CAO interspersed by 5-min of reperfusion (3CAO3) or a 15-min CAO followed by 10-min of reperfusion (1CAO15) or pharmacological preconditioning by 15-min infusion of exogenous adenosine (ADO; 10 mg/kg iv; Fig. 1). To study the effects of the PI3K–Akt–eNOS signaling pathway, sham- and preconditioned rats were pretreated intravenously with the PI3K inhibitor wortmannin [15µg/kg iv][15, 17] or the NOS inhibitor N-nitro-L-arginine [LNNA, 25 mg/kg iv].[5] Adenosine, LNNA and wortmannin were purchased from Sigma.

Mitochondrial Respiration. Mitochondrial respiration was studied in rats subjected to sham procedure, 3CAO3, 1CAO15 or ADO, with or without pretreatment with LNNA (Fig. 1). At the

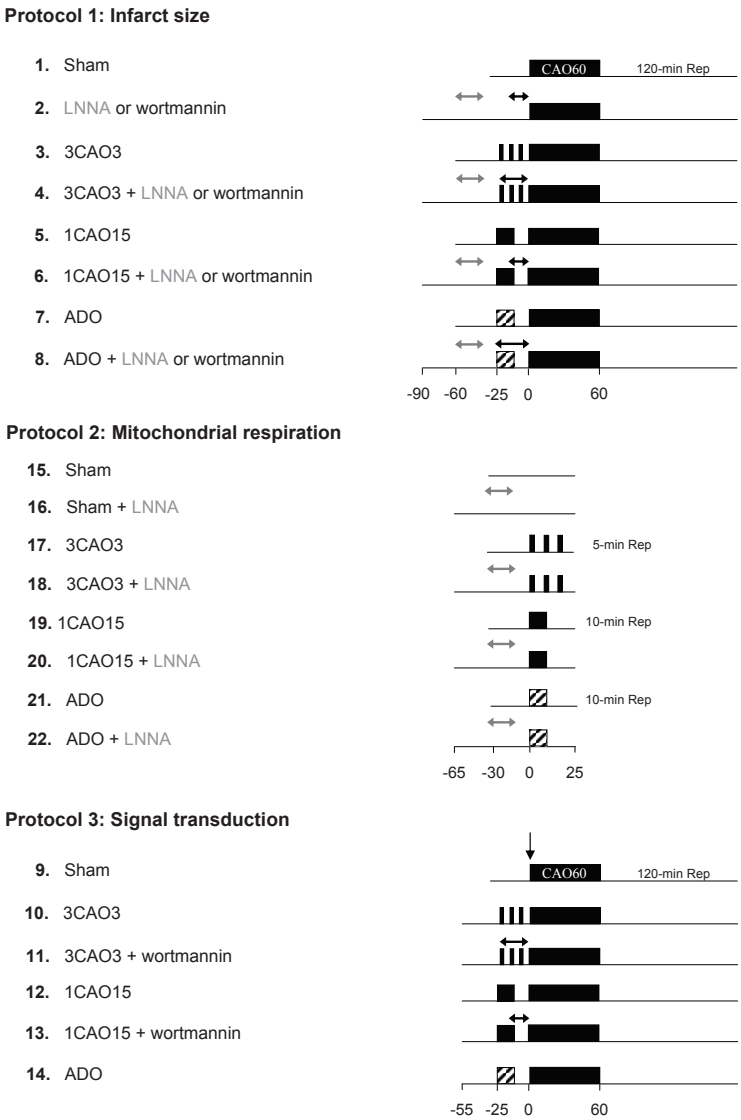

Fig. 1. Shown are the protocols for: 1) infarct size studies involving the different preconditioning protocols including the administration of wortmannin (↔) or LNNA (↔); 2) mitochondrial respiration studies (mitochondria were harvested at time point corresponding to completion of preconditioning protocol); 3) involvement of the signaling pathways in the different preconditioning protocols was studied by Western blotting by sacrificing animals at the time point corresponding with the onset of the 60-min CAO (↓).

time point corresponding with the onset of the 60-min CAO, hearts were quickly excised, the LAD area was dissected out and placed in ice cold mitochondrial isolation buffer (pH 7.15, containing 50 mM sucrose, 200 mM mannitol, 1 mM EGTA, 5 mM KH_2PO_4, 5 mM MOPS

and 0.1 % fatty acid free BSA), minced and mitochondria were isolated.[3] Mitochondria were subsequently suspended in mitochondrial respiration buffer (pH 7.15, containing 110 mM sucrose, 0.5 mM EGTA, 3 mM $MgCl_2$, 70 mM KCl, 10 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES and 0.1% fatty acid free BSA).[3] The oxygen consumption rate (nmol/min/mg protein) was measured at 30°C by high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria), in state-2 (in the absence of ADP) and in state-3 (in the presence of 0.5 mM ADP) using 10 mM succinate as a complex-II substrate. Respiratory control index (RCI) was calculated as state-3/state-2. Since barbiturate anesthesia inhibits complex-I activity,[18] we limited mitochondrial studies to complex-II-dependent respiration. Measurements were performed in the presence of the complex-I inhibitor rotenone.[19]

Signal transduction pathways. To determine protein levels (both phosphorylated protein and total protein), animals were sacrificed at the time point corresponding with the onset of the 60-min CAO (Fig. 1). Hearts were quickly excised, the LAD area dissected out and snap frozen in liquid nitrogen before being stored at -80°C. Approximately 150 mg of frozen left ventricular tissue was homogenized at liquid nitrogen temperature in a microdismembrator unit (B. Braun Biotech International) at 1700 rpm for 4 min in a Teflon vial with a Teflon coated sphere. The frozen powder was suspended in 20 volumes of cold Laemmli loading buffer, heated for 5 min at 95°C, sonicated in ice water in a Bioruptor (Diagenode) for 10 minutes at 30 seconds on/off intervals, and centrifuged for 1 min at 9700·g_{av}. Supernatant was removed Western blot analysis and protein determination using the RCDC protein assay (Bio-Rad Laboratories). Samples were stored at -80°C and were reheated for 5 min at 95°C before use. Proteins were separated by SDS-PAGE using 7.5%-15% gradient gels; 25 µg of protein/lane was applied onto the gels. Following electrophoresis proteins were blotted overnight at 40V onto PVDF membranes (Immobilon FL, Millipore). To check protein loading and transfer, blots were stained reversibly with Ponceau Red. Blots were pre-incubated in Odyssey blocking buffer (LI-COR Biosciences) diluted two times in PBS for 1 h at room temperature and incubated overnight at 4°C with diluted primary antibodies in PBS diluted blocking buffer containing 0.1% Tween-20.

All antibodies were purchased from Cell Signaling Technology except phospho-STAT1 Y701 (Santa Cruz Biotechnology). Antibodies that were used are Akt (rabbit polyclonal), phospho-Akt Ser^{473} and Thr^{308} (both mouse monoclonal), ERK1/2 (rabbit polyclonal), phospho-ERK1/2 (mouse polyclonal), STAT1 (rabbit polyclonal), STAT3 (rabbit monoclonal), phospho-STAT3 Tyr705 (mouse monoclonal), AMPKα (mouse monoclonal), phospho-AMPKα Thr172 (rabbit monoclonal) and phospho-STAT1 Y701 (mouse monoclonal).

Blots were probed for 1 h at room temperature with goat anti-mouse conjugated IRDye 800CW or goat anti-rabbit conjugated IRDye 680 secondary antibody (LI-COR Biosciences) in diluted blocking buffer supplemented with 0.1% Tween-20 and 0.01% SDS. After each incubation with antibodies the blots were washed extensively with 0.1% Tween-20 in PBS.

Fluorescent signals were detected and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Data Analysis and Presentation. IS and mitochondrial respiration were analyzed by ANOVA followed by Student-Newman-Keuls test. Statistical significance was accepted when $P<0.05$ (two-tailed). Data are mean±SEM.

Results

Mortality. Six out of 107 rats that entered the infarct protocols and five out of 131 rats that entered the mitochondrial respiration or signal transduction pathway protocols were excluded due to technical failure (≤1 rat per group), or acute pump failure (≤1 rat per group).

Infarct Size. IS produced by a 60-min CAO amounted to 62±2% of the area at risk in the sham-treated animals (Fig. 2). IS was less when rats were subjected to 3CAO3 (IS=45±2%), 1CAO15 (IS=42±5%) or ADO (IS=38±5%) prior to the 60-min CAO (all $P<0.05$ vs sham). Wortmannin, which did not affect IS in sham animals (IS=60±3%), abolished the cardioprotection by 1CAO15 (IS=60±2%), but had no effect on the cardioprotection by 3CAO3 (IS=50±3%) or ADO (IS=41±3%). In contrast, LNNA abolished cardioprotection by 3CAO3 (IS=61±2%), 1CAO15 (IS=60±2%) and ADO (IS=57±4%).

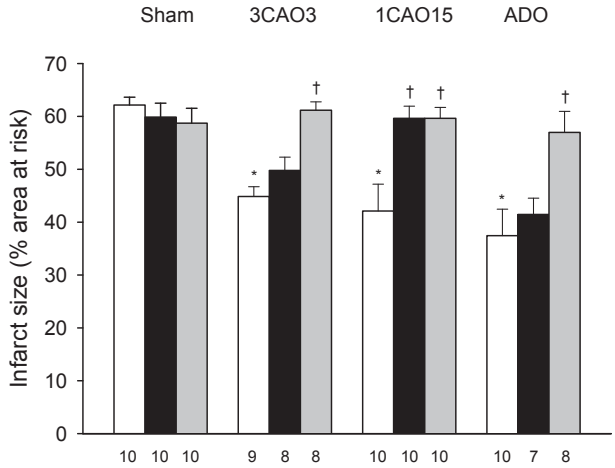

Fig. 2. The effects of preconditioning on myocardial infarct size are shown including treatment with wortmannin (black bars) and LNNA (grey bars). Data are mean±SEM. The number of animals is indicated below each bar. *$P<0.05$ vs corresponding Sham; †$P<0.05$ vs corresponding Control.

Mitochondrial Respiration. 3CAO3, 1CAO15 and ADO all resulted in a small decrease in RCI, secondary to an increase in state-2 respiration (Table 1). LNNA abolished the decrease in RCI

Table 1. Mitochondrial respiration.

		state-2 respiration (nmol O_2/min/mg protein)				state-3 respiration (nmol O_2/min/mg protein)				Respiratory Control Index (state-3/state-2)			
Group		Sham	3CAO3	1CAO15	ADO	Sham	3CAO3	1CAO15	ADO	Sham	3CAO3	1CAO15	ADO
Drug	Control	36±7	67±8*	77±14*	71±14*	106±19	164±24	180±34	173±36	3.1±0.1	2.4±0.1*	2.3±0.1*	2.4±0.1*
	LNNA	39±8	33±6†	37±10†	34±3†	116±24	106±18	108±32	103±10	3.2±0.2	3.3±0.1†	2.9±0.2†	3.0±0.1†

State-2 respiration using succinate; State-3 respiration using succinate+ADP; Preconditioning is performed by 3CAO3, 1CAO15 or ADO (see text); LNNA = N-nitro-L-arginine; Data are mean±SEM. The number of animals per group is 8-12. *P<0.05 vs corresponding Sham; †P<0.05 vs corresponding Control.

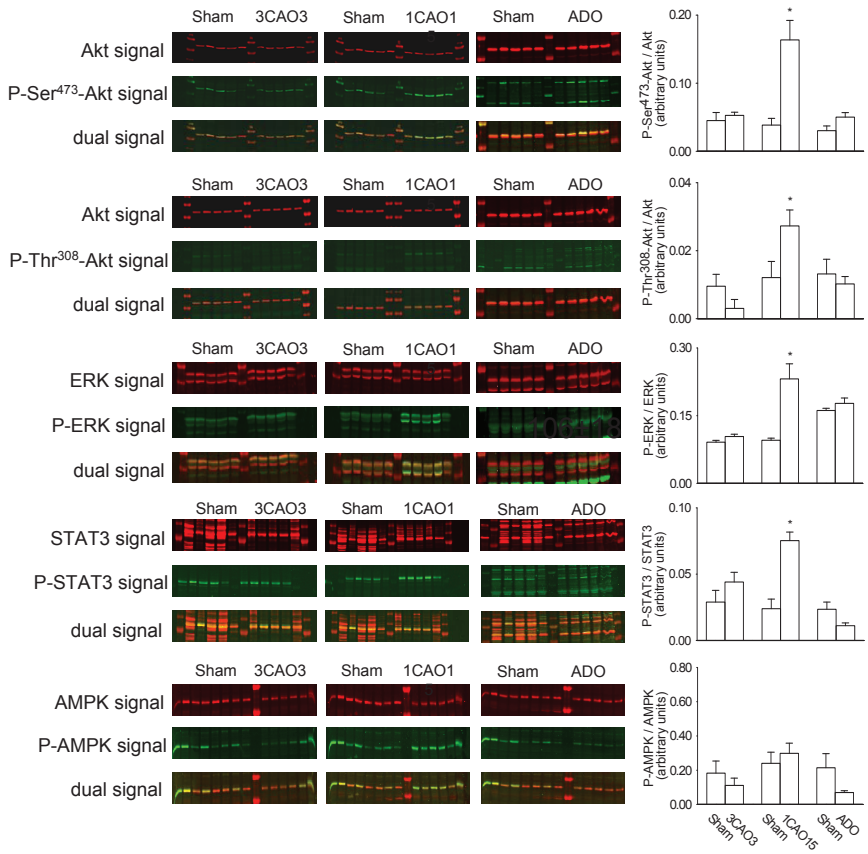

Fig. 3. The effects of preconditioning on protein phosphorylation. *A*: Western blots of Akt, ERK, STAT3 and AMPK for 3CAO3, 1CAO15 or ADO. *B*: effect of preconditioning on the normalized average data of these blots. Data are mean±SEM. A total of 5 animals per group is used. **P*<0.05 vs corresponding Sham.

produced by 3CAO3, 1CAO15 and ADO. IS was highly correlated with state-2 respiration (R^2 = 0.93; p<0.001) and with RCI (R^2 = 0.88; p<0.001).

Signal Transduction Pathways. Western blot data showed that 1CAO15, but not 3CAO3 or ADO, activated the RISK pathway reflected by the increased phosphorylation of both Ser^{473} and Thr^{308} sites of Akt and phosphorylation of ERK (Fig. 3). Wortmannin prevented the increases in phosphorylation of Akt (*P*<0.05) and ERK (*P*=0.06) produced by 1CAO15 (Fig. 4), corresponding with blockade of the effects of this stimulus on mitochondrial respiration and IS. Similarly, of the three stimuli only 1CAO15 activated the JAK/STAT pathway, with increased phosphorylation of STAT3 (Fig. 3), which was not affected by wortmannin (not shown). Finally, none of the stimuli had a significant effect on AMPK phosphorylation (Fig. 3).

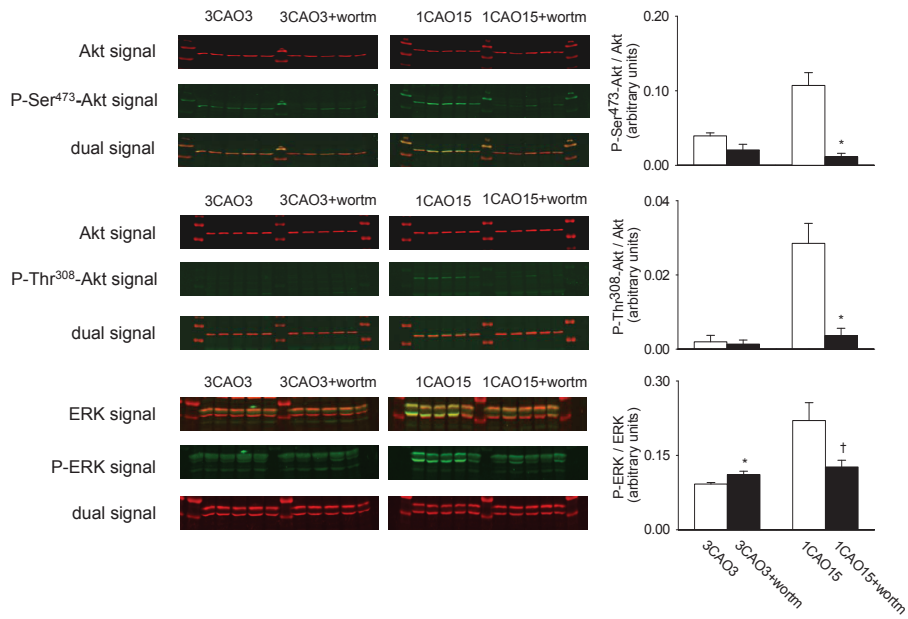

Fig. 4. The effects of IPC on protein phosphorylation. *A*: Western blots of Akt and ERK for 3CAO3 and 1CAO15 including treatment with wortmannin. *B*: effect of IPC on the normalized average data of these blots. Data are mean±SEM. A total of 5 animals per group is used. **P*<0.05 vs corresponding Sham; †*P*<0.05 vs corresponding Control.

Discussion

The major findings of the present study are: (*i*) preconditioning by the adenosine-independent stimulus 3CAO3, the endogenous adenosine-dependent stimulus 1CAO15 or intravenous infusion of exogenous adenosine all resulted in mild mitochondrial uncoupling and cardioprotection, which required intact NOS activity irrespective of the stimulus. (*ii*) In contrast, the cardioprotection by 1CAO15, but not by 3CAO3 or ADO, involved activation of the RISK pathway. *(iii)* Neither 3CAO3, nor ADO resulted in activation of the JAK/STAT pathway or AMPK. The implications of these findings will be discussed.

Signal Transduction Pathways

It is well recognized that IPC stimuli can employ highly diverse signaling pathways. For example, adenosine does not contribute to the IS limitation by 1CAO3 in swine or 3CAO3 in rats, whereas adenosine does contribute to the protection by 1CAO10 in swine[2] and 1CAO15 in rats.[4] Recently we further explored the signaling pathway of 1CAO15 and 3CAO3 and observed that the adenosine-dependent stimulus 1CAO15 resulted in opening of mitochondrial K^+_{ATP} channels and that the cardioprotection did not involve ROS.[3] In contrast, the adenosine-

independent stimulus 3CAO3 required ROS for its protection, but was not susceptible to pharmacological K^+_{ATP} channel blockade.[3] Similarly, pharmacological stimuli mimicking IPC can also recruit different signaling pathways. For example, the cardioprotection by infusion of adenosine, in contrast to bradykinin and acetylcholine, does not involve K^+_{ATP} channel opening or production of ROS in the *in vivo* rabbit heart.[1] Interestingly, endogenous adenosine (which is involved in the cardioprotection in the rabbit by 1CAO5) does involve ROS and K^+_{ATP} channels,[20] suggesting that endogenous and exogenous adenosine employ different pathways. Indeed, we found that exogenous adenosine, unlike 1CAO15, failed to increase myocardial interstitial adenosine levels,[5] and observed that exogenous ADO, unlike 1CAO15, mediated its effect in part by activation of a neurogenic pathway,[5] likely at a remote site.[6] These observations are consistent with the concept that various cardioprotective stimuli can employ highly diverse signaling pathways.

The RISK pathway. The RISK pathway has been implicated in the cardioprotection by preconditioning, both ischemic[7] as well as pharmacological.[8] However, not all stimuli result in activation of the RISK pathway.[9] In the present study the adenosine-dependent stimulus 1CAO15 increased phosphorylation of both Akt and ERK. In contrast, neither the adenosine-independent stimulus 3CAO3 nor exogenous ADO increased either Akt or ERK phosphorylation. Conversely, wortmannin blunted the phosphorylation of Akt and ERK and abolished the cardioprotection by 1CAO15, but had no effect on the cardioprotection of 3CAO3 or ADO. The results are consistent with reports that cardioprotection through endogenous adenosine is mediated via the RISK pathway.[10] In contrast, stimuli that fail to increase interstitial adenosine concentrations (3CAO3[3] and exogenous ADO[5]) are not dependent on activation of the RISK pathway.[14] Finally, the observation that the 1CAO15-induced increase in ERK phosphorylation was blunted by wortmannin suggests that ERK is located between Akt and NO synthase.[21]

The JAK/STAT pathway. The JAK/STAT pathway has been implicated in the cardioprotection by a variety of stimuli, including cytokines and growth factors, and has been shown to depend, in part, on ROS.[12, 22] IPC can activate different STATs,[11] of which STAT3 appears to be the most important isoform.[23, 24] Consequently, we investigated the effects of the ROS-dependent stimulus 3CAO3 on phosphorylation of STAT3. However, contrary to our hypothesis 1CAO15 but not 3CAO3 (or ADO) increased STAT3 phosphorylation. There is evidence that endogenous adenosine can activate the JAK/STAT pathway. Thus, IPC by 4 periods of 5 min of ischemia interspersed by 5 min of reperfusion (4CAO5), which results in a significant rise in interstitial adenosine,[25] resulted in STAT3 activation in the *in vivo* rat heart.[24] In addition, Lecour *et al*.[9] demonstrated in isolated mouse and rat hearts, that 2CAO5, which is similarly dependent on endogenous adenosine,[26, 27] also resulted in STAT3 activation. In contrast to these endogenous adenosine-dependent stimuli, 3CAO3 and ADO do not result in significant increases in interstitial adenosine levels and did not appear to activate STAT3. Interestingly, the 1CAO15-induced increase in STAT3 phosphorylation was not affected by pretreatment

with wortmannin. These findings could be interpreted to suggest that STAT3 activation alone is either not sufficient for cardioprotection by 1CAO15 or that STAT3 is located upstream of PI3K (see below).[28]

The AMPK pathway. Activated AMPK has a number of diverse actions during ischemia which act to maintain cellular energy stores.[29] The intracellular signaling pathways via which AMPK exerts its beneficial effects during ischemia-reperfusion remain incompletely understood, but may include NO[30] and sarcolemmal K^+_{ATP} channels.[31] Despite the involvement of NO in all three stimuli employed in the present study, levels of AMPK phosphorylation were not increased by any of the stimuli. At first glance our findings appear in stark contrast with observations in *in vivo*[32] and isolated rat hearts[13, 32] in which (simulated) ischemia resulted in a 30-500% increase in levels of AMPK phosphorylation. However, a recent study in the *in vivo* rat heart also failed to observe an increase in AMPK phosphorylation after 5 min of reperfusion following IPC by 2CAO5.[33] Interestingly, these authors reported a 3.5-fold increase in AMPK phosphorylation in tissue harvested at the end of the second CAO5 (i.e. no second period of reperfusion),[33] suggesting that AMPK phosphorylation was rapidly reversed upon reperfusion. Similarly, it is likely that in the present study AMPK phosphorylation also occurred during ischemia or infusion of ADO, but had normalized during reperfusion or washout of ADO at the time the tissue was harvested. Importantly, however, inhibition of AMPK by compound C, at a dose that inhibited AMPK-mediated changes in acetyl-coenzyme A carboxylase, did not mitigate the protective effects of 2CAO5 on lactate dehydrogenase release during index ischemia in the isolated rat heart.[13] These findings indicate that AMPK activation is not critical for the cardioprotection by IPC.[13]

Mitochondrial Respiration and Cardioprotection: critical role of NO

Mitochondrial proton (H^+) leak is characterized by a basal or induced permeability of the mitochondrial inner membrane, resulting in partial dissipation of the transmembrane electrochemical gradient ($\Delta\Psi_m$) and uncoupling of substrate oxidation from ATP synthesis (i.e. mild mitochondrial uncoupling).[34] A mild degree of uncoupling may represent a common characteristic of stress-resistant mitochondria within preconditioned myocardium.[3, 35] In support of this concept, cytoprotection of human Girardi cells and murine skeletal myotubes can be induced by both simulated ischemia and administration of adenosine or the K^+_{ATP}-channel opener diazoxide and in all cases the protective state is characterized by a mild degree of mitochondrial uncoupling.[36] In addition, mitochondrial uncouplers dinitrophenol or FCCP are cardioprotective in the isolated rat heart.[37, 38] The result of the present study, obtained *in vivo*, show that all three cardioprotective stimuli resulted in a reduction in RCI due to an increased state-2 respiration, indicating mild mitochondrial uncoupling. These findings are in agreement with previous observations in isolated,[39] and *in vivo* rat hearts[3, 40] and strongly suggest that mitochondria play a pivotal role in mediating cardioprotection by different stimuli.

The exact mechanism by which mitochondrial uncoupling can protect cardiomyocytes against ischemia-reperfusion damage is incompletely understood, but may include reduced mitochondrial matrix calcium overload and mitochondrial swelling leading to preserved energy production during prolonged ischemia and reperfusion.[41] Another mechanism could involve a reduction in pathological oxidative stress (as a results of mild inner mitochondrial membrane depolarization), thereby preventing sustained opening of the mitochondrial permeability transition pore (MPTP) and the consequent massive release of cytochrome c into the cytosol during sustained ischemia-reperfusion.[35] We observed in the present study that the cardioprotective and mitochondrial effects of all three stimuli depended critically on the bioavailability of NO. NO is known to activate the soluable guanylate cyclase (sGC) pathway, but can also act directly act on mitochondria, affording cardioprotection against ischemia reperfusion injury.[42] Thus, NO can reversibly inhibit electron entry into the electron transport chain, generate low levels of ROS to initiate cardioprotective cascades and inhibit cytochrome c peroxidase activity.[42] Importantly, NO has been shown to induce activation, translocation, and nitration of protein kinase C epsilon (PKCε),[43] which is important in preconditioning.[44] NO may also affect MPTP indirectly through activation of sGC leading to activation of protein kinase G, which may phosphorylate an unidentified component of the MPTP resulting in inhibition of MPTP opening.[45] Finally, NO can react with superoxide, forming peroxynitrite, inducing lipid peroxidation of the mitochondrial membrane to stimulate state-2 respiration.[46] Future studies are required to determine the molecular mechanism(s) via which NO increases state-2 respiration and decreases RCI and leads to cardioprotection in our *in vivo* rat model of myocardial infarction.

Integration and conclusions

Irrespective of the signaling pathway all three stimuli exerted their protective effects via NO and by influencing mitochondrial respiration. In Figure 5, we have schematically depicted how the different signal transduction pathways that are employed by 3CAO3, 1CAO15, and ADO may converge at the mitochondrion. ADO and 1CAO15 activate PKCε either directly via NO from eNOS in the endothelium, or indirectly via phospholipase C (PLC), Src-Tyrosine kinase (TyK) or PI3K and NOS of the cardiomyocyte, and subsequently allow K^+ influx into the mitochondrial matrix. This effect may act to reduce Ca^{2+} influx and mitochondrial swelling.[35, 41, 42] but can also result in an increased state-2 respiration and hence results in a small decrease in RCI.[46] The signal transduction route that is activated by the ROS generated by 3CAO3 involves TyK and PKC activation,[3] but does not involve the RISK-, JAK/STAT- or AMPK pathway and does not exert its mitochondrial effects through activation of mitoK^+_{ATP} channels.[3] In this respect it might be relevant that rat heart mitochondria contain a constitutive, mitochondrial NOS (mtNOS) that has a phosphorylation site linked to enzymatic regulation.[47] The role of mtNOS in the cardioprotection by the three cardioprotective stimuli awaits further investigation.

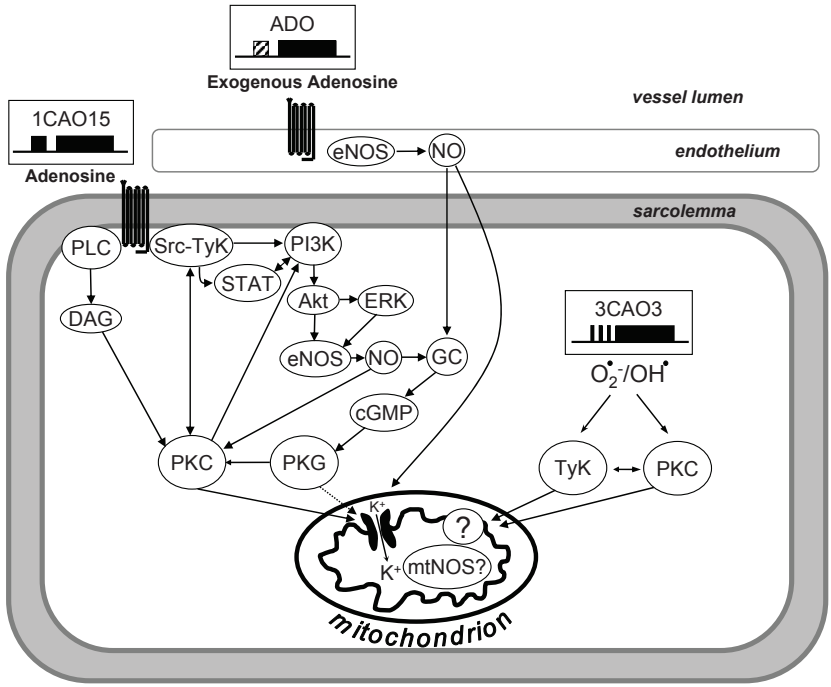

Fig. 5. Proposed scheme outlining the different signal transduction pathways from 3CAO3, 1CAO15 and ADO. All pathways converge at the mitochondrion to exhibit a protective effect on the myocardium.

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Acknowledgements

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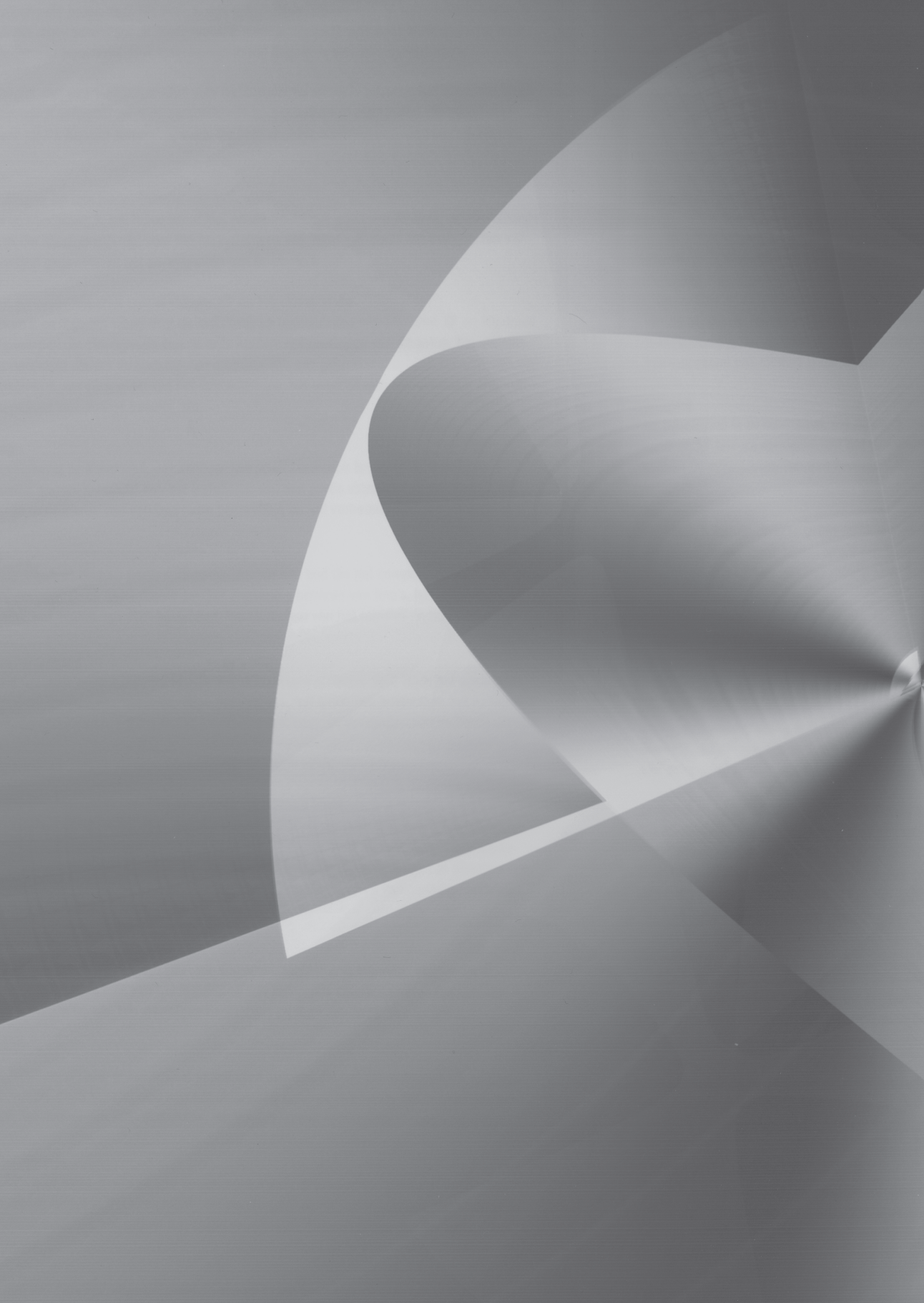

Chapter 5

Myocardium tolerant to an adenosine-dependent ischemic preconditioning stimulus can still be protected by stimuli that employ alternative signaling pathways

David A Liem, Maaike te Lintel Hekkert, Olivier C Manintveld, Frans Boomsma, Pieter D Verdouw and Dirk J Duncker.

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Abstract

Clinical studies on cardioprotection by pre-infarct angina are ambiguous, which may involve development of tolerance to repeated episodes of ischemia. However, not all preconditioning stimuli use identical signaling pathways, and since patients likely experience varying numbers of episodes of different degree and duration of pre-infarct angina, it is important to know whether myocardium tolerant to a particular preconditioning stimulus can still be protected by stimuli employing alternative signaling pathways. Consequently, we tested the hypothesis that development of tolerance to a particular stimulus does not affect cardioprotection by stimuli that employ different signaling pathways. Anesthetized rats underwent classical, remote or pharmacological preconditioning. Infarct size (IS), produced by a 60-min coronary artery occlusion (CAO), was determined after 120 min of reperfusion. Preconditioning by two 15-min periods of CAO, (2CAO15, an adenosine-dependent stimulus) limited IS from 69±2% to 37±6%, but when 2CAO15 was preceded by 4CAO15, protection by 2CAO15 was absent (IS=68±1%). This development of tolerance coincided with a loss of cardiac interstitial adenosine release, whereas two 15-min episodes of 200 µg/min intravenous adenosine infusion still elicited cardioprotection (IS=40±4%). Furthermore, cardioprotection was still produced when 4CAO15 was followed by either the adenosine-independent stimulus 3CAO3 (IS=50±8%), or the remote preconditioning stimulus of two 15-min periods of mesenteric artery occlusion (IS=49±6%). In conclusion, the development of tolerance to cardioprotection by an adenosine-dependent preconditioning stimulus still allows protection by ischemic stimuli or pharmacological intervention employing different signaling pathways.

Introduction

Ischemic preconditioning is the most powerful means of endogenous cardioprotection against irreversible cell injury in the experimental animal.[1, 2] However, clinical studies on IS limitation by brief anginal episodes preceding acute myocardial infarction are ambiguous,[3-9] which has been attributed to a loss of cardioprotection by ischemic preconditioning in the ageing[10-12] or pathological[13-15] heart. Another confounding factor could be the development of tolerance to ischemic preconditioning, i.e. the loss of cardioprotection when the same preconditioning stimulus is repetitively applied.[16-18] For example, Cohen *et al.*[16] demonstrated that in rabbits the cardioprotection produced by a single 5-min coronary artery occlusion (1CAO5) followed by 10 min of reperfusion was lost, when the CAO5 stimulus was applied at 30 min intervals for 8 hours during 3 days.

In recent years, it has become apparent that not all preconditioning stimuli employ the same signaling pathway to exert their cardioprotective action.[19-23] For instance, in the rat, cardioprotection by a single 15-min CAO followed by 10 min of reperfusion (1CAO15) is adenosine-dependent but does not involve reactive oxygen species (ROS), whereas the cardioprotection by 3 cycles of 3-min CAO interspersed by 5 min of reperfusion (3CAO3) depends on ROS,[23] but does not involve adenosine.[21, 24] The major aim of the present study was therefore to investigate whether tolerance that develops when the same ischemic preconditioning stimulus is applied repetitively also implies tolerance to a stimulus that employs a different signal transduction pathway. Hence, in the first part of the study we investigated whether tolerance to a particular (adenosine-dependent) preconditioning stimulus also affects the cardioprotection by a stimulus that employs an alternative (adenosine-independent) pathway. Myocardium cannot only be preconditioned by local myocardial ischemia, but also by brief ischemia in non-cardiac tissue such as the small intestine, kidneys and skeletal muscle[25-28] which, at least for the small intestine, involves a neurogenic pathway.[25, 26] Hence, in the second part we investigated whether the cardioprotection by remote preconditioning via a 15-min mesenteric artery occlusion (MAO15) is affected by the development of tolerance to a classical ischemic preconditioning stimulus.

Because tolerance to IPC has not yet been investigated in the rat, we first established a model for the development of tolerance based on our experience with the adenosine-dependent stimulus 1CAO15 in this species. Capitalizing on the observations by Vogt *et al.,*[29] who showed in pigs that progressive loss of adenosine production rendered myocardium tolerant to protection by 10-min coronary artery occlusions, but still responsive to exogenous adenosine, we also investigated whether loss of adenosine release also contributes to the development of tolerance in the rat heart and whether exogenous adenosine still induces protection once tolerance has developed.

Methods

Animals

Experiments were performed in ad libitum fed male Wistar rats (300-380 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 Erasmus University Rotterdam Animal Care Committee.

Surgical and Experimental Procedures

Pentobarbital-anesthetized (60 mg/kg intraperitoneally) 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.[26, 30] In the inferior caval vein a PE-50 catheter was placed for infusion of Haemaccel (Hoechst) to compensate for blood loss during surgery and to maintain central venous pressure during the experimental protocol, 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 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.[26, 30] After completion of surgery, a 30-min stabilization 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 via gently thumping on the thorax was successful within 2 min after onset of fibrillation. Occlusion and reperfusion were visually verified. In 13 additional rats, a CMA/20 microdialysis probe (Carnegie Medicine AB, Stockholm, Sweden; membrane 4 mm x 0,5 mm, cut-off: 20 kD) was implanted into the myocardial area at risk to determine myocardial interstitial adenosine levels [31]. Samples were collected during each 15-min CAO at a rate of 2 µl/min. At the conclusion of each experiment, probe recovery was determined *ex vivo* using a stock solution containing 100 µM adenosine, and found to be 14±1% (percentage of adenosine concentration in the stock solution recovered in the probe samples). All samples were stored at -50°C for later analysis. The adenosine concentrations in dialysate samples were determined by reversed phase high-performance liquid chromatography.[32]

Infarct size analysis

Infarct size was determined as previously described.[26, 30] Briefly, after 120 min of reperfusion the left coronary artery was re-occluded, immediately followed by intravenous infusion of 10 ml trypan blue (0.4%, Sigma Chemical Co) into the femoral vein to stain the normally perfused myocardium dark blue and delineate the non-stained area at risk. Subsequently, hearts were excised, rinsed in cold saline, and cut into slices of 2 mm thickness from apex

to base. From each slice the right ventricle was removed and the left ventricular area at risk (AR, non-stained) was dissected from the remaining left ventricular tissue. The AR was then incubated for 10 min in 37°C nitro-blue tetrazolium (Sigma Chemical Co; 1 mg/ml Sorensen buffer, pH 7.4), which stains viable tissue purple but leaves infarcted tissue unstained. After the infarcted area was isolated from the non-infarcted area, the different areas of the left ventricle were dried and weighed separately. Myocardial infarct size (IS) was computed as IA expressed as a percentage of AR.[26, 30]

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 ischemic preconditioning 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 multiple 15-min intravenous infusions of 200 µg/min adenosine separated by 15 min of wash-out (nADO15). Myocardial infarcts were produced by a 60-min CAO (index ischemia) and IS was determined after 120-min of reperfusion.[33]

Pilot experiments to develop a model for tolerance to classical ischemic preconditioning by an adenosine-dependent ischemic preconditioning stimulus.

Since there were no previous studies on tolerance to IPC in the rat heart, we first established (*i*) the number and timing of CAO15 required to elicit tolerance to IPC (Fig. 1). Based on these experiments (see Results section) 4CAO15 interspersed by 15 min of reperfusion and applied between 175 and 70 min prior to the 60-min index ischemia was selected to induce tolerance, while 2CAO15 separated by 15 min of reperfusion was used as the preconditioning stimulus.

Adenosine and the development of tolerance to preconditioning.

We first established whether the cardioprotection by 2CAO15, similar to 1CAO15,[21, 23] depends on adenosine receptor activation but not on ROS generation (Fig. 2A). For this purpose, we used the adenosine receptor antagonist 8-sulfo-phenyltheophylline (8-SPT, 50 mg/kg iv[21]) and the ROS scavenger mercapto-propionyl-glycine (MPG, 1 mg/kg/min iv[23]). Subsequently, we investigated whether loss of adenosine signaling could have contributed to the development of tolerance to 2CAO15 (Fig. 2B). For this purpose we measured interstitial adenosine levels during 4CAO15, and determined whether an exogenous adenosine infusion indeed reinstates protection in myocardium that has become tolerant to 4CAO15, by replacing the 2CAO15 by two episodes of ADO15 (4CAO15+2ADO15). Finally, we subjected rats to either one (1ADO15) or six (6ADO15) episodes of 15-min intravenous infusion of 200 µg/min ad-

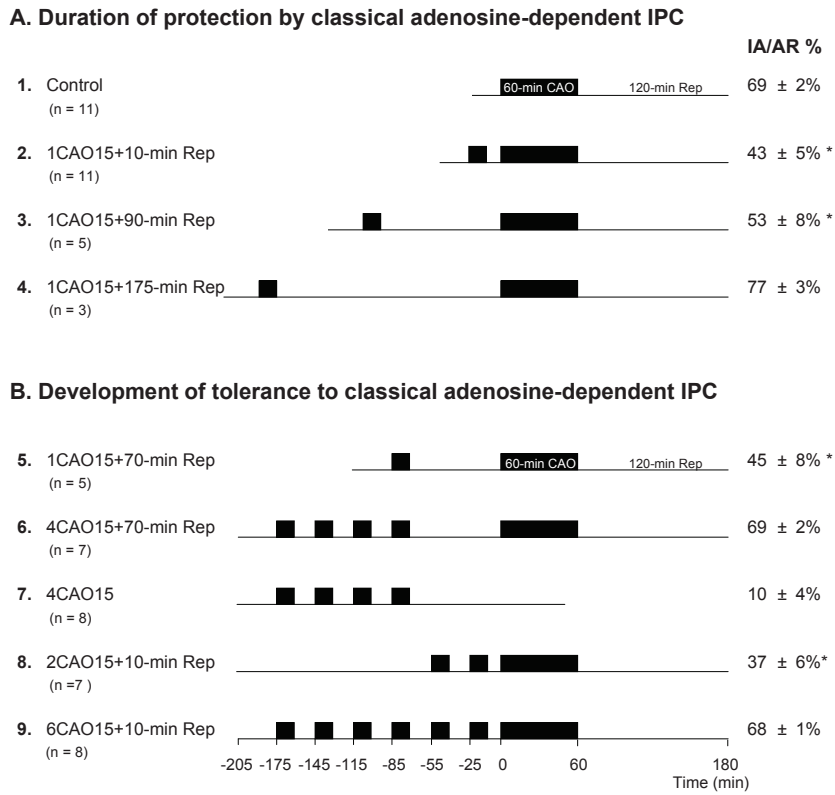

Figure 1. *Fig. 1A*: Time window of protection by CAO15. *Fig. 1B*: Model for the development of tolerance. IPC= ischemic preconditioning stimulus. Rep = reperfusion.
**P*<0.05 versus corresponding Control.

enosine (Fig. 2C), to determine whether repeated administration of exogenous adenosine leads to tolerance to its cardioprotection.[34-36]

Cross-tolerance between adenosine-dependent and other ischemic preconditioning stimuli.

To investigate whether the cardioprotection by the adenosine-independent preconditioning stimulus 3CAO3 or remote preconditioning are also lost after myocardium has become tolerant to the adenosine-dependent stimulus (2CAO15), we replaced the 2CAO15 by the adenosine-independent classical stimulus 3CAO3 (4CAO15+3CAO3; Fig. 3A), or by remote preconditioning with two episodes of MAO15 (4CAO15+2MAO15; Fig. 3B).

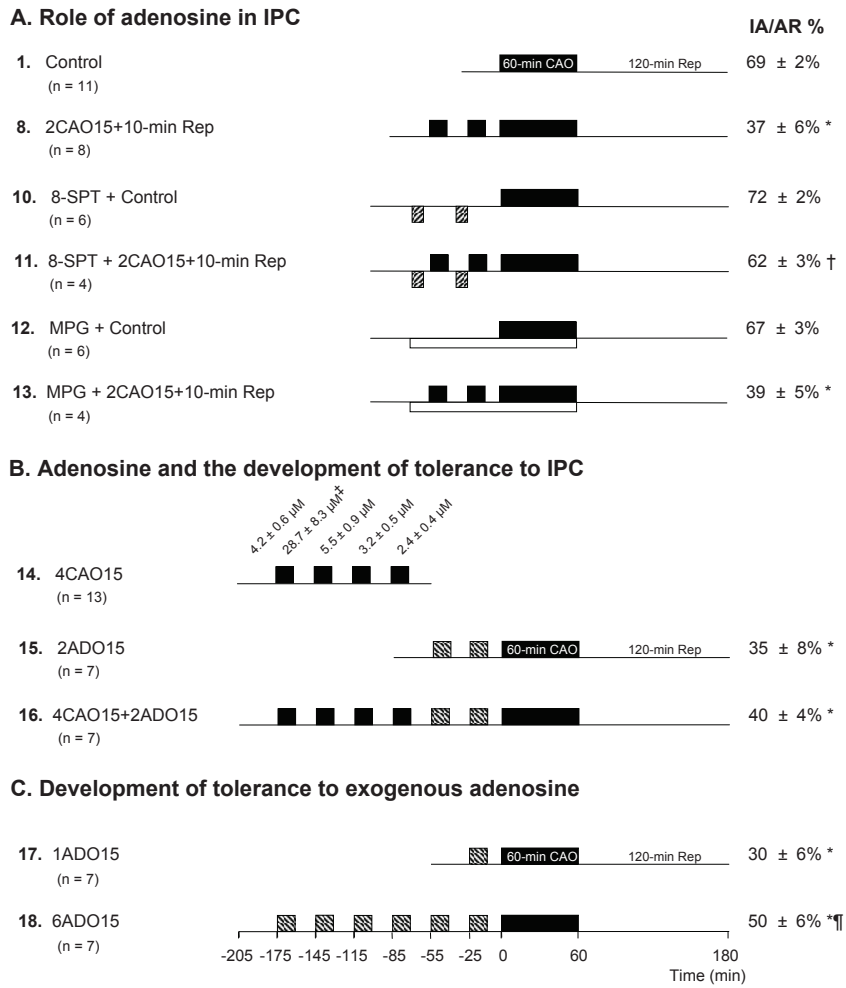

Figure 2. *Fig. 2A*: Role of adenosine in the cardioprotection by the classical IPC stimulus 2CAO15. 8-SPT = 8-S-sulfo-phenyltheophylline (▨), MPG = mercapto-propionyl-glycine (□). *Fig. 2B*: Role of adenosine in the development of tolerance to the classical IPC stimulus 2CAO15. The numbers shown in the protocol of group 14 represent the interstitial adenosine concentrations at baseline and during each of the 4 periods of 15-min coronary artery occlusions interspersed and followed by 15 min of reperfusion ($^{‡}P<0.05$ vs baseline concentrations). *Fig. 2C*: Development of tolerance to repeated infusions of exogenous adenosine (▧). IPC= ischemic preconditioning stimulus.
$^{*}P<0.05$ versus Control (group 1) or 4CAO15 + 70-min Rep (group 6); $^{†}P<0.05$ versus 2CAO15 + 10-min Rep. $^{‡}P<0.05$ versus baseline concentration. ¶P<0.05 6ADO15

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.

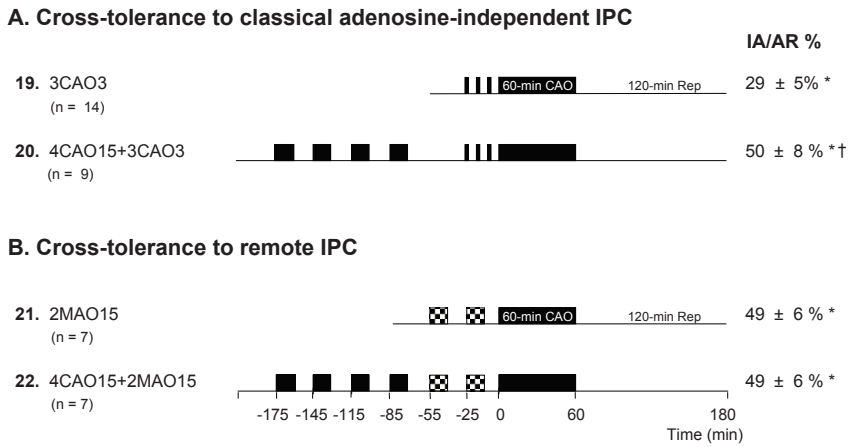

Figure 3. The classical adenosine-independent IPC stimulus 3CAO3 (Fig. 3A) and remote preconditioning stimulus (2MAO15 (⚄), Fig. 3B) can still afford protection when myocardium has become tolerant to 2CAO15. IPC= ischemic preconditioning stimulus.
*P<0.05 versus Control (group 1) or 4CAO15 + 70-min Rep (group 6); †P<0.05 versus corresponding stimulus without preceding 4CAO15.

Results

Mortality and exclusions

Of the 190 rats that entered the study, 21 rats were excluded because of sustained ventricular fibrillation during CAO or pump failure (no more than 3 rats per group), while 6 rats were excluded due to an area at risk <10% of the left ventricle.

Heart rate and arterial blood pressure

Table 1 shows the hemodynamic data for the various experimental groups. Importantly, there was no correlation between the rate-pressure product at the onset of the 60-min CAO and IS (r^2 = 0.003; P=0.55).

Area at risk

There were no intergroup differences in the area at risk (34±1% of the left ventricle; P=0.09) between the experimental groups.

Development of model for tolerance to classical ischemic preconditioning

Fig. 1A depicts that the protection by 1CAO15 was not affected when the reperfusion period between 1CAO15 and the 60-min index ischemia period was extended from 10 to 90 min, but was lost when further extended to 175 min. The protection by 1CAO15, when applied 70 min before the 60-min CAO, was abolished, however, when this stimulus was preceded by three

Table 1. Heart rate and arterial blood pressure

Experimental groups		60-min CAO		Reperfusion	
	Baseline	Before	End	15-min	120-min
1. *Control (n=11)*					
HR	367 ± 9	376 ± 15	392 ± 16	398 ± 17	425 ± 12*
MAP	92 ± 4	94 ± 5	91 ± 5	84 ± 5	75 ± 5*
2. *1CAO15 (n=11)*					
HR	357 ± 10	366 ± 9	377 ± 11	365 ± 10	408 ± 14*†
MAP	98 ± 3	95 ± 3	91 ± 4	79 ± 6*	70 ± 5*†
3. *1CAO15+90min Rep (n=5)*					
HR	382 ± 15	416 ± 14	400 ± 22	395 ± 63	365 ± 29
MAP	92 ± 1	91 ± 6	89 ± 7	74 ± 14	65 ± 17
4. *1CAO15+175min Rep (n=3)*					
HR	385 ± 11	453 ± 14	463 ± 10	460 ± 4	397 ± 38
MAP	95 ± 1	91 ± 6	85 ± 4	79 ± 6	62 ± 15
5. *1CAO15+70min Rep (n=5)*					
HR	357 ± 9	377 ± 14	386 ± 22	382 ± 26	401 ± 13
MAP	91 ± 5	96 ± 5	87 ± 5	82 ± 6	75 ± 8
6. *4CAO15+70min Rep (n=7)*					
HR	363 ± 11	408 ± 7*	402 ± 10*	406 ± 10*	397 ± 9
MAP	96 ± 4	82 ± 6	79 ± 6	78 ± 5	68 ± 5*
7. *2CAO15+10min Rep (n=8)*					
HR	352 ± 8	369 ± 6	368 ± 7	374 ± 8	409 ± 7*†
MAP	95 ± 4	95 ± 4	96 ± 5	93 ± 4	89 ± 5
8. *6CAO15+10min Rep (n=7)*					
HR	384 ± 21	415 ± 18	409 ± 21	408 ± 22	393 ± 26
MAP	103 ± 3	84 ± 3	74 ± 8*	72 ± 6*	64 ± 9*
9. 4CAO15 *(n=8)*					
HR	329 ± 9			383 ± 11*	411 ± 9*
MAP	95 ± 5			105 ± 4	100 ± 3
10. *8-SPT+Control (n=6)*					
HR	349 ± 5	340 ± 6	361 ± 9	367 ± 17	397 ± 24
MAP	104 ± 8	103 ± 11	89 ± 10	90 ± 10	83 ± 9
11. *8-SPT+2CAO15+10min Rep (n=4)*					
HR	345 ± 18	355 ± 17	359 ± 14	347 ± 11	364 ± 21
MAP	98 ± 4	96 ± 3	80 ± 3*†	79 ± 3*†	82 ± 5*†
12. *MPG+Control (n=6)*					
HR	360 ± 17	337 ± 13	351 ± 13	365 ± 9	375 ± 9
MAP	105 ± 13	73 ± 2	79 ± 10	76 ± 9	65 ± 9*
13. *MPG+2CAO15+10min Rep (n=4)*					
HR	334 ± 12	332 ± 16	340 ± 4	350 ± 5	382 ± 9*†
MAP	88 ± 11	53 ± 4*	62 ± 3*	66 ± 6	72 ± 4
15. 2ADO15 (n=7)					
HR	360 ± 7	376 ± 8	389 ± 7	391 ± 7	401 ± 11*
MAP	100 ± 4	109 ± 6	95 ± 8	95 ± 8	88 ± 8
16. 4CAO15+2ADO15 (n=7)					
HR	360 ± 6	412 ± 15*	422 ± 10*	413 ± 5	361 ± 35

Experimental groups		60-min CAO		Reperfusion	
	Baseline	Before	End	15-min	120-min
MAP	98 ± 5	86 ± 8	83 ± 6	78 ± 6	72 ± 8
17. *1ADO15 (n=7)*					
HR	357 ± 5	378 ± 16	384 ± 20	384 ± 22	401 ± 23
MAP	112 ± 5	128 ± 3*	111 ± 4†	108 ± 4†	107 ± 3†
18. *6ADO15 (n=7)*					
HR	344 ± 14	385 ± 10	393 ± 10*	389 ± 5	369 ± 6
MAP	100 ± 7	103 ± 4	76 ± 6*†	81 ± 16	70 ± 7*
19. 3CAO3 (n=14)					
HR	355 ± 6	368 ± 8	363 ± 17	370 ± 17	380 ± 18
MAP	93 ± 4	89 ± 5	78 ± 7	94 ± 7	77 ± 5
20. 4CAO15+3CAO3 (n=9)					
HR	341 ± 12	379 ± 10*	382 ± 9*	380 ± 10*	373 ± 11
MAP	94 ± 6	99 ± 6	89 ± 6	90 ± 6	80 ± 8
21. 2MAO15 (n=7)					
HR	364 ± 15	383 ± 15	365 ± 17	385 ± 18	355 ± 33
MAP	87 ± 5	97 ± 8	91 ± 5	86 ± 5	86 ± 4
22. 4CAO15+2MAO15 (n=7)					
HR	350 ± 13	355 ± 13	363 ± 18	350 ± 9	339 ± 35
MAP	96 ± 6	85 ± 7	79 ± 8	78 ± 6	76 ± 7

Values are means ± SEM. n CAO15, n episodes of 15 min of coronary artery occlusion (CAO); n ADO15, n episodes of 15 min of adenosine infusion; n MAO15, n episodes of mesenteric artery occlusion; Rep, reperfusion; HR, heart rate; MAP, mean aortic pressure. *P < 0.05 vs baseline. †P < 0.05 vs. before CAO.

additional CAO15's (4CAO15 + 70-min Rep, Fig. 1B). This figure also illustrates that 2CAO15 tended to be slightly more protective (IS = 37±6%) than 1CAO15 (IS = 43±5%, *P*=NS) and that the protection was abolished when preceded by the tolerance-inducing 4CAO15. This loss of protection by 2CAO15 was not due to cumulative necrosis induced by the preceding 4CAO15 (10±4%), because the combined IS of the 4CAO15 (10±4%) and the 2CAO15 + 60-min CAO (37±6%) amounted 47±7%, which was still significantly less than the IS produced by the 60-min CAO alone (69±2%; Fig. 1A) and the IS produced by 6CAO15 followed by 60-min CAO (68±1%; Fig. 1B).

Adenosine and the development of tolerance

The protection by 2CAO15 was virtually abolished by 8-SPT but not by MPG (Fig. 2A), demonstrating the critical role of adenosine in mediating the cardioprotection by 2CAO15 and its independence of ROS generation.

During the first CAO15 the average interstitial adenosine concentrations increased sevenfold, but during the second, third and fourth 2CAO15 the adenosine concentrations were no longer different from baseline (Fig. 2B). When 4CAO15 was followed by 2ADO15, IS produced by the 60-min CAO was limited to 40±4%. Since 10±4% of the area at risk had already become necrotic after the 4CAO15, the additional irreversible damage produced by the 60-min CAO

was [40±4] - [10±4%] which equals 30±5% of the area at risk. Taking into account that only 90±4% (100% - [10±4%]) of the area at risk was viable at the onset of the 60-min CAO, the percent of area at risk that became infarcted during the 60-min CAO amounted 33±6% of the viable area at risk ([30±5%] / [90±4%]). This degree of protection was not different from the infarct-size limitation by 2ADO15 alone, (IS= 35±8%; Fig. 2B). These findings indicate that exogenous adenosine still produces cardioprotection at a time that the myocardium has become tolerant to the protection by the adenosine-dependent stimulus 2CAO15.

Although the cardioprotection by exogenous adenosine was unperturbed in hearts tolerant to cardioprotection by 2CAO15, IS limitation by 6ADO15 was less than that by 1ADO15 (Fig. 2C), indicating that repeated adenosine infusions caused a blunting of its cardioprotective actions.

Cross-tolerance between adenosine-dependent and adenosine-independent classical IPC stimuli

When 4CAO15 preceded the adenosine-independent[21, 24] but ROS-dependent[23] 3CAO3 stimulus, IS limitation was still present, albeit less (IS=50±8%; $P<0.05$ vs Control), than the protection by 3CAO3 alone (IS=29±5%; Fig. 3A). Taking into account that IS was 10±4% after 4CAO15 alone, it was calculated (see above) that 44±9% of the area at risk that was viable at the onset of 3CAO3 became infarcted (*P*=NS vs. 3CAO3 alone). These findings indicate that myocardium that has become tolerant to the protection by an adenosine-dependent IPC stimulus, can still be protected by a classical adenosine-independent IPC stimulus.

Cross-tolerance between classical IPC and remote IPC stimuli

Remote myocardial preconditioning by 2MAO15 limited IS to 49±6% versus 69±2% in Control rats ($P<0.05$; Fig. 3B). When 4CAO15 preceded the 2MAO15 (4CAO15+2MAO15), IS was limited to 49±6% (43±7% of the area at risk that was viable at the onset of 2MAO15), which was not different from the cardioprotection by 2MAO15 alone (IS = 49±6%).

Discussion

The present study was undertaken to assess whether the development of tolerance to a particular ischemic preconditioning stimulus also affects the cardioprotection by stimuli that employ different mechanisms. The major findings can be summarized as follows: (*i*) Ischemic preconditioning by 2CAO15 resulted in potent cardioprotection against subsequent 60-min index ischemia in an adenosine-dependent manner. However, when 4CAO15 preceded the 2CAO15, the myocardium had become tolerant to the protection by 2CAO15. (*ii*) Development of tolerance coincided with loss of myocardial interstitial adenosine release. Although repeated infusion of adenosine was capable of producing tolerance as well, the loss of

adenosine release, in conjunction with the finding that exogenous adenosine still afforded protection following 4CAO15, is consistent with previous observations in pigs[29] that loss of adenosine release contributes to the development of tolerance. (*iii*) Myocardium that had become tolerant to 2CAO15, could still be protected by the adenosine-independent 3CAO3 stimulus, and by the remote preconditioning stimulus 2MAO15.

Development of tolerance to preconditioning in the rat heart

Cohen *et al*.[16] demonstrated in conscious rabbits that 5-min CAOs at 30-min intervals for 8 hours during 3 days resulted in tolerance. Iliodromitis *et al*.[17] showed in anesthetized rabbits that myocardial tolerance already started to develop after 4 cycles of 5 min CAO and 10 min of reperfusion. The present study shows that also in the rat heart tolerance develops after a limited number of brief CAOs. Because in our study we used coronary artery occlusions of 15 rather than 5 min, it could be argued that the loss of protection by 2CAO15 after 4CAO15 was caused by cumulative necrosis. This is, however, highly unlikely as the combined IS of 4CAO15 (10±4%) and 2CAO15 followed by 60-min CAO (37±6%) was significantly less (47±7%) than IS after 6CAO15 followed by 60-min CAO (68±1%). Moreover, the cardioprotection by 1CAO15 + 70-min Rep (IS = 45±8%) was prevented when 1CAO15 was preceded by three additional episodes of CAO15 (4CAO15+70-min Rep, IS=69±2%). This loss of protection can also not be explained by cumulative necrosis of the 3CAO15 (which in view of the infarct size by the 4CAO15 must have been smaller than 10±4%), and the 1CAO15 + 70-min Rep + 60-min CAO (IS=45±8%), which was still significantly less than 69±2% in the group subjected to 4CAO15 + 70-min Rep + 60-min CAO.

We established that, similar to 1CAO15[21], cardioprotection by 2CAO15 involves activation of adenosine receptors, whereas ROS do not play a role in either 1CAO15[23] or 2CAO15 (present study). In view of the similarly prominent role of endogenous adenosine in cardioprotection by 1CAO5 in rabbits[2] and 1CAO10 in swine[19], both a reduced adenosine receptor responsiveness[34, 35] and a progressive loss of adenosine production during repeated occlusions[17, 29] have been proposed as a mechanism underlying the development of tolerance. Although we found that repeated adenosine infusions are capable of blunting adenosine's cardioprotection, the observation that during the second, third and fourth CAO15, the myocardial interstitial levels of adenosine were no longer different from baseline is consistent with the hypothesis that a progressive loss of myocardial adenosine release contributes to the development of tolerance.[29] Moreover, similar to Vogt *et al*.,[29] we observed that intravenous infusion of adenosine could reinstate cardioprotection, suggesting that cardiac responsiveness to adenosine was maintained following 4CAO15.

Cross-tolerance to other IPC stimuli

The primary aim of the present study was to investigate whether jeopardized myocardium that has become tolerant to a particular preconditioning stimulus can still be rescued by

an ischemic stimulus that operates via a different mechanism. Cross-tolerance did not occur to remote preconditioning of the heart as the cardioprotection by brief intestinal ischemia, as 2MAO15 was not affected when this stimulus was preceded by 4CAO15. We have previously shown that MAO15 elicits cardioprotection via activation of a neurogenic pathway during early reperfusion of the mesenteric bed.[26] We have obtained evidence that adenosine receptor activation downstream of the neurogenic pathway, possibly in the myocardium,[25] contributes to remote IPC by 1MAO15. However, in two additional rats we did not observe an increase in myocardial interstitial adenosine levels either during (1.6±0.2 μM) or following (1.5±0.1 μM) MAO15, compared to "baseline" adenosine levels measured after the preceding 4CAO15 (2.2±0.9 μM). It must be emphasized that the myocardial adenosine concentrations represent the average concentrations of the 15-min microdialysis sampling period. We can therefore not exclude that a brief transient increase in myocardial adenosine concentration during early mesenteric artery reperfusion was masked. Alternatively, other mediators of remote preconditioning, including bradykinin,[37] calcitonin-gene related peptide,[38] and opioids,[39, 40] may also have contributed to the cardioprotection by remote IPC of hearts that have become tolerant. Future studies are needed to further investigate the role of these other mediators in the cardioprotection by remote IPC of myocardium made tolerant by 4CAO15 to the cardioprotection by 2CAO15.

Tolerance by 4CAO15 did also not abolish the cardioprotection by 3CAO3. If one assumes that the 15-min ischemia episode encompasses the signaling cascade triggered by the 3-min episode, one would expect a complete loss of cardioprotection by 3CAO3 in myocardium that had become tolerant to CAO15. However, we and others have shown that, unlike its involvement in CAO15,[21] adenosine is not involved in the cardioprotection by 3CAO3.[21, 24] Conversely, we have shown that the reactive oxygen species scavenger MPG attenuated the protection by 3COA3,[23] but left the protection by CAO15 unaffected. Hence, the partial loss of protection by 3CAO3 in myocardium tolerant to CAO15 is difficult to explain. Future studies, involving other triggers and mediators, are required to determine the molecular basis for this partial cross-tolerance to other classical preconditioning stimuli. Nonetheless, our data suggest that myocardium that has become tolerant to the protection by a stimulus employing a particular signal transduction pathway might still benefit from an IPC stimulus employing a different signal transduction pathway.

Clinical Relevance

Abundant evidence has been presented that ischemic preconditioning also occurs in man, using endpoints other than IS.[41-44] However, clinical studies on IS limitation by pre-infarct angina are discordant.[3-5, 7, 8] This has, at least in part, been ascribed to loss of preconditioning in the ageing [10-12] and pathological[3-9, 13-15] hearts. We hypothesized that development of tolerance might also contribute to the equivocal clinical findings, as multiple brief episodes of abrupt ischemia in the hours to days preceding a myocardial infarction render animal

hearts tolerant to the cardioprotective effects of preconditioning. However, rather than the repetitive bouts of brief ischemia of identical duration and severity that occur in the laboratory setting, patients are more likely to experience episodes of varying severity and duration of ischemia. The present study suggests that these patients could be less susceptible to the development of tolerance, due to recruitment of different signal transduction pathways by distinct stimuli. Our study also indicates that without a detailed knowledge of the number, severity and duration of the pre-infarct episodes of myocardial and/or remote organ ischemia it is impossible to classify patients as preconditioned or tolerant. Finally, the observation that administration of exogenous adenosine is still protective in hearts that have become tolerant to ischemic preconditioning, suggests that in patients with unstable angina administration of pharmacological agents that mimic preconditioning can still afford cardioprotection, at least in the (sub)acute setting.[34-36]

Acknowledgements

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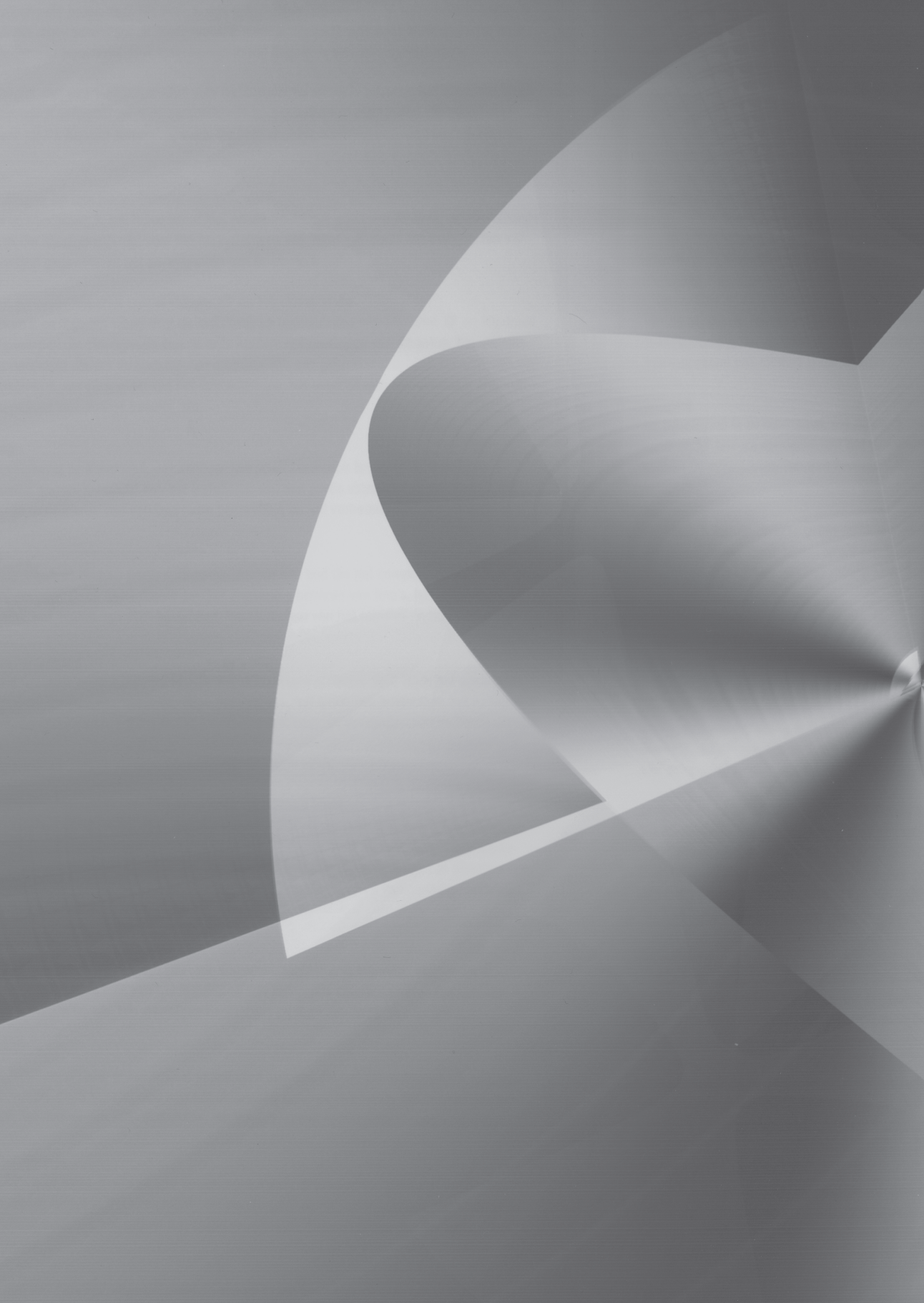

Chapter 6

Mitochondrial adaptations within chronically ischemic swine myocardium

Edward O McFalls, Wim Sluiter, Kees Schoonderwoerd, Olivier C Manintveld, Jos M Lamers, Karel Bezstarosti, Heleen M van Beusekom, Joseph Sikora, Herbert B Ward, Daphne Merkus and Dirk J Duncker.

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Abstract

Experimental evidence has emerged that myocardial ischemic preconditioning can prime the mitochondria into a "stress-resistant state", so that cell death is reduced following prolonged severe ischemia and reperfusion. Using a swine model of chronically ischemic myocardium, we tested the hypothesis that mitochondria within the ischemic territory have also acquired a protective phenotype. Eleven swine underwent a left thoracotomy with placement of an external constrictor around the proximal left anterior descending (LAD) artery. By 10 weeks, a severe stenosis of the LAD artery was documented by quantitative coronary angiography (92±2%). Animals were sacrificed and myocardium was extracted from the LAD and remote regions. Mitochondria were isolated from subendocardium and subepicardium from LAD and remote regions and state 2 (substrate alone) and state 3 (substrate plus ADP) respiration were assessed with a Clark electrode. Within the LAD subendocardium, the respiratory control index was 2.68±0.17 and was lower than the remote subendocardium (3.64±0.08; $P<0.05$). When exposed to 20 minutes anoxia with reoxygenation, the LAD region demonstrated a more preserved state 3 respiration compared with the remote region (99±14 versus 65±9 nmol O_2/mg respectively; $P<0.05$). In parallel mitochondrial experiments, chemiluminescence was detected with the probe coelenterazine and superoxide generation in the LAD region in the presence of antimycin A was 574±108 RLU/30sec/µg and was nearly 50% lower than the remote region (979±175 RLU/30sec/µg; $P<0.05$). Within the mitochondria, the expression of uncoupling uncoupling protein 2 (UCP-2) by western gels was 20% higher in the LAD region compared with the remote region ($P<0.05$) with no differences noted in UCP-3. In this swine model of chronic myocardial ischemia, isolated mitochondria from the ischemic tissue demonstrate preserved state 3 respiration following anoxia/reoxygenation, consistent with a stress-resistant state. This state is characterized by a mild degree of uncoupling under basal conditions and decreased superoxide generation. UCP-2 expression is enhanced in the mitochondria, providing a potential mechanism for these favorable mitochondrial adaptations.

Introduction

Among patients with advanced coronary heart disease and chronically ischemic myocardium, tissue can remain viable as noted by improved regional function following revascularization.[1] Although the mechanism of this chronic protection is unclear, the adaptations that occur in response to acute ischemia and reperfusion may provide some insight. Since the seminal observation of ischemic myocardial preconditioning in the anesthetized canine model,[2] there is emerging evidence that the mitochondria can be primed into a "stress-resistant state", so that cell death is reduced following prolonged, severe ischemia and reperfusion. Although the precise signal transduction pathway is unknown, mitochondria within preconditioned myocardium are altered in such a way, that the release of cytochrome c and pro-apoptotic factors, the accumulation of calcium and the generation of reactive oxygen species (ROS) following prolonged ischemia are reduced.[3-8] An attractive hypothesis has been proposed that the preconditioned phenotype is characterized by a mild degree of uncoupling within the inner membrane of the mitochondria, which attenuates the production of ROS following ischemia.[9-11] Expression and activation of uncoupling proteins within the inner membrane of mitochondria is one potential mechanism by which myocardial tissue can become protected against oxidant damage.[12-14] Within chronically ischemic myocardium, the mechanisms by which the mitochondria can adapt against repetitive oxidant stress are unclear. Accordingly, the present study was designed to test the hypothesis that mitochondria isolated from chronic ischemic myocardial tissue have acquired a "stress-resistant state", as noted by preserved mitochondrial integrity following *in vitro* anoxia. Secondly, we explored the potential mechanisms underlying this protection, in particular, mitochondrial uncoupling and attenuated maximal superoxide production. A swine model of chronic ischemia was used that with reduced regional function and increased glucose uptake relative to resting blood flow.[15]

Methods

This study was performed under the guidance of the animal care committee at the Erasmus MC and conforms to *Guide for the care and use of laboratory animals* published by the US National Institutes of Health (NIH publication No 85-23, 1996).

Animal Preparation

Eleven domestic pigs (~10 kg) were sedated with ketamine (30 mg/kg, im) and midazolam (1 mg/kg, im), ventilated and anesthetized with thiopental sodium (10 mg/kg via ear vein). Anesthesia was maintained with tentanylcitrate (10 µg/kg/h via ear vein). A left thoracotomy was performed in the 4th intercostal space, the LAD artery was dissected free and a C-shaped

occluder (3 mm in length and 1.4 mm in internal diameter) was secured around the vessel and gently closed with suture.[15] The pericardium and chest were closed in layers and animals were allowed to recover. Procainbenzylpenicilline 10 mg/kg and dihydrostreptomycinsulphate 12.5 mg/kg im (Streptoprocpen) was given for antibiotic prophylaxis and buprenorphine (0.3 mg/day, im) was provided for pain prophylaxis. Two animals died suddenly prior to the terminal study and no tissue data was available. Between 8 and 12 weeks following instrumentation, the remaining nine animals were sedated with ketamine (30 mg/kg, im) and midazolam (1 mg/kg, im) and 2D echocardiograms were acquired from the right parasternal and apical views to assess regional myocardial function. Regional wall thickening was measured from the anterior (LAD) and posterior (remote) walls and computed as the difference of end-systolic and end-diastolic wall thickness, expressed as a percent of end-diastolic thickness. End-diastole and end-systole were defined as the onset of the QRS and the frame with the smallest chamber size respectively. One week following the echocardiogram and after an overnight fast, animals were sedated with ketamine (30 mg/kg, im) and midazolam (1 mg/kg, im), ventilated and anesthetized with pentabarbitone (10-15 mg/kg/hour via ear vein). The right common carotid artery was cannulated with a 7F sheath, and selective coronary angiography of the left and right coronary arteries was performed. The stenosis of the LAD artery was determined by quantitative coronary angiography (% area stenosis) from the cranial left anterior oblique view. A mid-line sternotomy was then performed and the heart was extracted. The heart was sliced into 5 sections along the longitudinal axis and the two apical sections were placed in freshly prepared TTC solution for the detection of necrosis. The other sections were divided into subendocardial and subepicardial layers from the LAD and remote regions. In one animal, patchy necrosis was observed in the LAD subendocardium and tissue for mitochondria was harvested within the viable region. To assess gross changes in mitochondria by electronmicroscopy, endocardial and epicardial specimens from LAD and remote regions were fixed in glutaraldehyde, post-fixed with osmiumtetroxide and potassium ferricyanide, embedded in epon for ultrathin sectioning and counterstained with lead citrate.

Tissue Analysis

Mitochondrial Isolation. Fresh tissue was rapidly excised from subendocardial and subepicardial samples from the LAD and remote territories and placed in ice cold mitochondrial isolation buffer (MIB), pH 7.15, containing 50 mM Sucrose, 200 mM Mannitol, 1 mM EGTA. 5mM KH_2PO_2, 5 mM MOPS and 0.1 % Fatty acid free BSA.[16] Myocardium was excised free from fat and connective tissue, minced and a 5% homogenate was made. Tissue was homogenized in the same buffer (MIB). Fresh MIB was added and tissue separation was carried out in a glass homogenizer with a teflon pestle. Homogenates were centrifuged at 750 g for 10 minutes in order to pellet the cellular debris and mitochondria were collected from the supernatant. All centrifugations were performed in sorvall centrifuge tubes at 4° C. The supernatant was centrifuged two times, each at 8,000 g for ten minutes. The mitochondria

were collected and the protein concentration was determined. In general, the mitochondrial preparations contained about 5 mg of protein/ml and the citrate synthase activity amounted to 2.4 U/mg protein. The isolation procedure did not use nagarse and therefore, included only subsarcolemmal mitochondria.[17] To minimize the potential for impurities to collect in the mitochondrial membrane fraction, we used a lower centrifugation force of 8,000 g and avoided an isopycnic process that is known to create an osmotic stress. To ensure the purity of the mitochondrial isolates, we measured the differential expression of Cathepsin S (Abcam nr:ab18822, a goat polyclonal against lysozymes) and Prohibitin (Abcam nr:ab2996, a rabbit polyclonal against prohibitin) in the homogenate and mitochondrial membranes.

Mitochondrial Respiration. The mitochondria were suspended in mitochondrial respiration buffer (MRB) comprised of 110 mM Sucrose, 0.5 mM EGTA, 3 mM $MgCl_2$, 70 mM KCl, 10 mM KH_2PO_4, 20 mM Taurine, 20 mM HEPES and 0.1% fatty acid free BSA. They were then placed into the respiration chamber equipped with a Clark electrode to measure oxygen concentrations at 30°C. Once a steady state was achieved, respiration during state 2 (succinate 10 mM) and state 3 (with the addition of ADP, 0.5 mM) conditions was determined. The respiratory control index (RCI) was calculated by the ratio of state 3/state 2. In preliminary studies from non-instrumented pigs (n=2), state 2 respiration from mitochondria extracted from subendocardium tissue in the LAD and Remote regions were similar (35±1 and 34±2 nmol O2/min/mg respectively) while the RCI values in the two regions were 3.02±0.16 and 3.33±0.04 respectively. These RCI values are consistent with previous studies using the complex II substrate succinate.[18] Complex I substrates were not used in the initial mitochondrial studies because barbiturates were used to anesthetize the pigs and are known to inhibit the oxidation of complex I substrates.

Mitochondrial Function Following Anoxia-Reoxygenation. A separate group of mitochondria were used for these experiments. Prior to anoxia, succinate was added to the mitochondria in the polarograph, allowing the mitochondria to consume all of the oxygen (and the small amount of endogenous ADP) under state 2 conditions. After 20 min of anoxia, reoxygenation was allowed by opening the chamber to equilibrate with ambient oxygen and state 2 and state 3 respiration were determined.

Superoxide Measurement. A portion of the mitochondria was used to monitor superoxide release using the chemiluminescent probe coelenterazine (Calbiochem, La Jolla, CA).[19] Coelenterazine (0.25 μM) was added to a mitochondrial sample in a 96-well white-opaque microtiterplate within an EG&G Berthold MicroLumat LB 96P luminometer. Chemiluminescent measurements were acquired at room temperature after one minute, and the signal from the dye was integrated over 30 seconds following automatic injection of succinate (2 mM). Background signals were determined just prior to addition of the substrate and subtracted from the measurements. Signal measurements of chemiluminescence were obtained in the absence (basal conditions) and in the presence of antimycin A (200 ng/ml). With the latter, the spike in the superoxide generation from the Q_o, semiubiquinone at complex III was maximal.

Uncoupling Protein Assay. Another portion of the suspended mitochondria that were isolated from the subendocardial regions was stored at -80^{O} C for later analysis. After loading 10% SDS-PAGE gels with the same amount of mitochondrial protein (20 µg), immunoblots were performed by standard techniques and primary antibodies for UCP-2 and -3 were purchased from Alpha Diagnostic International. Western blots were analyzed in a densitometer and data were expressed as Arbitrary Units.

Glycogen. Samples from LAD and remote regions were dissolved in 30% KOH, precipitated with ethanol and hydrolyzed using amyloglucosidase. The glucose residues were measured with an NADP-linked spectrophotometric method by use of glucose 6-phosphate dehydrogenase and hexokinase.

Statistical Analysis

Results are expressed as means±SEM. Differences between LAD and remote regions were tested using Student's paired t-test. Multiple comparisons between subendocardial and subepicardial samples from LAD and remote regions were made by one-way ANOVA, using Fisher's least significant difference as the post hoc test. Due to considerable inter-animal variability, UCP content in the LAD subendocardium was expressed as a percentage of the remote subendocardium. A level of $P<0.05$ was considered statistically significant.

Results

Model of Chronic Myocardial Ischemia

One week prior to the terminal study, regional wall thickening was lower in the LAD compared with the remote region (Figure 1a). At the time of the terminal study, the stenosis in the LAD by quantitative coronary angiography was 92±2% (Figure 1b), with a complete occlusion and collateral formation noted in two pigs. Animals were fasted prior to sacrifice and transmural glycogen was higher in the LAD region, providing tissue evidence of chronic myocardial ischemia (Figure 1c). By electron microscopy, mitochondria from the subendocardial regions of the LAD and remote regions did not appear dissimilar in gross structure (Figure 1d).

Mitochondrial Respiration

Mitochondrial respiration was studied during state 2 (substrate alone) and state 3 (substrate plus ADP) conditions (Figure 2a). Within the LAD subendocardial region, mitochondria tended to have higher values for state 2 and lower values for state 3 respiration but the differences were not statistically significant (Figure 2b). The respiratory control index (RCI), defined by the ratio of state 3/state 2 was lower in the LAD subendocardial region however, indicative of a mild degree of uncoupling under basal conditions in the inner layer of the chronically ischemic myocardium (Figure 2c). Including data from the two pigs with the complete LAD

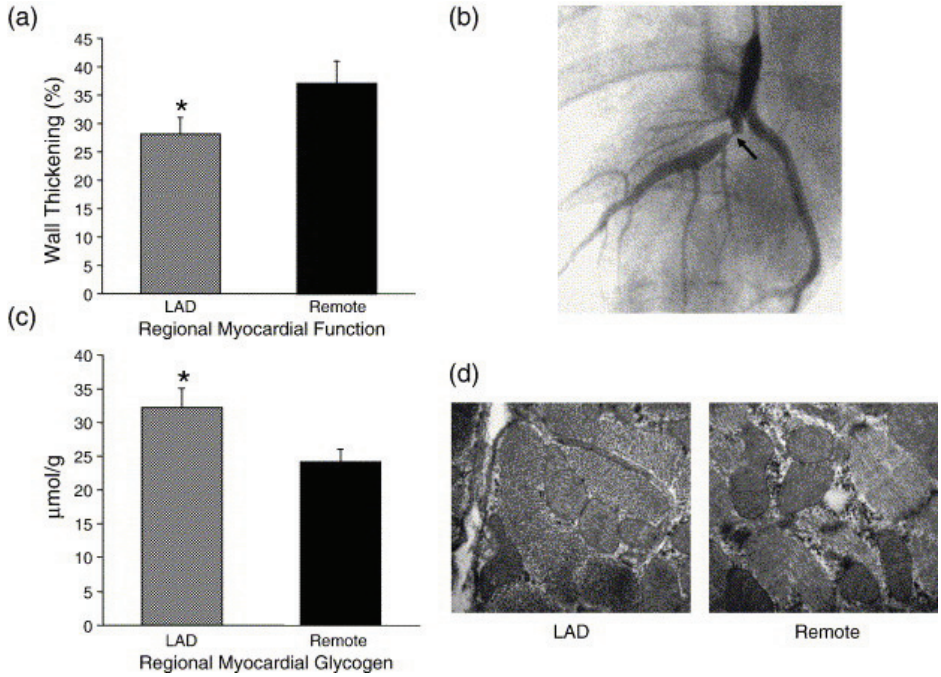

Figure 1: The swine model of chronic myocardial ischemia is characterized by (a) reduced regional wall thickening, (b) a severe stenosis in the proximal LAD artery, (c) increased myocardial glycogen in the fasted state (d) and no major ultrastructural changes in the involved mitochondria by electron microscopy.

occlusion and the one pig with patchy necrosis did not alter the results of these mitochondrial respiration studies.

Mitochondrial Superoxide Production

Measurements of superoxide were made over a 30 second period at baseline and following inhibition of complex III respiration with antimycin A (Figure 3a). Although superoxide production during a basal state did not differ between the LAD and remote regions, it was nearly 50% lower in the LAD region following antimycin A and could not be explained by regional differences in the concentration of MnSOD (Figure 3b-c). The differences in maximal superoxide production could also not be explained by regional differences in succinate dehydrogenase activity, which was 0.12±0.03 mU/U citrate synthase in the LAD region and 0.11±0.03 mU/U citrate synthase in the remote region (p=0.38). In addition, there was no regional difference in citrate synthase activity, which was 2.48±0.10 U/mg in the LAD region and 2.49±0.08 U/mg in the Remote region (NS). These data show that isolated mitochondria from the chronically ischemic myocardial regions produce lower levels of superoxide from ubisemiquinone during inhibition of complex III respiration with antimycin A.

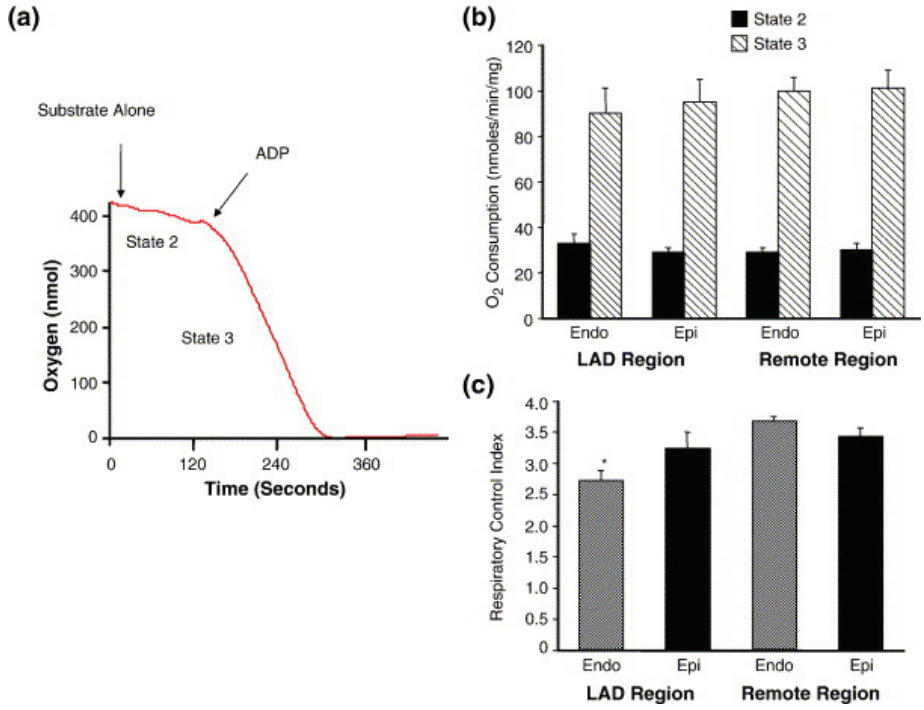

Figure 2: (a) A representative example of mitochondrial respiration is shown during state 2 (substrate without ADP) and state 3 (substrate with ADP) conditions. (b) Oxygen consumption is shown from isolated mitochondria from subendocardial (Endo) and subepicardial (Epi) tissue extracted from the chronically ischemic LAD and remote regions. (c) The respiratory control index (RCI) of the subendocardial (Endocardial) layer from the LAD region was lower than the LAD subepicardium (Epicardial) and the subendocardial and subepicardial layers from the corresponding remote region. Data are means±SEM; *P<0.05 versus all three additional regions.

Mitochondrial Resistance to Anoxia and Reoxygenation

Additional mitochondria from subendocardial samples from LAD and remote regions underwent 20 minutes of anoxia. Following reoxygenation, state 3 respiration was 99±14 nmol/min/mg in the LAD region and 65±9 nmol/min/mg in the remote region (P<0.05) while state 2 respiration was 36±3 in the LAD region and 29±2 nmol/min/mg in the remote region (NS). The RCI following anoxia and reoxygenation was 2.22±0.23 in the remote region and was significantly lower than the LAD region post-anoxia (2.76±0.27; P<0.05) as well as the basal value from the same region (P<0.05). These data support the contention that mitochondria from chronically ischemic myocardium have acquired a stress-resistant condition against anoxia and reoxygenation and that state is characterized by a lower that normal basal RCI and reduced superoxide production during complex III inhibition.

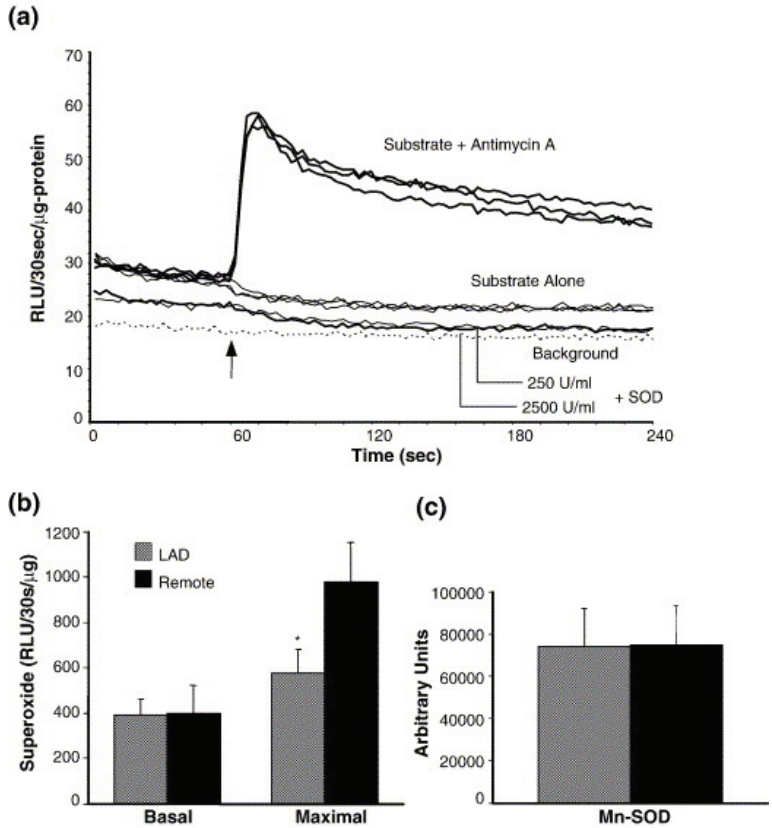

Figure 3: (a) Mitochondria were isolated from subendocardial and subepicardial layers from the LAD and remote regions and analyzed for superoxide generation by the chemiluminescent probe, coelenterazine. Superoxide was detected over a 30-second interval and was expressed as relative light units (RLU) normalized to µg of mitochondrial protein. Basal production was measured in the presence of succinate and maximal production was measured following administration of antimycin A. (b) The spike of superoxide production following inhibition of complex III respiration with antimycin A was 50% lower in the LAD region compared with the remote region and (c) the reduction in superoxide could not be explained by the expression of MnSOD. Data are means±SEM; *P<0.05 versus remote Region.

Mitochondrial uncoupling protein 2 and 3

Immunoblots for UCP-2 and -3 were obtained from isolated mitochondria from subendocardial tissue that was harvested from the LAD and remote regions. When normalized to the remote region, the chronically ischemic LAD region demonstrated a 20±11% (P<0.05) increase in the expression of UCP-2 with no change in expression of UCP-3 (Figure 4). Although there was a tendency for the degree of UCP-2 expression to correlate with state 2 respiration in the LAD subendocardial regions (R=0.57), this did not reach statistical significance (P=0.14).

To test the effect of deactivation of UCP-2 in the LAD subendocardium, the state 2 respiration and superoxide production were measured in 4 pigs prior to and following addition of

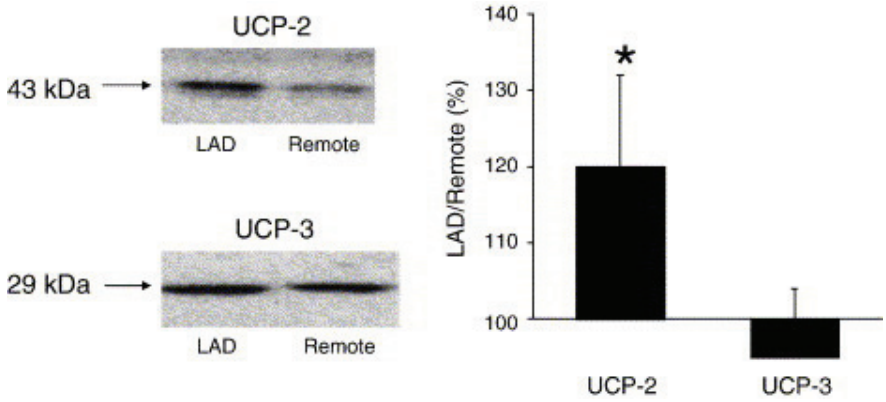

Figure 4: As shown by the representative gels and grouped data, uncoupling protein (UCP) 2 content in the mitochondria isolated from the subendocardial layer of the LAD region was increased relative to the remote region while no differences were noted in UCP-3 content. Data are means±SEM; *P<0.05 versus remote region.

GDP (1 mM). GDP reduced state 2 respiration from 56±12 nmol/min/g to 48±14 nmol/min/g (14% reduction) and increased superoxide production following inhibition of complex III with antimycin A from 126±20 RLU/30s/µg to 148±15 RLU/30s/µg (17% increase). Addition of FCCP (4 µg) induced a dramatic reduction in the superoxide production to 93±16 RLU/30s/µg and supports the notion that uncoupling reduces maximal complex III superoxide production.

Discussion

The principal findings of this study are that within chronically ischemic swine myocardium, mitochondria in the subendocardial layers have acquired a stress-resistant phenotype that is characterized by preserved state 3 respiration following in vitro anoxia and reoxygenation. In this protected state, the mitochondria demonstrate mild uncoupling (i.e. a lower respiratory control index), reduced superoxide production during inhibition of complex III respiration and enhanced expression of UCP-2. The latter may be an important adaptation in the chronically ischemic myocardial tissue that could favorably offset oxidant damage in response to repetitive ischemia.

Myocardial Adaptations and Chronic Myocardial Ischemia

Among patients with advanced coronary artery disease and chronically hypoperfused myocardial regions, tissue can remain viable as noted by improved regional function following revascularization.[1] Using dual tracers of blood flow and glucose, imaging with positron emission tomography (PET) has been a useful technique for identifying the presence of viable metabolically active myocardial tissue.[20] We have previously shown that this swine model

of chronic myocardial ischemia is also associated with increased glucose uptake relative to myocardial blood flow by PET at a time that regional function is decreased.[15] Interestingly, bioenergetics as measured by transmural ATP and creatine phosphate were preserved, suggesting that the balance between baseline energy expenditure and production is favorable, despite the presence of chronic ischemia.[15] By three months following instrumentation in a similar model, regional function by ultrasonic crystals to determine myocardial segment length shortening and blood flow by microspheres are both depressed, in the absence of significant necrosis and anaerobic glycolysis.[21] This finding highlights the notion that important adaptations occur in response to chronic myocardial ischemia, as previously identified in coronary artery resistance vessels in the original description of the model.[22]

The stimulus for the adaptations to chronic myocardial ischemia relates to the temporal reductions in vasodilator reserve which evolve during the transition from a state of chronic stunning with normal resting blood flow to a state of hibernation with reduced blood flow.[23] The molecular and morphological changes within hibernating myocardium from this model include a 10-fold increase in apoptosis with compensatory hypertrophy[24] and a decreased expression of the proteins that regulate calcium in the sarcoplasmic reticulum.[25] In the present study, the reduction in regional function, the severe coronary artery stenosis by coronary angiography and the elevated tissue levels of glycogen provide evidence of chronic myocardial ischemia in these animals. Increased glycogen has been observed in biopsy samples among patients with coronary artery disease undergoing bypass surgery and therefore has been identified as a marker of chronic myocardial ischemia.[26] Regional blood flow was not measured prior to sacrifice and although we cannot determine whether coronary blood flow reserve was exhausted, important adaptations occur within chronically ischemic myocardial tissue and precede the reduction in resting blood flow that is associated with hibernation.[27]

Mitochondrial Adaptations to Acute and Chronic Myocardial Ischemia

Since the seminal observation of ischemic myocardial preconditioning in the anesthetized canine model,[2] mitochondria have been identified as a potentially important pharmacological target for myocardial protection.[28, 29] The importance of mitochondrial function to cardioprotection has been demonstrated by the observations that isolated mitochondria from preconditioned myocardium retain the capacity to generate ATP following sustained ischemia and reperfusion[30] and are resistant to *in vitro* anoxia and reoxygenation.[31, 32] Importantly, the protection afforded by ischemic preconditioning is not limited to the early period following reperfusion but extends well beyond a 24-hour window.[33] Within this second window of protection, mitochondria have regained a protective phenotype that also results in less necrosis and apoptosis.[34, 35] It is possible that signaling kinases that are important in preconditioning are also activated in chronic hibernating myocardium.[36]

In the present study, mitochondria isolated from the chronically ischemic LAD subendocardium tolerated anoxia better than mitochondria isolated from the remote region as noted

by a more preserved state 3 respiration, suggesting maintained integrity of the mitochondrial respiratory chain following reoxygenation. These data show that mitochondria within chronically ischemic myocardium achieve a stress-resistant state, a finding that has also been observed in preconditioned myocardium. We found that the RCI of the mitochondria from the LAD subendocardium was lower than that of the remote subendocardium. A decrease in RCI might be due to a less efficient coupling of the oxidative phosphorylation. Mild uncoupling has been implicated in the protection by ischemic preconditioning[10, 11] which is supported by observations in isolated ventricular myocytes, that the uncoupling produced by the uncoupler dinitrophenol (DNP) protected the mitochondria against calcium overload and rigor following metabolic inhibition with reperfusion.[37] Also, in the isolated rat heart, DNP reduced infarct size produced by global total ischemia and reperfusion.[38] An interesting hypothesis has been proposed that a stress-resistant phenotype is caused by a modest degree of uncoupling within the inner membrane of the mitochondria from preconditioned hearts, so that during prolonged ischemia and reoxygenation, ROS production is attenuated.[9-11] The reduced ROS is likely due to minimization of the concentration of ubisemiquinone, so that exposure to oxygen following anoxia results in less superoxide formation.[9] The present study supports that concept, in that the ability to generate superoxide from ubisemiquinone was at least 50% lower in the mitochondria from the LAD subendocardium compared with those from the remote subendocardium.

The mechanism by which mitochondria from chronically ischemic myocardium achieve a stress-resistant state is unclear, but could be a result of the production of free radicals during intermittent supply-demand ischemia. In chick myocytes, superoxide is generated during hypoxia in the absence of oxygen and this process induces a state of protection against cell death following a subsequent prolonged period of hypoxia with reoxygenation.[8, 39] Within the LAD region, chronic ischemia was present and oxidant stress was likely increased at some point over the course of development of the model. In addition to a preconditioning effect, it is possible that mitochondria within repetitively ischemic myocardium maintain a chronic state of uncoupling, by activation and expression of uncoupling proteins.[40] Coenzyme Q is a co-factor for activation of uncoupling protein[41] and this could play a role in minimizing the accumulation of ubisemiquinone and subsequent superoxide generation. Alternatively, complex II is part of a multiprotein complex conferring mitochondrial ATP-sensitive K^+ ($mitoK^+_{ATP}$) channel activity and could be relevant to the observed protection[42] Inhibition of complex II leads to opening of the $mitoK^+_{ATP}$ and subsequently to dissipation of the mitochondrial membrane potential. While the capacity to generate superoxide will decrease at a sufficiently low mitochondrial membrane potential,[9] we did not find that a decreased expression of complex II in the LAD subendocardium can account for this effect.

There is emerging evidence that uncoupling proteins prevent cell injury following anoxia and reoxygenation. Within cardiac myocytes that have overexpressed UCP-2, the degree of superoxide generation following prolonged exposure to H_2O_2 and subsequent apoptosis was

attenuated.[12] Likewise, macrophages from UCP-2 knock-out mice have higher levels of ROS and as a result, increased toxoplasmacidic and bactericidal activity.[43] These data support the concept that increased expression of UCP-2 has a mechanistic link with the reduced superoxide generation in the mitochondria from the chronically ischemic myocardial tissue.[14, 44]

Methodological Considerations

Whether the mitochondria isolated from the LAD region were actually uncoupled may not necessarily be assumed by the changes in either state 2 respiration or the RCI. The presence of uncoupling is best determined by demonstration of altered respiratory rates over a series of membrane potentials, which was beyond the scope of this study. We cannot exclude the possibility that other proteins involved with the electron transport system in the inner membrane have been altered as a result of chronic myocardial ischemia and may also have contributed to the observed reductions in superoxide production following inhibition of complex III with antimycin A. A more thorough investigation of these possibilities is warranted in future studies. An additional consideration is that sedatives and anesthetics used during the terminal study might have altered the *ex vivo* mitochondrial studies. Pentobarbitone was used for general anesthesia and this has been shown to alter mitochondrial respiration related to complex I substrates. To compensate for that potential bias, we used only the complex II substrate succinate for the isolated mitochondrial studies and this may have underestimated the contribution of superoxide generated by the complex I electron transport chain. We also accept that administration of ketamine as a sedative may have enhanced mitochondrial protection in the isolated studies by virtue of a preconditioning-like effect. This effect would have altered the mitochondria in all regions however and therefore, would not explain our observed differences in mitochondria between LAD and remote regions.

Conclusions

In this study, mitochondria have been isolated from chronically ischemic myocardial tissue and demonstrate preserved state 3 respiration following in vitro anoxia and reoxygenation. In this stress-resistant state, they are characterized by a mild degree of uncoupling (as indicated by depressed respiratory control index) and by a reduced level of superoxide generation. The expression of uncoupling protein 2 is increased, suggesting a possible mechanism for these mitochondrial adaptations in response to chronic myocardial ischemia.

Acknowledgements

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Chapter 7

The tyrosine phosphatase inhibitor Bis(Maltolato)-Oxovanadium attenuates myocardial reperfusion injury by opening ATP-sensitive potassium channels

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

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Abstract

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. We tested the hypothesis that the organic vanadate compound bis(maltolato)-oxovanadium (BMOV) limits myocardial infarct size by attenuating reperfusion injury and investigated the underlying mechanism. Myocardial infarction was produced in 112 anesthetized rats by a 60-min coronary artery occlusion and infarct size was determined histochemically after 180 min of reperfusion. 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. We conclude that BMOV afforded significant cardioprotection principally by limiting reperfusion injury. The mode of action appears to be by opening of cardiac K^+_{ATP} channels via increased tyrosine phosphorylation.

Introduction

An increase in tyrosine residue phosphorylation via increased tyrosine kinase activity has been implicated in the signal transduction pathway of cardioprotection 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 white blood cell survival by inhibiting apoptosis.[4-6] Vanadate enhances tyrosine residue phosphorylation by inhibition of tyrosine phosphatase,[7, 8] suggesting that vanadate may be of therapeutic benefit in myocardial infarction, which may involve both apoptosis and necrosis.[9, 10] In support of this concept, Armstrong *et al.*[11] reported that serine threonine phosphatase inhibitors are highly effective in protecting isolated cardiomyocytes subjected to ischemia (without reperfusion), even when administered late (75 min) after onset of ischemia, suggesting that vanadate may not require administration prior to the onset of ischemia and might also act against reperfusion injury. However, to date *in vivo* studies on the cardioprotective effects of tyrosine phosphatase inhibitors are lacking.

The mechanism by which tyrosine phosphatase inhibitors exert their cardioprotective effects is incompletely understood. However, since K^+_{ATP} channels have been reported to be downstream targets of tyrosine kinase in the signalling pathway of ischemic preconditioning,[1, 2] we hypothesized that K^+_{ATP} channels contribute to the cardioprotection by vanadate. Finally, several studies including from our own laboratory, have reported that a brief period of ischemia,[12-15] or local intra-arterial infusion of adenosine[12-15] and bradykinin[16] in remote organs such as small intestine and kidneys can protect the myocardium by stimulation of afferent nerves in the remote ischemic organ that results in activation of a neurogenic pathway.[12, 13] We hypothesized that activation of this neurogenic pathway (which implies that compounds are not required to reach the area at risk), might also contribute to the cardioprotection by vanadate administered intravenously just prior to reperfusion.

In view of these considerations the present study was designed to investigate (*i*) whether pre-treatment with bis(maltolato)-oxovanadium (BMOV) is cardioprotective, (*ii*) whether BMOV treatment after the onset of occlusion, but just prior to reperfusion is still cardioprotective, and (*iii*) the mechanism of protection by BMOV, including the involvement of K^+_{ATP} channel opening and a neurogenic pathway. All studies were performed in anesthetized open-chest rats subjected to a 60-min coronary artery occlusion.

Methods

Guidelines for Animal Research. 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.[13, 14] 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.[15] 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.

All rats were subjected to 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.[12-15]

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.[12-15]

Effect of BMOV on Infarct Size. 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.

Mode of Action of BMOV. 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[1, 17]. Genistein was administered intravenously in doses of either 5 or 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 (to ensure sufficient

incubation time for glibenclamide in the area at risk)[18] and the second infusion starting at 45 min after the onset of occlusion.

The involvement of indirect protection of the heart by stimulation of afferent nerves in a remote organ and subsequent activation of a neurogenic pathway was investigated by studying the effect of intravenous 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.

Materials. 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) PBS (modified Sörensen). Genistein (5 and 10 mg/kg, Sigma Chemicals, St. Louis, MO) was dissolved in 0.3 ml of 95% ethanol and alkamuls EL-620 (Rhodia, Lyon, France) to which 0.3 ml physiologic saline was added. Glibenclamide (6 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.

Data Analysis and Presentation. Infarct size was analysed by one-way analysis of variance (ANOVA) followed by Dunnett's test. The importance of timing of administration of BMOV (pre-ischemia versus pre-reperfusion) was analysed by using two-way (timing x dose) ANOVA. 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.

Results

Exclusion Criteria. Of the 128 rats that entered the study, 13 rats were excluded because of sustained ventricular fibrillation during coronary artery occlusion (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.

Effect of BMOV on Infarct Size. There were no differences ($P=0.22$) between the areas at risk of the various experimental groups (40±1%, n=112). 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 (both $P>0.30$) as the corresponding doses administered before the 60-min CAO (Fig. 1, right panel).

Mode of Action of BMOV. 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 just before the 180-min reperfusion period (Fig. 2, right panel). The inhibition by genistein of the infarct size limitation by BMOV

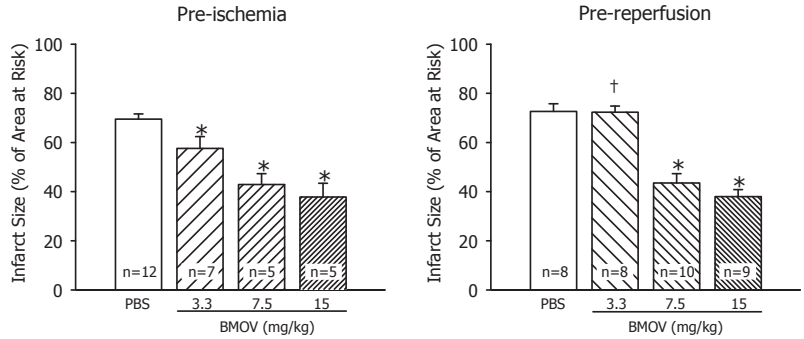

Figure 1. Effects of BMOV administered prior to ischemia (left panel) or reperfusion (right panel) on myocardial infarct size produced by a 60-min CAO. **P*<0.05 vs corresponding vehicle (PBS); †*P*<0.05 BMOV pre-reperfusion vs BMOV pre-CAO.

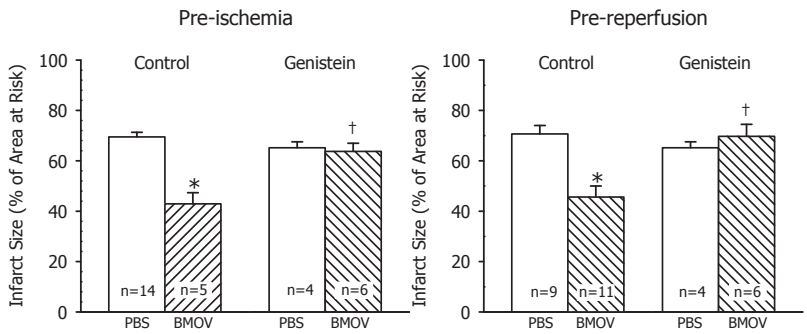

Figure 2. Effects of the tyrosine-kinase inhibitor genistein (10 mg/kg) iv, administered 15 min prior to reperfusion, on the cardioprotection by intravenous administration of 7.5 mg/kg BMOV administered either prior to ischemia (left panel) or prior to reperfusion (right panel). **P*<0.05 vs corresponding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

occurred in a dose-dependent fashion as 5 mg/kg only partly blocked the protection by BMOV administered just prior to reperfusion [infarct size 60±4% (n=5; data not shown in Fig. 2); *P*<0.05 versus both BMOV treated (44±3%) and PBS treated (70±2%) rats]. The cardioprotection by BMOV, administered prior to reperfusion, was also abolished by glibenclamide but not by hexamethonium (Fig. 3). Genistein (10 mg/kg; Fig. 2), glibenclamide (Fig. 3) and hexamethonium (Fig. 3) had no effect on infarct *per se*, which is in agreement with previous observations.[1, 17, 19, 20]

Hemodynamic Effects of BMOV. There were no differences in baseline values of heart rate (*P*=0.37) and mean arterial blood pressure (*P*=0.25) between any of the experimental groups (Table 1). In the PBS-treated groups, heart rate and mean aortic blood pressure remained

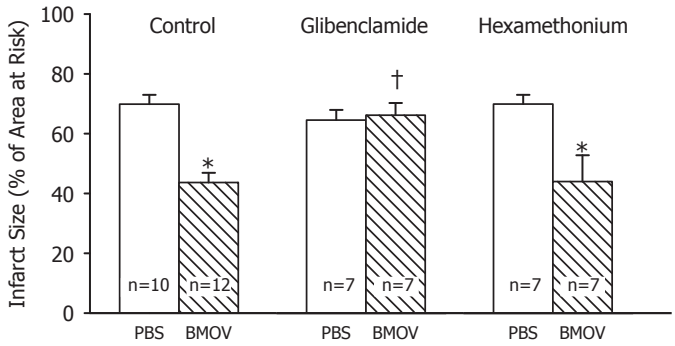

Figure 3. Effects of the K^+_{ATP} channel blocker glibenclamide (2 x 3 mg/kg iv) or the ganglion blocker hexamemethonium (20 mg/kg iv) on the cardioprotection by 7.5 mg/kg BMOV intravenously administered prior to reperfusion. **P*<0.05 vs vehicle (PBS); †*P*<0.05 vs corresponding control BMOV.

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 a transient and dose-dependent increase in mean arterial pressure, which was accompanied by a decrease in heart rate. Apart from these transient effects the hemodynamic responses to occlusion and reperfusion were not different from those in the vehicle-treated animals.

Pretreatment with 10 mg/kg genistein markedly attenuated the pressor response induced by 7.5 mg/kg BMOV (14±5 mmHg compared to 31±4 mmHg, *P*<0.05; Table 1). In contrast, the BMOV-induced increase in blood pressure was not altered by pretreatment with either glibenclamide (25±9 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.[12, 15, 21, 22] 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). Together, these findings indicate that the infarct size limitation by BMOV cannot be explained by a decrease in global left ventricular energy demands.

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 com-

Table 1 Heart rate and mean arterial blood pressure

	Pre-Coronary Artery Occlusion (min)			Coronary Artery Occlusion (min)			Reperfusion (min)			
	-25	-10	-1	30	50	60	15	60	120	180
Pre-Ischemia Treatment with BMOV										
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
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
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†
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*†
Pre-reperfusion Treatment with BMOV										
PBS pre-REP (n=8)										
HR	374±15	342±10*	338±9*	344±11*	341±7*	341±7*	349±8*	355±9*	373±13†	383±11†
MAP	97±2	99±3	97±3	95±4	96±5	100±5	94±4	92±5	89±7	89±3
BMOV 3.3 pre-REP (n=8)										
HR	387±18	368±10	362±9	364±5	364±3	343±10*	371±9	369±7	384±10	405±10†
MAP	99±5	95±3	87±4*	82±5*	93±4	105±4†‡	94±6	82±6*	76±5*	79±6*
BMOV 7.5 pre-REP (n=12)										
HR	373±7	340±7*	337±8*	345±10*	340±9*	285±8*†‡	38±10*	343±12*	369±10†	371±16†
MAP	101±4	97±2	97±2	95±3	96±3	127±4*†‡	96±2	87±4	84±3*	84±4*
BMOV 15 pre-REP (n=9)										
HR	367±12	338±6*	333±3*	339±5*	339±5	274±5*†‡	314±7*	344±9*	357±6†	379±4†
MAP	101±4	97±3	95±2	97±3	102±2	135±5*†‡	96± 3	93±4*	83±2*†	88±4*†

Mode of Action of BMOV										
Genistein (n=4)										
HR	366±6	368±10	369±13	388±18	388±16	394±13†	395±12†	410±12†	428±16†	441±10†
MAP	103±6	115±5	115±4	88±6†	91±5†	85±7†	90±5†	88±8†	88±6†	84±5*†
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*†
Genistein + BMOV 7.5 (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†
Glibenclamide (n=7)										
HR	370±11	351±10*	377±18	378±12	365±21	380±16	372±19	366±21	366±25	374±45
MAP	103±6	110±8*	111±8	96±7	104±6	106±6†	98±5†	95±6†	91±7†	86±12*†
Glibenclamide + BMOV 7.5 (n=7)										
HR	336±13	336±10	341±12	339±10	343±12	273±18*†‡	327±11	371±13	372±13	388±15*†
MAP	107±6	122±3*	125±2*	104±4	118±3	143±7*‡	106±6	97±5†	86±8†	94±6†
Hexamethonium (n=7)										
HR	338±10	338±4	332±4	340±6	328±7	319±8	339±8	336±7	338±8	337±9
MAP	96±4	100±3	92±5	92±4	73±3*†	77±4*†	84±5	86±7	84±8	82±9
Hexamethonium + BMOV 7.5 (n=7)										
HR	363±16	365±13	367±13	365±10	334±10†	98±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); MAP = mean arterial blood pressure (mmHg). Genistein was administered in a dose of 10 mg/kg (5 mg/kg not shown in Table 1).
Data are mean±SEM; *P<0.05 vs -25 min Pre-Coronary Artery Occlusion (Baseline); †P<0.05 vs -1 min Pre-Coronary Artery Occlusion; ‡P<0.05 60 min vs 50 min Coronary Artery Occlusion.

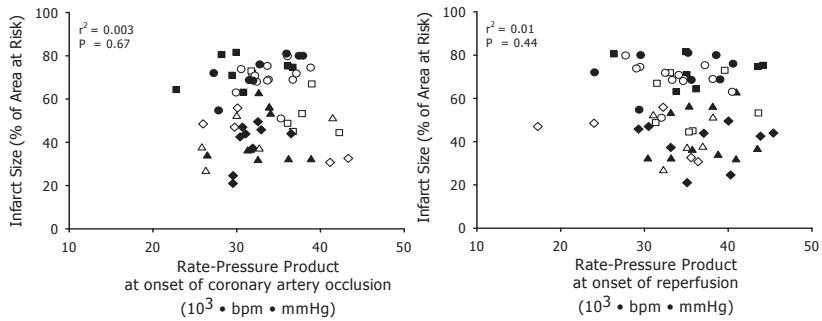

Figure 4. Lack of relation between the rate-pressure product at the onset coronary artery occlusion (left panel) and the onset of reperfusion (right panel) and myocardial infarct size in rats receiving PBS or BMOV prior to occlusion (open symbols) or prior to reperfusion (solid symbols). Data are presented as individual data points from animals presented in Figure 1. Circles denote PBS, squares denote 3.3 mg/kg BMOV, upward triangles denote 7.5 mg/kg BMOV, while diamonds denote 15 mg/kg BMOV, respectively.

pared 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.

Importance of Dosage and Timing of Administration of BMOV. Previous *in vitro* studies have indicated that vanadate possesses cardioprotective properties.[23] 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.[24] The results from this study 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.[24] Furthermore, ischemia lasted up to 15 min, which in buffer-perfused rodent hearts may already result in significant necrosis.[25] 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.

Takeuchi *et al* .[23] administered vanadate for a period of 3-4 weeks in a dose of 7.5 mg/kg *per os* per day to rabbits with pressure-overload induced left ventricular hypertrophy. When isolated hearts were then subjected to a 40 min period of global ischemia, vanadate-treated hearts showed reduced lactate release and improved post-ischemic recovery of left ventricular-developed pressure compared to vehicle-treated hypertrophied hearts. However, since in these experiments 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.[26]

The present 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. It could be argued that BMOV exerted at least part of its protective action by reducing ischemia injury during the last 10 min of the 60-min CAO. This is highly unlikely, in view of previous observations in our laboratory in the identical experimental model in which a 45-min CAO followed by 180 min of reperfusion already resulted in an infarct size of 61±7%, which was not significantly different from the infarct size of 70±2% produced by a 60-min CAO.[15] Consequently, any limitation of ischemia damage by BMOV, administered after 50 min of CAO, cannot explain the BMOV-induced limitation of infarct size to 43±5%. Taken together these findings suggest that BMOV exerts its effects principally during reperfusion, but that sufficiently high concentrations need to be present in the blood at the onset of reperfusion.

Mechanism of Cardioprotection by BMOV. The cardioprotective effect of vanadate in isolated buffer perfused rat hearts 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 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.[27] In contrast, under certain conditions *in vitro*, vanadate has been shown to be capable of generating free radicals.[28] 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.[29] Importantly, the role of reactive oxygen species in lethal reperfusion injury *in vivo* is still poorly understood, as studies on efficacy of scavengers of reactive oxygen species against reperfusion injury have been highly equivocal.[30, 31]

In the present *in vivo* study, the tyrosine kinase-inhibitor genistein dose-dependently 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 are consistent with the concept that BMOV is dependent on an intact tyrosine kinase activity during reperfusion, and also suggest that the tyrosine phosphorylation status is an important determinant 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.[23] Recent clinical trials indicate that a combination of glucose and insulin might increase the salvage of cardiomyocytes during early reperfusion.[32] 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 mito-

chondria 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.[33] Another mechanism by which an increase in tyrosine phosphorylation may exert cardioprotection could involve in opening of K^+_{ATP} channels, which is suggested by studies showing that ischemic preconditioning involves activation of tyrosine kinase and protein kinase C,[1, 3] and opening of K^+_{ATP} channels.[18] Although the sequence of involvement is still controversial,[34] studies in the rat heart suggest that kinases are principally involved early in preconditioning and act upstream of the K^+_{ATP} channels.[1, 3] In accordance with this concept, we observed that the K^+_{ATP} channel blocker glibenclamide, which had no effect on infarct size *per se*, abolished the cardioprotection by BMOV, suggesting that opening of K^+_{ATP} channels is involved in the actions of BMOV.[1, 35, 36] However, one could argue that glibenclamide antagonized the effects of BMOV by increasing myocardial susceptibility to ischemia (i.e. in a BMOV-independent manner), but that this went undetected in the control infarct group, because infarct size reaches a plateau at 60 min of coronary artery occlusion.[15] This scenario is, however, highly unlikely in view of a preliminary study from our laboratory in which we observed that the reduction in infarct size by ischemic preconditioning with three cycles of 3-min of coronary artery occlusion and interspersed by 5 min of reperfusion from 70±1% (in sham rats) to 25±4% ($P<0.05$), was not affected by glibenclamide (infarct size 28±8%).[19] Taken together, the findings in the present study are consistent with the concept that K^+_{ATP} channel activation during early reperfusion contributes to the protection by BMOV. Since glibenclamide blocks both the sarcolemmal and mitochondrial K^+_{ATP} channels,[37, 38] future studies are needed to determine the involvement of mitochondrial versus sarcolemmal K^+_{ATP} channels in the protection against reperfusion injury by BMOV.

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.[12, 13] 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. In patients with an impending myocardial infarction, early restoration of blood flow to jeopardized ischemic myocardium is compulsory for limiting infarct size. Despite its necessity, several investigators[39-41] have suggested that reperfusion causes irreversible myocardial damage by itself, beyond that inflicted by ischemia alone. This *"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, thereby resulting in

extension of myocardial infarction. Since in patients that encounter a myocardial infarction as the first symptom of ischemic heart disease, pharmacotherapy can only be applied after the coronary artery has occluded, there is a need for agents that are protective even when given after the onset of ischemia or just before reperfusion. Most cardioprotective agents developed to date require administration prior to the onset of ischemia in order to be effective.[31] The present study shows that BMOV, administered in a sufficiently high dose, is still highly cardioprotective when administered just prior to reperfusion.

Acknowledgements

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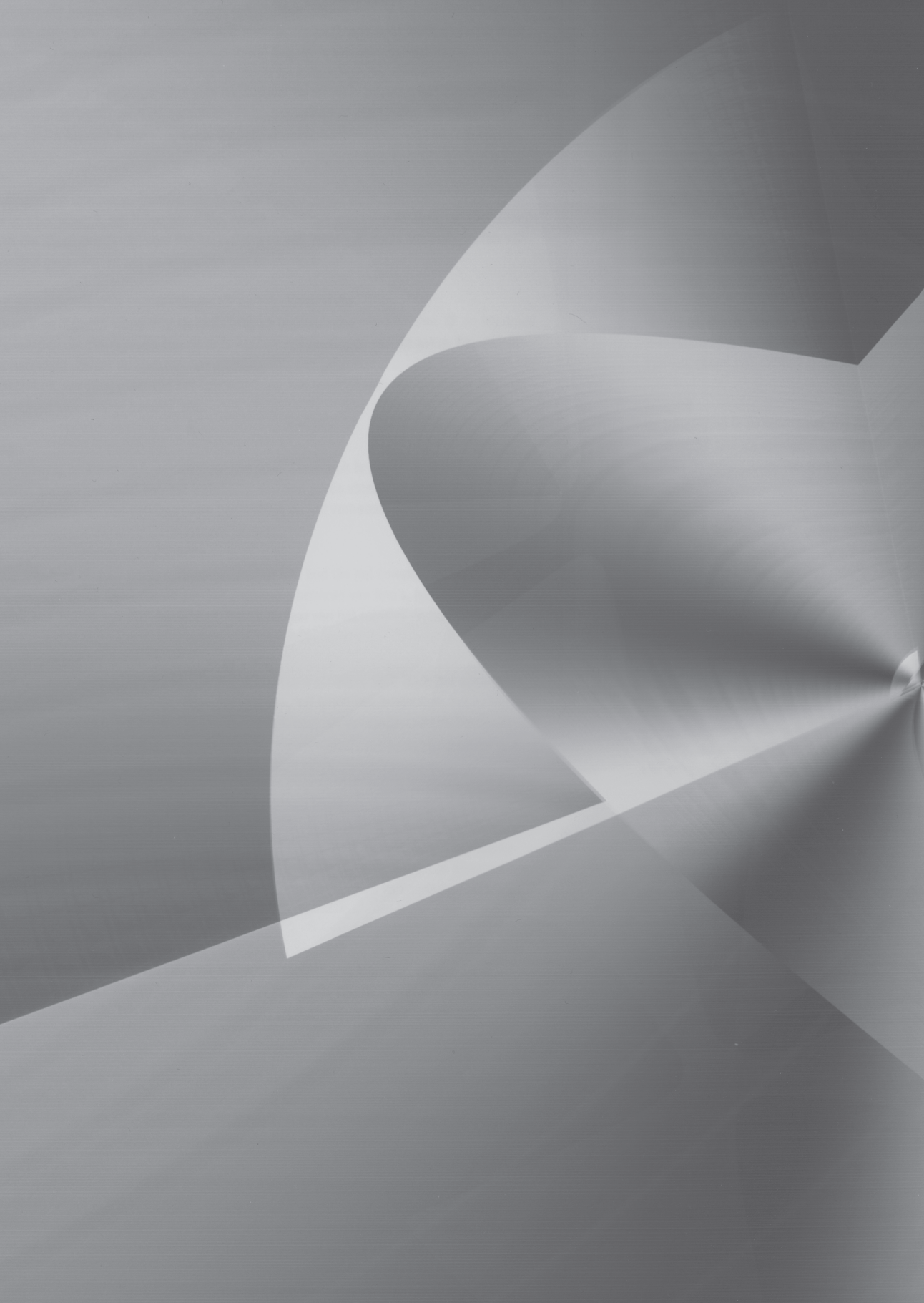

Chapter 8

Cardiac effects of postconditioning depend critically on the duration of index ischemia

Olivier C Manintveld, Maaike te Lintel Hekkert, Ewout J van den Bos, Grietje M Suurenbroek, Dick H Dekkers, Pieter D Verdouw, Jos M Lamers and Dirk J Duncker.

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Abstract

Postconditioning is known as the phenomenon whereby brief intermittent ischemia applied at the onset of reperfusion following index ischemia limits myocardial infarct size. While there is evidence that the algorithm of the postconditioning stimulus is an important determinant of the protective efficacy, the importance of the duration of index ischemia on the outcome of the effects of postconditioning has received little attention. Pentobarbital-anesthetized Wistar rats were therefore subjected to index ischemia produced by coronary artery occlusions (CAO) of varying duration (15- to 120-min) followed by reperfusion, without or with postconditioning produced by 3 cycles of 30-s of reperfusion and re-occlusion (3POC30). 3POC30 limited infarct size produced by 45-min CAO (CAO45) from 45±3% to 31±5%, and CAO60 from 60±3% to 47±6% (both $P \leq 0.05$). In contrast, 3POC30 increased infarct size produced by CAO15 from 3±1% to 19±6% and CAO30 from 36±6 to 48±4% (both $P \leq 0.05$). This deleterious effect of 3POC30 was not stimulus-sensitive as postconditioning with 3POC5 and 3POC15 after CAO30 also increased infarct size. The cardioprotection by 3POC30 after CAO60 was accompanied by an increased stimulation of Akt-phosphorylation after 7 min of reperfusion, and a 36% lower superoxide production, measured by dihydroethidium fluorescence after 2 hours of reperfusion. Consistent with these results, cardioprotection by 3POC30 was abolished by phosphatidylinositol-3-kinase (PI3K) inhibition, as well as NO synthase inhibition. The deleterious effect of 3POC30 after CAO15 was accompanied by an increased superoxide production with no change in Akt phosphorylation, and was not affected by NO synthase inhibition. In conclusion, the effect of cardiac postconditioning depends critically on the duration of the index ischemia and can be either beneficial or detrimental. These paradoxical effects of postconditioning may be related to the divergent effects on Akt phosphorylation and superoxide production.

Introduction

Restoration of blood flow to ischemic myocardium is a prerequisite to interrupt the development of irreversible damage, but the mode of its application is of crucial importance. For instance, while gradual reperfusion may attenuate deleterious effects, abrupt reperfusion has been shown not only to increase the incidence of reperfusion arrhythmias,[1] and aggravate stunning,[2] but even to contribute to the development of irreversible damage.[3-6] Zhao *et al*.[7] reported recently that abrupt reperfusion after a sustained coronary artery occlusion (CAO) can also limit irreversible damage provided that this was interrupted by a number of brief periods of abrupt coronary artery re-occlusion and abrupt reperfusion started within 30s after the initial abrupt reperfusion. The authors termed this phenomenon "postconditioning", because of its similarity to the multiple brief abrupt CAOs often employed in preconditioning protocols.[7] Subsequent studies confirmed this original observation made in intact canine hearts in *in vitro* and *in situ* rodent heart models.[8-13] These studies, which aimed to define the optimal algorithm of postconditioning, also revealed that in order to be effective the first re-occlusion has to be applied within 1-min after the onset of reperfusion,[8] while increasing the number of re-occlusion – reperfusion cycles beyond 4 cycles does not confer greater protection.[8, 9, 13] Nevertheless, the optimal duration of the re-occlusion and reperfusion periods of postconditioning is currently unknown, but most likely differ between animal species. Thus, while postconditioning with three 30s periods of abrupt CAO starting 30s after the initial reperfusion, and interspersed by 30s of abrupt reperfusion limited infarct size produced by a 60-min CAO in the dog[7, 14] it failed to afford cardioprotection against a 30-min CAO in the rat.[15] On basis of this single observation the authors suggested that briefer periods (i.e. 10-15s) of re-occlusion and reperfusion are required in smaller than in larger animals, in which 30s cycles are effective,[15] but conclusive evidence for this hypothesis is lacking. For instance, in discordance with this concept is a recent study by Schwartz and Lagranha[16] who showed in swine that three cycles of 30s of re-occlusion and reperfusion failed to limit infarct size produced by a 30-min CAO. Since postconditioning with the 30s algorithm was effective against a 60-min CAO[7, 14] but not against a 30-min CAO,[15] we hypothesized that the duration of the index ischemia also plays a major role in determining the effect of the postconditioning stimulus. Consequently, the aim of the present study was to investigate the influence of index ischemia duration (ranging from 15- to 120-min) on the protective effect of postconditioning. We subsequently investigated how the duration of index ischemia affected the role of potential mechanisms reported to be involved in the cardioprotection by postconditioning such as the activation of the PI3K-Akt-eNOS signaling pathway and the production of reactive oxygen species to explain our findings.

Methods

Animals

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 1996) and with prior approval of the Animal Care Committee of the Erasmus MC, University Medical Center Rotterdam.

Surgical and Experimental Procedures

Pentobarbital-anesthetized (60 mg/kg intraperitoneally) 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.[17-21] In the inferior caval vein a PE-50 catheter was placed for infusion of Gelofusin (5-10 ml; B. Braun, Melsungen AG) to maintain central venous pressure at 4-6 mmHg, and for administration of drugs. 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 occlusion of the vessel. A catheter was positioned in the abdominal cavity to allow intraperitoneal administration of pentobarbital for maintenance of anesthesia. Rectal temperature was maintained at 36.5-37.5˚C.[17-19]

Rats that fibrillated during occlusion or reperfusion were allowed to complete the protocol, provided that conversion to normal sinus rhythm occurred spontaneously within 1-min, or that defibrillation via gently thumping on the thorax was successful within 2-min after onset of fibrillation. Occlusion and reperfusion were visually verified. The area at risk (AR) and infarct area (IA) were determined after 120-min of reperfusion, using trypan blue and nitro-blue-tetrazolium staining, respectively [17-19]. Infarct size (IS) was calculated as IA/AR.

Experimental protocols/design

After completion of surgery, a 30-min stabilization period was allowed before animals were subjected to the experimental protocols (see Fig. 1 for an overview of the protocols) The duration of this stabilization period has been shown to be sufficiently long to exclude an effect of the surgical procedures on the development of infarct size.[22]

Protocol 1: Importance of the duration of index ischemia. Animals were subjected to periods of index ischemia consisting of a coronary artery occlusion varying between 15-min (CAO15) and 120-min (CAO120) in duration followed by abrupt reperfusion (control groups) or by postconditioning consisting of 3 cycles of 30s reperfusion and 30s of re-occlusion (3POC30).[7, 14, 16]

Protocol 2: Importance of the postconditioning stimulus. In another set of experiments we investigated the influence of the postconditioning stimulus. For this purpose, CAO30 and

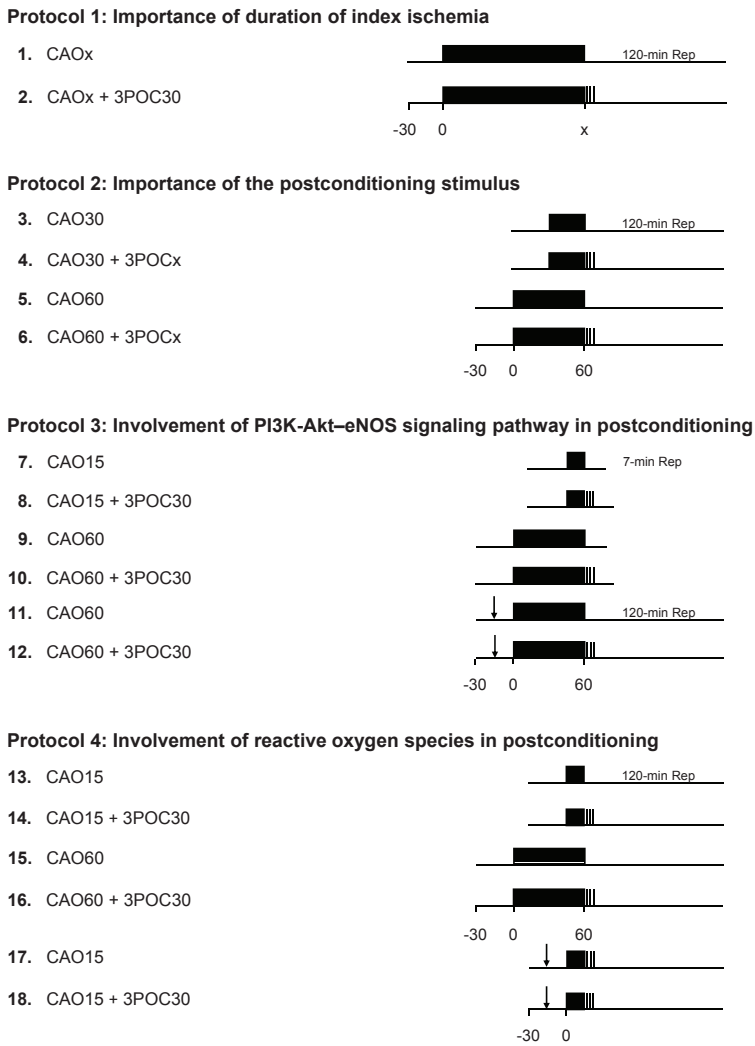

Fig. 1. Overview of all experimental protocols in the present study. Protocols 1 and 2 pertain to the study of the effect of postconditioning on infarct size, while protocols 3 and 4 pertain to the investigation of the potential mechanisms involved in postconditioning. CAOx denotes coronary occlusion durations ranging from 15 (CAO15) to 120 minutes (CAO120). 3POCx denotes different POC protocols including 3 cycles of 5, 15 or 30 seconds. The arrow indicates the time of administration of drugs.

CAO60 were followed by a postconditioning stimulus consisting of 3 cycles of 5s (3POC5), or 3 cycles of 15s (3POC15) of reperfusion and re-occlusion.

Protocol 3: Involvement of PI3K-Akt–eNOS signaling pathway in postconditioning. Four additional groups of rats were subjected to either 15- or 60-min of index ischemia followed by either abrupt reperfusion or 3POC30. After 7 min of abrupt reperfusion (control) or after postconditioning with 3POC30 followed by 7 min of reperfusion (3POC30),[10, 11] the AR was

dissected and snap frozen in liquid nitrogen within 30 s before being stored at -80°C. The 7 min reperfusion time point was employed based on previous POC studies[10, 12, 23-25] and in view of a previous *in vitro* study, in which Akt phosphorylation increased 4-5 fold within 10 min of reperfusion and remained elevated up to 60 min.[26] Furthermore, phosphorylation of both (Ser473)-Akt and (Thr308)-Akt has recently been shown to be obligatory for the full activation of Akt.[27] Consequenly, we determined P(Thr308)-Akt as well as P(Ser473)-Akt.

Additional rats were subjected to CAO60 with or without 3POC30 in the presence of the PI3K inhibitor wortmannin (15 µg/kg iv)[28] or the NO synthase inhibitor N-nitro-L-arginine (LNNA 25 mg/kg iv).[21]

Protocol 4: Involvement of reactive oxygen species in postconditioning. Four separate groups of animals were subjected to CAO15 or CAO60 followed by either abrupt reperfusion or 3POC30, while one group of control animals underwent only a sham procedure, i.e. without index ischemia. The four groups subjected to the 15-min or 60-min index ischemia were studied at the end of 120-min of reperfusion.[7]

Two additional group of rats underwent CAO15 either without or with 3POC30 (followed by 2 h of reperfusion) including treatment with the combination of ROS scavengers mercaptopropionyl-glycine (MPG; 60 mg/kg/h iv), N-acetylcysteine (250 mg/kg/h iv), and tempol (50 mg/kg/h iv). Infusion was started 15 min before the onset of the CAO15 and maintained throughout the experimental protocol. Starting 5 min prior to the onset of initial reperfusion, the infusion rates were doubled for 15 min.

Biochemical assays

Akt phosphorylation. Akt phosphorylation was determined in the area at risk harvested at 7 minutes of reperfusion. Approximately 40 mg of frozen tissue was homogenized at liquid nitrogen temperature in a microdismembrator unit (B. Braun Biotech International). The frozen powder was suspended in 20 volumes of cold Laemmli loading buffer,[29] and thereafter the suspensions were heated for 5 min at 95°C and sonicated in a Bioruptor. Protein determination was done using the RCDC protein assay (Bio-Rad Laboratories). Proteins were separated by SDS-PAGE and blotted onto PVDF membranes (Immunblot, Bio-Rad). Blots were pre-incubated in TTBS (10 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 0.1% Tween-20) supplemented with 5% BSA and incubated with diluted primary antibodies against Akt or phospho(Thr308)-Akt (rabbit polyclonal, Cell Signaling) or phospho(Ser473)-Akt (mouse monoclonal, New England Biolabs). Blots were probed with horseradish peroxidase conjugated goat or mouse anti-rabbit secondary antibody. Signals were visualized using Supersignal® West Femto Maximum Sensitivity Substrate (Pierce) and Hyperfilm™ ECL (Amersham Biosciences). Signal densities were quantified using a Bio-Rad calibrated GS-800 scanner.

Dihydroethidium (DHE) fluoresecence. Superoxide anion generation from ischemic-reperfused myocardium was determined using dihydroethidium (DHE) fluorescence.[7, 30] Hearts were excised and LV transmural tissue samples were placed in ice-cold saline, embedded in

OCT (while marking the area at risk), frozen in liquid nitrogen cooled isopentane and stored at -60°C. Tissue sections of 5 µm were cut using a cryostat, thaw-mounted on Fisher-Plus (Fisher Scientific) slides, and stained with 10 µM DHE at 37°C for 30 min. Fluorescent images were obtained with a 585 nm long-pass filter. Generation of superoxide by tissue was demonstrated by red fluorescent labeling. Images were analysed on a microscopy image analysis system (Impak C, Clemex vision Image analysis system, Clemex technologies, Quebec) on which a sub-routine has been written to assess the total fluorescence per slide in order to quantify the amount of radical damage. At least five determinations were performed in each group.

Data Analysis and Presentation

Infarct size data were analysed using two-way (duration of index ischemia x POC) ANOVA followed by post-hoc testing with Student-Newman-Keuls test. Heart rate and arterial blood pressure were analysed using two-way (time x treatment) ANOVA for repeated measures followed by Student-Newman-Keuls test. SDS-PAGE allows loading of only 12 samples per gel. Therefore, the signal intensities on the immunoblot, expressed as fold increase compared with corresponding controls on the same gel, were analyzed using Student-Newman-Keuls test. Statistical significance was accepted when $P \leq 0.05$. All data are presented as mean±SEM.

Results

Overall Mortality and Exclusion of Animals

Of the 271 rats that entered the study, 9 rats were excluded due to technical failure, 5 rats were excluded due to an area at risk <10% of the left ventricle and 4 rats died prematurely during the index ischemia because of pump failure (no more than 1 rat per group).

Importance of the duration of index ischemia

Infarct Size. There were no intergroup differences in the AR (40±1%; p=0.56) between the experimental groups (data not shown). Increasing the duration of index ischemia from 15- to 120-min produced a progressively greater infarct size in the animals of the control group, reaching a plateau after CAO60 (Figure 2). Postconditioning with 3POC30 was cardioprotective when the stimulus was applied after CAO45 and CAO60, but was ineffective when applied after coronary occlusions of longer duration (CAO90 and CAO120). Conversely, when the 3POC30 stimulus was applied following CAO15 and CAO30, infarct size was increased compared to their respective control groups (Figure 2).

Hemodynamics. Baseline heart rate and mean arterial blood pressure for all animals allocated to the infarct size studies were 339±3 bpm and 93±1 mmHg, with no significant differences in heart rate (p=0.12) and mean arterial blood pressure (p=0.60) between the

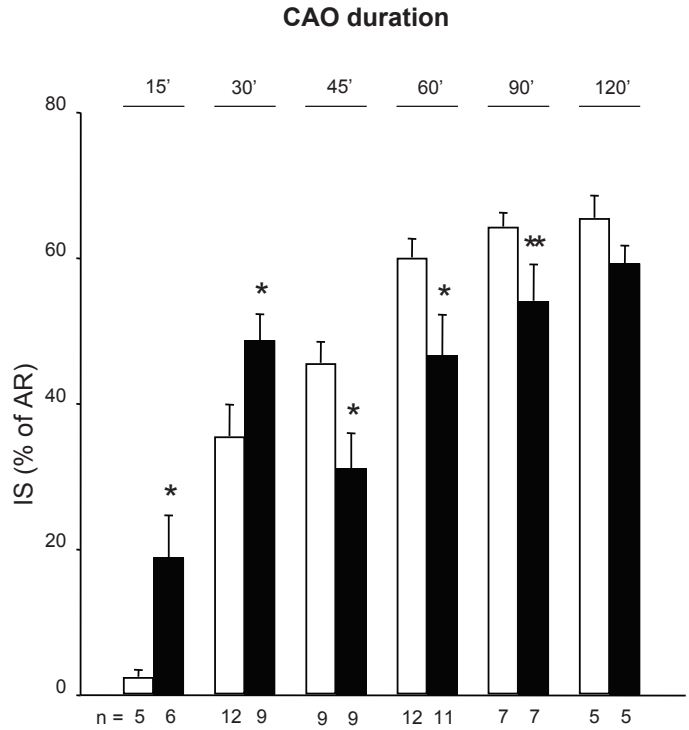

Fig. 2. Effect of postconditioning with 3POC30 on infarct size (IS, % of area at risk) produced by coronary artery occlusions of different duration. Data are mean±SEM. Number of animals is shown below each bar. **P*≤0.05 vs corresponding Control; ***P*=0.08 vs corresponding Control.

experimental groups (Table 1). Collateral blood flow measurements were not performed in the present study, in view of earlier reports that the rat heart is devoid of a significant collateral circulation in the heart.[31] Similar to previous observations from our laboratory,[20] stepwise regression analysis (with occlusion duration, 3POC30 stimulus, and rate-pressure product as independent variables) showed that there was no correlation between infarct size and the rate-pressure product either at the onset of index ischemia (*P* =0.61) or at the onset of initial reperfusion (*P*=0.17; data not shown).

Importance of the postconditioning stimulus

The additional experiments using a postconditioning stimulus with shorter reocclusion–reperfusion cycles revealed that this increase in infarct size by 3POC30 following CAO30 was not specific for this particular postconditioning stimulus, because infarct size was similarly increased when CAO30 was followed by 3POC5 (*P*=0.15 vs control) or 3POC15 (*P*<0.05 vs control; Figure 3). Furthermore, there was no significant difference between the different postconditioning stimuli (*P*>0.20). However there was a significant difference compared to control when all three postconditioning stimuli were combined compared to control

Table 1. Heart rate and arterial blood pressure of the rats studied for the effects of postconditioning with 3POC30 on infarct size

Experimental groups	n	Baseline	Occlusion		Reperfusion		
			15-min CAO	end CAO	15-min	60-min	120-min
CAO15	5						
HR		342 ± 17	348 ± 15	348 ± 15	348 ± 14	343 ± 14	356 ± 17
MAP		84 ± 9	91 ± 11	91 ± 11	79 ± 7	80 ± 9	78 ± 9
CAO15+POC	6						
HR		355 ± 10	363 ± 10	363 ± 10	356 ± 10	355 ± 8	371 ± 9
MAP		101 ± 5	100 ± 6	100 ± 6	98 ± 6	103 ± 6	99 ± 4
CAO30	12						
HR		359 ± 12	375 ± 11	363 ± 9	368 ± 11	373 ± 15	374 ± 15
MAP		93 ± 4	92 ± 7	89 ± 4	91 ± 4	87 ± 6	79 ± 7
CAO30+POC	9						
HR		337 ± 15	353 ± 14	349 ± 17	360 ± 15	366 ± 13	375 ± 13*
MAP		88 ± 6	87 ± 5	86 ± 4	87 ± 4	86 ± 6	78 ± 4
CAO45	9						
HR		356 ± 13	359 ± 9	354 ± 10	353 ± 12	357 ± 14	359 ± 14
MAP		90 ± 5	87 ± 6	85 ± 4	85 ± 4	81 ± 6	74 ± 6*
CAO45+POC	9						
HR		337 ± 10	348 ± 11	342 ± 8	346 ± 10	343 ± 10	361 ± 8*
MAP		95 ± 3	93 ± 5	97 ± 3	98 ± 3	92 ± 4	86 ± 5
CAO60	12						
HR		315 ± 7	326 ± 9	336 ± 11*	337 ± 8*	342 ± 8*	364 ± 11*
MAP		94 ± 3	101 ± 7	98 ± 4	96 ± 4	98 ± 5	93 ± 4
CAO60+POC	11						
HR		332 ± 9	349 ± 7*	347 ± 8*	345 ± 8	358 ± 9*	375 ± 11*
MAP		95 ± 4	99 ± 4	100 ± 4	99 ± 5	97 ± 5	93 ± 5
CAO90	7						
HR		327 ± 6	335 ± 7	327 ± 4	331 ± 4	341 ± 9	353 ± 10*
MAP		97 ± 2	99 ± 3	94 ± 3	91 ± 4	91 ± 4	77 ± 6*
CAO90+POC	7						
HR		343 ± 6	347 ± 6	330 ± 7	340 ± 5	358 ± 9	379 ± 10*
MAP		98 ± 7	95 ± 5	91 ± 5	97 ± 4	93 ± 6	89 ± 3
CAO120	5						
HR		350 ± 10	360 ± 15	357 ± 11	366 ± 11	376 ± 7	370 ± 16
MAP		92 ± 4	99 ± 4	92 ± 5	89 ± 6	88 ± 6	76 ± 7
CAO120+POC	5						
HR		337 ± 10	333 ± 11	344 ± 12	356 ± 10	367 ± 12	363 ± 10
MAP		94 ± 2	88 ± 3	83 ± 4	91 ± 6	87 ± 6	67 ± 7*

HR = heart rate; MAP = mean aortic pressure; Data are mean±SEM; *P<0.05 vs corresponding baseline

(P<0.01). Conversely, both 3POC5 (P=0.13) and 3POC15 (P= 0.18) failed to emulate the protection against CAO60 that was observed with 3POC30 (P<0.05). Also in the CAO60 groups there was no significant difference between the different postconditioning stimuli (P>0.20), whereas there was a significant difference compared to control when all three postconditioning stimuli were combined (P<0.05).

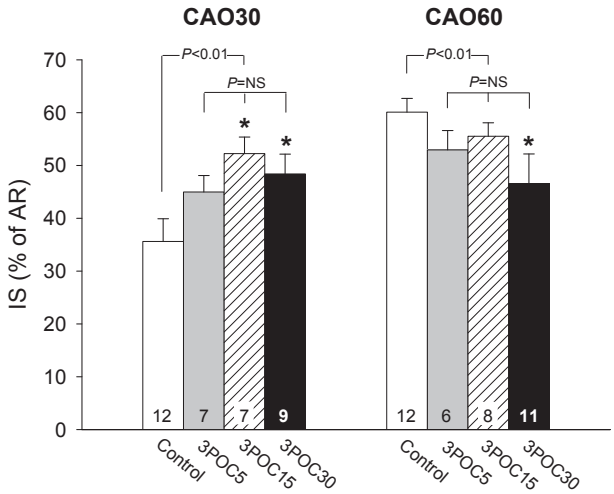

Fig. 3. Effect of postconditioning with 3POC5, 3POC15 and 3POC30 on infarct size (IS, % of area at risk) produced by CAO30 and CAO60. Data are mean±SEM. **P*≤0.05 vs corresponding Control; *P*=NS indicates non-significance for comparison between the three postconditioning stimuli.

Involvement of PI3K-Akt–eNOS signaling pathways in postconditioning

Akt phosphorylation. Total Akt was unchanged at 7 min of reperfusion after CAO15 as well as CAO60. However, there were marked increments in P(Thr308)-Akt (10- and 7-fold, respectively) and P(Ser473)-Akt (8- and 4-fold, respectively) (Figures 4A and 4B). Postconditioning with 3POC30 following CAO60 produced further increases in P(Thr308)-Akt but the further increase in P(Ser473)-Akt failed to reach statistical significance (Figure 4A and 4C); these increases were attenuated in the presence of the PI3K inhibitor wortmannin (example shown in Figure 4A). Conversely following CAO15, 3POC30 did not increase but rather tended to decrease levels of P(Thr308)-Akt and P(Ser473)-Akt (Figure 4C).

Infarct Size. Both wortmannin and the NO synthase inhibitor LNNA prevented the infarct size limitation by 3POC30 following CAO60 (Figure 5). These observations suggest that the PI3K-Akt signaling pathway mediated the cardioprotection by 3POC30 via an increase in NO synthase activity.

Hemodynamics. Wortmannin had no effect on heart rate and mean arterial blood pressure (data not shown). LNNA produced a 38±4 mmHg increase in mean aortic pressure, which was accompanied by a 19±3 bpm decrease in heart rate (both *P*≤0.05).

Involvement of reactive oxygen species in postconditioning

DHE Fluorescence. DHE reacts with superoxide anions to form ethidium bromide, which in turn intercalates with DNA to provide nuclear fluorescence as a marker for superoxide anion generation. As shown in Figure 6, DHE fluorescence was markedly enhanced at 120 min of reperfusion following CAO60 compared to sham; 3POC30 attenuated the DHE fluorescence

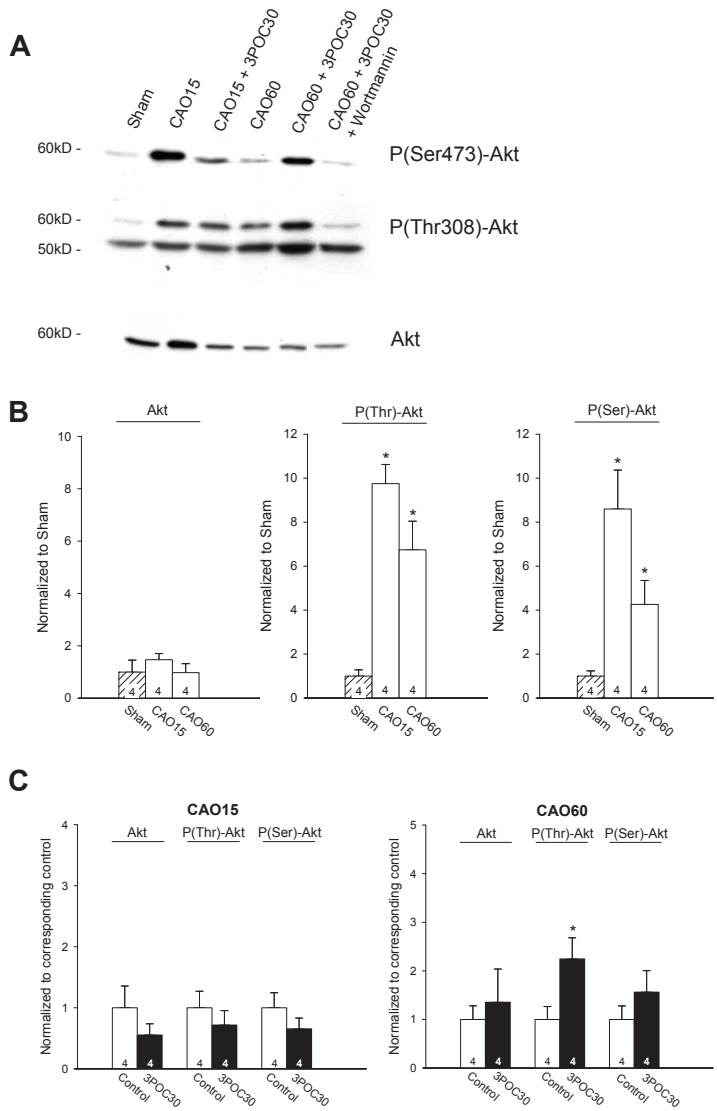

Fig. 4. Effect of postconditioning with 3POC30 on Akt phosphorylation. Panel A shows representative examples of western blots of Akt, phospho(Thr308)-Akt and phospho(Ser473)-Akt for CAO15 and CAO60 in the absence and in the presence of postconditioning with 3POC30 and Wortmannin. Panel B shows the effect of CAO15 and CAO60 on the normalized average data of these blots, while Panel C shows the normalized average data for rats undergoing CAO15 or CAO60 without (open bars) or with (solid bars) 3POC30. Data are mean±SEM; number of animals is shown in each bar. *$P \leq 0.05$ vs corresponding sham; †$P \leq 0.05$ POC vs corresponding CAO.

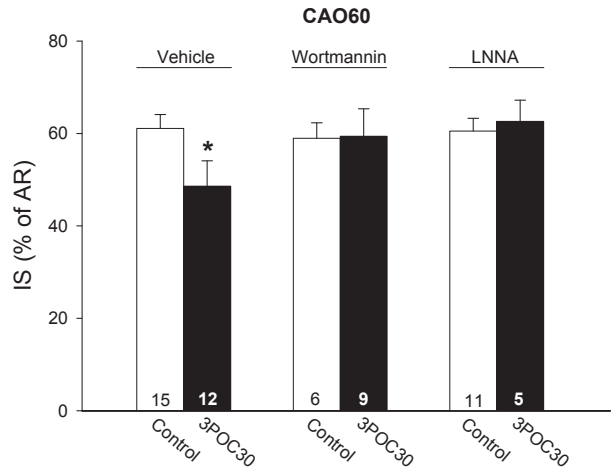

Fig. 5. Effect of wortmannin and LNNA on the cardioprotection by 3POC30 following CAO60. Open bars represent control infarcts; solid bars represent treatment with 3POC30. Data are mean±SEM; number of animals is shown in each bar. *$P \leq 0.05$ POC vs corresponding CAO60.

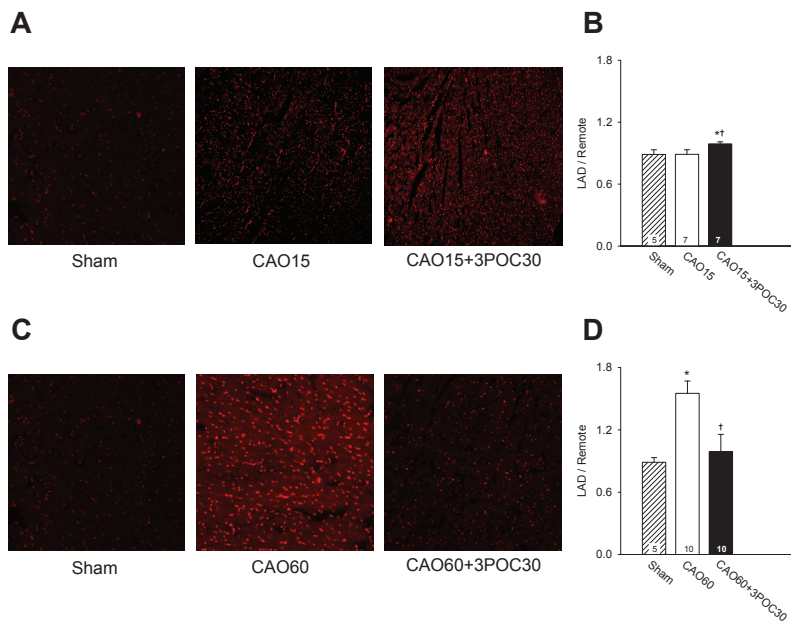

Fig. 6. Panels A and C show representative examples of DHE fluorescence (100x); Panels B and D show the average DHE fluorescence data (normalized to the remote left ventricular area) measured at 120 min of reperfusion in the myocardium at risk of rats treated with sham procedure (hatched bar), CAO15 (open bar) or CAO15 with 3POC30 (solid bar). Panel C shows representative examples and panel D shows average DHE fluorescence data (normalized to the remote left ventricular area) measured at 120 min of reperfusion in the myocardial area at risk of rats treated with either CAO60 (open bars) or CAO60 with 3POC30 (solid bars). Data are mean±SEM; number of animals is shown in each bar. *$P \leq 0.05$ vs sham; †$P \leq 0.05$ POC vs corresponding CAO.

at 120 min of reperfusion following CAO60 by 36% (Figure 6C and 6D). DHE fluorescence was not affected at 120 min of reperfusion by CAO15 compared to sham, which is consistent with observations that the burst of ROS occurs principally during the first few minutes of reperfusion following CAO15.[32] Conversely, 3POC30 following CAO15 increased DHE fluorescence at 120 min of reperfusion by 12%, compared to CAO15 alone.

Infarct Size. Treatment with a combination of ROS scavengers MPG – NAC – Tempol, which had no effect on infarct size produced by CAO15, abolished the increase in infarct size produced by 3POC30 after CAO15 (Figure 7). However, LNNA had no significant effect on the increase of infarct size produced by 3POC30 after CAO15.

Hemodynamics. Treatment with a combination of ROS scavengers MPG – NAC – Tempol produced an 11±4 mmHg decrease in mean aortic blood pressure (with no change in heart rate) 15 min after onset of the infusion, while an additional decrease in pressure of 18±4 mmHg occurred (accompanied by a 42±9 bpm increase in heart rate) during the 15-min that the infusion rates were doubled (all $P \leq 0.05$).

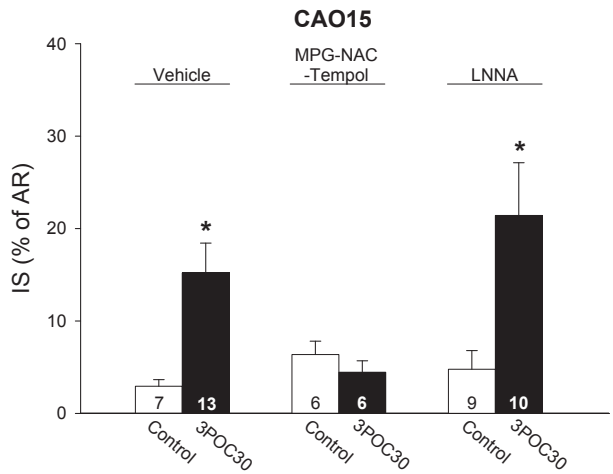

Fig. 7. Effect of a combination of the ROS scavengers MPG – NAC – Tempol and of the NO synthase inhibitor LNNA on infarct size produced by 3POC30 following CAO15. Open bars represent control infarcts; solid bars represent treatment with 3POC30. Data are mean±SEM; number of animals is shown in each bar. *$P \leq 0.05$ POC vs corresponding CAO15; †$P \leq 0.05$ vs corresponding Vehicle.

Discussion

The present results show that postconditioning of the intact rat heart by 3POC30 after CAO45 and CAO60 limits infarct size but is ineffective when applied following CAO90 and CAO120, and even aggravated infarct size when applied after CAO15 and CAO30. The detrimental ef-

fect of postconditioning on infarct size after these shorter periods of index ischemia was not stimulus-specific as postconditioning after CAO30 with 3POC5 or 3POC15 resulted in similar infarct size as postconditioning with 3POC30. The modest degree cardioprotection by 3POC30 (which was not afforded by 3POC5 or 3POC15) after CAO60 was accompanied by a further increase in stimulation of Akt phosphorylation and a reduced stimulation of superoxide production and abolished by PI3K- as well as NO synthase inhibition. The increase in infarct size by 3POC30 after CAO15 was accompanied by an increase in superoxide production, and a tendency to towards a decreased stimulation of Akt phosphorylation. Also at variance with 3POC30 after 60-min CAO, NO synthase inhibition did not affect infarct size when 3POC30 was applied after the shorter periods of index ischemia. The implications of these results will be discussed.

Importance of Index Ischemia Duration

Currently it is incompletely understood what the optimal duration of the re-occlusion and reperfusion periods of postconditioning is and to what extent its cardioprotective effect depends on the duration of index ischemia, experimental conditions or animal species.[15] For example, while four 30s periods of abrupt CAO starting 30s after the initial reperfusion, and interspersed by 30s of abrupt reperfusion limited infarct size produced by a 30-min CAO in the in situ rabbit heart,[9] it failed to afford significant protection in the isolated buffer perfused rabbit heart.[33] Furthermore, while three 30s periods of abrupt CAO starting 30s after the initial reperfusion, and interspersed by 30s of abrupt reperfusion limited infarct size produced by a 60-min CAO in the dog,[7, 14] it failed to afford cardioprotection against a 30-min CAO in the rat.[15] The latter authors proposed that in small rodents briefer periods (i.e. 10-15 s) of re-occlusion and reperfusion are required, whereas in larger animals 30 s cycles are effective.[15] In discordance with this notion, however, a recent study in swine showed that 3 cycles of 30s of re-occlusion and reperfusion failed to afford protection against infarct size produced by a 30-min CAO.[16] Since the 30s algorithm was effective against a 60-min CAO[7, 14] but not always against a 30-min CAO,[15, 16, 33] we hypothesized that another important determinant of the optimal algorithm could be the duration of index ischemia.

The present study demonstrates that the effect of postconditioning on myocardial infarct size in the rat heart depends critically on the duration of the index ischemia. Thus, while cardioprotection was observed with 3POC30 following the 45- and 60-min CAO, protection was lost with the longer occlusion durations of 90 and 120-min. This loss of protection likely reflects the progressive contribution of ischemic damage to infarction, with little contribution of reperfusion-injury, when the duration of index ischemia is prolonged. Paradoxically we observed that with 15-min CAO (which elicited negligible infarction under control conditions) as well as with 30-min CAO, 3POC30 aggravated irreversible damage. The increase in infarct size after postconditioning with 3POC30 was not a consequence of the application of this stimulus in a small animal model because postconditioning with 3POC5 or 3POC15, pur-

portedly appropriate stimuli for small animals,[15] also increased infarct size. Nevertheless, our data differ from that study[15] in which was shown that postconditioning with either 3POC10 or 3POC15 after 30-min CAO limited infarct size, while postconditioning with 3POC30 had no effect.[15] An explanation for these different observations is not readily found, but there are some differences between the two studies. First, although both studies employed male rats, we used Wistar rats whereas Vinten-Johansen *et al*.[15] used Sprague-Dawley rats. Another difference in experimental design is the use of isoflurane in addition to the pentobarbital anesthesia in the study by Vinten-Johansen *et al*.[15] This could be important, because it has been reported that isoflurane can protect the myocardium against irreversible reperfusion damage at least in part by activation of PI3K signaling.[34-36] Nevertheless, our study indicates that the effect of POC not only depends on the algorithm of POC but also on the duration of index ischemia. The present data may also help to understand why in pigs postconditioning CAO30 with 3POC30 was not cardioprotective,[16] while myocardial postconditioning in this species with the same stimulus after CAO75 was effective.[37] Furthermore, it may help to explain why in rats in one study 6POC10 afforded protection against CAO30,[15] but failed to protect against CAO60.[38]

Involvement of PI3K-Akt-eNOS Signaling Pathway and Reactive Oxygen Species

The novel finding that the effect of postconditioning on infarct size could be double-edged i.e. beneficial or detrimental, depending on the duration of the index ischema, warranted further investigation on the molecular mechanisms involved. There is already substantial evidence that several reperfusion injury survival kinase (RISK) pathways play a role in the cardioprotection by postconditioning.[15] For example, studies in the rabbit indicate that POC activates ERK1/2,[11] while the protection by postconditioning is abolished by the ERK1/2 inhibitor PD98059.[9, 11] On the other hand, the PI3K-Akt pro-survival pathway has been implicated in the protection in the isolated buffer-perfused rabbit[33] and rat[10] heart. The present study extends those observations to the *in vivo* rat heart and shows that postconditioning following CAO60 not only further increases Akt-phosphorylation, but also that the reduction of infarct size is abolished by the selective PI3K inhibitor wortmannin.[28] Further evidence for the involvement of the PI3K-Akt pro-survival pathway is the abolition of the postconditioning-induced protection by the NO synthase inhibitor LNNA, which corroborates previous findings in the *in vivo* rabbit heart.[9] How the PI3K-Akt-eNOS signaling pathway exerts its protection cannot be derived from the present observations, but there is evidence that prevention of mitochondrial permeability transition pore (MPTP) opening forms a crucial step in mediating the protection by postconditioning.[10, 12, 23-25] NO can inhibit opening of the MPTP directly but also indirectly by scavenging superoxide. In agreement with previous observations,[7, 8] observed in the present study that 3POC30 attenuated the superoxide anion production at 120 min of reperfusion which was likely due to an increased NO production by activation of the PI3K-Akt-eNOS signaling pathway.

Paradoxically, 3POC30 following CAO15 increased infarct size. This was, at variance with 3POC30 following CAO60, accompanied by an unchanged activity of the PI3K-Akt pro-survival pathway. Inhibition of NO synthase had no effect on the aggravation of irreversible injury produced by 3POC30, which is consistent with the lack of further increase of stimulation of the PI3K-Akt-eNOS pro-survival pathway. The apparent lack of NO synthase activation may also explain why we observed an increase, rather than a decrease in superoxide anion production at 120 min of reperfusion when POC followed CAO15. Administration of a combination of ROS scavengers abolished the damage by 3POC30 after CAO15, indicating that the increased oxidative stress produced by the intermittent re-occlusion and reperfusion sequences following the shorter periods (15- and 30-min) of index ischemia, contributes the increase in infarct size. Indeed, in the case of the 15-min index ischemia only 3% infarct size is observed under control conditions, which increased to 19% when 3POC30 was applied. The mechanism by which oxidative stress aggravates necrosis under this condition cannot be derived from the present study, and should be the subject of future investigations.

Conclusions

The effect of postconditioning on myocardial infarct size is ambiguous. Although most studies, including one in man[39] have shown that postconditioning is cardioprotective,[8-13] there are several reports that have failed to observe a cardioprotective effect.[15] This discrepancy has been ascribed to the employed POC algorithm in relation to the species studied. The present study demonstrates that the cardiac effects of postconditioning may even be detrimental and that this deleterious effect depended critically on the duration of the preceding period of index ischemia rather than the employed stimulus. These paradoxical effects of postconditioning are best explained by the distinct effects on Akt phosphorylation and superoxide anion production.

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Chapter 9

Protective as well as detrimental effects of postconditioning are lost in the preconditioned rat heart

Olivier C Manintveld, Maaike te Lintel Hekkert, Nathalie T van der Ploeg,
Dick H Dekkers, Pieter D Verdouw and Dirk J Duncker.

Submitted

Abstract

Mechanical modulation of coronary blood flow during early reperfusion, termed postconditioning (POC), can afford cardioprotection. However, since many patients with an impending myocardial infarction experience pre-infarct angina that may induce ischemic preconditioning (IPC) or tolerance to IPC, it is important to establish whether POC is still effective in preconditioned myocardium. Previous reports are ambiguous, possibly due to differences in preconditioning algorithm (activating divergent signaling pathways) and/or the duration of index ischemia (influencing the effect of postconditioning). Taking these considerations into account, we studied the interaction between IPC (by either 1CAO15 or 3CAO3) and POC, and investigated whether myocardium that had become tolerant to preconditioning also becomes resistant to the protection by POC. Myocardial infarction was produced in male pentobarbital-anesthetized Wistar rats via coronary artery occlusion (CAO) lasting either 60-min (CAO60) or 30-min (CAO30), followed by 120-min of reperfusion. Following CAO60, POC afforded cardioprotection that was comparable to IPC with either 1CAO15 or 3CAO3. POC did not afford additional protection when hearts had been subjected to IPC prior the CAO60, irrespective of the IPC algorithm. Nitric oxide (NO) synthase inhibition with N-nitro-L-arginine abolished the cardioprotection by POC, both IPC stimuli, and the combination of POC and either IPC stimulus. Following CAO30, POC paradoxically increased infarct size, which was prevented by IPC, irrespective of the IPC algorithm. Finally, myocardium tolerant to the cardioprotection by 1CAO15 was no longer protected by POC after CAO60. In conclusion, POC does not afford additional protection of preconditioned hearts after a 60-min index ischemia, irrespective of the IPC stimulus and irrespective of the presence of tolerance to IPC. This lack of additional protection may have its mechanistic basis in the observation that both POC and IPC stimuli are mediated via NO synthase. Finally, the myocardial damage produced by POC following a 30-min index ischemia can be prevented by either IPC stimulus. These findings indicate that the interaction between IPC and POC is highly dependent on the duration of index ischemia, but is independent of the IPC algorithm.

Introduction

Myocardial infarct size is an important determinant of subsequent cardiac remodelling and progression towards heart failure.[1, 2] Currently the only proven therapy to limit infarct size is early reperfusion therapy.[3-5] However, despite its clear benefits, reperfusion itself has been proposed to cause irreversible myocardial damage, termed "reperfusion injury", beyond that caused by the preceding period of ischemia, implying that optimization of reperfusion therapy could further limit infarct size.[3-5] Encouraging, albeit at times equivocal, results from experimental studies have prompted a large number of clinical trials investigating the therapeutic potential of pharmacological agents against reperfusion injury in the setting of acute myocardial infarction.[3-5] However, the results of these clinical trials generally have been disappointing. Several explanations for the discrepancy between experimental and clinical studies have been forwarded,[4] including the absence of underlying cardiovascular disease or risk factors thereof in the majority of experimental studies, while in patients risk factors or pre-existing symptomatic obstructive coronary artery disease are typically present.[4] In these patients, the resulting pre-infarct ischemia may either have resulted in myocardial preconditioning or in tolerance to preconditioning, due to repeated bouts of short periods of ischemia preceding infarction,[6, 7] or the presence of risk factors,[8] thereby potentially modifying the efficacy of pharmacological therapies aimed at reducing reperfusion injury that target similar signaling pathways as IPC.[9]

The discovery of the cardioprotective effect of one or multiple sequences of re-occlusion and reperfusion during early reperfusion (stuttering reperfusion)[10] or gradual reperfusion,[11-15] following a period of sustained ischemia, termed "postconditioning" (POC), has been proposed as an adjunctive strategy to optimize reperfusion therapy[16, 17]. Preliminary clinical trials have reported encouraging results,[18-22] and it is therefore important to determine whether pre-existing myocardial ischemia modulates the effects of POC on infarct size in order to allow proper interpretation of these and future clinical trials. The question whether POC can afford additional protection, i.e. further limit infarct size, when myocardium is already preconditioned has been addressed in a few experimental studies but results are ambiguous.[23-25] This could be due not only to differences in animal species, anesthesia regimen and algorithm of the postconditioning stimulus,[17] but also to the type of preconditioning stimulus employed[26, 27] and the duration of index ischemia.[28] For example, depending on the duration of index ischemia, POC can be protective, have no effect or even be detrimental.[28, 29] In light of these considerations, the first aim of the present study was to determine the interaction between IPC and POC taking into account the algorithm of the IPC stimulus. In view of the proposed role of NO in the signal transduction of both IPC[8, 30] and POC,[24, 25, 31] we investigated the obligatory role of NO in the cardioprotection by IPC and POC. Furthermore, we studied whether in myocardium that had become tolerant to IPC[6, 7] protection could be reinstated by POC. Finally, in view of recent observations in our laboratory that POC can increase infarct

size when applied following a brief period of index ischemia,[28] we investigated whether IPC could protect against the myocardial damage produced by POC and determined the role of NO herein.

Methods

Animals

Experiments were performed in 254 male Wistar rats (300-380 g) in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 86-23, revised 1996) and with prior approval of the Animal Care Committee of the Erasmus MC, University Medical Center Rotterdam.

Surgical and Experimental Procedures

Pentobarbital-anesthetized (60 mg/kg intraperitoneally) 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.[7, 28, 32] In the inferior caval vein a PE-50 catheter was placed for infusion of Gelofusin (5-10 ml; B. Braun, Melsungen AG) to maintain central venous pressure at 4-6 mmHg, and for administration of drugs. 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 occlusion of the vessel. A catheter was positioned in the abdominal cavity to allow intraperitoneal administration of pentobarbital for maintenance of anesthesia. Rectal temperature was maintained at 36.5-37.5˚C.[7, 28, 32] After completion of surgery, a 30-min stabilization period was allowed before animals were subjected to the experimental protocols. Rats that fibrillated during occlusion or reperfusion were allowed to complete the protocol, provided that conversion to normal sinus rhythm occurred spontaneously within 1-min, or that defibrillation via gently thumping on the thorax was successful within 2-min after onset of fibrillation. Occlusion and reperfusion were visually verified. The area at risk (AR) and infarct area (IA) were determined after 120-min of reperfusion, using Trypan Blue and nitro-blue-tetrazolium staining, respectively.[7, 28, 32] Infarct size (IS) was calculated as IA/ARx100%.

Experimental protocols

In the first series of experiments, the effect of POC after a 60-min coronary artery occlusion (CAO60) followed by 120-min of reperfusion was studied in animals that were preconditioned with either the adenosine-dependent (but reactive oxygen species (ROS)-independent) stimulus 1CAO15 or the ROS-dependent (but adenosine-independent) stimulus 3CAO3 (Figure 1, Protocol 1).[26, 27] Hearts were preconditioned by either a 15-min CAO preceding the

index ischemia by 10-min reperfusion (1CAO15) or three sequences of 3-min CAO and 5-min of reperfusion (3CAO3). POC was started 30 sec after termination of the index ischemia and consisted of 3 periods of 30 sec CAO separated by 30 sec of reperfusion.[28] To study the role of NO, sham- and conditioned rats were pretreated with the NO synthase inhibitor N-nitro-L-arginine (LNNA, 25 mg/kg iv).[28, 33]

In a second series of experiments the effect of POC on myocardium that was made tolerant to IPC was studied (Figure 1, Protocol 2). Tolerance to IPC was induced by 4CAO15 interspersed by 15 min of reperfusion and applied between 175 and 70 min prior to the 60-min index ischemia (Figure 1, Protocol 2).[7]

Finally, in view of recent observations that POC can increase infarct size when applied following a brief period of index ischemia,[28, 29] we investigated whether IPC could protect against the myocardial damage produced by POC after a 30-min CAO (CAO30) followed up by 120-min of reperfusion and determined the role of NO herein (Figure 1, Protocol 3).

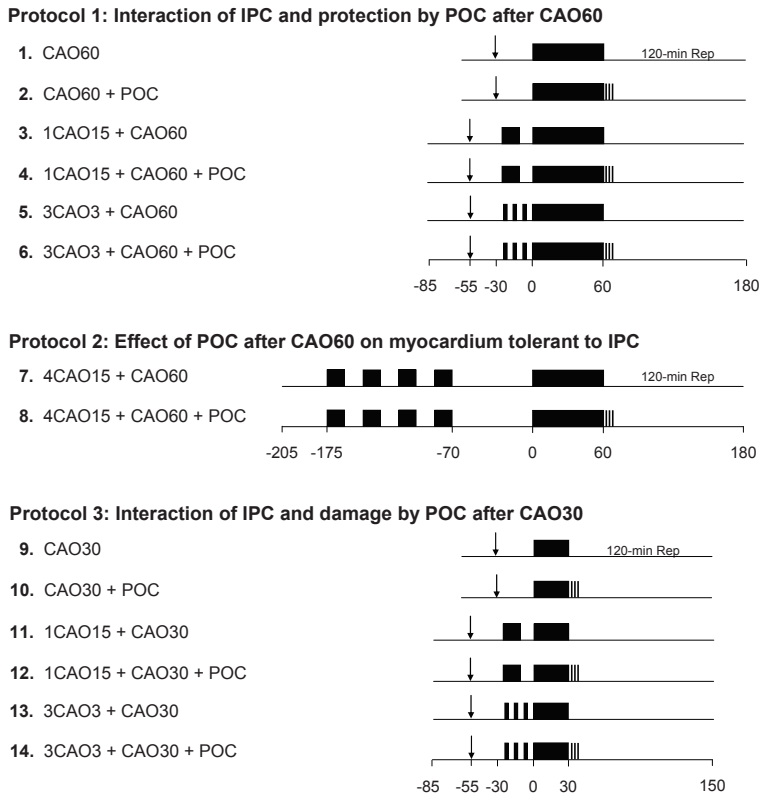

Fig. 1. Shown are the infarct size protocols involving a 60-min CAO or a 30-min CAO with different IPC stimuli with or without POC including the administration of LNNA (↓).

Data Analysis and Presentation

All data were analyzed using ANOVA for repeated (hemodynamics) or non-repeated (infarct size) measures, followed by post hoc testing with Student-Newman-Keuls test. Statistical significance was accepted when $P \leq 0.05$. All data are presented as mean±SEM.

Results

Mortality and Exclusions

Of the 254 rats that entered the study, 3 rats were excluded due to technical failure, 5 rats were excluded due to acute heart failure upon reperfusion (no more than 2 rats in a single experimental group), while 5 rats were excluded due to an AR <15% of the left ventricle.

Hemodynamic data

There were no significant differences in heart rate or mean aortic pressure at baseline between any of the treated groups undergoing CAO60 (Table 1), and only minor differences in the groups undergoing CAO30 (Table 2). LNNA produced an increase in blood pressure from 87±1 to 110±2 mmHg, which was accompanied by a decrease in heart rate from 320±2 to 289±2 bpm (both $p<0.05$). Importantly, there was no significant correlation between the rate-pressure product at the onset of the CAO and the corresponding IS (linear regression: $r^2 = 0.007$; $p=0.22$).

Infarct Size

There were no intergroup differences in AR (41±1% of the left ventricle; $p=0.46$) between the experimental groups. CAO60 amounted to an IS of 60±3%, while 1CAO15 limited IS to 44±5% and 3CAO3 to 43±3%; POC was equally cardioprotective (IS=46±5%) (for all stimuli: $P<0.05$ vs CAO60). Applying POC in myocardium preconditioned by either 1CAO15 or 3CAO3 did not afford additional protection over that produced by 1CAO15, 3CAO3 or POC alone, i.e. irrespective of the IPC algorithm. LNNA had no effect on IS itself (59±3%), but abolished the cardioprotective effect of 1CAO15, 3CAO3 or POC alone (60±2%, 61±2% and 62±2%, respectively) as well as that of the combination (1CAO15+POC: 57±3% and 3CAO3+POC 60±1%; Figure 2).

Secondly, we investigated the effect POC after CAO60 on myocardium made tolerant by 4CAO15 to the cardioprotection by 1CAO15.[7] POC resulted in an IS of 59±3%, which was not different from either IS in control rats undergoing CAO60 (60±3%) or IS in rats having undergone 4CAO15 followed by CAO60 (67±3%), indicating that POC failed to afford protection in myocardium tolerant to IPC (data not shown in Figure).

Finally, CAO30 resulted in an IS of 38±3%. IPC with either 1CAO15 or 3CAO3 limited IS to 21±3% and 19±4%, respectively, whereas CAO30+POC increased IS to 50±3% (all $P<0.05$ vs

Table 1. Heart rate and arterial blood pressure

Experimental groups	n	Baseline	Pre-CAO	End-CAO	15-min CR	60-min CR	120-min CR
Untreated groups							
CAO60 control	11						
HR		320 ± 5	313 ± 7	319 ± 6	320 ± 6	321 ± 5	335 ± 6*
MAP		94 ± 3	92 ± 3	89 ± 2	88 ± 2	88 ± 3	89 ± 4
1CAO15+CAO60	9						
HR		329 ± 5	328 ± 15	337 ± 15	337 ± 15	360 ± 17	350 ± 19
MAP		94 ± 5	87 ± 4	83 ± 5	85 ± 6	82 ± 6	74 ± 7*
3CAO3+CAO60	10						
HR		330 ± 16	334 ± 18	343 ± 21	341 ± 22	339 ± 24	346 ± 17
MAP		83 ± 4	90 ± 5	83 ± 6	87 ± 6	83 ± 6	72 ± 7*
CAO60+POC	12						
HR		327 ± 7	327 ± 7	342 ± 7*	342 ± 6*	355 ± 8*	365 ± 11*
MAP		94 ± 4	95 ± 3	99 ± 4	97 ± 4	93 ± 5	92 ± 5
1CAO15+CAO60+POC	10						
HR		341 ± 8	351 ± 7	354 ± 11	364 ± 11	373 ± 11*	395 ± 12*
MAP		92 ± 3	88 ± 5	90 ± 6	91 ± 7	84 ± 8	85 ± 5
3CAO3+CAO60+POC	8						
HR		348 ± 11	356 ± 14	366 ± 12	369 ± 12	377 ± 11*	396 ± 10*
MAP		94 ± 4	99 ± 4	94 ± 2	99 ± 3	93 ± 4	87 ± 4
LNNA treated groups							
CAO60 control	10						
HR		315 ± 6	294 ± 7	313 ± 11	310 ± 10	318 ± 12	308 ± 17
MAP		88 ± 3	136 ± 4*	101 ± 5	84 ± 7	75 ± 6	70 ± 6*
1CAO15+CAO60	11						
HR		322 ± 10	295 ± 10*	298 ± 11*	297 ± 13*	310 ± 15	303 ± 7*
MAP		90 ± 3	114 ± 10*	109 ± 7*	104 ± 8	94 ± 8	71 ± 8*
3CAO3+CAO60	8						
HR		307 ± 13	265 ± 17*	287 ± 21	293 ± 19	308 ± 18	321 ± 19
MAP		81 ± 2	122 ± 9*	95 ± 8	87 ± 8	75 ± 7	67 ± 9
CAO60+POC	10						
HR		335 ± 7	302 ± 5*	305 ± 7*	302 ± 5*	303 ± 6*	320 ± 5
MAP		95 ± 5	121 ± 7*	97 ± 5	88 ± 4	85 ± 5	79 ± 7
1CAO15+CAO60+POC	9						
HR		321 ± 10	291 ± 8*	293 ± 8*	296 ± 7*	296 ± 7*	321 ± 11
MAP		86 ± 2	100 ± 4*	88 ± 4	85 ± 6	76 ± 8	80 ± 7
3CAO3+CAO60+POC	6						
HR		307 ± 6	266 ± 2*	264 ± 5*	269 ± 6*	283 ± 6*	285 ± 8*
MAP		86 ± 4	113 ± 3*	98 ± 2	97 ± 3	87 ± 3	66 ± 8*

HR=heart rate; MAP=mean aortic pressure; CAO=coronary artery occlusion; CR=coronary reperfusion; Data are mean±SEM; *P<0.05 vs corresponding baseline

Table 2. Heart rate and arterial blood pressure

Experimental groups	n	Baseline	Pre-CAO	End-CAO	15-min CR	60-min CR	120-min CR
Untreated groups							
CAO30 control	11						
HR		344 ± 12	348 ± 14	349 ± 14	355 ± 15	357 ± 17	362 ± 17
MAP		83 ± 3	89 ± 3	85 ± 3	90 ± 3	87 ± 4	78 ± 6
1CAO15+CAO30	11						
HR		359 ± 12†	352 ± 13	354 ± 12	353 ± 15	345 ± 13	357 ± 13
MAP		90 ± 2	87 ± 3	88 ± 4	91 ± 4	91 ± 3	91 ± 5
3CAO3+CAO30	8						
HR		343 ± 7	337 ± 8	348 ± 8	338 ± 7	340 ± 4	349 ± 7
MAP		100 ± 4†	98 ± 4	97 ± 2	98 ± 3	99 ± 3	93 ± 8
CAO30+POC	11						
HR		335 ± 9	333 ± 12	343 ± 14	352 ± 14*	354 ± 11*	367 ± 12*
MAP		92 ± 4	89 ± 5	86 ± 3	88 ± 4	87 ± 5	79 ± 4*
1CAO15+CAO30+POC	11						
HR		345 ± 9	355 ± 11	355 ± 11	344 ± 10	344 ± 7	354 ± 9
MAP		93 ± 4	93 ± 3	91 ± 4	95 ± 2	92 ± 3	90 ± 4
3CAO3+CAO30+POC	7						
HR		346 ± 9	347 ± 8	342 ± 8	341 ± 7	335 ± 8	346 ± 7
MAP		98 ± 3†	104 ± 3	92 ± 4	98 ± 4	96 ± 2	98 ± 3
LNNA treated groups							
CAO30 control	8						
HR		329 ± 8	298 ± 4*	305 ± 5*	306 ± 5*	317 ± 11	316 ± 4
MAP		93 ± 5	113 ± 3*	103 ± 5	98 ± 5	93 ± 4	85 ± 5
1CAO15+CAO30	11						
HR		326 ± 7	299 ± 5*	295 ± 5*	297 ± 5*	318 ± 10	317 ± 6
MAP		85 ± 2	93 ± 5*	85 ± 4	86 ± 6	83 ± 5	72 ± 7*
3CAO3+CAO30	8						
HR		308 ± 7	275 ± 7*	279 ± 5*	280 ± 6*	283 ± 12*	279 ± 10*
MAP		78 ± 2	88 ± 5*	80 ± 5	77 ± 3	61 ± 5*	53 ± 4*
CAO30+POC	9						
HR		323 ± 4	293 ± 6*	297 ± 6*	295 ± 6*	302 ± 6*	313 ± 9
MAP		90 ± 4	114 ± 8*	99 ± 7	93 ± 7	93 ± 5	79 ± 8
1CAO15+CAO30+POC	9						
HR		322 ± 7	298 ± 5*	297 ± 5*	299 ± 5*	303 ± 5*	314 ± 8
MAP		85 ± 3	99 ± 6*	97 ± 4*	95 ± 4	93 ± 5	88 ± 6
3CAO3+CAO30+POC	6						
HR		317 ± 7	268 ± 6*	263 ± 9*	267 ± 8*	276 ± 6*	288 ± 11*
MAP		84 ± 3	110 ± 6*	91 ± 6	95 ± 7	79 ± 7	64 ± 6*

HR=heart rate; MAP=mean aortic pressure; Data are mean±SEM; *P<0.05 vs corresponding baseline; †P<0.05 vs baseline in 3CAO3+CAO30 pre-treated with LNNA.

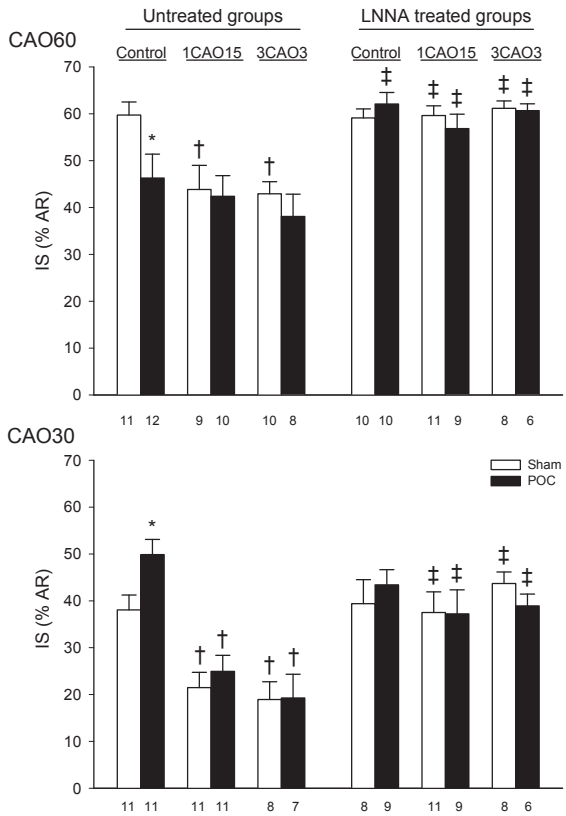

Fig. 2. The effects of CAO60 (upper panel) and CAO30 (bottom panel) after the different IPC stimuli on myocardial infarct size with or without POC are shown. Data are mean±SEM. The number of animals is indicated below each bar. **P*<0.05 vs POC in corresponding Sham; †*P*<0.05 vs corresponding Control; ‡P<0.05 vs corresponding untreated group.

CAO30). Combining IPC and POC nullified the increase in IS seen with POC irrespective of the employed IPC stimulus (1CAO15+POC: 26±4% and 3CAO3+POC 19±5%). LNNA, which did not affect IS by itself (38±3%), abolished the cardioprotective effect of 1CAO15 and 3CAO3 (39±5% and 37±4%, respectively), while the increase in IS by POC was virtually abolished (43±3%). LNNA blocked the protective effect when 1CAO15 and POC were combined (37±5% vs 25±3%) as well as the protective effect when 3CAO3 and POC were combined (39±3% vs 19±5%; Figure 2).

Discussion

The main findings of this study are: (*i*) Following CAO60, POC afforded cardioprotection that was comparable to IPC with either 1CAO15 or 3CAO3. (*ii*) POC did not afford additional protection when hearts had been subjected to IPC prior the CAO60, irrespective of the IPC algorithm. (*iii*) Furthermore, myocardium that had become tolerant to the cardioprotection by 1CAO15 was no longer protected by POC after CAO60. (*iv*) Inhibition of NO synthase abolished the cardioprotection by POC, IPC and the combination of IPC + POC after CAO60, irrespective of the IPC algorithm. (v) Following CAO30, the increase in IS produced by POC was prevented by IPC, which was mediated by NO and occurred independent of the IPC algorithm.

Influence of IPC algorithm on the interaction between IPC and POC. The question whether POC can afford additional protection, i.e. further limit infarct size, when myocardium is already preconditioned has been addressed in a few experimental studies but results are ambiguous,[23-25] with either no additional[23, 24] or additional cardioprotection[25] when IPC and POC were combined. These divergent findings could be due to a number of factors, including animal species, anesthesia regimen, algorithm of the postconditioning stimulus,[17] the type of preconditioning stimulus employed[26, 27] and the duration of index ischemia.[28] Indeed, all three studies were performed in different animal species under different experimental conditions, and differed in terms of algorithm of the POC stimulus, period of index ischemia and IPC stimulus (Table 3). We have previously shown that IPC can activate distinctly different signaling pathways.[26, 27] For example, IPC with 1CAO15 was found to be adenosine-dependent and ROS-independent, while IPC with 3CAO3 exerted its protective effect through ROS formation, but did not require adenosine receptor activation.[26, 27] Interestingly, both stimuli affect mitochondrial respiration,[27] which is likely mediated by NO (unpublished data from our laboratory), suggesting that these pathways ultimately converge at the level of NO-mediated modulation of mitochondrial respiration.[34] In light of these considerations, we specifically addressed in the present study the influence of the type of IPC stimulus on the cardioprotection by POC. We observed that irrespective of the transduction pathway employed by the IPC stimulus, POC did not afford additional cardioprotection after CAO60 in rat hearts subjected to IPC. The observation that inhibition of NO synthase abolished the cardioprotection by either IPC stimulus and by POC, either alone or combined, indicates that both IPC and POC stimuli require unperturbed NO signaling in order to exert their cardioprotection. Furthermore, these data could be interpreted to suggest that IPC already resulted in (near) maximum NO signaling so that POC could not stimulate this pathway further.

Although the present study fails to support the concept that POC affords additional protection when myocardium has been preconditioned prior to the index ischemia, it could be argued that a different POC algorithm (employing perhaps a different signaling pathway than IPC) might still be able to further limit infarct size in preconditioned myocardium.[17, 35] Indeed, in the rabbit heart in which POC (by four cycles of 30s reperfusion and 30s ischemia) and IPC

Table 3. Studies on the Interaction between POC and IPC against Infarction produced by Regional Myocardial Ischemia.

Studies	Experimental Set-up		IPC Stimulus (I'+R')	Index Ischemia (I'+R')	POC Stimulus (R"+I")	Infarct Size (% of Area at Risk)			
	Animal	Experimental Conditions				Control	POC	IPC	IPC +POC
Halkos *et al*.[1]	Dog	Morphine Chloralose anesthesia, Open-Chest	1x 5'+10'	60'+180'	3x 30"+30"	24±2	10±1*	13±2*	12±3*
Yang *et al*.[3]	Rabbit	Pentobarbital anesthesia, Open-Chest	1x 5'+10'	45'+180'	4x 30"+30"	62±2	40±4*	35±4*	23±3*†
Tsang *et al*.[2]	Rat	Isolated Buffer-Perfused	2x 5'+10'	35'+120'	6x 10"+10"	51±3	32±4*	28±2*	30±5*
Manintveld *et al*.	Rat	Pentobarbital anesthesia, Open-Chest	3x 3'+ 5' 1x 15'+10'	60'+120' 60'+120'	3x 30"+30" 3x 30"+30"	60±3	46±5*	46±2* 44±2*	38±5* 42±4*
			3x 3'+5' 1x 15'+10'	30'+120' 30'+120'	3x 30"+30" 3x 30"+30"	38±3	50±3*	19±4* 21±3*	19±5* 25±3*

IPC = ischemic preconditioning; POC = postconditioning; I = ischemia; R = reperfusion. *P<0.05 vs Control; †P<0.05 IPC+POC vs IPC

(by 1CAO5) afforded additive cardioprotection, POC activated extracellular signal-regulated kinase (ERK) without activating Akt,[25, 36] while IPC was ERK-independent.[25] Conversely, we have previously shown that the POC algorithm that was used in the present study resulted in activation of Akt following a 60-min CAO in rats, while its cardioprotection was blocked by wortmannin,[28] implicating a critical role for PI3K-Akt signaling. Interestingly, recent observations from our laboratory indicate that IPC by 1CAO15, but not by 3CAO3, is associated with activation of Akt and is blocked by wortmannin (unpublished data). The observation that the PI3K-Akt dependent POC algorithm did not afford additional protection in myocardium preconditioned with the PI3K-Akt independent 3CAO3 stimulus, suggests that other POC algorithms are also unlikely to enhance protection in preconditioned rat hearts.

Efficacy of POC in myocardium made tolerant to IPC. Several investigators have shown in experimental studies that myocardium that has been repeatedly exposed to brief episodes of ischemia can become tolerant to IPC.[6, 7, 37, 38] These observations suggest that some patients with a pending myocardial infarction may develop tolerance to protection as a result of repeated episodes of pre-infarct angina. Interestingly, we previously observed that in myocardium tolerant to the adenosine-dependent stimulus 1CAO15, could still benefit from IPC by a stimulus employing a different pathway, such as the ROS-dependent stimulus 3CAO3 or remote preconditioning of the heart by a brief mesenteric artery occlusion.[7] Furthermore, pharmacological preconditioning by exogenous adenosine, which acts in part via (remote) activation of neurogenic pathway,[33] could also overcome resistance to IPC.[7, 39] It is important to establish whether patients that are tolerant to IPC can still benefit from protection by POC during the revascularization procedure. Consequently, we investigated the cardioprotective

effect of POC in myocardium that had become tolerant to 1CAO15 by repeated episodes of the CAO15 stimulus (4CAO15). The observation that tolerance to IPC resulted in cross-tolerance to the cardioprotective effects of POC, may have important clinical implications. For example, our results suggest that patients enrolled in studies into the efficacy of POC during revascularization procedures may require stratification according to the absence or presence of pre-infarct angina, as the latter may be less responsive to POC therapy.

Influence of index ischemia on the interaction between IPC and POC. We have recently shown that POC affords cardioprotection following CAO60, but increases IS when applied following CAO30, observations that were independent of the POC algorithm used.[28] Consequently, in the present study we investigated the influence of the duration of index ischemia on the interaction between IPC and POC. The results of these experiments show that IPC protected against the detrimental effects of POC following CAO30. The protective effect of IPC against POC-induced myocardial damage was mediated by NO and was independent of the IPC algorithm.

Conclusions

The present study shows that POC does not afford additional protection after a long period of index ischemia when hearts have been preconditioned, which occurs irrespective of the signal transduction pathway employed by the IPC stimulus and irrespective of whether hearts have become tolerant to IPC or not. The lack of additional protection may have its mechanistic basis in the observation that POC and either IPC stimulus are critically dependent on unperturbed NO synthase activity. Finally, the myocardial damage produced by POC following a briefer period of index ischemia can be prevented by either IPC stimulus. These findings indicate that the interaction between IPC and POC is highly dependent on the duration of index ischemia, but is independent of the IPC algorithm.

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Chapter 10

General discussion and future perspectives

Summary

This thesis presents several studies focusing on the mechanisms of cardiac adaptation to acute ischemia and reperfusion in the rat (pre- and postconditioning) and to chronic ischemia in the pig (hibernation). **Chapter 1** encompasses an introduction into the characteristics and mechanisms of these phenomenona and presents the rationale for the individual studies described in Chapters 2 through 9.

Preconditioning

Mechanism of cardioprotection

In recent years, it has become apparent that not all preconditioning stimuli employ the same signaling pathway to exert their cardioprotective action.[1-5] Nonetheless, a frequently overlooked factor in studies addressing the mechanism of IPC is the nature of the IPC stimulus that leads to cardioprotection. Although different signal transduction pathways are activated by different stimuli, they may ultimately converge into a common cardioprotective phenotype.[6] Consequently, we investigated in **Chapter 2** the signal transduction pathways in two distinctly different IPC stimuli and assessed if they possessed a common cardioprotective phenotype. The major findings of that study are that IPC by 1CAO15 involves adenosine receptor stimulation which, via PKC and tyrosine kinase signaling, results in opening of mitoK^+_{ATP} channels. In contrast, IPC by 3CAO3 does not involve adenosine receptor stimulation and does not depend on opening of mitoK^+_{ATP}-channels, but involves the release of ROS, which then activate tyrosine kinase and PKC. However, both 1CAO15 and 3CAO3, despite using distinctly different signal transduction pathways, converge at the level of the mitochondria by increasing state-2 respiration and thus cause a decrease in respiratory control index.[7]

There is a general belief that that knowledge of the endogenous mediators involved in IPC can be used "to bottle" cardioprotection.[8] However, doubt has been expressed as to whether exogenous adenosine and endogenously released adenosine during IPC employ the same signaling pathways.[9-12] Therefore, we investigated in **Chapter 3** the effect of exogenous adenosine as a pharmacological preconditioning method (ADO) compared to that of the endogenous adenosine-dependent IPC stimulus 1CAO15, described in Chapter 2. The findings demonstrate that the early phase of cardioprotection by ADO is not associated with a detectable increase in myocardial interstitial purine concentrations, whereas 1CAO15 results in a 7-fold increase from baseline, depends critically on NO production, and involves the activation of a neurogenic pathway.[13] These findings indicate that ADO administered as adjunct therapy to reperfusion treatment in patients with a pending myocardial infarction may not require access to the jeopardized myocardium, but rather may initiate cardioprotection via the endothelium within the coronary circulation but also in part at remote extra-cardiac sites

(e.g. small intestines).[14, 15] It remains to be established if exogenous compounds activating other pharmacological targets identified in IPC or remote IPC follow similar signaling pathways as the endogenous mediators.

In light of the observations in Chapters 2 and 3 that both pharmacological and IPC stimuli can protect the myocardium by different pathways,[7, 13] we investigated the role of the PI3K–Akt–eNOS signaling pathway and ERK1/2, both components of the RISK pathway, in the cardioprotection by the three aforementioned preconditioning stimuli (3CAO3, 1CAO15 and ADO) in the *in vivo* rat heart. Since endogenous release of adenosine during IPC has been proposed to afford cardioprotection via increased activity of the RISK pathway during early reperfusion,[16] we hypothesized that the adenosine-dependent (but ROS-independent) stimulus 1CAO15 but not the adenosine-independent (but ROS-dependent) stimulus 3CAO3 involves activation of the RISK pathway. Conversely, the IPC-induced activation of the Janus kinase/transducer activator of transcription-3 (JAK/STAT) pathway[17, 18] and possibly also adenosine 5'-monophosphate-activated protein kinase (AMPK)[19] has been shown to depend on ROS signaling. Consequently, in **Chapter 4** we hypothesized that 3CAO3, but not 1CAO15, activates the JAK/STAT and AMPK pathway. Finally, because in the isolated rabbit heart cardioprotection by intravascular adenosine has been reported to be PI3K-independent,[20] we hypothesized that in the *in vivo* rat heart the RISK pathway is not involved in pharmacological preconditioning by intravenous infusion of exogenous adenosine. All three stimuli increased state-2 respiration thereby decreasing the respiratory control index, which was accompanied by a limitation of infarct size produced by a 60-min CAO. NO synthase inhibition abolished the mitochondrial effects and the cardioprotection by 3CAO3, 1CAO15 or ADO. In contrast, the PI3K inhibitor wortmannin blocked protection by 1CAO15, but did not affect protection by 3CAO3 or ADO. Western blotting confirmed that the RISK pathway was activated by 1CAO15, but not by 3CAO3 or ADO. The latter two stimuli also failed to activate the JAK/STAT or the AMPK pathway.

Based on the results obtained in Chapters 2-4, we have depicted the various signaling pathways the three cardioprotective stimuli in Figure 1. Inspection of Figure 1 demonstrates that whereas the signaling pattern in 1CAO15 is highly detailed, the pathways by which 3CAO3 and ADO modulate mitochondrial respiration are still incompletely understood. Future research should assess the following: A more detailed description of the signaling pathway involved in the cardioprotection by preconditioning with 3CAO3 or ADO is warranted. What are the remote extracardiac sites involved in the cardioprotection with ADO? What the mechanistic relationship between infarct size limitation after ADO and the cardioprotection afforded by remote IPC? What is the specific locus and source of the NO production in all three preconditioning stimuli? Finally, future studies are required to determine the molecular mechanism(s) via which NO increases state-2 respiration and decreases RCI and leads to cardioprotection in our *in vivo* rat model of myocardial infarction.

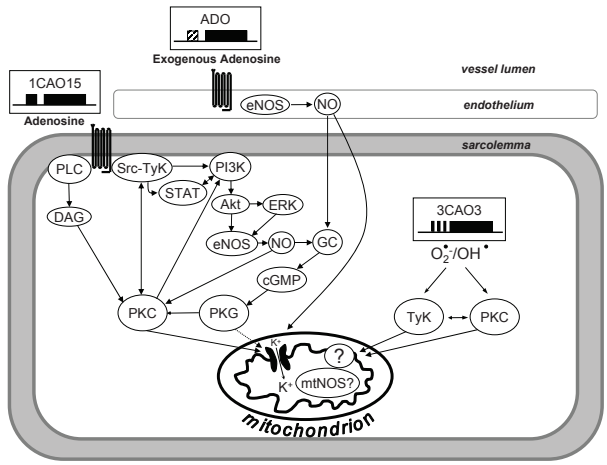

Fig. 1. Proposed scheme outlining the different signal transduction pathways from 3CAO3, 1CAO15 and ADO. All pathways converge at the mitochondrion to exhibit a protective effect on the myocardium.

Mechanism of tolerance to preconditioning

Repeated application of IPC stimuli can lead to the development of tolerance to cardioprotection.[21-23] The mechanism of myocardial tolerance to IPC 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 set up a model of tolerance to IPC using the CAO15 stimulus and investigated the underlying mechanisms. IPC by either 1CAO15 or 2CAO15 preceding CAO60 was cardioprotective, while 4CAO15 and 6CAO15 did not reduce infarct size. Using a microdialysis probe we measured adenosine levels during 1CAO15, as well as during 4CAO15. While the interstitial adenosine levels rose 7-fold during 1CAO15 (also see Chapter 3), interstitial adenosine levels were back to baseline during the fourth episode of ischemia of 4CAO15. This indicated that indeed there was a loss of adenosine production.[24] Secondly, we established that six periods of exogenous adenosine infusion (6ADO) resulted in a blunted cardioprotective effect compared to one or two episodes, implying a decreased responsiveness of adenosine receptors or downstream components.[24] However, jeopardized myocardium that has become tolerant to a particular preconditioning stimulus (4CAO15) could still be rescued by an ischemic stimulus that operates via a different mechanism. Thus, while 4CAO15 abolished the cardioprotective effect of the adenosine-dependent stimulus 2CAO15, protection could still be re-instated by repeated administration of exogenous adenosine (2ADO).[24] This implies that tolerance after 4CAO15 is not due to reduced adenosine receptor responsiveness. Specifically, cross-tolerance did not occur to remote preconditioning of the heart as cardioprotection by brief intestinal ischemia (2MAO) was also able to reduce infarct size.[24] Furthermore, IPC by 3CAO3, which employs an adenosine-independent signaling pathway, was also able to re-instate cardioprotection although its protection was slightly less compared to the application of 3CAO3 alone.[24] These

results suggest that patients could be less susceptible to the development of tolerance, due to recruitment of different signal transduction pathways by distinct stimuli. Since tolerance was initiated by repeated application of 1CAO15 and overcome by both 2ADO and 3CAO3, all being NO-dependent stimuli (see Chapters 2-4), it is highly unlikely tolerance develops at the level of NO. Future studies, involving other triggers and mediators, are required to determine the molecular basis for tolerance to other preconditioning stimuli, such as 3CAO3.

Abundant evidence has been presented that IPC also occurs in humans with the use of end points other than infarct size.[25-28] However, clinical studies on infarct size limitation by pre-infarct angina are discordant.[29-33] This has, at least in part, been ascribed to loss of the cardioprotective effect of preconditioning in the aging[34-37] and pathological hearts.[38-41] We hypothesized that the development of tolerance to IPC might also contribute to the equivocal clinical findings. However, rather than the repetitive bouts of brief ischemia of identical duration and severity that occur in the laboratory setting, patients are more likely to experience episodes of varying duration and severity of ischemia. The results in Chapter 5 suggest that these patients could be less susceptible to the development of tolerance as a result of recruitment of different signal transduction pathways by different preconditioning stimuli. This also indicates that, without a detailed knowledge of the number, severity and duration of the pre-infarct episodes of myocardial and/or remote organ ischemia, it is impossible to classify patients as preconditioned or tolerant to IPC. Finally, the observation that the administration of exogenous adenosine is still cardioprotective in hearts that have become tolerant to IPC suggests, that in patients with unstable angina, administration of pharmacological agents that mimic preconditioning can still afford cardioprotection, at least in the (sub)acute setting.[42-44]

Hibernation

Mechanism of cardioprotection

Although the mechanisms that lead to myocardial hibernation are still unclear, it is likely that IPC and hibernation share common signaling pathways that modify the severity of an energy supply/demand imbalance associated with limited blood flow. In a swine model of chronic hibernation there is an increased activation of p38 mitogen-activated protein kinase, enhanced GLUT4 translocation, and increased calcium-independent NO synthase activity, all of which have been observed in preconditioning.[45] As shown in Chapter 2-4, acute ischemia by IPC or pharmacological preconditioning by ADO can result in mitochondrial adaptations. However, the effects on mitochondria in myocardium under chronic ischemia (i.e. hibernation) had not been studied previously. Thus, in **Chapter 6** mitochondrial function in hibernating myocardium was investigated. The principal findings of this study are that within chronically ischemic swine myocardium, mitochondria in the subendocardial

layers have acquired a stress-resistant phenotype that is characterized by preserved state-3 respiration following in vitro anoxia and reoxygenation.[46] These mitochondria are characterized by a mild degree of uncoupling and by a reduced level of superoxide generation.[46] The expression of uncoupling protein 2 is slightly increased, suggesting a possible mechanism for these mitochondrial adaptations in response to chronic myocardial ischemia.[46] Future research is required to explore the effector mechanisms whereby the uncoupled phenotype is achieved. Additional studies are required to place a potential cytoprotective contribution of altered mitochondrial respiration into the proper context among the other cytoprotective mechanisms of down-regulation of energy demand and non-oxidative energy production that are also at work in the hibernating myocardium.[47]

A fundamental question is whether the mitochondria have acquired a program to reduce electron transport at the expense of limiting maximal oxygen consumption and possibly contraction.[48] It is also possible that alterations in the mitochondria that are initially programmed to reduce oxidant damage and prevent cell death in response to repetitive supply/demand ischemia, could act to limit maximal ATP production.[48] Page *et al*.[49] provide evidence that mitochondria in hibernating myocardium adapt in a way that reduces ROS production. Furthermore, they show that the proteomic phenotype of hibernating myocardium is dynamic and has similarities to global changes in energy substrate metabolism and function in the advanced failing heart.[49] In turn, these proteomic changes may limit oxidative injury and apoptosis and impact functional recovery after revascularization. Defining the factors that lead to the coordinated process to balance oxygen supply and expenditure, whether through mitochondrial adaptations or other signaling pathways, is critical for understanding the pathophysiology and advancing new therapies for hibernating myocardium and heart failure.[46, 47]

Pre- or postconditioning

Pharmacological cardioprotection

An increase in tyrosine residue phosphorylation via increased tyrosine kinase activity has been implicated in the signal transduction pathway of IPC.[4, 50, 51] Vanadate enhances tyrosine residue phosphorylation by inhibition of tyrosine phosphatase,[52, 53] suggesting that vanadate may be of therapeutic benefit in myocardial infarction.[54] Armstrong *et al*.[55] reported that serine threonine phosphatase inhibitors are highly effective in protecting isolated cardiomyocytes subjected to ischemia, even when administered late after onset of ischemia, suggesting that vanadate may not require administration prior to the onset of ischemia and might also act against reperfusion injury. In **Chapter 7** the aim was to investigate if a specific tyrosine phosphatase inhibitor bis(maltolato)-oxovanadium (BMOV), given either pre-ischemia or post-ischemia was affective in reducing infarct size, and what its mechanism of its protection

was. The major findings of that study are that pre-treatment with BMOV limits myocardial infarct size in a dose-dependent manner, while BMOV, administered in a sufficiently high dose, is still highly cardioprotective when administered just prior to reperfusion.[56] These findings suggest that BMOV exerts its effects principally during reperfusion, but that sufficiently high concentrations need to be present in the blood at the onset of reperfusion. Mechanistically, both tyrosine kinase inhibition and K^+_{ATP} channel blockade are involved in the cardioprotection by BMOV, while a neurogenic pathway is not involved.[56] Taken together, these findings are consistent with the concept that K^+_{ATP} channel activation during early reperfusion contributes to the protection by BMOV. Since, in IPC the K^+_{ATP} channel has been implicated as an important factor[57] and that IPC exerts its protective effects during reperfusion,[58] as well as the fact that POC can be blocked by the administration of glibenclamide (a K^+_{ATP} channel blocker), indicates that the K^+_{ATP} channel is important during reperfusion. Recently, it has been shown that vanadate activates the RISK pathway,[59] inhibits apoptosis[59] and targets the mitochondrion[60] to further describe the signalling pathways involved in its protection.

Most cardioprotective agents developed to date require administration before the onset of ischemia to be effective.[61] The present study shows that BMOV, administered in a sufficient high dose, is still highly cardioprotective when administered just before reperfusion. Future studies should assess both the timing, dosage as well as the duration of administration of compounds for the limitation of ischemia reperfusion injury. Thus, it might very well be that the lack of positive clinical results in reducing reperfusion injury is due to the fact that the drug was not dosed adequately. This may have led to insufficiently high drug concentrations in the area at risk prior to reperfusion. However, it needs to be established if the drug of interest has to reach the jeopardized myocardium or that it can protect the heart by activating a neurogenic pathway at extra-cardiac sites, as has been shown for exogenous adenosine in Chapter 3 of this thesis.[13, 15]

Postconditioning

Cardioprotection by mechanical modulation of reperfusion

A manner of influencing reperfusion itself is by applying POC (i.e. mechanical modulation) upon restoring reperfusion. However, not all reports on POC have shown to be in agreement with each other, especially regarding the ideal POC algorithm and period of index ischemia. Since POC with a 30 sec algorithm was effective against a 60-min CAO,[62, 63] but not against a 30-min CAO,[64, 65] we hypothesized that the duration of the index ischemia also plays a major role in determining the effect of the POC stimulus. Consequently, in **Chapter 8** we investigated the influence of index ischemia duration (ranging from 15- to 120-min) on the protective effect of POC. The present study demonstrates that the cardiac effects of POC may even be detrimental and that this deleterious effect depended critically on the dura-

tion of the preceding period of index ischemia rather than the employed stimulus.[66] These paradoxical effects of POC are best explained by the distinct effects on Akt phosphorylation and superoxide anion production.[66] While POC by 3 cycles of 30 sec of reperfusion and occlusion (3POC30) after CAO45 and CAO60 limits infarct size, it is ineffective when applied following CAO90 and CAO120, and even aggravates infarct size when applied after CAO15 and CAO30.[66] The detrimental effect of POC on infarct size after these shorter periods of index ischemia was not stimulus-specific as POC after CAO30 with shorter algorithms (3POC5 or 3POC15) resulted in similar infarct size as POC with 3POC30.[66] Cardioprotection by 3POC30 after CAO60, but not by 3POC5 or 3POC15, was accompanied by activation of the RISK pathway and reduced stimulation of superoxide production.[66] PI3K- and NO synthase inhibition blocked the protective effect of POC. Conversely, the increase in infarct size by 3POC30 after a 15-min CAO was accompanied by an increase in superoxide production, and a tendency towards a decreased stimulation of Akt phosphorylation.[66] Also at variance with 3POC30 after CAO60, NO synthase inhibition did not affect infarct size when 3POC30 was applied after a 15-min CAO.[66] The mechanism by which oxidative stress aggravates necrosis under this condition cannot be derived from the present study, and should be the subject of future investigations, since clinically this might be important.

Interestingly, Dow & Kloner[67] have shown that POC is not effective in female Sprague-Dawley rats. Utilizing various POC algorithms ranging from 10 sec to 30 sec with 4 to 20 cycles and 30 or 45 min of index ischemia no protective effects were seen utilizing different anesthetics. In fact, they also show that POC can result in an increase in infarct size. With an algorithm of 8 times 30 sec of reperfusion and occlusion infarct size tended to increase, which was significantly increased with an algorithm of 20 times 10 sec of reperfusion and occlusion compared to control. The results were not due to damage to the coronary arteries by repeated occlusions, since histology did not reveal any damage to the vessel wall. However, the authors did not discuss their observations of an increase in infarct size by their POC algorithm. Kaljusto *et al*.[68] have also shown in a multicenter laboratory study that POC in male pentobarbital- or isoflurane-anesthetized Wistar rat hearts also did not result in protection utilizing four different POC algorithms (3 cycles of 10, 15 or 30 sec or 2 cycles of 60 sec of reperfusion and ischemia) with different periods of index ischemia (30- or 40-min of CAO), while Tang *et al*.[69] have reported that in male Sprague-Dawley conscious rats the protective effect of POC (6 cycles of 30" of reperfusion and ischemia) waned after a 45-min CAO. The exact reason for the difference in outcome is not known, but confounding factors could be a difference in animal strains, gender, experimental model, duration of index ischemia or anesthesia regimen. However, we cannot exclude that it also might be due to the chosen POC algorithm. A multi center trial similar to Kaljusto *et al*.[68] is thus warranted which systematically assesses the influence of all the factors mentioned on the effect of POC. Table 1 gives a brief overview of these studies, including details from our own POC study.

Table 1. Studies in rats with different outcomes on infarct size after postconditioning.

Studies	Experimental Set-up			Index Ischemia (I'+R')	POC Stimulus (R"+I")	Infarct Size (% of Area at Risk)	
	Strain	Sex	Experimental Conditions			Control	POC
Dow *et al.*[67]	Sprague-Dawley	Female	Ketamine/Xylazine anesthesia, open-chest	30'+120'	8x 30"+30"	27±4	42±7
					4x 10"+10"		37±5
					20x 10"+10"		50±3*
				45'+120'	4x 20"+20"	43±3	47±4
					4x 10"+10"	45±5	47±4
			Pentobarbital/Isoflurane anesthesia, open-chest	30'+120'	4x 20"+20"	34±5	41±5
					4x 10"+10"	31±4	27±4
Kaljusto *et al.*[68]	Wistar	Male	Pentobarbital anesthesia, isolated hearts	30'+90'	2x 60"+60"	35±3	38±3
					3x 30"+30"		34±4
					3x 10"+10"		35±3
				40'+120'	3x 10"+10"	28±3	20±5
			Isoflurane, open-chest	30'+90'	3x 15"+15"	62±1	51±3*
				40'+180'	3x 10"+10"	36±6	24±4
Tang *et al.*[69]	Sprague-Dawley	Male	Ketamine/Xylazine/ Isoflurane anesthesia during instrumentation. Experiments performed in conscious rats.	30'+24hrs	6x 30"+30"	54±2	56±4
					6x 10"+10"		36±5*
					20x 10"+10"		29±5*
					60x 10"+10"		57±5
				45'+24hrs	20x 10"+10"	62±2	55±4
				60'+24hrs	20x 10"+10"	73±7	71±3
Manintveld *et al.*[66]	Wistar	Male	Pentobarbital anesthesia, open-chest	30'+120'	3x 30"+30"	36±4	48±4*
					3x 15"+15"		53±3*
					3x 5"+ 5"		45±3
				60'+120'	3x 60"+60"	60±3	58±4
					3x 30"+30"		47±6*
					3x 15"+15"		56±3
					3x 5"+ 5"		53±4

Interaction between pre- and postconditioning

Postconditioning of the preconditioned heart

The discovery of the cardioprotective effect of one or multiple sequences of re-occlusion and reperfusion during early reperfusion (stuttering reperfusion)[70] or gradual reperfusion,[71-75] following a period of sustained ischemia, termed "postconditioning" (POC), has been proposed as an adjunctive strategy to optimize reperfusion therapy.[64, 76] Preliminary clinical trials using POC have reported encouraging results,[77-81] and it therefore important to determine whether pre-existing myocardial ischemia modulates the effects of POC on infarct size in order to allow proper interpretation of these and future clinical trials. The question whether POC can afford additional protection, i.e. further limit infarct size, when myocardium is already preconditioned has been addressed in a few experimental studies but results are ambiguous.[63, 82, 83] This could be due not only to differences in animal species, anesthesia regimen and algorithm of the postconditioning stimulus,[64] but also to the type of preconditioning stimulus employed (see Chapters 2 and 4)[7, 84] and the duration of index ischemia (see Chapter 8).[66] For example, depending on the duration of index ischemia, POC can be protective, have no effect or even be detrimental.[66, 67] In light of these considerations, the first aim in **Chapter 9** was to determine the interaction between IPC and POC taking into account the algorithm of the IPC stimulus. In view of the proposed role of NO in the signal transduction of both IPC[85, 86] and POC,[82, 83, 87] we investigated the obligatory role of NO in the cardioprotection by IPC and POC. Furthermore, we studied whether in myocardium that had become tolerant to IPC (see Chapter 5)[21, 24] protection could be reinstated by POC. Finally, in view of recent observations in our laboratory that that POC can increase infarct size when applied following a brief period of index ischemia,[66] we investigated whether IPC could protect against the myocardial damage produced by POC and determined the role of NO herein. The main findings are: Firstly, after CAO60 POC affords cardioprotection similar to IPC with either 1CAO15 or 3CAO3. Secondly, after CAO60 there is no additional effect of POC on preconditioned myocardium, irrespective of the IPC stimulus. Thirdly, the increase in infarct size by POC after CAO30 was abolished in preconditioned myocardium, irrespective of the IPC stimulus. Fourthly, myocardium, in which IPC was prevented by administration of LNNA, does not benefit from POC. And finally, POC in myocardium that was made tolerant by 4CAO15 does not cause re-instatement of cardioprotection. The observation that tolerance to IPC resulted in cross-tolerance to the cardioprotective effects of POC, may have important clinical implications. For example, pre-infarct angina may either have resulted in myocardial preconditioning or in tolerance to preconditioning, due to repeated bouts of short periods of ischemia preceding infarction,[21, 24] thereby potentially modifying the efficacy of pharmacological therapies aimed at reducing reperfusion injury that target similar signaling pathways as IPC.[88] Our results also suggest that patients enrolled in studies into the efficacy of POC during revascularization procedures may require stratification according to the absence or presence of pre-infarct angina, as the latter

may be less responsive to POC therapy. Future studies should address if other POC algorithms do cause a further limitation of infarct size in preconditioned myocardium. Clinically, it is important to know if the cardioprotective effect of either pre- or postconditioning can be pharmacologically enhanced by compounds that target components identified in its protection (e.g. adenosine or BMOV, in respectively Chapters 3 and 7). This is especially important since many patients already have several co-morbidities at the time of myocardial infarction and are already on several prescribed medications that might restore the cardioprotective qualities of POC or even enhance its cardioprotection.

Future perspectives

Difficulties in translational medicine

While animal experiments are performed in young and naïve animals, without co-morbidity or concomitant medication, in a setting where occlusion and reperfusion are under total control, the reverse is true in patients. Many patients with an acute myocardial infarction (AMI) are older, have multiple risk factors, and usually take several drugs. In a sizable portion of patients with an AMI coronary occlusion may not be complete. Moreover, not rarely in human AMI a stuttering course with intermittent occlusion and reperfusion is present.[89] Indeed, previous angiographic studies of patients with an AMI have shown that, at the time of angiography, the infarct-related artery was not totally occluded in up to 35% of the patients.[89, 90] It was shown that the percentage of patients with residual anterograde flow in the infarct-related artery increases up to 50% presenting between 12 and 48 hours from symptom onset.[91] Preservation of residual blood flow in the infarct-related artery was found to be associated with a reduction in infarct size,[92, 93] better left ventricular function[94, 95] and a more favorable clinical outcome compared with total occlusion. While it is known that the severity of ischemia is dependent on the degree of residual flow as well as collateral flow,[96, 97] anterograde flow is measured, but collateral flow is rarely assessed. Furthermore, surrogate endpoints are used to estimate infarct size, while in experimental setting the infarct size can accurately be assessed. Also in clinical studies typically express infarct size as a percentage of the left ventricle, while in experimental studies infarct size is related to the anatomical area at risk, in greater infarct size variability. Future research should focus on creating animal models that more reliably resemble the clinical situation. For instance by treating hypercholesterolemic animals with a statin, since statin treatment started pre-ischemia has been shown to reduce infarct size in a rat model of ischemia reperfusion.[98, 99] Furthermore, numerous studies show that ACE-inhibition is also known to limit infarct size.[100-103] From a clinical perspective patient stratification is needed to better establish what patients to treat.

In the next paragraphs the potential clinical implications of preconditioning, hibernation and postconditioning will be discussed, including a role for mitochondria, and what can be done to advance their application in the clinical arena.

Mitochondria and cardioprotection

Mitochondrial adaptations have been described in both pre- and postconditioning, as well as in hibernation.[104, 105] Although many cellular processes are involved, mitochondria are the gatekeepers of life and death determining the fate of ischemic cardiomyocytes.[106] The mitochondrial permeability transition pore (MPTP) is the prime candidate to prevent the consequences of ischemia.[107] However, the molecular identity of mitochondrial channels is still elusive hindering their characterization as pharmaceutical targets.[108] Elucidating the mitochondrial switches that control the evolution of the ischemic injury towards recovery or loss of viability remains a challenge. This will help in identifying novel targets for cardio-protection that can be tested in the clinical arena. The next challenging step would then be to only target the affected organ, or even better to target only the mitochondria within that organ, to minimize side effects.

Ischemic preconditioning

Since the description of IPC remarkably few clinical applications have been found due to the fact that is nearly impossible to predict an ischemic event. In 1993, its application has been described in the setting of CABG surgery,[28] but since then has yielded very few positive results.[109] This could be due to the fact that patients were already preconditioned, tolerant to IPC or on medication that already exhibited on preconditioning effect. Nonetheless, recently remote IPC has also proven to be successful in patients undergoing CABG surgery.[110] Perhaps surgeons are hesitant to inflict further ischemic damage to an affected organ or regard the procedure as tedious and time consuming.[110] In this case remote IPC can be an attractive alternative, as has been shown in elective abdominal aortic aneurysm repair where remote IPC reduced post-operative myocardial and renal injury, as well as incidence of myocardial infarction.[111] Currently, five other trials are including patients looking at IPC or remote IPC in AMI or cardiac surgery. One of those studies is assessing the clinical effects, as well as the genomic response of remote IPC in cardiac surgery in children. This should further establish the role of (remote) IPC in clinical medicine. Remote IPC might be clinically relevant since many patients with an AMI exhibit peripheral vascular disease, which can cause remote IPC.

Myocardial hibernation

As we already know chronic myocardial dysfunction, present before coronary bypass, often improves after revascularization.[112-114] It has been shown that magnitude of improvement in left ventricular ejection fraction after revascularization is directly related to the extent of hibernating myocardium.[115] The presence and extent of viability is also significantly related

to the improvement of heart failure symptoms and quality of life after revascularization.[115] However, revascularization cannot be reached in all patients with myocardial hibernation. Hope is that mitochondrial targets identified in pre- and postconditioning can be targeted in myocardial hibernation. Furthermore, perhaps these targets can also be applied before, during or after revascularization to optimize the effect of revascularization in myocardial hibernation.

Ischemic postconditioning

With the description of POC[62] there has been a noticeable resurgence to reapply the knowledge we have gained with preconditioning in the past, together with the new found knowledge with POC. Now there is no more denying that reperfusion injury exists, thus making it a defined target to treat. POC clearly identifies reperfusion necrosis and has been reported in several animal species as well as in humans[116] having both short[77, 78] and long term benefits.[80, 117] Furthermore, both ischemic[118] as well as pharmacological POC[119, 120] have also found their way into the surgical arena.[121] Preliminary results in rats have shown that isolated hearts can still be protected after four hours of ischemia and a cardioplegic protocol.[122] Further *in vitro* and *in vivo* studies have to elucidate and clarify how POC can safely be applied in human cardiac transplantation.

In an attempt to resemble the clinical situation more closely POC has been applied in normo- and hypercholesterolemic pigs. POC reduced necrosis and area of no-reflow in normocholesterolemic pigs, while POC under the hypercholesterolemic condition did not.[123] The beneficial effect of POC on myocardial no-reflow could be due to its protection of endothelial function or simply a smaller infarct size. In contrast, in rabbits POC did not reduce infarct size, nor did it reduce the extent of the anatomic zone of no reflow even though two different algorithms of POC were applied.[124] Nevertheless, it should be noted that in the clinical studies with POC to date, patients exhibited all known risk factors, including hypercholesterolemia. For instance, remote ischemic pre- and postconditioning by transient limb ischemia has also shown to limit radial artery endothelial dysfunction in both healthy subjects as well as patients with atherosclerosis.[125] Yet a different algorithm of POC failed to prevent radial artery endothelial dysfunction in young and healthy non-smoking volunteers.[126] Nonetheless, Ma *et al*.[79, 127] have shown that POC was able to salvage coronary endothelial function besides reducing infarct size. This illustrates that more research is needed to fully elucidate the signal transduction pathways involved in its protection to fully exploit its protective qualities. Furthermore, pharmacological postconditioning would avoid the adverse consequences in vascular surgery associated with intermittent cross-clamping and provide a more simple method of myocardial protection following all cardiac procedures.

Because patients with pre-infarct angina were excluded in the first clinical studies with POC, it should be tested if patients with a short period of angina pectoris preceding an AMI can still benefit from POC. Furthermore, to date the clinical studies utilizing POC have only

included patients with an AMI exhibiting TIMI flow grade 0 at time of catheterization, which mimics the animal studies most closely. The next logical step would be to investigate in a clinical trial if POC is effective when TIMI flow grades above zero are included. Although it is questionable if it would be effective, since pre-clinical experience with POC has shown that it has to be initiated within one minute after reperfusion.[128] Finally, experimental evidence in dogs has shown that ischemic POC delays infarct development during reperfusion and extends the window for interventions.[129] Infarct size at 3 hours of reperfusion has reached 77% of ultimate infarct size, measured at 24 hours of reperfusion, while infarct size at 3 hours of reperfusion after ischemic POC results in an infarct size of 50% of final infarct size. Clinically this could mean that the time window to perform PCI in AMI can be extended when ischemic POC is applied. However, this still needs to be established.

Besides conventional treatments in AMI that target either ischemia-induced damage (e.g. early restoration of reperfusion), arrhythmias (e.g. β-blockers), or detrimental left ventricular remodeling (e.g. angiotensin converting enzyme inhibitors), no successful treatment has been clinically implemented thus far to limit reperfusion damage. However, POC can be applied easily to activate endogenous protective mechanisms to reduce reperfusion injury. Looking at the clinical studies to date the decrease in infarct size is ~30-40% compared to standard therapy.[117] This difference is not due to difference in prescribed medication since both control and treated groups received similar medication for their co-morbidities. Studies have clearly demonstrated that infarct size is a major determinant of prognosis after AMI [130, 131] and that heart failure is a common outcome after AMI, with a 5-year survival rate <50%.[132] A large scale trial with POC, instead of the small trials described thus far, should assess if these factors can be affected positively. The author of this thesis is currently setting up a clinical trial utilizing POC in AMI. This trial will be discussed in more detail in the next paragraph.

Future studies should address if combining remote IPC (or pharmacological IPC) with POC is able to further enhance the protective effects of these modalities and what algorithm of POC is most effective. Especially remote IPC might be clinically relevant since many patients with an AMI exhibit peripheral vascular disease, which can cause remote IPC. Finally, the application of remote POC[133, 134] or remote per(i)conditioning[135, 136] (i.e. brief periods of ischemia and reperfusion of a remote organ during sustained myocardial ischemia) should be assessed clinically. In animal studies remote per(i)conditioning has shown to limit infarct size as well as area of no-reflow.[135, 136] However, it is not known if its protective effects are equal in size to ischemic POC.

Pharmacological pre- and postconditioning

Many clinical trials, investigating the therapeutic potential of agents identified as important targets in preconditioning (and thus given pre-ischemically), have been performed after experimental evidence suggested that drugs are capable of limiting infarct size when given at the onset of reperfusion (i.e. pharmacological postconditioning). Nonetheless, these results

have been disappointing. Potential explanations for this discrepancy are numerous and are described in detail by several researchers[137-139] and has been discussed in the present thesis as well. However, there are small studies suggesting that pharmacological POC with adenosine is successful in surgery.[119, 120] If these results are confirmed in larger trials, then the next step would be to asses if the endogenous protection by ischemic pre- and postconditioning can be enhanced by pharmacological compounds that have been identified in ischemic pre- and postconditioning. In animal experiments it has been shown that adenosine administration can enhance the effects of IPC,[140-142] but this is not known for ischemic POC. Based on the fact that we have shown that exogenous adenosine and endogenous adenosine do not follow the same signaling pathway,[13] it could be that ischemic POC can be enhanced by concomitant treatment with adenosine. In order to introduce this in the clinic arena, the first step is to identify if ischemic pre- and postconditioning are effective, after which its effect can be compared to compounds that are either used by itself or in combination with ischemic pre- and postconditioning. The author of this thesis is involved in setting up a multi center trial under supervision of prof. dr. Wim van der Giessen in conjunction with the Interuniversity Cardiology Institute of the Netherlands (ICIN). This multi center trial will investigate if the therapeutic potential of ischemic POC in AMI can be confirmed, but also enhanced by intracoronary adenosine administration during the POC algorithm. Myocardial infarct size will be measured by delayed enhancement magnetic resonance imaging, besides serological markers of myocardial infarct size and inflammation.

A subject not discussed until now is myocardial protection with volatile anesthetics. They too have been shown to be protective both in pre- as well as in postconditioning[143, 144] and should regarded as another manner of pharmacological pre- or postconditioning. There is clinical evidence for anesthetic-induced preconditioning[145] and with a proteomic approach the molecular biology is being investigated.[146] In rats and rabbits isoflurane, used as a POC mimetic, has been shown to be NO-dependent[147] and prevent opening of the mitochondrial permeability transition pore.[148, 149] A meta-analysis on myocardial protection with volatile anesthetics during coronary artery bypass grafting concluded that there is some evidence of myocardial protection.[150] Larger adequately powered trials with agreed, defined outcomes need to be done to fully asses the possible beneficial effects of volatile anesthetics on the risk of myocardial infarction and mortality.

Application of postconditioning in other organs

The application of POC in an experimental setting has not gone unnoticed by those that study ischemia reperfusion injury in other organs. A few groups have reported that POC reduces infarct size in focal cerebral ischemia and improves deficits of short-term memory and motor coordination after global cerebral ischemia.[151] POC also reduces inflammation after focal brain ischemia.[152] Furthermore, co-application of ischemic pre- and postconditioning has been shown to provide additive neuroprotection against spinal cord ischemia in rab-

bits.[153] However, a successful clinical application still has to be found in these areas of interest. In patients with an ischemic stroke many patients do not reach the hospital in time to be treated with intravenous thrombolysis. However, angioplasty and stenting procedures are being performed more frequently than in the past as one of the treatment modalities for acute ischemic stroke patients.[154] If these are performed more routinely, than it must be a matter of time before the first clinical reports appear. Also, several papers have shown that reperfusion injury in the kidneys can also be limited by POC.[155-157] Surprisingly, no studies on (IPC or) POC have been performed in renal protection in humans.[158] A role for POC might be applicable in kidney transplantation, if cardioplegic protocol combined with POC leads to better results, as has been shown for the rat heart.[122] Further *in vitro* and *in vivo* studies have to elucidate and clarify how POC can safely be applied in human kidney transplantation.

Conclusions

The main challenge in the near future is to elucidate the key events in cardiomyocyte protection and translate these results in large clinical trials. After all, time is muscle, and muscle is life.[159] For future clinical trials we should not forget the lessons from the past where small positive trials ended up negative when performed in larger trials. We can possibly achieve this by using animal models that resemble the clinical situation more closely and by assessing the effects of preconditioning, postconditioning and hibernation in animals with co-morbidities, as well as in animals on different medications that are used clinically to date. Another approach would be to stratify patients according to the results obtained in different animal models.

Ischemia reperfusion injury to vital organs as the heart, brain and kidneys contribute to morbidity and mortality throughout the world. If pre- and postconditioning can successfully be applied in reducing injury of these organs, as it is already about to do for the heart, than this can result in a significant impact on clinical medicine and secondary also have large socio-economical consequences.

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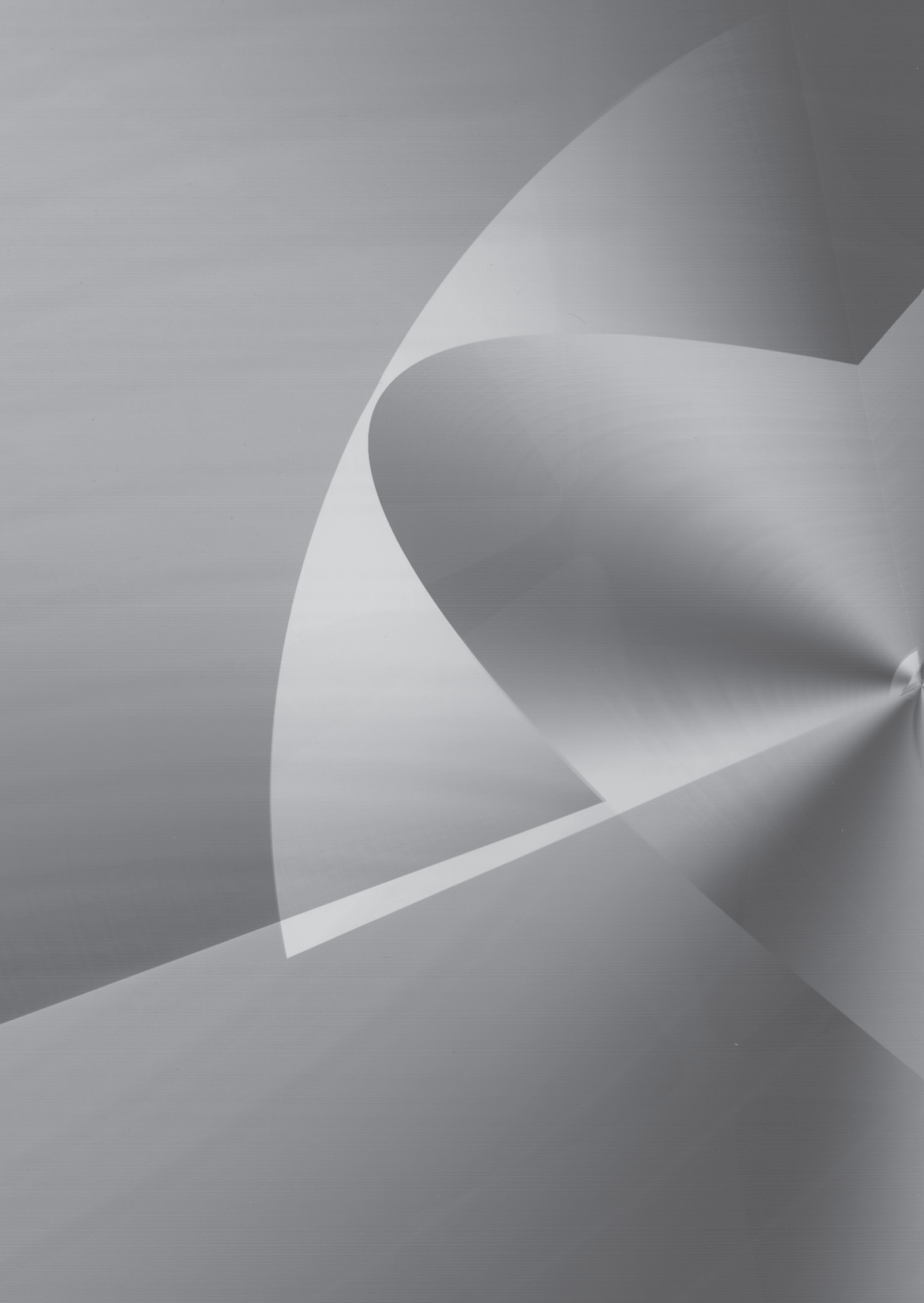

Chapter 11

Nederlandse samenvatting

Een hartinfarct of myocardinfarct, in de volksmond hartaanval, is het afsterven van een deel van de hartspier door onderbreking van de bloedtoevoer door de coronairen (kransslagaderen) naar het hart. Dit kan acuut leiden tot levensbedreigende hartritmestoornissen en op termijn tot hartfalen door onvoldoende pompwerking van het hart. Een hartinfarct ontstaat vrijwel altijd doordat zich op de plaats van een atherosclerotische plaque (aderverkalking) in een kransslagader een bloedstolsel vormt, waardoor de toevoer van bloed opeens wordt afgesneden en er zuurstof tekort van de hartspier optreedt. Dit wordt "ischemie" genoemd. Na een myocardinfarct ondergaat het overlevende hartspierweefsel een remodeleringsproces, in een poging te compenseren voor het verlies van hartspierweefsel. Op korte termijn werkt dit aanpassingsmechanisme positief voor de pompfunctie, maar op de langere termijn verhoogt dit de kans op hartfalen.

Hart- en vaatziekten vormen nog altijd de grootste doodsoorzaak in Nederland, met 42.522 sterfgevallen in 2006. Dat betekent dat per dag gemiddeld 116 mensen sterven aan hart- en vaatziekten. Uiteindelijk sterft één op de drie Nederlanders aan hart- of vaatziekten. Rond 70% van de mensen met ischemische hartziekten sterven als gevolg van een acuut myocardinfarct. Het aantal behandelingen van de kransslagaderen, waaronder dotterbehandelingen en stentplaatsingen blijft stijgen (33.678 in 2006), terwijl het aantal open-hartoperaties de laatste jaren min of meer stabiel blijft (ruim 15.000 in 2006). Momenteel zijn dit de enige effectieve manieren om het hart weer voldoende van zuurstofrijk bloed te voorzien.

In dit proefschrift worden de resultaten van enkele studies gepresenteerd waarin gekeken werd naar het mechanisme van aanpassing van het hart aan acuut zuurstoftekort in de rat en chronisch zuurstoftekort in het varken (hibernatie).

Gebleken is uit eerder onderzoek dat een kortdurende periode van zuurstoftekort, welke zelf geen schade berokkent aan het hart, voorafgaand aan een lange periode van zuurstoftekort, leidt tot een beperking van de grootte van het hartinfarct in vergelijking met wanneer deze kortdurende periode van zuurstoftekort niet vooraf aanwezig was. Dit wordt "ischemische preconditionering" (IPC) genoemd. Klinisch is dit in het geval van een patiënt met een acuut hartinfarct echter niet toe te passen aangezien een patiënt zich altijd zal presenteren nadat de klachten zijn begonnen. In een gecontroleerde setting op de operatiekamer zou dit echter wel toegepast kunnen worden in het kader van transplantatie chirurgie.

Indien het zuurstoftekort langzaam ontstaat en daarbij chronisch van aard wordt, is gebleken dat het hart zich hier ook op kan aanpassen indien het zuurstoftekort langzaam genoeg ontstaat. Dit wordt "hibernation" genoemd en heeft als gevolg dat de contractie en stofwisseling van het hart verminderen, waardoor hartspiercellen langer kunnen overleven.

Uiteindelijk is gebleken dat bij een acuut hartinfarct "reperfusie" (herstellen van de bloedvoorziening) de meest effectieve therapie is om de infarctgrootte te beperken. Dit kan echter zogenaamde "reperfusieschade" induceren, waarbij onder andere zuurstof radicalen (ROS) leiden tot extra schade. Door geleidelijk of stotterend de reperfusie te herstellen is gebleken dat de hoeveelheid zuurstofradicalen verminderd en de infarct grootte verder beperkt kan

worden. Dit kan door middel van mechanische modulatie gebeuren en heet "ischemische postconditionering". Juist dit heeft wel een potentieel grote klinische toepasbaarheid, aangezien bij een dotterprocedure in het kader van een acuut hartinfarct dit goed toegepast kan worden door geleidelijk de reperfusie te herstellen.

Hoofdstuk 1 geeft een introductie van de karakteristieken van de mechanismen van deze fenomenen en geven de rationale voor de studies welke beschreven zijn in hoofdstuk 2 t/m 9.

Preconditionering – mechanisme van bescherming

In de afgelopen jaren is duidelijk geworden dat niet alle preconditionering stimuli hetzelfde signaaltransductiepad volgen om hun beschermende effect uit te oefenen op het hart. In studies blijkt echter dat er geen rekening gehouden wordt met de verschillende soorten IPC stimuli, en wordt er van uit gegaan dat er slechts één mechanisme bestaat. In **Hoofdstuk 2** hebben we twee verschillende IPC stimuli vergeleken op hun bescherming van het hart. De uitkomsten van deze studie waren dat IPC door 1CAO15 (15-min coronair arterie occlusie) afhankelijk is van adenosine receptor stimulatie, welke via PKC en tyrosine kinase, resulteert in opening van het kalium-afhankelijke ATP-kanalen van het mitochondrion. Daarentegen, geeft IPC door 3CAO3 (3 maal 3-min coronair arterie occlusie) geen openening van de kalium-afhankelijke ATP-kanalen van het mitochondrion, maar genereert zuurstof radicalen, welke tyrosine kinase en PKC activeren. Echter, zowel 1CAO15 als 3CAO3, ondanks verschillende signaaltransductiepaden, convergeren op het mitochondrion, de energie fabriek van de cel, om hun beschermende effect uit te oefenen.

In het algemeen wordt aangenomen dat, indien we weten welke endogene mediatoren betrokken zijn bij IPC, we dit met exogene middelen kunnen nabootsen om het hart tegen ischemie te kunnen beschermen. Er is echter twijfel of exogeen adenosine en endogeen adenosine, wat vrij komt gedurende IPC, hetzelfde signaaltransductiepad activeren. Derhalve hebben we in **Hoofdstuk 3** het effect van exogene adenosine (ADO) vergeleken met de endogene adenosine-afhankelijke stimulus 1CAO15, beschreven in hoofdstuk 2. De bevindingen in deze studie tonen aan dat ADO niet leidt tot een stijging in de myocardiale interstitiële purine concentraties, terwijl 1CAO15 leidt tot een 7-voudige stijging ten opzichte van uitgangswaardes, afhankelijk is van NO productie, en afhankelijk is van een neurogeen pad. Dit zou betekenen dat ADO toegevoegd bij herstel van reperfusie in patiënten met een acuut hartinfarct niet direct in het hart aanwezig hoeft te zijn om zijn beschermende werking uit te oefenen. Mogelijkerwijs kan ADO zijn beschermende werking uiten via het endotheel in de kransslagaderen, dan wel gedeeltelijk via endotheel van bloedvaten buiten het hart (bijvoorbeeld de kleine darm). Het moet nog bekeken worden of andere exogene stoffen

hetzelfde signaaltransductiepaden activeren als de endogene stoffen die beschreven zijn bij IPC.

Naar aanleiding van de observaties in hoofdstuk 2 en 3 dat farmacolgische preconditionering (ADO) en IPC (1CAO15 en 3CAO3) het hart kunnen beschermen via verschillende signaaltransductiepaden, besloten we te kijken naar de rol van PI3K–Akt–eNOS en ERK1/2, beide componenten van het RISK signaaltransductiepad. Omdat endogeen adenosine vrij komt gedurende IPC en bescherming biedt door activatie van het RISK signaaltransductiepad gedurende de vroege reperfusie, hebben we de getest of de adenosine-afhankelijke (maar ROS-onafhankelijke) stimulus 1CAO15 in tegenstelling tot de adenosine-onafhankelijke (maar ROS-afhankelijke) stimulus 3CAO3 het RISK signaaltransductiepad activeert. Echter, andere signaaltransductiepaden, zoals het JAK/STAT en mogelijk ook het AMPK signaaltransductiepad zijn afhankelijk van ROS. Daarom onderzochten we in **Hoofdstuk 4** of 3CAO3, maar niet 1CAO15, juist deze signaaltransductiepaden zou activeren. Tenslotte, omdat in het geïsoleerde konijnenhart exogeen adenosine in een RISK-onafhankelijke manier zijn bescherming biedt, onderzochten we of in het rattenhart exogeen adenosine zijn bescherming biedt via het RISK signaaltransductiepad. Uiteindelijk zorgden alle drie de stimuli voor een toename van de mitochondriale ademhaling en afname van de mitochondriale koppeling, wat vergezeld ging met een afname van de infarct grootte. NO synthase inhibitie blokkeerde deze effecten op mitochondriaal niveau, maar blokkeerde ook de infarctbeperking op orgaan niveau. Waar het RISK signaaltransductiepadwel betrokkken was bij de bescherming door 1CAO15, was dit niet het geval voor 3CAO3 of ADO. Deze beide laatste stimuli konden evenmin het JAK/STAT of AMPK signaaltransductiepad activeren. Toekomstig onderzoek zal moeten aantonen hoe de signaaltransductiepaden bij deze stimuli precies lopen. Verder dient uitgezocht te worden welke plaatsen buiten het hart betrokken zijn in de bescherming door exogeen ADO. Uitgezocht dient te worden of er een gemeenschappelijk mechanisme is bij bescherming door ADO en IPC op een afstand in een ander orgaan dan het hart (remote IPC). Tenslotte, moeten toekomstige studies kijken naar de moleculaire mechanismen die via NO een toename geven van state-2 respiratie en een afname van de mitochondriale koppeling.

Mechanisme van tolerantie voor preconditionering

Herhaald toedienen van dezelfde IPC stimulus kan leiden tot tolerantie voor deze IPC stimulus, waardoor de beschermende werking er van teniet is gedaan. Het onderliggende mechanisme dat zorgt voor deze tolerantie is nog niet duidelijk bekend, maar zou kunnen berusten op verminderde adenosine productie, een verminderde adenosine receptor respons of in andere componenten in het signaaltransductiepad. In **Hoofdstuk 5** hebben we aangetoond dat IPC met zowel 1CAO15, als 2CAO15 leidt tot een beperking van het hartinfarct na een ischemie duur van 60-min (CAO60). Bij 6CAO15 bleek er echter geen sprake meer te zijn

van bescherming. Hiermee is voor het eerst in het rattenhart aangetoond dat tolerantie kan optreden door het herhaald toepassen van een IPC stimulus. Vervolgens hebben we met microdialyse interstitiële adenosine concentraties gemeten in het hart. Tijdens 1CAO15 was er sprake van een stijging van de adenosine spiegels (zie ook hoofdstuk 3), maar na 4CAO15 was de adenosine spiegel niet meer anders vergeleken met uitgangswaardes. Dit geeft aan dat er een verlies is van adenosine productie. Vervolgens hebben we aangetoond dat zes periodes van exogeen adenosine toediening (6ADO) leidt tot een beperking van de bescherming van adenosine in vergelijking met één of twee periodes van exogeen adenosine, waardoor de suggestie gewekt wordt dat er een verminderde respons is van adenosine receptoren of verder op gelegen componenten in het signaaltransductiepad. In additionele experimenten bleek echter dat na 4CAO15 tolerantie overwonnen kon worden door IPC met een ander signaaltransductiepad (3CAO3), dan wel remote IPC door darm ischemie, dan wel toediening van exogeen adenosine (2ADO). Deze resultaten suggeren dat de tolerantie na 4CAO15 niet het gevolg is van verminderde gevoeligheid van adenosine receptoren. Omdat de tolerantie van 4CAO15 overwonnen is door zowel 2ADO en 3CAO3, beide NO-afhankelijke stimuli (zie hoofdstuk 2-4), lijkt het onwaarschijnlijk dat deze tolerantie zich heeft ontwikkeld op het niveau van NO. Toekomstige studies zullen moeten kijken naar activatoren en mediatoren welke de moleculaire basis vormen van tolerantie voor andere IPC stimuli, zoals 3CAO3. De resultaten in hoofdstuk 5 suggeren dat patiënten minder makkelijk tolerant zouden kunnen worden door het recruteren van verschillende signaaltransductiepaden van verschillende IPC stimuli. Hierbij is ook duidelijk dat het nagenoeg onmogelijk is om patiënten als gepreconditioneerd of tolerant te omschrijven. Omdat in onze studie exogeen adenosine echter nog steeds bescherming kon geven in harten, welke tolerant waren voor IPC, is het nog goed mogelijk dat patiënten met onstabiele angina pectoris geholpen zouden kunnen worden met medicatie die preconditionering nabootsen.

Hibernatie – mechanisme van bescherming

Alhoewel de mechanismen die leiden tot hibernatie van hartspierweefsel nog niet geheel bekend zijn, is het waarschijnlijk dat IPC en hibernatie dezelfde signaaltransductiepaden delen welke in gang gezet worden door de zwaarte van energie vraag/aanbod welke geassocieerd is met een verminderde toevoer van bloed. In een varkensmodel van hibernatie is aangetoond dat p38MAPK geactiveerd is, GLUT4 translocatie plaatsvindt, en calcium-onafhankelijke toename van NO synthase activiteit, welke alle ook bij preconditionering beschreven zijn. Zoals aangetoond in hoofdstuk 2-4, kan acute ischemie door IPC of farmacologische preconditionering door ADO leiden tot mitochondriale aanpassingen. Echter, de effecten van chronische ischemie van het hart (hibernatie) op mitochondria is nog niet bestudeerd. Derhalve hebben we in **Hoofdstuk 6** gekeken naar de mitochondriale functie in

hibernerend hartspierweefsel. De belangrijkste bevindingen uit dit onderzoek zijn dat binnen chronisch ischemisch hartspierweefsel de mitochondria aan de binnenzijde van het hart een stress-resistent fenotype hebben aangenomen, welke gekarakteriseerd wordt door een maximale ademhaling na anoxie (geen zuurstof) en reoxygenatie in geïsoleerde hartspiercellen. Verder kenmerken deze mitochondria zich door een milde mate van ontkoppeling en een verminderd vermogen tot het produceren van superoxide. Voorts blijkt de expressie van uncoupling protein 2 licht te zijn verhoogd, wat een mogelijk mechanisme kan zijn voor de mitochondriale aanpassingen in respons op de chronische ischemie. Toekomstig onderzoek is nodig om te onderzoeken hoe de mitochondria zich kunnen ontkoppelen. Het expressie patroon van eiwitten van hibernerend hartspierweefsel is dynamisch en toont overeenkomsten met globale veranderingen in energie substraat metabolisme en functie in hartfalen. Het is goed mogelijk dat deze veranderingen oxidatieve schade en celdood verminderen en van invloed zijn op functionele verbetering na het herstellen van de bloedvoorziening. Toekomstig onderzoek moet deze factoren achterhalen om de pathofysiologie beter te begrijpen.

Farmacologische bescherming van het hart

Verondersteld wordt dat een verminderde tyrosine fosforylering, hetgeen leidt tot een toename in tyrosine kinase activiteit, een belangrijke rol speelt in het mechanisme van IPC. De stof vanadate versterkt tyrosine fosforylering door inhibitie van tyrosine fosfatase, waardoor dit mogelijk van nut zou kunnen zijn bij een myocard infarct. Het is zelfs aangetoond dat inhibitie van tyrosine fosfatase in een late fase van ischemie nog altijd beschermend kan werken tegen de ischemie in geïsoleerde hartspiercellen. In **Hoofdstuk 7** hebben we onderzocht of de tyrosine fosfatase inhibitor bis(maltolato)-oxovanadium (BMOV), gegeven pre- en post-ischemie leidt tot een reductie in infarct grootte. Eveneens onderzochten we hierbij het mechanisme van bescherming. De uitkomsten van deze studie toonden aan dat BMOV pre-ischemie het hart beschermde in een dosis-afhankelijke manier, terwijl het het pre-reperfusie beschermend werkt wanneer de concentratie BMOV hoog genoeg is. Hierdoor wordt de suggestie gewekt dat BMOV zijn effect primair gedurende de reperfusie heeft, maar dat dit in hoog genoege concentraties in het bloed aanwezig moet zijn op het moment van reperfusie. Mechanistisch bleken tyrosine kinase en ATP-gevoelige kalium kanalen van belang te zijn in de bescherming door BMOV. Er was hierbij geen sprake van de bescherming via een neurogeen pad, waardoor het blijkt dat BMOV het effect in het hart zelf bewerkt. Veel van de ontwikkelde stoffen die het hart moeten beschermen zijn enkel effectief indien ze gegeven worden voordat de ischemie start. De huidige studie toont echter aan dat het starten van BMOV net voor de reperfusie leidt tot een beperking van de grootte van het hartinfarct. Toekomstige studies zullen moeten kijken of de timing, dosis en de duur van de

toediening van stoffen, die de infarct grootte beperken, van belang zijn. Mogelijkerwijs is het zo dat het gebrek aan positieve klinische studies mede komt doordat de studie medicatie niet in een adequate dosering is gegeven. Hierbij dient echter wel aangetekend te worden dat een stof niet altijd het hart voorafgaande aan de reperfusie hoeft te bereiken om zijn beschermende werking te doen, zoals we hebben aangetoond voor ADO in hoofdstuk 3 van dit proefschrift.

Postconditionering - mechanische modulatie van reperfusie

Een manier om de reperfusie zelf te beïnvloeden is door postconditionering (POC) toe te passen op het moment dat de reperfusie hersteld wordt. Echter, niet alle gemelde resultaten van POC zijn in overeenstemming met elkaar, zeker wat betreft het ideale POC algoritme in relatie tot de duur van de lange coronair aterie occlusie (index ischemie). Andere onderzoeken hebben aangetoond dat POC met een 30 sec algoritme effectief is bij een occlusie duur van 60-min (CAO60), maar niet bij 30-min (CAO30). Derhalve hebben we getest of de duur van ischemie een belangrijke rol speelt in het efffect van POC. In **Hoofdstuk 8** hebben het effect van de duur van ischemie (varierend van 15- tot 120-min) bekeken op het beschermende effect van POC. Resultaten van deze studie tonen aan dat het effect van POC zelfs schadelijk kan zijn en dat het effect afhangt van de duur van de voorafgaande index ischemie en niet zo zeer van het POC algoritme. Waar POC met 3 cycli van 30 sec reperfusie en occlusie (3POC30) na CAO45 en CAO60 infarct grootte verkleind, is POC niet meer effectief bij CAO90 en CAO120, en vergroot POC zelfs het infarct wanneer toegepast na CAO15 en CAO30. Het negatieve effect van POC op infarct grootte na deze kortere periodes van ischemie waren niet stimulus afhankelijk, omdat POC na CAO30 met 3POC5 of 3POC15 (resp 3 cycli van 5 sec of 15 sec van reperfusie en occlusie) resulteerden in vergelijkbare infarct grootte als 3POC30. Bescherming van 3POC30 na CAO60, maar niet met 3POC5 of 3POC15, werd vergezeld met activatie van de RISK signaaltransductiepad en verminderde de stimulatie van superoxide productie. PI3K en NO synthase zijn betrokken bij de bescherming van POC. Daarentegen, de toename van infarct grootte met 3POC30 na CAO15, werd vergezeld met een toename van superoxide productie en een neiging tot verminderde stimulatie van Akt fosforylering. Ook in tegenstelling tot 3POC30 na CAO60 was dat NO synthase inhibitie niet van invloed was op de infarct grootte wanneer 3POC30 was toegepast na CAO15. Het mechanisme, waardoor oxidatieve stress leidt tot verergering van celdood onder deze condities kan niet worden bepaald in de huidige studie en moet onderwerp van toekomstig onderzoek zijn, aangezien dit klinisch van belang zou kunnen zijn.

Postconditionering van het gepreconditioneerde hart

De ontdekking van het beschermende effect van een of meerdere sequenties van reperfusie en occlusie gedurende vroege reperfusie of geleidelijke reperfusie na een periode van index ischemie, genaamd postconditionering (POC), wordt voorgesteld als strategie om de omstandigheden rondom reperfusie te optimaliseren. Kleine klinische trials hebben bemoedigende resultaten laten zien, en daarom is het belangrijk om te bepalen of pre-ischemisch hartweefsel het effect van POC beïnvloed om zodoende goed de resultaten van deze en toekomstige trials te kunnen interpreteren. De vraag of POC additionele bescherming kan geven, dat wil zeggen een minder groot infarct, wanneer het hartweefsel al gepreconditioneerd is, is geadresseerd in slechts enkele experimentele studies waarbij de resultaten uiteenlopen. Dit is mogelijk niet alleen door het verschil in diersoort, keuze van anesthesie en algoritme van de postconditionering stimulus, maar ook door de gekozen preconditionering stimulus (IPC, zie hoofdstuk 2 en 4) en de duur van de index ischemie (zie hoofdstuk 8). Met dit in gedachte, was in **Hoofdstuk 9** het doel om te kijken naar de interactie tussen IPC en POC, waarbij eveneens werd gekeken naar het effect van verschillende IPC stimuli. Omdat NO van belang is in zowel IPC als POC werd ook gekeken naar de rol van NO in de bescherming door IPC en POC. Tevens, werd gekeken naar hartspierweefsel wat tolerant was geworden voor IPC (zie hoofdstuk 5) en of de bescherming weer hersteld kon worden door POC. Als laatste, hebben we getest of IPC kon beschermen tegen de schade die door POC werd veroorzaakt, wanneer toegepast bij kortdurende ischemie (zie hoofdstuk 8) en keken naar de rol van NO hierin. De resultaten van deze studie zijn: Ten eerste, na CAO60 geeft POC een zelfde mate van bescherming als IPC met 1CAO15 of 3CAO3. Ten tweede, na CAO60 geeft POC geen additionele bescherming van gepreconditioneerd hartspierweefsel, onafhankelijk van de gebruikte IPC stimulus. Ten derde, de toename in infarct grootte door POC na CAO30 werd teniet gedaan in gepreconditioneerd hartspierweefsel, onafhankelijk van de gebruikte IPC stimulus. Ten vierde, hartspierweefsel, waarin IPC geblokkeerd werd door LNNA, kan niet meer beschermd worden door POC. Ten slotte, POC beschermt niet in hartspierweefsel welke tolerant is gemaakt door 4CAO15. Het feit dat tolerantie voor IPC resulteerde in cross-tolerantie voor de beschermende effecten van POC kan belangrijke klinische implicaties hebben. Bijvoorbeeld, pre-infarct angina kan geresulteerd hebben in preconditionering of tolerantie voor preconditionering door herhaalde periodes van angina, waardoor mogelijkerwijs farmacologische middelen gericht om reperfusie schade te beperken niet meer werkzaam zijn, omdat deze op vergelijkbare signaaltransductiepaden gericht zijn. Onze resultaten suggeren ook dat patiënten in klinische studies, die kijken naar het effect van POC gedurende revascularisatie procedures, gestratificeerd moeten worden naar de aan- en afwezigheid van pre-infarct angina aangezien de eerste groep mogelijk minder effect heeft van POC therapie. Toekomstige studies moeten kijken of andere POC algoritmes wel een verdere beperking van de infarct grootte geven in gepreconditioneerde harten. Klinisch, is het belangrijk om te weten of het

effect van pre- en postconditionering farmacologisch versterkt kan worden met middelen gericht op componenten ontdekt in hun bescherming (bijvoorbeeld adenosine of BMOV, in respectievelijk Hoofdstuk 3 en 7). Dit is met name van belang omdat veel patiënten al co-morbiditeit hebben op het moment van het infarct en al verschillende soorten medicatie gebruiken die mogelijk het beschermende effect van POC herstellen, dan wel versterken.

Toekomstperspectieven

Vrijwel alle experimenten over de effecten van pre- dan wel postconditionering op de grootte van een hartinfarct na kransslagader occlusie zijn uitgevoerd bij jonge gezonde dieren, die tevoren niet waren behandeld met medicamenten, zoals patiënten met ischemisch hartlijden die vaak wel gebruiken. Bovendien, heeft 35% van de patiënten met een hartinfarct, geen totale occlusie van een kransslagader, terwijl experimenteel wel altijd een totale occlusie wordt aangebracht. Soms is er bij patiënten sprake van herhaaldelijk open en dicht gaan van een kransvat, zodat er, bij wijze van spreken, sprake is van natuurlijke preconditionering. Tenslotte weten we, dat patiënten met ischemisch hartlijden meerdere risicofactoren met zich mee dragen, zoals hypertensie, hypercholesterolemie en diabetes mellitus. Zo is bijvoorbeeld al aangetoond, dat toediening van een statine, een beta-blokker of een ACE-remmer de infarct grootte beperkt na occlusie van een kransslagader.

Resultaten van klinische studies naar de mogelijke therapeutische effecten van stoffen, zoals adenosine, die betrokken zijn bij ischemische preconditionering, zijn teleurstellend wanneer deze stoffen na het moment van infarcering worden toegediend (farmacologische postconditionering). Echter, adenosine als farmacologische postconditionering blijkt wel een gunstig effect te hebben bij patiënten, die chirurgische behandeling ondergaan voor hun ischemisch hartlijden. Indien deze resultaten bevestigd kunnen worden in een grotere studie, dan zou de volgende stap moeten zijn om te kijken of de endogene bescherme door ischemische pre- en postconditionering versterkt kan worden door stoffen die betrokken zijn bij ischemische pre- en postconditionering. In dierproeven is reeds aangetoond dat exogeen adenosine de effecten van ischemische preconditionering kan versterken, maar dit is vooralsnog niet getest voor ischemische postconditionering.

Zelf hebben we aangetoond, dat exogeen toegediend adenosine en endogeen adenosine hun gunstige effect op infarctgrootte niet via hetzelfde signaaltransductiepad uitoefenen. Derhalve is het essentieel om uit te zoeken, wat de beste therapeutische strategie zou kunnen zijn: versterken van de effecten van endogeen adenosine dan wel toedienen van farmaca na het infarct. Bovenstaande overwegingen hebben ons er toe gebracht een multicentre studie in samenwerking met het Interuniversitair Cardiologisch Instituut (ICIN) op te zetten onder supervisie van prof. dr. Wim van der Giesen bij patiënten met een acuut hartinfarct, waarin met MRI infarctgrootte zal worden bepaald. Achtereenvolgens zullen de effecten van ische-

mische postconditionering met en zonder intracoronaire adenosine worden onderzocht. Bovendien zullen in bloed diverse markers voor infactgrootte en ontsteking worden bepaald. Bij de inclusie en exclusie van patiënten zal aandacht worden geschonken aan de medicatie, die patiënten vóór hun infarct gebruikten en zal medicatie na het infarct gestandaardiseerd worden. Er zullen 120 patiënten worden geïncludeerd door zes centra en de vervolgduur zal zes maanden bedragen. Nog in 2008 hopen we met de studie te beginnen, zodat de resultaten nog in 2009 beschikbaar kunnen zijn, als mooi voorbeeld van "translational research".

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Vele malen heb ik mogen constateren dat dit 90% transpiratie betreft en 10% inspiratie. Zeker gezien de tijd die het kost om te komen van het verkrijgen van data tot het accepteren van een manuscript. In tegenstelling tot de kliniek, werk je bij het onderzoek vaak solistisch, gaat het langzaam gepaard met vallen en opstaan. Pas echt duidelijk werd het contrast tussen kliniek en onderzoek, nadat ik na 2 jaar kliniek weer terug kwam om mijn onderzoek af te ronden. Gelukkig heb ik echter een groot gedeelte van mijn onderzoek hulp gehad van een aantal mensen. Graag wil ik van deze gelegenheid gebruik maken om enkele mensen te bedanken die betrokken zijn geweest bij het tot stand komen van mijn proefschrift.

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emeritus hoogleraar inmiddels helaas niet meer voeren. Maar bij is jou het preconditioneringsonderzoek wel ooit begonnen. Ik stel dan ook voor om jou de titel generator te geven.

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Maaike, je hebt me het instrumenteren geleerd van de ratten. Ongelooflijk met hoeveel geduld je dit kan uitleggen. Je hebt ook menig student deze techniek eigen kunnen maken. Uiteindelijk kan ik er niet onderuit te zeggen dat zonder jou dit proefschrift er niet was geweest. Dank voor al je julp en inzet. Je begon als stil jong meisje, maar laat inmiddels duidelijk niet meer het kaas van je brood af eten! Ik heb de hoop dat ik daar mede verantwoordelijk voor ben. Hiernaast moet ik ook Inge bedanken voor de extra hulp die ze heeft geboden. Dat heeft me veel tijd gescheeld. Het was door jou nooit saai op het lab.

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Voor mijn paranimfen: Beste Joep, al vanaf de Eureka week kennen we elkaar, maar pas echt goed toen we besloten om samen naar Australië te gaan om daar onderzoek te doen.

Dit beviel zo goed dat we het jaar daarna besloten nog eens te gaan, waardoor we in 2 jaar tijd bijna 9 maanden Down Under hebben gezeten. Een onvergetelijke tijd, die inmiddels al bijna 10 jaar achter ons ligt. In die tijd hebben we veel meegemaakt. Ik ben blij dat je je stekkie gevonden hebt in Groningen en dat je dit niet meer in je eentje is, maar met z'n tweeën! Bedankt, niet alleen voor het kritisch lezen van mijn artikelen (je bent immers een gigantische mierenneuker), maar ook voor het feit dat we vrienden zijn. Vrienden voor het leven, waar we dan ook wonen.

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Olivier Manintveld

"Now is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning".
Sir Winston Churchill (London, November 10th 1942)

List of publications

Full papers

1. Liem DA, Manintveld OC, Schoonderwoerd K, et al. Ischemic preconditioning modulates mitochondrial respiration, irrespective of the employed signal transduction pathway. Transl Res 2008;151(1):17-26.
2. Manintveld OC, Te Lintel Hekkert M, van den Bos EJ, et al. Cardiac effects of postconditioning depend critically on the duration of index ischemia. Am J Physiol Heart Circ Physiol 2007;292(3):H1551-60.
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Burrell LM, Droogh J, Man in 't Veld O, Rockell MD, Farina NK, Johnston CI
Antihypertensive and antihypertrophic effects of omapatrilat in SHR.
Poster; International Society of Hypertension Meeting, Milan, Italy, 1999

Balding LC, Risvanis J, Droogh JM, Man in 't Veld OC and Burrell LM
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Poster; International Society of Hypertension Meeting, Milan, Italy, 2002

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Poster; 48th Orthopaedic Research Society Meeting, New Orleans, USA, 2002

Van Zijderveld R, Manintveld OC, Mathura KR, de Smet MD, Ince C, Schlingemann RO
Orthogonal Polarisation Spectral Imanging of the conjunctival microcirculation in diabetes mellitus.
Poster; 75th ARVO meeting, Fort Lauderdale, Florida, USA, 7-9 May2003

Manintveld OC, Liem DA, Schoonderwoerd C, Sluiter W, McFalls EO, Heinen A, Verdouw PD, Duncker DJ Ischemic preconditioning produces mitochondrial uncoupling independent of the employed signal transduction pathway.
Oral presentation; 77th Scientific Sessions of the American Heart Association, New Orleans, USA, 7-10 November 2004

Curriculum Vitae

Olivier Christiaan Manintveld is op 12 september 1977 geboren te Rotterdam. Na in 1995 met succes het eindexamen te hebben afgelegd aan het Marnix Gymnasium te Rotterdam, studeerde hij geneeskunde aan de Erasmus Universiteit Rotterdam. Tijdens zijn studie heeft hij tweemaal onderzoek verricht naar de behandeling van hoge bloeddruk in het Austin & Repatriation Medical Centre aan universiteit van Melbourne te Australië (dr. L.M. Burrell). Zijn afstudeeronderzoek dat een onderdeel uitmaakt van het doctoraal examen werd eveneens verricht op deze afdeling. Het artsexamen werd behaald in 2001. Hierna heeft hij onderzoek verricht op de afdeling orthopedie in het Erasmus MC te Rotterdam (prof. dr. Weinans) en op de afdeling anesthesiologie in het AMC te Amsterdam (prof. dr. Ince). Uiteindelijk leidde het werk op de hartbewaking in het Erasmus MC er toe dat hij de overstap maakte naar de cardiologie. Hij startte dan ook in 2004 als tweede assistent-geneeskundige in opleiding tot klinisch onderzoeker (AGIKO) binnen het Thoraxcentrum van het Erasmus MC op de afdeling experimentele cardiologie (prof. dr. Duncker), waarbij hij een ZonMW-AGIKO stipendium verwierf. In 2005 en 2006 werd de vooropleiding Interne Geneeskunde in het Sint Franciscus Gasthuis te Rotterdam doorlopen (oud-opleider dr. Tjen en huidig opleider drs. Rietveld). Per juni 2008 zal hij de klinische opleiding tot cardioloog aanvangen. Allereerst in Dordrecht (opleider dr. Kofflard) en daarna in Rotterdam (oud-opleider prof. dr. Simoons en huidig opleider dr. Ten Cate). Hij woont samen met Marike Wabbijn en zal volgend jaar met haar trouwen.

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