Pharmacological Reduction of Infarct Size. In Search for a Drug that Mimics Ischemic Preconditioning.

PHARMACOLOGICAL REDUCTION OF INFARCT SIZE

IN SEARCH FOR A DRUG THAT MIMICS ISCHEMIC PRECONDITIONING

FARMACOLOGISCHE BENADERING VAN INFARCTGROOTTE BEPERKING

Proefschrift

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof. dr. P.W.C. Akkermans M.A.
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 26 Februari 1997 om 15:45 uur

door
SVEN MICHAEL ROHMANN
geboren te Frankfurt / Main

PROMOTIECOMMISSIE

Promotoren	Prof.dr. P.D. Verdouw
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Overige Leden	Prof.dr. A.J. Man in 't Veld
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ISBN 90-9010214-0

Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

Contents

Chapter 1

Introduction and Aim of the Thesis

In the late 1960s, when the high mortality of myocardial infarction had caused a high level of public awareness, the National Institutes of Health, USA, decided to fund projects directly related to the experimental treatment of infarcts, to develop animal models best suited to study infarct size after coronary occlusion, and to develop quantitative methods in animals and in man to measure infarcts. Many drugs, natural compounds, and physical methods were tested in the early phase of pragmatism and only a few forms of therapy met expectations when the measurements of infarct size became more precise. The most effective of these interventions, which is potentially able to reduce infarct size when applied within a certain window of time, is reperfusion.[1]

In the initiated basic pathophysiological studies two major determinant factors of the progression of infarct size had been identified, the myocardial oxygen consumption during coronary occlusion and the amount of collateral blood flow.[2] But although drugs were found able to effectively reduce myocardial oxygen consumption, their infarct size reducing properties remained limited since they were depending on the reperfusion of the ischemic myocardium.

In the early 1980s cardiologists became able to reopen freshly occluded coronary vessels,[3,4] thereby instituting reperfusion in a variety of patients. Nevertheless, sometimes the effect on myocardial infarct symptoms of vascular reopening procedures was very limited despite successfull reperfusion.[5] Either regional contractile function remained depressed for hours or even days after successful reperfusion, although the myocardium at risk was structurally salvaged - termed myocardial "stunning"[6] - or reperfusion had damaged the myocardium in addition to ischemia - termed "reperfusion injury".[7,8]

Following Heyndrickx's observation of long-lasting contractile dysfunction ("stunning") following a brief ischemic episode, several laboratories became interested in the mechanism of stunned myocardium[9,10,11] and it soon became apparent that multiple short coronary occlusions were not additive in their damaging effect but rather exerted, paradoxically, a protection in subsequent long occlusions. It was Jenning's group, who had termed this phenomenon "ischemic preconditioning".[12] They have shown that repeated 5 min coronary occlusions with 5 minutes of reperfusion interspersed in dogs condition the myocardium in such a way that a long occlusion, usually lethal for some myocytes at risk, does not lead to infarction, or it produces only very small ones. This finding contradicted the common opinion in the early 1980s. It found its basis in several reports, which implicated both severe depletion of adenosine triphosphate (ATP)[13] and accumulation of glycolytic intermediates[14] in the pathogenesis of lethal ischemic cell injury. Reimer et al[15] reported in 1986 that ATP resynthesis after a single episode of ischemia was very slow, taking as much as 4 days to recover after a 15-minute coronary artery occlusion. The delayed metabolic recovery led to

the assumption that repeated, brief episodes of ischemia, such as occur with angina pectoris, might cause cumulative ATP depletion and eventually result in myocardial necrosis. Using four cycles of 5 minutes of coronary artery occlusion followed by 5 minutes of reperfusion Jenning's group could neither observe a cumulative depletion of ATP nor cell death.[15] They concluded that intermittent reperfusion prevented cumulative injury by washing out ischemic catabolites, slowing down ATP depletion, and restoring the capacity for anaerobic glycolysis in subsequent ischemic episodes. These results obtained in dogs have also been reported by many other laboratories[16 17 18] and opened up a new avenue of investigation.

Aim of the thesis:

The thesis focuses on the pharmacological modulation of ischemic preconditioning. The first step was to establish an animal model, that allowed a high reproducibility of the size of the area at risk of ischemia and the subsequent myocardial infarction.[1] Furthermore, the model should be useful to assess the effects of drugs, which may be subsequently be used in clinical settings.

In ***chapter 2*** we tried to extend the observations in dogs of Murry et al.[12] to the pig heart which differs from that of the dog e.g. in the lack of a collateral circulation. Reduction of oxygen demand in the presence of a measurable collateral flow (in the canine heart up to 30% of normal flow) was believed to play a key role in preconditioning of canine myocardium. Since the porcine heart has no collateral blood flow, the reduction in myocardial oxygen demand, if of the same magnitude as in the dog (i.e. 30%) should produce different effects because the demand-supply ratio in pigs becomes infinitively high.

In pigs with nearly identical regions at ischemic risk, infarct size of porcine myocardium produced by a 60 min coronary artery occlusion preconditioned with two 10 min episodes of ischemia each followed by 30 min of reperfusion, was limited to about 20% of that in animals which were subjected to only 60 min of ischemia. Tracer microspheres were used to measure blood flow to the risk area, which was in every case virtually nil during ischemia. Obviously a different explanation for the effects of preconditioning had to be found.

We assumed that energy savings accruing from the acontractile units of stunned myocardium were responsible for the reduction in infarct. But this turned out not to be case. Firstly, the only moderate reduction of the measured myocardial oxygen consumption in our study was unable to explain the dramatic effect of preconditioning on infarct size.[19] Secondly, we attempted to prevent regional dysfunction by pretreating pigs with a calcium channel blocker prior to the preconditioning period. Swine myocardium, which was not stunned when pretreated with i.c. nifedipine although subjected to two cycles of 10 min coronary artery

occlusion and 30 min reperfusion, nevertheless exhibited a marked reduction in infarct size following 60 min of sustained ischemia.[II] The results implicated that myocardial stunning is not a prerequisite for ischemic preconditioning and that stunning and preconditioning are not related phenomena. These observations in pigs were perfectly in line with other reports in different species [20 21] including dogs, [22] mandating for other preconditioning mechanisms.

In the mid 1980 several dog studies were reported, [23 24 25] indicating that pre-administration of super oxide dismutase and catalase can blunt the preconditioning effects of brief ischemia, suggesting that oxygen free radical species produced during the initial episodes of brief ischemia might be important in the development of tolerance to subsequent longer episodes of ischemia. However, we could demonstrate that these observations can not be extended to the pig which differs from that of the dog by the absence of xanthine oxidase,[26] which is proposed to be a major source of oxygen free radicals in dogs.[27] Using SOD and catalase in relatively (with regard to the literature) high intravascular concentrations in the above mentioned preconditioning protocol, we observed a similar reduction of infarct size compared with only preconditioned pigs, and no alteration of stunning.[III] This is in line with further evidence by Koning et al [28]. In a two stage coronary occlusion model, which allowed no reperfusion between the partial (70% reduction of basal coronary blood flow) and the complete occlusion, the protective effect of ischemic preconditioning was observed. As oxygen free radical bursts are supposed to be related to reperfusion, this model questions a significant role of oxygen radicals in the course of ischemic preconditioning, too. Thus, most animal results favour preconditioning mechanisms independent of intravascular oxygen free radical formation.

In 1983, the ATP-sensitive potassium channel was discovered in guinea pig ventricular myocytes by Noma[29] using the patch-clamp technique. Noma found that the probability of this potassium channel being open was regulated primarily by intracelluar concentrations of ATP (= ATP-sensitive potassium channel). These channels have been shown to exist in other tissues, such as pancreas, central nervous system, smooth muscle, skeletal muscle, and kidney.[30 31] Subsequently, other important modulators of this channel have been identified, including pH, lactate, the ATP/ADP ratio, and nucleotides. Since the ATP-sensitive potassium channel is rapidly opened during ischemia or hypoxia and results in a marked shortening of action potential duration and rapid loss of contractile activity, Noma[29] proposed that these channels may play an important cardioprotective role.

As early as 1985 Lamping and Gross[32] reported about cardioprotective properties of nicorandil, a hybride between a nitrate and an opener of ATP-sensitive potassium channels. Interestingly, the cardioprotective properties seem neither to be attributable to the nitrate moiety of nicorandil[33 34] nor to its vasodilatory effects.[35] Auchampach et al [36 37] provided firm evidence that the positive effects of nicorandil on postischemic dysfunction, stunning, were a

result of opening ATP-sensitive potassium channels. The subsequent development of selective modulators of the ATP-sensitive potassium channel (that is, channel openers[38] such as cromakalim, aprikalim, and bimakalim and channel antagonists[39] [40] such as glibenclamide, tolbutamide, and sodium-5-hydroxydecanoate) allowed to obtain considerable evidence that opening of ATP-sensitive potassium channels is cardioprotective,[41] while blocking these channels is proischemic resulting in a delay of the subsequent recovery of myocardial contractile function or in an increase in the ultimate extent of myocardial infarction following ischemia and reperfusion.[42]

Chapter 3 describes the hemodynamic and cardioprotective effects of bimakalim,[43] a compound under development by Merck KGaA as an antianginal, cardioprotective agent.[44] It provides evidence that the results of the Gross group obtained in dogs[45] [46] can be extended to other mammalian species. Furthermore, it showed that ATP-sensitive potassium channel openers a) are only effective when applied prior to an ischemic insult and b) exhibit an effect on the myocardium independent of their hemodynamic actions. Thus, cardioselective potassium channel openers could be developed[47] for the prophylactic treatment of myocardial infarction,[48] which would lack the blood pressure lowering effects with subsequent reflex tachycardia of the first generation of potassium channel openers,[49] such as cromakalim[50] or bimakalim.

In ***chapter 4*** we evaluated the hemodynamic and cardioprotective properties of U-89232, about the first compound claimed to be a "cardioselective" potassium channel opener.[51] However, the inventors could not reverse the cardioprotective effect of U-89232 in rabbits using glibenclamide, which questions whether the cromakalim analog was still a potassium channel opener.[52] Using our pig model we could demonstrate that not only the cardioprotective effect of U-89232 is sensitive to glibenclamide and the effect is as pronounced as with the vasoactive potassium channel opener bimakalim, but that the cardioprotective properties can be observed in the complete absence of any hemodynamic changes.

Stimulated by the results of Gross and Auchampach,[53] who showed that intravenous administration of glibenclamide 10 minutes prior to or immediately upon reperfusion following the initial 5-minute ischemic period completely abolished the beneficial effect of ischemic preconditioning in anesthetized dogs, we used glibenclamide in our pig model to block either the effects of the potassium channel opener bimakalim or ischemic preconditioning.

Chapter 5 describes the effects of the pharmacological modulation of ATP-sensitive potassium channels on infarct size, myocardial stunning, and glycolytic breakdown products.

Interestingly, the effects of an ATP-sensitive potassium channel blocker on infarct size and myocardial stunning were divergent. While glibenclamide blocked the infarct size reducing effect of preconditioning it enhanced postischemic contractile dysfunction,[IV] which provides further evidence that ischemic preconditioning and myocardial stunning are not related phenomena.[54 55] Furthermore, for ease of comparison the cardioprotection by pharmacological opening of ATP-sensitive potassium channels and by ischemic preconditioning, the "original pig model" was modified. In the original model, preconditioning was established by two cycles of 10 minutes coronary artery occlusion while we now only used a single cycle. Consistent with observations in dogs,[56] a single cycle of ischemia was as protective as two,[V] which speaks in favour of the proposal that ischemic preconditiong is an all or nothing response to brief ischemia.[28 66]

Today there is little doubt that openers of ATP-sensitive potassium channels possess cardioprotective properties. They represent a new class of drugs that may offer exciting new therapeutic opportunities under conditions in which a reduction of consequences of an episode of ischemia is desirable.[57] Possible clinical applications include various forms of angina, myocardial infarction with early thrombolysis and cardiac surgery. However, their clinical use could be limited, since Cohen et al[58] reported that conscious rabbits can become tolerant to multiple episodes of ischemia. When the rabbits experienced a 5-minute coronary occlusion and 10 minutes of reperfusion before a 30 minute coronary artery occlusion, they were protected, but not if the sustained ischemia was preceeded by 40 to 65 five-minute occlusions during a 3- to 4-day period. If an ischemia-free interval of 2.5 to 3 days was interposed, protection was again evident. This observation is consistent with results obtained in anesthetized rats,[59] dogs,[60] and pigs.[61]

Chapter 6 provides evidence that ATP-sensitive potassium channels play an important role in the chain of events leading to cardioprotection and that pharmacological activation of ATP-sensitive potassium channels may overcome the limitations of preconditioning with ischemia. These results increase the clinical importance of potassium channel openers as they reassure the cardioprotective effectiveness of this pharmacological approach at any time.

Most of the preconditioning models used today incorporate complete restoration of myocardial reperfusion after the first ischemic episode in order to obtain preconditioning's protection.[62] Ovize et al [63] showed in dogs that moderate ischemia caused by a 50% reduction in myocardial blood flow failed to reduce infarct size during a subsequent 60 minute coronary artery occlusion unless intermittent reperfusion was allowed. These results supported the proposal by Murry et al[64] that ischemic preconditioning at least in part relies on the outwash of metabolites, such as lactate, thereby reducing the cellular load of catabolites during the subsequent sustained ischemic episode. However, the generalism of this proposal for all

mammalian species has been questioned, since it has been shown in pigs that preconditioning protection is possible without intermittent reperfusion.[65 66]

Nevertheless, the other intracellular alterations observed by Murry et al. have been confirmed by many other investigators in different species. They had all in common, that ischemic preconditioning resulted in reduced rates of glycolysis and high energy phosphate depletion, better preservation of pH, and myocardial ultrastructure during the sustained coronary artery occlusion.[67 68 69 70] In 1993 Steenbergen et al observed in isolated perfused rat hearts that preconditioning attenuated the increase of intracellular Ca^{2+}, Na^+, and H^+ during ischemia, most likely because of reduced stimulation of Na^+-H^+ and Na^+-Ca^{2+} [27] exchange. Their data suggested that pharmacological interventions that minimize ionic derangements during ischemia are associated with cardioprotection.

To investigate the role of the Na^+-H^+ exchanger in ischemia and reperfusion, the specific inhibitor Hoe 694 was used.[72] ***Chapter 7*** describes the effects of Hoe 694 in our established pig model. Hoe 694, when applied prior to 60 minutes of ischemia, reduced infarct size to the same extent as did ischemic preconditioning. The cardioprotective potential of Na^+-H^+ exchange inhibition has been confirmed by several investigators in pigs,[73] rabbits,[74] and rats.[75] Thus, when Hoe 694 was applied with the onset of reperfusion, it was able to significantly limit infarct size, providing evidence for the existence of injury. In none of the doses tested in pilot studies (0.3 mg - 21 mg/kg/15 min) Hoe 694 exerted any hemodynamic effect.

The pronounced cardioprotective properties in the absence of any hemodynamic effects make specific Na^+-H^+ exchange inhibitors very promising from a clinical point of view. Given as adjunct therapy in numerous reperfusion protocols and in the prevention of myocardial infarction these drugs may prove useful to limit ischemic damage and thereby reduce mortality of myocardial infarction.

Interestingly, Bugge and Ytrehus[76] noted that inhibition of the Na^+-H^+ exchange in the isolated rat heart is additive to the protection afforded by ischemic preconditioning. Although activation of protein kinase C (PKC) on one hand seems to be involved in mediating protective effects of ischemic preconditioning,[77 78] PKC may on the other hand activate the Na^+-H^+ exchanger.[79 80] This secondary activation of the Na^+-H^+ exchanger may counteract to some degree the protective effects associated with the initial PKC activation and may explain, why inhibition of the Na^+-H^+ exchanger could be of additional benefit to ischemic preconditioning as observed by Bugge and Ytrehus[76]. However, further experiments are necessary to elucidate this possibility, which could be of clinical importance.

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Chapter 2

Ischemic preconditioning reduces infarct size in swine myocardium

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Circulation Research 1990; 66: 1133-1142

Ischemic Preconditioning Reduces Infarct Size in Swine Myocardium

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We evaluated the hypothesis that stunning swine myocardium with brief ischemia reduces oxygen demand in the stunned region and increases tolerance of myocardium to longer periods of ischemia. Wall function was quantified with ultrasonic crystals aligned to measure wall thickening, and stunning was achieved with two cycles of left anterior descending coronary artery (LAD) occlusion (10 minutes) and reperfusion (30 minutes), after which the LAD was occluded for 60 minutes and reperfused for 90 minutes. Infarct size (as a percent of risk region) was then determined by incubating myocardium with *para*-nitro blue tetrazolium. Regional oxygen demand was measured as myocardial oxygen consumption before the 60-minute LAD occlusion in the stunned region; tracer microspheres were used to determine blood flow, and blood from the anterior interventricular vein and left atrium was used to calculate oxygen saturations. After the second reperfusion period, wall thickening in the stunned region was reduced to 1.4±2.4% compared with 36.7±2.5% (mean±SEM) before ischemia ($p<0.001$). Regional myocardial oxygen consumption after stunning (3.1±0.7 ml O_2/min/100 g) was no different from regional myocardial oxygen consumption before stunning (3.7±0.6 ml O_2/min/100 g). In the nine pigs "preconditioned" by stunning, infarct size was 10.4±6.3% of the risk region compared with 48.0±12.7% in the six control pigs subjected to 60 minutes of ischemia without prior stunning ($p<0.005$). The risk regions were similar (14.4±1.5% vs. 14.6±1.9% of the left ventricle, preconditioned vs. control pigs, respectively). We conclude that stunning swine myocardium with two cycles of a 10-minute LAD occlusion followed by reperfusion increases ischemic tolerance but that changes in regional demand in stunned myocardium do not predict the marked reduction in infarct size that follows a subsequent 60-minute period of ischemia. (*Circulation Research* 1990;66:1133–1142)

Myocardium subjected to continuous, severe ischemia does not suffer irreversible injury if reperfused within 15–20 minutes.[1,2] Repeated periods of ischemia of less than 15 minutes do not result in a cumulative injury if myocardium is reperfused between episodes of ischemia.[1,3] Paradoxically, repeated ischemia-reperfusion cycles render the myocardium more resistant to infarction during subsequent longer episodes of ischemia, a phenomenon that has been termed "ischemic preconditioning."[3] The mechanisms for ischemic preconditioning are not completely understood, although a number of important observations have been made. An initial period of brief ischemia followed by reperfusion has been shown to retard the consumption of high-energy phosphates during subsequent ischemic episodes.[4–8] Murry et al[3–5] have also observed a reduction in lactate accumulation in ischemic myocardium preconditioned with prior episodes of ischemia; this observation has led them to hypothesize that the increased ischemic tolerance accrues from preservation of energy resources during ischemia and/or from a reduction in the accumulation of toxic catabolites. This same group has implicated oxygen free radicals in the mechanism of preconditioning.[9] In a preliminary report, the protective effects of ischemic preconditioning were blocked by the administration of superoxide dismutase and catalase. This suggests that free radical generation during the initial ischemia-reperfusion cycles conditions the canine myocardium to the subsequent damage inflicted by 60 minutes of ischemia.[9]

Brief episodes of ischemia and reperfusion also produce transient contractile dysfunction,[10] termed "stunned myocardium,"[11] which in some models correlates with decreased myocardial oxygen consumption ($M\dot{V}O_2$) in the ischemic region during reperfusion.[12,13] In swine, the effects of stunning on $M\dot{V}O_2$ are controversial. Some investigators[14] have observed reduced $M\dot{V}O_2$; others[15] have reported no changes in

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Received May 11, 1989; accepted November 21, 1989.

$M\dot{V}O_2$ of stunned swine myocardium. We assessed $M\dot{V}O_2$ in stunned myocardium as a measure of regional demand; we hypothesized that the depressed contractile dysfunction that characterizes stunned myocardium reduces oxygen demand and that this contributes to the reduction in infarct size observed with ischemic preconditioning. Implicit in this hypothesis is the assumption that changes in regional demand before occlusion affect the evolution of necrosis during a subsequent ischemic episode, when metabolism becomes anaerobic and contractile effort is rapidly supplanted by systolic bulging.[10] However, it is known that variations in global left ventricular demand at the onset of occlusion affect the extent of necrosis during a subsequent ischemic episode.[16]

We selected swine myocardium because of its similarity to human myocardium in two important respects: there are few native collaterals,[17] and swine myocardium has no detectable xanthine oxidase, which is one likely source of oxygen free radicals.[18,19] Therefore, we subjected stunned swine myocardium to 60 minutes of ischemia to determine whether tissue without xanthine oxidase activity would show an increase in ischemic tolerance and to determine if an increase in ischemic tolerance could be predicted by a decrease in demand associated with stunned myocardium.

Materials and Methods

Experimental Preparation

Male mixed-breed Landrace-type domestic pigs weighing 19–23 kg were sedated with 2 mg/kg i.m. azaperone (Stresnil, Janssen Pharmaceutica, Neuss, FRG) 30 minutes before anesthesia with 30 mg/kg i.v. pentobarbital. After tracheostomy, the pigs were mechanically ventilated with a respirator (Mark 7, Bird Products, Palm Springs, California) on room air supplemented with 2 l oxygen per minute. Frequent arterial blood gases were measured to guide adjustment of ventilator settings. Both internal jugular veins were cannulated with polyethylene catheters. Anesthesia was maintained with continuous infusion of pentobarbital at 3 mg/min through one jugular catheter; the other was used for fluid and drug administration. The right femoral artery was cannulated with a polyethylene catheter, which was advanced to approximately the midaorta for continuous recording of arterial pressure and withdrawal of reference blood samples. The heart was exposed through a midline thoracotomy and suspended in a pericardial cradle. The left anterior descending coronary artery (LAD) was gently dissected free of surrounding tissue after the second branch. A loose ligature was placed around the vessel, which was subsequently occluded with a small vascular clip. A 27-gauge butterfly needle was introduced into the epicardial veins for collection of the blood samples used for calculation of regional venous oxygen saturations. The anterior interventricular vein, adjacent

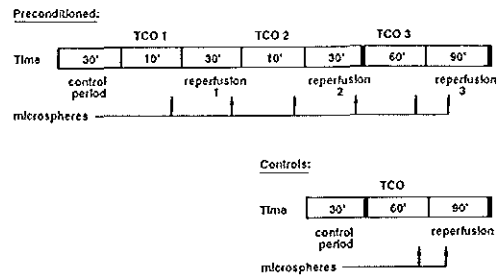

FIGURE 1. *Diagram of experimental design. Upper row of boxes contains coronary occlusion (TCO) and reperfusion times for preconditioned hearts; lower row of boxes contains TCO and reperfusion times for control hearts. Arrows indicate administration of tracer microspheres for blood flow determination. At the end of the final 90-minute reperfusion, biopsies for high-performance chromatography were taken, before heart excision and infarct size determination.*

to the occlusion site, was cannulated in a similar fashion to obtain oxygen saturations from the stunned region. A catheter was placed in the left atrium for injection of tracer microspheres and intermittent monitoring of left atrial pressure, which was used to guide fluid replacement. A stiff polyethylene catheter with multiple side holes was advanced over a wire into the right atrium and inserted retrograde between 1 and 2 cm into the terminal segment of the coronary sinus to allow collection of blood for the calculation of coronary sinus oxygen saturation. A micromanometer (Millar Instruments, Houston, Texas) was advanced via the carotid artery into the left ventricle for the continuous measurement of left ventricular pressure. Rectal temperature was monitored throughout the experiment, and the chest cavity was covered with a plastic sheet and kept warm with a heat lamp. Myocardial function in the ischemic area was measured with 5-MHz ultrasonic crystals aligned transmurally to measure wall thickness.[20] A brief (<10-second occlusion) was performed to identify the ischemic region, and the crystal pair was located centrally in the ischemic region. The inner crystal was advanced to the endocardium tangentially through a stab wound in the epicardium. The epicardial crystal, attached to a Dacron patch, was then positioned on the epicardium with the aid of an oscilloscope and secured with two sutures.

Experimental Design

The experimental design is depicted in Figure 1. The pigs were divided into two groups: preconditioned and control. Both groups underwent a 30-minute control period. Preconditioned pigs were then subjected to two 10-minute occlusions followed by 30-minute reperfusions before the final 60-minute occlusion and 90-minute reperfusion. Control pigs were subjected to only the 60-minute occlusion followed by 90 minutes of reperfusion.

Myocardial Blood Flow Measurements

Blood flow measurements were made with 10 μm tracer microspheres (New England Nuclear, Boston, Massachusetts) by using the reference withdrawal method.[21] Approximately 2 million spheres labeled with ^{113}Sn, ^{103}Ru, ^{46}Sc, ^{141}Ce, ^{57}Co, and ^{95}Nb were injected into the left atrium in random order. Reference blood was withdrawn at a constant rate (20 ml/min) from the aortic catheter. After risk region and infarct size assessments were made at the conclusion of the experiment, each ring of the left ventricle (approximately 1 cm in width) was placed in formalin. After allowing at least 72 hours for fixation, the rings were removed from formalin and cut into 4–16 sections depending on the size of the ring. Each section was further divided into approximately equal endocardial, midmyocardial, and epicardial samples, which were weighed (generally 50–300 mg) and placed into labeled tubes for gamma counting in a germanium solid-state well-type detector.[22] The compound spectrum was processed by a PDP-11/24 computer for generation of blood flow maps for each ring. From the first microsphere injection during ischemia, three perfusion patterns were discerned: normally perfused tissue, ischemic tissue (which averaged <10 ml/min/100 g of flow during occlusion), and mixed tissue. Blood flow during reperfusion in the previously identified ischemic tissue was used for calculation of regional ischemic $M\dot{V}O_2$, whereas blood flow from all tissue was used for calculation of global $M\dot{V}O_2$.

Risk Region and Infarct Size

After 90 minutes of reperfusion, the LAD was reoccluded, and 10 ml of 10% fluorescein dye was injected into the left atrial catheter. After 1–2 minutes, the pig was killed with a bolus injection of KCl, and the heart was excised. Warm 2% agarose was injected into the left ventricle via the aortic root, and the heart was placed on ice. After the agarose gelled, the right ventricle was cut away, and the left ventricle was "breadloafed" into 4–6 rings approximately 1 cm wide perpendicular to the LAD. The rings were weighed, and the risk region was traced onto an acetate sheet under a black light, which sharply defined the borders of the risk area, which was not perfused with fluorescein. Subsequently, the rings were incubated for 30–45 minutes in 0.125 g *para*-nitro blue tetrazolium (p-NBT) per liter of phosphate buffer (pH 7.1) at 37° C; p-NBT stains noninfarcted tissue deep blue and leaves infarcted tissue pale.[23] Rings were then photographed with a Polaroid MP-4 camera, and acetate tracings of the ring and infarcted region were planimetered with a graphics tablet. With these data, the percent risk region (area at risk divided by area of ring) was calculated for the top and bottom of each ring, and the average value for each ring was multiplied by the weight. The weights of the regions were summed and divided by the weight of the left ventricle to yield the percent of the left ventricle at risk and percent of the left ventricular risk region infarcted.

Myocardial Oxygen Consumption

$M\dot{V}O_2$ was determined in three regions of preconditioned hearts at three time points: during the control period, at 25 minutes after the first reperfusion, and at 25 minutes after the second reperfusion. The blood flow for calculation of control $M\dot{V}O_2$ was obtained from normal region flow during the first 10-minute occlusion (Figure 1). Oxygen saturation for global $M\dot{V}O_2$ determination was obtained from the coronary sinus and left atrium. Global and regional $M\dot{V}O_2$ at the first and second reperfusions were determined from blood flow measurements made concomitantly with the oxygen saturation measurements. The regional $M\dot{V}O_2$ represents oxygen consumption in the stunned region. Oxygen saturations were obtained from the anterior interventricular vein at the level of the occlusion. In six preconditioned pigs, local nonischemic $M\dot{V}O_2$ was obtained by calculating the oxygen saturations in a small vein draining the anterolateral wall of the left ventricle and by using the blood flow data from the first ring, which in all cases was outside the risk region. This served as a second control $M\dot{V}O_2$. All $M\dot{V}O_2$ values were calculated in the following fashion:

O_2 saturation = oxyhemoglobin/(hemoglobin + oxyhemoglobin)
O_2 content = 1.34 × hemoglobin × O_2 saturation
O_2 extraction = left atrial O_2 content − venous O_2 content
O_2 consumption = O_2 extraction × blood flow

The hemoglobin was measured on a Sysmex microcell counter (model CC180, Digitana AG, Hamburg, FRG) and was measured at the time of the second reperfusion. Blood samples for oxyhemoglobin concentrations were kept on ice until measured on blood gas analyzer (model AVL 995, Bad Homburg, FRG) within 10 minutes from the time the sample was drawn.

Biochemical Analysis

At the conclusion of the final reperfusion period, biopsies from the risk and the control area were taken with a high-speed biopsy drill and frozen within 10 seconds in liquid nitrogen until analysis. One half of the specimens was randomly selected from both the preconditioned and control groups for measurement of ATP, creatine phosphate (CP), ADP, and AMP by ion-paired high-pressure liquid chromatography. Briefly, the column used was a μBondapak C-18 (250 mm × 4 mm, 10 μm thick)(Waters, Millipore, Milford, Massachusetts). Buffers were 100 mM ammonium phosphate with 6 mM tetrabutylammonium hydrogen sulfate as buffer A and 30% acetonitrile plus 70% buffer A as buffer B (as modified by K. Hashizume in this laboratory). Step 1 was 98% buffer A and 2% buffer B for 5.5 minutes isocratically. Step

2 was from 98% to 0% buffer A and from 2% to 100% buffer B for 15 minutes on a linear gradient. Step 3 was 100% buffer B for 4.5 minutes isocratically, and then the column was reequilibrated with the same buffer as in step 1 for about 35 minutes.

Data Analysis

Continuous recording of hemodynamic and wall thickness data was made on a Graphtec recorder (Watanabe Instruments, Japan). Recordings at time points of interest were made at a paper speed of 50 mm/sec, and the data were collected from the record by hand. Wall thickness variables were averaged over five cycles at each time point and included end-diastolic wall thickness (EDWT, defined at 20 msec before peak negative dP/dt) and end-systolic wall thickness (ESWT, defined at the onset of peak positive dP/dt). These data were used to calculate the extent of wall thickening with the following formula: [(ESWT−EDWT)/EDWT]×100. Other variables include peak systolic pressure, mean arterial pressure, and heart rate. The double product was calculated by multiplying peak systolic pressure by heart rate.

Data are reported as mean±SEM. For comparison of variables across time between preconditioned and control groups, a two-factor analysis of variance (ANOVA) for repeated measures was used. When the ANOVA was significant, pairwise comparisons were made with Student's t test with the significance level for the p values corrected with the Bonferroni method. For comparison across time in the preconditioned group, a one-factor ANOVA of repeated measures was employed, with an identical post hoc testing strategy as for the two-factor ANOVA. A two-sample rank-sum test (Mann-Whitney U test) was used to test the differences between preconditioned and control animals with regard to risk region and the precent of the risk region infarcted.

Results

Twenty-five pigs were randomly divided between a control group (n=12) and a preconditioned group (n=13). Three preconditioned pigs (23%) were excluded from the study because of intractable fibrillation. A fourth preconditioned pig fibrillated at the very end of the study as the fluorescein was injected, after biopsy. Because the risk region could not be determined, this pig was not included in the infarct analysis. (The heart was cut and incubated with p-NBT and noted to have minimal infarction.) Two preconditioned pigs were excluded from $M\dot{V}O_2$ analysis because of technical problems with measurements (one with blood flow and the other with oxyhemoglobin concentrations). Of the 10 preconditioned pigs included in the study, nine were analyzed for infarct size, and eight were analyzed for oxygen consumption. Two control pigs were excluded because of fibrillation (16.7%); one was excluded for poor wall function in the control state, and the other was excluded because the risk area was only 6% of the left ventricle (13% of the risk region was noted to be infarcted in this pig). Two control pigs were excluded because of failure to demonstrate reperfusion with the microspheres; the pattern of reperfusion was consistent with spasm[24] or arterial thrombus in one animal, and in the second animal, the gamma counter failed to detect activity above background in any of the tissue specimens with reperfusion. (The experiment was otherwise completed, and 34% of the risk region was infarcted.) This left six pigs, which formed the control group.

Hemodynamic and Wall Function

Hemodynamic and wall thickness data are summarized in Table 1. The preconditioned pigs remained remarkably stable throughout each of the occlusions and reperfusions and did not vary significantly from the control pigs immediately before and during the 60-minute occlusion with regard to heart rate, mean arterial pressure, or double product. For preconditioned pigs, heart rate, mean arterial pressure, or double product did not vary from control values at any time point throughout the entire experiment. The changes in wall function through each of the brief occlusions and reperfusions for the preconditioned pigs are presented in Figure 2.

Myocardial Blood Flow

Myocardial blood flow values are summarized in Table 2. Myocardial blood flow for preconditioned and control pigs was not different during the 60-minute occlusion and subsequent reperfusion in either ischemic or nonischemic regions. There was no difference across time in endocardial, epicardial, or transmural blood flow by ANOVA in the nonischemic regions in both preconditioned and control pigs. For ischemic region myocardial blood flow comparisons within the preconditioned group, the reperfusion time points were grouped separately from the occlusion time points. Within the ischemic region, no differences were detected in endocardial, epicardial, or transmural blood flow during the three occlusions. The repeated-measures ANOVA was significant for the three reperfusion time points in the epicardial, endocardial, and transmural sections of ischemic tissue. Pairwise testing with the Bonferroni correction for differences during reperfusion in the ischemic region of preconditioned pigs revealed a significant reduction in endocardial ($p<0.005$), epicardial ($p<0.05$), and transmural blood flow ($p<0.01$) between the first and second reperfusion periods. Thus, as the myocardium became nearly akinetic in the second reperfusion period, blood flow decreased transmurally to this region.

Myocardial Oxygen Consumption

Global $M\dot{V}O_2$, local nonischemic $M\dot{V}O_2$, and regional (stunned) $M\dot{V}O_2$ is represented for preconditioned pigs before occlusion and at the end of the first and second reperfusion periods in Figure 3. Although a tendency for global and local nonischemic $M\dot{V}O_2$ to increase and for regional $M\dot{V}O_2$ to

TABLE 1. Hemodynamic and Wall Function Data

Time period	HR (beats/min)	MAP (mm Hg)	DP (/100)	EDWT (mm)	ESWT (mm)	ΔWT (%)
Preconditioned pigs (n=10)						
Control	96.0±3.1	61.7±2.7	72.6±3.8	5.2±0.2	7.0±0.3	36.7±2.5
TCO1	94.0±2.7	58.6±2.4	72.3±5.5	4.4±0.4	4.2±0.3*	−4.0±4.8*
REP1	96.5±2.4	57.1±3.6	68.7±4.8	5.1±0.2	5.9±0.3*	14.8±3.3*
TCO2	94.4±2.9	54.9±3.5	65.1±4.5	4.6±0.2	4.1±0.2*	−12.6±2.3*
REP2	100.5±4.9	54.9±4.6	67.5±6.6	5.2±0.2	5.3±0.2*	1.4±2.4*
TCO3	101.7±7.1	53.7±5.0	68.9±6.7	4.8±0.4	4.4±0.3*	−7.6±2.4*
REP3	118.9±6.3	49.9±6.8	78.0±10.9	6.8±0.5	5.4±0.8	−10.2±2.8*
Control pigs (n=6)						
Control	95.0±6.2	62.3±4.4	70.1±8.0	5.7±0.7	7.3±0.8	28.0±6.2
TCO	99.2±6.2	57.5±4.7	61.1±10.3	4.7±0.8	4.2±0.8	−13.0±3.5
REP	107.5±6.6	57.0±5.7	66.2±11.8	7.8±0.8	7.4±0.9	−6.0±4.1

Values are mean±SEM. HR, heart rate; MAP, mean arterial pressure; DP, double product (peak systolic pressure times HR); EDWT and ESWT, end-diastolic and end-systolic wall thickness, respectively, measured with ultrasonic crystals; ΔWT, change in wall thickness calculated by [(ESWT−EDWT)/EDWT]×100. Preconditioned and control pigs underwent a 30-minute control period (Control). The preconditioned group was then subjected to a 10-minute coronary occlusion (TCO1) followed by a 30-minute reperfusion (REP1) and a second 10-minute occlusion (TCO2) followed by a second 30-minute reperfusion (REP2); the group was then subjected to a final 60-minute occlusion (TCO3) followed by a 90-minute reperfusion (REP3). The control group was subjected only to a 60-minute occlusion (TCO) followed by a 90-minute reperfusion (REP). Values were obtained at 5 minutes into TCO1 and TCO2, at 25 minutes into REP1 and REP2, at 30 minutes into TCO3 and TCO, and at 15 minutes into REP3 and REP.

*Significant decrease at $p<0.001$ compared with all time points after the control period.

†Significant decrease at $p<0.005$ compared with all time points after the control period.

‡Significant decrease at $p<0.0001$ compared with control.

decrease is evident, the values at the first and second reperfusion periods were not significantly different from the control period for the stunned region. This finding is consistent with previously reported swine data.[15] To evaluate the dissociation evident in $M\dot{V}O_2$, we calculated the regional/global $M\dot{V}O_2$ ratio. This ratio was 1.0±0.1 before occlusion and decreased to 0.7±0.1 at the end of both the first and second reperfusion periods ($p<0.005$ by ANOVA of repeated measures). The relation between regional wall function and regional $M\dot{V}O_2$ was evaluated by plotting the $M\dot{V}O_2$ measured at each time point (the control period and first and second reperfusions) against the simultaneously obtained regional wall thickening, which is presented in Figure 4. The relation is described by linear regression with a slope of 5.0 (95% CI 0.7–9.4) and a y intercept of 2.1 ($r=0.47$, $p<0.05$). A linear regression of $M\dot{V}O_2$ against regional wall thickening during only the first and second reperfusions when the myocardium is stunned gives a similar slope (slope=5.2, $r=0.62$).

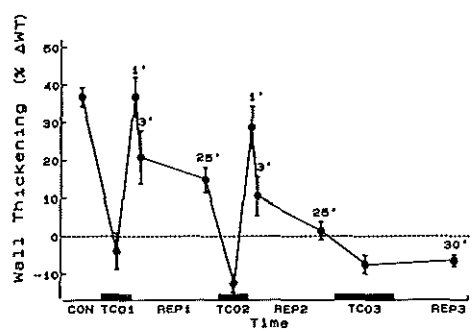

FIGURE 2. *Graph showing ischemic region wall thickening in preconditioned hearts measured with ultrasonic crystals. Wall thickening is calculated as follows: % ΔWT =[(ESWT−EDWT)/EDWT]×100, where ESWT and EDWT are end-systolic and end-diastolic wall thickness, respectively (see "Materials and Methods"). CON (control) is the 30-minute period before the 10-minute coronary occlusions (TCO1 and TCO2). REP1 and REP2 are the 30-minute reperfusion periods. TCO3 is the 60-minute occlusion followed by the final 90-minute reperfusion (REP3). Ischemic region wall function recovers immediately with reperfusion after the 10-minute occlusions, but at the onset of the 60-minute occlusion, this region is akinetic (stunned).*

Risk Region and Infarct Size

The risk region and infarct size expressed as a percentage of risk region are presented in Figure 5. The risk regions were virtually identical (14.4±1.5% for controls vs. 14.6±1.9% for preconditioned pigs, p=NS). The risk area was kept intentionally small by occluding the LAD after the second branch, because large risk areas were associated with an unacceptably high incidence of fibrillation in pilot studies with 45 minutes of LAD occlusion. The percent of the risk region infarcted was 48.0±12.7% for controls and 10.4±6.3% for preconditioned pigs ($p<0.005$ by Mann-Whitney U test). In preconditioned myocardium, the infarcted tissue was generally spotty and scattered throughout the risk region.

High-Performance Liquid Chromatography Data

Biopsy specimens from all experiments were divided randomly into two groups for biochemical

TABLE 2. Myocardial Blood Flow Determined With Tracer Microspheres

	Nonischemic blood flow (ml/min/100 g)			Ischemic blood flow (ml/min/100 g)		
	ENDO	EPI	TRANS	ENDO	EPI	TRANS
Preconditioned pigs (n=9)						
TCO1	103.8±16.1	111.2±8.7	106.7±11.6	5.1±1.2	9.8±1.9	6.8±1.0
REP1	123.7±32.1	115.0±16.0	117.8±23.9	132.8±37.3	123.5±23.2	125.5±28.7
TCO2	110.7±25.4	106.8±14.6	106.6±19.3	4.1±0.9	10.5±2.9	6.8±1.4
REP2	107.9±25.5	97.8±14.1	94.2±20.9	97.0±28.3	93.8±18.3*	93.8±22.0
TCO3	86.0±20.3	87.8±12.8	85.6±15.7	3.1±0.6	7.2±1.9	4.7±1.0
REP3	120.6±26.4	112.6±21.5	115.1±23.5	226.6±64.4	213.9±54.9	220.1±56.2
Control pigs (n=6)						
TCO	92.1±10.6	103.8±7.1	97.7±7.7	7.0±2.4	9.1±1.1	7.7±1.3
REP	106.2±10.2	97.1±5.4	100.9±6.9	149.6±22.5	193.9±6.7	167.9±15.0

Values are mean±SEM. ENDO, endocardial blood flow; EPI, epicardial blood flow; TRANS, transmural blood flow. For preconditioned pigs, values were obtained at 8 minutes into the first and second 10-minute coronary occlusions (TCO1 and TCO2, respectively), at 25 minutes into the first and second 30-minute reperfusions (REP1 and REP2, respectively), at 55 minutes into the 60-minute occlusion (TCO3), and at 25 minutes into the 90-minute reperfusion (REP3). For control pigs, values were obtained at 55 minutes into the 60-minute occlusion (TCO) and at 25 minutes into the 90-minute reperfusion (REP). Occlusion and reperfusion data were grouped separately for analysis.
*$p<0.05$ vs. REP1.
†$p<0.01$ vs. REP1.
‡$p<0.005$ vs. REP1.

analysis. One half was available for high-performance liquid chromatography analysis of CP, ATP, ADP, and AMP, with results reported in Table 3. All values are expressed as nanomoles per milligram of tissue (dry weight). The nonischemic region values were not different between preconditioned and control animals. CP in the ischemic region of preconditioned myocardium was equivalent to that in the nonischemic region at 90 minutes after the 60-minute ischemic period. However, CP remained depressed at less than half of the nonischemic region values in control hearts ($p<0.005$, paired t test). A similar trend is apparent in the ATP data although a statistically significant separation was not achieved.

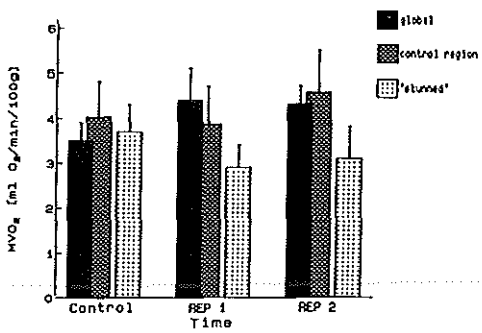

FIGURE 3. *Bar graph showing myocardial oxygen consumption (MVO_2) calculated from the entire left ventricle (global), a nonischemic control region in the circumflex distribution, and from the "stunned" region. Control is the 30-minute period before the first occlusion. REP 1 and REP 2 are the 30-minute reperfusion periods after the 10-minute occlusions. MVO_2 is measured at 25 minutes into REP 1 and REP 2.*

Discussion

Ischemic Preconditioning and Ischemic Tolerance

The primary objective of this study was to assess the effects of stunning on ischemic tolerance in swine myocardium. Myocardium was subjected to two 10-minute ischemic episodes, each followed by 30 minutes of reperfusion, which produced regional akinesis by the end of the second reperfusion period. Myocardium "preconditioned"[4] in this fashion has less than one quarter of the necrosis of myocardium that has not been stunned, despite the additional 20 minutes of ischemia in the preconditioned pigs. The differences in infarct size between preconditioned and control pigs are not attributable to differences in the traditional determinants of infarct size: risk regions and hemodynamics between the two groups

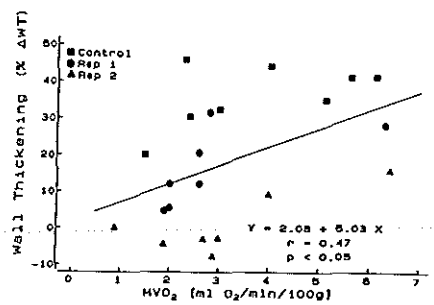

FIGURE 4. *Scatterplot of simultaneously obtained regional myocardial oxygen consumption (MVO_2) versus wall thickening (% ΔWT) for preconditioned pigs before ischemia (control) and at 25 minutes into each 30-minute reperfusion period (Rep 1 and Rep 2) after each 10-minute occlusion. Each experiment is represented by three time points.*

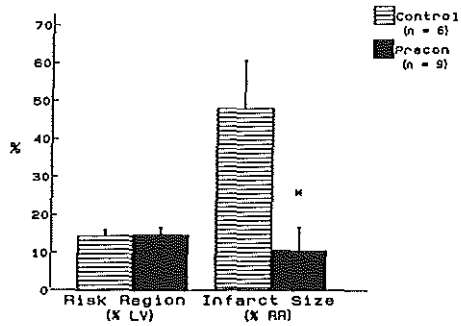

FIGURE 5. *Risk region (as percent of the left ventricle [% LV]) and infarct size (as percent of the risk region [% RR]) for preconditioned pigs and controls.* *$p<0.005$ *vs. controls by Mann-Whitney* U *test.*

were similar, and collateral transmural blood flow to the ischemic region in swine was minimal (<10 ml/min/100 g in these experiments).[1,24,25] These results are in close agreement with preconditioning effects reported by Murry et al,[3] who observed a 75% reduction in infarct size after 40 minutes of LAD occlusion in dogs preconditioned with four 5-minute occlusions followed by 5-minute reperfusions. Li et al[26] have also observed a substantial (10-fold) decrease in infarct size in dogs preconditioned with as little as 5 minutes of occlusion followed by 5 minutes of reperfusion before a 60-minute circumflex occlusion.

The mechanism of preconditioning is not completely understood although a number of researchers[4-8] have observed that ATP depletion is slowed after an initial episode of ischemia. Murry et al[3] have speculated that this, coupled with reduced catabolite accumulation after the initial periods of ischemia, could account for the protective effect of preconditioning. Furthermore, CP stores exhibit an "overshoot" with reperfusion after brief periods of ischemia,[27,28] making more energy stores available when entering a long period of ischemia. However, CP is depleted within minutes after the onset of ischemia, and the additional CP from the overshoot is probably not sufficient to sustain viability

TABLE 3. High-Energy Phosphates

Group	Creatine phosphate	ATP	ADP	AMP
Preconditioned pigs				
Ischemic	33.8±2.1	5.5±0.1	2.3±0.4	0.2±0.1
Nonischemic	35.9±3.0	20.4±0.3	2.9±0.4	0.3±0.1
Control pigs				
Ischemic	13.2±2.1*	4.0±0.9	1.9±0.2	0.2±0.1
Nonischemic	27.9±4.2	20.3±1.4	3.0±0.4	0.3±0.1

Values are mean±SEM and are in nanomoles per milligram of dry weight. Ischemic biopsies were taken from the center of the ischemic region 90 minutes after the final reperfusion; nonischemic specimens were taken from the lateral wall at the same time.
*$p<0.005$ vs. preconditioned ischemic region myocardium.

through 60 minutes of ischemia. Recently, it has been reported that preadministration of superoxide dismutase and catalase can blunt the preconditioning effects of brief ischemia; this finding suggests that oxygen free radical species produced during the initial episodes of brief ischemia might be important in the development of tolerance to subsequent longer episodes of ischemia.[9] Our demonstration of a preconditioning effect in myocardium devoid of xanthine oxidase activity[19] suggests either another source of free radicals (i.e., neutrophils[29]) or another mechanism for the preconditioning effects of brief ischemia in pigs.

We determined infarct size in myocardium reperfused for 90 minutes after the final occlusion period by incubating the myocardium with p-NBT. This established technique relies on the conversion of the tetrazolium salt to formazan in the presence of cofactors (nicotinamide adenine dinucleotides), which stain viable myocardium deep blue.[23] Infarcted tissue loses these cofactors during reperfusion and remains pale and easily distinguishable from viable myocardium. The duration of reperfusion periods used with this technique has been quite variable; 30 minutes of reperfusion is the minimum time reported for the delineation of infarcted tissue.[24] Recently, Fujiwara et al[30] compared 1-, 3-, and 7-hour reperfusion times after 1 hour of distal LAD occlusion in domestic swine and reported that 1 hour of reperfusion was adequate for the delineation of infarct size when cut specimens were incubated with p-NBT. In that study, infarct size was also determined with an immunohistochemical technique employing myoglobin antibodies, as well as with hematoxylin-eosin and Masson's trichrome staining of the myocardium. We have previously reported on the adequacy of p-NBT technique after 90 minutes of reperfusion, with ultrastructural verification of infarction.[23]

Myocardial Oxygen Consumption in Stunned Myocardium

A second objective of this study was to measure $M\dot{V}O_2$ in stunned myocardium. We hypothesized that preconditioning might result from stunning; with ischemic disengagement of the contractile apparatus, oxygen demand would be lowered entering a subsequent ischemic period. This requires an important assumption: variations in regional demand under aerobic conditions at the onset of occlusion affect the evolution of myocardial necrosis under anaerobic conditions during occlusion. Previous experiments[16] have demonstrated that variations in global demand before the onset of ischemia can alter the rate of necrosis after arterial occlusion, whereas alterations in $M\dot{V}O_2$ midway through a 90-minute occlusion do not affect the extent of necrosis. This finding suggests that the period immediately after occlusion in virgin myocardium is critical, which is supported by the observation of Neely and Feuvray[31] that there is a burst of anaerobic glycolysis immediately after occlusion in virgin myocardium, with slowing of aerobic

glycolytic flux as ischemic catabolites (lactate, nicotinamide adenine dinucleotide, and H^+) accumulate.[32,33] The data of Murry et al[5] indicate that the rate of ATP depletion and lactate production are greatest in the first 10 minutes after arterial occlusion. We hypothesized that continued contractile effort after occlusion was responsible for the accelerated anaerobic flux and that the rate of anaerobic flux could be slowed by stunning the myocardium. Our failure to observe a significant change in $M\dot{V}O_2$ in stunned myocardium suggests that despite near akinesis, oxidative metabolism remains relatively constant. Thus, the marked protective effect cannot be explained by an appreciable decrease in regional demand entering the ischemic period.

$M\dot{V}O_2$ of stunned myocardium has been a continuing source of controversy, with groups reporting that $M\dot{V}O_2$ is decreased,[12–14] unchanged,[15,34] or increased.[35] This may reflect species differences as well as the heterogeneity found in the contractile effort in myocardium that is labeled "stunned." A recent brief report suggests that regional $M\dot{V}O_2$ is minimally reduced until function is depressed to the onset of systolic bulge, at which point $M\dot{V}O_2$ falls off rapidly.[36] The akinetic stunned myocardium in the experiments reported here retains some residual contractile effort: the myocardium bulges 12% during the second occlusion but "recovers" to akinesis with the second reperfusion. Therefore, in addition to the basal metabolic requirements of myocardial tissue, which may account for one fourth to one third of resting $M\dot{V}O_2$,[37] there is the contractile effort to maintain akinesis.

With the necessity for small risk regions in these experiments, some function in the stunned region may artifactually result from "tethering" to normal myocardium, although Gallagher et al[38] have shown that this "functional border zone" extends for less than 0.5 cm in either direction from the perfusion boundary when ultrasonic crystals are aligned to measure wall thickness.

Although we report that regional $M\dot{V}O_2$ did not change significantly from the $M\dot{V}O_2$ before stunning, the trend was for gradually decreased $M\dot{V}O_2$ in the stunned myocardium. In addition to (stunned) regional $M\dot{V}O_2$, we calculated left ventricular global $M\dot{V}O_2$ from the coronary sinus oxygen saturation and mean blood flow to the entire left ventricle. In a subset of six pigs, a second control $M\dot{V}O_2$ was measured in a normally contracting region of myocardium in the left circumflex distribution. $M\dot{V}O_2$ in these control regions tended to increase after coronary occlusion. A change in hemodynamics did not account for this, although it is known that the nonischemic regions of the myocardium compensate with increased contractility during occlusion[39] and that this may in turn account for the increased $M\dot{V}O_2$ in this region. When (stunned) regional $M\dot{V}O_2$ was compared with the simultaneously obtained global $M\dot{V}O_2$, regional $M\dot{V}O_2$ fell from 100% of global $M\dot{V}O_2$ before occlusion to 70% of the global $M\dot{V}O_2$ with stunning ($p<0.005$). This was accompanied by a fall in transmural blood flow from the first to the second reperfusion period; this fall is consistent with downregulation of blood flow in response to decreased oxygen demand.[13] Regional $M\dot{V}O_2$ was weakly correlated with regional wall function ($r=0.47$, $p<0.05$).

$M\dot{V}O_2$ before occlusion is relatively low (3.5 ± 0.4 ml O_2/min/100 g) in this model. This is less than we have previously reported for swine using the Bretschneider equation,[24] although it is consistent with other reports of measured $M\dot{V}O_2$ in swine.[14] This may be a function of an open-chest preparation[34] but also may reflect inclusion of small quantities of right ventricular venous effluent in our measurements of oxyhemoglobin from the interventricular vein and coronary sinus. The right ventricle has a lower oxygen consumption,[40] which may have elevated the oxyhemoglobin concentrations that we measured. In addition, oxygen saturations from the stunned region are included in the calculations of global $M\dot{V}O_2$ during the first and second reperfusion periods.

High-Energy Phosphates

CP levels from the preconditioned myocardium at 90 minutes of reperfusion were twice that of myocardium subjected to a single 60-minute episode of ischemia, despite an additional 20 minutes of ischemia. Our conclusion is that preconditioned myocardium is viable after 60 minutes of ischemia and is able to resume oxidative phosphorylation and replete CP stores. It is less likely that preconditioned myocardium can preserve significant quantities of CP, which under normal circumstances is rapidly depleted after the onset of ischemia.[33] ATP and ADP were depleted to the same degree at 90 minutes after the 60-minute occlusion in both preconditioned and control myocardium. Regeneration of creatine kinase occurs quickly in stunned myocardium[28]; however, ATP and ADP resynthesis requires days after brief ischemia.[41]

Summary

In summary, we demonstrated that two brief cycles of ischemia and reperfusion increase the ischemic tolerance of swine myocardium during a subsequent 60-minute coronary occlusion. In addition to reducing the volume of infarcted myocardium, preconditioning with ischemia was associated with restoration of CP stores 90 minutes after a 60-minute episode of ischemia. $M\dot{V}O_2$ in stunned myocardium was decreased when compared with simultaneously measured global left ventricular $M\dot{V}O_2$; however, it was not decreased when compared with preischemic regional $M\dot{V}O_2$. This suggests that increases in ischemic tolerance are independent of changes in regional oxygen demand associated with stunning. The precise mechanisms by which ischemically primed myocardium can protect itself remain unclear. The extent of the infarct size reduction exceeds any that has been produced with pharmacological interventions and warrants further careful investigation.

Acknowledgments

The authors acknowledge Bernd Winkler for his technical support with the microspheres, Sybille Jürgens for her assistance with surgery, and Koyuki Hashizume and Linda Morrow for their work with the high-performance liquid chromatography. We thank Kim Gallagher and Thomas McClanahan for providing us with the ultrasonic crystals that were used for these experiments.

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KEY WORDS • stunning • infarct size • myocardial oxygen consumption • reperfusion injury • preconditioning

Chapter 3

Effect of bimakalim (EMD 52692), an opener of ATP sensitive potassium channels, on infarct size, coronary blood flow, regional wall function, and oxygen consumption in swine

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Cardiovascular Research 1994; 28: 858-863

Effect of bimakalim (EMD 52692), an opener of ATP sensitive potassium channels, on infarct size, coronary blood flow, regional wall function, and oxygen consumption in swine

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Objective: The aim was to assess whether bimakalim, an opener of ATP sensitive potassium channels, can reduce infarct size in swine myocardium. **Methods:** Experiments were performed in open chest pigs subjected to a 60 min occlusion of a branch of the left anterior descending coronary artery and to 2 h reperfusion. Five groups of animals were studied. In seven animals bimakalim infusion (3 $\mu g \cdot kg^{-1}$ bolus over 5 min followed by 0.1 $\mu g \cdot kg^{-1} \cdot min^{-1}$) was started at 45 min of coronary occlusion and continued until 60 min of reperfusion (group A), while in seven other animals the bimakalim infusion was started 15 min before occlusion and also ended at 60 min of reperfusion (group B). In a further seven animals bimakalim infusion was started 15 min before coronary occlusion, but was stopped at the onset of ischaemia (group C). In the fourth group of animals ($n = 7$), a hydralazine infusion (0.2 $mg \cdot kg^{-1}$ over 15 min) was started 15 min before the occlusion and also terminated at the start of occlusion. The dose of hydralazine was chosen such that it lowered arterial pressure to the same extent as bimakalim. A fifth group of animals ($n = 7$) received the vehicle and served as controls. At the end of the protocol, infarct size (as percent of risk region) was determined by incubating myocardium with p-nitrobluetetrazolium. Regional myocardial oxygen consumption ($M\dot{V}O_2$) was calculated as the product of coronary blood flow (electromagnetic flowmeter) and the difference in the oxygen contents of the aorta and the interventricular vein accompanying the left anterior descending coronary artery. Regional wall function was quantified with ultrasonic crystals aligned to measure wall thickening (%ΔWT). **Results:** In all pigs in which bimakalim treatment was started prior to the 60 min coronary occlusion, infarct size was significantly reduced [B: 22.4(SEM 4.5)%; C: 35.3(6.6)%] compared with 60.4(5.2)% in pigs subjected to 60 min of ischaemia only ($p < 0.05$); drug-induced potassium channel opening during reperfusion had no effect [A: 56.6(4.1)%]. Treatment with hydralazine did not reduce infarct size [59.4(4.3)%]. Neither drug altered %ΔWT; however, they reduced $M\dot{V}O_2$ by 36.5% in B, by 27.1% in C, and by 14.6% in the hydralazine group. **Conclusions:** Bimakalim treatment prior to the onset of a 60 min coronary occlusion increases the tolerance of pig myocardium to ischaemia. The data are consistent with the hypothesis that bimakalim reduces infarct size by activation of cardiac ATP sensitive potassium channels and not through unloading of the heart because of its vasodilator effects.
Cardiovascular Research 1994;28:858-863

Potassium channel activators are a chemically diverse group of compounds that are thought to open ATP sensitive potassium channels, thereby relaxing smooth muscle[1 2] and shortening cardiac action potentials.[3 4] These effects of potassium channel activators are antagonised by glibenclamide, a relatively selective blocker of ATP sensitive potassium channels in a number of tissue types.[5 6] There is growing evidence that these potassium channel activating compounds have an anti-ischaemic effect, as pinacidil and cromakalim have been shown to improve postreperfusion function and to reduce the release of lactate dehydrogenase (LDH) in an in vitro model of global ischaemia. The protective actions were fully reversed by glibenclamide.[6] Furthermore, it has been proposed that potassium channel activation is involved in the phenomenon termed "ischaemic preconditioning", in which brief periods of coronary artery occlusion followed by reperfusion increase the tolerance of myocardium to subsequent longer episodes of ischaemia.[7] In ischaemically preconditioned myocardium the development of myocardial necrosis during a subsequent coronary occlusion is markedly delayed.[8 9] The availability of agonists and antagonists for ATP sensitive potassium channels enabled Gross and Auchampach[10] to study this in more detail; the ATP sensitive potassium channel antagonist glibenclamide blocked the protective effect of preconditioning, while the ATP sensitive potassium channel opener RP 52891 mimicked it in canine myocardium.

We have recently reported that bimakalim,[11] another potassium channel activator under development as an antianginal drug, reduces infarct size in pigs. In this species bimakalim has been shown to decrease arterial blood pressure due to systemic vasodilatation, to increase heart rate, and to exert no effect on left ventricular filling pressure or myocardial contractility.[12] The present study was performed to analyse the nature of the cardioprotective effect of bimakalim. In order to test whether the protective effect is

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related to alterations of myocardial contractility, coronary blood flow, or oxygen consumption ($M\dot{V}O_2$) in healthy tissue prior to infarction we started bimakalim treatment before or during ischaemia and compared its effects with those of an equihypotensive dose of the vasodilator hydralazine.

Methods

The investigation conforms with the *Guide for the care and use of laboratory animals* published by the US National Institutes of Health (NIH publication No 85-23, revised 1985).

Experimental design

All 35 pigs underwent a 60 min left anterior descending coronary artery occlusion followed by 120 min of reperfusion. The animals were divided into five groups. The design is depicted in fig 1. Vehicle treated animals served as controls. Bimakalim treatment was performed as follows: group A (n = 7) received bimakalim starting 45 min after coronary occlusion and continued during the first 60 min of reperfusion; in group B (n = 7) bimakalim was infused from 15 min before occlusion until 60 min after start of reperfusion: animals in group C (n = 7) received bimakalim only for 15 min before the onset of ischaemia. Hydralazine (n = 7) was given in the same way as bimakalim in group C.

Experimental preparation

Thirty five mixed breed Landrace-type domestic pigs of either sex weighing 23 and 29 kg were premedicated with ketamine-HCl (500 mg intramuscularly) 15 min prior to anaesthesia with intravenous chloralose (100 mg·kg^{-1}). Following tracheotomy pigs were mechanically ventilated with nitrous oxide supplemented with oxygen at a ratio of 2:1. In order to guide adjustment of ventilator settings arterial blood gases were measured repeatedly. Anaesthesia was maintained by continuous infusion of pentobarbitone at 3 mg·kg^{-1}·h^{-1} through a catheter in the right jugular vein. Arterial blood was sampled through a catheter placed in the right femoral artery, while the right brachial artery was cannulated with a polyethylene catheter, which was advanced to the ascending aorta for continuous recording of arterial pressure. An 8F Millar micromanometer was advanced via the carotid artery into the left ventricle for continuous measurement of left ventricular pressure. The heart was exposed through a midline thoracotomy and suspended in a pericardial cradle. The proximal part of the second branch of the left anterior descending coronary artery was carefully dissected free over a length of 4 mm and a loose ligature was placed around the vessel which could be occluded with a small vascular clip. Electromagnetic flow probes were situated on the ascending aorta and the left anterior descending coronary artery proximal to the occlusion site. A Teflon catheter (outer diameter 0.8 mm) was placed into the epicardial vein draining the area at risk. Rectal temperature was monitored throughout the experiment and the chest cavity was covered with a plastic sheet and kept warm with a heat lamp to keep body temperature between 37°C and 38°C. Myocardial function in the ischaemic area was measured with 5 mHz ultrasonic crystals aligned transmurally to measure wall thickness. A brief (<10 s) occlusion was performed to identify the ischaemic region and the crystal pair was located centrally in this region. The inner crystal was advanced to the endocardium tangentially through a stab wound in the epicardium. The epicardial crystal, attached to a Dacron patch, was positioned on the epicardium with the aid of an oscilloscope, and secured with three sutures. Following a stabilisation period of 30 min after surgery the experimental protocol was started.

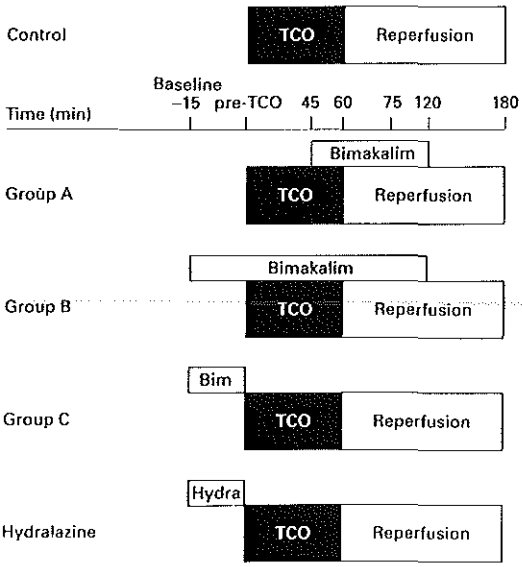

Figure 1 Design of the study. Bim = bimakalim; TCO = total coronary artery occlusion; Hydra = hydralazine.

Risk region and infarct size

Following a reperfusion period of 2 h, the coronary artery was reoccluded and 10 ml of 10% fluorescein dye were injected into the left atrium. After 1-2 min the pig was killed by electrical fibrillation with a 9 V battery and the heart excised. Warm 2% agarose was injected into the ventricle via the aortic root and the heart was kept on ice. After the agarose had congealed, the right ventricle was removed and the left ventricle was sliced into four to six rings perpendicular to the left anterior descending coronary artery. Subsequently, the rings were weighed and the risk region traced onto an acetate sheet under blacklight, which sharply defined the borders of the risk area not perfused with fluorescein. Incubation for 30 min in 1.25% paranitrobluetetrazolium in phosphate buffer (pH 7.1) at 37°C delineated non-infarcted tissue (deep blue) and infarcted tissue (pale). Rings were then photographed with a Polaroid camera and acetate tracings of the ring and infarcted region were determined by planimetry. The percent risk region (area at risk/area of ring) was calculated for the top and bottom of each ring and the mean value for each ring was multiplied by the weight. The weights of regions were summed and divided by the weight of the left ventricle to yield the percentage of the left ventricle at risk (%RR) and percentage of the left ventricular risk region infarcted.

Myocardial oxygen consumption

Arterial and coronary venous blood samples were obtained anaerobically for determination of pH, Po_2, and Pco_2 by withdrawal of 3.0 ml of blood from the aortic and anterior interventricular vein catheters simultaneously. Haemoglobin content, O_2 saturation, blood Po_2, and pH, were measured by a computer aided blood gas analyser.

Oxygen consumption in the region of myocardium perfused by the left anterior descending coronary artery was computed by multiplying the arteriovenous oxygen difference by coronary blood flow, determined with the flowmeter probe proximal to the occlusion site. Since anterior interventricular vein blood flow corresponds to left anterior descending coronary artery influx,[13] this method allows determination of myocardial oxygen consumption in the region perfused by this vessel.[14]

Lactate extraction

At control, at 3 min of reperfusion, and at 120 min of reperfusion simultaneous arterial and coronary venous blood samples were obtained for measurement of lactate concentration. Lactate extraction of the myocardium was calculated according to McFalls *et al*[15]:

$$\% \text{ lactate extraction} = [(\text{arterial minus venous lactate})/\text{arterial lactate}] \times 100$$

Administration of bimakalim

Animals in the treatment groups received bimakalim dissolved in 1-2-propandiol and isotonic saline, while the pigs in the control group received vehicle (1-2-propandiol and isotonic saline) corresponding to the time course of treatment of group B. Bimakalim was supplied as a sterile, non-pyrogenic, freeze dried solid. Prior to each experiment it was dissolved and mixed in a 37°C warm sodium chloride solution for injection (0.9%). The bimakalim solution was given as a continuous infusion via a femoral vein at a dose of 0.1 μg·kg^{-1}·min^{-1} preceded by a bolus injection of 3 μg·kg^{-1} over 5 min.

Data analysis

Continuous recording of haemodynamic and wall thickness data was made on a paper recorder and on a computer aided recording system. Recordings at time points of interest were made at a paper speed of 100 mm·s^{-1}, and the wall thickness data were collected from the recordings. Wall thickness variables were averaged over 10 cycles at each time point and included systolic wall thickness (ESWT, defined at 20 ms before peak negative dP/dt), and end diastolic wall thickness (EDWT, defined at the onset of peak positive dP/dt). These data were used to calculate wall thickening (%ΔWT) with the following formula: (ESWT-EDWT)/EDWT × 100. Other variables included peak left ventricular systolic pressure (LVSP, mm Hg), mean arterial pressure (MAP, mm Hg), and heart rate (beats·min^{-1}). The coronary vascular resistance

(dyn·s·cm^{-5}) was calculated as: diastolic aortic blood pressure × 80/coronary blood flow; and the total peripheral resistance: MAP × 80/aortic blood flow.

Data are reported as mean(SEM). For comparison of variables across time between the groups, a two factor analysis of variance (ANOVA) of repeated measures was used. When the ANOVA was significant, pairwise comparisons were made with the Student's t test with the significance level for the p values corrected with the Bonferroni method. For comparison across time in each of the bimakalim or hydralazine treated groups or the control group, a one factor ANOVA of repeated measures was employed, with a post-hoc testing strategy identical to that of the two factor ANOVA. A two sample rank sum test (Mann-Whitney U) was used to test the differences of the bimakalim v hydralazine, bimakalim v control, and hydralazine v control animals with regard to the risk region and the percentage of the risk region infarcted.

Results

Risk region and infarct size

The risk region and infarct size are depicted in fig 2. The risk regions were virtually identical in all groups. The risk area was intentionally kept small (10% of left ventricular wall volume), because in pilot studies larger risk areas were associated with an unacceptably high incidence of ventricular fibrillation. The percentage of the risk region infarcted was 60.4(SEM 5.2)% for controls. Starting drug treatment after 45 min of occlusion did not alter infarct size [group A, 56.6(4.2)%], but infusing bimakalim before ischaemia reduced infarct size by about 50% [group B, 22.4(4.5)%; group C, 35.3(6.6)%; $p < 0.05$]. The difference in infarct size between group B and C was not significant ($p = 0.1$). Pretreatment with hydralazine did not affect infarct size [59.4(4.3)%; NS], which was therefore significantly different as compared with that after bimakalim treatment in groups B and C.

Haemodynamics and wall function

Haemodynamic and wall thickness data are summarised in the table. The control pigs remained haemodynamically stable throughout the occlusion and reperfusion period and did not vary significantly from baseline with regard to heart rate, mean arterial pressure, or double product; only during the hyperaemic response (reperfusion at 3 min) was heart rate significantly increased, while mean arterial pressure and double product decreased.

Infusion of bimakalim at 45 min of total coronary occlusion (group A) affected haemodynamics when comparing the time points 30 min of total coronary occlusion (before bimakalim treatment) with 60 min of occlusion (15 min of bimakalim treatment): heart rate increased [from 115(3) to 127(6) beats·min^{-1}, $p < 0.05$] and mean arterial pressure decreased [from 84(6) to 60(6) mm Hg, $p < 0.05$], while regional wall function (%ΔWT) slightly improved, from −4.8(1.6)% to +2.4(2.1), $p < 0.05$.

The effects on haemodynamics and wall function of bimakalim infused prior to the onset of ischaemia (group C) in comparison with the hydralazine group are depicted as percent change from baseline in fig 3. In the two groups pretreatment non-significantly increased heart rate and significantly lowered mean arterial pressure ($p < 0.01$). Although both bimakalim and hydralazine significantly decreased coronary vascular resistance [bimakalim group C, from 177(27) to 101(18) dyn·s·cm^{-5} before occlusion; hydralazine, from 189(18) to 126(14) dyn·s·cm^{-5} before occlusion; $p < 0.01$], only bimakalim increased myocardial blood flow [group B, from 36(9) to 40(8) ml·min^{-1}; group C, from 37(5) to 44(9) ml·min^{-1}]. Before the onset of the 60 min coronary occlusion %ΔWT was not significantly different in the bimakalim or the hydralazine treated regions compared with baseline values. Neither bimakalim nor hydralazine significantly altered cardiac output or maximum left ventricular dP/dt.

In a subset of 20 pigs the time elapsed until bulging (%ΔWT < 0%) was measurable in the first minute after coronary occlusion. In 15 cases a wall function analysis was not possible due to premature ventricular beats. The untreated group consisted of five animals in the control group and four in the bimakalim group A, while the bimakalim treated group consisted of seven pigs from the bimakalim groups B and C. In the hydralazine group four animals were

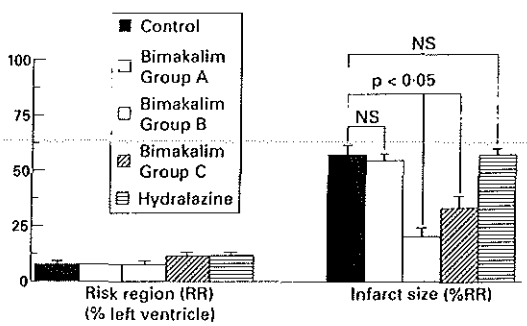

Figure 2 Area at risk (AAR) expressed as percent of the left ventricle (LV) and myocardial infarct size expressed as percent of AAR with and without bimakalim treatment. Columns are means, bars = SEM, $n = 7$ in all groups. Treatment with bimakalim significantly reduced infarct size provided that it was started prior to coronary occlusion.

Haemodynamic variables, regional wall function (percent Δ wall thickening), and global cardiac performance variables assessed at baseline, directly before the 60 min coronary occlusion (Pre-60'TCO), at 60 min of occlusion (60'TCO), at 3 min of reperfusion (Rep 3'), and at the end of the protocol (Rep 120').

	Baseline	*Pre-60'TCO*	*60'TCO*	*Rep 3'*	*Rep 120'*
			Heart rate (beats·min^{-1})		
Control	114(3)	114(3)	107(7)	111(7)	113(8)
Group A	116(3)	116(3)	127(6)*	131(6)*	159(9)*
Group B	106(7)	113(7)	124(7)*	132(5)*	143(8)*
Group C	102(11)	113(8)	116(9)	118(7)	127(13)
Hydra	100(8)	109(9)	120(11)	105(13)	119(8)
			Mean arterial pressure (mm Hg)		
Control	87(6)	87(6)	71(4)	60(4)	71(6)
Group A	92(6)	92(6)	60(6)*	61(7)	57(5)*
Group B	86(7)	58(4)*	54(5)*	59(6)	52(3)*
Group C	92(2)	61(4)*	71(6)	62(5)	70(3)
Hydra	92(4)	64(6)*	68(8)	67(7)	74(9)
			Wall function (%ΔWT)		
Control	26.2(3.3)	26.2(3.3)	−4.1(2.6)	−5.7(1.0)	−4.8(1.5)
Group A	27.7(3.2)	27.7(3.2)	2.4(2.1)	−1.9(2.1)	4.5(1.2)
Group B	23.1(3.5)	23.9(4.4)	−0.7(1.4)	−2.4(1.5)	6.5(2.4)
Group C	23.4(1.8)	23.6(2.2)	−3.8(2.4)	−4.6(4.5)	−2.9(2.1)
Hydra	23.0(4.5)	24.2(5.0)	−2.6(3.6)	−14.1(5.9)	−8.6(4.9)
			LV dP/dt_{max}		
Control	1423(122)	1423(122)	1149(111)	959(30)	1023(59)
Group A	2151(286)	2151(286)	1656(147)*	1648(188)*	2456(372)†
Group B	1503(138)	1332(138)	1410(86)*	1484(109)*	1610(101)*
Group C	1837(258)	1889(337)	1845(348)	1378(202)*	1782(288)*
Hydra	1694(117)	1762(80)	1714(135)*	1659(147)*	1622(209)*
			Cardiac output (ml·min^{-1}·10 kg^{-1})		
Control	967(62)	967(62)	855(59)	764(75)	739(62)
Group A	900(112)	900(112)	858(121)	851(123)	930(115)
Group B	928(81)	877(74)	931(91)	877(43)	803(63)
Group C	851(70)	846(82)	758(72)	690(43)	684(83)
Hydra	852(111)	953(111)	869(95)	874(94)	704(103)

TCO = total coronary occlusion; Rep = reperfusion; Hydra = hydralazine.
*$p < 0.05$, †$p < 0.01$ v controls.

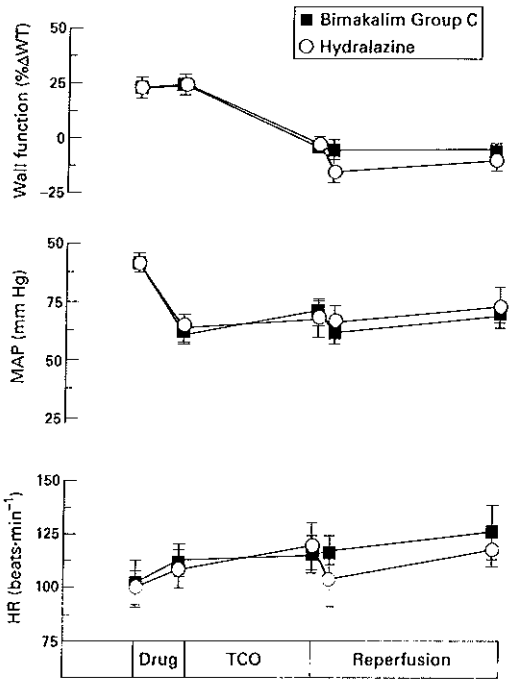

Figure 3 Heart rate (HR), mean arterial pressure (MAP), and regional wall function (%ΔWT) during the experimental protocol in the hydralazine (n = 7) and the bimakalim group C (n = 7). There were no significant differences between the groups throughout the experiment.

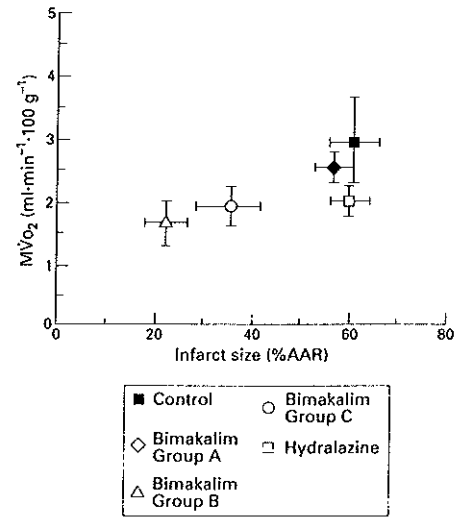

Figure 4 Scatterplot of regional oxygen consumption ($M\dot{V}O_2$) in the left anterior descending coronary artery bed prior to the onset of 60' coronary occlusion versus subsequent infarct size. In all groups n = 7. There is no significant correlation between $M\dot{V}O_2$ and infarct size. AAR = area at risk.

included in the analysis. There was a tendency towards a slowed myocardial depression in the pigs treated with hydralazine [20.3(2.0) s] or bimakalim [22.6(2.5) s] compared with untreated animals [15.2(1.7) s].

Myocardial oxygen consumption ($M\dot{V}O_2$)

Regional $M\dot{V}O_2$ was assessed at baseline and before the 60 min coronary occlusion. Treatment before ischaemia significantly decreased $M\dot{V}O_2$ by 36.5% in the bimakalim group B [from 2.60(0.32) to 1.65(0.36) ml·min^{-1}·100 g^{-1}, $p < 0.05$], by 27.1% in the bimakalim group C [from 2.65(0.29) to 1.93(0.31) ml·min^{-1}·100 g^{-1}, $p < 0.05$], and by 14.6% in the hydralazine group [from 2.32(0.23) to 1.98(0.09) ml·min^{-1}·100 g^{-1}, $p < 0.05$]. $M\dot{V}O_2$ measured prior to 60 min of ischaemia was plotted against subsequent infarct size. No significant positive correlation was found ($y = 1.29 + 0.02x$; $r = 0.37$; NS). Regional $M\dot{V}O_2$ was virtually identical in the bimakalim or hydralazine treated groups prior to the onset of ischaemia, although subsequent infarct size was significantly larger in hydralazine treated animals compared with bimakalim pretreated pigs (fig 4).

Lactate extraction

The percent lactate extraction was assessed at baseline, during the peak of reactive hyperaemia (approximately 3 min of reperfusion), and at the end of the protocol. There were no significant differences between groups, although the mean negative percent lactate extraction during reactive hyperaemia was less than 40% of the controls [−157(56)%] if the animals were pretreated with bimakalim [group B: −56(26)%: group C: −59(16)%]. Pretreatment with hydralazine was not associated with such a trend [−127(26)%].

Discussion

The objective of this study was the assessment of the effect of bimakalim, an activator of ATP sensitive potassium channels, on ischaemic tolerance in pig hearts. Bimakalim reduced infarct size by approximately one half provided that its infusion was started prior to the coronary artery occlusion. Bimakalim infusion towards the end of the occlusion period and continued into the reperfusion period was ineffective. Our findings are in line with results published by Lamping *et al.*[16] These investigators showed that nicorandil, in doses that produced a 20-25 mm Hg decrease in mean arterial pressure, significantly reduced myocardial infarct size (from 34% to 18%) in dogs subjected to two hours of coronary artery occlusion followed by 30 minutes of reperfusion. Grover *et al*[6] obtained similar results with cromakalim and a high dose of pinacidil injected into the coronary circulation. In the absence of peripheral haemodynamic effects, a marked reduction in infarct size was observed in anaesthetised dogs subjected to 90 minutes of left circumflex coronary artery occlusion followed by five hours of reperfusion. Similarly, Auchampach *et al*,[17] using anaesthetised dogs, found a 40-50% decrease in myocardial infarct size for RP 52891 after 90 minutes of left circumflex coronary artery occlusion followed by five hours of reperfusion. In the latter two studies the protective effect occurred independently of changes in peripheral haemodynamics, area at risk, or collateral blood flow, suggesting that potassium channel activators have a direct effect on the heart.

In order to evaluate whether the differences in infarct size observed in our study are related to drug induced changes of the well known determinants of infarct size,[18] namely size of risk region,[19] haemodynamic variables,[20] and collateral blood

flow,[21] the vasodilator hydralazine[22] was used to induce cardiovascular changes similar to those of bimakalim. Apparently, the cardioprotective effect of bimakalim cannot be explained in this way, for the following reasons. (1) The risk regions of all groups were virtually identical. (2) Bimakalim and hydralazine were given in equihypotensive doses; neither drug showed any significant difference with regard to regional wall function, regional oxygen consumption, heart rate, left ventricular dP/dt_{max}, and cardiac output, either at the onset of ischaemia or during the experimental protocol (fig 3). Nevertheless, bimakalim was capable of reducing infarct size by about 50%, while hydralazine was totally ineffective. Therefore unloading of the heart is unlikely to account for the cardioprotective effect of bimakalim treatment. (3) It has been shown that pig hearts lack pre-existing collateral vessels,[8] in contrast to dogs, excluding such vessels as a target of the action of bimakalim. This conclusion carries considerable weight, since previous studies in dogs had revealed a rather selective dilatation of coronary collateral vessels by potassium channel activators.[23]

It is well known that the plateau phase of the cardiac action potential shortens markedly during metabolic depression elicited by ischaemia.[24] The more rapid repolarisation of the action potential could be due to a decrease in inward current, or an increase in outward current, or a combination of these changes. Voltage clamp studies imply that the primary alteration in membrane currents during ischaemia is the development of a very large time independent outward K^+ conductance, resulting from the activation of ATP sensitive potassium channels.[25] Noma[26] postulated that ATP sensitive potassium channel opening and a shortening of the action potential with the subsequent rapid decline in myocardial function may preserve cellular ATP and delay irreversible ischaemic injury.

In our study the total dose of bimakalim (4 $\mu g \cdot kg^{-1}$) given before the onset of ischaemia was not negatively inotropic in the normoxic myocardium, which precludes a saving of high energy phosphates prior to the 60 minute coronary occlusion. If preservation of high energy phosphates through a decrease in myocardial contractility plays a role, it has to occur immediately after the onset of ischaemia.

To preserve energy in early phase of ischaemia, ATP sensitive potassium channel openers must shut down contractile function more rapidly than would occur with ischaemia alone. There is evidence that under hypoxic conditions lower doses of these drugs are sufficient to open ATP sensitive potassium channels, with a subsequent decrease in contractility.[27] This would allow, for example, bimakalim to become more potent in ischaemia. This sensitisation of the myocardium to potassium channel openers may be related to a decrease in ATP concentration in a subsarcolemmal compartment, that is, in close vicinity of the ATP sensitive potassium channels.[28] If one mechanism for the cardioprotective action of potassium channel openers is sparing of high energy phosphates because of rapid cardiac arrest, one should be able to demonstrate with these drugs an acceleration in the decline of mechanical activity as soon as ischaemia starts to develop. In fact, Mitani *et al*[29] observed in globally ischaemic rat hearts that during ischaemia nicorandil decreases the time to mechanical arrest by 21%, while glibenclamide increased it by 42%. However, in our pig model we did not observe changes in mechanical activity during the first minute of ischemia; regional wall function did not show an accelerated decline in a subset of bimakalim treated pigs. Nevertheless, bimakalim treatment significantly reduced infarct size.

In pigs pretreated with bimakalim the lactate washout during the first minutes of reperfusion after 60 minutes of coronary artery occlusion was about 50% less as compared with controls or hydralazine treated animals. This could give rise to the assumption that under ischaemic conditions opening of ATP sensitive potassium channels triggers a process that slows down glycolysis and in this way retards the development of acidosis. However, the accuracy of our lactate measurements was limited because the cannulated epicardial vein drained infarcted, salvaged, and non-ischaemic regions and this might be responsible for the large scatter of the lactate data. Despite this scatter, the difference in lactate washout between bimakalim and hydralazine treated pigs almost reached statistical significance ($p = 0.06$). There is evidence in another model of cardioprotection that the rate of glycolysis plays an important role. Murry and coworkers[30] showed in a canine model of coronary artery occlusion and reperfusion ("preconditioning") that the rates of glycogen breakdown and ATP use during ischaemia were significantly reduced in the cardioprotected hearts. Recently, Wolfe *et al*[31] found in a rat model of preconditioning that a reduced rate of glycolysis and attenuation of intracellular acidosis during ischaemia appear to be important factors in delaying irreversible injury and reducing infarct size. The importance of attenuating acidosis is confirmed by results of Kida and coworkers,[32] who showed that preservation of pH contributes to the infarct size reducing effect of ischaemic preconditioning in pigs. We recently presented evidence that myocardial preconditioning in the pig heart is mediated by opening of ATP sensitive potassium channels and can be blocked by glibenclamide.[33] Similar results were obtained by Gross and Auchampach in a canine model.[10] All these experimental findings suggest that drug induced opening of ATP sensitive potassium channels may set in motion a process that ultimately leads to myocardial protection and is therefore of significant therapeutic relevance.

Conclusion

Bimakalim, an activator of ATP sensitive potassium channels, reduced infarct size in anaesthetised pigs with acute coronary artery occlusion, provided that the drug was given prior to the onset of cardiac ischaemia. There is evidence, albeit indirect, that haemodynamic or coronary vascular factors are not responsible for the cardioprotective effect of bimakalim. We therefore conclude that the potassium channel activator induces ischaemic tolerance in pig myocardium through a direct effect on cardiac cells, most likely through opening of ATP sensitive potassium channels.

Key terms: infarct size; bimakalim; hydralazine; ATP sensitive potassium channels; myocardial oxygen consumption; myocardial lactate extraction.

Received 29 July 1993; accepted 27 September 1993. Time for primary review 25 days.

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Chapter 4

In swine myocardium the infarct size reduction induced by U-89232 is glibenclamide-sensitive. Evidence that U-89232 is a cardioselective opener of ATP-sensitive potassium channels.

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In swine myocardium the infarct size reduction induced by U-89232 is glibenclamide-sensitive. Evidence that U-89232 is a cardioselective opener of ATP-sensitive potassium channels.

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Objective: We determined if U-89232, a derivative of the ATP-sensitive potassium (K_{ATP}) channel opener cromakalim, is cardioselective and whether its action on the myocardium is still sensitive to glibenclamide. **Methods:** Experiments were performed in open chest pigs subjected to a 60 min occlusion of the left anterior descending coronary artery (LADCA) and to 2 h of reperfusion. Four groups of animals were studied (n = 6 each). Animals received either U-89232 3mg/kg i.v. over 15 min (U) or glibenclamide, a selective K_{ATP} channel blocker, 1mg/kg i.v. over 15 min (GLI) prior to the LADCA occlusion. In the GLI+U group, first glibenclamide (1mg/kg/15min) and then U-89232 (3mg/kg/15min) were infused prior to the 60 min of ischemia. Saline treated animals served as controls (CON). Hemodynamic parameters were continuously monitored. Regional contractile wall function was quantified with ultrasonic crystals aligned to measure wall thickening. At the end of the protocol, infarct size (IS, as percent of risk region) was determined by incubating the myocardium with p-nitrobluetetrazolium. **Results:** With comparable myocardium at risk, infusion of U-89232 prior to 60 min of LADCA occlusion significantly reduced infarct size (IS: 18.5±3.7%, $p<0.001$ vs. 63.2±3.3% for the controls), while glibenclamide had no effect on infarct size (IS: 69.5±4.4%). The administration of glibenclamide prior to U-89232 infusion blocked the infarct size reducing effect of U-89232 (IS: 61.2±9.1, n.s. vs controls and $p<0.001$ vs. U). Infusion of U-89232 had no effect on hemodynamic parameters or on regional wall function. **Conclusions:** At least in a pig model, U-89232 appears to be a cardioselective K_{ATP} channel opener, since in the absence of hemodynamic alterations it exhibits a profound cardioprotective effect, which is fully reversible by blocking K_{ATP} channels.

Numerous studies have shown that structurally diverse openers of ATP sensitive potassium channels (K_{ATP}), namely e.g. nicorandil[1], pinacidil[2], cromakalim[3], aprikalim[4], and bimakalim[5], can protect ischemic myocardial tissue. The fact that structurally unrelated K_{ATP} channel openers protect with a similar profile indicates a common mechanism which may involve an interaction with the K_{ATP} channel[6 7]. Further confirmation of this mechanism was shown by the reversal of the cardioprotective effects of the K_{ATP} channel openers by selective blockers of this channel[8 9], such as glibenclamide[10]. These data provide evidence that K_{ATP} channel opening serves as an endogenous protective mechanism and several studies showing a potential protective role for K_{ATP} channels in ischemic preconditioning further support this possibility[11 12 13]. However, in some clinical situations K_{ATP} channel openers, may not be ideal cardioprotective drugs since their direct vasodilatory properties[14] could reduce cardiac output and thereby coronary perfusion pressure, although it has been reported in anesthetized dogs that aprikalim[4] as well as bimakalim[15] are able to induce cardioprotection in doses, which have no effect on blood pressure and heart rate.

Most recently, there have been reports about cromakalim analogs which are relatively devoid of peripheral vasodilator activity even in high doses, but afford a pronounced cardioprotection[16 17]. The more "cardioselective" opening of K_{ATP} channels may not only improve the therapeutic window, in which K_{ATP} channel openers can effectively be used to treat myocardial infarction without potentially inducing hypotension and subsequent reflex tachycardia[18], but also suggests that channel subtypes may exist.

While the protection induced by the cromakalim analog BMS-180448 can be reversed by glibenclamide[19], the infarct size reducing effect of another cromakalim derivative U-89232 was reported not to be glibenclamide sensitive[20]. These data, however, would question whether K_{ATP} channel opening is still the mechanism by which the cardioselective cromakalim analogs induce the reported cardioprotection.

It was the aim of the present study to evaluate the hemodynamic and cardioprotective properties of U-89232 in a pig model, in which a peripheral vasodilatory active cromakalim derivative, bimakalim, had been characterized[21] and to test whether the reported cardioprotection is sensitive to glibenclamide.

Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No 85-23, revised 1985).

Experimental Design:

All 24 pigs underwent a 60 min left anterior descending coronary artery (LADCA) occlusion followed by 120 min of reperfusion. The animals were randomly assigned to one of four groups. They received either U-89232 3 mg/kg i.v. over 15 min (U) or glibenclamide 1 mg/kg i.v. over 15 min (GLI) prior to the LADCA occlusion. In the GLI+U group first glibenclamide (1mg/kg/15min) and then U-89232 (3mg/kg/15min) were infused prior to the 60 min of ischemia. Vehicle treated animals served as controls (CON). The design is depicted in Figure 1.

Experimental Preparation:

Twenty-four mixed breed Landrace-type domestic pigs of either sex weighing between 24 and 31 kg were premedicated with ketamine-HCl (10mg/kg i.m.) 15 min prior to anesthesia with i.v. bolus injection of sodium pentobarbital (30 mg/kg). Following tracheotomy pigs were mechanically ventilated with an animal respirator (Rhema, Hofheim/FRG) on nitrous oxide supplemented with oxygen at a ratio of 2:1. In order to guide adjustment of ventilator settings arterial blood gases were measured repeatedly. Anesthesia was maintained by continous infusion of sodium pentobarbital (3mg/kg/h). Arterial blood was sampled through a catheter placed in the right femoral artery, while the right brachial artery was cannulated with a polyethylene catheter, which was advanced to the ascending aorta for continuous recording of arterial pressure. An 8 French Millar micromanometer was advanced via the carotid artery into the left ventricle for continuous measurement of left ventricular pressure. The heart was exposed through a midline thoracotomy and suspended in a pericardial cradle. Distally of its second branch the (LADCA) was carefully dissected free over a length of 4 mm and a loose ligature was placed around the

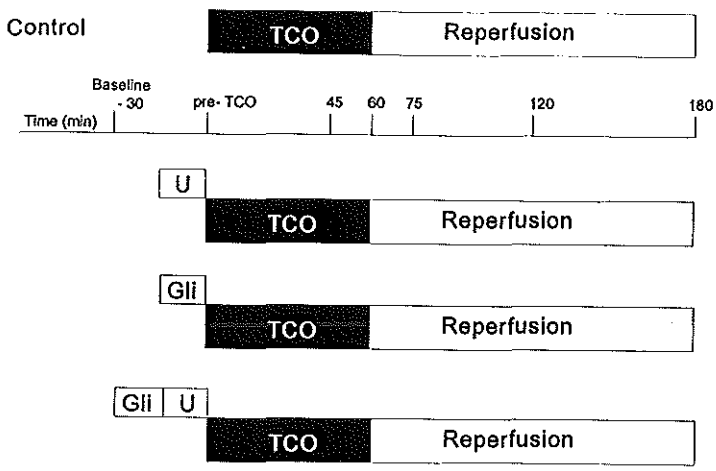

Figure 1. Design of the study. TCO= total coronary artery occlusion.

vessel which could be occluded with a small vascular clip. Electromagnetic flow probes (Flo-Probe, Spectramed Inc., Oxnard, California 93030, USA) were situated on the ascending aorta and the LADCA proximal to the occlusion site. A Teflon catheter (outer diameter 0.8 mm) was placed into the epicardial vein draining the area at risk. Rectal temperature was monitored throughout the experiment and the chest cavity was covered with a plastic sheet and kept warm with a heat lamp to keep body temperature between 37°C and 38°C. Myocardial function in the ischaemic area was measured with 5 mHz ultrasonic crystals aligned transmurally to measure wall thickness. A brief (< 10 second) occlusion was performed to identify the ischaemic region and the crystal pair was located centrally in the ischaemic region. The inner crystal was advanced to the endocardium tangentially through a stab wound in the epicardium. The epicardial crystal, attached to a Dacron patch, was positioned on the epicardium with the aid of an oscilloscope, and secured with three sutures. After a stabilisation period of 30 min after surgery the experimental protocol was started.

Risk Region and Infarct Size:

Following a reperfusion period of two hours the LADCA was reoccluded and 10 ml of 10% fluorescein dye were injected into the left atrium. After one to two min the pig was killed by electrical fibrillation with a 9-V battery and the heart excised. Warm 2% agarose was injected into the ventricle via the aortic root and the heart was kept on ice. After the agarose had congealed, the right ventricle was removed and the left ventricle was sliced into four to six rings perpendicular to the LADCA. Subsequently, the rings were weighed and the risk region traced onto an acetate sheet under blacklight, which sharply defined the borders of the risk area not perfused with fluorescein. Incubation for 30 min in 1.25% paranitrobluetetrazolium in phosphate buffer (pH 7.1) at 37°C delineated noninfarcted tissue (deep blue) and infarcted tissue (pale). The size of the area at risk and the infarcted region were determined by planimetry. The % risk region (area at risk / area of ring) was calculated for the top and bottom of each ring and the mean value for each ring was multiplied by the weight. The weights of regions were summed and divided by the weight of the left ventricle to yield the % of the left ventricle at risk (%RR) and % of the left ventricular risk region infarcted (% IS).

Administration of U-89232 and glibenclamide:

Animals in the treatment groups received U-89232 dissolved in isotonic saline, while the pigs in the control group received isotonic saline only. Prior to each experiment the compound was dissolved and mixed in 37°C warm saline. U-89232 (3mg/kg) was administered as a continuous infusion over 15 min via a femoral vein. Glibenclamide (1 mg/kg) was dissolved in 1.2 propandiol and was infused intravenously over 15 minutes.

Table 1. Hemodynamics, coronary blood flow, and regional contractile wall function assessed at baseline are presented in column 1. Changes from baseline directly before the 60 min LADCA occlusion (Pre-TCO), 60 min of LADCA occlusion (TCO), at reactive hyperemia (about 3 min of reperfusion; RH), and at the end of the protocol (120' REP) are listed.

	Baseline Values	Pre-TCO Δ %	60' TCO Δ %	RH Δ %	15' REP Δ %	120' REP Δ %
Heart rate (beats / min						
Control	111 ± 5	-1.0 ± 1.2	1.1 ± 5.2	1.4 ± 6.5	5.7 ± 5.4	13.4 ± 12.3
U	113 ± 9	-3.7 ± 2.4	-6.2 ± 4.6	5.0 ± 9.3	-6.4 ± 9.6	13.0 ± 7.1
Gli	108 ± 11	-4.9 ± 3.7	-2.3 ± 7.9	6.5 ± 15.5	9.1 ± 15.2	19.2 ± 14.0
Gli + U	100 ± 8	6.0 ± 10.6	6.3 ± 8.4	13.5 ± 9.4	10.3 ± 7.0	25.9 ± 11.3
Mean arterial pressure (mmHg)						
Control	92 ± 6	-3.9 ± 2.6	-19.3 ± 6.5	-30.3 ± 6.2	-24.7 ± 7.2	-14.7 ± 3.1
U	96 ± 7	8.7 ± 2.7	-11.8 ± 4.7	-28.8 ± 8.7	-20.0 ± 9.3	-7.5 ±7.1
Gli	98 ± 6	20.7 ± 2.2	2.3 ± 6.4*	-12.9 ± 4.6	-19.8 ± 8.7	-15.5 ± 6.9
Gli + U	102 ± 4	1.2 ± 5.3	-8.5 ± 7.6	-21.1 ± 11.9	-16.1 ± 8.6	-13.4 ± 8.8
Double product [(mmHg x bpm) / 100]						
Control	120 ± 11	-4.3 ± 3.0	-21.3 ± 7.5	-31.3 ± 5.3	-21.4 ± 9.9	-0.1 ± 10.8
U	117 ± 8	5.3 ± 4.6	-12.0 ± 8.9	-20.0 ± 14.4	-26.5 ± 9.1	8.8 ± 9.5
Gli	120 ± 12	16.7 ± 4.7	0.6 ± 12.0	-7.6 ± 12.0	-12.7 ± 11.6	-2.0 ± 10.3
Gli + U	117 ± 7	8.8 ± 12.0	0.4 ± 11.8	-7.8 ± 16.0	-5.2 ± 10.7	6.1 ± 21.6
LV dP / dt_{max} (mmHg / s)						
Control	1720 ± 157	-0.6 ± 1.4	-15.6 ± 7.2	-26.2 ± 12.7	-20 ± 9.1	-17.9 ± 4.3
U	1698 ± 144	2.6 ± 3.2	-22.2 ± 5.7	-41.4 ± 5.1	-35.1 ± 7.3	-17.2 ± 9.1
Gli	1862 ± 132	-4.4 ± 2.8	-13.6 ± 10.5	-27.9 ± 8.3	-28.4 ± 8.2	-26.4 ± 9.0
Gli + U	1625 ± 104	-8.9 ± 12.2	-12.9 ± 12.6	-30.5 ± 18.7	-16.9 ± 18.1	-23.8 ± 9.8
Wall function (%Δ wall thickening)						
Control	32 ± 3	-0.9 ± 0.9	-120.8 ± 5.3	-122.9 ± 8.9	-123.1 ± 8.2	-122.4 ± 3.0
U	29 ± 7	-2.6 ± 3.0	-105.0 ± 8.0	-125.3 ± 17.5	-142.2 ± 14.2	-127.8 ± 14.0
Gli	27 ± 3	4.0 ± 13.1	-132.4 ± 16.6	-146.8 ± 13.3	-116.6 ± 6.7	-125.5 ± 6.8
Gli + U	30 ± 5	-0.9 ± 8.5	-104.7 ± 10.2	-132.3 ± 21.1	-134.3 ± 18.7	-132.7 ± 14.4
CBF (ml / min)						
Control	36 ± 4	-2.9 ± 3.2	-52.7 ± 6.2	17.4 ± 16.5	0.8 ± 23.0	5.6 ± 15.6
U	47 ± 9	5.2 ± 6.1	-45.2 ± 8.0	6.9 ± 10.8	6.4 ± 15.5	-2.5 ± 21.5
Gli	32 ± 4	-1.7 ± 4.1	-55.3 ± 5.0	45.3 ± 20.0	6.5 ± 14.1	-9.3 ± 13.4
Gli + U	42 ± 5	-10.1 ± 12.5	-43.4 ± 8.4	22.1 ± 17.4	12.1 ± 22.6	0.1 ± 19.3
CVR x 10^3 (dyn x s x cm^{-5})						
Control	183 ± 24	-1.5 ± 3.6	85.1 ± 39.1*	-40 ± 4.4	-12 ± 17.1	-11.9 ± 10.0
U	153 ± 26	4.9 ± 6.0	76.7 ± 35.6*	-35.6 ± 4.6	-26.3 ± 4.8	6.6 ± 16.8
Gli	225 ± 38	25.0 ± 4.3	127.3 ± 28.9*	-36.7 ± 7.8	-22.7 ± 7.5	-2.9 ± 9.2
Gli + U	175 ± 30	19.7 ± 15.0	79.7 ± 41.2*	-36.0 ± 5.5	-18.4 ± 13.1	0.5 ± 8.0
COI (ml / min / 10kg)						
Control	987 ± 81	-1.2 ± 1.2	-6.3 ± 1.6	-10.4 ± 7.6	-3.6 ± 5.6	-17.0 ± 4.6
U	1020 ± 59	1.6 ± 3.6	-13.7 ± 6.0	-35.8 ± 7.2	-28.4 ± 9.4	-17.4 ± 11.3
Gli	916 ± 19	-8.2 ± 8.7	-24.7 ± 3.6*	-37.1 ± 4.4*	-38.0 ± 2.9*	-30.3 ± 8.7
Gli + U	990 ± 71	-3.8 ± 8.8	-11.9 ± 6.0	-31.0 ± 8.6	-16.2 ± 7.4	-16.6 ± 6.7
TPR x 10^3 (dyn x s x cm^{-5})						
Control	2.9 ± 0.3	- 2.8 ± 2.3	-13.9 ± 6.7	-20.7 ± 7.4	-21.3 ± 8.0	4.7 ± 8.7
U	3.3 ± 0.4	7.6 ± 4.8	5.1 ± 11.2	13.9 ± 13.7	15.0 ± 13.6*	22.3 ± 19.8
Gli	3.5 ± 0.3	38.0 ± 16.4	35.7 ± 4.3*	39.5 ± 5.9*	28.2 ± 10.4*	28.2 ± 15.4
Gli + U	3.1 ± 0.3	9.0 ± 12.0	4.7 ± 8.42	13.0 ± 7.2	0.7 ± 7.3	12.8 ± 11.7

*= $p<0.05$ compared with baseline. Abbr.: MAP = mean arterial blood pressure, LV = left ventricular

Data analysis:

Continuous recording of hemodynamic and wall thickness data was made on a paper recorder (Gould) and on a computer-aided registration system (IFD, Mescher, Mülheim/Ruhr, FRG). Recordings at timepoints of interest were made at a paper speed of 100mm/sec, and the wall thickness data were collected from the recordings. Wall thickness variables were averaged over 10 cycles at each timepoint and included end diastolic wall thickness (EDWT, defined as the point when left ventricular dP/dt started its rapid upstroke after crossing the zeroline), and end systolic wall thickness (ESWT, defined as the point of maximal wall thickness within 20 ms before peak negative left ventricular dP/dt). These data were used to calculate wall thickening (%delta WT) with the following formula: ((ESWT-EDWT) / EDWT) x 100. Other variables included peak left ventricular systolic pressure (LVSP, mmHg), mean arterial pressure (MAP, mmHg), and heart rate (HR, beats/min). The double product (DP) was calculated by heart rate x peak systolic pressure. Flow-Probes™ (Spectramed Inc.) were used for measurement of aortic and coronary blood flows. The zero baseline (zero value and zero balance) was electronically calibrated by the IFD computer system, which allows non-occlusive zero adjustments. The coronary vascular resistance (dyn x sec x cm^{-5}) was calculated as diastolic aortic blood pressure x 80 / mean coronary blood flow; analogously, the total peripherial resistance was calculated as MAP x 80 / mean aortic blood flow.

Statistics:

For comparison of variables across time between the groups, a two factor analysis of variance (ANOVA) of repeated measures was used. When the ANOVA was significant, pairwise comparisons were made with the Student's t-test with the significance level for the p values corrected with the Bonferroni method. For comparison across time in the control group, a one factor ANOVA of repeated measures was employed, with a post-hoc testing strategy identical to that of the two factor ANOVA. A two sample rank sum test (Mann-Whitney-U) was used to test the differences between two groups with regard to the risk region and the % of the risk region infarcted. Data are reported as mean±SEM.

Results

During the protocol, no more than a single shock to defibrillate the pig heart was given. Ventricular fibrillations, which required electrical defibrillation, were observed in one pig in the control group, in none of the animals in the U-89232 group, in one pig in the glibenclamide group and in one in the Gli+U group. In consequence, no pig had to be excluded from data analysis.

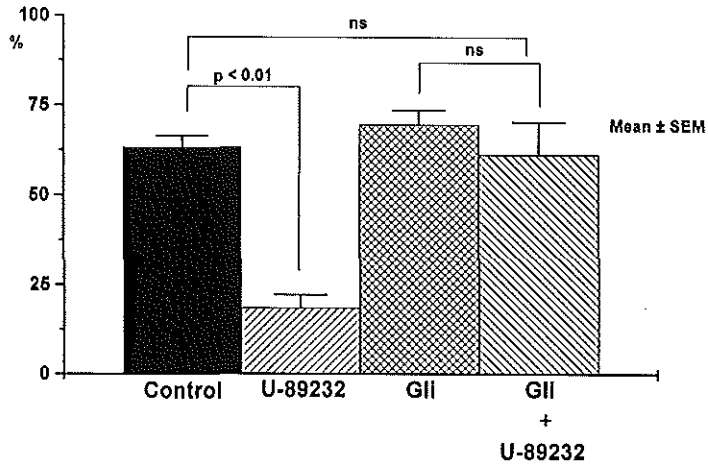

Figure 2. Plot of the mean±SEM of the myocardial infarct size values expressed as percent of the risk region (RR). All groups n = 6.

Risk Region and Infarct Size:

The risk regions (expressed as % of the left ventricle) were virtually identical in all groups (Control, 12.0±2.1%; GLI, 13.7±2.3%; U, 11.9±1.8%; GLI+U, 12.5±1.2%). The risk area was intentionally kept small (10% - 15% of left ventricular wall volume), because in pilot studies larger risk areas were associated with a high incidence of ventricular fibrillation.

The percentage of the risk region infarcted was 63.2±3.3% for the controls. Infusion of U-89232 prior to 60 min of LADCA occlusion significantly reduced infarct size (18.5±3.7%, $p<0.001$ vs. controls), while glibenclamide had no effect on infarct size (69.5±4.4%). The administration of glibenclamide prior to U-89232 infusion blocked the infarct size reducing effect of U-89232 (61.2±9.1, n.s. vs controls and $p<0.001$ vs. U).

Hemodynamics and wall function: The control pigs remained hemodynamically stable throughout the occlusion and reperfusion period and did not vary significantly from baseline with regard to heart rate, mean arterial pressure or double product.

Infusion of U-89232 did not significantly alter mean arterial pressure, double product, regional wall function or any other measured hemodynamic parameter (table 1). Glibenclamide significantly increased coronary vascular resistance, tended to rise mean arterial blood pressure, but did not significantly affect regional wall function (table 1).

Discussion

Our results indicate that U-89232 is a cardioselective K_{ATP}-channel opener, since its protective

actions are glibenclamide-sensitive and appear in the absence of hemodynamic changes. This would put U-89232 in line with other more cardioselective cromakalim analogs, such as BMS-180448.

In our pig model U-89232 reduced infarct size by 70%, which is in the range reported for other K_{ATP}-channel openers[4 8], e.g. bimakalim, another cromakalim derivative, reduces in the same pig model infarct size by 50% - 70%[21]. In addition, the noted effect on infarct size is very much consistent with the observations made by Norman et al.[22] or Toombs et al.[23] ; in anesthetized rabbits with comparable myocardium at risk U-89232 reduced infarct size significantly by about 50% . Furthermore, in line with their rabbit results, we observed no alterations of any of the measured hemodynamic parameters by U-89232 while using a dosage, which is highly significantly cardioprotective. Thereby U-89232 fullfills some important criteria, necessary to achieve the claim "cardioselective"[24].

The protective effect of U-89232, however, observed in rabbits was not influenced by glibenclamide[20 22]. This would rule out that the protective effects of U-89232 on the myocardium are mediated by activation of K_{ATP}-channels, since blocking these channels by glibenclamide abolishs the protective effect, as it has been shown with other K_{ATP}-channel opener studied[8 9 13]. In our pig model, glibenclamide (1mg/kg/15min), in line with previously published reports[25], affected coronary vasculature, but not infarct size. The same dose of glibenclamide, which has previously been reported to block the effects of bimakalim[13], has been used in this study, too. In contrast to the results obtained in rabbits[20 22], in pigs glibenclamide was able to completely antagonize the infarct size reducing effect of U-89232, thereby favouring a K_{ATP}-channel involving mechanism. The discrepancy between the observations might be attributable either to the animal model or to the dosage of U-89232 used.

Several reports state that in rats[26 27] and rabbits[28] glibenclamide itself is able to increase infarct size, thereby biasing direct comparisons between control and treated animals[29]. This may raise some doubts whether rat or rabbit models are the best suited to evaluate the protective potential of K_{ATP}-channel openers, since only in pigs and dogs their effects on K -channels are unequivocally[30]. However, Toombs et al. could demonstrate that in their rabbit model glibenclamide had no such effect, since using glibenclamide they could completely block the protective effect of ischemic preconditioning, an endogenous protective mechanism that may involve activation of K_{ATP}-channels. Furthermore, they were able to completely block the protective effects of cromakalim, while in the same model the same dose of glibenclamide did not affect U-89232's protection[20].

As the more cardioselective K_{ATP} channel openers do not allow to titrate doses by their impact on blood pressure, the only way to find an effective dose is to measure infarct size. In rabbits Toombs et al.[20] infused 20μg/kg/30min to observe an infarct size reduction by maximally 50%,

which is close to the dose range reported for other K_{ATP} channel openers[2]. In contrast, in the pig U-89232 exhibited a marked reduction of infarct size (70%) at a dose of 3 mg/kg/15min and, furthermore, in some pilot dose finding experiments we observed no reduction in infarct size at a dose below 1mg/kg/15min. Whether this reflects species differences between rabbits and pigs with regard to U-89232's binding affinity to cardiac K_{ATP} channels is speculative.

Toombs et al.[23] concluded from the bulk of evidence they have obtained in the rabbit, that the mechanism of action of U-89232 is most likely due to the ability to affect cardiac electrophysiology, since U-89232 decreased the myocardial action potential duration (APD)[16 23]. This electrophysiological feature is very commonly observed with high doses of K_{ATP} channel openers[32 33] and is supposed to be sensitive to K_{ATP} channel blockers[34 35].

The relevance of APD shortening in the cascade leading to cardioprotection, however, is questionable. Yao and Gross[15] showed that it is possible to find an intracoronary dose of bimakalim which reduced infarct size, but had no effect on epicardial monophasic APD. This suggests that significant APD shortening is not necessary for cardioprotection to occur. In line with these findings, Grover et al.[18] reported that the cardioprotective effect of another cardioselective cromakalim derivative, BMS-180448, is independent of APD shortening. Whether the mechanism of action of U-89232 or the affinity of U-89232 towards K_{ATP} channels is different between species can not be excluded or confirmed by our study, but at least in the swine myocardium the protective mechanism appears to involve K_{ATP}-channels

Acknowledgements

The authors thank Andreas Kaufmann and Ina Seibel for their excellent technical assistance.

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Chapter 5

Involvement of ATP-sensitive potassium channels in preconditioning protection

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Basic Research in Cardiology 1994; 89: 563-576

Involvement of ATP-sensitive potassium channels in preconditioning protection*

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Summary: Single or multiple brief periods of ischemia (preconditioning, PC) have been shown to protect the myocardium from infarction during a subsequent more prolonged ischemic insult. To test the hypothesis that opening of ATP-sensitive potassium channels (K_{ATP}) is involved in this mechanism, either bimakalim, a K_{ATP} channel opener, or glibenclamide, a K_{ATP} channel blocker, were administered to mimic or to block preconditioning protection in barbital-anesthetized pigs. PC was elicited by a single period of 10 min left anterior descending coronary artery (LADCA) occlusion followed by 15 min of reperfusion before the LADCA was reoccluded for 60 min. Instead of PC, bimakalim infusion was started 15 min before the 60 min LADCA occlusion (TCO) and stopped with the onset of ischemia. Glibenclamide was administered either for 10 min prior to the PC protocol, before bimakalim infusion, or before TCO. Regional wall function was quantified with ultrasonic crystals aligned to measure wall thickening (%ΔWT). At the end of the protocol, infarct size was determined by incubating myocardium with p-nitrobluetetrazolium.

In seven preconditioned pigs, infarct size was 9.9 ± 5.1% of the risk region compared with 65.9 ± 6.0% in the seven control pigs subjected to 60 min of ischemia only ($p<0.001$). In seven pigs treated with bimakalim, infarct size was reduced to 35.3 ± 6.6 ($p<0.05$ vs. controls). Blocking ATP-sensitive potassium channels with glibenclamide prior to PC abolished its protective effect (infarct size, 62.2 ± 4.5%; $p<0.001$ vs. PC alone). Glibenclamide also antagonized the protective effect of bimakalim (infarct size, 55.2 ± 4.0%), but did not affect infarct size, when solely administered prior to the prolonged ischemic period (62.2 ± 4.3%). We conclude that in swine myocardium K_{ATP} channels are involved in the protective effect of ischemic preconditioning, since glibenclamide completely abolished the protective effect of preconditioning, while bimakalim could – at least in part – mimic it.

Key words: Preconditioning – stunning – infarct size – ATP-sensitive potassium channels – glibenclamide

Introduction

Pretreating myocardium with brief periods of coronary artery occlusion and reperfusion increases myocardial tolerance to a subsequent prolonged episode of ischemia, a phenomenon, which has been termed "ischemic preconditioning" (19). Ischemically preconditioned myocardium greatly slows the rate of myocardial necrosis during a subsequent coronary occlusion (14), reduces the incidence of reperfusion arrhythmias (10, 27), and decreases autonomic denervation (35). Protection by preconditioning the myocardium with a sublethal period of ischemia is manifest in every species examined to

* Dedicated to Prof. Dr. H.-J. Langmann on the occassion of his 70th birthday.

date, including man (34). Recent studies indicate that occupancy of A_1-type adenosine receptors and /or the activation of ATP-sensitive potassium channels might be the trigger for the preconditioning response. Liu et al. (15) have reported that the protection against infarction that is afforded by preconditioning is mediated by A_1 adenosine receptors. Kirsch and coworkers (11) provided evidence that ATP-sensitive potassium channels are functionally coupled via G_i proteins to A_1 receptors. Accordingly, Gross and Auchampach (6) reported that in dogs the ATP-sensitive potassium channel blocker glibenclamide blocked the protective effect of preconditioning, while the ATP-sensitive potassium channel opener RP 52891 could mimic it.

However, the importance of K_{ATP} channel involvement in preconditioning's protection became highly controversial since Thornton and Downey were unable to block ischemic preconditioning protection against infarction with glibenclamide in rabbit hearts (28). Glibenclamide also failed to alter preconditioning in their second low collateral blood flow model, the rat heart (16). In addition, the results of Kitzen et al. (12) in a canine model indicate that potassium channel openers can fail to protect myocardium from ischemia-reperfusion injury.

Prompted by studies performed in seemingly identical rabbit models which have yielded opposing results (28, 29), a second element was added to the controversy. Downey (4) raises the point that involvement of K_{ATP} channels in preconditioning may be sensitive to the type of anesthetic used. He indicates from prepublication data that glibenclamide blocks preconditioning under ketamine-xylazine anesthesia, but fails to show similar blockade when pentobarbitone is used.

To address the above-mentioned points, we have chosen a porcine model of ischemia and reperfusion, from which it is known that 1) preconditioning cardioprotection is inducible in the presence of pentobarbitone anesthesia (26) and 2) preexisting collateral blood flow is low (26), allowing comparisons with other low collateral blood flow models such as rabbits and rats. Thus, the aim of the study was to evaluate in this porcine model whether bimakalim, an opener of K_{ATP} channels, is able to mimic preconditioning and whether glibenclamide is able to block the cardioprotective effects of both ischemic preconditioning and bimakalim.

Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No 85-23, revised 1985).

Experimental design

Fifty-six pigs were assigned to one of six groups; controls (n = 9), preconditioning (PC, n = 9), treatment with Bimakalim (BIM, n = 9, the results of the animals in this group have been described before (24)), and treatment with glibenclamide prior to the preconditioning protocol (Gli + PC; n = 10), to the application of bimakalim (Gli +BIM, n = 9), or before the onset of 60 min of ischemia (Gli, n = 10). The experimental design of the six groups is depicted in Fig. 1. All animals were subjected to a 60 min LADCA occlusion followed by 120 min of reperfusion. Vehicle-treated animals served as controls. Preconditioning was elicited by a single cycle of 10 min LADCA occlusion

followed by 15 min of reperfusion. Bimakalim (Bim; 4µg/kg/15 min) was administered for 15 min before the onset of 60 min of ischemia. Glibenclamide (1 mg/kg/10 min) was infused over 10 min either before the onset of 60 min LADCA occlusion (Gli), or before starting the bimakalim treatment (Gli + Bim), or before the preconditioning period (Gli + PC).

Experimental preparation

Fifty-six mixed breed Landrace-type domestic pigs of either sex between 25 and 29 kg were premedicated with ketamine-HCl (500 mg i.m.) 15 min prior to anesthesia with i.v. chloralose (100 mg/kg). Following tracheotomy pigs were mechanically ventilated with a Rhema animal respirator (Hofheim/FRG) on nitrous oxide supplemented with oxygen at a ratio of 2:1. In order to guide adjustment of ventilator settings arterial blood gases were measured repeatedly. Anesthesia was maintained by continuous infusion of pentobarbital at 3 mg/kg/h through a catheter in the right jugular vein. Arterial blood was sampled through a catheter placed in the right femoral artery, while the right brachial artery was cannulated with a polyethylene catheter, which was advanced to the ascending aorta for continuous recording of arterial pressure. A 8 French Millar micromanometer was advanced via the carotid artery into the left ventricle for continuous measurement of left ventricular pressure. The heart was exposed through a midline thoracotomy and suspended in a pericardial cradle. The proximal part of the second branch of the left anterior descending coronary artery (LADCA) was carefully dissected free over a length of 4 mm and a loose ligature was placed around the vessel which could be occluded with a small vascular clip. Electromagnetic flow probes were situated on the ascending aorta and the LADCA proximal to the occlusion site. A Teflon catheter (outer diameter 0.8 mm) was placed into the epicardial vein draining the area at risk. Rectal temperature was monitored throughout the experiment and the chest cavity was covered with a plastic

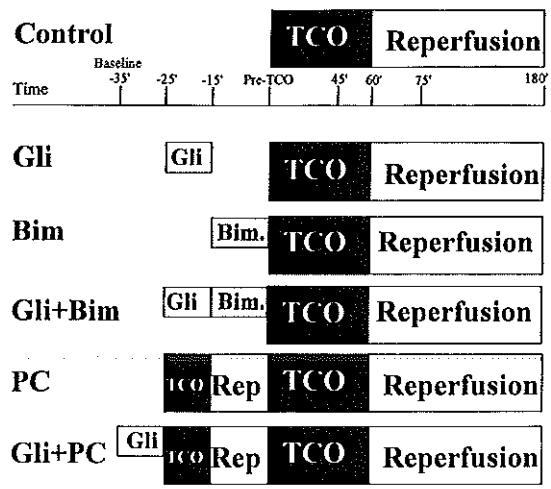

Fig. 1. Design of the study (Bim = bimakalim, TCO = total coronary artery occlusion).

sheet and kept warm with a heat lamp to keep body temperature between 37° and 38°C. Myocardial function in the ischemic area was measured with 5 MHz ultrasonic crystals aligned transmurally to measure wall thickness. A brief (< 10 s) occlusion was performed to identify the ischemic region and the crystal pair was located centrally in the ischemic region. The inner crystal was advanced to the endocardium tangentially through a stab wound in the epicardium. The epicardial crystal, attached to a Dacron patch, was positioned on the epicardium with the aid of an oscilloscope, and secured with three sutures. After a stabilization period of 30 min after surgery the experimental protocol was started.

Risk region and infarct size

Following a reperfusion period of 2 h the LADCA was reoccluded and 10 ml of 10% fluorescein dye were injected into the left atrium. After 1 to 2 min the pig was killed by electrical fibrillation with a 9-V battery and the heart excised. Warm 2% agarose was injected into the ventricle via the aortic root and the heart was kept on ice. After the agarose had congealed, the right ventricle was removed and the left ventricle was sliced into four to six rings perpendicular to the LADCA. Subsequently, the rings were weighed and the risk region traced onto an acetate sheet under blacklight, which sharply defined the borders of the risk area not perfused with fluorescein. Incubation for 30 min in 1.25% paranitrobluetetrazolium in phosphate buffer (pH 7.1) at 37°C delineated noninfarcted tissue (deep blue) and infarcted tissue (pale). Rings were then photographed with Polaroid MP-4 camera and acetate tracings of the ring and infarcted region were determined by planimetry. The percent risk region (area at risk / area of ring) was calculated for the top and bottom of each ring and the mean value for each ring was multiplied by the weight. The weights of regions were summed and divided by the weight of the left ventricle to yield the percent of the left ventricle at risk (%AAR) and percent of infarction in the left ventricular area at risk.

Lactate extraction

Lactate production has been a reliable index for the presence of anaerobic glycolysis (5), and percentage lactate extraction has been shown to correlate well with tissue gradients of lactate (17). At baseline, at the peak reactive hyperemia (about 3 min of final reperfusion), and at 120 min of reperfusion arterial and coronary venous blood samples were simultaneously obtained for measuring lactate concentration. Since anterior interventricular vein blood flow corresponds to LADCA influx (3), this method allowed determination of changes in myocardial lactate extraction in the region perfused by the LADCA. Lactate values were calculated in the following fashion:

% lactate extraction = [(arterial minus venous lactate)/arterial lactate] × 100.

Administration of drugs

Animals in the treatment group received bimakalim dissolved in 1,2-propandiol (0.05%) and isotonic saline, while the pigs in the control group received an equal volume of the vehicle at corresponding time points. Bimakalim was supplied as sterile, nonpyrogenic freeze-dried solid. Prior to each experiment bimakalim was dissolved and mixed in

a 38° C warm sodium chloride solution for injection (0.9%). The bimakalim solution was administered at a rate of 3μg/kg over 5 min followed by a continuous infusion of 1μg/kg over 10 min via one femoral vein.

Glibenclamide was purchased from Sigma (St. Louis, Missouri, USA) and dissolved as well as administered in an identical fashion as described above for bimakalim.

Data analysis

Continuous recording of hemodynamic and wall thickness data was made on a recorder and on a computer-aided registration system (IFD, Mescher, Mülheim/Ruhr, FRG). Recordings at timepoints of interest were made at a paper speed of 100 mm/s, and the wall thickness data were collected from the recordings. Wall thickness variables were averaged over 10 cycles at each timepoint and included end diastolic wall thickness (EDWT, defined as the point when left ventricular dP/dt started its rapid upstroke after crossing the zeroline), and end systolic wall thickness (ESWT, defined as the point of maximal wall thickness within 20 ms before peak negative left ventricular dP/dt). These data were used to calculate the extent of wall thickening (% ΔWT) with the following formula: ((ESWT-EDWT) / EDWT)*100. Other variables include peak systolic pressure (PSP, mmHg) from the Millar, mean arterial pressure (MAP, mmHg), and heart rate (HR, beats/minute). The double product (DP) was calculated by multiplying PSP × HR. The coronary resistance (in dyn) was calculated as follows: Diastolic blood pressure × 80 / coronary blood flow; analogous, the total peripherial resistence: MAP × 80 / aortic blood flow.

Results

Two control, two preconditioned, two BIM, and two GLI + BIM treated pigs, as well as three GLI, and three GLI + Precon animals were excluded from the study because of intractable fibrillation. Thus, 42 pigs completed the protocol and were used in data analysis.

Risk region and infarct size

The infarct size expressed as a percentage of risk region is presented in Fig. 2. The risk regions were virtually identical (Control, 12.4 ± 2.4; GLI, 13.4 ± 1.8%, BIM, 11.5 ± 2.5%; GLI + BIM, 11.9 ± 1.5%; Precon, 11.6 ± 1.8%; GLI + Precon, 13.4 ± 1.6%). The risk area was kept intentionally small, because in pilot studies large risk areas (> 20% of the left ventricle) were associated with an unacceptably high incidence of ventricular fibrillations. The percent of the risk region infarcted was 65.9 ± 6.0% for controls vs. 9.9 ± 5.1% for preconditioned pigs ($p < 0.001$). Bimakalim treatment reduced infarct size (35.3 ± 6.6%; $p < 0.05$ vs. control), but significantly less than ischemic preconditioning. There was no significant difference between the three glibenclamide groups (GLI, 58.7 ± 5.9%; GLI + BIM, 55.2 ± 4.0%; GLI + Precon, 62.2 ± 4.5%; n.s.) and the control group.

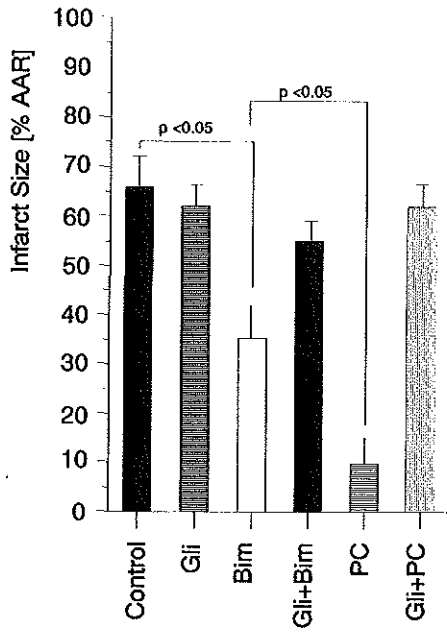

Fig. 2. Myocardial infarct size expressed as percent of the left ventricular area at risk (AAR), results are mean ± SEM. All groups n = 7.

Hemodynamics and wall function

Hemodynamic data are summarized in Table 1. Baseline values were not different for the different groups. Bimakalim treatment decreased mean arterial pressure and increased left ventricular dP/dt_{max}, while ischemic preconditioning did not significantly alter hemodynamic parameters. Glibenclamide treatment tended to increase mean arterial pressure independent of whether pigs underwent preconditioning, bimakalim treatment, or just infusion of vehicle. Cardiac output was neither different from baseline values nor between groups at the onset of the 60 min LADCA occlusion.

Before the onset of the 60 min LADCA occlusion regional wall function was significantly reduced by about 50% in preconditioned pigs compared with control or bimakalim treated animals. Treatment with glibenclamide further reduced regional wall function following the ischemic preconditioning stimulus ($-86\%; p < 0.05$ vs. PC), while glibenclamide administration alone or in combination with bimakalim did not affect wall function (see Table 2). After 60 min of LADCA occlusion, wall function remained depressed to the same extent in all groups.

Coronary blood flow

Coronary blood flow (CBF) and coronary vascular resistance (CVR) values are summarized in Table 3.

Table 1. Hemodynamics and global cardiac performance parameters. (n = 7 for all groups; * = $p < 0.05$ compared with corresponding time value in the control group; # = $p < 0.05$ compared with corresponding time value in the group not treated with glibenclamide. Pre TCO = before onset of 60 min LADCA occlusion (TCO), RH = reactive hyperemia (3 min of reperfusion), Rep = reperfusion.).

Heart Rate [beats/min]					
	Baseline	Pre TCO	60' TCO	RH	120' Rep
Control	112 ± 4	115 ± 5	107 ± 7	110 ± 7	127 ± 10
Gli	108 ± 9	103 ± 9	102 ± 3	110 ± 6	124 ± 4
Bim	102 ± 11	113 ± 8	116 ± 9	118 ± 7	127 ± 13
Gli+Bim	106 ± 8	116 ± 5	108 ± 7	134 ± 6*	134 ± 10
PC	112 ± 7	114 ± 6	112 ± 8	124 ± 8*	111 ± 13
Gli+PC	111 ± 8	97 ± 6#	108 ± 10	143 ± 20*	115 ± 6
MAP[mmHg]					
	Baseline	Pre TCO	60' TCO	RH	120' Rep
Control	93 ± 7	91 ± 6	67 ± 4	62 ± 2	77 ± 8
Gli	99 ± 5	109 ± 7*#	102 ± 7*#	85 ± 7*#	87 ± 8
Bim	92 ± 2	61 ± 4*	71 ± 6	62 ± 5	70 ± 3
Gli+Bim	94 ± 4	78 ± 7*#	81 ± 5*	73 ± 3#	70 ± 6
PC	95 ± 9	86 ± 9	80 ± 6*	61 ± 6	75 ± 10
Gli+PC	104 ± 4	106 ± 7#	94 ± 5*#	69 ± 6	93 ± 6#
Double Product [(mmHg*bpm)/100]					
	Baseline	Pre TCO	60' TCO	RH	120' Rep
Control	118 ± 9	118 ± 9	98 ± 7	81 ± 6	116 ± 15
Gli	121 ± 9	158 ± 29*#	116 ± 7#	107 ± 12#	122 ± 11
Bim	103 ± 6	86 ± 7*	95 ± 7	87 ± 7	106 ± 11
Gli+Bim	112 ± 6	109 ± 11#	111 ± 11	112 ± 7#	115 ± 16
PC	135 ± 15	114 ± 10	107 ± 10	91 ± 5	102 ± 18
Gli+PC	136 ± 15	130 ± 14	116 ± 11	108 ± 14	124 ± 10
LV dP/dt_{max} [mmHg/sec]					
	Baseline	Pre TCO	60' TCO	RH	120' Rep
Control	1224 ± 115	1116 ± 108	1078 ± 83	874 ± 53	1029 ± 65
Gli	1335 ± 135	1522 ± 136*#	1558 ± 74*#	1272 ± 85*#	1460 ± 159*#
Bim	1373 ± 258	1889 ± 337*	1845 ± 348*	1378 ± 202*	1382 ± 288*
Gli+Bim	1341 ± 44	1601 ± 48*	1731 ± 116*	1550 ± 95*	1317 ± 70*
PC	1369 ± 84	1374 ± 132*#	1669 ± 112*	1199 ± 147*	1397 ± 181*
Gli+PC	1322 ± 66	1447 ± 90*	1238 ± 90*#	900 ± 95	1018 ± 152
Cardiac Output [ml/min/10 kg]					
	Baseline	Pre TCO	60' TCO	RH	120' Rep
Control	963 ± 63	865 ± 80	741 ± 76	751 ± 56	755 ± 63
Gli	844 ± 35	767 ± 66	623 ± 28*#	509 ± 28*#	596 ± 68*#
Bim	851 ± 70	846 ± 82	785 ± 72	690 ± 43	684 ± 83
Gli+Bim	829 ± 53	784 ± 45	765 ± 44	676 ± 40	691 ± 41
PC	1024 ± 154	1080 ± 157	848 ± 128	698 ± 80	823 ± 115
Gli+PC	845 ± 91	835 ± 67	624 ± 96	435 ± 90*#	689 ± 111

In preconditioned animals after 10 min of LADCA occlusion and 15 min of reperfusion CBF was significantly reduced ($p < 0.05$ vs. control), paralleled by a significant increase in CVR (+32%). Although glibenclamide infusion prior to preconditioning

Table 2. Wall function (as %Δ wall thickening) in the LADCA region (n = 7 for all groups; * = $p < 0.05$ compared with corresponding time value in the control group; # = $p < 0.05$ compared with corresponding time value in the group not treated with glibenclamide).

% ΔWT					
	Baseline	Pre TCO	60' TCO	RH	120' Rep
Control	25 ± 3	24 ± 3	− 8 ± 3	−10 ± 2	−7 ± 3
Gli	26 ± 3	28 ± 5	− 8 ± 2	−11 ± 2	−8 ± 2
Bim	23 ± 2	24 ± 2	− 4 ± 2	− 5 ± 5	−3 ± 2
Gli+Bim	23 ± 2	21 ± 2	−10 ± 2#	−14 ± 5	−6 ± 2
PC	27 ± 3	14 ± 6*	− 6 ± 7	− 5 ± 5	−2 ± 6
Gli+PC	23 ± 3	3 ± 3*#	− 2 ± 4	− 8 ± 2	−5 ± 1

Table 3. Coronary blood flow (CBF) and coronary vascular resistance (CVR) at five time points during the experimental protocol (n = 7 for all groups; * = $p < 0.05$ compared with corresponding time value in the control group; # = $p < 0.05$ compared with corresponding time value in the group not treated with glibenclamide).

CBF [ml/min]					
	Baseline	Pre TCO	60' TCO	RH	120' Rep
Control	32 ± 3	35 ± 4	17 ± 3	53 ± 6	29 ± 7
Gli	33 ± 3	31 ± 2	14 ± 2	46 ± 2	29 ± 3
Bim	37 ± 5	44 ± 6	15 ± 3	51 ± 7	45 ± 8
Gli+Bim	39 ± 5	42 ± 7	13 ± 4	63 ± 8	44 ± 4
PC	35 ± 2	22 ± 3	16 ± 2	40 ± 2*	23 ± 3
Gli+PC	38 ± 5	31 ± 6#	16 ± 6	51 ± 6#	32 ± 4#
CVR [dyn*sec*cm^{-5}]					
	Baseline	Pre TCO	60' TCO	RH	120' Rep
Control	208 ± 24	206 ± 24	299 ± 61	138 ± 20	210 ± 47
Gli	221 ± 28	276 ± 31*#	423 ± 67	115 ± 14	219 ± 37
Bim	197 ± 27	101 ± 18*	189 ± 23*	100 ± 10*	87 ± 9*
Gli+Bim	183 ± 25	135 ± 20*	277 ± 41#	92 ± 13*	112 ± 9*#
PC	229 ± 44	303 ± 69*	350 ± 58	138 ± 18	239 ± 47
Gli+PC	182 ± 26	249 ± 43	288 ± 74	76 ± 9*#	186 ± 35

period resulted in nearly akinesis in the LADCA region before the onset of the 60 min LADCA occlusion, it did not significantly decrease CBF.

Bimakalim treatment did not alter CBF to the subsequent ischemic region, but significantly reduced CVR by 49%. Pretreatment with glibenclamide at the given dose only partially antagonized the bimakalim-induced vascular effects before the onset of 60 min of LADCA occlusion, e.g. CVR still declined by 26%.

Glibenclamide treatment alone resulted in a little less pronounced hyperemic response (3 min of reperfusion) after 60 min of ischemia compared with control animals, but in combination with preconditioning of bimakalim CBF was increased.

Table 4. Venous blood glucose levels.

Glucose [mM/L]	Baseline	Pre TCO	60' TCO	RH	120' Rep
Control	4.7 ± 0.5	4.7 ± 0.5	4.1 ± 0.5	3.7 ± 0.4	3.9 ± 0.5
Gli	4.8 ± 0.4	4.4 ± 0.4	4.5 ± 0.4	4.1 ± 0.4	3.7 ± 0.4
Bim	4.6 ± 0.2	4.7 ± 0.2	5.3 ± 0.2	4.9 ± 0.2	5.3 ± 0.3
Gli+Bim	4.6 ± 0.6	4.3 ± 0.7	4.7 ± 1.1	4.3 ± 0.9	3.6 ± 0.9
PC	3.9 ± 0.4	3.9 ± 0.4	3.8 ± 0.4	3.3 ± 0.3	3.9 ± 0.4
Gli+PC	3.9 ± 0.5	3.7 ± 0.8	3.7 ± 0.7	3.4 ± 0.7	2.7 ± 0.7

Lactate and glucose analysis

The venous glucose concentrations are listed in Table 4. There were no significant differences by the ANOVA between glibenclamide treated animals and corresponding time values in the control, preconditioned, or bimakalim treated groups.

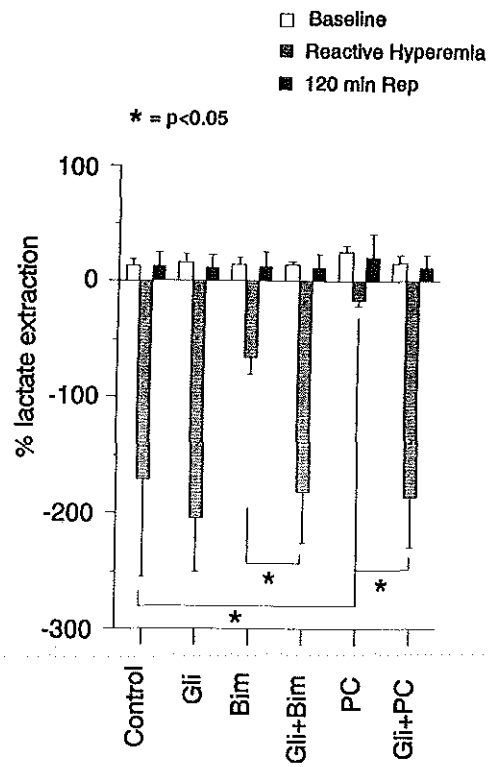

Fig. 3. Percent lactate extraction (mean ± SEM) in the LADCA region at baseline, during reactive hyperemia (3 min of reperfusion), and at the end of the protocol (120 min of reperfusion). All groups n = 7.

The extent of lactate wash-out after 60 min of LADCA occlusion was different between groups (see Fig. 3). While all glibenclamide treated hearts showed no significant different lactate wash-out, it was reduced by about 60% when pretreated with bimakalim. In preconditioned animals an even more reduced lactate wash-out was observed after the 60 min of ischemia ($p < 0.001$ vs. control; $p < 0.05$ vs. bimakalim treatment), which was fully reversible by the pretreatment with glibenclamide.

Discussion

The objective of this study was to evaluate in pigs the hypothesis that activation of ATP-sensitive potassium channels (K_{ATP}) is involved in the phenomenon of ischemic preconditioning. The opener of K_{ATP} channels, bimakalim, was able to reduce infarct size about 50%, albeit not to the same extent as ischemic preconditioning (80%), while the K_{ATP} channel blocker, glibenclamide, could fully prevent both the protective effects of bimakalim and ischemic preconditioning. Thereby, this study extends the hypothesis of K_{ATP} channel involvement in preconditioning to a large animal model which lacks significant collateral blood flow and confirms results previously obtained in the dog and some rabbit studies.

ATP-sensitive potassium channels in infarct size limitation

Pig hearts, when subjected to a 10-min ischemic episode of the LADCA region followed by 15 min of reperfusion, acquired not only regional dyskinesis, but also protection against a further episode of ischemia. Myocardium preconditioned in this fashion had much less necrosis than myocardium that has not previously been made ischemic, despite the additional 10 min of ischemia in the preconditioned pigs. This is in line with results obtained in dogs showing that a single occlusion seems to be equally effective as six or 12 5-min occlusive episodes (14). Consistently, in our study in pigs a single, preconditioning period of 10 min of ischemia followed by 15 min of reperfusion was as protective as the previously reported two cycles of 10 min of ischemia and 30 min of reperfusion (26). These observations imply that ischemic preconditioning is consistent with an "all or nothing" response (13), which may correlate with an on or off switching of K_{ATP} channels.

Similar to preconditioning with ischemia, bimakalim treatment was associated with a significant decrease in infarct size. This confirms in pigs the results of Grover et al. (7) and Auchampach et al. (29) that K_{ATP} channel openers are able to significantly reduce infarct size. In both dog models the protective effect occurred independent of any changes in peripheral hemodynamics, area at risk, or collateral blood flow, suggesting that potassium channel agonists have a direct cardioprotective effect.

The results from Murry and coworkers (20) provide evidence that a reduced accumulation of glycolytic intermediates (such as lactate, H+, and NADH) may be responsible for the protective effect of preconditioning. Lactate accumulation has been reported to cause marked alterations in mitochondrial structure (1), which were significantly reduced in dogs preconditioned with ischemia. The combined effects of ischemic catabolites may act as an osmotic load to the cell, the reduction of this osmotic load may be protective by preventing osmotically induced rupture of cell membranes. In our study, the bimakalim treated pigs released only half the amount of lactate after 60 min of ischemia as did the

control pigs, which would support the concept that reduction in the osmotic load during coronary artery occlusion results in signs of reduced ischemia (9, 24). Thus, preconditioned pigs showed an even more decreased lactate wash-out after the final occlusion (about 1/10 the amount of control animals). Accordingly, the infarct in preconditioned myocardium was only one-third the size measured in bimakalim treated myocardium. Consistent with our results, Gross and Auchampach (6) found in their dog model ischemic preconditioning much more potent to reduce infarct size (from 28% to 6%; 79% reduction) than the K_{ATP} channel opener RP 52891 (to 13%; 54% reduction).

The reason why short ischemia followed by reperfusion can prevent cumulative injury to a greater extent than drug-induced opening of K_{ATP} channels is unknown. However, during an interspersed reperfusion not only potentially harmful catabolites are washed out, but also adenosine, which may contribute to the salutary effects of ischemic preconditioning. In dogs intracoronary administration of an A_1-selective adenosine agonist (R-PIA) resulted in a 44% infarct size reduction (8). Infusion of a K_{ATP} channel blocker abolished the protective effect of A_1-adenosine receptor stimulation in dogs (8) and in pigs (31), providing evidence that preconditioning may be mediated by K_{ATP} channel activation, which sensitivity is modulated by A_1-receptor stimulation. This concept is supported by the observation that K_{ATP} channels are completely inhibited at much lower ATP levels than the intracellular ATP concentration seen at the early stage of ischemia (22). Therefore, the K_{ATP} channel has to be sensitized to smaller changes in the ATP-level as a response to short ischemia. One possible explanation is that adenosine is involved; adenosine released during short ischemia decreases the K_{ATP} channel sensitivity via interaction with a G_i protein. This may be a route by which channel activity could be increased during brief ischemia and could explain the data supporting the adenosine hypothesis of preconditioning.

Additionally, adenosine release from myocytes, K_{ATP} channel opening, as well as ischemic preconditioning seem to result in a slower accumulation of ischemic catabolites such as lactate. Reimer and Jennings's group did show that the metabolism during ischemia, namely rate of ATP depletion, glycogen utilization, and lactate accumulation during a later episode of ischemia is slowed by both, pretreatment with ischemic preconditioning (20) and intracoronary adenosine (30).

Glibenclamide to prevent infarct size reduction

In contrast to K_{ATP} channel activation, preventing K_{ATP} channel opening by its blocker is expected to prevent the protective effect of preconditioning or bimakalim treatment resulting in a tolerance which is no longer different from the tolerance of animals undergoing a long period of ischemia only. Indeed, in our study glibenclamide abolished the infarct size-reducing effect of ischemic preconditioning as well as of bimakalim. Importantly, treatment with a K_{ATP} channel blocker did not increase infarct size when administered to pigs undergoing 60 min of ischemia and 120 min of reperfusion only. This is in contrast to the results obtained in rabbits by Thornton et al. (28), but consistent with the data obtained in dogs (6) or in pigs (31). Furthermore, the antagonistic effects of glibenclamide on infarct size in the canine model of Gross and Auchampach (6) as well as in our swine model occured independently of differences in hemodynamics, coronary collateral blood flow, or size of the ischemic bed.

Another possibility to explain the differences between the rabbit and dog/pig models may result from a different sensitivity of pancreatic cells to glibenclamide. Glibencla-

mide is known to increase insulin and to decrease blood glucose levels, which may affect preconditioning. Omar et al. (23) showed in an isolated rabbit heart model that preconditioning protection occurs only in the presence of high glucose concentrations in perfusate during reperfusion. With the 1 mg/kg dose of glibenclamide, no significant hypoglycemia (mean −8.3%) was induced in the pig model and, presumably, as inferred from glucose levels, insulin was not greatly increased. However, Gross and Auchampach (6) observed a decrease by maximal 16% in the blood glucose levels when infusing glibenclamide (0.3 mg/kg/10 min) in dogs. In the rabbit Thornton et al. (28) registered with all three doses of glibenclamide used (0.15 mg/kg, 0.3 mg/kg, and 3.0 mg/kg) a significant drop in blood glucose levels, ranging from 23% to 38%. That the effect on blood glucose was less pronounced in pigs than reported for dogs and rabbits might be due to species related differences in the pancreatic cells to glibenclamide. Additionally, our pigs fasted for 24 h before they were instrumented and the blood glucose levels could have been lowered at baseline, which might reduce the response of pancreatic cells to glibenclamide, too.

In a prelimenary study in pigs, glibenclamide, in a dose of 1 mg/kg, was able to prolong action potential duration by about 20% in non-ischemic myocardium. In this model, bimakalim (4μg/kg/15 min) shortened action potential duration by 15%–20%, which could be fully reversed by glibenclamide (1 mg/kg). We have no data yet in pigs about the influence of K_{ATP} channel openers on action potential duration under ischemic conditions, but Yao et al. could show in a canine model that bimakalim accelerates (33) while glibenclamide blocks (32) the shortening of the action potential duration induced by ischemia.

In our study glibenclamide worsened the postischemic contractile dysfunction after 10 min of LADCA occlusion ("stunning") as compared with preconditioning alone. This is a new finding in pigs and is further evidence from a functional point of view that blocking K_{ATP} channels in the course of ischemia and reperfusion is detrimental. Furthermore, the results imply that there is a clear dissociation between myocardial stunning and preconditioning, since one would expect the Glib + PC group to have small infarcts if these two phenomena were related. This is in line with results obtained in dogs (21) and rabbits (18) demonstrating that myocardial stunning is insufficient to cause preconditioning protection.

Taken together, these results suggest an important involvement of the ATP-sensitive potassium channel in the preconditioning phenomenon in swine myocardium, because blocking these channels by glibenclamide completely abolished the protective effect of ischemic preconditioning as well as of bimakalim, an opener of the K_{ATP} channels. Substituting preconditioning by bimakalim infusion did not exhibit the same profound infarct size-reducing effect as did preconditioning with ischemia. This suggests that the full protective effect of ischemic preconditioning is multifactorial, however, involving the opening of ATP-sensitive potassium channels. The precise mechanisms by which ischemically primed myocardium can protect itself remains unclear. The extent of the infarct size reduction induced by ischemic preconditioning exceeds any that has been produced with pharmacological interventions and warrants further investigation.

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Received March 21, 1994
revision accepted August 22, 1994

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Chapter 6

Renewal of ischemic preconditioning's protection is possible by activation of K_{ATP}-channels

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Renewal of Ischemic Preconditioning's Protection is possible by Activation of K_{ATP}-channels

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Objective: Brief cycles of coronary artery occlusion that precede a sustained occlusion do not aggravate infarction but paradoxically increase the ischemic tolerance of the myocardium. This protective effect is lost when the time between the first ischemic event and the sustained ischemic episode is prolonged. We assessed whether bimakalim, an opener of ATP sensitive potassium channels (K_{ATP}) or a second preconditioning cycle can renew ischemic preconditioning protection. **Methods:** Studies were performed in pentobarbitone anesthetized, open chest pigs which were subjected to a 60 min occlusion (TCO) of the left anterior descending coronary artery (LADCA) followed by two hours of reperfusion (REP). Six groups of animals were studied. In two groups either a preconditioning cycle of 10 min TCO followed by 15 min REP was performed (PC15) or bimakalim (Bim) was infused (4μg/kg) over 10 min prior to the 60 min occlusion. In the third group REP following preconditioning with 10 min TCO was prolonged to 100 min (PC100). In the PC+PC group a second cycle of 10 min TCO followed by 15 min REP was performed 100 min after the first cycle, while in the PC+Bim group bimakalim was infused instead of the second preconditioning cycle. In the control group (n = 7) animals received vehicle only. At the end of the protocol, infarct size (IS, as % of the left ventricular risk region) was determined by the p-nitroblue-tetrazolium method. **Results:** A single cycle of 10 min TCO is highly protective when followed by 15 min of REP (IS: 9.9±5.1% $p<0.001$ vs. 66.3±5.6% of controls), but this potection was completely lost in all animals if REP was prolonged to 100 min (IS: 61.6±5.2%). With a second preconditioning cycle renewal of the protection was only possible in 2 out of 7 pigs (IS: 40.9±7.7% $p<0.05$ vs. controls and vs. PC15). Applying the K_{ATP} channel opener instead, full protection was achieved in every pig (IS: 21.6±3.5% $p<0.001$ vs. control n.s. vs. PC15). **Conclusion:** Protection by ischemic preconditioning in pigs was lost if the intermittent reperfusion period was prolonged to 100 min. The renewal of the protective effect is only possible in part by a second preconditioning cycle, most likely depending on the remaining myocardial adenosine pool, but can be fully restored by applying bimakalim. The results implicate that a K_{ATP}-channel opener may be clinically very useful in coronary heart disease as it is reliably able to re-establish the endogenous protection provided by ischemic preconditioning.

Ischemic preconditioning is an endogenous protective mechanism in which brief periods of myocardial ischemia and reperfusion render the myocardium resistant to a subsequent more sustained ischemic insult[1]. This phenomenon has been shown to exist in most mammalian species including man[2]. Although the protective effect could be confirmed in every species tested, there still exists controversy over the initiating event and subsequent subcellular mechanisms leading to cardioprotection.

In anesthetized dogs Gross and coworkers[3] descibed prevention of ischemic preconditioning after blockade of K_{ATP}-channels with glibenclamide. They also reported that administration of the K_{ATP}-channel opener EMD 52692 (bimakalim) provided tolerance to subsequent ischemia[4] [5]. Gross, therefore, theorized that activation of the K_{ATP}-channel is importantly involved in mediating ischemic preconditioning. We[6] [7] have confirmed these data in anesthetized pigs, thereby excluding a role of coronary collaterals.

Today there is little doubt that openers of ATP-sensitive potassium channels possess cardioprotective properties[8]. They represent a new class of drugs that may offer exciting new therapeutic opportunities under conditions in which a reduction of consequences of an ischemic episode is desirable[9]. Possible clinical applications include various forms of angina, myocardial infarction with early thrombolysis and cardiac surgery. However, their clinical use could be limited, since it has been shown in rats, rabbits, pigs, and dogs that preconditioning protection can be lost when the intermittent reperfusion period between the initial preconditioning ischemia and the subsequent sustained ischemic episode is prolonged[10] [11] [12] [13].

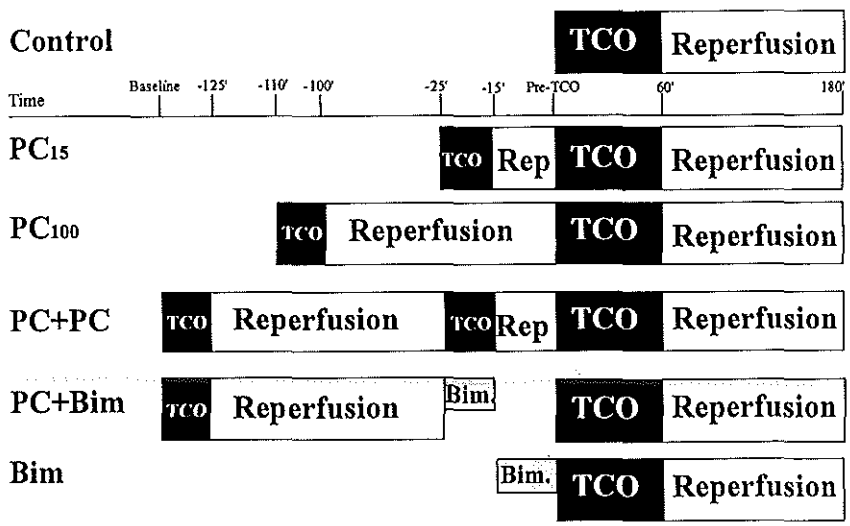

Figure 1. Design of the study. TCO= total coronary artery occlusion.

It was the aim of this study to confirm these results in our pig model, in which bimakalim has been shown to be able to mimic preconditioning's protection. Furthermore, we wanted to evaluate to which extent a second preconditioning event or the pharmacological activation the K_{ATP} channel by bimakalim is able to re-establish the protection.

Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No 85-23, revised 1985).

Experimental Design:

The design is depicted in figure 1. All 42 pigs underwent a 60 min left anterior descending coronary artery (LADCA) occlusion followed by 120 min of reperfusion. The animals were divided into six groups. Ischemic preconditioning was induced by 10 min of LADCA occlusion followed either by 15 min of reperfusion (PC15) or by 100 min of reperfusion (PC100). In the PC+PC group 100 min after the first preconditioning ischemia a second 10 min LADCA occlusion was performed, while in PC+Bim animals received an i.v. infusion of bimakalim (4 μg/kg/10min) after 100 min of reperfusion instead of the second preconditioning occlusion of the PC+PC group. Furthermore, to demonstrate that an effective dose of bimakalim able to limit infarct size was used, bimakalim was infused (4 μg/kg/15min i.v.) prior to the 60 min LADCA occlusion. Vehicle treated animals served as controls (CON). The results of two groups, BIM and PC15, have been used already in another publication[7], but since all experiments were performed at the same time and in the same laboratory they were included in this study.

Experimental Preparation:

Fourty-two mixed breed Landrace-type domestic pigs of either sex weighing between 23 and 29 kg were premedicated with ketamine-HCl (10mg/kg i.m.) 15 min prior to anesthesia with i.v. bolus injection of sodium pentobarbital (30 mg/kg). Following tracheotomy pigs were mechanically ventilated with an animal respirator (Rhema, Hofheim/FRG) on nitrous oxide supplemented with oxygen at a ratio of 2:1. In order to guide adjustment of ventilator settings arterial blood gases were measured repeatedly. Anesthesia was maintained by continous infusion of sodium pentobarbital (3mg/kg/h). Arterial blood was sampled through a catheter placed in the right femoral artery, while the right brachial artery was cannulated with a polyethylene catheter, which was advanced to the ascending aorta for continuous recording of

arterial pressure. An 8 French Millar micromanometer was advanced via the carotid artery into the left ventricle for continuous measurement of left ventricular pressure. The heart was exposed through a midline thoracotomy and suspended in a pericardial cradle. Distally of its second branch the (LADCA) was carefully dissected free over a length of 4 mm and a loose ligature was placed around the vessel which could be occluded with a small vascular clip. Electromagnetic flow probes were situated on the ascending aorta and the LADCA proximal to the

Table 1 Hemodynamics assessed at baseline, directly before the 60 min LADCA occlusion (Pre-TCO), 60 min of LADCA occlusion (TCO), at reactive hyperemia (about 3 min of reperfusion; RH), and at the end of the protocol (120' REP).

Heart Rate [beats/min]					
	Baseline	**PreTCO**	**60'TCO**	**RH**	**120'Rep**
Control	102 ± 3	103 ± 4	111 ± 4	114 ± 4	128 ± 10
PC_{15}	112 ± 7	114 ± 6	112 ± 8	124 ± 8	111 ± 13
PC_{100}	96 ± 7	98 ± 10	101 ± 11	117 ± 8	120 ± 13
PC+PC	99 ± 5	99 ± 4	109 ± 4	120 ± 3	121 ± 6
PC+Bim	97 ± 9	105 ± 6	121 ± 15	120 ± 13	122 ± 9
Bim	102 ± 11	113 ± 8	116 ± 9	118 ± 7	127 ± 13
Mean Arterial Pressure [mmHg]					
	Baseline	**PreTCO**	**60'TCO**	**RH**	**120'Rep**
Control	95 ±6	93 ±6	70 ±3	63 ±2	80 ±7
PC_{15}	95 ±9	86 ±9	80 ±6	61 ±6	75 ±10
PC_{100}	104 ±4	96 ±6	93 ±7*	80 ±8*	92 ±7
PC+PC	99 ±6	97 ±6	91 ±4*	71 ±4*	88 ±4
PC+Bim	103 ±7	70 ±4*	76 ±4	64 ±5	78 ±4
Bim	92 ±2	61 ±4*	71 ±6	62 ±5	70 ±3
Double Product [(mmHg*bpm)/100]					
	Baseline	**PreTCO**	**60'TCO**	**RH**	**120'Rep**
Control	117 ± 7	112 ± 8	95 ±4	85 ±3	120 ±13
PC_{15}	135 ±15	114 ± 10	107 ±10	91 ±5	102 ±18
PC_{100}	116 ± 10	102 ± 14	112 ±14	110 ±8*	130 ±16
PC+PC	106 ±7	112 ± 6	116 ±6	100 ±4*	126 ±10
PC+Bim	113 ± 8	94 ±6*	112 ±13	93 ±6	118 ±11
Bim	103 ±6	86 ± 7*	95 ±7	87 ±7	106 ±11
$LVdP/dt_{max}$ [mmHg/sec]					
	Baseline	**PreTCO**	**60'TCO**	**RH**	**120'Rep**
Control	1435 ±114	1367 ±104	1076 ±84	869 ±53	1018 ±64
PC_{15}	1369 ±84	1374 ±132	1669 ±112*	1199 ±147*	1397 ±181*
PC_{100}	1565 ±187	1520 ±167	1469 ±146*	1168 ±207*	1357 ±161*
PC+PC	1564 ±95	1463 ±81	1244 ±62*	991 ±67	1430 ±109*
PC+Bim	1365 ±74	1529 ±98	1394 ±84*	1162 ±116*	1471 ±111*
Bim	1373 ±258	1889 ±337	1845 ±348*	1378 ±202*	1382 ±288*
Cardiac Output [ml/min/10 kg]					
	Baseline	**PreTCO**	**60'TCO**	**RH**	**120'Rep**
Control	969 ± 66	923 ± 64	839 ± 76	691 ± 53	771 ± 77
PC_{15}	1024 ± 154	1080 ± 157	848 ± 128	698 ± 80	823 ± 115
PC_{100}	1078 ± 40	992 ± 106	890 ± 89	748 ± 126	776 ± 135
PC+PC	950 ± 35	896 ± 32	818 ± 40	558 ± 48*	795± 67
PC+Bim	1065 ± 62	1087 ± 49*	1114 ± 56*	883 ± 124*	1093 ± 112*
Bim	851 ± 70	864 ± 82	785 ± 72	690 ± 43	684 ± 83

*= $p<0.05$ compared with control animals at the same timepoint. (Abbr.: MAP = mean arterial blood pressure, LV = left ventricular)

occlusion site. A Teflon catheter (outer diameter 0.8 mm) was placed into the epicardial vein draining the area at risk. Rectal temperature was monitored throughout the experiment and the chest cavity was covered with a plastic sheet and kept warm with a heat lamp to keep body temperature between 37°C and 38°C. Myocardial function in the ischaemic area was measured with 5 mHz ultrasonic crystals aligned transmurally to measure wall thickness. A brief (< 10 second) occlusion was performed to identify the ischaemic region and the crystal pair was located centrally in the ischaemic region. The inner crystal was advanced to the endocardium tangentially through a stab wound in the epicardium. The epicardial crystal, attached to a Dacron patch, was positioned on the epicardium with the aid of an oscilloscope, and secured with three sutures. After a stabilisation period of 30 min after surgery the experimental protocol was started.

Risk Region and Infarct Size:

Following a reperfusion period of two hours the LADCA was reoccluded and 10 ml of 10% fluorescein dye were injected into the left atrium. After one to two min the pig was killed by electrical fibrillation with a 9-V battery and the heart excised. Warm 2% agarose was injected into the ventricle via the aortic root and the heart was kept on ice. After the agarose had congealed, the right ventricle was removed and the left ventricle was sliced into four to six rings perpendicular to the LADCA. Subsequently, the rings were weighed and the risk region traced onto an acetate sheet under blacklight, which sharply defined the borders of the risk area

Table 2. Coronary blood flow (CBF) and calculated coronary vascular resistance (CVR) during the protocol.

CBF [ml/min]					
	Baseline	**PreTCO**	**60'TCO**	**RH**	**120'Rep**
Control	36 ± 3	37 ± 3	17 ± 3	48 ± 6	31 ± 6
PC_{15}	35 ± 2	22 ± 3*	16 ± 2	40 ± 2	23 ± 3
PC_{100}	40 ± 4	36 ± 3	20 ± 2	58 ± 11	41 ± 3
PC+PC	37 ± 1	33 ± 2	22 ± 2	49 ± 3	37 ± 2
PC+Bim	39 ± 2	35 ± 3	21 ± 3	55 ± 7	57 ± 7*
Bim	37 ± 5	44 ± 6	15 ± 3	51 ± 7	45 ± 8
CVR [dyn*sec*cm^{-5}]					
	Baseline	**PreTCO**	**60'TCO**	**RH**	**120'Rep**
Control	201 ± 24	198 ± 22	306 ± 66	135 ± 21	206 ± 48
PC_{15}	229 ± 44	303 ± 69*	350 ± 58	138 ± 18	239 ± 47
PC_{100}	165 ± 20	171 ± 17*	240 ± 26	101 ± 14	148 ± 10*
PC+PC	182 ± 11	197 ± 19	307 ± 54	101 ± 9*	158± 6*
PC+Bim	179 ± 11	129 ± 12*	207 ± 28*	97 ± 10	93 ± 11*
Bim	197 ± 27	101 ± 18*	189 ± 23*	100 ± 10*	87 ± 9*

*= $p<0.05$ compared with control animals at the same timepoint, time points (see table 1).

Table 3. Regional wall function (%WT = % delta wall thickening) in the LADCA area subsequently made ischemic for 60 min.

	Baseline	PreTCO	60'TCO	RH	120'Rep
Control	28 ± 3	25 ± 3	- 6 ± 3	- 11 ± 2	- 9 ± 3
PC_{15}	27 ± 3	14 ± 6*	- 6 ± 7	- 5 ± 5	- 2 ± 6
PC_{109}	32 ± 3	23 ± 5	- 5 ± 2	- 9 ± 3	- 8 ± 2
PC+PC	33 ± 3	14 ± 3*	- 5 ± 2	- 8 ± 3	- 6± 1
PC+Bim	33 ± 4	24 ± 3	- 6 ± 2	- 12 ± 3	- 4 ± 4
Bim	23 ± 2	24 ± 2	- 4 ± 2	- 5 ± 5	- 3 ± 2

*= $p<0.05$ compared with control animals at the same timepoint, time points (see table 1).

not perfused with fluorescein. Incubation for 30 min in 1.25% paranitrobluetetrazolium in phosphate buffer (pH 7.1) at 37°C delineated noninfarcted tissue (deep blue) and infarcted tissue (pale). The size of the area at risk and the infarcted region were determined by planimetry. The % risk region (area at risk / area of ring) was calculated for the top and bottom of each ring and the mean value for each ring was multiplied by the weight. The weights of regions were summed and divided by the weight of the left ventricle to yield the % of the left ventricle at risk (%RR) and % of the left ventricular risk region infarcted (%IS).

Administration of bimakalim:

Animals in the treatment groups received bimakalim dissolved in 1-2-propandiol and isotonic saline, while the pigs in the control group received vehicle (1-2-propandiol and isotonic saline). Prior to each experiment bimakalim was dissolved and mixed in a 37°C warm saline. Bimakalim 4μg/kg min was administered as a continuous infusion via a femoral vein either over 10 min (PC+Bim) or over 15 min (Bim).

Data analysis:

Continuous recording of hemodynamic and wall thickness data was made on a paper recorder (Gould) and on a computer-aided registration system (IFD, Mescher, Mülheim/Ruhr, FRG). Recordings at timepoints of interest were made at a paper speed of 100mm/sec, and the wall thickness data were collected from the recordings. Wall thickness variables were averaged over 10 cycles at each timepoint and included end diastolic wall thickness (EDWT, defined as the point when left ventricular dP/dt started its rapid upstroke after crossing the zeroline), and end systolic wall thickness (ESWT, defined as the point of maximal wall thickness within 20 ms before peak negative left ventricular dP/dt). These data were used to calculate wall thickening (%delta WT) with the following formula: ((ESWT-EDWT) / EDWT) x 100. Other variables included peak left ventricular systolic pressure (LVSP, mmHg), mean arterial pressure (MAP, mmHg), and heart rate (HR, beats/min). The coronary vascular resistance (dyn x sec x cm^{-5}) was calculated as diastolic aortic blood pressure x 80 / coronary blood flow; analogously, the total peripherial resistance was calculated as MAP x 80 / aortic blood flow.

cm^{-5}) was calculated as diastolic aortic blood pressure x 80 / coronary blood flow; analogously, the total peripherial resistance was calculated as MAP x 80 / aortic blood flow.
For comparison of variables across time between the groups, a two factor analysis of variance (ANOVA) of repeated measures was used. When the ANOVA was significant, pairwise comparisons were made with the Student's t-test with the significance level for the p values corrected with the Bonferroni method. For comparison across time in the control group, a one factor ANOVA of repeated measures was used, with a post-hoc testing strategy identical to that of the two factor ANOVA. A two sample rank sum test (Mann-Whitney-U) was used to test the differences between two groups with regard to the risk region and the % of the risk region infarcted. Data are reported as mean±SEM.

Results

Risk Region and Infarct Size:

The risk regions (expressed as % of the left ventricle) were virtually identical in all groups (Control, 12.6±2.4%; Bim, 11.5±1.0%; PC15, 11.6±1.8%; PC100, 12.3±0.7%; PC+Bim, 12.6±1.0%; PC+PC, 11.1±0.6%). The risk area was intentionally kept small (10% - 15% of left ventricular wall volume), because in pilot studies larger risk areas were associated with a high incidence of ventricular fibrillation.
The percentage of the risk region infarcted was 66.3±5.6% for the controls. Starting bimakalim treatment as well as the preconditioning cycle prior to 60 min of LADCA occlusion significantly reduced infarct size (Bim, 35.3±6.6% and PC15, 9.9±5.1%, $p<0.05$ and $p<0.01$ vs. controls, respectively). When the reperfusion period between the preconditioning cycle was prolonged from 15 min to 100 min, the infarct size limiting effect was lost (61.6±5.2%). A second preconditioning cycle given at that time point restored protection only in part (40.9±7.7, $p<0.05$ vs controls and $p<0.05$ vs. PC15). Analyzing the individual infarct sizes (depicted in figure 2), a full or some protection in the PC+PC group was achieved in 4 pigs, while at least 3 animals showed no protection. Bimakalim was able to re-establish protection in every case (21.6 ±3.5%, $p<0.001$ vs control, n.s. vs. PC15 and Bim, $p<0.05$ vs PC+PC).

Hemodynamics and wall function: The control pigs remained hemodynamically stable throughout the occlusion and reperfusion period and did not vary significantly from baseline with regard to heart rate, mean arterial pressure or double product.
Infusion of bimakalim significantly reduced mean arterial pressure and the double product, while the preconditioning cycle(s) did not significantly affect global hemodynamics (table 1).

Bimakalim significantly reduced coronary vascular resistance, but did not significantly increase coronary blood flow when assessed prior to the 60 min of LADCA occlusion (table 2).

Wall function in the LAD region was significantly reduced in the PC15 and PC+PC group prior to the 60 min LADCA occlusion (table 3), but not with any other intervention. Comparing the time course of postischemic dysfunction after the first preconditioning cycle (see table 3), regional wall function shows a stepwise recovery over time. Within 120 min of reperfusion after the 60 min LADCA occlusion no recovery of function in any of the groups was observed.

Discussion

It is currently being hoped that ischemic preconditioning will have clinical importance[14]. Therefore, the results by Cohen et al.[13] indicating that it is possible to become tolerant to multiple episodes of ischemic preconditiong were alerting. In conscious rabbits cardioprotection waned after multiple 5-minute coronary occlusion have occured but did

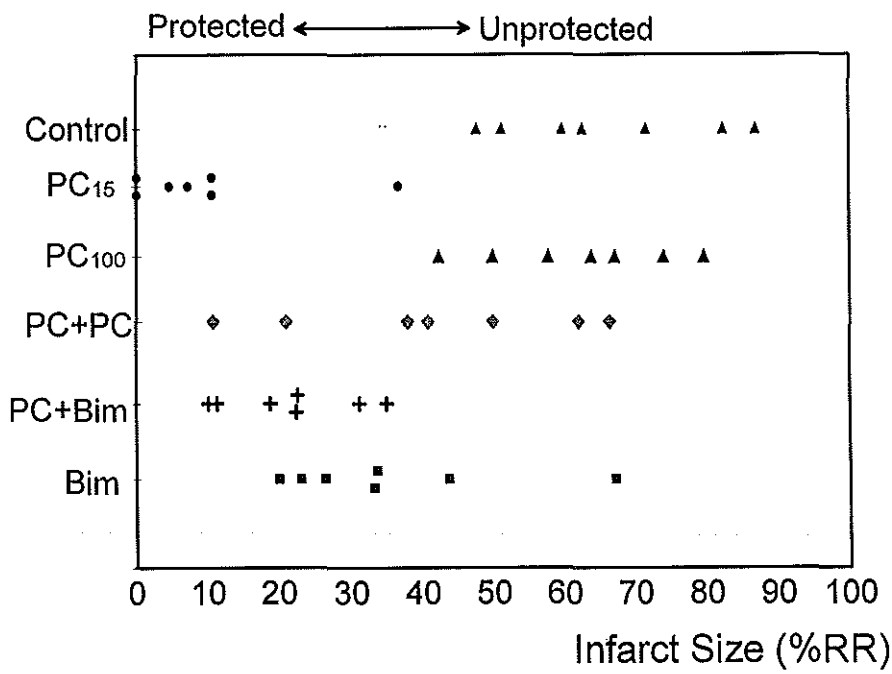

Figure 2. Plot of myocardial infarct size expressed as percent of the risk region (RR) in each individual animal. All groups n = 7. The dotted line indicates the border towards protection basing on the maximal infarct size value in the PC15 group.

reappear after an ischemia-free period.

In our pig study the cardioprotective effect was completely lost, when the reperfusion period between the initial ischemic event and the subsequent sustained ischemia was prolonged to 100 minutes. This is consistent with results obtained in anesthetized rats[12], dogs[11], and in pigs[10], all indicating that by prolonging reperfusion the myocardium looses its protection.

Sack et al.[10] could show in pigs that preconditioning's protection could be renewed by a second cycle of two 10 min occlusions seperated by 30 min of reperfusion after 4 days but not 60 min after the first preconditioning cycle. Their results suggested that the release of a mediator from an exhaustible pool, most likely adenosine, is necessary to activate a chain of events leading to cardioprotection.

In contrast to Sack et al.[10] we only used a single cycle of 10 min of ischemia instead of two cycles of 10 min of ischemia. While the induced cardioprotective effect appears to be independant of the number of preceeding cycles of ischemia, which is in line with results obtained in dogs[15], the effect on the cardiac adenosine pool could be different. A single cycle of 10 min of ischemia might not be severe enough to exhaust myocardial adenosine pools in every case, but two cycles (20 min) may do so. Henrichs et al.[16] could demonstrate in dogs that there is a significant difference in myocardial adenine nucleoside content following a single 5 min occlusion and two 5 min occlusions. A different amount of preceeding adenosine release might explain why we observed a renewal of preconditioning's protection in 2 out of 7 animals, while Sack et al.[10] did not. However, both pig studies favour a mechanism involving adenosine as a primary mediator to initiate cardioprotective effects, although both studies did not measure adenosine release.

Thus, a leading hypothesis regarding the initiating events leading to cardioprotection, as been forwarded by Downey and coworkers[17], proposes that the mechanism of preconditioning involves activation of adenosine receptors, stimulation of G_i proteins, activation of phospholipase C and the formation of diacylglycerol. This results in the activation and translocation of protein kinase C (PKC) and the resultant phosphorylation of an unknown effector protein. Evidence in support of this theory was obtained from in vitro and in vivo rabbit models, in which various antagonists and agonists along this pathway were found to prevent and mimic ischemic preconditioning, respectively.

Recently, in dog[18] as well as in pig model[8] it has been shown that adenosine mediates its cardioprotection via a cardiac K_{ATP}-channel linked mechanism, naming the K_{ATP} -channel the since unknown effector. However, Yao and Gross[18] observed in dogs, that, despite the efficacy of adenosine in mimicking the effects of preconditioning, its protective effects did not persist as long as those of preconditioning. In their experiment, the adenosine induced cardioprotection totally disappeared 60 minutes after administration, whereas ischemic

preconditioning persisted more than 1 hour[18]. How preconditioned dog hearts maintain K_{ATP}-channel activation to memorize the preconditioning for 1 hour or longer, therefore, cannot be explained by adenosine alone.

Ytrehus et al.[20] showed in rabbit hearts that activation of protein kinase C may be involved in the memory phase of ischemic preconditioning, while several laboratories[21 22] demonstrated that stimulation of protein kinase C activates the K_{ATP}-channel. Since 5 min of ischemia is sufficient to activate protein kinase C[23], it appears possible that even short preconditioning stimuli lead to activation of PKC, which may translate into opening and persistent activation of K_{ATP}-channels[24].

Furthermore, using human atrial cells it has been demonstrated that protection can be induced by activation of PKC and by the opening of K_{ATP}-channels and that the protection induced by PKC activation and preconditioning can be blocked by blocking the K_{ATP}-channel[25].

The smaller infarcts in the preconditioned or bimakalim treated animals did not translate into a better recovery of regional wall function. This may be attributable either to the fact that the ischemic burden in each group has been too large to see a sign of recovery within the relatively short reperfusion period of two hours or that the reperfusion period was too short to allow the area at risk to overcome myocardial stunning[11]. However, reperfusion periods of more than 60 min are sufficient to allow precise measurement of infarct size[26], which was the primary endpoint of this study.

In our pig study pharmacological activation of the K_{ATP}-channel could bypass the initial adenosine dependant pathways and thereby fully reinstalled the protective effect of preconditioning. This provides further evidence that the mechanism of preconditiong may initially act via adenosine, PKC, etc. but relies on the action of the K_{ATP}-channel as the end effector.

This hypothesis is supported by two most recent observations. Firstly, in pigs Vogt et al.[27] could overcome the deficit in endogenous adenosine production post-preconditioning by substituting with a local intramyocardial application of an adenosine A_1-agonist, thereby reinstalling preconditioning's protection. Secondly, in dogs Yao et al.[28] blocked the K_{ATP}-channels after 1 h of reperfusion with glibenclamide, which abolished the otherwise cardioprotective activation of K_{ATP}-channels elicited by 10 min of preconditioning ischemia

Acknowledgements:

We wish to thank Andreas Kaufmann and Claus Fuchs for excellent technical assistance with the animal preparation and for help in the data analysis.

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Chapter 7

Preischaemic as well as postischaemic application of a Na^+/H^+ exchange inhibitor reduces infarct size in pigs

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Cardiovascular Research 1995; 30: 945-951

Preischaemic as well as postischaemic application of a Na^+/H^+ exchange inhibitor reduces infarct size in pigs

Sven Rohmann, Heinrich Weygandt, Klaus-Otto Minck

Background: During reperfusion of ischaemic myocardium, Na^+/H^+ exchange promotes recovery from acidosis resulting in an accumulation of intracellular Na^+. This leads to calcium overload via Na^+/Ca^{2+} exchange and might result in cell necrosis contributing to reperfusion injury. **Methods and Results:** We assessed whether HOE 694, a specific inhibitor of Na^+/H^+ exchange, is able to reduce infarct size in swine myocardium. Experiments were performed in pentobarbitone anaesthetized, open chest pigs which were subjected to a 60 min occlusion of the left anterior descending coronary artery (LADCA) followed by two hours of reperfusion. Three groups of animals were studied. In the pre-reperfusion group (pre-REP, n=7) HOE 694 infusion (7 mg/kg/15min) was started at 45 min of occlusion of the LADCA and continued until the end of occlusion, while in pre-occlusion group (pre-TCO, n=7) HOE 694 infusion was started 15 min before occlusion and stopped at the onset of ischaemia. In the control group (n=7) animals received vehicle alone. At the end of the protocol, infarct size (as % of the left ventricular risk region) was determined by the p-nitro-blue-tetrazolium method. Treatment with HOE 694 prior to the ischaemic insult or upon reperfusion significantly reduced infarct size (4.1%(1.4%), $p<0.01$ and 38.2%(5.8%), $p<0.05$, respectively), compared with 77.7%(4.0%) in the control group. However, infarct size was significantly more reduced in the pre-TCO group than in the pre-REP group ($p<0.05$). **Conclusion:** Treatment with HOE 694 leads to a significant reduction in infarct size; even when administered after the onset of ischaemia. Thus, inhibition of Na^+/H^+ exchange was able to limit cell necrosis. This implicates an important role for Na^+/H^+ exchange in the pathogenesis of infarct expansion and provides evidence that reperfusion injury exists. However, HOE 694 was even more effective when given before ischaemia, indicating an additional protective effect during ischaemia which might be due to slowing down of a vicious cycle, that consumes ATP and generates H^+.

In the last decade several intracellular processes have been identified as key players involved in the imbalance of cellular homeostasis in cardiac tissue. In the course of myocardial ischemia and reperfusion these include: decreased intracellular pH[1], intracellular Na^+ overload[2], and intracellular Ca^{2+} overload[3].

Na^+/H^+ exchange represents the most important mechanism for myocardial pH regulation in ischaemic and reperfused tissue[4 5 6]. Myocardial ischaemia is known to result in acidosis due to a retention of H^+ from glycolytic ATP turnover, accumulation of CO_2 and glycolytic degradation products such as lactate, and net ATP breakdown[7]. The Na^+/H^+ exchanger of cardiac myocytes is stimulated by intracellular acidosis, resulting in H^+ extrusion and Na^+ influx[8]. Scholz et al.[9] have proposed that such an influx of Na^+ stimulates the Na^+/K^+-ATPase, which could accelerate ATP depletion and cellular injury in tissue that is metabolically compromised by ischaemia. Stimulation of the Na^+/H^+ exchanger by intracellular acidosis and its detrimental consequences may, however, be confined to the early minutes of ischaemia. The Na^+/H^+ exchanger has been reported to be inhibited by extracellular acidosis[10], which may exceed intracellular acidosis within 10 minutes of ischaemia[11].

During reperfusion of ischemic myocardium the effect of the Na^+/H^+ exchanger may be substantial, too. While the Na^+/H^+ exchanger is inhibited by extracellular acidosis during ischemia, Lazdunski et al.[4] provided evidence that a rapid washout of extracellular H^+ during reperfusion reactivates the Na^+/H^+ exchanger, which results in a marked influx of Na^+. Such an influx of Na^+, in the face of Na^+/K^+-ATPase inhibition due to ATP depletion caused by the preceeding ischaemia, may lead to a dramatic increase in intracellular Na^+ concentration. This sets in motion the Na^+/Ca^{2+} exchange favouring an excessive increase in the intracellular Ca^{2+} concentration (Ca^{2+}-overload)[12 13], which has been implicated as a crucial contributing factor in reperfusion injury [14].

Based on the preceeding discussion, it is evident that activation of the Na^+/H^+ exchanger, although leading to rapid restoration of normal pH, could result in a paradoxical acceleration of tissue injury. To evaluate the pathophysiological role of the Na^+/H^+ exchanger in infarct size expansion, we applied its specific inhibitor HOE 694 [(3-methylsulfonyl-4-piperidinobenzoyl)guanidine methanesulfonate][15] either prior to 60 minutes of ischaemia or

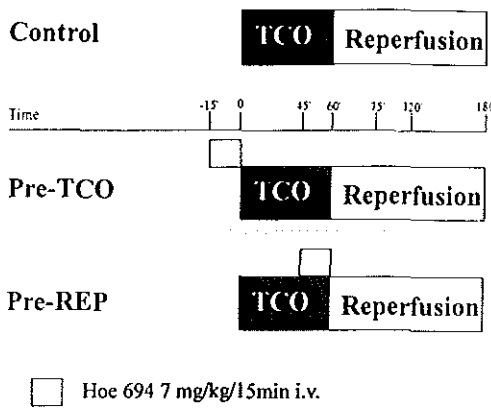

Figure 1. Design of the study. TCO = total coronary artery occlusion.

prior to 120 minutes of reperfusion in a pig model of myocardial ischaemia and measured infarct size at the end of the experiment.

Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No 85-23, revised 1985).

Experimental design

All 21 pigs underwent a 60 min left anterior descending coronary artery (LADCA) occlusion followed by 120 min of reperfusion. The animals were divided into three groups in random order. The design is depicted in figure 1. Vehicle treated animals served as controls (CON). HOE 694 was infused (7 mg/kg/15min i.v.) either prior to the 60 min LADCA occlusion (Pre-TCO) or prior to 120 min of reperfusion (Pre-REP). The dose of HOE 694 was chosen according to the study of Sack et al.[16] demonstrating that with a bolus injection of 7 mg/kg plasma levels can be achieved adequate for the inhibition of the Na^+/H^+ exchanger.

Experimental Preparation

Twenty-one mixed breed Landrace-type domestic pigs of either sex weighing between 23 and 29 kg were premedicated with ketamine-HCl (10mg/kg i.m.) 15 min prior to anaesthesia with i.v. bolus injection of sodium pentobarbital (30 mg/kg). Following tracheotomy pigs were mechanically ventilated with a animal respirator (Rhema, Hofheim/FRG) on nitrous oxide supplemented with oxygen at a ratio of 2:1. In order to guide adjustment of ventilator settings arterial blood gases were measured repeatedly. Anaesthesia was maintained by continuous infusion of sodium pentobarbital at 3 mg/kg/h through a catheter in the right jugular vein. Arterial blood was sampled through a catheter placed in the right femoral artery, while the right brachial artery was cannulated with a polyethylene catheter, which was advanced to the ascending aorta for continuous recording of arterial pressure. A 8 French Millar micromanometer was advanced via the carotid artery into the left ventricle for continuous measurement of left ventricular pressure. The heart was exposed through a midline thoracotomy and suspended in a pericardial cradle. Distally of its second branch the (LADCA) was carefully dissected free over a length of 4 mm and a loose ligature was placed around the vessel which could be occluded with a small vascular clip. Electromagnetic flow probes were situated on the ascending aorta and the LADCA proximal to the occlusion site. A Teflon catheter (outer diameter 0.8 mm) was placed into the epicardial vein draining the area at risk. Rectal temperature was monitored throughout the experiment and the chest cavity was covered with a plastic sheet and kept warm with a heat lamp to keep body temperature

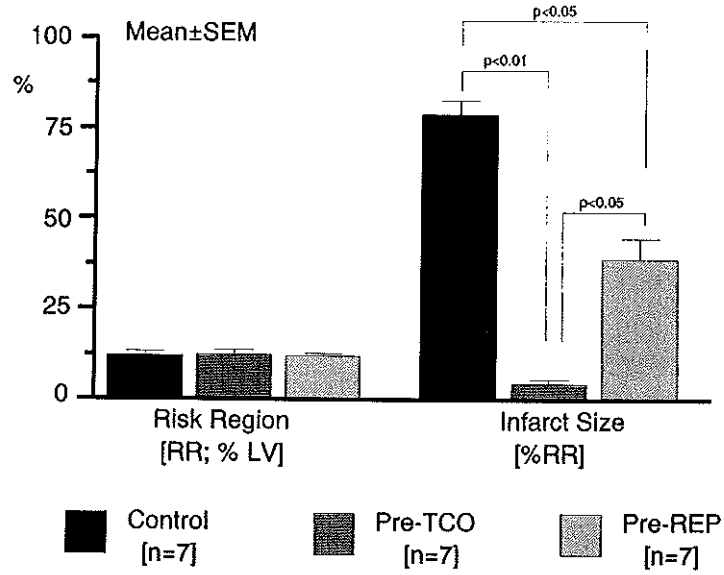

Figure 2. Risk region (RR) expressed as percent of the left ventricle (LV) and myocardial infarct size expressed as percent of RR with and without HOE 694 treatment, results are mean±SEM. All groups n = 7.

between 37°C and 38°C. Myocardial function in the ischaemic area was measured with two 5 mHz ultrasonic crystals aligned transmurally to measure wall thickness. A brief (< 10 second) occlusion was performed to identify the ischaemic region and the crystal pair was located centrally in the ischaemic region. The inner crystal was advanced to the endocardium tangentially through a stab wound in the epicardium. The epicardial crystal, attached to a Dacron patch, was positioned on the epicardium with the aid of an oscilloscope, and secured with three sutures. After a stabilisation period of 30 min after surgery the experimental protocol was started.

Risk Region and Infarct Size

Following a reperfusion period of two hours the LADCA was reoccluded and 10 ml of 10% fluorescein dye (fluorescein sodium solved in saline, E. Merck) were injected into the left atrium. After one to two min the pig was killed by electrical fibrillation with a 9-V battery and the heart excised. Warm 2% agarose was injected into the ventricle via the aortic root and the heart was kept on ice. After the agarose had congealed, the right ventricle was removed and the left ventricle was sliced into four to six rings perpendicular to the LADCA. Subsequently, the rings were weighed and the risk region traced onto an acetate sheet under blacklight,

which sharply defined the borders of the risk region not perfused with fluorescein. Incubation for 30 min in 1.25% paranitrobluetetrazolium in phosphate buffer (pH 7.1) at 37° C delineated noninfarcted tissue (deep blue) and infarcted tissue (pale). The size of the risk region and the infarcted region were determined by planimetry. The % risk region (risk region / area of ring) was calculated for the top and bottom of each ring and the mean value for each ring was multiplied by the weight. The weights of regions were summed and divided by the weight of the left ventricle to yield the % of the left ventricle at risk (%RR) and % of the left ventricular risk region infarcted (%IS).

Myocardial Oxygen Consumption

Arterial and coronary venous blood samples were obtained anaerobically for determination of pH, pO_2, and pCO_2 by withdrawal of 3.0 ml of blood from the aortic and anterior interventricular vein catheters simultaneously.

Haemoglobin content, O_2 saturation, blood PO_2, and pH, were measured by a computer-aided blood gas analyser (ABL 300 Radiometer, Copenhagen, Denmark).

Oxygen consumption in the region of myocardium perfused by the LADCA was computed by multiplying the arteriovenous oxygen difference by coronary blood flow determined with the flowmeter probe proximal to the occlusion site. Since anterior interventricular vein blood flow corresponds to LADCA influx[17], this method allows determination of myocardial oxygen consumption in the region perfused by the LADCA[18].

Administration of HOE 694

Animals in the treatment groups received HOE 694 (E.Merck) dissolved in isotonic saline, while the pigs in the control group received isotonic saline. Prior to each experiment HOE 694 was dissolved and mixed in a 37°C warm saline. The HOE 694 solution was administered as a continuous infusion over 15 min via a femoral vein.

Biochemical analysis of HOE 694 in plasma samples

The plasma samples were stored at -20°C in plastic centrifuge tubes until analysis. Plasma was extracted according to the method of Sack et al.[16], using EMD 80 563 as internal standard. All chemicals used were of analytical grade (E. Merck). The HPLC equipment consisted of autosampler (Merck Hitachi AS2000A), HPLC pump (Merck Hitachi L6020A), UV-detector (Merck Hitachi L4000), integrator (Merck Hitachi D-2500), and a column (E. Merck 60 RP Select B) in the dimension 125 x 4 mm. Flow rate of the modified buffer, 0.05M, pH 4.0 (CH_3CN/NH_4PO_4 (40/60)) and dodecane-1-sulfone-sodium-salt (0.5 mM) was 1.2 ml/min.

Retention time was 2.7 min for HOE 694 and 4.7 min for the internal standard at the described conditions. The limit of quantification was to 40 ng/ml plasma.

Data analysis

Continuous recording of haemodynamic and wall thickness data was made on a paper recorder (Gould) and on a computer-aided registration system (IFD, Mescher, Müllheim/Ruhr, FRG). Recordings at timepoints of interest were made at a paper speed of 100mm/sec, and the wall thickness data were collected from the recordings. Wall thickness variables were averaged over 10 cycles at each timepoint and included end diastolic wall thickness (EDWT, defined as the point when left ventricular dP/dt started its rapid upstroke after crossing the zeroline), and end systolic wall thickness (ESWT, defined as the point of maximal wall thickness within 20 ms before peak negative left ventricular dP/dt). These data were used to calculate wall thickening (%ΔWT) with the following formula: ((ESWT-EDWT) / EDWT) x 100. Other variables included peak left ventricular systolic pressure (LVSP, mmHg), mean arterial pressure (MAP, mmHg), and heart rate (HR, beats/min). The coronary vascular resistance (dyn x sec x cm^{-5}) was calculated as diastolic aortic blood pressure x 80 / coronary blood flow; analogously, the total peripheral resistance was calculated as MAP x 80 / aortic blood flow.

Table 1
Haemodynamics assessed at baseline, directly before the 60 min LADCA occlusion (Pre-TCO), at 45 min and 60 min of LADCA occlusion (TCO), at reactive hyperaemia (about 3 min of reperfusion; RH), at 15 min of reperfusion (15'REP) and at the end of the protocol (120'REP)

	Baseline	Pre-TCO	45'TCO	60'TCO	RH	15'REP	120'REP
Heart rate (beats / min)							
Control	113±5	115±6	122±6	119±8	119±10	116±7	116±11
Pre-TCO	106±6	106±6	111±7	108±7	108±8	108±8	108±10
Pre-REP	105±8	106±8	109±8	103±8	108±6	111±6	114±9
MAP (mmHg)							
Control	92±5	89±4	88±5	89±6	86±6	83±7	89±6
Pre-TCO	99±4	91±2	87±3	87±4	81±5	88±6	97±6
Pre-REP	96±3	95±3	96±6	96±7	89±7	95±8	95±9
*Double product [(mmHg * bpm) / 100]*							
Control	120±12	118±10	110±12	99±10	88±8	96±11	106±10
Pre-TCO	123±9	114±5	115±7	111±8	103±10	110±9	122±14
Pre-REP	122±14	123±14	125±17	113±17	114±16	119±19	126±22
LV dP/dt_{max} (mmHg / s)							
Control	1944±139	1945±158	1767±197	1774±189	1876±219	1836±200	1848±217
Pre-TCO	1903±158	1991±123	1798±134	1705±114	1655±95	1568±108	1818±153
Pre-REP	2211±340	2126±356	1945±350	1942±264	1750±267	1795±328	1736±359
Cardiac output (ml / min / 10 kg)							
Control	997±70	996±82	937±99	959±174	929±107	942±117	855±105
Pre-TCO	851±77	884±76	883±130	839±97	802±110	963±104	792±104
Pre-REP	1127±110	1119±110	1053±105	958±83	912±70	932±99	749±93

MAP = mean arterial blood pressure; LV = left ventricular.

Data are reported as mean±SEM. For comparison of variables across time between the groups, a two factor analysis of variance (ANOVA) of repeated measures was used. When the ANOVA was significant, pairwise comparisons were made with the Student's t-test with the significance level for the p-values corrected with the Bonferroni method. For comparison across time in each of the individual groups, a one factor ANOVA of repeated measures was employed, with a post-hoc testing strategy identical to that of the two factor ANOVA. A two sample rank sum test (Mann-Whitney-U) was used to test the differences between two groups with regard to the risk region and the % of the risk region infarcted.

Results

Risk Region and Infarct Size

The risk region and infarct size are depicted in figure 2. The risk regions were virtually identical in all groups. The risk region was intentionally kept small (10% - 15% of left ventricular wall volume), because in pilot studies larger risk regions were associated with an unacceptably high incidence of ventricular fibrillation. The percentage of the risk region infarcted was 77.7% (4.0%) for the controls. Starting drug treatment after 45 min of LADCA occlusion reduced infarct size by 51% [38.2(5.8%)]. Infusing HOE 694 before ischaemia reduced infarct size by 95% [4.1±(1.4%)]. The difference in infarct size between Pre-TCO and Pre-REP was significant ($p<0.05$).

Haemodynamics, wall function, and myocardial oxygen consumption (MVO_2)

The control pigs remained haemodynamically stable throughout the occlusion and reperfusion period and did not vary significantly from baseline with regard to heart rate, mean arterial pressure or double product.

Infusion of HOE 694 did not affect haemodynamics (table 1) or coronary blood flow (table 2), neither in the Pre-TCO group nor in the Pre-REP group.

Comparing baseline values with values assessed directly before occlusion in the Pre-TCO

Table 2

Coronary blood flow (CBF) and calculated coronary vascular resistance (CVR) during the protocol (* = $P < 0.05$ compared with control animals at the same timepoint (for time points, see Table 1)

	Baseline	Pre-TCO	45'TCO	60'TCO	RH	15'REP	120'REP
CBF (ml / min)							
Control	54±4	54±3	31±5	32±4	78±6	65±9	66±10
Pre-TCO	45±5	43±5	27±5	27±4	73±7	50±11	34±*
Pre-REP	51±7	50±7	34±7	31±6	66±8	70±11	54±11
*CVR (dyn * s * cm^{-5})*							
Control	123±11	116±8	226±35	212±24	78±9	100±14	109±16
Pre-TCO	163±18	160±19	283±53	248±37	82±11	152±32 *	263±56 *
Pre-REP	146±28	148±30	238±59	245±52	102±15	106±20	148±25

group, it was noted that none of the parameters changed significantly. Wall function (%ΔWT) in the subsequently ischaemic region did not change at all (table 3) and regional MVO_2 did not decline significantly (from 4.98(0.51) to 4.01(0.76) ml/min/100g).

During reperfusion the post-ischaemic depression of regional wall function (%ΔWT) was identical in all three groups (table 3), despite the significant differences in infarct size. In addition, after 120 min of reperfusion no differences with regard to global or regional cardiac performance were observed between the three groups.

Plasma levels of HOE 694

Table 4 summarizes the plasma levels of HOE 694 throughout the experiment. There was no difference in peak plasma levels following i.v. infusion between the two treatment groups (8.9(1.3) µg/ml vs. 9.7(1.6) µg/ml, n.s.). These values correspond to a plasma concentration of about 2.5 x 10^{-5} M with respect to a molecular weight of 360 for HOE 694, which is sufficient for a selective inhibition of the Na^+/H^+ exchanger.

Discussion

In the present study, we have investigated the effect of HOE 694, a specific inhibitor of the Na^+/H^+ exchanger, on ischaemic and postischaemic damage in anaesthetized pigs.

The major controversy regarding the nature of reperfusion injury is whether injury at the time of reperfusion merely represents an acceleration of damage that would in any case have occurred or whether there is a specific additional injury caused by reperfusion itself. Opie[19]

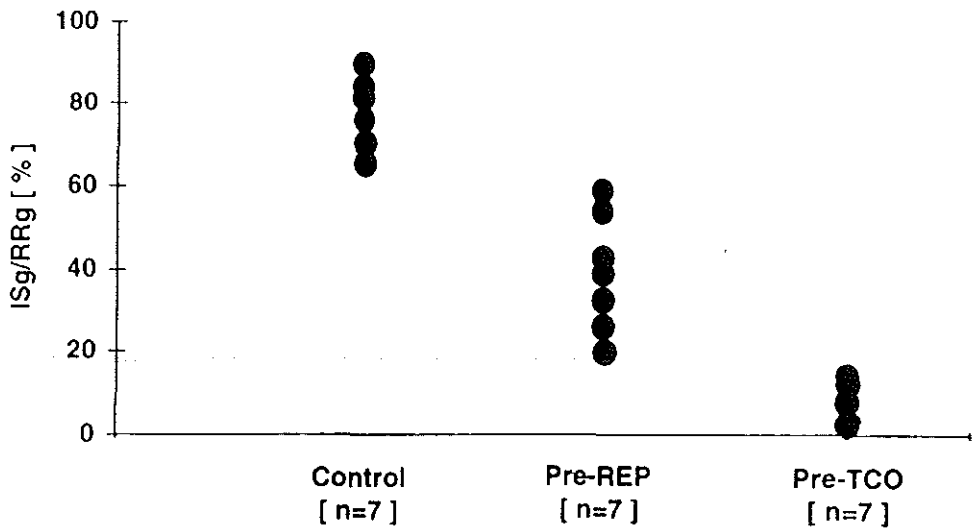

Figure 3. Infarct size in the individual animal in the control and HOE 694 treatment groups.

Table 3
Regional wall function (%Δ wall thickening) in the LADCA area subsequently made ischemic for 60 min (* $P < 0.05$ compared with control animals at the same timepoint; for time points, see Table 1)

	Baseline	Pre-TCO	45'TCO	60'TCO	RH	15'REP	120'REP
Control	32±4	33±5	−1±2	−2±2	0±2	−2±3	0±3
Pre-TCO	31±5	31±5	−2±2	−1±2	−2±3	−1±4	0±2
Pre-REP	38±4	39±3	−9±3 *	−7±3	−10±2 *	−6±3	−5±2

Table 4
Plasma levels of HOE 694 (μg/ml) throughout the experiment in the two treatment groups [the timepoints of sampling are identical to those in Table 1; 0 = below the limit of quantification (40 ng/ml)]

	Baseline	Pre-TCO	45'TCO	60'TCO	RH	15'REP	60'REP	120'REP
Pre-TCO	0	8.9±1.3	1.6±0.1	1.3±0.2	1.3±0.2	0.9±0.1	0.6±0.1	0.4±0.1
Pre-REP	0	0	0	9.7±1.6	4.1±0.6	1.8±0.1	0.8±0.1	0.4±0.1

emphasized that firm evidence for reperfusion injury would require proof that a reperfusion-associated event can be diminished in severity by an intervention given only at the time of reperfusion. In fact, inhibition of the Na^+/H^+ exchanger has been shown to prevent some reperfusion-associated events. HOE 694, when used during reperfusion, decreased the incidence of reperfusion arrhythmias[20 21] and myocardial stunning[20]. However, there is divided opinion whether these acute, transitory events can be regarded as markers of reperfusion injury. Jennings and colleagues[22] argued that "reperfusion injury should be restricted to mean cell death caused by reperfusion." In this way, our study is the first to show that selective inhibition of the Na^+/H^+ exchanger by HOE 694 during reperfusion limits irreversible cell damage and thereby reduces infarct size measured at the end of 120 min of reperfusion. Thus, this study provides evidence that reperfusion injury does exist.

HOE 694 has been characterized as a potent inhibitor of the Na^+/H^+ exchanger in erythrocytes, thrombocytes, bovine aortic endothelial cells, and in endothelial cells of porcine brain capillaries[23], with a high affinity for the NHE-1 isoform[24]. We administered 7 mg/kg/15 min of HOE 694 i.v., which is a dose resulting in plasma levels of Hoe 694 sufficient to limit reperfusion arrhythmias and stunning as shown in a pig model of two cycles of 10 min LADCA occlusion followed by 4 h of reperfusion[16]. In line with this pig study, HOE 694 did neither have an effect on haemodynamic parameters nor on cardiac contractility of our animals. This suggests that this agent is able to be cardioprotective without an effect on haemodynamic parameters. As the left ventricular ischemic regions were virtually identical and pigs are known to have no preexisting collaterals[25], any effect on infarct size in this model must be a direct drug action on the myocardium.

The precise way by which Na^+/H^+ exchange mediates reperfusion injury can not be deducted from our study. On one hand, while activation of the Na^+/H^+ exchange can restore intracellular pH following an acid load, the concomitant increase in Na^+ can aggravate existing

derangements of ionic homeostasis, particularly with respect to calcium overload[2], and may result in exacerbation and acceleration of tissue injury[18]. In addition, the reduced cellular Na^+ influx would require less Na^+ ions to be extruded by the Na^+/K^+ pump, with consequently less ATP consumption[15] and less generation of protons. On the other hand, the Na^+/H^+ exchange has been shown to participate in the activation of both platelets[26 27] and neutrophils[28], which are supposed to participate in reperfusion injury[29].

Less generation of protons during ischemia has been shown to be protective[30] and a longer preservation of normal pH has been stated to be at least in part responsible for the infarct size reducing effect of preconditioning in pig hearts[31]. E.g. changes in intracellular pH might influence the activity of phospholipases[32] or the opening probability of ATP-sensitive potassium channels[33]. Both are systems many authors believe to play a role in ischaemia-reperfusion injury.

Thus, the cardioprotective effect of HOE 694 was most pronounced when the drug was present prior to the ischaemic insult and during reperfusion. Inhibition of Na^+/H^+ exchange during ischaemia and reperfusion reduced infarct size by 95%, which is in the range of protection reported for ischemic preconditioning[34]. Furthermore, inhibition of Na^+/H^+ exchange in our model was more effective than e.g. a treatment with an ATP-sensitive potassium channel opener[35].

HOE 694 had a more pronounced protective effect when present during ischaemia and reperfusion compared with being administered during reperfusion only. This is in line with most other previous reports, in which Na^+/H^+ exchange inhibition was observed to be most protective when initiated before the ischaemic period[36 20]. The pronounced protective effect during ischaemia is surprising since the detrimental consequences of Na^+/H^+ exchanger stimulation should be confined to the time prior to the development of significant extracellular acidosis. Most recently Bugge and Ytrehus[37] reported in a isolated rat heart study that EIPA did not protect the heart when given only during reperfusion, but was protective when given prior to the onset of regional ischemia. This difference between the two rather specific Na^+/H^+ exchange inhibitors is most likely a dose effect, since we learned in pilot dose finding studies that 10 times higher dosages of a Na^+/H^+ exchange inhibitor are necessary to protect during reperfusion than during ischemia. While Na^+/H^+ exchange inhibitors of the HOE 694 type showed no side effects even in higher doses, Bugge & Ytrehus were limited to a certain dose to avoid the EIPA typical interactions with the sodium or calcium channels.

Our study implies that the action of HOE 694 during the first minutes of ischaemia is sufficient to protect the heart. This is supported by the observation that in early ischaemia intracellular Na^+ rises rapidly[38 39], which can, at least partially, be suppressed by the unspecific Na^+/H^+ exchange inhibitor amiloride[40]. Inhibition of Na^+/H^+ exchange may delay or prevent the rapid Na^+ loading in early ischaemia and thereby Ca^{2+} loading via the Na^+-Ca^{2+} exchanger.

Consequently, Hendrikx et al.[36] observed in isolated perfused rabbit hearts a marked delay in the onset of ischaemic contracture after HOE 694 treatment.

Interestingly, in our study we did not observe a difference in postischaemic regional wall function, despite the marked differences in infarct size. On one hand, acidification of the myocyte shuts down myocardial contractility[41] and thereby might save energy, while, on the other hand, activation of the Na^+/H^+ exchange induces an alkalinisation of the cell[42] thereby allowing to restore contractility. Blocking alkalinisation by Hoe 694 might result in a longer lasting detoriation of contractility, although the myocyte is viable. Studies with longer reperfusion periods (< 12 h) would be necessary to address the question of the time course of recovery of myocardial function after treatment with a Na^+/H^+ exchange inhibitor.

Conclusions

Treatment with HOE 694 results in a marked reduction in infarct size. This beneficial effect is best explained by the decrease in Na^+ influx during ischaemia as well as during reperfusion, resulting in less energy expenditure during ischaemia and a decrease in calcium overload during reperfusion in HOE 694-treated pig hearts.

Thus, in contrast to many other interventions, Na^+/H^+ exchange inhibition provides a pronounced infarct size reducing effect even when only administered post-ischaemically. We propose, therefore, that activation of the Na^+/H^+ exchanger contributes to reperfusion injury and that inhibition of the exchanger will be of benefit in reduction of such injury.

The protective properties of specific Na^+/H^+ exchange inhibitors offer substantial clinical promise for the use of these agents as adjunct therapy in numerous reperfusion protocols and for the prevention of myocardial infarction.

Acknowledgments

We wish to thank Matthias Schranz, Renate Siegel and Werner Trumpfheller for excellent technical assistance with the animal preparation and Claus Fuchs for help in the data analysis.

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Chapter 8

Summary

In this thesis are represented the results of our studies on ischemic preconditioning and on drugs supposed to interact with its mechanisms. Murry et al[1] termed 1986 a phenomenon "ischemic preconditioning", which described the effect of short ischemic episodes on infarct size following a sustained occlusion of the coronary artery. In their dog model infarct size was 29% in control animals subjected to a 40 minutes coronary artery occlusion, but only 7% when the 40 minutes occlusion was preceded by 4 cycles of 5 minutes coronary artery occlusion and 5 minutes of reperfusion. These results implied that brief ischemia, which does not lead to myocardial damage by itself, is able to trigger an endogenous mechanism, which conditions the myocardium to better tolerate ischemia.[2]

In chapter 2 we extended the observations by Murry et al. to the pig heart. In pigs with nearly identical risk regions, preconditioning with two episodes of ten minutes ischemia, each followed by 30 minutes of reperfusion, reduced infarct size from 48% of the control myocardium subjected to 60 minutes of coronary artery occlusion to 10%. This nearly 80% reduction of infarct size did neither accrue from a recruitment of collaterals nor from energy savings due to concommitant myocardial stunning.

Since then it has been shown that preconditioning exists in every species in which it has been investigated including man, and research has focused on evaluating the modalities of ischemic preconditioning.[3] It became apparent that ischemic preconditioning can be stimulated not only by ischemia, but by e.g. heat stress,[4] myocardial stretch,[5] rapid ventricular pacing,[6] and transient occlusion of a mesenteric or renal artery.[7] The variety of stimuli may point towards a common end effector, which could be the ATP-sensitive potassium channel. Gross and coworkers showed in various experiments in dogs, that blockade of ATP-sensitive potassium channels blocks the protection afforded by ischemic preconditioning independent of whether the stimulus was ischemia,[8] an adenosine-mimetic,[9] or acetylcholine![10] If the ATP-sensitive potassium channels would be the common end effector, it would be the target to pharmacologically induce preconditioning protection.[11]

Using the ATP-sensitive potassium channel opener bimakalim, we could show in pigs that bimakalim is able to reduce infarct size nearly to the same extent as ischemic preconditioning and found first evidence, that the peripheral vasodilatory effect observed with our cardioprotective dose was not necessary for its protective effects. An observation, which was substantiated by experiments using another opener of ATP-sensitive potassium channels, U-89232, which is almost completely devoid of vasodilatory properties. The protective effects of all three, ischemic preconditioning, bimakalim, and U-89232, could be blocked when the selective antagonist of ATP-sensitive potassium channels, glibenclamide, was infused (chapters 3 to 5).

With the increasing body of knowledge about ischemic preconditioning in well defined experimental settings, the question rises whether ischemic preconditioning is clinically relevant.[12] When a great variety of stimuli can precondition the heart, the heart of a patient frequently suffering from angina pectoris attacks should be preconditioned all the time. Cohen et al[13] showed that this might not be the case, since he reported that conscious rabbits can become tolerant to multiple episodes of ischemia and lose the preconditioning protection. When the rabbits experienced a 5-minute coronary occlusion and 10 minutes of reperfusion before a 30 minute coronary artery occlusion, they were protected, but not if the sustained ischemia was preceded by 40 to 65 five-minute occlusions during a 3- to 4-day period. If an ischemia-free interval of 2.5 to 3 days was interposed, protection was again evident. Their observation is consistent with results obtained in anesthetized rats,[14] dogs,[15] and pigs.[16] From a clinical point of view, these results implicate that stimulation by ischemia may not be sufficient to achieve protection all the time.

In chapter 6 we could not only confirm some of the previously made observations in our pig model, but, by applying bimakalim, we could completely reinstall the protection, whith has been lost with ischemic preconditioning and could only in part be re-established by a second preconditioning cycle of 10 minutes of ischemia and 15 minutes of reperfusion. These results add to the clinical importance of potassium channel openers as they allow the reinstatement of the cardioprotection by a pharmacological approach at a timepoint when this is not possible by ischemic preconditioning. Future studies should also investigate the effectiveness of potassium channel openers, when myocardium has become unresponsive to ischemic preconditioning due to a larger number of sequences of brief ischemia and reperfusion.[13] It is quite feasible that in such studies the potassium channel opener, because of its pharmacodynamic properties, should be applied during the occlusion-reperfusion sequences.

From a mechanistic point of view, these results implicate that, although ischemic preconditioning provides a very potent means to protect the heart, its efficacy can pharmacologically be optimized.

To investigate the role of the Na^+-H^+ exchanger in our pig model of ischemia and reperfusion, we used its specific inhibitor HOE 694.[17] HOE 694, when applied prior to 60 minutes of ischemia, reduced infarct size to the same extent as did ischemic preconditioning (chapter 7). The profound cardioprotective potential of Na^+-H^+ exchange inhibition has been confirmed by several investigators in pigs,[18] rabbits,[19] and rats.[20] However, we were not able to investigate an effect of HOE 694 additive to ischemic preconditioning, since the protection afforded by ischemic preconditioning in our pig model was so profound that an additive effect was not measurable.

Most investigators observed cardioprotection only when the Na^+-H^+ exchange inhibitor was applied prior to the onset of ischemia. Interestingly in our study, the application of HOE 694 at the onset of reperfusion significantly limited infarct size, while administration of an ATP-sensitive potassium channel opener at that timepoint is ineffective (e.g. see chapter 3). This effect of Na^+-H^+ exchange inhibition during reperfusion appears to be highly dose dependent, as Klein et al[24] did not observe protection with a dose of 3 mg/kg of HOE 694, while we observed protection with a dose of 7 mg/kg.

The in our study observed additional benefit during treatment only upon reperfusion, which cannot be observed with potassium channel openers, favors the idea that Na^+-H^+ exchange inhibitors can be clinically very valuable to optimize the protection afforded by ischemic preconditioning and reperfusion protocols. However, more experimental studies are necessary to elucidate the possible synergistic role of ATP-sensitive potassium channel openers and Na^+-H^+ exchange inhibitors in the treatment of acute myocardial infarction.

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Chapter 9

Samenvatting

Dit proefschrift is samengesteld uit een aantal studies over ischemische preconditionering, waarbij zijn inbegrepen studies met farmaca die ischemische preconditionering nabootsen en waarbij de acties van de farmacon mogelijk kunnen bijdragen tot het begrijpen van de mechanismen die bij ischemische preconditionering zijn betrokken. Murry en medewerkers[1] zijn de eersten geweest die de term ischemische preconditionering gebruikten toen ze waarnamen dat in genarcotiseerde honden de grootte van het infarct na een 40 minuten durende afsluiting van een kransslagader was afgenomen van 29% tot 7%, wanneer die 40 minuten durende kransslagader afsluiting werd voorafgegaan door 4 cycli van 5 minuten durende afsluiting van de betreffende kransslagader en 5 minuten reperfusie. Downey[2] concludeerde dat het hart in staat was onherstelbare schade door langdurig zuurstofgebrek te beperken door een endogeen mechanisme dat getriggerd werd door korte perioden van zuurstofgebrek, die op zichzelf niet tot onherstelbare schade leiden.
In hoofdstuk 2 is aangetoond dat ischemische preconditionering niet alleen bij het hondenhart maar ook bij het varkenshart optreedt. Om tot ischemische preconditionering te komen werden in genarcotiseerde varkens 2 cycli van 10 minuten durende kransslagader afsluiting en 30 minuten reperfusie gebruikt. Infarctgrootte na een 60 minuten durende kransslagader afsluiting was na ischemische preconditionering 10% tegen 48% in varkens die niet gepreconditioneerd waren voordat de kransslagader voor 60 minuten werd afgesloten. Omdat varkens in tegenstelling tot honden geen coronair collateralen hebben, kon de laatste als een factor die bijdroeg aan de verminderde infarctgrootte worden uitgesloten. Besparing van de energiebehoefte van het hart, ten gevolge van het optreden van stunning na de ischemische preconditionering, was een andere factor die kon worden uitgesloten.
Sindsdien is aangetoond dat ischemische preconditionering optreedt in elke species waarin het fenomeen is onderzocht. Dit geldt ook voor de mens. Onderzoek was naast het vinden van het mechanisme gericht op het vinden van andere stimuli die het hart konden beschermen[3]. Een aantal van deze stimuli zijn inductie van heat shock eiwitten door thermische stress[4], stretch[5], ventrikel pacen[6] en kortdurende afsluiting van arterien die andere organen dan het hart van bloed voorzien.[7] Deze verscheidenheid van stimuli zou erop kunnen duiden dat deze stimuli een gemeenschappelijk doelwit hebben. Activatie van ATP afhankelijke kalium kanalen zou zo'n gemeenschappelijke target kunnen zijn. Gross en medewerkers hebben aangetoond dat blokkade van deze kalium kanalen de bescherming door ischemische preconditionering opheffen ongeacht welke preconditioneringsstimulus werd gebruikt[8-10]. Wanneer deze kalium kanalen inderdaad zo'n gemeenschappelijk eindpunt is van de cascade van processen die het fenomeen bewerkstelligen, dan is het mogelijk het fenomeen ook farmacologisch na te bootsen[11]. Door gebruik te maken van de ATP afhankelijke kalium kanaal opener bimakalim werd aangetoond dat in varkens de infarctgrootte bijna evenveel was afgenomen als na ischemische preconditionering wanneer een kransslagader gedurende 60 minuten werd

afgesloten. Eigenschappen als vasodilatatie van de coronair en perifere bloedvaten speelden hierbij geen rol. Deze bevindingen zijn ondersteund door gebruik te maken van een andere kalium kanaal opener, U-89232, een farmacon dat effectief was zonder dat er vasodilatatie optrad. Behandeling met glibenclamide, een blokker van de kalium kanalen voorkwam zowel de bescherming door ischemische preconditionering als dat van de beide farmaca (hoofdstukken 3 en 5).

Aangezien het hart beschermd kan worden door een groot aantal stimuli rijst de vraag of ischemische preconditionering klinisch relevant is[12]. Het is namelijk heel goed mogelijk dat harten van patienten met kransslagader aandoeningen bijna continu beschermd worden door deze stimuli. In dat geval zal toediening van een farmacon nog nauwelijks de bescherming kunnen verhogen, tenzij via een geheel ander mechanisme. Er zijn aanwijzingen dat harten niet altijd beschermd worden door ischemische preconditionering. Zo hebben Cohen en onderzoekers[13] aangetoond dat wakkere konijnen resistent worden voor ischemische preconditioneringsstimuli, wanneer deze met een hoge frequentie worden aangeboden. Dus wanneer konijnen werden blootgesteld aan een enkele 5 minuten kransslagader afsluiting en 10 minuten later die kransslagader voor 30 minuten werd afgesloten, waren de harten beschermd. Dit bleek echter niet het geval wanneer 40-65 van die stimuli werden aangeboden over een periode van 3 tot 4 dagen. Na een ischemische-vrije periode van 2.5 tot 3 dagen was het weer mogelijk harten te preconditioneren. Deze waarnemingen zijn in overeenstemming met waarnemingen in genarcotiseerde ratten,[14] honden[15] en varken[16]. In hoofdstuk 6 is aangetoond dat op een moment dat het in genarcotiseerde varkens niet mogelijk is de harten volledig te beschermen door een nieuwe ischemische preconditioneringsstimulus het toch mogelijk is de harten te beschermen met behulp van bimakalim. Dit resultaat draagt dus bij aan het klinisch belang van ATP afhankelijke kalium kanaal openers, aangezien het met deze farmaca mogelijk is het hart te beschermen, wanneer dit niet kan door toediening van een nieuwe ischemische preconditioneringsstimulans. Toekomstige studies zullen moeten nagaan of de kalium kanaal openers ook nog effectief zijn wanneer harten resistent zijn geworden door een groot aantal stimuli over een korte periode toe te dienen, zoals in de proeven bij Cohen en medewerkers.[13]

Vanuit een mechanisch standpunt betekent dit dat de bescherming door ischemische preconditionering geoptimaliseerd kan worden met behulp van farmaca. Dit is nog verder onderzocht door gebruik te maken van de natrium-waterstof exchange inhibitor HOE 694 in het genarcotiseerde varkensmodel.[17] Het bleek dat HOE 694 even effectief was als ischemische preconditionering in het beperken van de infarctgrootte. De bescherming door beide stimuli was zo groot dat het niet mogelijk was om na te gaan of er sprake kan zijn van een synergistisch effect. De resultaten van onze experimenten zijn in overeenstemming met de bescherming beschreven door anderen in in vivo en in vitro modellen.[18-20] In deze studies werd

bescherming alleen waargenomen wanneer de inhibitor voor het begin van de ischemische periode werd toegediend. In de huidige studie was HOE 694 ook effectief, hoewel minder, wanneer het werd toegediend bij het begin van de reperfusie. Het is van belang op te merken dat de kalium kanaal opener in onze studies ook alleen effectief was wanneer het voor het begin van de 60 minuten ischemie werd toegediend (zie hoofdstuk 3). De reden voor de effectiviteit tijdens reperfusie kan gelegen zijn in de dosis die gebruikt werd (7 mg/kg) aangezien Klein en medewerkers[24] geen bescherming tijdens reperfusie vonden met een dosis van 3 mg/kg. De eigenschap dat toediening tijdens reperfusie ook nog bescherming kan bieden, geeft een duidelijke meerwaarde voor de natrium-waterstof exchange inhibitoren en geeft aanleiding tot verdere studies onder andere het nagaan van een synergetische actie van ATP afhankelijke kalium kanaal opener en natrium-waterstof exchange inhibitoren.

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Acknowledgements

Many thanks go to all the people who contributed directly and indirectly to this thesis during the past years.

I am specially grateful to my promotor Prof.dr. P.D. Verdouw and co-promotor Prof.dr. P. Schelling, who provided me with a challenging opportunity and an all-time available expertise.

Prof.dr. A.J. Man in 't Veld, Prof.dr. P.R. Saxena and dr. J.W. de Jong are thanked for reviewing the manuscript and their suggestions.

Special thanks are dedicated to dr. L.K. Soei and drs. S. de Zeeuw for accepting to be my 'paranymphs'.

I thank dr. I. Lues, dr. C.-J. Schmittges, and prof. W. Schaper for creating working environments, which allowed me to succeed.

I am grateful to dr. K.-H. Becker for teaching a "youngster" how to manoeuvre in a drug company.

Very warm-hearted thanks are given to mr. H. Weygandt for his very close collaboration and outstanding commitment during all the years. Many times his great technical expertise found solutions, where many others would have failed. Thanks also to the "cardioprotection lab team", namely M. Barthelme, C. Fuchs, A. Kaufmann, M. Schranz, R. Siege and W. Trumpfheller for their technical support.

Thanks to all the colleagues at Merck, especially drs. K.-O. Minck, Z. Fotev, S. Bernatat-Danielowski and at the Max-Planck Institute, especially drs. R. Schott and E. Braun for their omnipresent help and for all the fun we had.

Curriculum vitae

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From 1981 on I studied medicine at the University of Mainz, Germany. In 1987 I obtained my M.D. (Approbation) and received my doctorship in clinical medicine (Promotion).

In the years 1988 and 1989 I worked as a post-doc stipendiate of the Max-Planck Gesellschaft at the Max-Planck-Institute of Cardiovascular Research, Bad Nauheim, Germany (director: Prof. W. Schaper).

From 1990 on I spent two years at the II Medical Clinic of the University of Mainz (director: Prof. J. Meyer), learning internal medicine and clinical cardiology and heading the clinical research group "infective endocarditis" under the supervision of Prof. R. Erbel.

Since 1992 I am working at Merck KGaA, Darmstadt, Germany. From January 1992 until April 1995 I was responsible for cardiovascular research laboratories, dealing mostly with studies in larger animals concerning acute myocardial ischemia and infarction.

In April 1995 I became a member of the corporate strategic marketing department as a product manager for the first ATP-sensitive potassium channel opener on the market, nicorandil, and as a marketing evaluation manager for new cardiovascular products to be developed at Merck KGaA.