MYOCARDIAL PRECONDITIONING IN SWINE

MYOCARDIAL PRECONDITIONING IN SWINE

MYOCARDIALE PRECONDITIONERING IN VARKENS

Proefschrift

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Contents

aan mijn ouders
en aan Ron

Chapter 1

Introduction and aim of the thesis

The rhythmic contraction of the heart pumps blood to the peripheral organs and tissues of the body and provides them with oxygen and nutrients. The energy that is required by the heart for basal metabolism (20%), electromechanical coupling (5%) and contraction (75%) is derived from energy rich phosphate bonds as in adenosine triphosphate (ATP). Since the heart has limited stores of ATP, its constant regeneration from adenosine diphosphate (ADP) is required. ATP can be generated either aerobically (oxidative phosphorylation via the tricarboxylic acid cycle, high yield) or anaerobically (via glycolysis, low yield). Anaerobic glycolysis can at best provide 5-10% of the ATP requirements under basal conditions, so that the heart must rely principally on oxidative phosphorylation for ATP generation. Under basal conditions the heart already extracts 70-80% of the coronary arterial oxygen content. Consequently, reductions in coronary blood supply will result in almost equivalent reductions in oxygen supply. Clinically a decrease in oxygen supply can result from narrowing of a proximal coronary artery segment, vasospasm, thrombus formation, or a combination of these factors. The consequences of an acute decrease in oxygen supply include 1) diminished contractile function, 2) cardiac arrhythmias, and 3) myocardial cell death (infarct). Onset and occurrence of myocardial cell death depends on factors such as species, residual flow via collateral vessels and oxygen demand at the onset of occlusion. Myocardium can, therefore, be salvaged anywhere between one hour (swine, rabbit) and three hours (dogs, humans) after the onset of occlusion. To salvage jeopardized myocardium it is mandatory to reinstate blood flow to the heart (reperfusion), but understanding and subsequent manipulation of factors that affect the progression of myocardial cell death could delay infarction so that more myocardium is salvaged when reperfusion is achieved.

The concept of infarct size limitation[1,2] has its basis in early human autopsy studies which reported that myocardial infarcts were significantly smaller than the myocardial region supplied by the occluded coronary artery,[3-5] and in experimental studies reporting that brief (up to 20 min) periods of ischemia are tolerated without development of necrosis.[6] Fuelled by emerging concepts of the determinants of infarct size, of which collateral blood flow and energy utilization of the ischemic myocardial segment were the most important, many pharmacological studies were undertaken in an attempt to limit infarct size. Early experimental studies were often able to show an infarct size limiting effect of a drug, but later studies could not reproduce these results when more rigorous investigative methods were used. Consequently, myocardial reperfusion is clinically the only infarct-size reduction therapy currently in use.

Experimental interest in the concept of infarct size limitation was revived in 1986 when Murry et al[7] first described the phenomenon "Ischemic Preconditioning" when they observed that multiple brief ischemic episodes separated by brief periods of reperfusion reduced infarct size produced by a sustained period of myocardial ischemia, despite an increase in total ischemia time. More specifically they found that in dog hearts infarct size (defined as the ratio of infarcted area and the anatomical area at risk) was reduced from 29% to 7%, when a 40 min

total coronary artery occlusion was preceded by four sequences of 5 min occlusion and 5 min of reperfusion. These findings could not be explained by enhanced recruitment of coronary collateral blood flow. However, during the 40 min total coronary artery occlusion depletion of high energy phosphates in the preconditioned animals was slower than in the control animals. Interestingly, infarct size limitation was lost when the duration of the coronary occlusion was extended to three hours, indicating that preconditioning only *delayed* myocardial necrosis[7] and therefore could only enhance myocardial salvage when reperfusion was reinstated.

Since the classical study of Murry et al a vast number of studies exploring the phenomenon of ischemic preconditioning has been published. These studies can be divided into two groups: 1) Descriptive studies (e.g. what stimuli produce preconditioning?, how long does the protective effect last? and 2) Mechanistic studies (mediators and cellular mechanisms of preconditioning). For example, it is now clear that the duration of the protective effect of ischemic preconditioning is limited in time,[8] but can be reinstated after an ischemia-free period.[9,10] A classical preconditioning stimulus can be described as a single brief coronary artery occlusion. This approach necessitates an intervening reperfusion before the sustained occlusion for the preconditioning stimulus to be effective (as it would otherwise be a sustained occlusion period). Ovize et al[11] showed that a partial coronary artery occlusion could be equally effective in producing a protective effect although intermittent reperfusion was still necessary. Until now most studies have focused on manipulating the supply side of the myocardial oxygen balance to obtain preconditioning. However, evidence is emerging that an increase in oxygen demand can also produce preconditioning. Thus, a short period of ventricular pacing protected the heart against the arrhythmogenic effect of a longer coronary artery occlusion.[12] Also, an acute increase in left ventricular filling pressure by volume loading was shown to decrease infarct size.[11] Early mechanistic studies showed that myocardial stunning was not the mechanism underlying preconditioning as the protective effect of a brief coronary artery occlusion had dissipated at a time when impaired contractile function was still present.[8] Downey and co-workers made the first important step toward revealing the mechanisms of ischemic preconditioning, when they described the role of adenosine in mediating the phenomenon.[13] Other mediators/mechanisms of preconditioning that are currently under investigation are K^+_{ATP} channels, adrenergic receptors, muscarinic receptors and protein kinase C. Despite the tremendous wealth of information on preconditioning that has emerged in the last decade, the exact mechanism of preconditioning and particularly its clinical relevance are still incompletely understood.

Experimental animal

All experiments in this dissertation have been performed in domestic pigs (25-35 kg), which were anesthetized throughout the experimental protocol. Several reviews have emphasized the value of the pig in cardiovascular research.[14] For studies on myocardial preconditioning the pig

model poses several advantages over other, particularly smaller, animal models. First, the size of the heart is large enough to monitor regional contractile function and to allow myocardial tissue sampling to monitor oxidative metabolism during the experimental protocol and to accurately determine infarct size in two layers of the left ventricle. Thus, the protective effect of a preconditioning stimulus can be related to reduction in myocardial contractile function and to the metabolic state of the myocardium. Second, the anatomy of porcine coronary circulation is quite similar to that of man in that there are no collaterals between the perfusion areas of the three main coronary arteries. Consequently, infarct size variability due to variable collateral blood flow, which is common in species like the dog, is likely to be minimal in pigs. Furthermore, the absence of a significant collateral circulation allows the study of ischemic preconditioning by partial flow reductions in the pig. Partial flow reductions are easy to monitor and to adjust on line with Doppler flow measurements on the supplying coronary artery. The presence of a coronary collateral circulation can perturb the results of such studies as flow measurements on the supplying coronary artery do not correctly represent myocardial flow.

AIM OF THE THESIS

In this thesis, the results of our studies on ischemic preconditioning in domestic pigs are presented. The rationale for these studies stems from several earlier observations.

First, it has been well established that in dogs the relation between infarct size (IS) and the anatomical area at risk (AR) is linear but not proportional, i.e. the regression line describing the relation between IS and AR is linear but has a positive intercept on the AR-axis.[15] Such a relation implies that the IS/AR ratio, which is the index commonly used to express infarct size, is not a constant but depends on AR. In the dog, this intercept can be explained, at least in part, by collateral blood flow. If a positive intercept would also be present in a collateral deficient species like the pig, this could severely limit the use of IS/AR in assessing infarct size and myocardial protection. Consequently, we determined the relationship between the anatomical area at risk and the infarcted area in the pig.

Second, it is well established that most of the protective effect of a brief period of ischemia is lost when the reperfusion period separating the brief and sustained coronary artery occlusion exceeds two hours.[7] The relation between the extent of protection by ischemic preconditioning and the duration of the intervening reperfusion period is not well established, although some data suggest that the protective effect disappears gradually and quite early in the intervening reperfusion period.[16] We studied the time course of the protective effect of a brief period of ischemia to determine the time window of protection and whether protection wears off gradually or disappears abruptly.

Third, the necessity of a period of reperfusion between the brief and sustained total coronary artery occlusions to elicit ischemic preconditioning is self-evident; otherwise the duration of the sustained coronary artery occlusion will be merely increased. It is less obvious, however, whether a partial coronary artery occlusion can lead to ischemic preconditioning without the need for an

intervening reperfusion period. If myocardium can be preconditioned with a partial coronary artery occlusion, the question naturally arises whether this protection is equal for the inner and outer halves of the myocardium as it is well established that a coronary flow reduction affects perfusion of the epicardial layers less dramatically than perfusion of the endocardial layers with consequently more severe ischemia in the latter. Thus we assessed the transmural distribution of infarct size after a coronary artery was occluded for 60 min immediately following a period in which coronary blood flow was reduced to a fixed percentage of baseline.

Fourth, Ovize et al[17] have shown that stretch produced by acute volume loading precondition the myocardium without the need for ischemia suggesting that ischemia is not obligatory for the induction of preconditioning. We have extended these observations and investigated whether other non-ischemic stimuli are also capable to precondition the myocardium.

Finally, although there is much experimental evidence for the existence of ischemic myocardial preconditioning, there is still no direct proof that ischemic preconditioning exists in man, when infarct size is taken as endpoint. The reason that the clinical relevance of ischemic preconditioning remains incompletely understood is several-fold. First, in man infarct size is usually assessed from enzyme leakage. Although enzymes such as creatine phosphorase-MB are relatively cardiac specific, estimates are rather inaccurate compared to the measurements in the animal laboratory. Second, it is next to impossible to determine the duration of a coronary artery occlusion as neither the onset of occlusion nor the time that reperfusion starts can be determined accurately. Multiple episodes of reversible ischemia are not uncommon in man, however, and those induced by repeated stress tests, and balloon inflations during angioplasty are even fairly reproducible. These interventions could be tools to investigate whether the human myocardium can adapt to ischemic stress. We have reviewed the literature in order to find evidence for the occurrence of ischemic preconditioning in humans.

Brief description of the content of the thesis

In chapter two the effects of multiple intermittent episodes of brief coronary occlusions on regional myocardial function were studied. The multiple occlusion-reperfusion sequences do not lead to myocardial necrosis, but reversible abnormalities in myocardial contractile performance were observed. In this model the effects of the anti-anginal drug trimetazidine were studied to determine if this compound alters the time course of the regional myocardial function responses to the brief occlusion-reperfusion sequences.

In chapter three the techniques used to identify and quantify the area at risk and delineate viable from necrotic myocardium are presented. In addition the methodological and physiological determinants of infarct size are discussed. Infarct size is usually expressed as the ratio of the infarcted area and the area at risk. Such an index is only valid if this ratio is independent of the area at risk, i.e. infarcted area and area at risk are proportional. To assess if the relationship is indeed proportional, data are presented for both control and preconditioned animals in which the site of occlusion was varied to produce variable areas at risk.

In chapter four the time course of the protective effect of a single 10 min total coronary artery occlusion was studied to determine the duration of preconditioning and to determine whether the protective effect dissappears gradually or abruptly. For this purpose the duration of the intervening reperfusion period that separated the 10 min preconditioning period and the ischemic period was varied between 15 and 240 min.

In view of the clinical relevance of preconditioning the question arose if it was possible to trigger preconditioning with a partial coronary artery occlusion without intermittent reperfusion before the onset of the sustained occlusion, as in many patients thrombus development may give rise to severe ischemia before the artery is totally occluded. This was studied in chapter five in which a 30 min 70% flow reduction preconditioning stimulus immediately preceded the sustained ischemia episode.

In chapter six we further explored the myocardial preconditioning afforded by a partial occlusion immediately preceding the sustained ischemic period without an intervening period of reperfusion. Specifically, we determined whether the protection afforded by a partial occlusion depended on the severity and the duration of the flow reduction and whether the magnitude of the protection was inhomogeneously distributed across the left ventricular wall. The latter is not unlikely, as a partial coronary artery occlusion compromises perfusion of the endocardial layers more severely than perfusion of the epicardial layers, causing different degrees in the severity of ischemia. It could thus be that a partial occlusion would result in protection in the inner but not in the outer layers of the myocardium.

In chapters four, five and six brief periods of ischemia were applied to study myocardial protection. In chapter seven we employed rapid ventricular pacing as a preconditioning stimulus and studied its effects on myocardial infarct size. Specifically, periods of ventricular pacing with and without an intervening period of spontaneous sinus rhythm prior to the sustained ischemic period were studied. In addition, the role of myocardial ATP-sensitive K^+-channel activation and induction of myocardial ischemia in the protection afforded by ventricular pacing were determined.

In chapter 8, we will review the literature for evidence that adaptation to ischemic stress and ischemic preconditioning occurs in man. Chapters nine (English) and ten (Dutch) contain a general discussion and summary of this thesis.

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Chapter 2

Intracoronary trimetazidine does not improve recovery of regional function in a porcine model of repeated ischemia

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SUMMARY

We evaluated the effect of trimetazidine (TMZ) on recovery of regional cardiac function in anesthetized open-chest pigs, subjected to fifteen two-minutes occlusions of the left anterior descending coronary artery, separated by two minutes of reperfusion and a 120 min recovery period. Regional myocardial function was evaluated by sonomicrometry-derived segment lengthening and the area enclosed by the left ventricular pressure-segment length loop (external work EW) in animals, which received either an intracoronary infusion of TMZ (33 $\mu g.kg^{-1}.min^{-1}$, $n = 6$) or saline (1 $ml.min^{-1}$, $n = 7$), starting 15 min before the first occlusion and ending 2 min after the fifteenth occlusion. In addition, myocardial malondialdehyde production to evaluate oxygen free radical production, oxygen consumption and the ATP, ADP and AMP content as well as the energy charge were determined at regular time intervals.

In control pigs the sequences of occlusion-reperfusion did not affect systemic hemodynamics, except for the $LVdP/dt_{max}$, which decreased by 11% during the interventions and did not recover during the following reperfusion period of 2 hours (78% of baseline, $p < 0.05$). Systolic segment length shortening and EW were increased at the end of the first occlusion-reperfusion cycle, decreased gradually during the remainder of the occlusion-reperfusion periods and did not improve during the recovery period. Energy charge and myocardial blood flow were not impaired, but oxygen consumption was decreased during the recovery period. The malondialdeyde data did not provide evidence for production of oxygen free radicals. TMZ decreased $LVdP/dt_{max}$ by 6% ($p < 0.05$) and caused a twofold increase in post-systolic segment shortening ($p < 0.05$) before the first occlusion, but did not influence the hemodynamic responses, the changes in regional cardiac function and the metabolic events produced by repetitive regional ischemia.

Key words. trimetazidine, myocardial ischemia, reperfusion, regional myocardial perfusion and function, external work, oxygen free radicals, high energy phosphates

INTRODUCTION

The effectiveness of the 1-(2,3,4-trimethoxybenzyl)-piperazine dihydrochloride, trimetazidine in increasing the ischemic threshold during exercise tolerance testing without obvious changes in hemodynamic parameters has led to further investigations concerning the mechanisms by which the drug exerts its action [1-3]. A number of properties, mainly demonstrated in isolated organs and tissues, have been identified that could explain the cardioprotective activity of trimetazidine: 1) reduced intracellular acidosis during ischemia [4,5], 2) accelerated adenine nucleotide rephosphorylation [4,6], 3) decreased membrane content of peroxidized lipids [7], 4) diminished cellular oedema and preservation of oxydative phosphorylation during reperfusion. The latter two arguments indicate a reduction in oxygen free radical production [8].

Kober et al [9] demonstrated recently that intracoronary administration of trimetazidine delayed the development and reduced the magnitude of ECG-changes in patients undergoing percutaneous transluminal coronary angioplasty (PTCA) during balloon inflation. In this study it was not investigated whether this was also accompanied by an improved recovery of regional cardiac function after reperfusion was reinstated. Several studies suggest that in particular the diastolic function shows an impaired recovery after repeated brief (2-4 min) coronary artery occlusions [10,11].

In the present study, we, therefore, investigated the effects of trimetazidine on the recovery of regional cardiac function after multiple two-minutes-occlusions separated by two minutes of reperfusion, mimicking a clinical PTCA protocol. Since other studies [7,8] have indicated that trimetazidine or its metabolite(s) may reduce the formation of oxygen free radicals, a potential mechanism for myocardial stunning [12], we also determined myocardial malondialdehyde (MDA) production to investigate whether formation of oxygen free radicals plays a role in this model.

METHODS

Surgical procedure

After an overnight fast, cross-bred Landrace x Yorkshire pigs of either sex (25-32 kg) were sedated with 25 $mg.kg^{-1}$ ketamine (A.U.V., Cuijck, The Netherlands) i.m., anesthetized with 18 $mg.kg^{-1}$ pentobarbital (Sanofi, Paris, France) i.v., intubated and connected to a respirator for intermittent positive pressure ventilation with a mixture of oxygen and nitrogen. By adjusting respiratory rate, tidal volume and the fraction of oxygen in the inspired mixture, arterial blood gases and pH were kept within the normal limits. The temperature of the animal was kept around 37°C by using an electrical blanket. Anesthesia was maintained with a continuous infusion of pentobarbital (10-15 $mg.kg^{-1}.h^{-1}$, i.v.). Saline and haemaccel (Behringwerke A.G., Marburg, Germany) were infused to maintain the fluid balance via catheters, inserted into the external jugular vein. Catheters were also positioned via both femoral arteries into the descending aorta for measurement of central aortic blood pressure and withdrawal of blood samples for determination of blood gases and malondialdehyde concentrations. A 7F Sensodyn microtransducer catheter (B. Braun Medical B.V., Uden, The Netherlands) was advanced into the left ventricle (LV) via the carotid artery to monitor left ventricular pressure and its first derivative (LVdP/dt). Following administration of 4 mg pancuronium bromide (Organon Teknika B.V., Boxtel, The Netherlands) i.v., a midline thoracotomy was performed, the left mammary vessels were ligated and the second and third left ribs were partly removed to facilitate additional instrumentation. An electromagnetic flow probe (Skalar, Delft, The Netherlands) was placed around the ascending aorta and the left atrial appendage was cannulated for injection of $1\text{-}2.10^{6}$

microspheres, 15 ± 1 (SD) μm in diameter (NEN Company, Dreieich, Germany), labelled with either ^{95}NB, ^{103}Ru, ^{113}Sn, ^{46}Sc or ^{141}Ce, to determine regional myocardial blood flow using the reference blood sample technique. For the measurement of regional segment shortening by sonomicrometry (Triton Technology Inc., San Diego, CA, USA) ultrasonic crystals (Sonotek Corporation, Del Mar, CA, USA) were positioned in the layers of the left ventricle subendocardium, covering the interventional area as well as a control area. The proximal left anterior descending coronary artery (LADCA) was dissected free to apply intermittent occlusions with an atraumatic clamp and a proximally placed electromagnetic flow probe. Distal to this site a small cannula (0.8 mm outer diameter) was inserted into the LADCA for the administration of either trimetazidine or saline. The vein accompanying the LADCA was cannulated for the determination of coronary venous blood gases, malondialdehyde and hemoglobin concentrations. At the end of the experiment the interventional area was identified by an intra-atrial injection of methylene blue after the LADCA had been occluded again. The pigs were then sacrificed with an overdose of pentobarbital, the heart was excised and processed to determine regional myocardial blood flow.

Experimental procedure and data analysis

After surgical preparation the pigs were allowed to stabilize for 45 minutes. Prior to the intervention protocol, which consisted of 15 sequences of 2 min occlusion and 2 min reperfusion, pigs either received an intracoronary infusion of saline or trimetazidine (33 $\mu g.kg^{-1}.min^{-1}$) at a rate of 1 $ml.min^{-1}$, which started 15 min before the first occlusion and continued until the end of reperfusion 15 (at 2 min of the final reperfusion period), which was then followed by two hours of recovery. At scheduled intervals recordings were made of systemic hemodynamic variables and regional myocardial function, arterial and coronary venous blood samples were drawn to quantify oxygen consumption and malondialdehyde production, biopsies were taken for the determination of high energy phosphates and microspheres were injected to obtain regional blood flow data.

From the segment length tracings were calculated (Fig. 1) (i) regional systolic segment shortening as SS(%)=100 x (EDL - ESL)/EDL, in which EDL and ESL are the segment length at end-diastole and end-systole respectively, (ii) regional post-systolic segment shortening (PSS) as PSS(%)=100(%) x (ESL - minL)/EDL, in which minL is the segment length at maximal contraction, (iii) normalized regional velocity of contraction (VC) as $VC(mm.s^{-1})$ = 10(mm) (EDL-ESL/EDL)/$t_{systole}$ in which $t_{systole}$ equals the duration of left ventricular ejection and (iv) the relaxation time (t_{50}) as the duration of the period (ms) in which 50% of total segment lengthening was achieved. Left ventricular pressure and myocardial segmenth length were digitized (sample rate 250 Hz) using an 8 bits AD-converter (Tiepie Engineering, Leeuwarden, The Netherlands) on an IBM PC-computer to calculate the area enclosed by the pressure-segment length loop over a full cardiac cycle [13], which is an index of regional myocardial work [14].

High energy phosphates were measured in transmural myocardial biopsies, taken with a Tru-Cut needle (Travenol Laboratories Inc., Deerfield, Illinois, USA) from the intermittent ischemic area and the adjacent control area. Biopsies were immediately dipped into 0.9% NaCl at 0^0C to remove adherent blood, frozen in liquid nitrogen (within 10 s) and stored until analysis at -80^0C [15].

MDA formation was measured in blood samples which were placed on ice directly after sampling, centrifuged at 0^0C to obtain plasma and stored at -80^0C until analysis. The

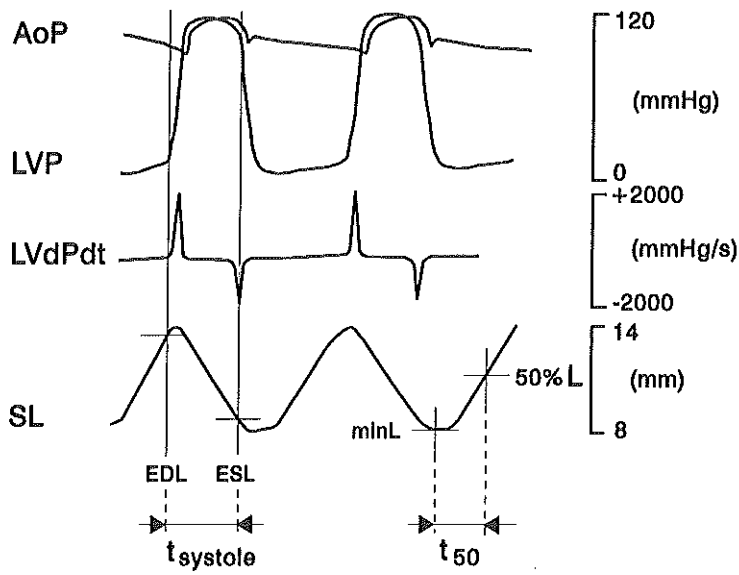

Figure 1 Calculation of regional segment function data from the segment length tracings. AoP = aortic pressure; LVP = left ventricular pressure; LVdPdt = first derivative of LVP; SL = segment length; EDL = end-diastolic length; ESL = end-systolic length; minL = minimum length (at maximal contraction); 50%L = length at 50% relaxation; tsystole = duration of left ventricular ejection; t50 = time-period in which 50% of the relaxation is achieved.

MDA content of the plasma samples was measured as previously described [16].

Oxygen consumption (MVO_2) and MDA production were calculated as the product of regional transmural myocardial blood flow and the difference in the arterial and coronary venous contents. Energy charge was calculated as (ATP + 0.5 ADP)/[ATP + ADP + AMP] as defined by Atkinson [17].

Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was performed by use of a parametric two-way analysis of variance (randomized block design), followed by the Duncan new multiple range test. Statistical significance was accepted at $p < 0.05$ (two-tailed).

Drugs

Except for the anesthetic drugs, only trimetazidine (courtesy of Dr C. Harpey, I.R.I.S., Courbevoie, France) dissolved in isotonic saline was used.

RESULTS

Systemic hemodynamics

Two min after the last occlusion, $LVdP/dt_{max}$ had decreased to 85-90% of the preocclusion

value, but none of the other cardiovascular variables were significantly affected (Table 1). During the following 15 min, there was an additional decrease in $LVdP/dt_{max}$ to 80% of its preocclusion value ($p<0.05$), not followed by any sign of recovery. Hence, at the end of the recovery period, the values of all variables except for that of $LVdP/dt_{max}$ were similar to those observed before the first occlusion.

Infusion of trimetazidine did not alter heart rate, mean arterial blood pressure, cardiac output and left ventricular end-diastolic pressure, but decreased $LVdP/dt_{max}$ by 6% ($p< 0.05$). The drug did not modify the changes in the hemodynamic variables during the occlusion-reperfusion protocol. During the recovery period, in constrast with the untreated animals, there was a complete recovery of $LVdP/dt_{max}$ in the trimetazidine-treated animals (Table 1).

Regional myocardial wall function

In the untreated animals, the first occlusion caused a complete loss of systolic segment shortening (SS) of the myocardium perfused by the LADCA, but there was a complete recovery during reperfusion (Fig. 2). As a matter of fact SS was even slightly increased to above baseline. During the subsequent occlusion-reperfusion cycles there was again a complete loss of function during occlusion, while recovery became only partial during reperfusion. Consequently, two minutes after the fifteenth occlusion SS had decreased from 18 ± 2% at baseline to 13 ± 2% ($p < 0.05$) and further declined to 9 ± 1% ($p<0.05$) during the following 15 min. There were no further changes in SS during the remainder of the reperfusion period. Post-systolic shortening (PSS) was absent at baseline and also after the first occlusion-reperfusion sequence, but gradually increased during the following occlusion-reperfusion sequences (4 ± 1% after the fifteenth sequence). There were no further changes in PSS during the recovery period. The changes in the velocity of systolic shortening were parallel to those in SS, because heart rate and the duration of systole did not change. The relaxation time was not affected by the first occlusion-reperfusion sequence, but gradually shortened during the following sequences, to be prolonged again during the 120 min recovery period. In the adjacent control region SS decreased from 17 ± 1% at baseline to 13 ± 1% two min after the last occlusion period and did not change during the ensuing recovery period of 120 min. PSS never became significantly different from zero and the changes in the velocity of shortening again paralleled those in SS, while relaxation time was not affected.

Intracoronary infusion of trimetazidine had no significant effect on SS, neither before and during the occlusion-reperfusion periods, nor during the 120 min recovery period (Fig. 2). The same was true for velocity of shortening and the relaxation time. PSS, however, increased from 1 ± 0.3% to 4 ± 0.4% ($p < 0.05$), which was neither enhanced by the repetitive occlusions, nor attenuated during the recovery period.

External work

In the untreated animals, the index reflecting the external work performed by the myocardium perfused by the LADCA (231 ± 31 mmHg.mm at baseline) decreased to virtually zero during the occlusions, but was unchanged at the end of the first, fifth, tenth and fifteenth reperfusion. During the following 120 min recovery period there was a decline to 151 ± 18 mmHg.mm ($p<0.05$) after 15 min, which did not improve during the remainder of the observation period (140 ± 16 mmHg.mm after 120 min).

Trimetazidine administration tended to decrease external work in the intervention region (231 ± 33 mmHg.mm at baseline and 217 ± 8 mmHg.mm after 10 min intracoronary infusion, $p = 0.16$). During the following occlusion-reperfusion sequences, the changes in the trimetazidine

animals resembled closely those in the untreated animals (199 ± 38 mmHg.mm after the last occlusion-reperfusion cycle and 178 ± 36 mmHg.mm after 120 min of recovery).

Myocardial blood flow

Baseline values of the untreated animals and the trimetazidine-treated animals of the myocardium perfused by the LADCA were very similar (158 ± 13 $ml.min^{-1}.100g^{-1}$ and 160 ± 18 $ml.min^{-1}.100g^{-1}$, respectively). In either group of animals, transmural blood flow and its distribution over the subendocardial and subepicardial layers were not different from baseline, both at 15 min and 60 min, of the recovery period (not shown). There were also no changes in the perfusion of the myocardium outside the distribution area of the LADCA (not shown).

Oxygen consumption of the myocardium perfused by the left anterior descending coronary artery

Fifteen minutes after the last of 15 sequences of occlusion and reperfusion, myocardial oxygen consumption (MVO_2) of the LADCA-perfused myocardium was reduced from 486 ± 31 $\mu mol.min^{-1}.g^{-1}$ to 326 ± 44 $\mu mol.min^{-1}.g^{-1}$ ($p< 0.05$) and did not fully recover during the following 60 min of reperfusion (394 ± 20 $\mu mol.min^{-1}.g^{-1}$, $p< 0.05$). Trimetazidine did not modify this pattern, as MVO_2 had decreased from 467 ± 48 $\mu mol.min^{-1}.g^{-1}$ to 362 ± 86 $\mu mol.min^{-1}.g^{-1}$ ($p< 0.05$) at 15 min of reperfusion in the treated animals and was only 372 ± 27 $\mu mol.min^{-1}.g^{-1}$ after 60 min of reperfusion.

Myocardial high energy phosphate levels

In the untreated animals, myocardial ATP content after the first five occlusion-reperfusion sequences (28 ± 2 µmol/g protein) was not different from the values measured at baseline (28 ± 3 µgmol/g protein), but during the following ten occlusion-reperfusion sequences there was a gradual decrease to 23 ± 2 µmol/g protein ($p<0.05$). There was no recovery during the first 15 min of reperfusion (20 ± 2 µmol/g protein).

In the trimetazidine-treated animals (32 ± 2 µmol/g protein at baseline) a similar pattern in the changes in ATP content was observed (29 ± 3, 26 ± 3 and 27 ± 3 µgmol/g protein, at the end of the fifth and fifteenth occlusion-reperfusion sequence and after 15 minutes of recovery, respectively).

For both groups of animals, the total adenine nucleotide levels followed the same pattern as the ATP levels. CP levels had increased significantly ($p<0.05$) from 37 ± 7 µmol/g protein at baseline to 63 ± 14 µmol/g protein after the fifth occlusion-reperfusion cycle in the untreated animals and from 37 ± 4 µmol/g protein at baseline to 62 ± 9 µmol/g protein in the treated animals ($p<0.05$). In both groups there was a slight decline during the recovery period (to 54 ± 9 µmol/g protein and 57 ± 10 µmol/g protein in the untreated and treated animals at 15 min of reperfusion, respectively). The energy charge (0.91 ± 0.09 at baseline for both groups) was not significantly affected during the reperfusion periods.

Myocardial malondialdehyde production

In neither the untreated animals (0.02 ± 0.09 $nmol.ml^{-1}$, 0.02 ± 0.12 $nmol.ml^{-1}$ and 0.04 ± 0.08 $nmol.ml^{-1}$ at baseline, 2 min and 10 min after the last occlusion respectively) nor in the trimetazidine treated animals (-0.02 ± 0.07 $nmol.ml^{-1}$, -0.08 ± 0.02 $nmol.ml^{-1}$ and -0.05 ± 0.08 $nmol.ml^{-1}$ at baseline, 2 min and 10 min after the last occlusion, respectively) did we observe any significant production of MDA.

Table 1 Systemic hemodynamic effects induced by 15 consecutive cycles of 2 min occlusion and 2 min reperfusion in untreated (C, n = 7) and with trimetazidine-treated (n = 6) open-chest anesthetized pigs. Trimetazidine infusion (33 $\mu g.kg^{-1}.min^{-1}$) started 15 minutes prior to the first occlusion and lasted until 2 min after the last occlusion.

				recovery after last occlusion (min)			
		Pre-trimetazidine	Pre-occlusion	2	15	60	120
HR	C	111 ± 7	112 ± 7	109 ± 5	108 ± 6	108 ± 7	111 ± 8
		105 ± 7	104 ± 8	106 ± 6	106 ± 5	108 ± 5	104 ± 6
MAP	C	97 ± 4	98 ± 3	94 ± 5	93 ± 6	91 ± 2	98 ± 6
		91 ± 3	92 ± 3	85 ± 2	84 ± 2°	82 ± 2*°	83 ± 5
$LVdP/dt_{max}$	C	2180 ± 190	2360 ± 160	2070 ± 110	1850 ± 170*	1840 ± 180*	1890 ± 210*
		2250 ± 250	2090 ± 210*	1950 ± 210	1880 ± 230*	2000 ± 310	2480 ± 300
LVEDP	C	14 ± 1	14 ± 1	14 ± 1	14 ± 1	13 ± 1	13 ± 1
		12 ± 2	13 ± 2	14 ± 2	14 ± 2	14 ± 2	12 ± 2
CO	C	2.6 ± 0.3	2.6 ± 0.2	2.5 ± 0.2	2.5 ± 0.2	2.4 ± 0.3	2.3 ± 0.2
		2.3 ± 0.2	2.5 ± 0.3	2.5 ± 0.2	2.5 ± 0.2	2.4 ± 0.2	2.2 ± 0.3
SVR	C	40 ± 3	40 ± 4	39 ± 3	38 ± 3	44 ± 5	46 ± 6
		40 ± 3	38 ± 4	35 ± 2	34 ± 2	36 ± 3	39 ± 4

Trimetazidine infusion (33 µg/kg/min) started 15 minutes prior to the first occlusion and lasted until 2 minutes after the last occlusion.
HR = heart rate ($beats.min^{-1}$); MAP = mean arterial blood pressure (mmHg); $LVdP/dt_{max}$ = maximal rate of rise of left ventricular pressure ($mmHg.s^{-1}$); LVEDP = left ventricular end-diastolic pressure (mmHg); CO = cardiac output ($l.min^{-1}$); SVR = systemic vascular resistance ($mmHg.min.l^{-1}$). Data have been expressed as mean ± SEM. * $P<0.05$ versus baseline; ° $P<0.05$ versus trimetazidine

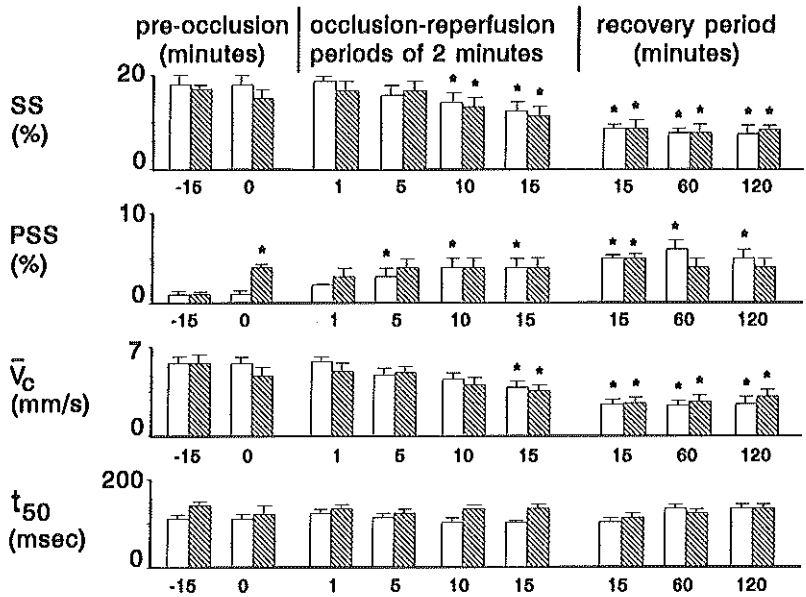

Figure 2 Effect of 15 consecutive occlusion-reperfusion periods of 2 minutes each in untreated (open columns, n=7) and trimetazidine-treated (hatched columns, n=6) animals on segment length changes (SS), post-systolic segment length changes (PSS), velocity of contraction (V_C) and relaxation time (t_{50}). Data are shown from 15 minutes and 0 minutes before the first occlusion and from the end of the first (1), fifth (5), tenth (10) and fifteenth (15) occlusion-reperfusion period and from 15 minutes (15), 60 minutes (60) and 120 minutes of reperfusion during the recovery period. Trimetazidine was administrated before and during the occlusion-reperfusion periods. * $p < 0.05$ versus minus 15 minutes (-15) before the first occlusion. Data are presented as mean ± s.e. mean.

DISCUSSION

The model used in the present study mimicks the clinical PTCA in so far that multiple short lasting coronary artery occlusions separated by 2 min of reperfusions were used. In the clinical setting the effects of PTCA on myocardial wall motion are not well defined as the duration and number of occlusions may vary considerably. A further complicating factor may be that in experimental animals, hearts are used which have not been submitted to myocardial ischemia before, while in the clinical setting the myocardium has usually experienced several episodes of myocardial ischemia and may already have an abnormal wall motion, and possibly, metabolism because of the presence of an existing chronic obstruction. Hence, phenomena such as ischemic preconditioning and hibernation may be confounding factors in clinical investigations. It is therefore not surprising that Jaski et al. [10] reported that after 4-6 balloon inflations each lasting less than 1 min, both systolic and diastolic wall motion returned to normal within 5 min, while Wijns et al. [11], reported an impaired diastolic function in the presence of a normal systolic

function following a similar study protocol involving a larger number of occlusions. It therefore appears that diastolic function may be more sensitive to multiple shortlasting occlusions than systolic function.

In the present study, systolic segment shortening completely recovered during the first reperfusion period. As a matter of fact there was even a slight increase in regional myocardial function and external work, which is consistent with earlier observations [18]. This may be, but is not necessarily [19] related to the hyperemic response. The aforementioned study [18] also showed that after such a shortlasting occlusion, stunning does not develop when perfusion is maintained. In the present study, myocardial stunning gradually developed during the following occlusion-reperfusion sequences and was most pronounced after reperfusion was established for 15 min. Our observation that regional wall function completely recovered after the first occlusion, but became impaired during the following episodes of ischemia-reperfusion appears to contradict clinical studies, which suggest, using electrocardiographic and metabolic abnormalities, that the first occlusion is the most damaging [20].

Kober et al. [9] ignoring the first occlusion, observed that electrocardiographic abnormalities were similar during the second and third occlusion in patients undergoing PTCA. When trimetazidine (6 mg) was administered intravenously before the third occlusion, the signs of ischemia were less during balloon inflation. Using a study protocol in which demand ischemia developed rather than supply ischemia as in the study of Kober et al. [9], Sellier et al. [1] and Dalla-Volta et al. [2] also showed less evidence of ischemia based on electrocardiographic abnormalities. The present study failed to demonstrate an effect of trimetazidine on recovery of function during reperfusion following repeated brief coronary artery occlusions. This does not necessarily contradict the observation by Kober et al. [9], considering the poor relation between functional and electrocardiographic changes [21].

Myocardial oxygen consumption (the product of coronary blood flow and difference in arterial and coronary venous oxygen contents) was not affected by trimetazidine, which is in accordance with the concept that trimetazidine does not influence the hemodynamic variables determining myocardial oxygen demand, but it contrasts the suggestion that trimetazidine enhances the utilization of the available oxygen [4]. In this in vivo model, also at variance with the results found in isolated rat heart [4] and in isolated myocardial mitochondria [8], high energy phosphates were not better preserved or restored in the presence of trimetazidine. Previously, it was shown that trimetazidine can reduce ischemia-induced acidosis in myocardium [4]. Development of acidosis is believed to contribute to the deregulation of myocardial Ca^{2+} homeostasis via a coupled operation of the Na^+/H^+ and the Na^+/Ca^{2+} exchangers [4,5]. In the present investigation, we did not study any variable reflecting tissue acidosis or Ca^{2+} overload and a possible occurrence of a beneficial effect of trimetazidine on tissue acidosis or Ca^{2+} overload can therefore not be excluded.

MDA is one of the endproducts of the reaction of endogenously generated free radicals with membrane polyunsaturated fatty acids, but its formation in the myocardium could not be consistently demonstrated in the coronary effluent during the repetitive occlusion-reperfusion periods in the present study. This could be due to the short duration of the occlusions (2 min) as we have demonstrated previously that MDA production occurred in anesthetized pigs which were subjected to 5 min coronary artery occlusions [16]. On the other hand, there are two studies which showed that trimetazidine attenuated oxygen free radical generation during ischemia-reperfusion [22,23]. It should be noted that in these studies, the most convincing evidence for free radical generation and an inhibitory effect of trimetazidine on this harmful process was

obtained using spintrapping agents such as N-tert-butyl alpha phenylnitrone and electron paramagnetic resonance spectroscopy (ESR) in liquid nitrogen frozen myocardial biopsies. In one study, the changes in the myocardial homogenate MDA content corresponded with the changes in ESR spectra [23]. In the present study, we determined MDA concentrations only in arterial and coronary venous blood, and no spintrapping agents for detecting the earliest products during oxygen free radical generation were used. We can therefore not make a definite statement, concerning the generation of oxygen free radicals in our study. Nevertheless, the impairment of regional wall function during reperfusion, which may be a result of oxygen-free radical induced damage [12], was not affected by trimetazidine in this particular model of ischemia and reperfusion.

The question may arise whether the lack of effect of trimetazidine is due to the experimental design, the choice of the experimental animal, the dose or route of the drug or a combination of these factors. The lack of any significant effect of trimetazidine on systemic hemodynamic variables is consistent with earlier observations using intravenous doses. It is unlikely that the intracoronary dose used in this study, was too low considering the intravenous dose used in the study by Kober et al. [9]. It is also unlikely that the duration of the administration of trimetazidine was the cause for the lack of effect on regional wall function, as no improved recovery was seen when the duration of trimetazidine administration was extended until the end of the 120 min recovery period (unpublished observations in 5 animals).

In conclusion trimetazidine, in this in vivo model, did not affect hemodynamic, metabolic and functional responses to fifteen repetitive short-lasting occlusions.

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Chapter 3

Determination and Determinants of the Area at Risk and Infarct Area

A prerequisite for the assessment of the protective effect of myocardial preconditioning on infarct size is accurate identification of the area of myocardial necrosis and the anatomical area at risk. In patients infarct size is estimated indirectly from the kinetics of the CPK plasma concentrations, electrocardiograms and radionuclide imaging techniques. In contrast, in experimental animals myocardial infarct size can be determined microscopically by quantitative histology. This technique is time consuming and a sharp delineation between viable and necrotic tissue can be made only 12 hours after the onset of ischemia. An alternative method is the use of a histochemical staining technique to visualize early myocardial infarcts macroscopically.

Identification of myocardial necrosis

Early studies reported that within hours after the onset of myocardial infarction plasma levels of several myocardial enzymes increase, which is associated with a decrease in enzyme levels in the infarcted area of the heart, as these enzymes leak out of dead myocytes. These observations led to the development of histochemical staining techniques to delineate between viable and necrotic myocardial tissue in the gross identification of early myocardial infarcts.[1-3] Using triphenyltetrazolium chloride (TTC), an oxidation-reduction indicator, it was possible to recognize infarcts 4 to 24 hours after the onset of coronary artery occlusion. Normal myocardium reduced TTC to a brick red formazan stain, whereas the infarcted tissue remained unstained.[2] Nachlas and Shnitka[3] used another tetrazolium salt nitroblue tetrazolium (NBT) to identify myocardial necrosis, which reportedly provided a better contrast between normal (dark blue) and necrotic (pale) myocardium than TTC staining. Moreover, myocardial infarction could be identified as early as 2 to 8 hours after the onset of coronary artery occlusion. Both, TTC and NBT have gained wide acceptance for infarct size determinations, each of which offers distinct advantages and disadvantages. For example, with NBT the demarcation between infarct and viable tissue appears more distinct than with TTC. On the other hand, TTC gains easy tissue access as it is able to permeate through the cell membrane and can thus be injected intravenously

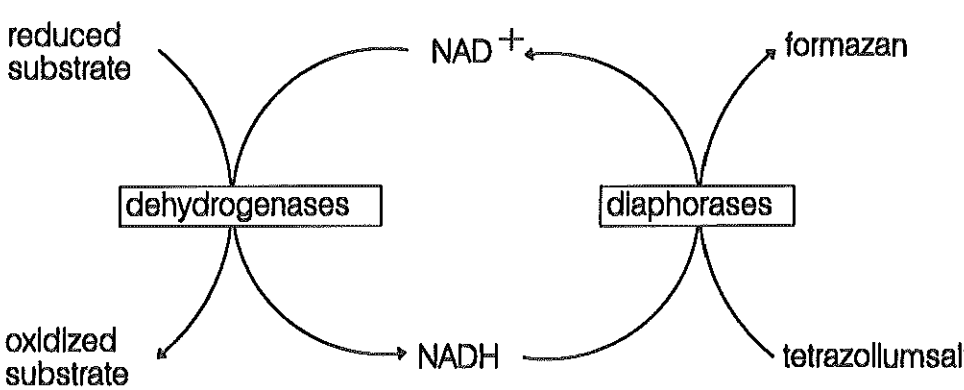

Figure 1 An enzymatic cycle as the basis of the NBT stain for identifying myocardial infarction

or into the coronary arteries. With NBT infusion is not possible because the compound cannot cross the cell membrane. It therefore has the disadvantage that it can only be applied to cut surfaces of cells.

The histochemical staining technique is based on an enzymatic cycling of oxidation-reduction reactions (Fig. 1). Tetrazolium salts are converted to the colored formazan by the uptake of 2 electrons and 1 proton with the help of membrane-bound diaphorases (enzymes of the respiratory chain) and NADH. The resultant NAD is reduced back to NADH by a dehydrogenase. Nachlas and Shnitka[3] explained the basic mechanism of the NBT staining by alterations in dehydrogenase activity. However, they also considered that loss of coenzymes and substrates may be of importance in early infarcts. Klein et al[4] showed that loss of the coenzyme NAD played a key role in identifying early myocardial infarction with NBT. In contrast to a 66% reduction in concentration of total NAD (sum of NAD and NADH) the activities of the tissue dehydrogenases had not changed after 4 hour of ischemia, while NBT staining revealed an infarction which could be made "viable" again (false-positive staining) by the addition of coenzymes. In older infarcts (more than 12 hours) loss of coenzymes is joined by marked decreased activities of dehydrogenases and diaphorases. Schaper et al[5] demonstrated that infarct size measured with para-NBT correlated very well with the size determined by histological examination. In another study Schaper et al[6] showed that no significant differences were found between infarcts which were reperfused for 48 hours and those reperfused for only 90 minutes. Schaper et al concluded that the short period of reperfusion produced washout of the necessary enzymes, subsequently shown to be the coenzymes by Klein et al[5]. Fujiwara et al[7] confirmed these findings as they observed that in pigs myocardial necrosis could be demarcated with NBT as early as 1 hour after the onset of reperfusion following a one hour coronary artery occlusion. If reperfusion was not allowed NBT staining needed 4-8 hours to obtain a clear demarcation between necrotic and viable tissue, which agrees well with the time reported by Nachlas and Shnitka[3]. These findings are also in accordance with the suggestion that reperfusion accelerates the washout of enzymes and coenzymes needed in the histochemical staining of viable myocardium.

Quantitative determination of the area at risk and infarct size

In this thesis hearts of open-chest pigs were subjected to a one hour total coronary artery occlusion to produce myocardial infarction of reproducible size (control animals). Two hours of reperfusion were allowed to enable reliable determination of infarct size with the NBT staining method. Preconditioning stimuli were given prior to the one hour occlusion and 2 hours of reperfusion.

To demarcate the area at risk perfused by the occluded artery from the control area the coronary artery was reoccluded at the former site of the coronary artery occlusion at the end of each protocol and 15 ml of 10 % (w/w) solution of fluorescein sodium (Sigma Chemical Co, St. Louis, USA) were injected into the left atrium to demarcate the area at risk by negative staining from fluorescein. After induction of ventricular fibrillation with a 9V battery the heart was

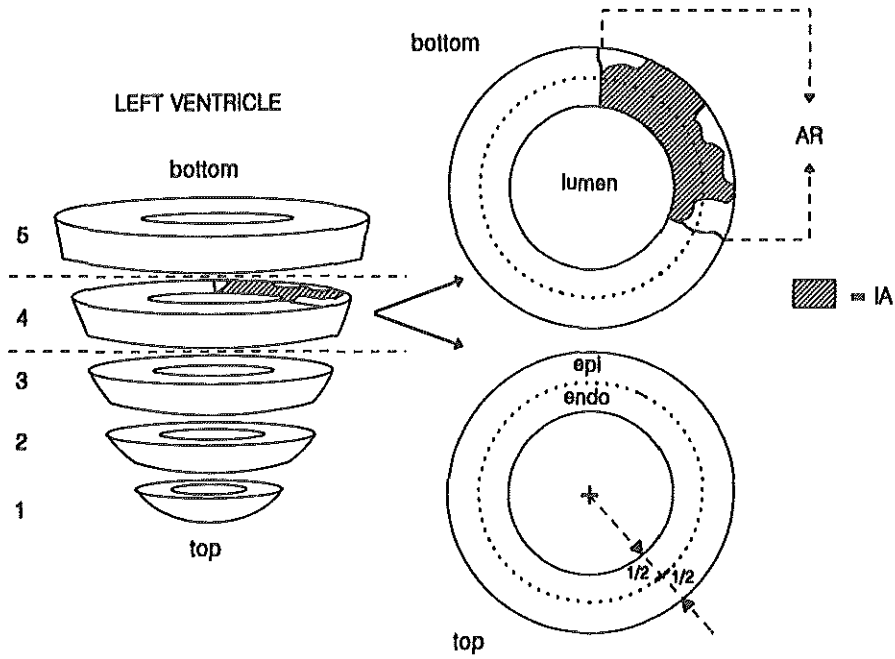

Figure 2 Segmental subdivision of the left ventricle, assessment of the risk area and the infarct area by fluorescein and NBT staining respectively and division of the left ventricular wall into endocardial and epicardial layers of equal thickness.

excised and both atria, the right ventricular free wall and the left ventricular epicardial fat were removed. The remaining left ventricle was filled with alginate impression material (BayerDental, Leverkusen, Germany) to preserve the shape of the left ventricle, cooled in crushed ice and sliced parallel to the atrioventricular groove into 5 segments (Fig. 2). The cut surface(s) of each segment and the demarcated areas at risk (AR) was (were) then traced on an acetate sheet under a black light. The 5 ventricular segments were subsequently incubated for 30 min in 0.125 g para-nitrobluetetrazolium (Sigma Chemicals Co., St. Louis, USA) per liter of phosphate buffer (pH 7.1) at 37°C, while the slices were turned upside down after 15 minutes of incubation for equal treatment of top and bottom side. As a result viable tissue was stained deeply blue and the non-stained pale infarcted tissue was traced on the acetate sheet. The tracings of the ring, risk area and infarct area were computed by planimetry. With the obtained data the risk area fraction (area at risk/area of ring) and the infarcted area fraction (infarcted area/area of ring) were calculated for the top and bottom of each ring and the mean value for each ring was multiplied by the weight. The weight of the risk area and infarcted area was summed and divided by the weight of the left ventricle to yield the fraction of the left ventricle at risk and the fraction of the left ventricle infarcted. In subsequent studies this method was extended to a further subdivision,

every traced segmental surface was subsequently subdivided into an endocardial and epicardial layer by adding a line that divided the myocardial wall into two layers of equal thickness. The endocardial, epicardial and total area were determined for the top and bottom of each segment. The endocardial and epicardial fraction of each segment were computed as the average of top and bottom. The endocardial fraction and the epicardial fraction of each segment were multiplied by the total weight of that slice to yield the endocardial and epicardial weight of the slice, respectively. The area at risk (fraction of ring area) and the infarcted area (fraction of ring area) were calculated separately for the endocardial, epicardial and total area for the top and bottom of each segment and averaged. These fractions were multiplied by the endocardial, epicardial and total weight of the segment to yield the weight of the endocardial, epicardial and total area at risk or infarcted. The endocardial, epicardial and total weights of the area at risk and the infarcted area of the 5 segments were summed separately and divided by the weight of the left ventricle to yield the endocardial, epicardial and total fraction of the left ventricle at risk and infarcted, respectively.

Determinants of infarct size

Duration of Occlusion. A major determinant of infarct size is the time during which the myocardium is deprived of blood flow. Thus, in dogs myocardium was salvageable up to three hours after the onset of coronary artery occlusion.[8-10] In swine this time window is much narrower as within 75 min most of the myocardium was irreversibly damaged.[10-13] This difference in time course between species is, at least in part, related to the amount of collateral blood flow available to the myocardium distal to the occlusion site. Thus, in dogs collateral blood flow can be as high as 0.4 ml/min/g,[14] whereas in pigs collateral blood flow is usually less than 0.07 ml/min/g.[10,13]

Area at Risk. Early canine studies of the effects of coronary artery occlusion on myocardial infarction used occlusions at an anatomically identical site, expressing infarct size as a percent of the left ventricle. However, variability in the pattern of distribution of terminal arterial branches can result in substantial variability of the myocardial mass perfused by the occluded arterial segment.[15] To take into account this anatomic variability in coronary vascular distribution, infarct size is generally expressed as a percent of the area at risk. However, several investigators[16-20] have shown that in both anesthetized and conscious dogs infarct area as percent of left ventricular mass (IA/LV) and area at risk as percentage of left ventricular mass (AR/LV) are highly linearly related, but that the regression has a positive intercept on the area at risk axis. Hence, the ratio between the infarct area and the area at risk is not a constant, but is dependent on the area at risk. Comparing the results of studies in which different areas at risk have been used may therefore lead to misinterpretation of the quantitative data.

To illustrate this problem the relationships between IA and AR of two groups of pigs presented in chapter 5 have been depicted in figure 3. One group of animals had a 60 min occlusion of the left anterior descending coronary artery at varying sites and 2 hours of

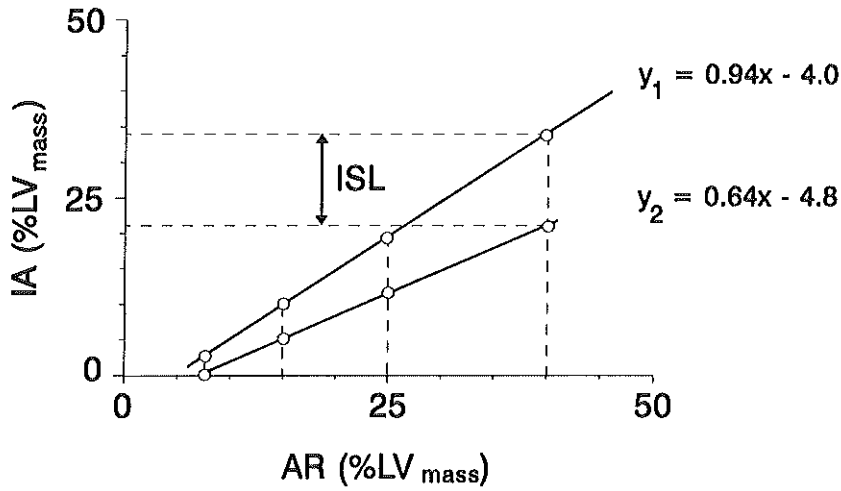

Figure 3 The linear regression lines (solid lines) for the control group (y_1 = 0.94x - 4.0) and a preconditioned group (y_2 = 0.68x - 4.8). The vertical dashed lines indicate that for the specified area at risk (AR, x) the effect of preconditioning can be calculated by substracting y_2 (infarct area (IA) of the preconditioned group) from y_1 (IA of the control group) to yield the infarct size limitation (ISL).

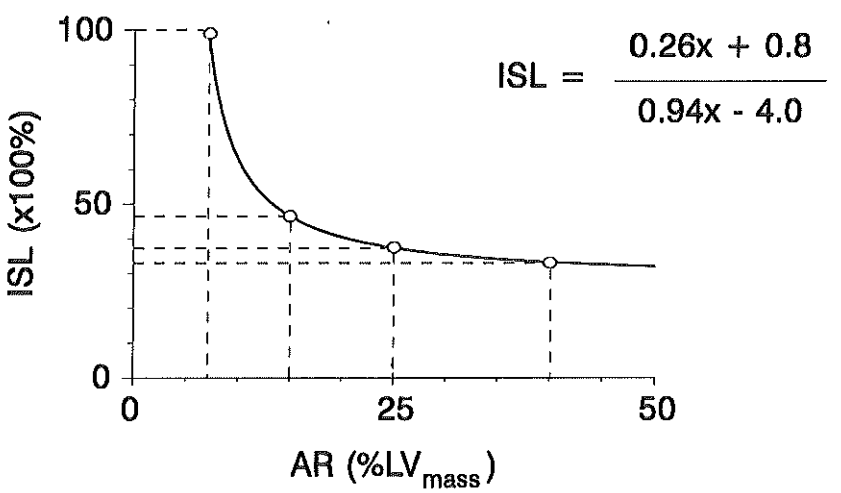

Figure 4 Relation between infarct size limitation (ISL) because of preconditioning and the area at risk (AR). For each AR infarct size limitation is calculated as $(y_1 - y_2)/y_1 \times 100\%$ in which y_1 and y_2 are the infarct area for the control and the preconditioned animals as predicted from the respective regression equations y_1 and y_2 (Fig 3).

reperfusion. The relationship between IA (y-axis) and AR (x-axis) can be described by the equation y = 0.94x - 4.0. The other animals had an intervention which preceded the 60 min coronary artery occlusion and which had a protective effect on the myocardium resulting in a smaller infarct area than could be predicted from the equation from the first group. The relationship for the intervention group could be described by the equation y = 0.68x - 4.8.

To determine the infarct size limitation (ISL) for the varying areas at risk within the intervention group its observed linear regression equation should be substracted from the linear regression equation for the control group and than divided by the linear regression equation for the control group to yield the salvaged myocardium for varying areas at risk as a fraction of the predicted dead myocardium if no protection had been applied. A new equation is generated which has been plotted as a funtion of the area at risk in figure 4. From this graph it can easily be seen that small variations in the low range of areas at risk can give rise to huge differences in infarct size limiting effects and thus may give rise to confusing results when comparing ratios between IA and AR only. When larger areas at risk are taken into account the infarct size limitation varies only slightly with an increase or decrease of the area at risk.

The reason for the positive intercept is unclear, but it is of interest that the value found in this thesis in control pig studies is very similar to the value for the intercept in the anesthetized dogs.[20] The possibility that infarct size development in small areas at risk progresses less rapidly than in larger areas at risk can therefore not be excluded. This has been suggested for the dog and been explained by its collateral flow. The latter is, however, absent in the pig. In conclusion the non-zero intercept of the regression equations requires a careful use of the infarct size-area at risk ratio because this ratio depends on the area at risk itself.

Collateral Blood Flow to the Area at Risk. In species with substantial and variable degrees of native collateral circulation such as the dog, the magnitude of collateral blood flow to the ischemic region exerts a potent effect on infarct size.[8,10,13,17,18,21,22,23] In species like rabbit and particularly pigs the collateral blood flow to the area at risk is negligible, which results in less infarct size variability.

Myocardial Oxygen Demand. The role of myocardial oxygen demand at the onset of occlusion as a determinant of infarct size is controversial. Both a positive correlation[10,24,25] and no significant relationship[14,15,24,26] between myocardial oxygen demand, estimated by the product of heart rate and LV systolic pressure, and infarct size as a percent of the area at risk have been reported in dogs. Studies of the influence of myocardial oxygen demand on infarct size in dogs are complicated by the effects of heart rate and blood pressure on collateral flow. Thus, an increase in heart rate can decrease collateral blood flow and redistribute collateral flow away from the subendocardium, while an increase in blood pressure can increase collateral blood flow by increasing the collateral driving pressure.[27] However, even when multiple stepwise linear regression was performed to assess the contribution of the rate-pressure product to infarct size, independent of its effects on collateral flow, the results were variable with both positive[24] and negative results.[23] In species with minimal collateral blood flow such as rabbit, infarct size

expressed as a percent of the area at risk was not significantly correlated with the rate-pressure product.[28] Garcia-Dorado et al[29] studied the effects of an increase in myocardial oxygen demand on infarct size expressed as a percent of the area at risk produced by a 60 min occlusion of the left anterior descending coronary artery in open-chest swine. Increasing the rate-pressure product by atrial pacing had no effect on infarct size, while increasing the arterial blood pressure caused a small increase in infarct size from 61% in the control group to 74% in the hypertensive group ($P<0.05$). The increase in arterial pressure was produced by intravenous infusion of the α_1-adrenergic receptor agonist methoxamine; this was associated with significantly elevated left ventricular end-diastolic pressure which might also have influenced infarct size. The myocardium ceases to contract early after onset of occlusion so that energy utilization is confined mainly to maintenance of ionic homeostasis and basal metabolism. Under these conditions the rate-pressure product may no longer reflect the energy demands of the ischemic myocardium.

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Chapter 4

Is myocardial infarct size limitation by ischemic preconditioning an "all or nothing" phenomenon?

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SUMMARY

The protective effect of a short lasting coronary artery occlusion on the development of myocardial necrosis during a subsequent longer lasting occlusion has only a limited duration ("ischemic preconditioning")[1,2]. It has not been established, however, how the magnitude of this protective effect changes with time during the reperfusion period following the preconditioning stimulus. Because this knowledge may help elucidating the mechanism underlying ischemic preconditioning we varied the duration of the reperfusion period following the preconditioning stimulus. Experiments were performed in pigs, a species in which the infarct size limiting effect of ischemic preconditioning has been demonstrated[3,4], while interpretation of the results will not be complicated by a variable residual myocardial blood flow after a coronary artery has been occluded, as functional coronary collaterals are absent.

MATERIALS AND METHODS

Fasted open-chest pentobarbital anesthetized pigs (28-32 kg) were randomly assigned to different experimental groups and instrumented as described earlier[5]. The control group (A) involved a 60 min coronary artery occlusion (CAO) followed by 120 min of reperfusion. In all other animals the 60 min CAO was preceded by a single 10 min CAO. In these animals the reperfusion period (R) between the 10 min and the 60 min CAO's was 15 min (B), 60 min (C), 120 min (D), 180 min (E), or 240 min (F). In each group the area at risk (AR) of the individual animals was varied by occluding the left anterior descending coronary artery or its branches at different sites. During the experimental protocol, continuous recordings were made of the ECG, systemic hemodynamic variables and systolic segment length shortening (SS), while needle biopsies for determination of ATP, ADP, creatine, creatine phosphate and energy charge were collected at intervals. At the end of the experimental protocol AR was identified and the infarcted area (IA) was determined[6].

RESULTS AND DISCUSSION

In the control group (A) SS decreased from 16 ± 1% to 2 ± 1% during the 60 min CAO. This loss in function was independent of AR. During the subsequent 120 min R there was no recovery of function (3 ± 1%). In these animals, IA was linearly related to AR (Figure 1). In the other groups the 10 min CAO also resulted in a loss of SS (from 19 ± 2% to 4 ± 2%) and a partial recovery (to 12 ± 1%) independent of the duration of R. In B and C the slope of the line describing the relation between IA and AR, was significantly reduced compared to the slope found for A, indicating infarct size limitation (Figure 1). In D and E the protective effect of preconditioning was lost in several animals, while still conspicuously present in others (Figure 1). Preconditioning was completely lost in all but one of the animals of F. IA/AR proved not to be related to any of the metabolic variables prior to 60 min CAO (or the change from baseline). In Figure 2 the lack of relation between ATP/ADP and IA/AR has been depicted (Figure 2).

Our working hypothesis was that the protective effect of preconditioning would decrease gradually as the duration of the reperfusion period between 10 min CAO and 60 min CAO was extended. Such an observation would be consistent with a decrease in the concentration of a substance (circulating or present in tissue) responsible for the infarct size limitation. Our data, however, did not reveal such a gradual decrease when the reperfusion period was extended to 120 min or longer. Our data may therefore point towards an "all or nothing" phenomenon, which suggest a different type of mechanism.

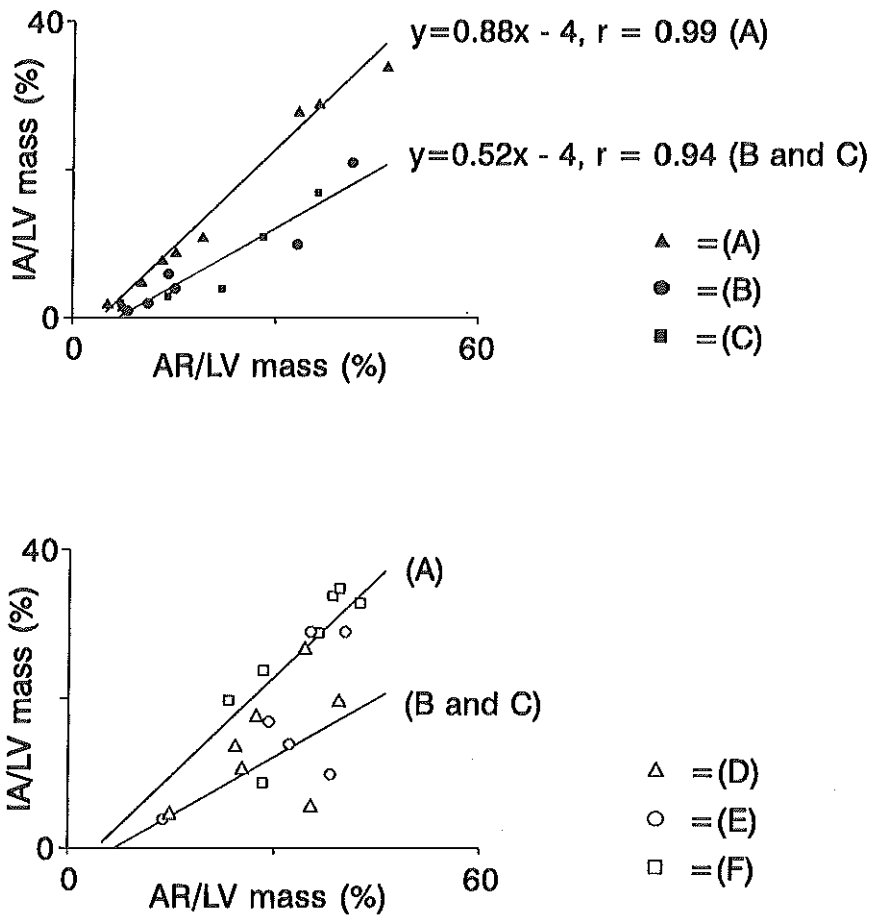

Figure 1 **Top:** The relationships between the infarcted area (IA and the area at risk (AR), both presented as percentage of the left ventricular mass, of the control animals (Group A), which had a 60 min coronary artery occlusion (CAO), and the preconditioned animals of Groups B and C, which had a preceding 10 min CAO followed by 15 min and 60 min reperfusion, respectively, have been depicted. **Bottom:** The regression lines for Group A and Groups B and C are shown, together with the individual data of the animals, which had a preconditioning stimulus followed by 120 min (D), 180 min (E), or 240 min (F) reperfusion.

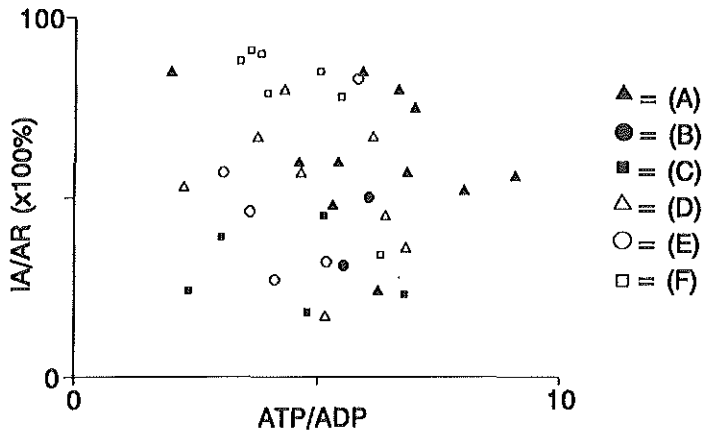

Figure 2 The relation between the infarcted area (IA) as percentage of the area at risk (AR) and ATP/ADP prior to the 60 min coronary artery occlusion (CAO). Data are from the control animals (A; 60 min CAO) and the animals in which the 60 min CAO was preceded by a 10 min CAO preconditioning stimulus followed by reperfusion periods of 15 min (B), 60 min (C), 120 min (D), 180 min (E) or 240 min (F).

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Chapter 5

Ischaemic preconditioning by partial occlusion without intermittent reperfusion

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SUMMARY

Objective: To investigate whether ischaemic preconditioning can be obtained by a partial coronary artery occlusion without intermittent reperfusion. *Methods:* In 7 anaesthetized open-chest pigs, the flow in the proximal left anterior descending coronary artery (LADCA) was reduced to 30% of baseline during 30 min before the vessel was occluded completely for 60 min (60 min TCO). After two hours of reperfusion (REP), the area at risk (AR) and infarct size (IS) were determined using standard procedures. Infarct sizes were compared to those observed in control animals (n = 12), which were subjected to 60 min TCO and two hours of REP, and to IS determined in animals preconditioned by 10 min TCO with either 15 min (n = 10) or 60 min (n = 5) of REP before the 60 min TCO and two hours REP. In the last 3 groups of animals, AR was varied by occluding the LADCA or its branches at different sites. *Results:* In the control animals IS was linearly related (r = 0.99, $p<0.001$) to AR with a positive intercept on the AR-axis: IS/LV_{mass} (x 100%) = 0.88 AR/LV_{mass} (x 100%) - 3.6. At comparable AR, the IS of the animals preconditioned with a 10 min TCO was less than for the control animals. For the animals preconditioned with 10 min TCO and 15 min REP, the relationship between IS and AR was again linear (r = 0.88) and had also a positive intercept on the AR-axis: IS/LV_{mass} (x 100%) = 0.68 AR/LV_{mass} (x 100%) - 4.8. All animals with the flow reduction to 30% of baseline immediately preceding the 60 min TCO had infarct sizes smaller ($p<0.05$) than predicted from the regression equation for the control animals, but the infarct size limitation could not be simply related to variables such as changes in regional systolic and post-systolic segment length shortening, ATP or ADP during the partial occlusion period. *Conclusion:* Myocardium can be preconditioned with a flow reduction to 30% of baseline for 30 min without intermittent reperfusion (two-stage Harris model). The positive intercept on the AR-axis of the IS-AR relation warrants caution of the use of IS/AR as an index for infarct size limitation.

INTRODUCTION

The standard protocol to induce ischaemic preconditioning involves one or more brief coronary artery occlusions before that artery is occluded for a prolonged period of time. This method has been shown to be effective in reducing infarct size in dogs[1,2], pigs[3,4], rabbits[5] and rats[6], and in several of these species also in reducing the incidence of ventricular arrhythmias and ventricular fibrillation during the longlasting coronary artery occlusion and subsequent myocardial reperfusion.[7-9] In a recent study, Ovize *et al*[10] showed that moderate ischaemia induced by a 50% reduction in myocardial blood flow lasting 15 min also reduced infarct size during a subsequent 60 min total coronary artery occlusion when myocardial reperfusion was restored completely between the ischaemic preconditioning stimulus and the subsequent 60 min total occlusion. In that study the partial flow reduction did not lead to infarct size limitation during the subsequent 60 min total coronary artery occlusion, when myocardial reperfusion was not restored between the partial and total coronary artery occlusion periods. The reason for this last prerequisite remains unclear and, because only one degree of flow reduction for one period of time was used, cannot be generalized to partial occlusions which differ in duration and degree of flow reduction. In this respect it is of interest that over 40 years ago, Harris used a two-stage coronary artery occlusion to prevent the large incidence of death because of ventricular fibrillation during the first 30 min after an abrupt coronary artery occlusion.[11] His first step was to occlude a coronary artery partially for 30 min, thereby allowing a (not measured) reduced perfusion, before the artery was occluded completely for a prolonged period of time. It was reasoned that during these 30 min the hypoperfusion reduced the severity of ischaemia and

thereby prevented the conduction disturbances (because of reentry and enhanced automaticity), that give rise to the high incidence of ventricular fibrillation when the artery is occluded completely. It was never considered why these lethal arrhythmias should not occur when the artery was occluded completely after the 30 min of partial occlusion.

The current insight in the effects of ischaemic preconditioning invites an alternative hypothesis: the myocardium becomes preconditioned during the 30 min partial coronary artery occlusion, and the high incidence of ventricular fibrillation usually observed during an acute total occlusion is decreased when the artery is totally occluded after 30 min. If this would be true, it is also possible that myocardial infarct size is reduced when a complete occlusion is preceded by a partial occlusion. Considering the above, the failure of Ovize *et al*[10] to observe an infarct size limiting effect using a two-stage coronary artery occlusion could be due to the short duration of the partial occlusion in their study. In the present series of experiments in pigs, a species with only a minimal collateral circulation, we have evaluated this hypothesis by using a 30 min partial occlusion of the proximal left anterior descending coronary artery before occluding the artery completely without intermittent reperfusion (two-stage coronary artery occlusion). We used a flow reduction (to 30% of baseline), which reduces the incidence of ventricular fibrillation during the first 30 min by more than 70%.[12]

The infarct sizes of the animals with the two-stage coronary artery occlusion were compared to those of a control group (60 min total coronary artery occlusion) and to those of two groups of animals which were preconditioned with a single 10 min total coronary artery occlusion and either 15 min or 60 min of myocardial reperfusion before the 60 min total coronary artery occlusion. Several groups of investigators have shown that after an acute coronary artery occlusion, the relationship between infarct size (IS) and area at risk (AR) is linear but has a positive intercept on the AR-axis.[13-15] This implies that the use of IS/AR may lead to erroneous conclusions when studies with different area at risks are compared. In the last three groups of animals we therefore varied the size of the area at risk to determine whether the infarct size limitation by ischaemic preconditioning also depends on the area at risk.

METHODS

Experimental groups

The control group consisted of 12 animals which were subjected to a 60 min total coronary artery occlusion (60 min TCO) using an atraumatic clamp. Fifteen animals were preconditioned with a single 10 min total coronary artery occlusion (10 min TCO) before the vessel was occluded for 60 min. The reperfusion period between the 10 min TCO and the 60 min TCO was 15 min (15 min REP) in 10 and 60 min (60 min REP) in 5 animals. In these animals the site of occlusion (left anterior descending coronary artery or its branches) were varied to create areas at risk (AR) which were different for each animal. The last group consisted of 7 animals, in which the flow in the proximal left anterior descending coronary artery was reduced to approximately 30% of baseline during 30 min by using a balloon occluder driven by a micrometer (Hamilton Co., Reno, Nevada, U.S.A.), before the vessel was occluded for 60 min without intermittent reperfusion (two-stage coronary artery occlusion). In this last group of animals the area at risk could not be varied because of the length of the vessel segment needed for the placement of the flow probe and the balloon occluder (see below).

Surgical procedure

All experiments were performed in accordance with the "Guiding principles in the care and

use of animals" as approved by the Council of the American Physiological Society and under the regulations of the Animal Care Committee of the Erasmus University Rotterdam.

Domestic Yorkshire-Landrace pigs (25-35 kg, HVC, Hedel) were anaesthetized with pentobarbital and instrumented for measurement of arterial and left ventricular pressure and control of arterial blood gases as described elsewhere.[17] Following administration of pancuronium bromide (4 mg i.v., Organon Teknika B.V., Boxtel, The Netherlands), a midline thoracotomy was performed, the left mammary vessels were ligated and parts of the second and third left rib were removed to facilitate further instrumentation. An electromagnetic flow probe (Skalar, Delft, The Netherlands) was placed around the ascending aorta to measure cardiac output. In 27 animals, the left anterior descending coronary artery (LADCA) or its side branches were dissected free from the surrounding tissue to induce ischaemia with different areas at risk. In 7 animals a proximal segment of the LADCA was dissected free to accommodate a probe for pulsed Doppler flow velocimetry (Crystal Biotech Inc., Hopkinton, Md, U.S.A.) and an inflatable balloon (R.E. Jones, Silver Spring, Md Maryland, U.S.A.) to occlude the vessel in two stages. One pair of ultrasonic crystals (Sonotek Corporation, Del Mar, Ca, USA) was positioned into the layers of the left ventricular mesocardium in the distribution area of the LADCA, while a separate pair was placed into the myocardium remote from the ischaemic segment for the measurement of regional segment length shortening by sonomicrometry (Triton Technology Inc., San Diego, Ca, USA).[17]

Experimental procedures

After completion of the instrumentation, a stabilization period of at least 30 min was allowed before the animals were subjected to the experimental protocols (see experimental groups). When necessary, small adjustments were made in the volume of the balloon to keep the flow at its reduced value during the 30 min partial coronary artery occlusion in the 7 animals in which the LADCA flow was reduced to 30% of baseline. In all animals, the ischaemic myocardium was reperfused for two hours at the end of the 60 min TCO.

In the animals with the partial occlusion, ATP and ADP were determined in biopsies taken prior and at the end of the 30 min partial occlusion period.[17] Systemic haemodynamic variables and regional segment length changes were recorded throughout the experimental protocols. In case of ventricular fibrillation, the animals were promptly (within 30 s) defibrillated using DC countershock (15-30 Watt) and the animals were allowed to complete the experimental protocol. This procedure does not cause irreversible damage[18].

Area at risk and infarct size

Validation of the methods to determine the area at risk and infarct size has been described extensively[19,20], while details have been reported by Rohmann *et al.*[21] Briefly the area at risk was identified by an intra-arterial injection of 15 ml of a 10 % (w/w) solution of fluorescein sodium (Sigma Chemical Co, St. Louis, USA) after the LADCA had been reoccluded. After induction of ventricular fibrillation with a 9V battery the heart was excised and both atria, the right ventricular free wall and the left ventricular epicardial fat were removed. The remaining left ventricle was filled with alginate impression material (BayerDental, Leverkusen, Germany), cooled in crushed ice and sliced parallel to the atrioventricular groove into 5 segments. The cut surface(s) of each segment and the demarcated areas at risk (AR) were then traced on an acetate sheet. The viable myocardium was then stained deeply blue by incubating the segments for 30 min in 0.125 g para-nitrobluetetrazolium (Sigma Chemicals Co., St. Louis, USA) per liter of

phosphate buffer (pH 7.1) at 37°C. The non-stained pale infarcted tissue was traced onto the acetate sheet and the area at risk (percentage of area of ring) and the infarcted tissue (percentage of area of ring) were calculated for the top and bottom of each ring and the mean 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 percentage of the left ventricle at risk (AR/LV_{mass}), the percentage of the left ventricle that had become infarcted (IS/LV_{mass}) and the percentage of the area at risk that had become infarcted (IS/AR).

Data analysis and presentation

The relationship between IS/LV_{mass} and AR/LV_{mass} was determined for the control animals and the animals preconditioned with 10 min TCO and 15 min REP in order to determine whether the size of the area at risk is important for the protective effect of ischaemic preconditioning. For each of the animals, which underwent the two-stage coronary artery occlusion, infarct size limitation was calculated as $(IS_{pred} - IS_{obs})/IS_{pred}$ (x 100%) in which IS_{pred} and IS_{obs} are the infarct sizes predicted from the regression equation of the control group on the basis of the weight of the area at risk and the actually observed infarct size, respectively.

From the segment length tracings were calculated (i) regional systolic segment shortening as SS(%) = 100 x (EDL - ESL)/EDL, in which EDL and ESL are the segment length at end-diastole and end-systole, respectively, and (ii) post-systolic segment length shortening as PSS(%) = 100 x (ESL - minimal segment length)/EDL. Haemodynamic and regional myocardial function data have been presented as mean (SEM). Statistical significance for the changes in the cardiovascular variables were determined by the paired Student t-test.

RESULTS

Ventricular fibrillation

Table 1 shows that ventricular fibrillation did not occur more frequently in the control group than in the 10 min TCO preconditioning groups. Because the occurrence of ventricular fibrillation depends strongly on variables such as the size of the area at risk, which was varied intentionally in these series of experiments, we also analysed the relation between the incidence of ventricular fibrillation and the area at risk independent of preconditioning. Ventricular fibrillation occurred in 7 of the 9 (78%) animals in which AR/LV_{mass} was more than 30%, while it occurred in 1 of the 13 (7%) animals in which AR/LV_{mass} was less than 20% ($p<0.05$). Ventricular fibrillation did not occur during the partial flow reduction, but was still observed in 3 of the 7 animals (AR/LV_{mass} between 20% and 30%) during the 60 min TCO. All animals were defibrillated successfully within 30 seconds and kept in the study.

Infarct size area at risk relationships in the control animals and the animals preconditioned with a single 10 min TCO

In the control group, there was a highly linear relationship ($r = 0.99$, $p<0.001$) between IS/LV_{mass} and AR/LV_{mass}, which could be described with IS/LV_{mass} (x 100%) = 0.88 AR/LV_{mass} (x 100%) - 3.6. The same figure also shows the individual data for the animals which were preconditioned with 10 min TCO and 15 min REP. In these animals the relationship (IS/LV_{mass} (x 100%) = 0.68 AR/LV_{mass} (x 100%) - 4.8, not shown in figure) proved again to be linear ($r = 0.88$, $p<0.05$) with a less steeper slope ($p<0.05$), but with a similar positive intercept on the AR-axis as found for the control animals. The protective effect of the 10 min TCO was still present when the duration of REP was extended to 60 min (fig 1).

Table 1 Ventricular fibrillation in all pigs

Groups	(n)	Preconditioning		60 min TCO	120 min REP
		occlusion	reperfusion		
Control	12	-	-	3	2
10 min TCO + 15 min REP	10	0	2	4	3
10 min TCO + 60 min REP	5	1	0	4	1
30 min 30% Flow	7	0	-	3	0

TCO = Total coronary artery occlusion, REP = Reperfusion, 30 min 30% Flow = 30 min 70% flow reduction without reperfusion before the 60 min TCO

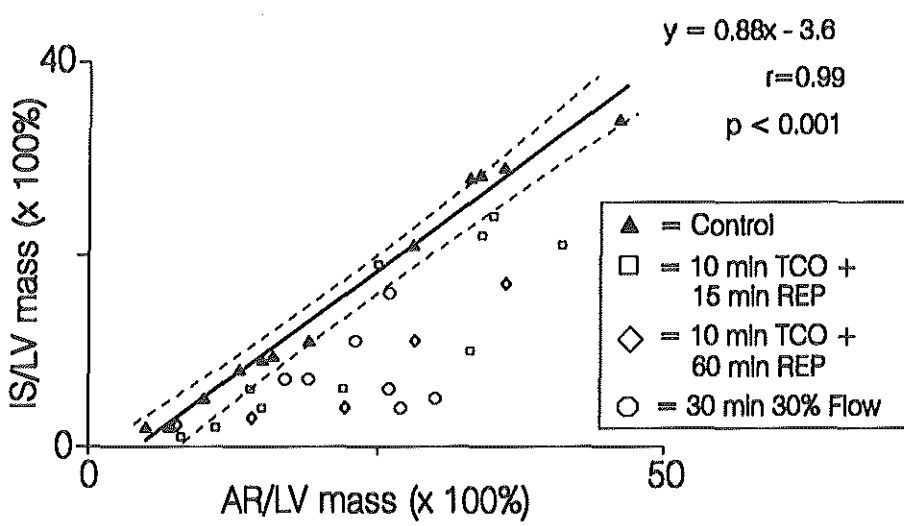

Figure 1 Individual data points determining the relation between the infarct size (IS), expressed as percentage of left ventricular mass (LV_{mass}) and the area at risk (AR), also expressed as percentage of LV_{mass} are shown for the four groups of animals which underwent a 60 min total coronary artery occlusion without (Control or after a preconditioning stimulus). TCO = total coronary artery occlusion; REP = reperfusion; 30 min 30% Flow = 30 min reduction of flow to 30% of baseline. The solid line is the linear regression line for the control group, while the dashed lines indicate the 95% confidence interval.

Infarct size after a two-stage coronary artery occlusion

The individual infarct size data of the animals with the 30 min reduction in flow to 30% of baseline (30 min 30% Flow) preceding the 60 min total coronary artery occlusion (two-stage coronary artery occlusion) have also been depicted in fig 1. The most striking feature is that the infarct sizes of these animals were smaller than predicted from the regression line and the 95% confidence interval of the control animals.

Factors possibly related to the preconditioning effect of the partial occlusion

Baseline values of heart rate (HR), mean arterial blood pressure (MAP), $LVdP/dt_{max}$, left ventricular end-diastolic pressure (LVEDP) and cardiac output (CO) were 105(4) bpm, 90(1) mmHg, 2270(130) $mmHg.s^{-1}$, 12(1) mmHg and 3.0(0.1) $l.min^{-1}$, respectively. The haemodynamic responses to the 60 min TCO of the individual animals in the control group and the groups conditioned with the single 10 min TCO varied considerably depending on the size of the area at risk. The major differences were in the $LVdP/dt_{max}$, which decreased up to 40% in the animals in which AR/LV_{mass} exceeded 30%, but was less than 15% when AR/LV_{mass} was less than 10%.

MAP, $LVdP/dt_{max}$ and CO had decreased to 86(3) mmHg, 1400(50) $mmHg.s^{-1}$ and 2.3(0.2) $l.min^{-1}$ (all $p<0.05$ vs baseline) at the end of the 30 min 30% Flow , while LVEDP had increased ($p<0.05$) to 23(3) mmHg ($p<0.05$) and heart rate did not change. The subsequent 60 min TCO did not lead to further changes (90(3)mmHg, 1320(50)$mmHg.s^{-1}$, 2.2(0.1)$l.min^{-1}$ and 97(7)bpm, respectively). During the two hour reperfusion period only the LVEDP (15(2) mmHg) recovered, as MAP (82(3)mmHg), $LVdP/dt_{max}$ (1330(170)$mmHg.s^{-1}$) and CO (1.8(0.1)$l.min^{-1}$) remained depressed (all $p<0.05$), while HR had increased to 125(5) bpm ($p<0.05$).

Analysis of the pooled (global haemodynamic) data of all groups of animals did not reveal a significant relationship between IS/AR and variables such the heart rate-pressure product at the beginning of the 60 min TCO ($r = 0.22$). This proved also to be true for the data of the separate groups (values for the control group and the two-stage coronary artery groups were $r = 0.36$ and $r = 0.22$, respectively).

Baseline values for the SS of the myocardium perfused by the LADCA were 19(1)%. In the control group SS decreased to 2(1)% during the 60 min TCO and did not recover during the 2 hours REP (3(1)%). During the 10 min TCO preconditioning stimulus, SS was also abolished but had recovered to 11(2)% and 13(1)% (both $p<0.05$ vs baseline) after the 15 min REP and 60 min REP, respectively. At the end of the subsequent 60 min TCO, SS was 2(1)% and 3(1)% for the 15 min REP and 60 min REP, respectively and did not recover during the 2 hours REP (2(1)% and 3(1)%, respectively). In the animals with the two-stage coronary artery occlusion SS had decreased to 2(2)% ($p<0.05$) after 30 min 30% Flow. During the subsequent 60 min TCO there was a further decrease to -1(1)% ($p<0.05$), while there was no sign of recovery during the 2 hours REP (-2(1)%, $p<0.05$ vs baseline). During the 70% flow reduction the loss in systolic segment length shortening was accompanied by the appearance of the post-systolic segment length shortening PSS (from 3(1)% to 11(2)%, $p<0.05$). There were no further changes in PSS during the further course of the experiment.

Because partial flow reductions of the animals in the two-stage coronary artery group was in the range between 61% and 78% of baseline (68 ± 3%), we investigated whether there was a relation between the flow reduction and IS/AR. This proved not to be the case ($r = 0.02$). Figure 2 shows that there were also no significant correlations between IS/AR and the

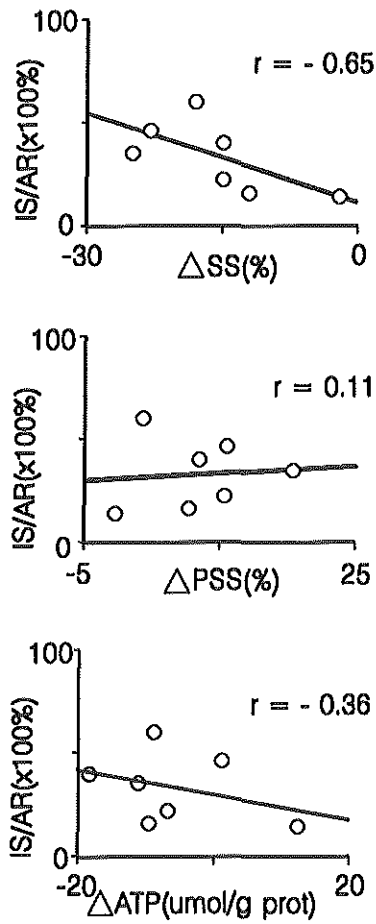

Figure 2 Relation between infarct size/area at risk (IS/AR) and the changes in systolic segment length shortening (SS), post-systolic segment length shortening (PSS) and ATP during the 30 min 30% Flow preceding the 60 min total coronary artery occlusion.

changes in SS, PSS and ATP, observed during the 30 min 30% Flow. There were also only weak correlations between *infarct size limitation* (see data analysis) and the changes from baseline prior to the 60 min total coronary artery occlusion in SS (r = 0.71), PSS (r = -0.32), ATP (r = 0.44), ADP (r = 0.42) and ATP/ADP (r = -0.08).

DISCUSSION

Relationships between infarct size and area at risk for control pigs and pigs preconditioned with a single 10 min TCO

In both anaesthetized and conscious dogs, IS and AR (both expressed as percentage of left ventricular mass) are highly linearly related with a positive intercept on the AR-axis.[13,14] Because of this non-zero intercept, the ratio between IS and the AR is not a constant, but depends on the AR. In the present study we found a highly linear relationship with similar positive intercepts on the AR-axis for our control pigs, as well as for the pigs preconditioned with a single 10 min TCO. The slope for the preconditioned animals was significantly less than for the control animals indicating that infarct size development was less. The reason for the positive intercept is unclear. It is unlikely that in the present study insertion of the crystals contributed significantly to the non-zero intercept as non-zero intercepts have also been found in studies in which no crystals were implanted.[13] The possibility that infarct size development in small areas at risk progresses less rapidly than in larger areas at risk can therefore not be excluded. This has indeed been suggested for the dog and been explained by its collateral flow. This now appears to be an unlikely explanation as not only in the pig, but also in the rabbit[15,22], an other species with a lack of coronary collaterals, the IS-AR relationship is linear with a positive intercept on the AR-axis. In this respect it is of interest that in the rat, a species with a much smaller heart, the relation between IS and AR appears to have a zero intercept (unpublished observations from our laboratory).

Irrespective of its cause, the non-zero intercept makes IS/AR highly dependent on AR. Using the linear regression equations of the control group and the group preconditioned with a 10 min TCO + 15 min REP, it can be easily calculated that infarct size limitation by preconditioning $(IS_{control} - IS_{precond})/IS_{control} \times (100\%)$ is 62% when AR is 10% of the LV_{mass}, but only 28% if AR is 25% of the LV_{mass}. This large variability may easily lead to erroneous conclusions when studies are compared in which different sizes of the area at risk have been used.

Preconditioning with a partial occlusion without intermittent reperfusion

Up till now complete restoration of myocardial reperfusion was necessary to obtain ischaemic preconditioning (see ref. 23). This requirement appears to be further supported by the study of Ovize *et al*[10], who showed that moderate ischaemia caused by a 50% reduction in myocardial blood flow lasting for 15 min failed to reduce infarct size during a subsequent 60 min TCO unless intermittent reperfusion was allowed. In the present study we now show that infarct size can be limited by a partial occlusion without intermittent reperfusion. The two-stage occlusion model proved to be as effective as the standard model in which preconditioning is elicited by brief total occlusion separated from the longlasting occlusion by a period of complete reperfusion (fig 1). Similar to other investigators[15], we found a very poor correlation between IS/AR and global haemodynamic variables such as the heart rate-pressure product just prior to the 60 min TCO. There were also only weak correlations with the loss in function and some metabolic measures such as changes in ATP and ATP/ADP. It must be remarked, however, that we measured total ADP content, which is almost entirely bound or compartmented in the myocyte. From thermodynamic point of view, the free cytosolic ADP concentration is a more valid of measure of myocardial energetics, but its calculation using the creatine kinase mass action ratio would have required the measurement of creatine and creatinephosphate.

The need for reperfusion in the earlier studies has remained unclear as some phenomena directly related to reperfusion such as hyperaemia (accompanied by washout of metabolites

accumulated during ischaemia), free radical generation and myocardial stunning have been discarded.[23-25] However, several groups of investigators have shown that the tolerance of normal healthy myocardium can also increase by preceding transient ischaemic periods in adjacent myocardium[26] or kidneys.[27] This may point towards a role for circulating substances, not necessarily released by the transient ischaemic tissue.

It has been well established that metabolism of the ischaemic segment changes continuously during a fixed reduction in coronary blood flow, while the function remains depressed at the same level. Studies from several laboratories, including our own, have shown that ATP breakdown, myocardial lactate production and efflux of potassium ions increase gradually during the early period of a fixed flow reduction, but that there is a normalization of ischaemia-induced metabolic changes as the hypoperfusion is prolonged.[12,28-31] If the metabolic state of the myocardium is important for preconditioning, the duration and severity of the flow reduction may be critical in obtaining this protective action. In the study of Ovize et al.[10], the combination of the duration and the degree of flow reduction may have not been sufficient. Several other mechanisms (adenosine hypothesis, opening of ATP sensitive potassium channels, preservation of high energy phosphates, etc. for an extensive review see ref. 22) have been proposed to explain myocardial preconditioning. The design of the present study does not provide any evidence in favour of against any of these hypothesis.

Limitations

During the 30 min 30% Flow, the volume of the inflated balloon had to be adjusted occasionally to keep the flow at its reduced value. Although this was not a frequent phenomenon, we can not exclude entirely that the small flow oscillations have contributed to the preconditioning effect. In this respect, it is worth mentioning that Ovize *et al*[24] have reported that cyclic flow variations could lead to preconditioning. In their study[24] the flow oscillations were very large, however, and reperfusion was restored between the flow reductions. The model in that study therefore resembles more closely that of the multiple complete occlusion-reperfusion sequences. Furthermore, we have tested only one flow reduction (68 ± 3%) for a fixed period of time (30 min) and have therefore no information about infarct size limitation when other combinations of duration and degree of flow reductions are used.[10]

Conclusion

Infarct size can be limited with a two-stage coronary artery occlusion without intermittent reperfusion. This last observation brings the phenomenon of ischaemic preconditioning closer to the clinical setting as in many patients thrombus development may give rise to severe ischaemia before the artery is totally occluded. The model also suggests that complete washout of metabolites, accumulated during ischaemia, is not required to obtain preconditioning.

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Chapter 6

Endocardial and Epicardial Infarct Size Limitation by Preconditioning with a Partial Coronary Artery Occlusion Without Intermittent Reperfusion

Importance of the Severity and Duration of Flow Reduction

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ABSTRACT

Background. One or more brief total coronary artery occlusions can limit myocardial infarct size produced by a subsequent sustained occlusion. Recently, we reported that a partial coronary artery occlusion immediately preceding a sustained coronary artery occlusion could also limit infarct size. In the present study we investigated (*i*) whether the protection against infarction exerted by partial coronary artery occlusions depends on the severity and(or) duration of the flow reduction and (*ii*) whether the protection afforded by the partial coronary artery occlusions varies in the different myocardial layers.

Methods and Results. Studies were performed in 63 open-chest pigs. Using standard staining techniques, the left ventricular area at risk (AR) and infarct area (IA) expressed as a percentage of the left ventricular (LV) mass were determined for the endocardial half (IA_{endo}/LV_{endo} and AR_{endo}/LV_{endo}) and epicardial half of the left ventricle (IA_{epi}/LV_{epi} en AR_{epi}/LV_{epi}). The results of eight groups of animals are presented. In control animals that underwent 60 min of total coronary artery occlusion (TCO) followed by 120 min or reperfusion (Rep) there were a highly linear relations in the endocardium (r=0.98, $P<0.001$) and epicardium (r=0.97, $P<0.001$) between IA/LV and AR/LV which could be described by the equations $IA_{endo}/LV_{endo}(\bullet 100\%) = 1.01 \bullet AR_{endo}/LV_{endo}(\bullet 100\%) - 4.6$ and by $IA_{epi}/LV_{epi}(\bullet 100\%) = 0.91 \bullet AR_{epi}/LV_{epi}(\bullet 100\%) - 4.1$. A second group of animals underwent a single 10 min TCO and 15 min of Rep prior to the 60 min TCO and 120 min Rep. Infarct area in both the endocardium and epicardium were again linearly related with the area at risk, with a positive intercept on the AR/LV axis that was similar to that of the control group. Preconditioning with 10 min TCO and 15 min Rep decreased infarct size, so that the slopes of the linear relations in both the endocardium (0.63) and epicardium (0.57) were less steep compared to the control group (both $P<0.05$). The magnitude of protection was not different between the inner and outer half. Two groups of pigs were subjected to either 30 or 90 min of 70% reduction of coronary blood flow (FR) immediately preceding the 60 min TCO and 120 min Rep, *without* intermittent reperfusion. Thirty min of 70% FR decreased infarct size in both the endo- and epicardial half, so that the degree of protection was not different between the inner and outer half. Ninety min of 70% FR also resulted in infarct size limitation in both layers ($P<0.05$), but the protection was greatest in the epicardium ($P<0.01$ vs endocardium). Ninety min of 70% FR alone, without the 60 min TCO, resulted in infarction in both endo- and epicardium with the greatest infarct size in the endocardium ($P<0.01$ endocardium vs epicardium). Thus, the observation that 90 min of 70% FR resulted in less infarct size reduction (compared to the control group) in the endocardial than in the epicardial half was likely due to a greater degree of irreversible damage in the endocardium already produced by this duration of severe flow reduction. Endocardial and epicardial infarct size in animals subjected to either 30 or 90 min of 30% FR prior to the 60 min TCO was not different from that in the control group, indicating that exposure to such mild flow reductions fails to limit irreversible ischemic damage during a subsequent 60 min TCO and 120 min Rep. Ninety min of 30% FR without the 60 min TCO did not produce myocardial necrosis in either the outer or inner half of the left ventricle.

Conclusions. Thirty or ninety min of severe (70%) but not mild (30%) coronary flow reductions protected against myocardial infarction produced by a subsequent total coronary artery occlusion, suggesting the presence of an ischemic threshold for myocardial preconditioning. Infarct size limitation afforded by a single 10 min TCO and 15 min Rep was distributed homogeneously across the left ventricular wall. In contrast, the transmural distribution of infarct size limitation by a 70% coronary flow reduction was influenced by the duration of flow reduction. Thus, whereas 30 min of 70% flow reduction produced similar decreases in infarct size in the inner- and outer half of the left ventricle, 90 min of 70% flow reduction preferentially limited epicardial infarct size. These findings suggest that perfusion abnormalities immediately preceding a coronary artery occlusion in patients may be an important source of infarct size variability.

INTRODUCTION

In a large number of species, including dogs[1,2], pigs[3-5] rabbits[6,7] and rats,[8,9] one or more brief total coronary artery occlusions can limit the irreversible damage produced by a subsequent sustained occlusion. Recently, it was reported that partial coronary artery occlusions can also precondition the myocardium.[10,11] Thus, Ovize et al.[10] observed in dogs that the development of myocardial necrosis during 60 min total coronary artery occlusion was attenuated, when coronary blood flow was reduced by 50% for 15 min preceding the sustained coronary artery occlusion. In that study 15 min of complete reperfusion between the graded coronary artery stenosis and the sustained total coronary occlusion was necessary to obtain the reduction in infarct size. In contrast, we observed in pigs that a 70% coronary flow reduction that lasted 30 min protected the myocardium during a subsequent 60 min total coronary artery occlusion *without* the need of intermittent reperfusion.[11] These findings suggest that the severity and/or duration of the flow reduction plays a critical role in eliciting the protective effect.

Although ischemic preconditioning has been the topic of many studies, none of them investigated the transmural distribution of infarct size limitation afforded by the ischemic stimulus. It is well established that a partial coronary artery occlusion affects perfusion of subendocardial and subepicardial layers differently, with the largest decreases occurring in the subendocardial layers.[12-17] Thus, differences in severity of ischemia across the left ventricular wall could produce different degrees of protection for different myocardial layers. To address these questions we investigated (*i*) whether the protection exerted by partial coronary artery occlusions depends on the severity and(or) duration of the flow reduction and (*ii*) whether the protection afforded by the partial coronary artery occlusions varies in the different myocardial layers. These issues are of particular clinical interest, since preconditioning with partial occlusions mimics more closely the condition of patients suffering from coronary artery disease than the abrupt brief total occlusion and reperfusion sequences. To compare the protective effects of the flow reduction models with a classical model of myocardial preconditioning we also analyzed the subendocardial and subepicardial distribution of previously published transmural infarct size data

of eleven pigs that had been preconditioned with a single 10 min coronary artery occlusion.[11]

METHODS

All experiments were performed in accordance with the "Guiding principles in the care and use of animals" as approved by the Council of the American Physiological Society and under the regulations of the Animal Care Committee of the Erasmus University Rotterdam.

Surgical Procedures

Domestic Yorkshire-Landrace pigs (25-35 kg, HVC, Hedel, The Netherlands) were anesthetized with pentobarbital and instrumented for measurement of arterial and left ventricular pressure and control of arterial blood gases.[18] After administration of pancuronium bromide (4 mg i.v., Organon Teknika B.V., Boxtel, The Netherlands), a midline thoracotomy was performed and an electromagnetic flow probe (Skalar, Delft, The Netherlands) was placed around the ascending aorta to measure cardiac output. In the control animals the left anterior descending

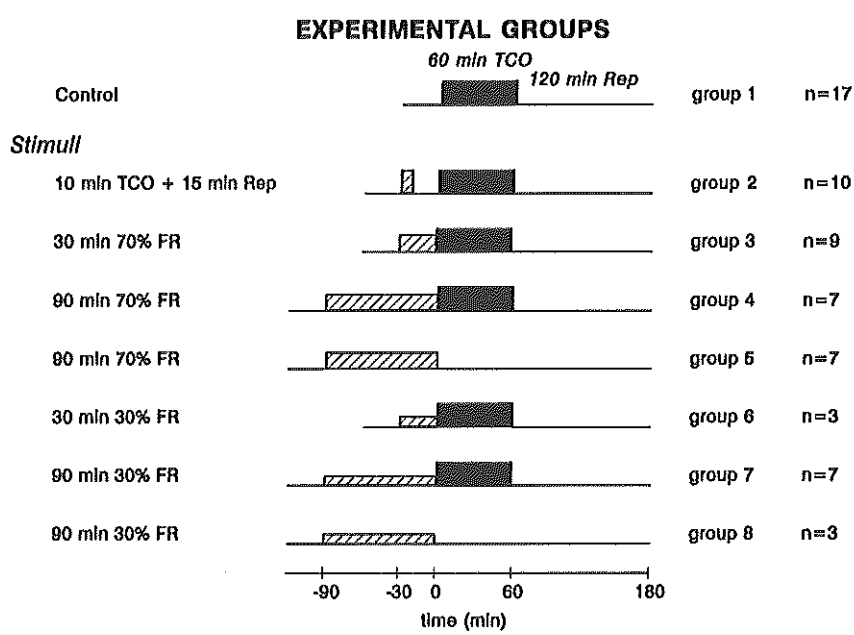

Figure 1 Experimental protocol of the 8 groups of animals in which the distribution of infarction size was determined. The 60 min total coronary artery occlusion (60 min TCO) has been indicated in black. Flow reductions have been indicated with a cross-hatch pattern. TCO = total coronary artery occlusion, Rep = reperfusion, FR = flow reduction.

coronary artery (LADCA) or its diagonal branches supplying the left ventricular anterior wall were dissected free from the surrounding tissue to allow placement of a microvascular clamp. The anatomic location of the occlusion site was varied to create areas at risk of different sizes.[11] In the animals which underwent a partial occlusion preceding the total coronary artery occlusion, a proximal segment of the LADCA was dissected free for the placement of a probe for pulsed Doppler flow velocimetry (Crystal Biotech, Hopkinton, MD, U.S.A.) and an inflatable balloon (R.E. Jones, Silver Spring, MD, USA) to occlude the vessel in two stages.

Regional Myocardial Contractile Function

One pair of ultrasonic crystals (Sonotek Corporation, Del Mar, CA, USA) was positioned into the subendocardial layers of the left ventricular myocardium in the distribution region of the LADCA for the measurement of segment shortening by sonomicrometry (Triton Technology, San Diego, CA, USA). Another pair of crystals was placed into the myocardium perfused by the left circumflex coronary artery, remote from the ischemic region. From the segment length tracings the segment length at the end of diastole (EDL, onset of positive ascending aortic flow) and the length at the end of systole (ESL, positive aortic flow crossing the zero flow line) were determined and regional systolic segment shortening was computed as:

$$SS(\%) = 100 \times (EDL - ESL)/EDL,$$

while post-systolic segment length shortening was computed as:

$$PSS(\%) = 100 \times (ESL - \text{minimal segment length})/EDL.$$

Experimental Protocols

After completion of the instrumentation, a stabilization period of at least 30 min was allowed before the animals were subjected to the experimental protocols. Systemic hemodynamic variables and regional segment length changes were recorded throughout the experimental protocols. In case of ventricular fibrillation defibrillation was started within 30 s, using DC countershocks (15-30 Watt). If defibrillation was succesful within 2 min animals were allowed to complete the experimental protocol, since this procedure does not produce irreversible damage.[19] Animals in which defibrillation could not be accomplished within 2 min were excluded from further study.

The results of eight groups of animals are presented (Fig 1). Eighteen animals underwent a single 60 min total coronary artery occlusion (TCO), followed by 120 min of reperfusion (Rep). Eleven animals underwent a 60 min TCO preceded by a single 10 min TCO and 15 min Rep. Four groups underwent a partial coronary artery occlusion of either 30 min or 90 min, before the artery was occluded completely for 60 min without intermittent reperfusion. The partial occlusions were chosen such that coronary blood flow was reduced by either 30% or 70% of baseline. When necessary, small adjustments were made in the volume of the balloon to keep the flow at its reduced value during the partial coronary artery occlusion period. In all animals the ischemic myocardium was reperfused for 120 min following the 60 min total coronary artery

occlusion. Because it is conceivable that the preconditioning stimulus of either 90 min of 70% coronary flow reduction (FR) or 90 min of 30% FR could already lead to necrosis, we also analyzed two groups of animals that underwent 90 min of either 70% FR (n=7) or 30% FR (n=3), followed by 120 min Rep without the 60 min of total coronary artery occlusion (Fig 1). Transmural infarct size data of 12 animals of the control group and 10 animals that were preconditioned with a single 10 min total coronary artery occlusion, as well as 7 animals with 30 min of 70% FR have been presented in an earlier study.[11]

Area at Risk and Infarct Area

Validation of the methods to determine the area at risk and infarct area has been described extensively.[5,20,21] Briefly, following reocclusion of the LADCA the area at risk was identified by an intra-atrial injection of 15 ml of a 10 % (w/w) solution of fluorescein sodium (Sigma Chemical Co, St. Louis, USA). Ventricular fibrillation was produced with a 9V battery and the heart was excised. Both atria, the right ventricular free wall and the left ventricular epicardial fat were removed. The left ventricle (LV) was filled with alginate impression material (BayerDental, Leverkussen, Germany), cooled in crushed ice and sliced parallel to the atrioventricular groove into 5 segments. The cut surface(s) of each segment and the demarcated areas at risk (AR) were then traced on a transparent acetate sheet under a black light . The viable myocardium was then stained deeply blue by incubating the segments for 30 min in 0.125 g para-nitrobluetetrazolium (Sigma Chemicals Co., St. Louis, USA) per liter of phosphate buffer (pH 7.1) at 37°C and the non-stained pale infarcted tissue was traced onto the acetate sheet. The surface of each ring of was subsequently subdivided into a subendocardial (inner) half and subepicardial (outer) half by drawing a line which divided the myocardial wall into two layers of equal thickness. Division into two layers was done as it provides information on the transmural distribution of infarct size, yet preserving sufficient accuracy of infarct size determination in the two halves. Surface areas of the subendocardial and subepicardial halves, and of the subendocardial and subepicardial areas at risk and infarct areas (IA) were determined and averaged for the top and bottom of each individual ring. Then the fraction of the ring that was infarcted and at risk was multiplied by the weight of the ring to yield the weight of the infarct area and area at risk for that ring. The weights of the subendocardial and subepicardial halves and the total weight of each ring were then summed to yield the LV_{endo}, LV_{epi} and total LV masses. The weights of the endocardial, epicardial and total areas at risk of each ring were summed to yield the total AR_{endo}, AR_{epi} and total AR masses; the weights of the endocardial, epicardial and total infarct areas of each ring were summed to yield IA_{endo}, IA_{epi} and total IA masses. Endocardial, epicardial and total IA and AR data were expressed as a percentage of LV_{endo}, LV_{epi} and total LV masses, respectively.

Data Analysis and Presentation

Infarct size data have been presented by plotting the IA/LV against the AR/LV for the inner

and outer half and for the whole left ventricular wall. Linear regression analysis was performed to determine the relation between endocardial and epicardial IA/LV and AR/LV in the control group and the animals preconditioned with 10 min TCO and 15 min of Rep. For the animals that underwent the two-stage coronary artery occlusion the individual data are presented. Intergroup differences in IA_{endo}/LV_{endo}, IA_{epi}/LV_{epi}, or total IA/LV were analyzed by analysis of covariance (ANCOVA), with AR_{endo}/LV_{endo}, AR_{epi}/LV_{epi} or total AR/LV as a covariate. Intragroup differences between IA_{endo}/LV_{endo} and IA_{epi}/LV_{epi} were analyzed using ANCOVA for repeated measures, with AR_{endo}/LV_{endo} and AR_{epi}/LV_{epi} as covariates. The incidence of ventricular fibrillation was analyzed by Fisher's exact test (two-tailed). Hemodynamic data were analyzed with ANOVA followed by unpaired t-test. Values are expressed as mean ± SEM.

RESULTS

Mortality and Exclusions

In the control group (60 min TCO) one animal was excluded because defibrillation during the 60 min TCO was unsuccessful within 1min after onset of ventricular fibrillation. Two animals which underwent a 30% FR (one pig for 30 min and the other for 90 min) could also not be defibrillated during the 60 min TCO period. Two animals died after unsuccesful defibrillation, during 70% FR (after 15 min and 20 min of flow reduction, respectively), while one animal was excluded from further study because of technical failure of the balloon occluder. One animal was excluded from the 90 min 70% FR group because of unsuccesful reperfusion after 60 min TCO.

Ventricular Fibrillation

Table 1 shows that during the 60 min TCO ventricular fibrillation occurred in 7 of the 18 control animals, and in 5 of the 11 animals that had been subjected to 10 min TCO and 15 Rep (P=NS). Animals that fibrillated in these two groups during 60 min TCO had larger areas at risk than animals that did not fibrillate (34±3% and 18±2% of the left ventricle, respectively, $P<0.01$). Thirty min of 70% FR also failed to exert a protective effect on the incidence of ventricular fibrillation during the subsequent 60 min TCO (area at risk 27±2% of the left ventricle), but ventricular fibrillation during the 60 min TCO in the animals that were precondtioned with 90 min 70% FR was absent (0 of 8, $P<0.05$ vs control group, area at risk 36±3% of the left ventricle). In contrast, the incidence of ventricular fibrillation during the 60 min TCO in the group preconditioned with either 30 or 90 min of 30% FR (area at risk 35±3% of the left ventricle) was not different from the control group. Thus, while the protective effect of 90 min of 70% FR could not be explained by differences in area at risk, the findings indicate that severity and duration of the partial flow reduction critically determine its protection against ventricular fibrillation duration a sustained ischemic episode. Upon reperfusion ventricular fibrillation was absent or rare, which is in agreement with studies

Table 1 Ventricular fibrillation in all pigs

Groups	Preconditioning Stimulus	60 min TCO	Reperfusion
(1) Control	-	7(18)°	2(17)°
(2) 10 min TCO + 15 min Rep + 60 min TCO	2(11)$^{+,x}$	5(11)°	3(10)$^{\circ,+}$
(3) 30 min 70% FR + 60 min TCO	0(9)	3(9)	0(9)
(4) 90 min 70% FR + 60 min TCO	1(9)	0(8)	0(7)
(5) 90 min 70% FR	1(8)	-	0(7)
(6) 30 min 30% FR + 60 min TCO	2(4)^{x}	1(4)^{x}	0(3)
(7) 90 min 30% FR + 60 min TCO	0(8)	6(8)	0(7)
(8) 90 min 30% FR	0(3)	-	0(3)

Between parentheses are the total numbers of animals per group at that moment still in the study. TCO = Total coronary artery occlusion, Rep = Reperfusion, FR = Coronary Flow Reduction, ° one pig fibrillated during both 60 min TCO and Reperfusion, $^{+}$ one pig fibrillated during both the preconditioning stimulus and Reperfusion, ^{x} one pig fibrillated during both the preconditioning stimulus and 60 min TCO

showing that ventricular fibrillation occurs predominantly after TCO's with a duration between 10 min and 30 min.[25,26]

Infarct Area - Area at Risk Relation in Control Pigs and Pigs Preconditioned with a Single 10 min TCO

In the control group there was a highly linear relation ($r=0.98$, $P<0.001$) between IA_{endo}/LV_{endo} and AR_{endo}/LV_{endo} which could be described by the equation IA_{endo}/LV_{endo} (•100%) = 1.01•AR_{endo}/LV_{endo} (•100%) - 4.6 (Fig 2). In the epicardial half of the left ventricle we also observed a linear relationship ($r=0.97$, $P<0.001$) which could be described by the equation IA_{epi}/LV_{epi} (•100%) = 0.91•AR_{epi}/LV_{epi} (•100%) - 4.1 (Fig 2). ANCOVA (with AR_{endo}/LV_{endo} and AR_{epi}/LV_{epi} as covariates) revealed that IA_{endo}/LV_{endo} was larger than IA_{epi}/LV_{epi} ($P<0.05$).

Preconditioning with 10 min TCO and 15 min Rep decreased infarct size in both the inner and outer half of the left ventricle. The infarct areas in both the endocardium and epicardium were again linearly related with the areas at risk, with a positive intercept on the AR/LV axis that was similar to that of the control group. In contrast, slopes of the linear relation in both the endocardium and epicardium were less steep compared to the control group (both $P<0.05$). ANCOVA (with AR_{endo}/LV_{endo} or AR_{epi}/LV_{epi} as covariates) also indicated that IA_{endo}/LV_{endo} and IA_{epi}/LV_{epi} were smaller in the 10 min TCO and 15 min Rep group compared to the control group (both $P<0.01$). The degree of protection afforded by 10 min TCO and 15 min Rep in the inner and outer halves of the ventricle was of identical magnitude ($P=0.60$).

Infarct Area - Area at Risk Relation in Pigs Undergoing Two Stage Coronary Artery Occlusion 70% Coronary Artery Flow Reduction

Transmural Infarct Size. When the 60 min TCO was preceded by 30 min of 70% coronary flow reduction (FR), transmural infarct size was significantly smaller compared to the control group (Fig 3). Extending the duration of the 70% FR to 90 min produced similar protection against infarction compared to the 30 min 70% FR animals. Thus, even though 90 min of 70% FR without the 60 min TCO had already resulted in considerable myocardial necrosis the individual transmural data points remained below the regression line of the control group ($P<0.01$), indicating significant protection against irreversible myocardial damage produced by the subsequent 60 min TCO (Fig 3).

Distribution of Infarct Size. Thirty min of 70% FR decreased infarct size in both endo- and epicardial halves compared to the control group ($P<0.01$) (Fig 4). The protection produced by the 30 min 70% FR tended to be greater in the epicardium than in the endocardium but this failed to reach statistical significane ($P=0.13$). When the duration of 70% FR prior to the 60 min TCO was extended from 30 min to 90 min, infarct size limitation in the outer half of the

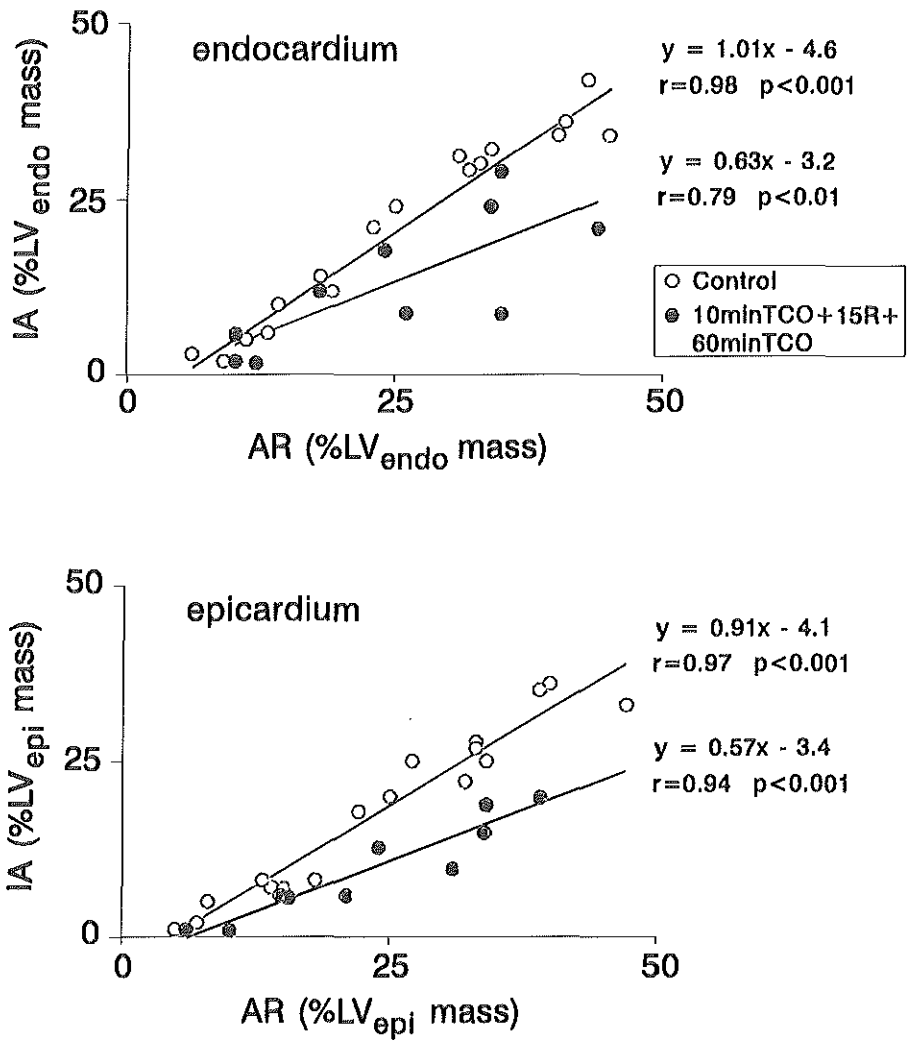

Figure 2 Individual data points determining the relation between infarcted area and area at risk in the endocardium (upper panel) and in the epicardium (lower panel), both expressed as a percentage of left ventricular endocardial (Lvendo) or epicardial mass (Lvepi). Shown are the relationships in the control group (60 min total coronary occlusion, TCO) (open circles) and in the animals preconditioned with 10 min TCO and 15 min Rep prior to the 60 min TCO (closed circles). Infarct size limitations afforded by the 10 min TCO and 15 min Rep was of similar magnitude in the two myocardial layers.

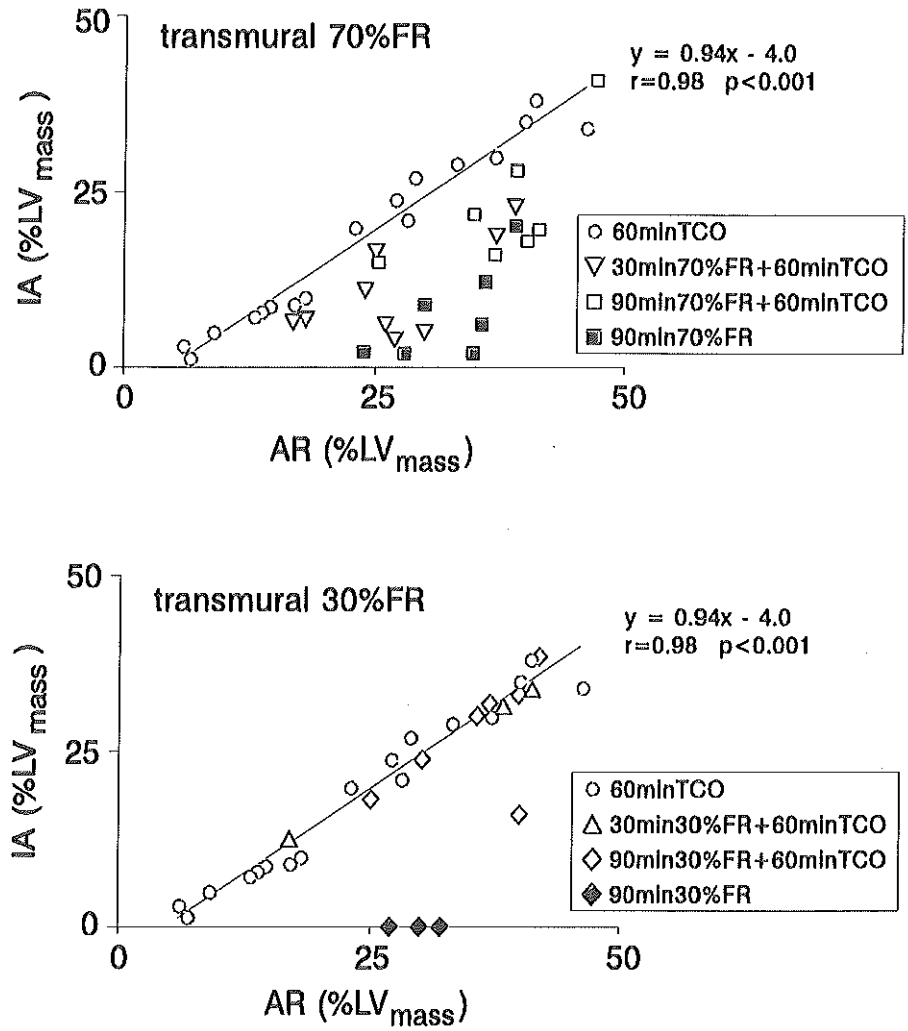

Figure 3 Individual transmural data points determining the relations between infarcted area (IA), and the area at risk (AR), both expressed as a percent of the left ventricular mass (LV_{mass}). In the upper panel are shown the regression line and individual data points for the control group (60 min total coronary occlusion), TCO) (open circles), and individual data points for animals subjected to 30 min of 70% coronary flow reduction (FR) followed by 60 min TCO (open triangle pointing downward), 90 min of 70% FR followed by 60 min TCO (open squares), and 90 min of 70% FR without the 60 min TCO (closed squares). In the lower panel are shown the relations in the control group (60 min TCO) (open circles), 30 min of 30% FR followed by 60 min TCO (open diamonds), and 90 min of 30% FR without the 60 min TCO (closed diamonds). Ninety min of 30% FR, which itself did not result in infarction, did not limit infarct size produced by 60 min TCO. In contrast, 70% which itself resulted in significant infarction after 90 min, provided protection against the irreversible damage produced by 60 min TCO.

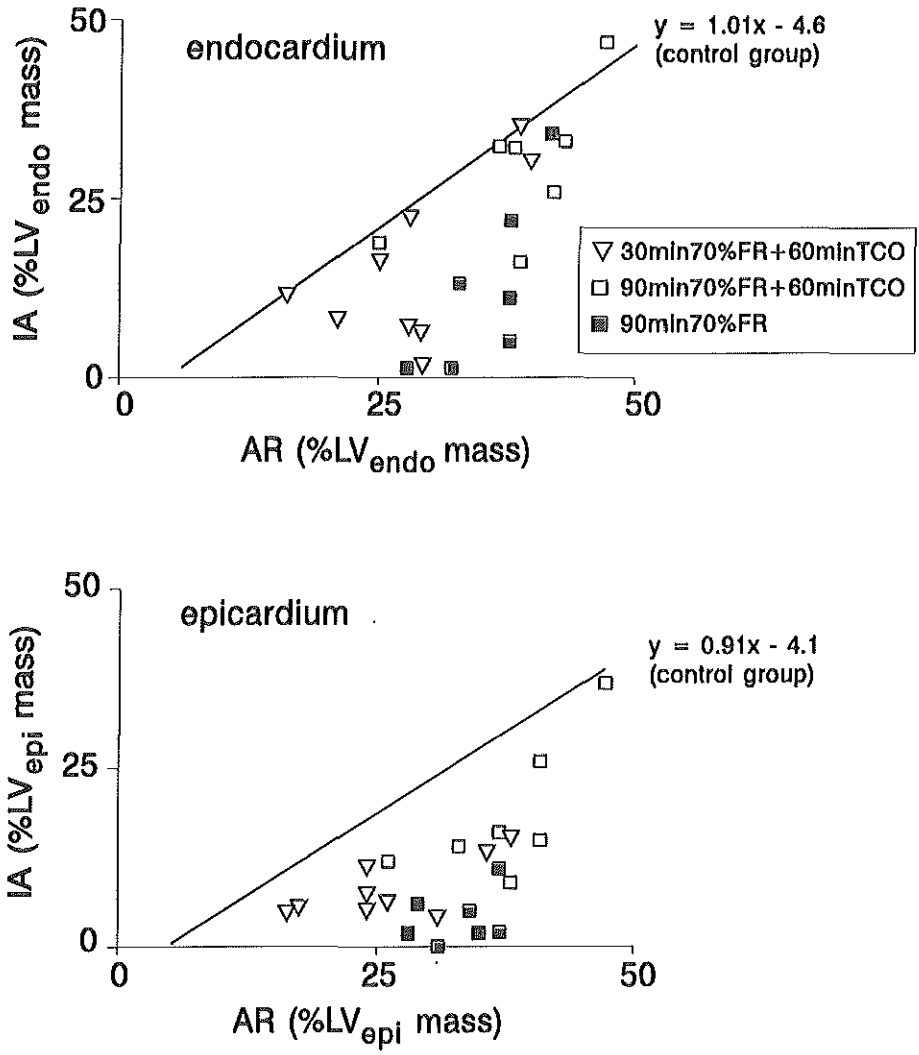

Figure 4 Individual data points determining the relations between infarcted area (IA) in the endocardium (upper panel) or epicardium (lower panel), expressed as a percent of the left ventricular endocardial mass or epicardial mass (LV_{endo} mass or LV_{epi} mass), and the endocardial or epicardial area at risk (AR), expressed as a percent of the LV_{endo} mass or LV_{epi} mass. Shown are the regression line and the equation describing the relations in the control group (60 min total coronary occlusion, TCO), and the individual data points in animals subjected to 30 min of 70% FR followed by 60 min TCO (open triangles), 90 min of 70% FR followed by 60 min TCO (open squares), and 90 min of 70% FR without the 60 min TCO (closed squares). Ninety min of 70% FR, which itself produced some infarction after 90 min, provided protection against irreversible damage caused by 60 min TCO. This protective effect was similar in endo- and epicardium when animals were exposed to 70% FR for 30 min, but was significantly greater in the epicardium when the flow reduction was present for 90 min prior to the 60 min TCO.

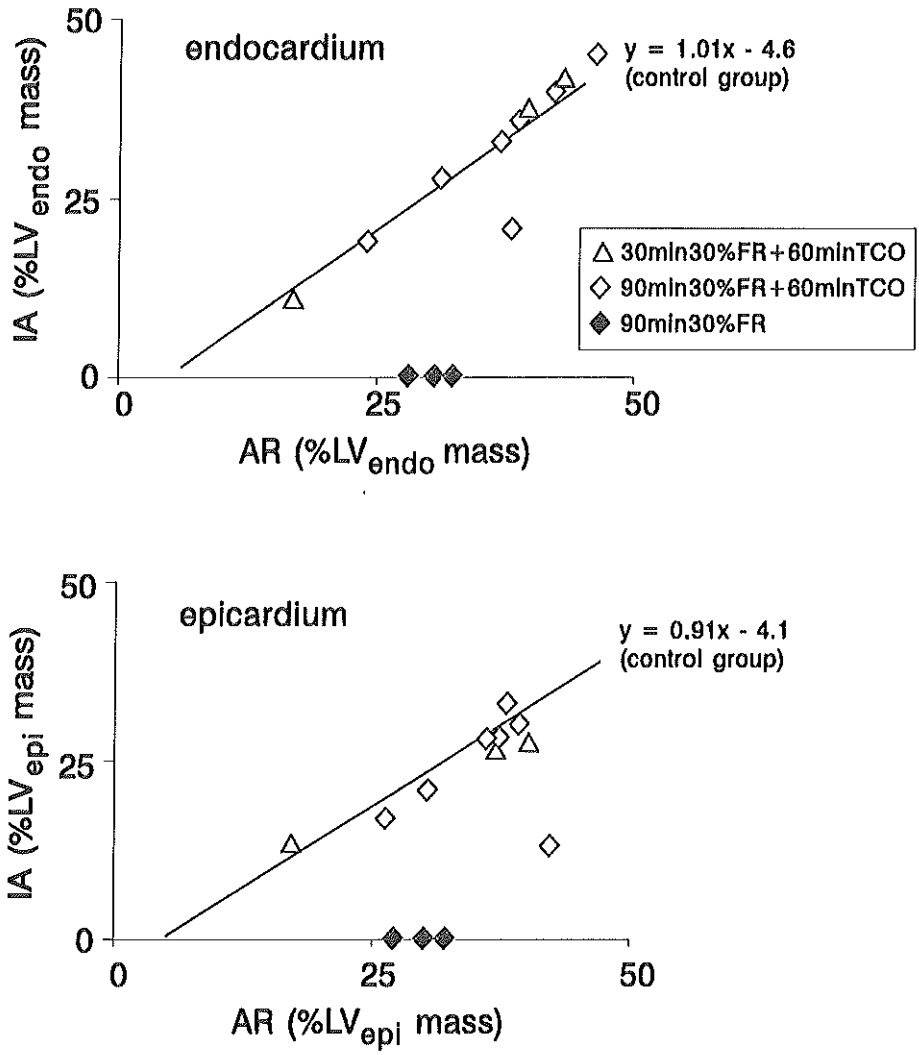

Figure 5 Individual data points determining the relations between infarct area (IA) in the endocardium (upper panel) or epicardium (lower panel), expressed as a percent of the left ventricular endocardial mass or epicardial mass (LV_{endo} mass or LV_{epi} mass), and the endocardial or epicardial area at risk (AR), expressed as a percent of the LV endo mass or LV epi mass. Shown are the regression line and the equation describing the relationship in the control group (60 min total coronary occlusion, TCO), and the individual data points in animals subjected to 30 min of 30% flow reduction (FR) followed by 60 min TCO (open triangles), in animals subjected to 90 min of 30% FR without the 60 min TCO (closed diamonds). Ninety min of 30% FR, which itself did not result in infarction, failed to limit infarct size in either endo- or epicardium produced by 60 min TCO.

left ventricle was similar to that observed with the 30 min 70% FR. Infarct size limitation was also present in the endocardium ($P<0.05$ vs control group), but the degree of protection produced by 90 min of 70% FR was greater in the epicardium than in the endocardium ($P<0.01$). Ninety min of 70% FR alone resulted in infarction in both endo- and epicardium, with the greatest infarct size in the endocardium ($P<0.01$ endocardium vs epicardium). The addition of 60 min of TCO increased infarct size slightly further in both endocardium ($P<0.05$) and epicardium ($P<0.01$), so that the magnitude of additional necrosis produced by the 60 min TCO was not different between endocardium and epicardium ($P>0.20$). Thus, the observation that 90 min of 70% FR resulted in less infarct size reduction (compared to the control group) in the endocardium than in the epicardium was likely due to a greater degree of irreversible damage in the endocardium already produced by this duration of severe flow reduction.

30% Coronary Artery Flow Reduction

Transmural Infarct Size. Transmural infarct size in animals subjected to 30 min of 30% FR prior to the 60 min TCO was identical to that of the control group (Fig 3). Extending the period of flow reduction to 90 min did also not alter infarct size produced by 60 min of TCO, indicating that exposure to such mild flow reductions fails to limit irreversible ischemic damage during a subsequent 60 min TCO. Ninety min of 30% FR without the 60 min TCO did not produce myocardial necrosis (Fig 3).

Distribution of Infarct Size. Endocardial and epicardial infarct size in animals subjected to 30 or 90 min of 30% FR prior to the 60 min TCO were identical to those of the control group (Fig 5). Ninety min of 30% FR without the 60 min TCO did not produce myocardial necrosis in either the outer or inner half of the left ventricle.

Systemic Hemodynamic Variables

Baseline values of heart rate (109±3 beats/min), mean arterial blood pressure (88±1 mmHg), cardiac output (2.8±0.1 L/min), or the product of heart rate and systolic arterial pressure (11890±340 beats•mmHg/min) were not different between the eight experimental groups (n=63). In the control group and the 10 min TCO + 15 min Rep group, mean arterial pressure and heart rate did not change significantly in response to the 60 min TCO, but cardiac output decreased to 2.4±0.1 L/min ($P<0.01$). At the end of 120 min of Rep none of the hemodynamic variables showed significant recovery towards baseline levels.

In the groups in which the animals were subjected to 70% FR heart rate and mean aortic pressure did not change from baseline, but cardiac output and $LVdP/dt_{max}$ decreased to 2.4±0.2 L/min and 1510±110 mmHg/s, respectively (both $P<0.05$), at 30 min. During the remainder of the 90 min 70% FR and during the subsequent 60 min TCO no further changes in any of the systemic hemodynamic variables occurred. At the end of 120 min of Rep, cardiac

output and $LVdP/dt_{max}$ remained depressed while heart rate increased to 138±6 beats/min ($P<0.01$ vs baseline).

In the groups in which pigs were subjected to 30% flow reduction, significant hemodynamic changes did not occur in response to either 30 min or 90 min of 30% flow reduction. The subsequent 60 min TCO did not alter mean arterial pressure, but increased heart rate to 132±9 beats/min and decreased cardiac output to 1.9±0.1 L/min and $LVdP/dt_{max}$ to 1820±140 mmHg/s (all variables $P<0.05$ vs baseline). At the end of 120 min Rep, cardiac output and $LVdP/dt_{max}$ remained depressed; heart rate had increased further to 145±10 beats/min ($P<0.05$ vs 60 min TCO).

Relation to Infarct Size. Analysis of covariance with the infarct area as dependent factor, the experimental groups as independent factor, and the area at risk and rate pressure-product (either at baseline or at the onset of 60 min TCO) as covariates did not reveal a significant correlation between the rate-pressure product ($P>0.09$) and infarcted area. Regression analysis did also not reveal a significant correlation between IA/AR and the rate-pressure product at baseline ($r=0.12$) or at the onset of the 60 min TCO ($r=0.29$). Similar results were obtained when heart rate or systolic pressure were entered as covariates into the ANCOVA, or into the regression analysis.

Myocardial Contractile Function

At baseline systolic segment shortening in the myocardial regions perfused by the left anterior descending and the left circumflex coronary artery were 17±1% and 16±1%, respectively. Post-systolic segment shortening was minimal in all experimental groups (1±0.2%) under baseline conditions. In the control group segment shortening in the area perfused by the LADCA decreased to 1±1% during the 60 min TCO and did not recover during 120 min Rep (2±1%). The decrease in systolic shortening was accompanied by the appearance of post-systolic segment shortening (5±1% at the end 60 min TCO, $P<0.01$), which was not further altered during 120 min of Rep (4±1%). After 10 min of TCO and 15 min Rep, segment shortening had decreased to 11±2% ($P<0.01$), with a further depression to 2±1% ($P<0.01$) during the subsequent 60 min TCO and no recovery at the end of 120 min Rep. At 30 min of 70% FR systolic shortening was 2±1% ($P<0.01$), which did not change during the remainder of the 90 min period of FR (2±1%). No significant further deterioration of systolic function occurred during the subsequent 60 min TCO (0±1%) or 120 min Rep (-1±1%). Post-systolic shortening had increased to 11±1% at 30 min of 70% FR with no additonal changes during the remainder of the 90 min FR period (9±1%). At 30 min and 90 min of 30% FR systolic shortening had decreased to 12±2% and 13±2%, respectively (both $P<0.01$ vs baseline), while systolic shortening in the control region was not altered. During the subsequent 60 min TCO systolic shortening was further reduced to 0±1% and to -1±1% at 120 min Rep. Post-systolic shortening increased to 4±2% ($P<0.05$) at 30 min but was no

longer significant at 90 min of 30% flow reduction (2±1%).

Relation to Infarct Size. There was no correlation between the loss of systolic wall thickening produced by the precondtioning stimuli and the infarct size. Similarly, we did not observe a correlation between systolic segment shortening at the end of 120 min Rep and the IA/AR, indicating that ischemic preconditioning did not lead to improved functional recovery during the first 120 min of reperfusion. In contrast, an inverse relation was observed between the magnitude of post-systolic thickening (a marker of myocardial tissue viability) of the anterior wall at the end of reperfusion and transmural IA/AR ($r=0.54$, $P<0.001$) (Fig 6). Analysis of animals with an area at risk ≥20% of the left ventricle showed a slight improvement in the correlation ($r=0.62$).

DISCUSSION

The present study has yielded several new findings. 1) Infarct size limitation afforded by a single 10 min total coronary artery occlusion and 15 min reperfusion preceding a 60 min total coronary artery occlusion is distributed homogeneously across the left ventricular wall in pigs. 2) Infarct size was also limited by a 70% coronary blood flow reduction for 30 or 90 min prior to a 60 min total coronary artery occlusion without the need of intermittent reperfusion. The transmural distribution of this protective effect depended critically on the duration of flow reduction. Thus, whereas 30 min of 70% flow reduction produced similar decreases in infarct size in the inner- and outer half of the left ventricle, 90 min of 70% flow reduction preferentially limited infarct size in the epicardium. 3) In contrast, protection against myocardial infarction or ventricular fibrillation was not afforded by either 30 or 90 min of 30% flow reduction. 4) The incidence of ventricular fibrillation during the 60 min total coronary artery occlusion was not altered by the classical preconditioning stimulus, but was significantly reduced when 90 min of 70% flow reduction preceded the sustained ischemic period. The implications of these findings will be discussed in detail.

Ischemic preconditioning and short-term recovery of contractile function

In the present study we did not observe a correlation between systolic segment shortening at the end of 120 min Rep and IA/AR, indicating that ischemic preconditioning did not improve recovery of systolic function recovery during the first 120 min of reperfusion. In the present study we observed an inverse relation between the magnitude of post-systolic thickening of the anterior wall at the end of 120 min reperfusion and transmural IA/AR. This agrees well with previous observations that post-systolic segment shortening is a marker of myocardial viability[22]

Ischemic preconditioning and ventricular fibrillation during subsequent sustained ischemia

Studies in rats reported that single or multiple brief total coronary artery occlusions decrease the incidence of ventricular fibrillation during a subsequent sustained period of ischemia.[23,24]

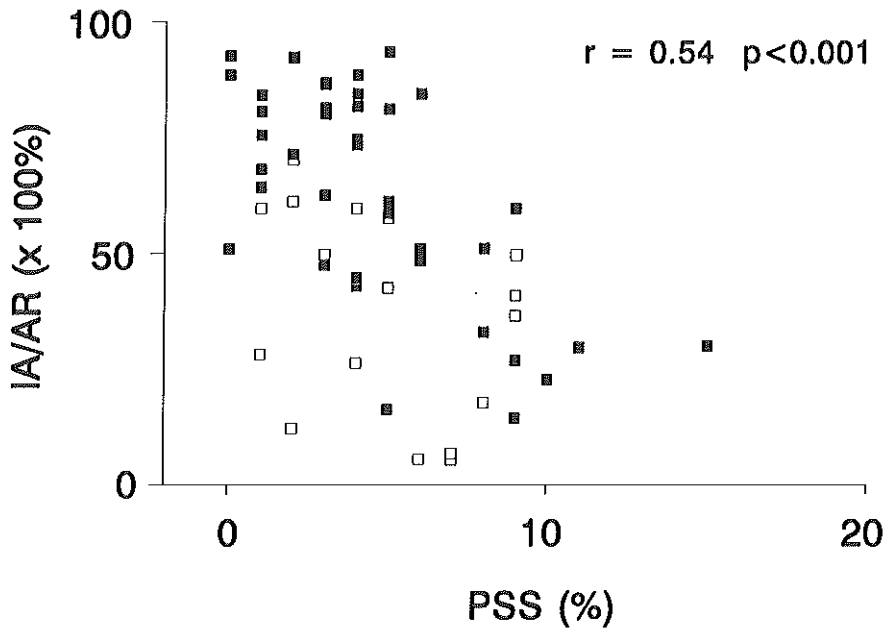

Figure 6 Relations between post-systolic segment shortening (PSS) measured at the end of reperfusion and transmural infarct area expressed as a percent of the area at risk (IA/AR). Shown are data from the six experimental groups that underwent 60 min of total coronary artery occlusion (TCO) and the animals that were subjected to 90 min of 70% flow reduction (FR) without 60 min TCO. In closed circles are shown the animals with AR greater or equal to 20% and in open circles are shown animals with AR<20%. Regression analysis of all animals showed a moderate but statistically significant correlation (r=0.54, p<0.0001); analysis of the animals with AR greater or equal to 20% improved the correlation slightly further (r=0.63, p<0.0001).

Also, a preliminary study in pigs reported that a single 5 min total coronary artery occlusion followed by 30 min of reperfusion reduced the incidence of ventricular fibrillation during a 30 min occlusion.[24] In contrast, we previously observed that a 10 min coronary artery occlusion and 15 min reperfusion was ineffective against ventricular fibrillation during the 60 min coronary occlusion.[11] An explanation for the different results in our study and that of Parratt and Vegh[24] is not readily found but could be due to a number of factors such as different durations of the preconditioning stimulus and the intermittent reperfusion periods and the areas at risk. In the present study a partial coronary artery occlusion immediately preceding the sutained period of ischemia (analogous to Harris' two-stage coronary artery occlusion)[25] significantly suppressed the occurrence of ventricular fibrillation provided that the duration and severity of flow reduction were sufficient. These findings are in agreement with earlier studies from our laboratory in partial flow reductions can decrease the incidence of ventricular

fibrillation during a sustained coronay artery occlusion.[25]

To study the effects of preconditioning on infarct size, a 60 min total coronary artery occlusion was used that resulted in significant myocardial necrosis. Since ventricular fibrillation during reperfusion in pigs occurs predominantly after occlusions of 10-30 min[25,26] these arrhythmias were rare in the present study. Consequently, the efficacy of preconditioning on fibrillation during reperfusion could not be assessed.

Transmural Distribution of Myocardial Infarct Size

Relation Between Infarct Area and Area at Risk in Control Animals and Animals Subjected to Classical Preconditioning.

In anesthetized and awake dogs, IA and AR (both expressed as percent of left ventricular mass) are linearly related with a positive intercept on the AR-axis.[27,28] In the present study we found a highly linear relation with a positive intercept on the AR-axis in both the endocardial and epicardial half of the left ventricle in pigs subjected to 60 min of total coronary artery occlusion. The reason for the positive intercept is incompletely understood, but it is possible that infarct size development in small areas at risk progresses less rapidly than in larger areas at risk. The intercept in dogs has been explained on the basis of progressive increases in collateral flow as the area at risk becomes smaller.[27,28] This is an unlikely explanation in rabbits[29] and pigs[11] since these species lack significant coronary collateral circulation. Interestingly, the AR intercept in dogs is considerably higher in the epicardium than in the endocardium likely because collateral flow is highest in the outer layer.[30] In contrast, we found similar intercepts in the inner and outer halves of the left ventricle, which correlates well with the transmurally homogeneous blood flow reductions distal to a total coronary artery occlusion in pigs.[31,32]

In dogs myocardial infarction occurs preferentially in the subendocardial layers, due to the transmural gradient of collateral blood flow. But also in pigs, in which total coronary artery occlusions result in transmurally homogeneous blood flow reductions, infarction progresses from inner to outer layer[32,33] possibly due to higher energy demands in the inner layers. In agreement with these findings we observed that for a given area at risk endocardial infarct area produced by 60 min total coronary artery occlusion was slightly larger than infarct area in the epicardial half of the left ventricle.

In pigs that were preconditioned by a 10 min total coronary artery occlusion and by 15 min of reperfusion the relation between infarct area and area at risk was linear, with a similar AR-intercept but with a lower slope of the relation than the animals in the control group. The decrease in slope was similar in the endo- and epicardium indicating that the protection afforded by the 10 min occlusion was identical in the inner and outer half of the left ventricle.

Partial Coronary Artery Occlusion: Importance of Severity and Duration of Flow Reduction for the Distribution of Infarct Size Limitation.

Recently, Ovize *et al*[10] reported that moderate myocardial ischaemia in dogs caused by a 50% reduction in myocardial blood flow lasting for 15 or 25 min failed to reduce infarct size during a subsequent 60 min TCO unless intermittent reperfusion was allowed. In contrast, infarct size could be limited by 30 min of 70% coronary flow reduction without intermittent reperfusion in pigs.[11] The present study extends these findings and indicates that a partial coronary artery occlusion can be as effective as the classical model in which preconditioning is elicited by a brief total occlusion separated from the sustained occlusion by a period of complete reperfusion.

The magnitude of protection was not different between 30 min or 90 min exposure to the coronary flow reductions, but critically depended on the severity of the flow reduction. Thus, a 70% reduction of coronary flow preceding the 60 min of TCO reduced infarct size, whereas infarct size limitation was not observed when the myocardium was first subjected to 30% reduction in coronary artery flow. The protection produced by the 30 min 70% flow reduction was not different between endo- and epicardium. However, when the duration of 70% flow reduction prior to the 60 min TCO was extended from 30 min to 90 min, infarct size limitation in the endocardial half was significantly smaller than that in the epicardial half of the left ventricle. 90 min of 70% flow without the 60 min TCO produced larger infarct size in the endocardium than in the epicardium, which is likely associated with more severe flow reductions in the endo- than in the epicardium distal to a coronary artery stenosis. The magnitude of additional necrosis produced by the 60 min TCO was not different between endocardium and epicardium. Thus, the observation that 90 min of 70% flow reduction resulted in less infarct size reduction (compared to the control group) in the endocardium than in the epicardium was due to a greater degree of irreversible damage in the endocardium already produced by this duration of severe flow reduction.

Ninety min of 30% flow reduction alone did not result in necrosis in either epi- or endocardium, whereas 90 min of 70% flow reduction produced necrosis in the endocardium but also in the epicardium. This suggests that the decrease in epicardial flow during 70% coronary artery flow reduction was more severe than the decrease in endocardial flow during 30% coronary artery flow reduction. In the present study we did not measure the transmural distribution of myocardial blood flow. Previous studies, including from our laboratory, reported that a 70% coronary flow reduction in pigs is associated with approximately 80% reduction in blood flow to the inner half and approximately 60% reduction in blood flow to the outer half of the left ventricle.[14,15] In contrast, a 25-30% reduction in total myocardial flow resulted in approximately 20% flow reduction to the epicardial half and approximately 40% flow reduction to the endocardial half.[12,13,17,38] Thus, the epicardial flow deficit during 70% coronary artery flow reduction was likely to be more severe than the endocardial flow deficit associated with 30% flow reduction.

While ischemia was not severe enough to produce myocardial necrosis after 90 min, a 30% reduction coronary blood flow *did* result in ischemia as indicated by a 25% decrease in systolic segment shortening. It is well established that metabolism of an ischemic segment changes continuously during a fixed reduction in coronary blood flow, while contractile function remains depressed. Studies from several laboratories, including our own, have shown that myocardial lactate production and efflux of potassium ions increase during the early period of a fixed flow reduction, but that there is a normalization of ischemia-induced metabolic changes as the hypoperfusion is prolonged.[34-38] Specifically, a 30-40% coronary blood flow reduction in pigs produces metabolic abnormalities (increase in myocardial lactate content and production and a decrease in myocardial phosphocreatine levels) that reach a nadir at approximately 15 min after the onset of flow reduction followed by significant recovery towards baseline within 60 min.[17,34,38] Thus, lack of necrosis after 90 min of 30% FR despite continuing myocardial hypoperfusion and hypofunction could be due to metabolic adaptations. The metabolic recovery also suggests that a period of 30% flow reduction in pigs lasting longer than 90 min is not likely to produce myocardial necrosis.

The findings of the present study are compatible with the hypothesis that an ischemic stimulus can produce myocardial preconditioning only when the degree of ischemia *per se* is severe enough so that if the myocardium would be exposed long enough to the stimulus it would produce myocardial necrosis. Thus, a total coronary occlusion (requiring intermittent reperfusion) and a 70% coronary flow reduction protected the myocardium against a subsequent sustained ischemic insult. In contrast, when the degree of ischemia was too mild to produce myocardial necrosis, protection against myocardial infarction was absent. In view of the metabolic adaptations that have been described in response to prolonged periods of 30% flow reductions, the present study suggests that myocardial preconditioning and the metabolic adaptations to mild ischemia ("hibernation") have separate mechanisms as hibernation itself does not lead to infarct size limitation when the artery becomes subsequently totally occluded. An alternative explanation could be that preconditioning requires a threshold stimulus such as activation of protein kinase C which is thought to be the intracellular target for several mechanisms that can precondition the myocardium.[39,40] Thus, while the flow reduction itself may not reach the threshold level of stimulation, addition of another subthreshold stimulus (e.g. a low dose of a K^+_{ATP} channel opener) could result in protection as the threshold stimulus for preconditoning is reached by the simultaneous presence of two stimuli. This hypothesis is supported by findings that both a 90 s coronary artery occlusion and a low dose of aprikalim failed to precondition the myocardium, but simultaneous exposure of the myocardium to the two stimuli resulted in a synergistic myocardial protection during a period of sustained ischemia.[41] A threshold phenomenon would also explain why we previously observed that ischemic preconditioning in pigs is an all or nothing phenomenon.[42] Thus, protection did not appear to be lost gradually as the duration of reperfusion between the preconditioning stimulus and the sustained occlusion was extended from 1 to 4 hours at one hour intervals. Rather,

when the duration between the previous 10 min occlusion and the sustained occlusion in an individual animal was sufficient the animal was as effectively protected as at 15 min after the 10 min occlusion. However, when too much time had elapsed and the degree of stimulation decreased below the threshold level for that animal protection was abruptly and completely lost.

Clinical Relevance

Until recently, myocardial preconditoning was studied using brief total coronary artery occlusions followed by complete reperfusion. These abrupt total occlusion and reperfusion sequences are useful for the study of basic mechanisms of myocardial precondtioning but do not reflect the clinical situation where patients with myocardial infarction often have significant coronary artery lesions associated with (transient) reductions in coronary blood flow. Study of the infarct size limiting effects of partial coronary artery occlusions may help to determine whether patients can benefit from preconditioning. The present study shows that a partial flow reduction can be a preconditioning stimulus that is as effective as the classical brief total occlusion and reperfusion sequence, provided that the severity of flow reduction is sufficient. Our data also suggest that mild flow reductions, even when sustained for a long period of time, may not produce sufficient stimulation to precondition the myocardium. However, it is possible that the addition of another subthreshold stimulus, e.g. a low dose of a K^+_{ATP} channel opener, could result in protection. Our findings may also have implications for interpretation of studies in which the effects of reperfusion therapy on infarct size are evaluated, as the myocardial perfusion status immediately preceding the coronary artery occlusion may be an important source of infarct size variability.

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Chapter 7

Rapid Ventricular Pacing Produces Myocardial Preconditioning by Non-ischemic Activation of K^+_{ATP} Channels

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ABSTRACT

Background. Rapid ventricular pacing reduces ventricular arrhythmias during a subsequent sustained period of ischemia and reperfusion. We investigated whether rapid ventricular pacing also limits myocardial infarction and determined the role of myocardial ischemia and activation of K^+_{ATP} channels in the protection afforded by ventricular pacing.

Methods and Results. Myocardial infarction was produced by a 60 min coronary artery occlusion in open-chest pigs. Infarct size of pigs subjected to 10 min of ventricular pacing at 200 beats per min followed by 15 min of normal sinus rhythm prior to the occlusion (83±2% of the area at risk, mean±SEM) was not different from control infarct size (85±2%). Thirty min pacing followed by 15 min sinus rhythm resulted in marginal albeit significant reductions in infarct size (72±2%, $P<0.05$ versus control). In contrast, 30 min pacing immediately preceding the occlusion without intervening sinus rhythm resulted in considerable limitation of infarct size (62±4%, $P<0.05$). The K^+_{ATP} channel blocker glibenclamide abolished the protection by pacing (78±5%, P=NS). K^+_{ATP} channel activation did not appear to involve ischemia: (*i*) myocardial endo/epi blood flow ratio was 1.07±0.08, (*ii*) phosphocreatine and ATP levels and arterial-coronary venous differences in pH and P_{CO2} were unchanged, (*iii*) end-systolic segment length did not increase and post-systolic shortening was not observed during pacing, and (*iv*) systolic shortening recovered immediately to baseline levels and coronary reactive hyperemia was absent following cessation of pacing.

Conclusions. Ventricular pacing preconditioned myocardium via non-ischemic activation of K^+_{ATP} channels.

INTRODUCTION

Myocardial preconditioning can be induced by a variety of ischemic stimuli. Thus, one or more brief total[1] or partial[2, 3] coronary artery occlusions can limit infarct size produced by a sustained ischemic period. Moreover, infarct size can be limited by transient ischemia in adjacent myocardium[4] or even different organs.[5,6] In all these studies a temporary interruption of oxygen supply either within or outside the myocardial region of interest was required to produce preconditioning. Recent studies suggest that non-ischemic stimuli may also precondition the myocardium. Thus, Ovize et al[7] reported that an increase in left ventricular wall stretch produced by acute volume overload protected the myocardium against infarction during a subsequent 60 min coronary artery occlusion. Also two consecutive 2 min periods of rapid ventricular pacing in open-chest dogs reduced the incidence of ventricular arrhythmias during and immediately following a subsequent 25 min coronary artery occlusion[8]. In contrast, Marber et al[9] failed to show a protective effect of a single five min period of rapid atrial pacing against myocardial infarction in the rabbit heart. To date no study has addressed the effect of *rapid ventricular pacing* on infarct size development produced by a sustained coronary artery occlusion.

In the present study we therefore investigated whether rapid ventricular pacing preceding a 60 min total coronary artery occlusion (60min TCO) altered infarct size development in open-chest pigs. In two groups of animals we studied the effects of either 10 min or 30 min of rapid ventricular pacing followed by 15 min of normal sinus rhythm on infarct size produced by 60min TCO, analogous to the classical preconditioning model of a brief ischemic stimulus followed by reperfusion. In view of our earlier findings that ischemia produced by a partial coronary artery occlusion can precondition the myocardium without the need for intermittent reperfusion[3], we also studied a third group of animals in which the 30 min rapid ventricular pacing period preceded the 60min TCO without normal sinus rhythm. If rapid ventricular pacing produces protection by inducing ischemia,[8,10] protection is likely to be distributed heterogeneously across the left ventricular wall because ischemia would occur predominantly in the inner layers. Consequently, infarct size was also determined for the outer and inner halves of the left ventricle. To explore the mechanism of preconditioning produced by rapid ventricular pacing, we investigated if ischemia occurred in animals subjected to 30 min of rapid ventricular pacing followed by 180 min of normal sinus rhythm *without* 60min TCO. Also, in view of evidence that ventricular pacing can activate ventricular K^+ channels,[11, 12] and that activation of K^+_{ATP} channels is cardioprotective in pigs,[13,14] we also studied the role of K^+_{ATP} channels in preconditioning induced by rapid ventricular pacing.

Methods

All experiments were performed in accordance with the "Guiding principles in the care and use of animals" as approved by the Council of the American Physiological Society and under the regulations of the Animal Care Committee of the Erasmus University Rotterdam.

Experimental Groups

Studies were performed in a total of 46 pigs assigned to six experimental groups (Fig 1). In five groups, animals underwent a 60 min total coronary artery occlusion (60min TCO) followed by 120 min of reperfusion. Eleven animals served as control and underwent only a single 60min TCO. Two groups of animals underwent a 60min TCO preceded by either 10 min (n=4) or 30 min (n=6) of rapid left ventricular pacing (RVP) at 200 bpm and 15 min of normal sinus rhythm. In 18 animals the 60min TCO was preceded by 30min RVP at 200 bpm without an intermittent period of normal sinus rhythm; 7 of these animals were pretreated with glibenclamide (1 mg/kg, iv) 10 min before the start of RVP. This dose of glibenclamide was chosen as it was previously shown to block preconditioning by a single 10 min coronary occlusion in pigs.[15] In the latter two groups ventricular pacing was terminated immediately (< 10 s) following the start of the 60 min left anterior descending coronary artery (LADCA) ligation. To evaluate wether RVP produced myocardial ischemia, wall function in the distribution area of the LADCA, high energy phosphates, oxygen consumption and regional

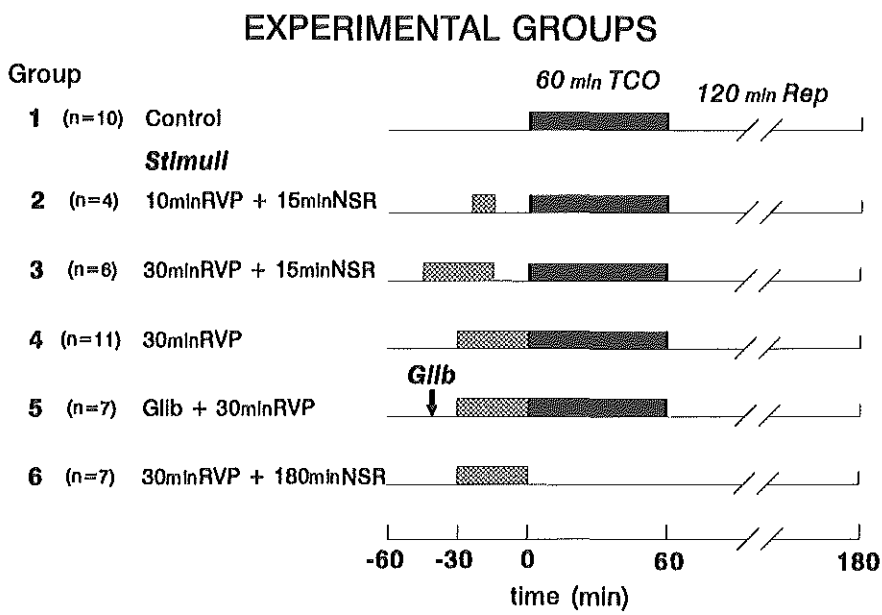

Figure 1 Experimental groups in which the effect of rapid ventricular pacing on infarct size after a 60 min total coronary artery occlusion was determined. Filled bar = 60 min total coronary artery occlusion (60min TCO), hatched bar = rapid ventricular pacing (RVP). NSR = normal sinus rhythm, Rep = reperfusion, Glib = glibenclamide (1 mg/k, iv).

myocardial blood flow were obtained in seven animals throughout a 30 min period of left ventricular pacing at 200 bpm followed by 180 min of normal sinus rhythm.

Surgical procedure

Domestic Yorkshire-Landrace pigs (25-35 kg, HVC, Hedel, The Netherlands) were sedated with ketamine (20 mg/kg, im), anesthetized with pentobarbital (25 mg/kg, iv) and instrumented for measurement of arterial and left ventricular pressure and control of arterial blood gases.[16] Following administration of pancuronium bromide (4 mg i.v., Organon Teknika B.V., Boxtel, The Netherlands) and a midline thoracotomy, an electromagnetic flow probe (Skalar, Delft, The Netherlands) was placed around the ascending aorta to measure cardiac output (Fig 2). The left anterior descending coronary artery (LADCA) was dissected free from the surrounding tissue to allow placement of a microvascular clamp (groups 1-5) and a Doppler flow probe (Crystal Biotech Inc., Hopkinton, MD, U.S.A.) (groups 2-6). In the animals that underwent RVP an electrode was attached to the anteriolateral left ventricular wall close in the vicinity of the apex for stimulation of the myocardium by electrical monophasic stimuli with

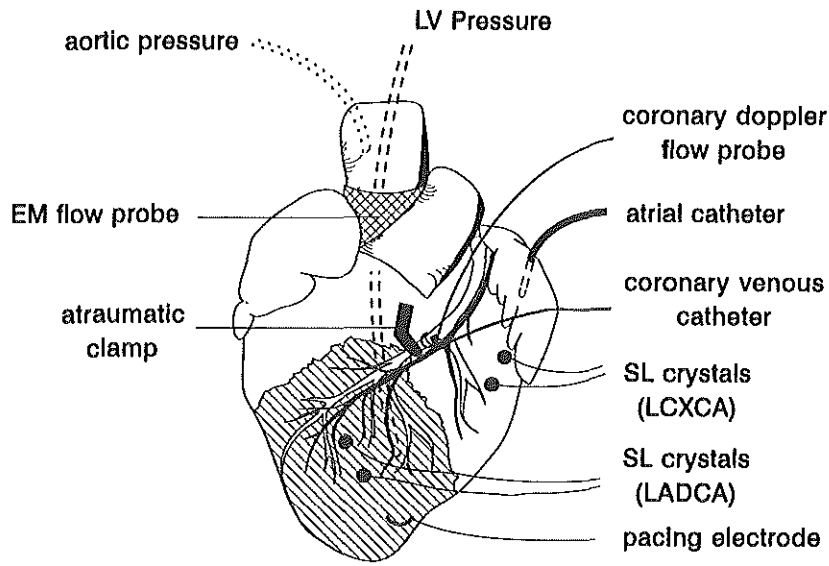

Figure 2 Schematic presentation of the experimental model. The pacing electrode was inserted into the superficial layers of the anteriolateral wall of the left ventricle. Pairs of ultrasonic crystals were inserted into the mesocardial layers of the left anterior descending coronary artery (LADCA) area and the left circumflex coronary artery (LCXCA) area. The LADCA was occluded at the site of the clamp causing the shaded area to become ischemic. The site of the clamp was varied in the control animals to create a range of areas at risk. LV = left ventricle, EM = electromagnetic, SL = segment length.

an amplitude of 2 mA and a frequency of 3.33 Hz. A small cannula was inserted into the vein accompanying the LADCA for the withdrawal of local venous blood for the determination of blood gases.

Regional Myocardial Function

In all six groups, pairs of ultrasonic crystals (Sonotek Corporation, Del Mar, CA, USA) were positioned into the midmyocardial layers of the left ventricle in the distribution areas of the LADCA and the left circumflex coronary artery (LCXCA) for the measurement of regional segment shortening by sonomicrometry[14,16] (Triton Technology Inc., San Diego, CA, USA)(Fig 2). From the segment length tracings systolic segment length at the end of diastole (EDL, onset of positive ascending aortic flow) and the length at the end of systole (ESL, end of positive aortic flow) were determined and regional systolic segment shortening (SS) was computed as:

$$SS(\%) = 100 \cdot (EDL - ESL)/EDL,$$

and post-systolic segment shortening (PSS) was calculated as:

$$PSS(\%) = 100 \cdot (ESL - \text{minimum segment length})/EDL.$$

Regional Myocardial Blood Flows

In the animals of group 6 (Fig 1) we also investigated the effects of rapid ventricular pacing on the distribution of transmural myocardial blood flow. For this purpose, the left atrial appendage was cannulated for injection of 1-2 $*10^6$microspheres, 15 ± 1 (SD) μm in diameter (NEN Company, Dreieich, Germany), labelled with either ^{95}NB, ^{103}Ru, ^{113}Sn, ^{46}Sc or ^{141}Ce. Processing of myocardial tissue samples and computation of blood flow data have been described earlier.[15]

High Energy Phosphate Metabolism

High energy phosphates were measured in transmural myocardial biopsies, taken with a Tru-Cut needle (Travenol Laboratories Inc., Deerfield, IL, USA) from the area perfused by the left circumflex coronary artery (LCXCA) at baseline and immediately before the 60min TCO. This procedure allowed assessment of the effects of ventricular pacing on high energy phosphate metabolism without interfering with the infarct size determination in the area perfused by the LADCA. Biopsies were immediately dipped into 0.9% NaCl at 0°C to remove adherant blood, frozen in liquid nitrogen (within 10 s) and stored until analysis at -80°C. Adenine nucleotides (ATP, ADP, AMP), creatine (Cr) and creatine phosphate (CrP) were measured by isocratic ion-pairing high performance liquid chromatography as previously described.[15] From these measurements CrP/Cr and CrP/ATP ratios were calculated to estimate changes in oxidative phophorylation potential. Energy charge was calculated as ([ATP + 0.5 ADP])/[ATP + ADP + AMP].[17]

Experimental protocols

After completion of the instrumentation, 5,000 I.U. of heparin were administered intravenously and a stabilization period of at least 30 min was allowed before baseline data were obtained of systemic hemodynamic variables, coronary blood flow and regional segment length changes. The animals were then subjected to one of the six study groups (Fig 1). In case of ventricular fibrillation defibrillation using DC countershocks (15-30 Watt) was started within 10 s. If defibrillation could not be accomplished within 2 min, animals were excluded from further study. Throughout the experimental protocol body core temperature was rigorously controlled with a heating pad to maintain temperature within a narrow range (37-38°C) to minimize temperature-induced infarct size variability.[18,19]

In the animals of groups 3 and 6 arterial and coronary venous blood samples for the determination of oxygen content and pH were withdrawn at baseline, at 10 min and 30min RVP and at 2, 5 and 15 min of normal sinus rhythm. In the animals of group 6 measurements were also made at 60, 120 and 180 min of normal sinus rhythm. Myocardial biopsies for the measurement of high energy phosphate levels in the LCXCA perfused area were obtained at baseline and at 30min RVP. The effects of RVP on the distribution of myocardial blood flow were determined in group 6 with radioactive microspheres at baseline and at 30min RVP.

Area at risk and infarct size

Validation of the methods to determine the area at risk and infarct size has been described extensively.[14,20] Briefly, following reocclusion of the LADCA the area at risk was identified by an intra-artrial injection of 30 ml of a 5 % (w/w) solution of fluorescein sodium (Sigma Chemical Co, St. Louis, USA). Ventricular fibrillation was then induced with a 9V battery and the heart was excised. Both atria, the right ventricular free wall and the left ventricular epicardial fat were removed. The left ventricle was filled with alginate impression material (BayerDental, Leverkusen, Germany), cooled in crushed ice and sliced parallel to the atrioventricular groove into 5 segments. The cut surface(s) of each segment and the demarcated areas at risk (AR) was (were) then traced on an acetate sheet under UV light. The viable myocardium was then stained deeply blue by incubating the segments for 20 min in 0.125 g para-nitrobluetetrazolium (Sigma Chemicals Co., St. Louis, USA) per liter of phosphate buffer (pH 7.1) at 37°C. The non-stained pale infarcted tissue was traced onto the acetate sheet. The surface of each ring was subdivided into an endocardial (inner) half and an epicardial (outer) half by drawing a line which divided the myocardial wall into only two layers of equal thickness. Division into two layers was done as it provides information on the transmural distribution of infarct size, yet preserving sufficient accuracy of infarct size determination in the two halves. Surface areas of the subendocardial and subepicardial halves, and of the subendocardial and subepicardial areas at risk and infarct areas (IA) were determined and averaged for the apical and basal side of each individual ring. The fraction of the ring that was infarcted and at risk were then multiplied by the weight of the ring to yield the weight of the area at risk and infarct area for that ring. Subsequently, the weights of the subendocardial and subepicardial halves and the total weight of each ring were summed to yield the LV_{endo}, LV_{epi} and total LV mass. The weights of the endocardial, epicardial and total areas at risk of each ring were summed to yield AR_{endo}, AR_{epi} and total AR mass; the weights of the endocardial, epicardial and total infarct areas of each ring were added to yield IA_{endo}, IA_{epi} and total IA mass. Endocardial, epicardial and total AR and IA data were expressed as a percentage of LV_{endo}, LV_{epi} and total LV mass, respectively.

Data analysis

Infarct size data have been presented by plotting the IA against AR for the endocardial and epicardial half and for the whole left ventricular wall. Linear regression analysis was performed to determine the relation between endocardial and epicardial IA and AR in the control group. For all experimental groups individual infarct size data points are presented. Intergroup differences between IA_{endo}, IA_{epi} or total IA were analyzed by analysis of covariance (ANCOVA), with AR_{endo}, AR_{epi} or total AR as covariate. When a significant effect was observed comparisons between individual groups were made with ANCOVA followed by modified Bonferroni procedure to correct for multiple comparisons. Intragroup differences between IA_{endo} and IA_{epi} were analyzed using ANCOVA for repeated measurements, with

AR_{endo} and AR_{epi} as covariates. The effect of rapid ventricular pacing on the incidence of ventricular fibrillation during 60min TCO was analyzed by Fisher's exact test.

Hemodynamic and regional myocardial function data were analyzed by two-way ANOVA followed by either paired t-test (intragroup) or unpaired t-test (intergroup) with modified Bonferroni procedure to correct for multiple comparisons. A P value less than 0.05 was considered statistically significant (two-tailed). Data are presented as Mean ± SEM.

Results

Ventricular Fibrillation and Mortality

In the control group one animal was excluded because of unsuccessful defibrillation during the 60min TCO. All other animals that fibrillated were defibrillated successfully. Table 1 shows that ventricular fibrillation occurred in 7 of the 11 control animals during the 60min TCO. The incidence of ventricular fibrillation in all groups that were subjected to rapid ventricular pacing before the 60min TCO (groups 2-5) was not significantly different from the control group indicating that in this model RVP did not protect against ventricular fibrillation during the subsequent coronary artery occlusion. Ventricular fibrillation was rare when reperfusion was reinstated which is in agreement with previous observations that in pigs ventricular fibrillation during reperfusion occurs predominantly following 10-30 min coronary artery occlusions.[21]

Table 1. Ventricular fibrillation

Experimental groups	60 min TCO	Reperfusion
Control	7(11)°	1(10)°
10 min RVP + 15 NSR + 60 min TCO	1(4)	0(4)
30 min RVP + 15 NSR + 60 min TCO	4(6)	0(6)
30 min RVP + 60 min TCO	6(11)	0(11)
Glib + 30 min RVP + 60 min TCO	2(7)	0(7)

In parentheses the total number of animals per group at the onset of each intervention is indicated. TCO = Total coronary artery occlusion; RVP = rapid ventricular pacing at 200 beats per minute; NSR = normal sinus rhythm; Glib = Glibenclamide 1 mg/kg i.v. as a bolus 10 min prior to the onset of RVP; ° one pig fibrillated during both 60 min TCO and Reperfusion

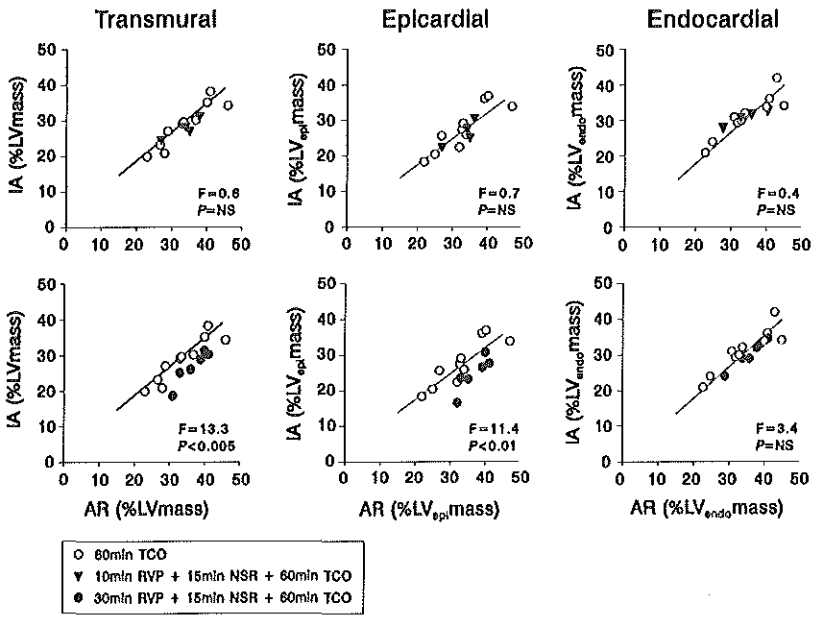

Figure 3 Effects of rapid ventricular pacing on the transmural, endocardial and epicardial relations between infarct area (IA) and area at risk (AR). Shown are the relations in the control group (60 min of total coronary artery occlusion, 60min TCO) and animals subjected to either 10 or 30 min of rapid ventricular pacing (RVP) separated from the 60min TCO by 15 min of normal sinus rhythm (15min NSR). Note that while 10min RVP had no effect on transmural infarct size (F=0.6, P=NS), 30min RVP produced a small, but statistically significant decreases in transmural infarct size (F=13.3, $P<0.005$). This was due to a selective reduction in epicardial infarct size (F=11.4, $P<0.01$), as endocardial infarct size was not significantly altered (F=3.4, P=NS). NS=not significant.

Infarct Area - Area at Risk Relation

Mean areas at risk (expressed as percentage of left ventricular mass) for the five experimental groups of animals which underwent the 60min TCO were not different from each other (34±2%, 34±2%, 37±2%, 31±2% and 31±3% for groups 1,2,3,4 and 5, respectively; F=1.0, P=0.41).

In the 10 control animals transmural infarct area was linearly related with the area at risk (r=0.92, $P<0.001$; Fig 3). Separation of the left ventricular wall into two layers of equal thickness revealed a highly linear relation in both endocardial half (r=0.91, $P<0.001$) and epicardial half (r=0.88, $P<0.001$) of the left ventricle. Ten min of RVP, separated from the 60min TCO by a 15 min period of normal sinus rhythm, failed to reduce transmural, epicardial and endocardial infarct size compared to the control group (Fig 3). When the

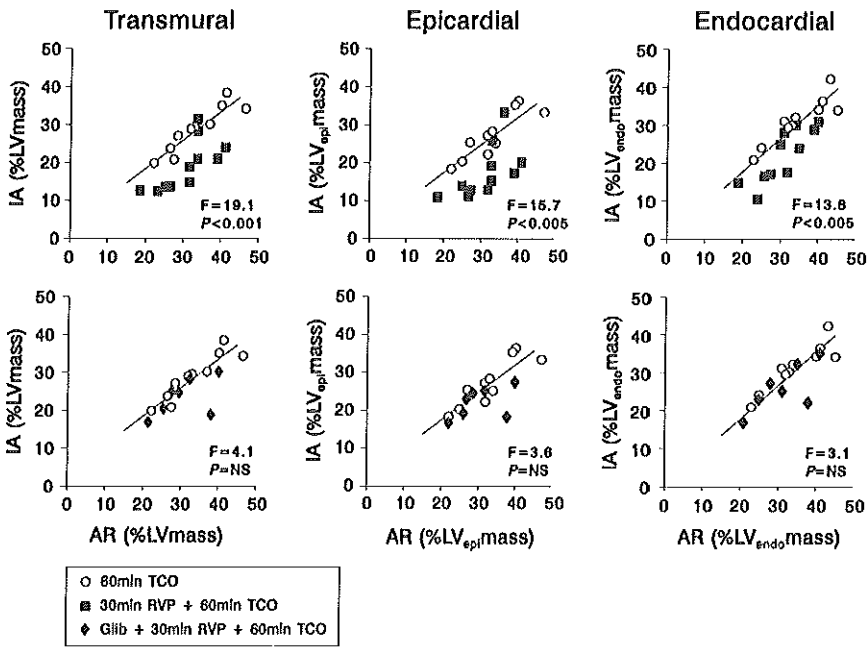

Figure 4 Effects of rapid ventricular pacing on the transmural, endocardial and epicardial relations between infarct area (IA) and area at risk (AR). In the top panels are shown the control animals (60 min of total coronary artery occlusion, 60min TCO) and the animals subjected to 30 min of rapid ventricular pacing (RVP) immediately preceding the 60min TCO. 30min RVP produced significant reductions in transmural ($F=19.1$, $P<0.001$), epicardial ($F=15.7$, $P<0.005$) and endocardial ($F=13.8$, $P<0.005$) infarct size. The lower panels illustrate that pretreatment with glibenclamide (Glib, 1 mg/kg, intravenous) prevented the protective effects of rapid ventricular pacing. NS=not significant.

period of rapid venticular pacing was extended to 30 min, infarct size in the endocardial half was again not significantly different from that in the control group ($F=3.4$, $P=0.09$), but now small albeit statistically significant reductions in epicardial ($F=11.4$, $P<0.01$) and transmural ($F=13.3$, $P<0.005$) infarct size were observed (Fig 3). In this group of animals the transmural IA/AR ratio was also significantly lower than that in the control group ($72\pm2\%$ versus $85\pm2\%$, $P<0.01$).

The transmural IA in eight of the eleven animals that underwent 30min RVP immediately followed by 60min TCO was located well below the regression line describing the relation between IA and AR in the control group (Fig 4). The IA/AR of this group of animals was $62\pm4\%$ ($P<0.01$ versus control group). ANCOVA showed that 30min RVP immediately followed by 60min TCO significantly reduced infarcted area for a given area at risk compared

to the control group ($F=19.1$, $P<0.0005$). Further analysis indicated that the infarct size reduction was not different between subepicardium and subendocardium (Fig 4). Pretreatment with glibenclamide abolished the protective effect of 30min RVP in both the endocardial and epicardial halves (Figs 4). This is further illustrated by the IA/AR ratio which was 78±5% (P=NS versus control group and $P<0.05$ versus 30min RVP + 60min TCO).

Hemodynamic Responses to Rapid Ventricular Pacing and Total Coronary Artery Occlusion (Groups 1-5)

In the five groups that underwent 60min TCO (groups 1-5), there were no significant differences between heart rate (108±3 bpm, n=38), mean aortic pressure (85±1 mmHg), cardiac output (2.8±0.1 L/min), $LVdP/dt_{max}$ (1910±100 mmHg/s) or LV end-diastolic pressure (9±1 mmHg) at baseline. Rapid ventricular pacing in groups 2,3 and 4 was associated with immediate decreases in mean arterial blood pressure (38±3 %), cardiac output (41±2%) and stroke volume (69±2%), while systemic vascular resistance, left ventricular end diastolic pressure and $LVdP/dt_{max}$ remained unchanged. This hemodynamic profile was maintained during the remainder of the ventricular pacing period and was not different between the three groups. In the animals of groups 2 and 3, in which pacing was terminated without an immediate occlusion of the LADCA, all variables returned to baseline within 1 min of normal sinus rhythm except for heart rate which remained slightly (~15 bpm) elevated during the 15 min following cessation of ventricular pacing. In groups 1-4 (n=31), total coronary artery occlusion resulted in decreases in cardiac output (15±3%) and increments in heart rate (15±4%) and LV end-diastolic pressure (37±8%) compared to baseline values (all $P<0.05$), with no significant decrease in mean aortic pressure (4±3%); these responses were not different between the four groups. None of the hemodynamic variables recovered significantly toward baseline levels during 120 min of reperfusion.

Glibenclamide produced modest increments in left ventricular end-diastolic pressure from 9±1 to 11±3 mmHg ($P<0.05$) and mean aortic pressure from 84±3 to 95±4 mmHg (P=0.055). The latter was due to systemic vasoconstriction as cardiac output was not altered by the K^+_{ATP} channel blocker. Glibenclamide had no effect on LADCA blood flow, LADCA vascular resistance or systolic segment shortening in the LADCA perfused segment. Pretreatment with glibenclamide enhanced the pacing-induced decreases in mean aortic pressure (56±4%), cardiac output (62±3%), and coronary blood flow (40±6%) but had no effect on hemodynamic changes produced by 60min TCO.

Effect of Rapid Ventricular Pacing on Myocardial Performance (Groups 3 and 6)

Rapid ventricular pacing in groups 3 and 6 was associated with immediate decreases in mean arterial blood pressure and cardiac output and hence myocardial work, while $LVdP/dt_{max}$, systemic vascular resistance, and left ventricular end diastolic pressure were

TABLE 2. Systemic hemodynamics at baseline, during 30 minutes of rapid ventricular pacing and during subsequent 15 min of normal sinus rhythm.

Variable	Baseline	Rapid Ventricular Pacing (min)		Normal Sinus Rhythm (min)				
		10	30	0.5	1	2	5	15
HR	108 ± 4	200 ± 0*	200 ± 0*	137 ± 4*	133 ± 3*	132 ± 3*	126 ± 3*	119 ± 3*
CO	2.8 ± 0.2	1.8 ± 0.1*	1.9 ± 0.1*	3.1 ± 0.2	3.2 ± 0.3	3.1 ± 0.1	2.9 ± 0.1	2.6 ± 0.1
SV	26 ± 2	9 ± 1*	9 ± 1*	23 ± 1	24 ± 2	23 ± 1	23 ± 1*	22 ± 1*
MAP	83 ± 2	55 ± 3*	61 ± 2*	94 ± 4*	97 ± 4*	97 ± 3*	93 ± 2*	84 ± 2
SVR	31 ± 1	31 ± 2	33 ± 2	32 ± 3	32 ± 4	31 ± 1	33 ± 1	33 ± 1
$LVdP/dt_{max}$	1780 ± 130	1980 ± 220	2010 ± 160	1770± 150	1820 ± 130	1840 ± 100	1910 ± 160	1600 ± 140
LVEDP	9 ± 1	7 ± 1	8 ± 1	8 ± 1	8 ± 1	8 ± 1	8 ± 1	8 ± 1
MW	231 ± 12	102 ± 9*	116 ± 9*	294 ± 25*	312 ± 30*	302 ± 14*	271 ± 12*	218 ± 10

HR = heart rate (bpm); CO = cardiac output (L/min); SV = stroke volume (ml); MAP = mean arterial pressure (mmHg); SVR = systemic vascular resistance (mmHg/L/min); $LVdP/dt_{max}$ = maximum rise in left ventricular pressure (mmHg/s); LVEDP = left ventricular end diastolic blood pressure (mmHg); MW = myocardial work, MAP•CO (mmHg•L/min).

Data are mean±SEM, n=13; * $P<0.05$ vs baseline.

maintained (Table 2). Thirty min of rapid ventricular pacing decreased coronary blood flow by 20±5% (n=13) accompanied by a small increase in myocardial oxygen extraction from 72±2 % to 76±2 % ($P<0.05$, Table 3). Oxygen consumption per gram of myocardium tended to decrease during RVP, but this failed to reach levels of statistical significances. Microsphere data revealed that the subendocardial to subepicardial blood flow ratio at 30min RVP was maintained well above unity (1.07±0.08, n=6) although absolute levels were slightly lower than at baseline (1.23±0.07) ($P<0.05$). Coronary vascular resistance (calculated as mean arterial pressure divided by coronary blood flow per g of myocardium) was also maintained during pacing. Fractional systolic shortening decreased markedly in both the LADCA and the LCXCA perfused segments. However, this was due to a marked decrease in end-diastolic length of both the LADCA (17±1%) and the LCXCA (15±2%) perfused segments as end-systolic length of both LADCA (4±1%) and LCXCA (5±1%) segments decreased slightly (Table 3). Furthermore the decrease in systolic shortening during RVP was not accompanied by the appearance of post-systolic shortening. Throughout the pacing protocol the arterial-coronary venous differences in pH (0.04±0.01 and 0.04±0.01 at baseline and 30min RVP, respectively) and in pCO_2 (11.4±0.5 mmHg and 10.2±0.9 mmHg at baseline and 30min RVP, respectively) were maintained. In further support of aerobic metabolism we also did not observe decreases in ATP levels (36.3±1.4 μmol/g protein at baseline vs 36.5±1.4 μmol/g protein at 30min RVP), CrP/Cr ratio (1.24±0.12 vs 1.36±0.12), CrP/ATP ratio (1.52±0.26 vs 1.65±0.29) or energy charge (0.922±0.003 vs 0.924±0.003), at 30min RVP versus baseline, respectively.

Immediately after RVP was stopped systemic hemodynamic variables recovered to baseline values except for heart rate which remained slightly elevated following restoration to normal sinus rhythm. Mean aortic pressure increased to levels slightly higher than baseline during the first minute but had recovered to baseline levels at 15 min after cessation of rapid ventricular pacing. During the first minute of post-pacing systolic shortening in both the LADCA and LCXCA perfused segments recovered to baseline values, although this was followed by a slight decrease in systolic thickening in the LCXCA area during the remainder of the protocol. Because reactive hyperemia was also absent these findings indicate that 30min RVP was not associated with myocardial ischemia.

Discussion

The present study has yielded several important findings: 1) infarct size after 60 min coronary artery occlusion is limited when the occlusion is immediately preceded by a period of rapid ventricular pacing. 2) In contrast, when the period of rapid ventricular pacing was separated from the 60 min total coronary artery occlusion period by 15 min of normal sinus

TABLE 3. Regional myocardial performance at baseline, during and following 30 minutes of rapid ventricular pacing and during subsequent 15 min of normal sinus rhythm.

Variable	Baseline	Rapid Ventricular Pacing (min)		Normal Sinus Rhythm (min)				
		10	30	0.5	1	2	5	15
LADCA								
CBF	1.73 ± 0.18	1.47 ± 0.16	1.38 ± 0.18*	1.93 ± 0.21	1.91 ± 0.21	1.77 ± 0.20	1.70 ± 0.20	1.58 ± 0.21
CVR	0.55 ± 0.06	0.47 ± 0.08	0.52 ± 0.06	0.54 ± 0.05	0.56 ± 0.06	0.61 ± 0.06	0.62 ± 0.06	0.62 ± 0.06
$cvPO_2$	23.8±0.8	22.2±0.9	23.1±1.5	--	--	26.7±1.6*	24.8±1.1	23.5±0.8
O_2 extraction	72±2	76±2*	76±2*	--	--	68±4	71±2	73±1
MVO_2	6.84 ± 0.78	6.16 ± 0.73	6.01 ± 0.87	--	--	6.47 ± 0.90	6.93 ± 0.87	6.62 ± 1.02
EDL	9.50±0.23	7.88±0.18*	7.89±0.21*	9.86±0.26	9.87±0.24	9.71±0.21	9.59±0.20	9.34±0.22
ESL	7.96±0.20	7.72±0.17*	7.70±0.19*	8.33±0.24	8.22±0.21	8.19±0.17*	8.07±0.16	7.96±0.17
SS (%)	16.2 ± 0.9	2.8 ± 1.2*	2.5 ± 1.4*	14.8 ± 0.8	15.8 ± 0.9	15.6 ± 0.7	15.8 ± 0.5	14.8 ± 0.8
PSS (%)	1.3 ± 0.6	0.6 ± 0.4	0.8 ± 0.5	3.3 ± 0.9	2.4 ± 0.6	2.1 ± 0.5	2.3 ± 0.7*	1.4 ± 0.5
LCXCA								
EDL	10.87±0.35	9.19±0.41*	9.16±0.39*	10.86±0.44	10.85±0.49	10.79±0.38	10.73±0.36	10.58±0.36*
ESL	9.31±0.32	8.70±0.38*	8.71±0.31*	9.43±0.46	9.61±0.48	9.54±0.33*	9.43±0.32*	9.37±0.31
SS (%)	14.8 ± 1.5	9.7 ± 2.4*	9.8 ± 2.3*	12.7 ± 1.6	12.9 ± 1.5	10.8 ± 1.3*	12.1 ± 1.3*	11.6 ± 1.2*
PSS (%)	0.7 ± 0.4	0.2 ± 0.2	0.1 ± 0.1	0.4 ± 0.2	0.6 ± 0.3	0.6 ± 0.2	0.4 ± 0.2	0.4 ± 0.2

LADCA = left anterior descending coronary artery; LCXCA = left circumflex coronary artery; CBF = coronary blood flow ($ml \cdot min^{-1} \cdot g^{-1}$); CVR = coronary vascular resistance ($mmHg \cdot ml^{-1} \cdot min \cdot g$); $cvPO_2$ = coronary venous partial O_2 pressure (mmHg); O_2 extraction = O_2 extraction of the LADCA area (% of arterial O_2 content); MVO_2 = O_2-consumption of the LADCA area ($\mu l \cdot min^{-1} \cdot g^{-1}$); EDL= end diastolic segment length (mm); ESL= end systolic segment length (mm); SS = segment shortening; PSS = post-systolic segment shortening.
Data are mean±SEM, n=13; * $P<0.05$ vs baseline.

rhythm the protective effect of rapid ventricular pacing was nearly completely lost. 3) pretreatment with glibenclamide abolished the protective effect of rapid ventricular pacing suggesting the involvement of activation of K^+_{ATP} channels in the preconditioning and 4) the pacing-induced activation of K^+_{ATP} channels was not due to myocardial ischemia.

The protection afforded by rapid ventricular pacing against irreversible myocardial damage produced by a sustained period of ischemia was reversed by glibenclamide indicating that K^+_{ATP} channels mediate, at least in part, the protective mechanism of rapid ventricular pacing. Since K^+_{ATP} channel blockade inhibits ischemic preconditioning in several species including rabbits,[22] dogs[23] and swine,[15,24] it could be hypothesized that ventricular pacing produced preconditioning via induction of myocardial ischemia. In support of this hypothesis Vegh et al[8] and Szilvassy et al[10] reported that ventricular pacing produced myocardial ischemia as judged from myocardial ST-segment elevation. In contrast, in the open-chest pig model used in the present study we failed to observe evidence of myocardial ischemia during rapid ventricular pacing at 200 bpm: (*i*) transmural myocardial blood flow during rapid ventricular pacing remained equally distributed across the inner and outer layers of the left ventricular wall, (*ii*) the decrease in systolic shortening was entirely due to a decrease in end-diastolic length, not an increase in end-systolic length, (*iii*) development of post systolic shortening was not observed,[25] and (*iv*) no changes were observed in myocardial ATP and phosphocreatine levels, energy charge and arterial or coronary venous pH levels.[26] Furthermore, following restoration to normal sinus rhythm evidence for myocardial ischemia during the preceding period of rapid ventricular pacing was also absent because (*v*) reactive hyperemia did not occur, (*vi*) coronary venous oxygen tension was minimally affected following restoration to normal sinus rhythm, (*vii*) systolic segment shortening recovered instantaneously to baseline levels at which it was maintained throughout the subsequent 180 min normal sinus rhythm period and (*viii*) there was no sustained post-systolic shortening during normal sinus rhythm suggesting that post-ischemic myocardial stunning did not occur. These findings fail to support the occurrence of significant myocardial ischemia in the present study. Although we cannot entirely exclude the occurrence of subtle subendocardial ischemia, this certainly would have been insufficient to induce ischemic preconditioning as Ovize et al.[2] have shown that a 25 min 50% flow reduction immediately preceding a 60 min total coronary artery occlusion (resulting in total loss of contractile function in the area perfused by the partially occluded coronary artery) failed to limit infarct size. In addition, we recently observed that 30 or 90 min periods of 30% coronary blood flow reduction, associated with a 25% decrease in systolic segment shortening (due to an increase in end-systolic length), did not protect the myocardium against infarction produced by 60 min of total coronary artery occlusion immediately following the 30% flow reduction (unpublished data from our laboratory). Therefore if some endocardial might have gone undetected it is highly unlikely that this was responsible for the protective effect produced by rapid ventricular pacing.

Although the exact mechanism of K^+_{ATP} channel activation by ventricular pacing cannot be

determined from the present study, there is ample evidence that ventricular pacing is capable of activating transient outward K^+ currents. Thus, Geller and Rosen[12] observed that an increase in electric activation rate of canine ventricular slabs from 90 to 130 pulses per min shortened the action potential. The action potential shortening persisted for several min after the activation rate was lowered to 90 pulses per min, indicative of myocardial "memory" for the activation stimulus. The persistent shortening of the action potential could be antagonized by blockade of the transient outward K^+ current. Although the specific role of K^+_{ATP} channels was not investigated in that study, our findings that after 15 min after cessation of 30 min of rapid ventricular pacing a small but statistically significant reduction in infarct size occurred, suggest that K^+_{ATP} channel activation by ventricular pacing may also display memory. Interestingly, Geller and Rosen[12] reported that cardiac memory in isolated slabs of canine ventricular myocardium was only produced when the activation sequence was abnormal (stimulation from the lateral side of the preparation, i.e. perpendicular to the fiber axis, mimicking ventricular pacing[11]) but not when the activation sequence was normal (stimulation from the basal end of the preparation in the direction of the fibers, mimicking atrial pacing). This could explain why Marber et al[9] failed to observe a protective effect of 5 min of *atrial* pacing followed by 10 min of normal sinus rhythm on myocardial infarct size in rabbit hearts.

Ventricular pacing afforded myocardial protection which was slightly greater in the epicardial half than in the endocardial half when pacing was followed immediately by the 60 min coronary artery occlusion. In addition, protection was marginal in the subepicardium but absent in the subendocardium when a 15 min period of normal sinus rhythm was allowed between the pacing period and the sustained occlusion. There is evidence that the K^+ channels are heterogeneously distributed across the left ventricular wall. Geller and Rosen[12] reported that transient outward repolarizing K^+ currents in the epicardium increased more than in the endocardium during altered myocardial activation sequence. Also, Litovsky and Antzelevitch[27] reported that acetylcholine sensitive K^+ channels are present in the epicardium but not in the endocardium. The distribution of K^+_{ATP} channels is presently unknown, but our findings that 30 min of rapid ventricular pacing followed immediately by a sustained period of ischemia limited infarct size in both endo- and epicardium indicate that ventricular pacing can stimulate both endocardial and epicardial K^+_{ATP} channels. Following cessation of pacing the protective effect appeared to be lost more rapidly in the endocardium than in the epicardium. There is evidence that exposure of the myocardium to repeated periods of ventricular pacing progressively prolongs cardiac memory.[11] It is thus possible that repeated bouts of rapid ventricular pacing in pigs could have resulted in greater infarct size limitation compared to a single episode when pacing was separated by 15 min from the sustained coronary artery occlusion.

Conclusions

Thirty min of rapid ventricular pacing decreased myocardial infarct size produced by a 60 min total coronary artery occlusion in open-chest pigs. The magnitude of protection was greatest when the period of ventricular pacing immediately preceded the sustained period of ischemia, as the protection was nearly completely lost when 15 min of normal sinus rhythm separated the rapid ventricular pacing period from the sustained occlusion. The protective effect of pacing was abolished by K^+_{ATP} channel blockade indicating that K^+_{ATP} channel activation is involved in the mechanism of protection. Since we failed to observe significant myocardial ischemia during rapid ventricular pacing, it appears that K^+_{ATP} channel activation was produced via a non-ischemic mechanism, possibly an alteration in ventricular activation sequence.

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Chapter 8

Ischemic preconditioning: modalities and clinical relevance

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Introduction

Some fifteen years ago several groups of investigators paid considerable attention to the reproducibility of metabolic and functional changes in animal models of repeated reversible ischemia. If reproducible, these models could then be used for the evaluation of pharmacological interventions with the animals serving as their own control. The results were rather disappointing, however. For instance, we found that myocardial lactate production and ATP breakdown were much less during the second of two periods of ischemia produced by identical coronary flow reductions, which were separated by 40 min of complete reperfusion [1]. Also in patients undergoing a cardiac catheterization for suspected coronary artery disease, myocardial lactate production and inosine release was less during the second of two identical atrial pacing stress tests [2,3]. In addition to these functional and metabolic parameters the incidence of ventricular arrhythmias during occlusion and early reperfusion also proved to be less during the later occlusion-reperfusion sequences. From these and many other observations it has become clear that adaptive processes occur in models of repeated reversible ischemia, but the mechanism(s) leading to these adaptations were not further investigated at that time [4,5].

Less than a decade ago Murry *et al* reported that infarct size was 29% in dogs subjected to a 40 min coronary artery occlusion but only 7% when the 40 min occlusion was preceded by 4 cycles of 5 min coronary artery occlusion and 5 min of reperfusion [6]. The protective effect of these brief periods of myocardial ischemia on development of infarct size during a sustained coronary artery occlusion was termed "ischemic preconditioning" and has now been confirmed in a large number of other laboratory animals [7] including pigs [8], rabbits [9] and rats [10].

Protection by preconditioning has not only been connected with infarct size limitation but also with enhanced recovery of regional cardiac contractile function and anti-arrhythmic activity. For instance, Cave and Hearse have shown that in globally ischemic isolated rat hearts recovery of contractile function is enhanced when these hearts are preconditioned by brief episodes of global ischemia [11]. Furthermore, the number of ventricular arrhythmias, and more importantly the incidence of ventricular fibrillation, is reduced in preconditioned rats during a sustained coronary artery occlusion and reperfusion [12-14]. Because the incidence of reperfusion arrhythmias is highest after coronary artery occlusions lasting between 10 and 30 min, which are too short to lead to infarction, the effect of ischemic preconditioning on infarct size versus its effect on reperfusion arrhythmias and recovery of contractile function is usually studied in separate models. In contrast to the effect on infarct size, the anti-arrhythmic component of preconditioning has been demonstrated primarily in the rat. However, in other animal species this aspect of ischemic preconditioning appears to be less effective [4,6].

In this overview we will restrict the experimental evidence of the protective effect of preconditioning to infarct size limitation and outline the problems that exist in obtaining clinical evidence for this phenomenon. However, clinical studies, exclusively dealing with

aspects of preconditioning in models of reversible ischemia will be discussed. The limitations in applying the results of these studies as evidence for protection against irreversible damage will be emphasized. Finally, we will address the possibilities that preconditioning can be exploited in man.

Features of experimental studies on ischemic preconditioning with infarct size as endpoint

There are four distinct phases in ischemic preconditioning experiments (Fig. 1), which can be characterized as (i) the preconditioning stimulus, (ii) the intervening reperfusion period, (iii) the sustained coronary artery occlusion, which is followed by (iv) a sustained reperfusion period at the end of which the anatomical area at risk and infarcted area are determined. Important features of each of these phases have been summarized in Table 1 and will be discussed briefly below [6-9, 15-55].

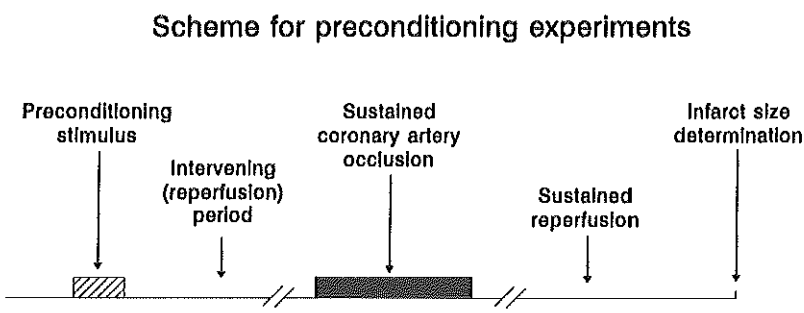

Figure 1 Scheme depicting the five different phases in experimental preconditioning protocols. In table 1 several of the characteristics of these phases have been tabulated.

The preconditioning stimulus

Single or multiple brief total coronary artery occlusions varying from 2 to 10 min in duration have been used to precondition the myocardium. The minimum stimulus to elicit preconditioning is not sharply defined and may depend on the species. Ovize *et al* reported that a 2.5 min left circumflex coronary artery occlusion was already sufficient to precondition the myocardium [15]. Li *et al* have shown that in dogs a single 5 min coronary artery occlusion followed by 10 min of reperfusion was as effective as 12 sequences of 5 min occlusion and 10 min of reperfusion [16].However, two different subthreshold stimuli can produce preconditioning. Thus, Yao and Gross reported that while a brief coronary artery occlusion and low dose of the K^+_{ATP} channel activator Bimakalim (see mechanisms) had no effect on infarct size when given separately, combined administration of these stimuli in dogs resulted in significant infarct size limitation [40]. Not only total coronary artery occlusions, but clinically even more relevant, also partial coronary artery occlusions can precondition the

Table 1. Features of preconditioning experiments with infarct size as endpoint.

Preconditioning stimulus

Ischemic

- single or multiple total coronary artery occlusions lasting from 2 to 10 min (supply ischemia) [6-9, 15-18]
- severe partial coronary artery occlusions (supply ischemia) [19-21]
- moderate partial coronary artery occlusions + adrenergic stimulation (demand ischemia) [22]
- moderate fixed stenosis + endothelial injury [23]

Non-ischemic

- heat stress (heat shock proteins) [24, 25]
- brief total occlusion of coronary artery supplying adjacent myocardium (remote preconditioning) [26]
- left ventricular volume loading [27]
- rapid ventricular pacing [28]
- transient hypoxia [29]
- transient occlusion of renal artery? (remote preconditioning) [30, 31]

Pharmacological substances

- K^+_{ATP} channel openers [32-34], adenosine [35, 36], Protein kinase C activators [37-39]
- decreased threshold for ischemic preconditioning by subthreshold K^+_{ATP} channel activation [40]

Intervening (reperfusion) period

- mandatory following total occlusion (self evident)
  1 min - 2 hours first window of protection [6, 17, 41-44]
  24 hours - ? second window of protection [45, 46]
- not necessary following severe partial occlusions [20, 21]
- not necessary following rapid ventricular pacing [28]

Sustained coronary artery occlusion

- duration limited from 30 min to 90 min (species specific) [6, 47]

Sustained reperfusion

- mandatory following the sustained coronary artery occlusion (self evident)

Infarct size determination

- area at risk determination (*in-vivo* by negative staining fluoresceine, Evans blue etc.)
- infarct area determination *ex vivo* with histochemistry Tetrazolium staining after reperfusion of at least 90 min [48]
- infarct size expressed as percentage of the area at risk is a reliable index only for areas at risk larger than 20% of the left ventricular mass [28, 49-51]

Other aspects of preconditioning

- controversy about the loss of the protective effect of preconditioning during first window (gradual decrease or "all or nothing" phenomenon) [43, 44, 52]
- different endo- and epicardial distribution of the protective effect due to the different time course of the development of infarction in the endo- and epicardial regions [21]
- preconditioning can be reinstated after the protection is lost [42, 53]
- tolerance develops with chronically applied preconditioning stimuli [54]
- preconditioning has been demonstrated in hypertrophic hearts [55]
- preconditioning has been shown in awake as well as anaesthetized animals [54]

myocardium [19-21]. It appears, however, that the flow reduction must be sufficiently large (approximately 50%) to produce preconditioning.

While most investigators have used an impaired blood supply to induce preconditioning, Iwamoto *et al* reported that preconditioning can also be obtained without a reduction in absolute supply [22]. This group of investigators showed that when myocardial oxygen demand was augmented by stimulation of the left stellate cardiac nerve, the myocardium became preconditioned when the accompanying increase in coronary blood flow was prevented. When the flow was allowed to increase, thereby leaving the myocardial oxygen supply demand balance intact, the increase in adrenergic activity did not trigger cardioprotection. These data are consistent with the observations by Marber *et al* who failed to observe limitation of infarct size when rabbits hearts were subjected to 5 min of rapid atrial pacing [56].

In the "classical" ischemic preconditioning studies the myocardium is subjected to a brief period of ischemia before the infarct is produced. However, there is evidence that the myocardium can also be preconditioned by ischemia in a remote region of the left ventricle or forms of stress that do not cause ischemia. Thus, Przyklenk *et al* reported that a brief total coronary artery occlusion preconditions not only the myocardium within its perfusion territory but also protects the myocardium outside its territory representing an example of remote ischemic preconditioning [26]. Another example of remote ischemic preconditioning is the reduction in myocardial infarct size, which is observed when a renal artery is transiently occluded prior to the coronary artery occlusion [30, 31]. Ovize *et al* [27] described that stretching the myocardium by volume loading reduces infarct size during a subsequent coronary artery occlusion via a mechanism not involving ischemia. Their observations could provide an explanation for the remote ischemic preconditioning as severe regional ischemic contractile dysfunction leads to stretching of the adjacent non-ischemic myocardium [26]. In our laboratory we have shown that 30 min of rapid ventricular pacing at 200 beats min^{-1} limited infarct size during a subsequent 60 min coronary artery occlusion [28]. The effect was most pronounced when no intervening period of normal sinus rhythm was used between the ventricular pacing period and the sustained coronary artery occlusion. The protection by ventricular pacing did not involve ischemia as in a separate series of experiments it was shown that high energy phosphates were not depleted during the ventricular pacing period, while systolic segment shortening recovered immediately (no stunning) and reactive hyperemia was absent after ventricular pacing was terminated. The mechanism by which ventricular pacing protected the myocardium likely involved activation of K^+_{ATP} channels as pretreatment with the K^+_{ATP} channel inhibitor glibenclamide prevented the protection by ventricular pacing. That not all forms of stress are capable of preconditioning the myocardium is suggested by the studies employing left stellate cardiac nerve stimulation or atrial pacing as preconditioning stimuli [22,56]. Interestingly, Zhu *et al* even reported an increase in infarct size when rats had been chronically exposed to cigarette smoke [57].

The intervening reperfusion period

When myocardium is preconditioned with a total coronary artery occlusion, it is self evident that an intervening reperfusion period is mandatory as otherwise the period of sustained ischemia would merely be increased. It is still unclear what minimal duration of reperfusion is required, but 1 to 2 minutes of complete reperfusion between the preconditioning stimulus and the sustained coronary artery occlusion may well be sufficient to trigger myocardial preconditioning [41]. With respect to the maximum length of the intervening reperfusion period, it is now clear that ischemic preconditioning is a transient phenomenon. Thus, with reperfusion periods exceeding two hours preconditioned animals develop infarcts which are not different from that of control animals. However, some investigators reported that when the duration of the intervening reperfusion period is increased to 24 hours the myocardium may again become preconditioned [45,46]. This reappearance of protection has been called the second window of protection (SWOP) to discriminate it from the first window of protection (FWOP) or classical preconditioning which exists during the first two hours after a preconditioning stimulus has been applied. However, the experimental evidence for this second window of protection is not as convincing as that for the first window of protection and is therefore still a point of debate [58].

The necessitity of an intervening reperfusion period has been a point of discussion when myocardium is preconditioned by a partial coronary artery occlusion. Ovize *et al* could not trigger preconditioning without a period of complete reperfusion when they used a 50% flow reduction which lasted 15 min. The authors concluded that complete reperfusion is mandatory for preconditioning to occur [19]. In contrast to their findings in dogs [19], we found that in pigs a 70% flow reduction which was maintained for 30 min without an intervening reperfusion period *did* reduce infarct size produced by a 60 min total coronary artery occlusion [20]. This two-stage coronary artery occlusion model is very similar to the model which was initially employed by Harris to reduce the high incidence of ventricular fibrillation during the first 30 min of a total coronary artery occlusion [59].

In pigs, which lack a significant innate coronary collateral circulation we observed that preconditioning by a 10 min total coronary artery occlusion caused almost identical reductions in the inner (endocardial) and outer (epicardial) half of the myocardium. Conversely, after preconditioning with a 70% flow reduction protection was greater in the epicardial than in the endocardial half [21]. Consistent with the observation by Ovize *et al* [19] we found that infarct size after the 60 min total coronary artery occlusion was not reduced when only a 30% flow reduction was used to precondition the myocardium, even when the duration of the flow reduction was increased to 90 min [21]. No studies have yet investigated whether myocardium is also preconditioned when reperfusion during the intervening period after a brief total coronary artery occlusion is incomplete. These data would be of significant clinical importance as they are likely to mimic the clinical situation (e.g. residual coronary stenosis following thrombolysis) more closely than the abrupt occlusion-reperfusion protocols.

The sustained total coronary artery occlusion

Studies in dogs suggest that with sustained coronary artery occlusions lasting longer than 90 min the protective effect of the preconditioning stimulus is lost [6]. The protective effect of preconditioning should therefore be considered to be a shift in the time course of infarct size development (Fig. 2), and will not likely reduce infarct size when the duration of the sustained occlusion exceeds a certain limit [6,47].

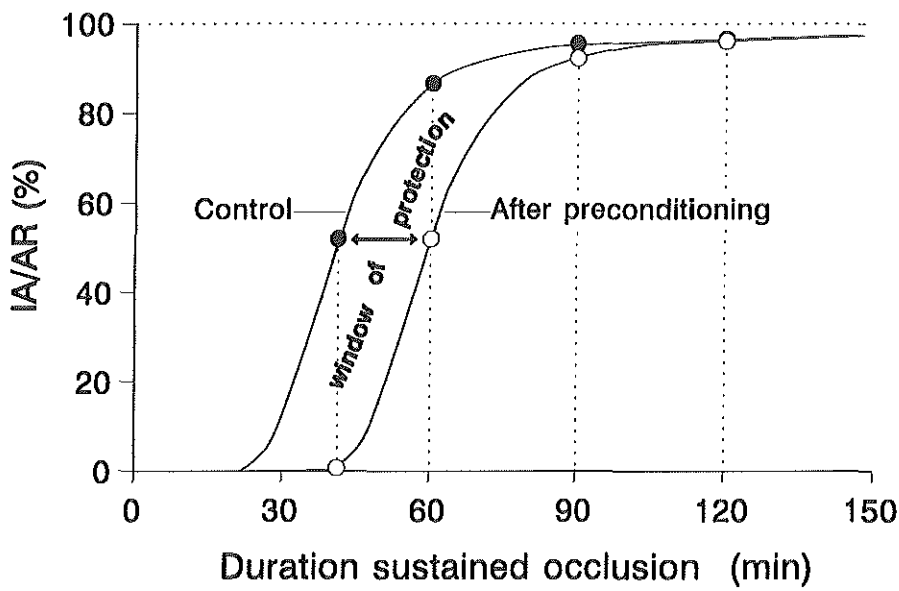

Figure 2 Schematic presentation of the effect ischemic preconditioning by a brief total coronary artery occlusion on myocardial infarct size produced by a sustained coronary artery occlusion. Preconditioning results in a rightward shift of the time infarct size relation indicating a delay in cell death (time window of protection is ~20 min). Depending on the duration of the sustained coronary occlusion the amount of protection can be close to 100% (40 min), 40% (60 min) or neglibible (>90 min). The shape and steepness of the curve are species dependent. It is currently unclear whether different preconditioning stimuli (duration of brief occlusion, rapid ventricular pacing, stretch) produce different time windows of protection.

The sustained reperfusion period

The histochemical staining techniques for the determination of infarct size require a prolonged period of complete reperfusion (minimal duration of 90 min) to obtain accurate measurements [48]. It has been suggested that a decrease in infarct size determined during the early phase of reperfusion (<6 hours) produced by using pharmacological interventions may be lost when the duration of reperfusion is prolonged to 3 days [60]. Analogous to the observation with pharmacological interventions it could be hypothesized that ischemic

preconditioning merely postpones myocardial cell death during the reperfusion period, in particular since most studies of myocardial preconditioning use brief periods of reperfusion (<4 hours).That the protective effect of preconditioning persists and is not only a delay of cell death during the reperfusion period follows from studies in dogs [6] and rabbits [65] that allowed 3-4 days of reperfusion prior to infarct size determination.

Assessment of infarct size

In experimental studies the ratio of the infarcted area and the area at risk is routinely used to assess the efficacy of ischemic preconditioning. In such studies the anatomical area at risk is defined as the myocardium supplied by the coronary artery which has been occluded for a sustained period of time to produce a myocardial infarction. The area at risk is usually delineated from the remaining myocardium by reoccluding the coronary artery and infusion of fluoresceine- or Evans blue dye into the left atrium at the end of the experiment. The infarcted area is then determined by excising the heart and staining the vital myocardium with tetrazolium salts (NAD-dependent dehydrogenase staining). Several groups of investigators have shown that in animals both with and without an extensive coronary collateral circulation the relation between infarcted area (IA) and anatomic area at risk (AR) is highly linear, but not proportional [20,49-51]. In other words the equation relating these two variables can be described as $IA = a\ AR + b$. This implies that the IA/AR ratio is not a constant but depends on AR ($IA/AR = a + b/AR$). Only when the area at risk exceeds 20% of the left ventricular mass the term b/AR is negligible compared to a [49]. Comparisons between studies which have used different areas at risk must therefore be performed cautiously to avoid erroneous conclusions.

Other aspects of preconditioning

Natural decay of protective effect of preconditioning.

Because the protection by ischemic preconditioning is lost after a two hour intervening reperfusion period the question arises whether a gradual decrease in the amount of protection occurs as the duration of the intervening reperfusion period increases. Some studies [43,44] indeed suggest a gradual decrease already during the first hour of the intervening reperfusion period. Our studies in pigs do not support such a hypothesis as we observed that after a single 10 min total coronary artery occlusion the amount of protection was the same for intervening reperfusion periods of 15 min and 60 min [52]. Following a two hour reperfusion period most animals developed infarcts of a similar size as in the control animals, but a few animals had infarct sizes which were not different from the preconditioned animals with 15 or 60 min of intervening reperfusion. These observations are more consistent with "an all or nothing" phenomenon in the individual animal. The available evidence suggests that averaging data for a group of animals may give the impression that the protective effect of preconditioning wanes gradually, at a time when individual data points suggest an all or nothing type of response.

Development of tolerance to continuously applied preconditioning stimuli and reinstatement of preconditioning after it has been lost.

It appears that a stimulus given immediately after preconditioning is lost will reinstate the cardioprotective action [42,43,53], but that a stimulus given while the myocardium is still protected does not prolong the protective effect beyond that produced by the initial stimulus [61]. This observation could have significant clinical importance as most patients are likely to have several periods of ischemia prior to developing myocardial infarction.

Numerous examples can be found in the literature about the development of tolerance upon continuous infusion of drugs (tachyphylaxis), with the tolerance to nitrates being the most widely described. Since patients may have multiple episodes of ischemia each day for a prolonged period of time, the question arises whether the protective action of ischemic preconditioning persists when multiple sequences of brief occlusions and reperfusions are continued for a period of hours to days. Cohen *et al* addressed this issue in conscious rabbits which were preconditioned using 5 min occlusions at 30 min intervals for 8 hours every day [54]. In this model infarct size was reduced from 38% to 6% when a 30 min coronary artery occlusion was preceded by a preconditioning stimulus consisting of a single 5 min coronary artery occlusion separated from the sustained occlusion by an intervening reperfusion period of 30 min. After 3 to 4 days of these repetitive occlusions, myocardial infarct size produced by the 30 min occlusion was not different (27%) from that of the control group. These authors also showed that a couple of days after these repetitive occlusions, preconditioning could be recaptured with a single 5 min occlusion. It is yet unknown how relevant the model used by Cohen *et al* is for the clinical situation as such a large array of multiple occlusions is unlikely to occur in patients with ischemic heart disease. It is, however, quite feasible that preconditioning may already be lost after a much lower number of these ischemic episodes which would enhance the clinical relevance of these observations. An equally important observation is that preconditioning could be reinstated after an ischemic free period. Another important feature of this study is that it was the first preconditioning study performed in conscious animals, thereby eliminating possible interference from the effects of acute surgical trauma and anaesthesia on reflex pathways and the autonomic nervous system.

Ischemic preconditioning in hypertrophic hearts.

Classical ischemic preconditioning studies have mostly employed animals with a normal heart. However, many patients suffering from a myocardial infarction are older than 50 years and are likely to have hearts which are quite different from those in which ischemic preconditioning has been demonstrated. For instance, the Framingham study revealed that left ventricular hypertrophy occurs in 12-40% of subjects aged 50 years and older [62]. Hence, in order to increase its potential clinical relevance, ischemic preconditioning should also be demonstrated in animal models of left ventricular hypertrophy. Thus, Speechly-Dick *et al* studied ischemic preconditioning in rats with left ventricular hypertrophy induced by

concurrent administration of deoxycorticosterone acetate (DOCA) and saline for 4 weeks [55]. The infarct to risk area volume ratio was considerably lower in the hypertrophied animals which had been preconditioned by a 5 min coronary artery occlusion separated from the 30 min sustained occlusion by 10 min of reperfusion (19% vs 67% in the control group).

Possible mechanisms of preconditioning

Ischemic preconditioning has been demonstrated in several species which lack a significant coronary collateral circulation, and thus recruitment of collateral blood flow by the brief ischemic periods can be excluded as a potential mechanism for ischemic preconditioning [8, 32]. The original investigators [6] as well as others [63] reported reduced rates of glycolysis and high energy phosphate depletion as well as better preservation of pH and myocardial ultrastructure during the sustained coronary artery occlusion when preceded by brief periods of reversible ischemia. The reduced rate of energy utilization was initially believed to result from a decrease in myocardial energy requirements secondary to the depressed contractile function produced by the brief period of reversible ischemia (i.e. myocardial stunning). However, Matsuda *et al* demonstrated that the myocardium remained protected when systolic segment shortening (a marker for regional cardiac function) in stunned myocardium was recruited by infusion of dobutamine within the allocated 2 hours interval between the short and the longlasting coronary artery occlusions [64]. In agreement with their findings Miura *et al* failed to observe a correlation between the degree of stunning and infarct size limitation produced by preconditioning [65]. These studies indicate that myocardial stunning is not a prerequisite for triggering cardioprotection.

It was not until Downey and co-workers proposed a role for adenosine in preconditioning, that a large number of studies began to focus on the (sub)cellular mechanisms [35,37,66]. Inhibition of mitochondrial ATPase, free radicals, and the endothelium derived substances prostacyclin and nitric oxide are examples of mechanisms that have at some point been suggested to play a role, but which may now be considered as minor when infarct size is taken as the endpoint [7,67,68]. Mechanisms that are currently ascribed a pivotal role in ischemic preconditioning are adenosine [4,6,35,36], K^+_{ATP} channel activation [32-34,69], and G-protein/protein kinase C activation [37-39]. Most of the evidence has been obtained by using substances which either mimic or inhibit the effect of preconditioning. It is beyond the scope of this review to discuss the possible mechanisms of preconditioning in detail and Fig. 3 therefore only provides a scheme for some of the proposed mechanisms. For a more detailed description of the mechanisms of preconditioning the reader is referred to one of the many extensive reviews recently published [5,7,37,67,68].

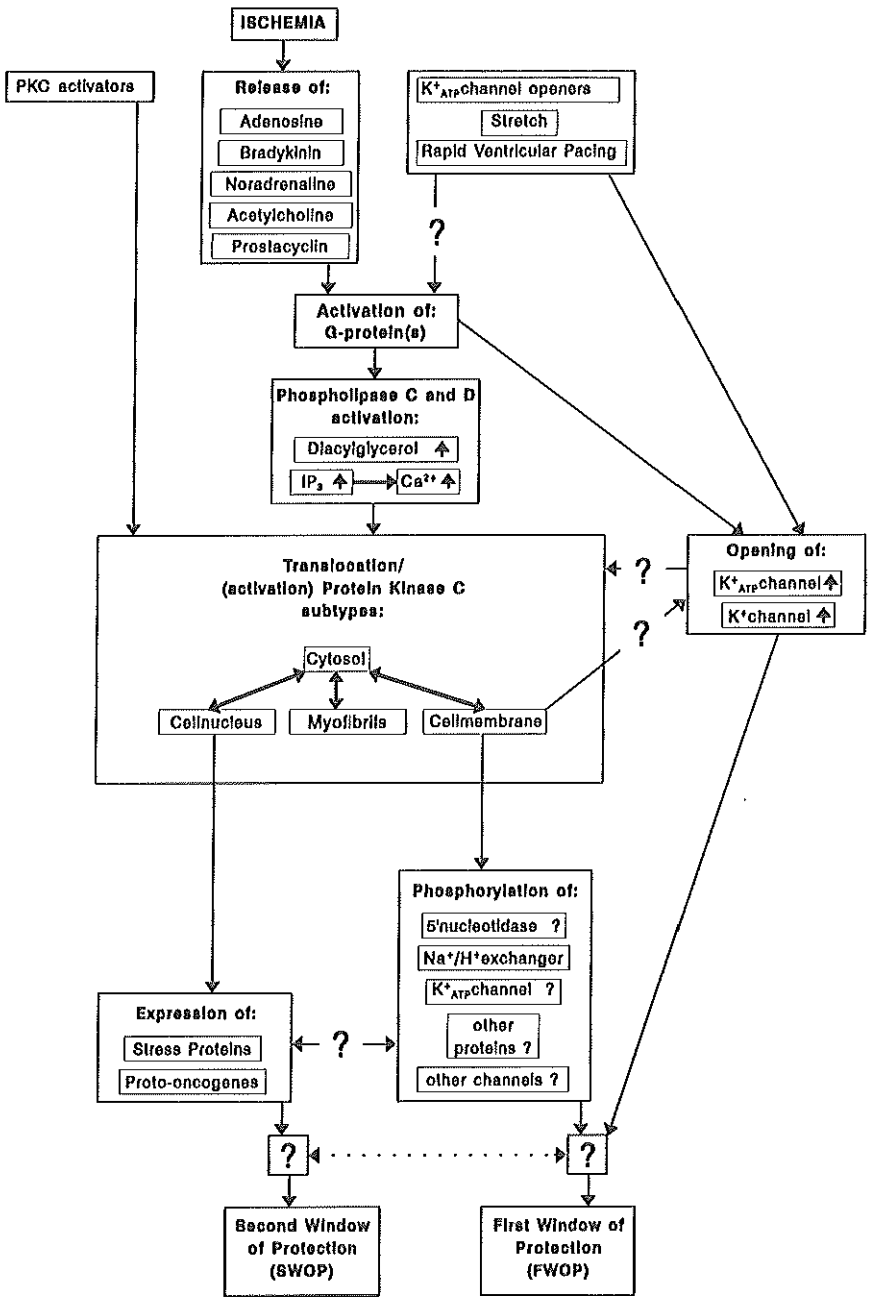

Figure 3 Scheme depicting possible mechanisms in (ischemic) preconditioning.

Evidence for the existence of ischemic preconditioning in patients

It is self evident that no clinical study meets the strict conditions outlined for the experimental studies in Table 1. Consequently, direct evidence for the occurrence of ischemic preconditioning with infarct size as endpoint in man is not available. Table 2 lists the potential pitfalls in obtaining indirect evidence for the occurrence of preconditioning in man.

Table 2. Problems with obtaining evidence for preconditioning in man using infarct size as endpoint.

Preconditioning stimulus
- unknown if the duration and severity of anginal attacks are sufficient to precondition the myocardium
- unknown if silent ischemia can precondition the myocardium
- medication and other forms of stress may interfere with the potential protective effect of the brief ischemic periods [treatment with K^+_{ATP} channel openers for hypertension or treatment with K^+_{ATP} channel blockers (Glibenclamide) for diabetes mellitus type II]

Intervening reperfusion period
- reperfusion following anginal attacks may be incomplete and variable in duration

Sustained coronary artery occlusion
- occlusion that produces infarction may be incomplete and variable in severity
- collateral bloodflow to the area at risk is unknown
- onset and duration of occlusion cannot be accurately defined

Reperfusion following the sustained coronary artery occlusion
- onset of reperfusion cannot be accurately defined
- reperfusion may be incomplete due to pre-existent coronary artery stenosis

Determination of infarct size
- "infarct size" should be related to the anatomical area at risk; area at risk is usually not determined
- enzyme leakage, preservation of left ventricular function or survival are often used as endpoints; these may not accurately reflect the extent of the infarcted area

A main source of error lies in the inability to accurately define the onset, duration and the completeness of the different occlusion and reperfusion phases as outlined in Fig 1. Thus, a confounding factor is that both the onset of occlusion and the beginning of reperfusion (and also whether these are complete) are not precisely known as this does not necessarily coincide with the onset or relief of anginal pain or changes in the electrocardiogram. The extent of coronary collateralization is another important determinant of infarct size, which cannot be quantified with sufficient degree of accuracy. Also a shortcoming of clinical studies is the inability to accurately determine infarct size, for which often indirect measures (enzyme leakage, survival or left ventricular function) have to be relied on. Although the area at risk can be determined, most often it is not and consequently it is impossible to relate the amount

of necrosis to the area at risk. Thus, because of methodological limitations comparison of infarct size between different groups of patients remains limited to comparing such variables as total enzyme leakage without taking into account the area at risk and the duration of the occlusion. Finally, patients may be on medication (e.g. aminophylline, K^+_{ATP} channel modulators) that are capable of mimicking or inhibiting ischemic preconditoning.

In view of these limitations of human studies on myocardial infarction we will in the following paragraphs not only review infarction studies, but also studies in which multiple periods of ischemia have been investigated usually as part of a diagnostic or therapeutic intervention. A number of these repeated ischemia studies have attempted to unravel the mechanisms underlying the myocardial adaptation triggered by the first period of ischemia. If it could be shown that the mechanisms responsible for ischemic preconditioning (infarct size limitation) are the same as the mechanisms responsible for myocardial adaptation (lesser signs of ischemia during the second of two periods of reversible ischemia) this would provide strong, though indirect, evidence that ischemic preconditioning may occur in the human heart.

Lawson [70] and Kloner and Yellon [71] have identified several patient categories in which the phenomenon of ischemic adaptation can be demonstrated. These groups are: (*i*) patients which suffer an acute myocardial infarction preceded by periods of angina pectoris [71-83] (Table 3). Because reperfusion following the sustained period of ischemia is mandatory if preconditioning is to be able to limit infarct size. These authors have differentiated between studies performed during the pre- and thrombolytic eras, (*ii*) patients who have undergone two or more episodes of reversible ischemia as part of diagnostic or therapeutic interventions. These interventions include repeated exercise stress tests [85-84] and atrial pacing stress tests [2,3,88-89] and percutaneous transluminal coronary angioplasty (PTCA) [91-99], (*iii*) patients undergoing arterial cross-clamping as part of cardiac surgery [100], and (*iv*) *in-vitro* studies in samples obtained from human hearts [101,102] (Table 4).

Angina prior to an acute myocardial infarction

The clinically most relevant situation for studying ischemic preconditioning is that of the patients who experience anginal attacks shortly before they suffer an acute myocardial infarction. These anginal episodes may be caused by exercise-induced oxygen supply-demand mismatch or by cyclic flow variations resulting from repeated platelet formation and dispersion, situations by which ischemic preconditioning can be induced in the laboratory [22, 23].

Because the protective effect of ischemic preconditioning is not seen after permanent occlusions, studies must be stratified according to whether they have been performed before or after the introduction of thrombolytic therapy and emergency coronary angioplasty. Table 3 shows that positive as well as negative results have been reported which cannot be explained by the absence or presence of reperfusion by thrombolysis or acute percutaneous transluminal coronary angioplasty. Importantly, none of the studies reported positive results on long

Table 4. Myocardial adaptation to repeated episodes of reversible ischemia in man.

Adaptation to ischemia	References
Exercise stress test ("Warm up" and "Walk through" phenomenon)	
decreased intensity of pain	[84]
increased time to onset of pain	[85,86]
less ST segment changes	[85,86]
decreased anaerobic myocardial metabolism	[86]
decreased myocardial O_2-demand (systemic hemodynamics)	[84,85]
decreased myocardial O_2-consumption	[86]
increased coronary bloodflow	[85]
Atrial pacing stress test	
Positive results	
decreased intensity of pain	[87, 88]
increased time to onset of pain	[87]
less ST segment changes	[3, 88, 89]
decreased anaerobic myocardial metabolism	[3, 88, 89]
decreased myocardial O_2-consumption	[88]
decreased myocardial O_2-demand (systemic hemodynamics)	[3, 89]
Negative results	
similar intensity of pain	[2]
similar time to onset of pain	[2]
similar ST segment changes	[87]
similar anaerobic myocardial metabolism	[2]
similar myocardial O_2-consumption	[2]
similar myocardial O_2-demand (systemic hemodynamics)	[87]
Angioplasty procedures	
Positive results	
decreased intensity of pain	[90-94]
less ST segment changes	[90-92,94-96]
decreased anaerobic myocardial metbolism	[91]
improved global hemodynamics	[91,92]
Negative results	
similar ST segment changes	[97-99]
similar global hemodynamics	[93]
similar anaerobic myocardial metabolism	[99]
Aortic cross clamp during cardiac surgery	
slowing of high energy phosphates degradation	[100]
In vitro studies	
Isolated human right atrial trabeculae	
less deterioration of contractile function	[101]
Human ventricular myocytes	
decreased cell death, decreased H^+, preserved aerobic myocardial metabolism	[102]

term survival. This may be attributed to a greater prevalence of severe coronary artery disease and other risk factors such as age, smoking and presence of hypertension. Furthermore, it cannot be excluded that in a number of patients, because of the frequency of ischemic episodes prior to the occurrence of myocardial infarction tolerance to ischemic preconditioning might have developed [54]. Based on the currently available evidence it is premature to conclude that angina prior to myocardial infarction limits infarct size. Until more sensitive measures of infarct size and area at risk become available, and the incidence and duration and severity of occlusions can be more accurately determined, this question will likely remain unanswered.

Repeated episodes of ischemia

Acute tolerance to angina pectoris: the 'warm up' and 'walk through' phenomenon.

In a large number of patients the first anginal attack in the morning is more severe than those occurring during the later hours of the day ('warm up' phenomenon). A circadian variation in the autonomic tone is a generally accepted mechanism to explain this observation [103,104]. In view of the concept of myocardial adaptation an alternative explanation could be that the less severe signs of ischemia during these later episodes are the result of adaptation triggered by the first episode. It has also been shown that exercise-induced ischemia is less severe during the second of two identical exercise tests when these are separated by a short recovery period [84-86]. In one of these studies, myocardial oxygen consumption at a given workload was less, suggesting a role for metabolic adaptation [83].

At the present time it is unclear if exercise when stopped at the earliest signs of myocardial ischemia (as is often the case) is sufficient to protect the myocardium against irreversible damage during the development of myocardial infarction [19,20,22,28].

Atrial pacing stress test.

During atrial pacing stress tests the myocardial oxygen balance can become disturbed by the increase in heart rate. The ensuing ischemia results from both a decrease in diastolic duration, with myocardial blood flow being distributed away from the subendocardium, and a modest increase in myocardial oxygen demand. Early on it was recognized that the metabolic, electrocardiographic and functional responses to two pacing stress tests were not always reproducible when repeated at a short time interval. Thus in several studies, though not all, it was shown that during the second test time to onset of angina was prolonged, while ST-segment changes and metabolic and functional changes were less compared at comparable heart rates (Table 4). These findings are compatible with the hypothesis that the myocardium can adapt to ischemia. However, since pacing-induced ischemia may be too brief in duration and not severe enough to elicit ischemic preconditioning these studies of atrial pacing stess tests must be viewed with caution.

Angioplasty procedures.

During angioplasty the myocardium is exposed to brief multiple periods of ischemia of which the duration can be well controlled. A serious disadvantage is that the occlusion periods are rather short and that differences in myocardial perfusion during balloon deflation may be caused by a reduction in the severity of the lesion and during subsequent balloon inflations by recruitment of collateral flow. Moreover, the balloon inflations do not cause irreversible damage and the endpoint of these studies, such as intensity of pain, ST segment depression and lactate production, are therefore markers of reversible forms of ischemia.

Several studies have reported that the changes occurring during the second and third balloon inflations indicate less severe signs of ischemia than during the first inflation. This is in itself not a surprising finding as it has also been shown for atrial pacing stress tests in patients undergoing a diagnostic catheterisation for suspected coronary artery disease. Not all studies have, however, demonstrated that an adaptative mechanism becomes operative after the first inflation, which can be explained by the fact that the myocardium of these patients may already have been adapted because of previous ischemic episodes [61] or had become resistant [54]. In addition, the duration of inflation in the studies of De Bruyne *et al* [93] and Oldroyd *et al* [99] might have been too short (60 sec) [15] to produce adaptation. In a preliminary study Kerensky *et al* [96] reported that after pretreatment with intracoronary adenosine, the magnitude of ST-segment changes was no longer different between the first and second inflation. Their findings could suggest that adenosine pretreatment adapted the myocardium analogous to its role in ischemic preconditioning (infarct size limitation). Of interest is that pretreatment of patients with glibenclamide completely abolished the attenuation of intracoronary electrographic chagnes during the second balloon inflation [94], suggesting that activation of K^+_{ATP} channels which are involved in ischemic preconditioning also modulate the myocardial adaptation process to these brief periods of reversible ischemia.

Studies in human myocardial samples

Left ventricular biopsies collected during cardiac surgery.

Yellon *et al* [100] recently demonstrated metabolic adaptative processes during aortic-cross clamping in patients undergoing cardiac surgery. The advantage of this last model is that in dealing with global rather than regional ischemia, the importance of the coronary collateral circulation as a confounding factor is eliminated. In their study the authors assigned 14 patients randomly to a sustained period of global ischemia consisting of 10 min cross-clamping either without (control) or with two preceding sequences of 3 min cross-clamping and two min of reperfusion (preconditioning). As expected, adenosine triphosphate levels were decreased (by approximately 30%) after the preconditioning protocol and thus lower than in the control group when the 10 min cross-clamping period was started. At the end of this 10 min period of global ischemia, the adenosine triphosphate levels in the preconditioned group were higher than in the control group. This was due to the absence of further decreases in adenosine

triphosphate in the preconditioned group, while the adenosine triphosphate levels decreased by 60-70% in the control group. These observations are in agreement with the original observations in dogs by Murry *et al* who showed that ischemic preconditioning slows energy metabolism during a sustained coronary artery occlusion [4, 6]. The study by Yellon *et al* [100] shows metabolic features of myocardial preconditioning in man that are consistent with those found in the preconditioning experiments in the animal laboratory, providing direct evidence that in man the myocardium can *adapt* to ischemic stress. However, there is no proof that slowing of the energy metabolism is the critical factor in limiting necrosis when the duration of the ischemic episode is prolonged. Whether the metabolic adaptations will ultimately lead to *limitation of infarct size* (true preconditioning) still remains to be answered.

Right atrial trabeculae.

Walker *et al* [101] suspended human right atrial trabeculae in an organ bath and preconditioned one group with 3 min of pacing (180 pulses/min) while superfusing the trabeculae with a hypoxic and substrate-free buffer. As an intervening reperfusion period the trabeculae were paced at 60 pulses/min for 10 min in a reoxygenated buffer with substrate. This preconditioned group and a control group which did not receive prior treatment were then subjected to pacing (180 pulses/min) for 90 min during superfusion with a hypoxic and substrate-free buffer (comparable to "the sustained coronary artery occlusion" period). This was followed by 120 min of reoxygenation and pacing at 60 pulses/min. At the end of this reoxygenation period recovery of developed tension was twice as large in the preconditioned group. It may be argued that pacing in a hypoxic substrate-free medium is not the same as *in vivo* ischemia produced by total coronary artery occlusions. We have shown, however, that it is also possible to precondition myocardium by partial coronary artery occlusions during which there is also a (limited) wash-out of metabolites [20,21].

Isolated human cardiomyocytes.

In cultured myocytes grown from ventricular myocytes obtained during cardiac surgery have been preconditioned with 20 min of anoxia followed by 20 min of normoxia before they were subjected to 90 min of anoxia [102]. After 30 min of normoxia, these preconditioned cells had a higher survival rate and less uptake of trypan blue than a group of cells which underwent only the test procedure. The supernatant of the preconditioned cells had also lower concentrations of hydrogen, lactate and lactic dehydrogenase. Models of isolated cultured cells are attractive as potential mechanism(s) of preconditioning can be studied under rigorously controlled conditions without any interference of other cells. The latter may also be a disadvantage as events in adjacent myocardial cells and even other organs could lead to cardioprotection.

Summary and Conclusion

The protective effect of a brief period of reversible ischemia against the development of irreversible damage has now convincingly been demonstrated in a large number of animal species. The protective state is temporary but may have a biphasic pattern as some studies suggest that the protective state of a preconditioning stimulus disappears after a few hours, but reappears after 24-36 hours. There is evidence that myocardium can also be preconditioned by a partial coronary artery stenosis, even without the need of an intervening reperfusion period. The severity of the flow reduction during the partial occlusion appears to be important and the transmural distribution of protection may not be homogeneous. In the animal laboratory myocardial preconditioning can also be obtained by transient ischemia in other organs as well as by non-ischemic stimuli such as stretch and rapid ventricular pacing. These models may help to bridge the gap between the classical abrupt occlusion-reperfusion models which have limited clinical applicability and the clinical situation.

There is evidence to suggest that the protection afforded by ischemic preconditioning could extend into the coronary circulation, in particular the endothelium of which the function can become impaired following longer periods of myocardial ischemia and reperfusion. Defily and Chilian [105] reported that endothelium-dependent vasodilation was depressed in isolated coronary microvessels from dogs subjected to 60 min of coronary artery occlusion and reperfused for 90 min. A 10 min coronary artery occlusion preceding the 60 min occlusion preserved endothelium-dependent vasodilation. In contrast, Bauer *et al* [106] failed to observe a protective effect of four episodes of 5 min occlusions on the loss of endothelium-dependent vasodilation produced by a 60 min coronary artery occlusion in dogs. Thus, at the present time the question whether preconditioning protects against coronary vascular damage remains unanswered.

Direct clinical evidence for the classical preconditioning phenomenon with infarct size limitation as endpoint cannot be obtained but a number of patient groups have been identified in which adaptation to ischemia has been demonstrated by enhanced recovery of function or preservation of high energy phosphates in models of repeated ischemia such as angioplasty and aortic cross-clamping during cardiac surgery. Evidence is accumulating that mechanisms which are operative in experimental ischemic preconditioning (infarct size limitation) are also operative in the clinical models of repeated reversible ischemia. Insight into the mechanisms responsible for ischemic preconditioning could potentially help to develop pharmacological agents which mimic preconditioning. This is especially attractive as several of the ischemic episodes may be too short or not severe enough to trigger preconditioning. By a synergistic or additive action combination of such a stimulus and low dose of pharmacological agent might result in a protective action [40]. If these agents were also to be used for treating cardiovascular conditions, such as the K^+_{ATP} channel activator nicorandil for the treatment of angina pectoris, the cardioprotective effect could be a beneficial side effect. The currently available protein kinase C activators are oncogenic, it may be envisioned that, in the future

with the recognition and better understanding of the different subtypes new Protein kinase C activators may become available which can be applied for cardioprotection without negative side-effects. The hearts of patients who regularly experience episodes of ischemia may be in a more or less permanent state of preconditioning afforded by one of these stimuli. Alternatively, resistance may have developed. In this situation it is quite feasible that additional protection by a pharmacological agent cannot be accomplished at that time. It is reassuring, however, that preconditioning can be reinstated immediately after the cardioprotection is lost and that it can also be demonstrated in hearts with pathologic conditions such as hypertrophy.

In view of the observations that cardioprotection may also be produced by transient ischemia in other organs and even some forms of stress which do not lead to myocardial ischemia, it could be envisioned that ischemic preconditioning is only one component of a general form of cardioprotection.

Acknowledgements

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Chapter 9

General Discussion and Summary

[A more detailed version of this chapter will be published as "Novel approaches to myocardial preconditioning in pigs" in Myocardial Preconditioning, Wainwright C.L. and Parratt J. (eds), Austin, Texas, RG Landes Co.]

In this thesis are presented the results of our studies on ischaemic preconditioning in domestic pigs. A number of earlier observations have guided these studies. First, it has been well established that in dogs the relation between infarct size (IS) and the anatomical area at risk (AR) is linear but not proportional, i.e. the regression line describing the relation between IS and AR is linear but has a positive intercept on the AR-axis.[1] Such a relation implies that the IS/AR ratio, which is the index commonly used to express infarct size, is not a constant but depends on AR. As was shown in this thesis, a positive intercept is also present in swine which can severely limit the use of IS/AR in assessing infarct size and myocardial protection. Secondly, it is well established that most of the protective effect of a brief period of ischaemia is lost when the intervening reperfusion period separating the brief and sustained coronary artery occlusions exceeds two hours.[2] The relation between the extent of protection by ischaemic preconditioning and the duration of the intervening reperfusion period is not well established, although some data suggest that the preconditioning stimulus starts to lose some of its efficacy already very early in the intervening reperfusion period.[3] Thirdly, the necessity of a period of reperfusion between the brief and sustained *total* coronary artery occlusions to elicit ischaemic preconditioning is self-evident; otherwise the duration of the sustained coronary artery occlusion will be merely increased. It is less obvious, however, whether a *partial* coronary artery occlusion can lead to ischaemic preconditioning without the need for an intervening reperfusion period. Finally, Ovize et al[4] have shown that stretch can precondition the myocardium without the need for ischaemia. We have extended this observation and investigated whether other non-ischaemic stimuli are also capable to precondition myocardium.

Relation between infarct size and area at risk in pigs (Chapter 4 and 5)

The effect of ischaemic preconditioning is usually evaluated by the ratio of infarct size (IS) and the anatomical area at risk (AR). This index (IS/AR) can be used without restriction only when infarct size development occurs independent of the size of the area at risk. For anaesthetized as well as conscious dogs, it has been shown that the linear relation between infarct size and area at risk has a positive intercept on the AR-axis.[2] Because dogs can have an extensive coronary collateral circulation, the positive intercept can be easily ascribed to collateral blood flow. Consequently, in this species infarct size correlates well with the amount of collateral blood flow to the area at risk. If collateral blood flow is indeed the cause of this positive intercept the relation between infarct size and area at risk should be proportional in pigs, a species with a negligible native coronary collateral circulation. In open-chest anaesthetized pigs we have occluded the left anterior descending coronary artery or its branches at different locations, thereby intentionally creating a wide range in the size of the area at risk.[5,6] In 17 animals that underwent a 60 min coronary artery occlusion we found that the relation between infarct size and area at risk was highly linear ($r=0.99$, $P<0.001$) and could be described by $IS=0.97AR - 4.5$, in which IS and AR are both expressed as percent of left ventricular mass (Fig. 1, upper panel).

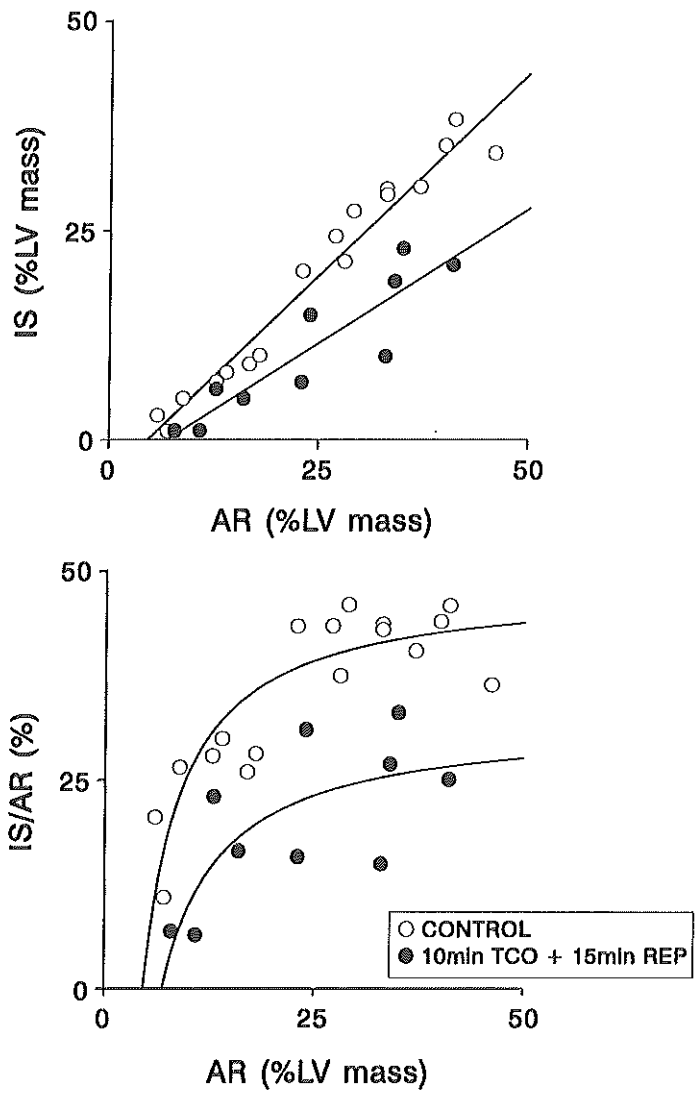

Figure 1 Relation between infarct size (IS) determined 2 hours after a 60 min total coronary artery occlusion (60 min TCO), and the area at risk (AR) for a group of control animals and a group of animals preconditioned by a 10 min total coronary artery occlusion which was separated from the 60 min TCO by 15 min of complete reperfusion (upper panel). Due to the positive intercept on the AR-axis the IS/AR of these two groups (upper panel) of the relation between IS and AR of animals strongly depends on AR for AR less than 20% of the left ventricular mass (LVmass) (lower panel). (adapted from Koning et al[5,6,11]).

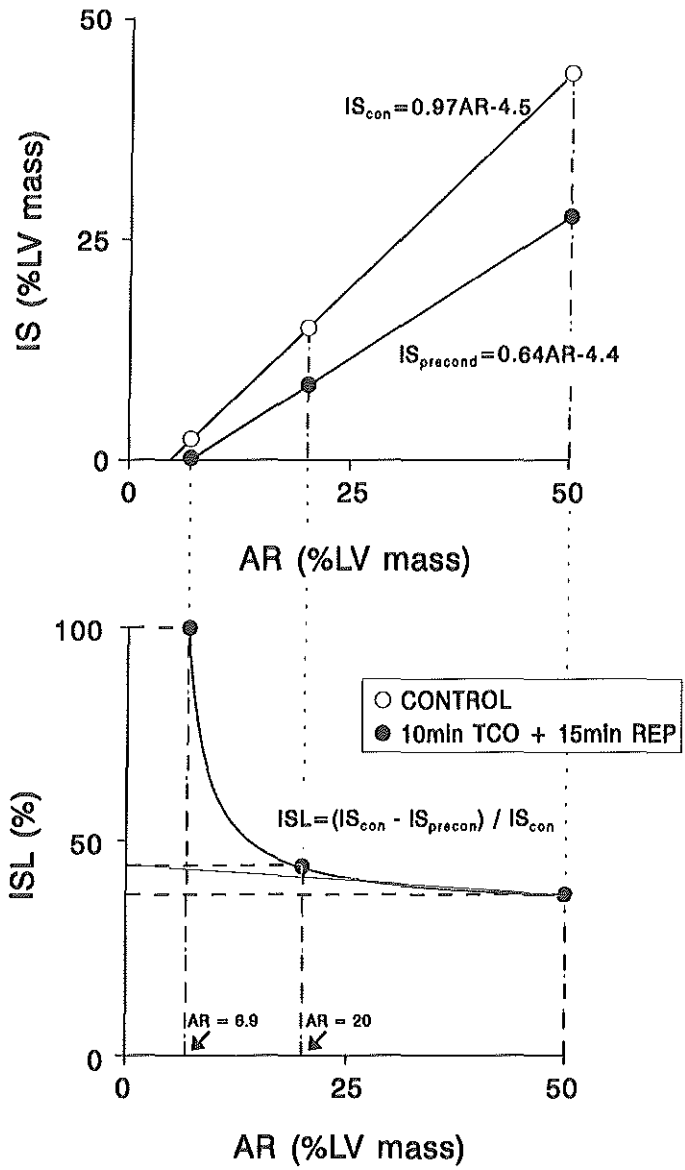

Figure 2 Regression lines for the control (con) and preconditioned (precond) pigs, as depicted in figure 1, are shown in the upper panel. The lower panel shows the infarct size limitation (ISL) afforded by the 10 min TCO + 15 min REP preconditioning stimulus. ISL (%) was computed as $(IS_{con} - IS_{precond})/IS_{con}$, where IS_{con} and $ISL_{precond}$ are the infarct size (IS) of the control group and the preconditioned group, respectively.

Thus, similar to the findings in dogs, we also observed a positive AR-intercept in a species with minimal coronary collaterals. In support of our findings, Ytrehus et al[7] recently showed that also in rabbits, the relation between infarct size and area at risk can be described by a linear regression equation with a positive intercept on the AR-axis.

In a subsequent series of experiments we investigated how the relation between infarct size and area at risk is modified by ischaemic preconditioning. For this purpose animals were preconditioned with a 10 min total coronary artery occlusion which was separated from the 60 min occlusion by 15 min of reperfusion.[5,6] In these preconditioned animals the linear relation between infarct size and area at risk was rotated downward with a lower slope but with a similar positive intercept on the AR-axis as in the control animals (IS=0.64AR-4.4: Fig. 1, upper panel). The implication of these findings is that in both control and preconditioned animals IS/AR depends on AR (Fig. 1, lower panel). From this figure it can be easily deducted that in particular for areas at risk less than 20% of the left ventricular mass IS/AR is highly sensitive to the size of the area at risk. Figure 2 illustrates the infarct size limitation, defined as the difference in infarct sizes (predicted from the regression lines) of the control and the preconditioned animals expressed as a percent of the infarct size of the control animals [$(IS_{control} - IS_{preconditioned})/IS_{control}$]. This figure clearly demonstrates that an interstudy comparison of the protective effect of ischaemic preconditioning can easily lead to erroneous conclusions when in these studies different areas at risk have been used. This may be especially true for the pig in which (in order to reduce the incidence of ventricular fibrillation) smaller areas at risk are quite often preferred.

Transmural distribution of infarct size in pigs (Chapter 6 and 7)

In order to assess whether the endocardial and the epicardial halves benefit equally from ischaemic preconditioning, we divided the myocardium in two layers of equal thickness and related these infarcts to the corresponding areas at risk. The results revealed linear relations with similar positive intercepts on the AR-axis, for the endocardial and epicardial half of the left ventricle in control pigs subjected to a 60 min total coronary artery occlusion (Fig. 3). In dogs, IS and AR are also linearly related, but in contrast to pigs, the AR-intercept in dogs is considerable higher in the epicardium than in the endocardium.[8] The consequent heterogeneity of transmural infarct size distribution in dogs is likely in part due to the transmural gradient of collateral blood flow in the dog heart. In pigs, in which total coronary artery occlusions result in transmurally homogeneous myocardial blood flow reductions, infarction also progresses from inner to outer layer[9,10], possibly due to higher energy demands in the inner layers. In agreement with these findings we observed that for a given area at risk the infarct size produced by the 60 min total coronary artery occlusion was slightly (but significant by ANCOVA) larger in the endocardial than in the epicardial half of the left ventricle.[11]

In the pigs preconditioned by the 10 min total coronary occlusion and 15 min reperfusion the relation between infarct area and area at risk of the endocardial and the epicardial halves were also linear. The AR-intercepts were the same as in the control animals but with lower slopes. The

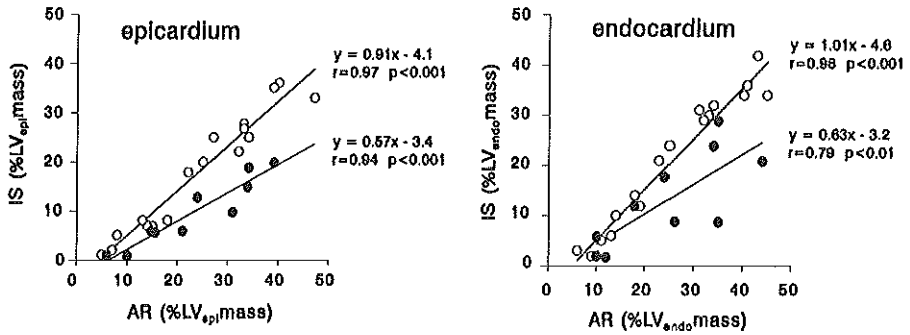

Figure 3 Transmural distribution of infarct size (IS) for control pigs (0, 60 min TCO only) and for pigs preconditioned by 10 min TCO + 15 min REP prior to the 60minTCO ( ). Note that the infarct size reduction was similar in the epi- and endocardial halves of the left ventricular wall, but that the scatter of the data points around the regression line was considerably less in the epicardial than in the endocardial half of the preconditioned animals. (Adapted from Koning et al[5,6,11]).

decrease in slope was the same for the endo- and epicardium indicating that the protection was similar in the inner and outer half of the left ventricle.[11]

The duration of protection afforded by ischaemic preconditioning (Chapter 4)

To determine the time course of protection we prolonged the intervening reperfusion period between the brief and sustained coronary artery occlusions from 15 min to 60 min. We hypothesized that if the preconditioning stimulus already starts to lose its efficacy early in the intervening reperfusion period, the regression line relating infarct size and area at risk would gradually rotate upwards towards the control line when the duration of the intervening reperfusion period is extended. With the 60 min reperfusion period the relation between infarct size and the area at risk remained virtually unchanged compared to the 15 min reperfusion period, indicating that in pigs a 10 min preconditioning stimulus does not start to lose its efficacy during the first hour of reperfusion.[5] A further prolongation of the intervening reperfusion period up to 2 hours revealed that the infarct size of most animals was no longer different from the control animals. However, the infarct size of a few animals remained well below the regression line of the control animals.[5] Because in the control animals all IS-AR data points are very close to the regression line it appears that the preconditioned animals with the IS-AR data points well below the regression line of the control group were still preconditioned. Infarct size determinations for animals with even longer intervening reperfusion periods showed that a few animals, although a progressively smaller percentage, remained preconditioned up to 5 hours after the brief period of ischaemia. It thus appears that in contrast to the earlier observations in rabbits,[3] (i) the efficacy of preconditioning in pigs remains virtually unchanged during the first hour, (ii) most pigs have lost protection by ischaemic preconditioning at two hours and (iii) a few pigs may remain protected up to five hours.

Ischaemic preconditioning with a partial coronary artery occlusion without intervening reperfusion (Chapter 5 and 6)

In all "classical" preconditioning experiments unimpeded coronary blood flow was restored after a brief total occlusion before the coronary artery was occluded for a sustained period of time. This period of reperfusion is obviously necessary because otherwise the duration of sustained ischaemia will merely be increased. A different situation may arise if myocardium could be preconditioned by a partial coronary artery occlusion. Harris was the first who applied a partial occlusion for 30 min (without measuring residual coronary artery blood flow) prior to a complete occlusion which lasted several hours (two-stage coronary artery occlusion) and observed a decrease in the high incidence of ventricular fibrillation usually occurring during the first 30 min after the onset of a total coronary artery occlusion.[12] This reduction in the incidence of ventricular fibrillation has been explained by the lesser severity of ischaemia during the partial occlusion period. The onset of the subsequent total occlusion (at 30 min) was considered beyond the time-point when most lethal arrhythmias occur. It was never considered why these lethal arrhythmias would *not* occur after the artery was occluded completely. Current insight into the effects of ischaemic preconditioning[13] offers an alternative hypothesis: the partial occlusion preconditions the myocardium thereby attenuating the incidence of ventricular fibrillation during the subsequent total occlusion. The first attempt to induce myocardial preconditioning by a two-stage coronary artery occlusion was by Ovize et al, who reduced coronary blood flow by 50% for 15 min before occluding the coronary artery completely without an intervening reperfusion period between the flow reduction and the complete coronary artery occlusion.[14] Infarct size in these animals was not different from that of a group of control animals which underwent only the sustained total coronary artery occlusion. Increasing the duration of the flow reduction to 25 min did not affect the results. Infarct size was markedly reduced, however, when these investigators allowed 10 min of complete reperfusion between the 15 min 50% coronary flow reduction and the sustained artery occlusion. The authors therefore concluded that myocardium could be preconditioned by a partial coronary artery occlusion but that an intervening period of reperfusion was mandatory to elicit the protective action.

We hypothesized that in the study of Ovize et al[14] the flow reduction may not have been severe enough or the duration too short for preconditioning to occur and therefore first reduced blood flow in the left anterior descending coronary by 70% for 30 min before occluding the artery for 60 min without an intervening reperfusion period. Figure 4 shows that all animals which underwent this two-stage coronary artery occlusion had transmural infarct sizes smaller than in the control group. When the duration of the 70% flow reduction period was extended from 30 min to 90 min before the coronary artery was occluded completely, transmural infarct sizes were still smaller than in the control groups, even though the 90 min 70% flow reduction per se had already caused some irreversible damage. In contrast a 30 min 30% flow reduction preceding the 60 min total coronary artery occlusion without an intervening period of reperfusion did not limit infarct size.[11] Findings were also negative when the duration of the 30% flow reduction was

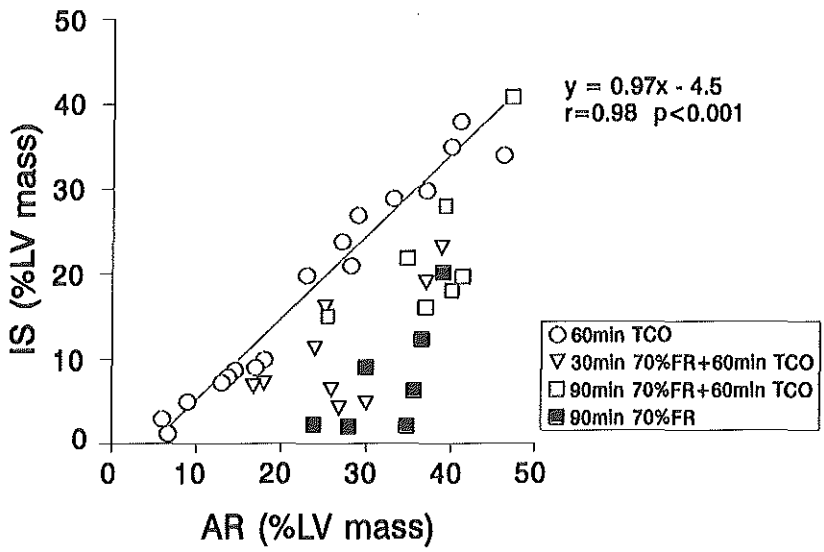

Figure 4 Effect of ischaemic preconditioning with a 70% coronary flow reduction (70%FR) without an intervening period of reperfusion on myocardial infarct size produced by a 60 min total coronary artery occlusion (60minTCO). Shown are the regression line and individual data points for the control group (60minTCO) and individual data points for animals subjected to a 30min70%FR followed by 60minTCO, a 90min70%FR followed by 60minTCO and a 90min70%FR without the 60minTCO. Note that although the 90min70%FR itself resulted in significant infarction after 90 min, it still provided protection against the irreversible damage produced by the subsequent 60minTCO (adapted from Koning et al.[11])

increased to 90 min. It therefore appears that myocardium can be preconditioned by a flow reduction without the need for an intervening reperfusion period, but that the severity of flow reduction is critical.

We also hypothesized that the protection by ischaemic preconditioning would most likely not be homogeneously distributed when a partial coronary artery occlusion was used as a stimulus. Because the flow deficit in the endocardial layers would be greater, it is there that ischaemia would be more severe than in the epicardial layers. If, as has been proposed, there is a threshold for ischaemia before preconditioning can occur it can be envisioned that with moderate flow reductions the endocardium could be preconditioned while the epicardium was exposed to a subthreshold stimulus. On the other hand, the subendocardium may already start to develop irreversible damage because of the larger flow deficit[14] as was observed when the coronary artery flow was reduced by 70% for 90 min. When we analyzed the endocardial (inner 50%) and the epicardial (outer 50%) infarct size we found that in the animals in which the induction of preconditioning was attempted by a 30% flow reduction, neither the endocardial nor the epicardial half were protected.[11] In the animals subjected to the 30 min 70% flow reduction

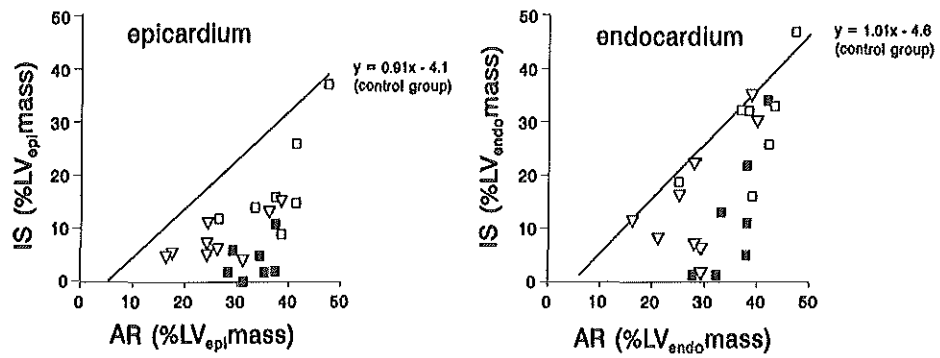

Figure 5 Effect of ischaemic preconditioning with a 70% coronary flow reduction (70%FR) without an intervening period of reperfusion on the transmural distribution of myocardial infarct size produced by a 60 min total coronary artery occlusion (60minTCO). Shown are the regression line and individual data points for the control group (60minTCO) and individual data points for animals subjected to a 30min70%FR followed by 60minTCO, a 90min70%FR followed by 60minTCO and a 90min70%FR without the 60minTCO. For details of the legends see Fig. 4. (adapted from Koning et al.[11])

infarcts were smaller in the epicardial than in the endocardial half, but both were less than the infarcts in the corresponding halves of the control animals (Fig. 5). The 90 min 70% flow reduction caused some irreversible damage, but this occurred predominantly in the endocardial half. Nevertheless, the endocardial infarcts after the subsequent 60 min total coronary artery occlusion were still smaller than in the control group, although the difference was less prominent than in the epicardial half. Thus, in contrast to classical preconditioning with a total coronary artery occlusion (which in our hands affords transmurally homogeneous protection) partial occlusions produced preferential epicardial protection. These findings may be of clinical relevance, since a large group of patients suffering from myocardial infarction will have pre-existing coronary artery lesions which may provide variably flow reductions prior to the occlusion by a thrombus.

Myocardial protection by a period of rapid ventricular pacing (Chapter 7)

Przyklenk et al have shown that myocardium can be preconditioned by a brief coronary artery occlusion supplying the adjacent myocardium demonstrating that myocardium does not have to become ischaemic itself to increase its tolerance to irreversible ischaemic damage.[15] This protection could be due to stretch of this virgin myocardium in response to the ischaemic dysfunction in the adjacent area. Further support for this hypothesis came from the same group of investigators when they showed that stretching the myocardium, obtained by volume loading, also attenuated infarct size during a sustained coronary artery occlusion without the prerequisite of ischaemia.[4] The protection by "stretch" could be blocked by pretreatment with gadolinium, an inhibitor of stretch-activated cation channels. In search of preconditioning myocardium by other types of stress we have used rapid ventricular pacing. The rationale for this approach is the

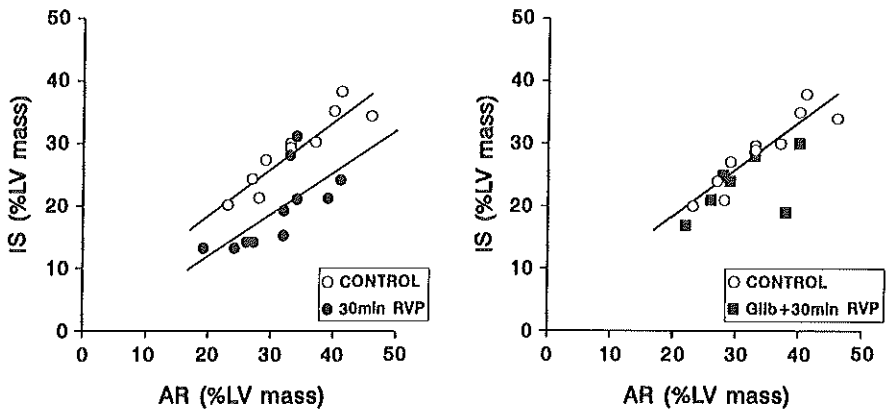

Figure 6 Effect of 30 min of rapid ventricular pacing at 200 bpm (30minRVP) on myocardial infarct size produced by a 60 min total coronary artery occlusion. Ventricular pacing was stopped after the onset of the coronary occlusion (left panel). The panel on the right shows that the protective effect of 30minRVP could be blocked by pretreatment with the K^+_{ATP} channel inhibitor glibenclamide (Glib, 1 mg/kg, iv). (adapted from Koning et al.[17])

observation by Vegh et al, that rapid ventricular pacing reduced the incidence of ventricular fibrillation during and after subsequent coronary artery occlusion.[16]

In four animals we found that 10 min of rapid ventricular pacing at 200 bpm separated from a 60 min total coronary artery occlusion by 15 min of normal sinus rhythm did not limit infarct size. When in six other animals the duration of rapid ventricular pacing was increased to 30 min there was a small but significant limitation of infarct size in the epicardial half but not in the endocardial half.[17] A much more striking effect was observed when the intervening period of normal sinus rhythm was abolished. Thus, when at the end of a 30 min period of rapid ventricular pacing, the left anterior descending coronary artery was occluded and the pacemaker was switched off immediately thereafter, infarct size in both the epicardial and the endocardial half were significantly reduced compared to that of the control group (Fig. 6 and Fig. 7). Pretreatment with glibenclamide abolished the protection by ventricular pacing in all but one of the animals, suggesting a role for K^+_{ATP} channel activation analogous to the preconditioning by a brief total coronary artery occlusion in pigs.[18-20] It would seem reasonable to assume that ventricular pacing-induced ischaemia played a major role in eliciting the protection, since several groups of investigators have shown that ventricular pacing produces changes in the electrocardiogram similar to those observed with ischaemia.[16,21] We therefore evaluated the effects of 30 min of ventricular pacing on global and regional cardiac performance.[17] In a separate group of pigs we established that ventricular pacing (i) did not affect transmural blood flow and its distribution of the transmural layers, (ii) did not widen the arterial coronary venous differences in pH and pCO_2, (iii) left high energy phosphate levels and energy charge unchanged, (iv) did not cause coronary vasodilation during pacing, (v) did not result in reactive hyperaemia after pacing was stopped and

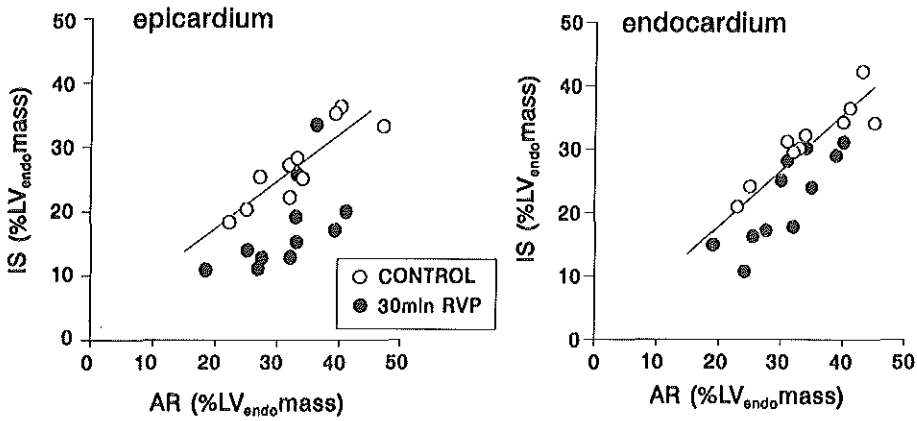

Figure 7 Effect of 30 min of rapid ventricular pacing at 200 bpm (30minRVP) on the transmural distribution of myocardial infarct size produced by a 60 min total coronary artery occlusion. Ventricular pacing was stopped after the onset of the coronary occlusion. Note that the protective effect on the epicardial half (left panel) was more pronounced than on the endocardial half (right panel). (adapted from Koning et al.[22])

(vi) did not prevent the immediate return of regional cardiac function after pacing was stopped. We feel that these findings fail to indicate the presence of myocardial ischaemia. Even, if some subendocardial ischaemia would have gone undetected, it would not have been severe enough to precondition myocardium via a pathway involving ischaemia. This conclusion is based on our findings that a 30% reduction in coronary blood flow does not lead to myocardial protection,[11] but produces significant metabolic and functional signs of ischaemia.

Evidence for the existence of ischemic preconditioning in humans (Chapter 8)

Direct clinical evidence for the classical preconditioning phenomenon with infarct size limitation as endpoint cannot be obtained. However, a number of patient groups have been identified in which adaptation to ischemia has been demonstrated by enhanced recovery of function or preservation of high energy phosphates in models of repeated ischemia such as angioplasty and aortic cross-clamping during cardiac surgery. Evidence is accumulating that mechanisms which are operative in experimental ischemic preconditioning (infarct size limitation) are also operative in the clinical models of repeated reversible ischemia. Insight into the mechanisms responsible for ischemic preconditioning could potentially help to develop pharmacological agents which mimic preconditioning. This is especially attractive as several of the ischemic episodes may be too short or not severe enough to trigger preconditioning. By a synergistic or additive action combination of such a stimulus and low dose of pharmacological agent might result in a protective action.[22] If these agents were also to be used for treating cardiovascular conditions, such as the K^+_{ATP} channel activator nicorandil for the treatment of

angina pectoris, the cardioprotective effect could be a beneficial side effect. The currently available protein kinase C activators are oncogenic. However, it may be envisioned that in the future, with the recognition and better understanding of the different subtypes, new Protein kinase C activators may become available which can be applied for cardioprotection without negative side-effects. The hearts of patients who regularly experience episodes of ischemia may be in a more or less permanent state of preconditioning afforded by one of these stimuli. Conversely, resistance may develop in such patients. In these situations it is quite feasible that additional protection by a pharmacological agent cannot be accomplished at that time. It is reassuring, however, that preconditioning can be reinstated immediately after the cardioprotection is lost and that it can also be demonstrated in hearts with pathologic conditions such as hypertrophy.

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Chapter 10

Samenvatting

Het vermogen van myocard (hartspierweefsel) om zich aan te passen aan ischemische (zuurstof tekort) stress is 15 jaar geleden al aangetoond in zowel experimentele als klinische studies. Hierin maakte men gebruik van herhaalde perioden van myocard ischemie, die werden opgewekt door de coronaire doorbloeding te reduceren. Zo is in varkens aangetoond dat het vrijkomen van metabole producten (markers van ischemie) tijdens de tweede van twee opeenvolgende perioden van ischemie veel geringer is. Ook bij patiënten werd dit fenomeen waargenomen. Destijds zijn echter geen pogingen ondernomen de gewijzigde reactie op de tweede ischemische periode te verklaren. Het doel van die studies was namelijk modellen te ontwikkelen waarbij reproduceerbare veranderingen in de verschillende "markers" van ischemie zouden optreden. In dergelijke modellen zou de patiënt of het proefdier als zijn eigen controle kunnen dienen in het evalueren van de effecten van farmacologische interventies. Daarop volgende studies naar de metabole en functionele gevolgen van meervoudige korte perioden van ischemie, zoals ook beschreven staat in hoofdstuk 2, toonden eveneens aan dat er geen cumulatieve verliezen waren van energierijke fosfaten en regionale contractiele functie in het myocardweefsel. Kennelijk vindt er na een periode van myocard ischemie een adaptatie in het myocard plaats, die tot doel heeft de integriteit van het myocard te bewaren.

Myocardiale adaptatie begon pas meer aandacht te krijgen toen Murry et al. in 1986 in honden aantoonden dat de infarctgrootte van het ischemische risicogebied afnam van 29% naar 7% wanneer een kransslagader afsluiting van 40 minuten werd vooraf gegaan door 4 cycli van 5 minuten occlusie en 5 minuten reperfusie. Inmiddels is ook voor een groot aantal andere diersoorten, zoals varkens, konijnen en ratten, aangetoond dat het mogelijk is om het myocard door middel van korte periodes van ischemie te beschermen tegen het ontwikkelen van onherstelbare schade ten gevolge van een langdurige afsluiting van een kransslagader ("ischemische preconditionering"). De bescherming die geboden wordt door ischemische preconditionering is echter aan een aantal beperkingen onderhevig. Zo kan het beschermende effect van ischemische preconditionering niet meer worden aangetoond als de langdurige kransslagader afsluiting langer duurt dan 90 minuten. Tevens heeft de bescherming een beperkte duur en verdwijnt wanneer tussen de preconditioneringsstimulus en de langdurige occlusie meer dan 2 uur verstrijkt.

In dit proefschrift zijn de resultaten van een aantal studies naar ischemische preconditionering bij varkens beschreven. In honden is reeds duidelijk vast komen te staan dat de relatie tussen infarctgrootte (infarct-size, IS) en het anatomische risicogebied (AR) wel lineair, maar niet proportioneel is, d.w.z. de regressielijn die de relatie beschrijft tussen IS en AR is lineair maar heeft een positief intercept op de AR-as ofwel bij een klein risicogebied treedt er geen infarct op. Dit houdt in dat de IS/AR ratio, de gebruikelijke index om infarctgrootte uit te drukken, niet constant is, maar afhankelijk is van AR. Zoals beschreven is in hoofdstuk 3 kan dit in ernstige mate het gebruik van de IS/AR ratio beperken bij het vaststellen van infarctgrootte en myocardiale bescherming. In hoofdstuk vier wordt beschreven hoe het beschermende effect van een korte periode van ischemie grotendeels verloren gaat wanneer de reperfusieperiode (herstelde

doorbloeding) die de korte en de langdurige occlusie periode scheidt langer duurt dan 2 uur. De relatie tussen de duur van de bescherming door ischemische preconditionering en de duur van de tussenliggende reperfusie periode is nog niet volledig duidelijk. Er zijn studies die suggereren dat het beschermende effect van de preconditioneringsstimulus al heel snel in de tussenliggende reperfusieperiode begint te verdwijnen. De noodzaak van een reperfusie periode tussen de korte en de langdurige volledige kransslagader afsluiting om ischemische preconditionering op te wekken is vanzelfsprekend, aangezien de duur van de langdurige kransslagader afsluiting anders simpelweg verlengd zou worden. Het is echter minder duidelijk of een gedeeltelijke afsluiting van een kransslagader ook kan leiden tot ischemische preconditionering (zonder een tussenliggende reperfusie periode). Indien dit mogelijk blijkt is het vervolgens de vraag of deze bescherming gelijk is voor de buitenste (epicard) en binnenste (endocard) helft van het myocard, omdat de doorbloeding van de endocardiale laag van de linker ventrikelwand het meest vermindert. Teneinde een antwoord op die vraag te geven bepaalden we de transmurale distributie van de infarctgrootte veroorzaakt door een 60 minuten durende volledige kransslagader afsluiting direct voorafgegaan door gedeeltelijke kransslagader afsluitingen (hoofdstuk 5 en 6).

Relatie tussen infarctgrootte en risicogebied in varkens

Het effect van ischemische preconditionering wordt gewoonlijk geëvalueerd aan de hand van de ratio van infarctgrootte (IS) en het anatomische risicogebied (AR) dat verstoken is van perfusie. Deze index (IS/AR) mag alleen zonder restricties worden gebruikt als de ontwikkeling van de infarctgrootte onafhankelijk is van de grootte van het risicogebied. Voor zowel genarcotiseerde als wakkere honden is echter aangetoond dat de lineaire relatie tussen infarctgrootte en risicogebied een positief intercept heeft op de AR-as. Honden kunnen echter een uitgebreide coronair collateraal circulatie hebben. Dit kan het positieve intercept gemakkelijk verklaren, omdat het risicogebied via deze collateralen van bloed kan worden voorzien. Indien de collaterale bloedtoevoer inderdaad de oorzaak is van het positieve intercept moet de relatie tussen infarctgrootte en risicogebied in varkens, een diersoort met een verwaarloosbare collateraal circulatie in het hart, proportioneel zijn. Daartoe werd in hoofdstuk 4 en 5 de grootte van het risicogebied gevarieerd door op verschillende plaatsen de arteria coronaria anterior descendens zelf of één of meer van zijn zijtakken af te sluiten. Wij vonden dat na een 60 minuten durende kransslagader afsluiting de relatie tussen infarctgrootte en risicogebied zeer lineair was ($r=0.99$, $P<0.001$; waarbij $IS=0.97AR-4.5$), en dat deze wederom een positief AR-intercept vertoonde.

In hoofdstuk 3 en 4 werd o.a. gekeken naar hoe de relatie tussen infarctgrootte en risicogebied wordt gewijzigd door ischemische preconditionering. Hiertoe werden de varkens gepreconditioneerd middels een 10 minuten durende volledige kransslagader afsluiting, die van een 60 minuten durende volledige afsluiting was gescheiden door 15 minuten reperfusie. In de gepreconditioneerde varkens bleek de lineaire relatie tussen infarctgrootte en risicogebied

een lagere richtingscoëfficient te bezitten dan bij de controle dieren (IS = 0.64AR-4.4), maar met een vrijwel identiek positief intercept op de AR-as. Dus zowel in de controle als in de gepreconditioneerde dieren is de IS/AR ratio afhankelijk van AR. De IS/AR ratio bleek met name zeer gevoelig te zijn voor de grootte van het risicogebied tot 20% van de linker ventrikel. Een directe vergelijking van resultaten uit verschillende studies kan dus gemakkelijk leiden tot verkeerde conclusies indien verschillende risicogebieden zijn bestudeerd. Dit geldt in hoge mate voor het varken, waarbij vaak bewust de voorkeur wordt gegeven aan kleinere risicogebieden teneinde de kans op het optreden van fatale ventrikel ritmestoornissen te verminderen.

Transmurale distributie van infarctgrootte in varkens

In hoofdstuk 6 werd de transmurale distributie (i.e. de verdeling over endocardiale en epicardiale lagen) van het beschermende effect van preconditionering in de linker ventrikelwand bestudeerd. Lineaire relaties met een positief AR-intercept werden in zowel de endocardiale als de epicardiale helften van de linker ventrikelwand gevonden in controle varkens die een 60 minuten durende volledige kransslagader afsluiting hadden ondergaan. In honden zijn de infarctgrootte en het anatomisch risicogebied ook lineair gerelateerd, maar in tegenstelling tot varkens is het AR-intercept bij honden beduidend groter in het epicard dan in het endocard. Dit is waarschijnlijk gedeeltelijk een gevolg van de transmurale gradiënt in collaterale bloedtoevoer in het hondehart, die het grootst is in het epicard. In varkens, waar een volledige kransslagader afsluiting resulteert in een transmuraal homogene afname van de myocardiale bloedtoevoer, ontwikkelt het infarct zich ook van de binnenste naar de buitenste laag, mogelijk ten gevolge van een groter energieverbruik en daarom een snellere reductie van de energievoorraad in de binnenste lagen. In overeenstemming hiermee vonden wij dat voor een gegeven anatomisch risicogebied de infarctgrootte veroorzaakt door de 60 minuten durende volledige kransslagader afsluiting, iets groter was in de endocardiale helft dan in de epicardiale helft van de linker ventrikel.

Ook in varkens gepreconditioneerd middels een 10 minuten durende kransslagader afsluiting en gevolgd door 15 minuten reperfusie, bleek de relatie tussen infarctgrootte en risicogebied van de endocardiale en de epicardiale helften lineair. Terwijl de AR-intercepten gelijk waren aan de intercepten in de controle dieren, waren de regressie coëfficienten van de relaties kleiner. De afname van de regressie coëfficient was gelijk voor endocard en epicard, wat wijst op een vrijwel identieke bescherming van de binnenste en buitenste laag van de linker ventrikel door preconditionering.

De duur van de bescherming ten gevolge van preconditionering

Om de duur van de bescherming te bestuderen werd in hoofdstuk 4 de tussenliggende reperfusieperiode tussen de korte en de langdurige kransslagader afsluiting verlengd van 15 minuten naar 1 uur. De hypothese was dat als de preconditioneringsstimulus zijn effect al vroeg

in de tussenliggende reperfusie periode begint te verliezen, de regressielijn die de relatie van infarctgrootte en het anatomisch risicogebied beschrijft geleidelijk opwaarts zou roteren in de richting van de controle lijn wanneer de tussenliggende reperfusieperiode wordt verlengd. Een 1 uur durende reperfusieperiode resulteerde in een vrijwel identieke relatie tussen de infarctgrootte en het risicogebied vergeleken met 15 minuten reperfusie, wat erop duidt dat het effect van een 10 minuten durende preconditioneringsstimulus in varkens gedurende het eerste uur reperfusie nog niet verloren is gegaan. Wanneer de duur van de tussenliggende reperfusieperiode tot 2 uur werd verlengd, bleek de infarctgrootte in de meeste dieren niet langer te verschillen van de infarctgrootte in de controle dieren. In enkele dieren bleef de infarctgrootte echter nog steeds ruim onder de regressielijn van de controle dieren. Aangezien de IS-AR punten van de controle groep weinig variatie vertoonden, is het zeer waarschijnlijk dat de dieren met IS-AR punten ruim onder de regressielijn nog steeds gepreconditioneerd waren na 2 uur reperfusie. Wanneer de tussenliggende reperfusieperiode nog verder verlengd werd tot 5 uur, bleek dat enkele dieren (hoewel een progressief afnemend percentage) nog steeds beschermd waren tot zelfs 5 uur na de korte ischemische periode. Hieruit kan men concluderen dat (i) het effect van preconditionering in varkens nagenoeg onveranderd blijft gedurende het eerste uur, (ii) de meeste varkens de bescherming door ischemische preconditionering na 2 uur hebben verloren en (iii) enkele varkens tot 5 uur na de preconditioneringsstimulus beschermd blijven. Dit suggereert dat ischemische preconditionering een "alles of niets" fenomeen is.

Ischemische preconditionering door een gedeeltelijke kransslagader afsluiting zonder tussenliggende periode van reperfusie

In alle "klassieke" preconditionerings experimenten wordt de kransslagader doorbloeding hersteld voordat de coronaire arterie voor een langdurige periode volledig wordt afgesloten. Voor het preconditioneren van het hartspierweefsel door een gedeeltelijke afsluiting van een kransslagader ligt dit mogelijk anders, omdat in dit geval de resterende doorbloeding voldoende zou kunnen zijn om het preconditioneren plaats te laten vinden. Harris was de eerste die gedurende 30 minuten een gedeeltelijke afsluiting toepaste (zonder overigens de resterende kransslagader doorbloeding te quantificeren) voorafgaand aan een volledige afsluiting van enkele uren. Harris beschreef een afname van de hoge incidentie van ventrikel fibrilleren die normaal gesproken optreedt gedurende de eerste 30 minuten na het volledig afsluiten van een coronair arterie. Deze afname in de incidentie van ventrikel fibrilleren werd uitgelegd door de mindere ernst van de ischemie gedurende de periode van gedeeltelijke afsluiting. De start van de daaropvolgende totale occlusie (na 30 minuten) werd beschouwd als komende na de tijdsduur waarbinnen de meeste ventrikel arrhytmiëen optreden. Huidige inzichten in de effecten van ischemische preconditionering leveren een alternatieve hypothese op: de gedeeltelijke afsluiting preconditioneert het myocard, waarbij de kans van ventrikel fibrilleren tijdens de daaropvolgende volledige afsluiting wordt verminderd. Het beschermende effect van een gedeeltelijke kransslagader afsluiting werd voor het eerst bestudeerd door Ovize et al door gedurende 15

minuten de kransslagader doorbloeding met 50% te reduceren alvorens de kransslagader volledig af te sluiten. De infarctgrootte in deze dieren verschilde niet van die in een controle groep, waarin de honden alleen de langdurige kransslagader afsluiting hadden ondergaan. Ook een 25 minuten durende 50% kransslagader afsluiting had geen effect op de infarctgrootte. Een afname van de infarctgrootte werd alleen waargenomen indien 10 minuten van volledige reperfusie werd toegestaan tussen de 15 minuten 50% reductie van de kransslagader doorbloeding en de langdurige kransslagader afsluiting. De auteurs concludeerden dat myocard kon worden gepreconditioneerd door een partiële occlusie, maar dat een tussenliggende reperfusieperiode obligaat was om een beschermend effect te verkrijgen.

We veronderstelden dat in de studie door Ovize et al de reductie in doorbloeding niet ernstig genoeg was of te kort duurde om preconditionering op te wekken. In hoofdstuk 6 reduceerden we de bloedtoevoer in de arteria coronaria anterior descendens eerst gedurende 30 minuten met 70% voordat de kransslagader direct aansluitend (dus zonder tussenliggende reperfusie) voor 60 minuten werd afgesloten. Alle dieren die aan dit regime werden onderworpen hadden beduidend kleinere infarcten dan de controle groep. Wanneer de duur van de periode van de 70% doorbloedingsreductie van 30 minuten werd verlengd naar 90 minuten, bleken de gevonden infarcten nog steeds kleiner dan de infarcten bij de controle dieren ondanks het feit dat de 90 minuten 70% reductie van de bloedtoevoer op zichzelf al had geresulteerd in enige irreversibele schade. In tegenstelling tot de beschermende effecten van 70% doorbloedingsreducties, beperkte een 30 of 90 minuten durende 30% reductie van de kransslagader doorbloeding de infarctgrootte niet. Deze bevindingen suggereren dat myocard kan worden gepreconditioneerd door een reductie in bloedtoevoer zonder een tussenliggende periode van reperfusie, maar dat een voldoende mate van doorbloedingsreductie, waarbij de stimulus een zekere drempelwaarde overschreidt, van cruciaal belang is.

Tijdens een partiële afsluiting van een kransslagader is de afname van bloedtoevoer distaal van de afsluiting niet homogeen verdeeld over de wand van de hartspier. In de endocardiale lagen neemt de bloedtoevoer meer af dan in de epicardiale lagen, waardoor de ischemie het ernstigst is in het endocard. Indien, zoals gesuggereerd is, er een drempel bestaat voor ischemische preconditionering die een voldoende sterke prikkel vereist, kan men zich voorstellen dat met een 30% reductie van de kransslagader doorbloeding het endocard zou kunnen worden gepreconditioneerd, terwijl het epicard slechts blootgesteld geweest zou zijn aan een stimulus die niet boven de drempelwaarde uitkomt. Aan de andere kant kan zich bij een 70% doorbloedingsvermindering subendocardiaal al irreversibele schade ontwikkelen, vanwege de grotere afname in bloedtoevoer, zodat de bescherming gedeeltelijk teniet wordt gedaan. Wanneer we de endocardiale (binnenste 50%) en de epicardiale (buitenste 50%) infarctgrootte analyseerden, bleek dat in de dieren die onderworpen waren aan 30 minuten 70% afname van de bloedtoevoer in beide helften de infarcten kleiner waren dan in de corresponderende helften van de controle dieren. Echter wij constateren dat de infarctgroottebeperking in de epicardiale helft groter was dan in de endocardiale helft. In tegenstelling tot klassieke preconditionering met een

totale occlusie van een coronair arterie (die in onze proeven een transmuraal homogene protectie biedt) resulteerde een 70% vermindering in de kransslagader doorbloeding in een voorkeur voor epicardiale protectie.

Myocardiale bescherming door een periode van snel ventrikel pacen

Przyklenk et al hebben aangetoond dat het hartspierweefsel gepreconditioneerd kan worden door een kort durende afsluiting van een kransslagader die een aangrenzend deel van de hartspier van bloed voorziet. Dit duidt erop dat myocard niet zelf ter plaatse ischemisch hoeft te worden om toch een verhoogde bescherming tegen ischemische schade te verkrijgen. De bescherming kan het gevolg zijn van rek van dit niet ischemische hartspierweefsel in respons op een verslechtering van contractiefunctie in het aangrenzende ischemische gebied. Ondersteuning voor deze hypothese kwam van dezelfde groep onderzoekers die aantoonden dat het rekken van de hartspier verkregen door acute volume belasting, ook leidde tot een vermindering van infarctgrootte ten gevolge van een langdurige kransslagader afsluiting zonder dat hiervoor ischemie nodig was. De bescherming door "rek" kon worden geblokkeerd door voorbehandeling met gadolinium, een remmer van rek-geactiveerde kation-kanalen. In hoofdstuk 7 is beschreven hoe hoogfrequente elektrische ventrikel stimulatie resulterend in een hartfrequentie van 200 slagen per minuut preconditionering opwekt. De gedachte achter deze benadering is de observatie door Vegh et al dat elektrische ventrikel stimulatie de incidentie van ventrikel fibrilleren verminderde gedurende en direct volgend op een afsluiting van een kransslagader. Tien minuten elektrische ventrikel stimulatie met 200 slagen per minuut, gescheiden van een 60 minuten durende coronair arterie occlusie door 15 minuten normaal sinusritme, had geen effect op de infarctgrootte. Wanneer de ventrikel stimulatie dertig minuten duurde resulteerde dit in een geringe, maar significante beperking van de infarctgrootte in de epicardiale, maar niet in de endocardiale helft. Een hogere mate van infarctverkleining werd bereikt als de tussenliggende periode van normaal sinusritme achterwege werd gelaten. Dus wanneer aan het einde van de 30 minuten elektrische ventrikel stimulatie de arteria coronaria anterior descendens werd afgesloten, nadat de elektrische stimulator (pacemaker) was afgezet, dan waren de infarctgroottes in zowel de epicardiale als de endocardiale helft beduidend kleiner in vergelijking met de controle groep. Voorbehandeling met de ATP-gevoelige kalium-kanaal antagonist glibenclamide resulteerde bij alle dieren, op één na, in het uitblijven van de beschermende effecten van ventrikel stimulatie. De centrale rol van de ATP-gevoelige kalium-kanalen bij ventrikel stimulatie is analoog aan diens rol bij preconditionering door een korte totale kransslagader afsluiting bij varkens. Er werd veelal aangenomen dat hoogfrequente ventrikel stimulatie ischemie gaf, welke de beschermende werking van ventrikel stimulatie zou verklaren. Deze aanname is gebaseerd op de door ventrikel stimulatie geinduceerde veranderingen in het electrocardiogram, welke vergelijkbaar zijn met de veranderingen zoals die worden waargenomen tijdens ischemie. Metingen aan het hart gedurende en volgend op 30 minuten van ventrikel stimulatie toonden aan dat de ventrikel stimulatie (i) geen effect had op de transmurale verdeling van de doorbloeding van de linker ventrikelwand,

(ii) de coronair arterio-veneuze pH en pCO_2 gradiënten en (iii) de energierijke fosfaatverbindingen en de "energielading" onveranderd liet. Verdere aanwijzingen dat ischemie niet optrad tijdens ventrikel stimulatie zijn (iv) de afwezigheid van een reactieve hyperemie en (v) het directe herstel van de regionale hartspierfunctie na het uitzetten van de pacemaker. Maar zelfs als enige subendocardiale ischemie optrad, welke door de metingen niet werd geregistreerd, dan nog zou deze niet ernstig genoeg zijn om de hartspier te preconditioneren. Deze conclusie is gebaseerd op onze bevindingen dat een 30% vermindering in kransslagader doorbloeding niet leidt tot myocardiale bescherming (hoofdstuk 6), maar wel resulteerde in anaeroob metabolisme met significante veranderingen van de metabole en functionele "markers" van ischemie.

In dit proefschrift is aangetoond dat het gebruik van de ratio infarctgrootte over anatomisch risicogebied om de mate van bescherming door ischemische preconditionering vast te stellen niet feilloos is, omdat de ratio afhankelijk is van de grootte van het anatomische risicogebied. Ook is bewijs aangevoerd dat niet alleen volledige maar ook gedeeltelijke kransslagader afsluitingen de hartspier kunnen preconditioneren. In het door ons gehanteerde model bleek ook dat een tussenliggende reperfusieperiode niet noodzakelijk is na de gedeeltelijke kransslagaderafsluiting. De bescherming welke wordt geleverd door een gedeeltelijke afsluiting is groter in de epicardiale dan in de endocardiale helft van de linker ventrikelwand, wat in tegenstelling is met de transmuraal homogeen verdeelde bescherming veroorzaakt door een korte volledige afsluiting van een kransslagader. Hoogfrequente elektrische ventrikel stimulatie is in staat myocard te preconditioneren, hoewel het "geheugen" voor bescherming geïnduceerd door ventrikel stimulatie veel korter is dan dat voor een korte afsluiting van een kransslagader. Activatie van K^+_{ATP} kanalen, via niet-ischemische weg lijkt een centrale rol te spelen in het beschermingsmechanisme van ventrikel stimulatie. Omdat de hartspier ook door andere vormen van niet-ischemische belasting kan worden gepreconditioneerd, waaronder rek en mogelijk ook ischemie in andere organen, zoals de nier, lijkt het dat lokale ischemie slechts een methode is om stress op te wekken in het myocard, welke vervolgens beschermt tegen irreversibele schade door een langdurige totale afsluiting van een kransslagader. Echter de vraag blijft welke vormen van stress preconditionering van het hart zouden kunnen geven, omdat stress door bijvoorbeeld een chronische blootstelling aan tabaksrook de infarctgrootte ten gevolge van een kransslagader afsluiting juist doet toenemen.

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Curriculum Vitae

Monique Martine Gertrude Koning was born on January 17 1960 in Vlaardingen, The Netherlands. She obtained her Medical Degree at the Erasmus University Rotterdam, Rotterdam, The Netherlands in 1988. From 1989 till the end of 1994 she has spent 4 years working at the Laboratory of Experimental Cardiology, Thoraxcenter, Erasmus University Rotterdam, under the supervision of Dr. P.D. Verdouw, professor of Experimental Cardiology, which has resulted in this thesis.

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